The role of brain PPAR[gamma] in regulation of energy balance and glucose homeostasis

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THE ROLE OF BRAIN PPARγ IN REGULATION OF ENERGY BALANCE AND GLUCOSE HOMEOSTASIS

by

Madeliene Stump

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience in the Graduate College of The University of Iowa

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To my husband Aaron David Stump
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Soli Deo gloria!
ABSTRACT

The Peroxisome Proliferator-Activated Receptor gamma (PPARγ), a master regulator of adipogenesis, has been shown to influence energy balance through its actions in the brain rather than in the adipose tissue alone. Deletion of PPARγ in mouse brain results in resistance to weight gain in response to high fat diet. Activation of PPARγ leads to change in the firing pattern of melanocortin system neurons (POMC and AgRP), which are critical for energy homeostasis. To determine the effects of modulation of brain PPARγ on food intake and energy expenditure we generated a novel transgenic mouse model in which a dominant-negative (DN) mutant form of PPARγ (P467L) or a wild type (WT) form that is conditionally expressed in either the entire central nervous system (CNS) or specifically in POMC or AgRP neurons. Interference with brain PPARγ results in impaired insulin and glucose regulation. This in turn has significant implications in altering the growth rate and metabolic homeostasis. In light of the well-established role of PPARγ in regulating insulin sensitivity, this is the first report implicating brain PPARγ in controlling peripheral insulin levels. Overexpression of the WT PPARγ in the CNS leads to failure to thrive and early death due to microcephaly and severe distortion of brain architecture with notable agenesis of the corpus callosum. Our results show that the levels of PPARγ in the brain are tightly regulated and perturbations leading to “too much” or “too little” functional PPARγ result in major shifts in structural organization of the brain or metabolic balance.

The herein presented data show that chronic interference with the function of neuronal PPARγ affects energy balance only under certain dietary conditions and through specific neuronal populations. We show that POMC, but not AgRP neurons, are particularly sensitive to modulation of PPARγ activity. These observations give support to the notion that cellular adaptations in POMC neurons, driven by PPARγ, represent critical components in the regulation of metabolic homeostasis.
PUBLIC ABSTRACT

Today we are faced with the first generation of American children who will have shorter life span than their parents. This is largely due to the staggering rates of obesity and type 2 diabetes mellitus. As of recently we have learned that type 2 diabetes develops not only because of dysfunction of peripheral organs, as it was previously thought, but also because of disruption of the ability of the brain to respond appropriately to signals of hunger or fullness. In my research I have developed several new mouse models which were used to identify the mechanisms that cause dysfunction of the brain regions critical for energy balance and thus make the whole organism vulnerable to the effects of the high fat western diet so popular today.
# TABLE OF CONTENTS

TABLE OF FIGURES........................................................................................................ x

CHAPTER I. INTRODUCTION............................................................................................. 1
  The Nuclear Receptor Superfamily.................................................................................. 3
  PPARs: Isotypes, Structure and Mechanism of Action..................................................... 4
    Isotypes.......................................................................................................................... 4
    Structure.......................................................................................................................... 4
    Mechanism of Action........................................................................................................ 5
    Post-translational Modification........................................................................................ 5
  The Emerging Concept of a PPAR Triad.......................................................................... 6
  PPARα.............................................................................................................................. 7
  PPARδ.............................................................................................................................. 8
  PPARγ............................................................................................................................. 9
    Isoforms: Genomic Characterization, Tissue Distribution, and Target Genes................. 9
    Ligands and Mechanism of Transcriptional Regulation by PPARγ................................. 11
    Role of PPARγ in Peripheral Control of Metabolism: Adipogenesis, Lipid Storage, and Insulin Sensitivity .................................................................................................................. 12
    PPARγ Polymorphisms.................................................................................................... 17
    Role of PPARγ in Central Nervous System (CNS) Diseases.......................................... 19
    Role of PPARγ in the Central Nervous System Control of Metabolism......................... 23
  Summary ........................................................................................................................ 28

CHAPTER II. GENERATION AND VALIDATION OF TRANSGENIC MOUSE MODELS ............................................................................................................................. 32
  Introduction...................................................................................................................... 32
  Materials and Methods.................................................................................................... 33
    Construction of Inducible Transgene Expression System.............................................. 33
    Cell Culture Validation Experiments.............................................................................. 34
    Generation of Transgenic Mice...................................................................................... 34
    Breeding and Maintenance of Transgenic Lines............................................................ 34
    Liver-Specific Transgene Activation Experiments.......................................................... 35
Brain-Specific Transgene Activation: Intracerebral Injection Experiments .......... 35
Skeletal Muscle-Specific Transgene Activation: Electroporation Experiments ...... 36
Western Blotting .................................................................................................. 37
Quantitative Real Time PCR (qRT-PCR) Analysis ........................................ 37
Immunohistochemistry ....................................................................................... 38
Confocal Imaging ................................................................................................ 39
Results ................................................................................................................ 39
Expression of Transgenic Proteins in Heterologous Cells ................................ 39
Expression of Transgenic Proteins in Mice ......................................................... 41
Induction of hPPARγ-WT Expression in the Brain Upregulates Classic Gene Targets .......................................................... 42
Transgenic Proteins Express in POMC Neurons of DT Mice ......................... 43
Discussion ........................................................................................................... 44

CHAPTER III. EFFECTS OF INTERFERECE WITH BRAIN PPARγ FUNCTION ON ENERGY BALANCE AND GLUCOSE REGULATION................................. 59
Introduction ......................................................................................................... 59
Material and Methods ........................................................................................ 61
Generation of Conditional hPPARγ Transgenic Mice ........................................ 61
Western Blotting for Transgenic PPARγ Protein .............................................. 61
Quantitative Real Time PCR (qRT-PCR) Analysis ............................................ 62
Mice and Diet ....................................................................................................... 63
Measurement of Body Composition ................................................................. 63
Measurement of Energy Expenditure ................................................................. 63
Fasting Glucose Measurements ........................................................................ 64
Glucose and Insulin Tolerance Tests ................................................................. 64
Plasma Measurements ....................................................................................... 65
Statistics .............................................................................................................. 65
Results ................................................................................................................ 65
Generation and Validation of NestinCre/PPARγ-P467L Mice ......................... 65
Overexpression of DN PPARγ in the Nervous System Alters Energy Balance ... 66
Discussion ........................................................................................................... 71
CHAPTER IV. THE ROLE OF PPARγ IN THE CONTROL OF ENERGY BALANCE BY THE MELANOCORTIN SYSTEM NEURONS

Introduction ......................................................................................................................... 90
Material and Methods ......................................................................................................... 92
Mice and Diet ......................................................................................................................... 92
Hypothalamic Immunohistochemistry ............................................................................... 93
Quantitative Real Time PCR (qRT-PCR) Analysis ............................................................. 94
Measurement of Body Composition .................................................................................... 95
Measurement of Energy Expenditure ................................................................................... 96
Fasting Glucose Measurements .......................................................................................... 96
Leptin Sensitivity ................................................................................................................ 96
Peripheral Injection of Rosiglitazone ................................................................................. 97
Statistics ............................................................................................................................. 97
Results .................................................................................................................................. 97
Generation and Validation of POMC\textsuperscript{Cre}/PPARγ-P467L and WT Mice .......... 97
Overexpression of PPARγ-WT in POMC Neurons Leads to Increased Sensitivity to Rosiglitazone-Induced Weight Gain ................................................................. 98
Overexpression of PPARγ-P467L Specifically in POMC Neurons Alters Energy Balance Only under Certain Dietary Conditions ................................................................. 99
Overexpression of PPARγ-WT Specifically in POMC Neurons Does Not Alter Energy Balance under Various Dietary Conditions ......................................................... 103
Overexpression of PPARγ-P467L Specifically in AgRP Neurons Does Not Alter Energy Balance under Various Dietary Conditions ......................................................... 104
Discussion .......................................................................................................................... 104

CHAPTER V. OVEREXPRESSION OF PPARγ IN THE BRAIN LEADS TO SEVERE MICROCEPHALY, GROWTH RETARDATION AND EARLY DEATH

Introduction ......................................................................................................................... 125
Materials and Methods ....................................................................................................... 128
Generation and Characterization of Conditional hPPARγ Transgenic Mice ................. 128
Determination of Cre-Mediated Recombination in Brains of Nestin\textsuperscript{Cre}/PPARγ-WT Double Transgenic Mice by PCR ......................................................... 128
Western Blotting for Transgenic PPARγ Protein ............................................................... 129
Quantitative Real Time PCR (qRT-PCR) Analysis .............................................. 130
Histological Analysis of Brain Tissue .................................................................. 130
Results .................................................................................................................... 131
Nestin\textsuperscript{Cre}/PPAR\textgamma-WT Mouse Model Validation Studies ....................... 131
Overexpression of PPAR\textgamma-WT in the Nervous System Leads to Significant Growth Retardation and Structural Brain Abnormalities ...................... 132
Discussion ............................................................................................................. 132

CHAPTER VI. GENERAL DISCUSSION............................................................... 141
Interference with PPAR\textgamma in the Brain: Lessons from Transgenic Mouse Models ..... 141
New Therapeutic Interventions Targeting PPAR\textgamma ............................................. 145

REFERENCES ....................................................................................................... 148
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Human PPARγ Isoforms</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic of PPARγ’s Mechanisms of Action</td>
</tr>
<tr>
<td>2.1</td>
<td>Inducible Transgene Expression System</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic Representation of the CAG-PPAR-WT (P467L)-T Transgene vector</td>
</tr>
<tr>
<td>2.3</td>
<td>The Red Reported TdTomato Is Expressed Only in the Presence of Cre-Recombinase, Demonstrating a Functional LoxP-STOP-LoxP Sequence</td>
</tr>
<tr>
<td>2.4</td>
<td>Expression of hPPARγ Protein</td>
</tr>
<tr>
<td>2.5</td>
<td>Functional Validation of CAG-hPPARγ-WT (P467L) Constructs</td>
</tr>
<tr>
<td>2.6</td>
<td>Transgene Induction in Liver</td>
</tr>
<tr>
<td>2.7</td>
<td>Transgene Induction in Skeletal Muscle</td>
</tr>
<tr>
<td>2.8</td>
<td>Transgene Induction in Skeletal Muscle</td>
</tr>
<tr>
<td>2.9</td>
<td>Transgene Induction in Brain</td>
</tr>
<tr>
<td>2.10</td>
<td>Transgene Expression in Brains of Nestin&lt;sup&gt;Cre&lt;/sup&gt;/PPAR-P467L Double Transgenic Mice</td>
</tr>
<tr>
<td>2.11</td>
<td>Transgene Induction in Brains of Nestin&lt;sup&gt;Cre&lt;/sup&gt;/PPAR-P467L Double Transgenic Mice</td>
</tr>
<tr>
<td>2.12</td>
<td>Global Overexpression of PPARγ-WT in the Brain Upregulates Target Genes</td>
</tr>
<tr>
<td>2.13</td>
<td>Transgene Expression in POMC&lt;sup&gt;Cre&lt;/sup&gt;/PPAR-P467L Double Transgenic Mice</td>
</tr>
<tr>
<td>3.1</td>
<td>Transgene Induction in Brains of Nestin&lt;sup&gt;Cre&lt;/sup&gt;/PPAR-P467L Double Transgenic Mice</td>
</tr>
<tr>
<td>3.2</td>
<td>Male Nestin&lt;sup&gt;Cre&lt;/sup&gt;/PPARγ-P467L Mice Resistant to Weight Gain in Response to 60% High Fat Diet (HFD) Treatment</td>
</tr>
<tr>
<td>3.3</td>
<td>Body Composition Characteristics of Male Nestin&lt;sup&gt;Cre&lt;/sup&gt;/PPARγ-P467L Mice after 15 Weeks of 60% (HFD) Treatment</td>
</tr>
</tbody>
</table>
Figure 3.4. Female Nestin^{Cre}/PPAR\textgamma-P467L Show No Difference in Body Composition after 15 Weeks of 60\% HFD Treatment ......................................................... 80

Figure 3.5. Organ Weights in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice after 15 Weeks of 60\% HFD Treatment ............................................................. 81

Figure 3.6. Adipose Depots Weights in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice after 15 Weeks of 60\% HFD Treatment ........................................... 82

Figure 3.7. Energy Expenditure in Response to HFD Treatment in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice ................................................................. 83

Figure 3.8. No Effect of 15 Weeks of 60\% HFD Treatment in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice ........................................................................ 84

Figure 3.9. No Difference in Plasma Leptin Levels in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice and Controls After 15 Weeks of HFD Treatment .... 85

Figure 3.10. Fasting Glucose Levels in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice and Controls After 15 Weeks of HFD Treatment ........................................ 86

Figure 3.11. Severe Fed State Hypoinsulinemia in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice after HFD Treatment ....................................................... 87

Figure 3.12. Impaired Glucose Handling in Nestin^{Cre}/PPAR\textgamma-P467L Male Mice after HFD Treatment .......................................................................... 88

Figure 3.13. Body Weight Response to Isocaloric 10\% fat Control Diet in Nestin^{Cre}/PPAR\textgamma-P467L Mice ................................................................. 89

Figure 4.1. RT-qPCR Analysis of Brain Punches from POMC^{Cre}/PPAR\textgamma-P467L and POMC^{Cre}/PPAR\textgamma-WT Mice ..................................................................... 109

Figure 4.2. RT-qPCR Analysis of Transgene Expression in Peripheral Tissues from POMC^{Cre}/PPAR\textgamma-P467L Mice ..................................................... 110

Figure 4.3. Peripheral Administration of Rosiglitazone Leads to Increased Body Weight in POMC^{Cre}/PPAR\textgamma-WT but not in POMC^{Cre}/PPAR\textgamma-P467L Mice .............................................. 111

Figure 4.4. Twenty-five Weeks of 60\% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in POMC^{Cre}/PPAR\textgamma-P467L Mice ........................................ 112

Figure 4.5. POMC^{Cre}/PPAR\textgamma-P467L Male Mice Show No Difference in Leptin Sensitivity after 25 Weeks of 60\% HFD Treatment ........................................ 113

Figure 4.6. Twenty-five Weeks of 60\% HFD Treatment Results in no Difference in Body Composition ................................................................. 114
Figure 4.7. Twenty-five Weeks of 60% HFD Treatment Results in no Difference in Fasting Glucose Levels ................................................................. 115

Figure 4.8. Twenty-five Weeks of 60% HFD Treatment Results in no Difference in Organ and Adipose Tissue Weights ................................................................. 116

Figure 4.9. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Treatment Leads to Increased Body Weight (BW) in POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Male but not Female Mice ................................................................. 117

Figure 4.10. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Treatment Leads to Increased Fat Mass in POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Male Mice .......... 118

Figure 4.11. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Results in No Difference in Body Composition of POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Female Mice..... 119

Figure 4.12. POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Male Mice Show No Difference in Leptin Sensitivity after 25 Weeks of 10% Fat Isocaloric-Match Control Diet ................. 120

Figure 4.13. No Difference in the Level of Expression of Energy Balance Regulating Genes in the Medial Ventral Hypothalamus of POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Males and Littermate Control Mice after 25 Weeks of 10% Fat Isocaloric-Match Control Diet .... 121

Figure 4.14. Interference with PPAR\(\gamma\) in POMC Neurons Does Not Lead to Changes in Energy Expenditure or Locomotor Activity ............................................ 122

Figure 4.15. Twenty-five Weeks of 60% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in POMC\(^{\text{Cre}}\)/PPAR\(\gamma\)-WT Mice ............................................. 123

Figure 4.16. Twenty-five Weeks of 60% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in AgRP\(^{\text{Cre}}\)/PPAR\(\gamma\)-P467L Mice ............................................. 124

Figure 5.1. Representative PCR Analysis for Recombination (Deletion) Event in Brains of Nestin\(^{\text{Cre}}\)/PPAR\(\gamma\)-WT Mice ................................................................. 135

Figure 5.2. Representative Western Blot Demonstrating Overexpression of Transgenic PPAR\(\gamma\)-WT Protein in the Brain of Nestin\(^{\text{Cre}}\)/PPAR\(\gamma\)-WT Mouse ......................... 136

Figure 5.3. Overexpression of PPAR\(\gamma\)-WT in the Brain Leads to Severe Postnatal Growth Restriction .......................................................................................... 137

Figure 5.4. Overexpression of PPAR\(\gamma\)-WT in the Brain Leads to Severe Microcephaly138

Figure 5.5. Overexpression of PPAR\(\gamma\)-WT in the Brain Leads to Agenesis of the Corpus Callosum ........................................................................................................ 139

Figure 5.6. Abnormal Brain Phenotype Following Overexpression of PPAR-WT Is Characterized by Different Levels of Severity .......................................................... 140
CHAPTER I.

INTRODUCTION

Once considered a problem in developed countries alone, obesity has now become a global pandemic\(^1\). In June 2013 the American Medical Association classified obesity as a disease\(^2\). This decision was aimed at enhancing efforts to minimize the incidence of obesity and work toward treatments. The number of overweight adults in the world has risen from 857 million in 1980 to 2.1 billion in 2013\(^3\). As of 2010 the consequences of this rise have been cited as 3.4 million deaths, 4% years of life lost, and 4% of disability-adjusted life-years worldwide\(^4\).

Obesity does not affect only adults as its newest victims, especially in developed countries, are children and adolescents\(^3\). The National Health and Nutrition Examination Survey in the US estimated that 17% of 6-19 year olds were obese in 2010\(^5\). In this population obesity has a very serious negative impact on both psychological and physical health. Negative body image is frequently experienced by obese adolescents and leads to low self-esteem and depression\(^6,7\). Excess weight during childhood is a predisposing factor for increased risk of death in adulthood\(^8\). The seriousness of the now obesity pandemic was starkly stated by the former Surgeon General Richard Carmona: “Because of the increasing rates of obesity, unhealthy eating habits and physical inactivity, we may see the first generation that will be less healthy and have a shorter life expectancy than their parents.”\(^9\)

“Energy balance” is defined by the laws of thermodynamics as the relationship of “energy or calories in” versus “energy or calories out”. This relationship determines whether weight is gained, lost or stays the same. When the input of calories exceeds those burned, the end result is accumulation of excess body fat. Obesity becomes a medical
problem when health conditions such as hypertension, insulin resistance, and dyslipidemia begin to develop as well. These together are referred to as metabolic syndrome or syndrome X\textsuperscript{10}. Once diagnosed with metabolic syndrome patients are at a significantly (5 fold) increased risk of development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (2-fold) as early as 5 years after diagnosis\textsuperscript{10}. There is an additional three fold increased risk of stroke as well as myocardial infarction\textsuperscript{11}, compared to healthy individuals, even in the absence of history of cardiac problems\textsuperscript{12}. In children and adolescents obesity leads to similar health problems which previously were only observed in adults. Today obese pediatric patients present with high blood pressure\textsuperscript{13}, type 2 diabetes\textsuperscript{14} and increased blood cholesterol\textsuperscript{15}.

The prevalence of obesity and the ensuing metabolic syndrome cast a grim prognosis on the health of our society as the longevity of the next generation is now in jeopardy. In their review Evans and colleagues\textsuperscript{16} eloquently explore the point that the development of obesity is indeed a “complex” process. This complexity stems from the fact that adipose tissue is not simply a “storage depot” for excess calories\textsuperscript{16}. Fat is an organ that exists in an intricate signaling equilibrium with many tissues in the body such as the liver, skeletal muscle as well as the energy homeostasis regions in the brain\textsuperscript{16}. Herein is found the major reason that despite improved understanding of how this equilibrium is maintained, there is still no effective therapy against the development of obesity or preventing the associated medical sequelae. Peroxisome proliferator activated receptor gamma (PPARγ), a “master regulator of adipocyte function”\textsuperscript{17, 18} whose synthetic ligands are used as potent insulin sensitizers in the treatment of T2DM, has been implicated as a key modulator of the mechanisms involved in the development of obesity. Interestingly, it was recently discovered that PPARγ modulates susceptibility to high fat diet through its actions in the brain. This dissertation work addresses the role of brain PPARγ in the control of energy balance. The introductory chapter provides an overview of the existing literature on the physiological role of PPARγ. Chapter 2 will detail the generation and validation of
the transgenic mouse models used in experiments described in subsequent chapters. In the third chapter I will analyze and discuss results from my research to address the role of global brain PPARγ function in regulation of energy balance. Chapter 4 will examine the role of neuron-specific interference with PPARγ and its effects on energy homeostasis. Chapter 5 will include an analysis and discussion of data from my research into the role of PPARγ in brain development. In the final chapter I will present a general discussion and perspectives on the future of the field investigating how brain PPARγ affects energy balance as well as its role in development.

**The Nuclear Receptor Superfamily**

The nuclear hormone receptor proteins are ligand-activated transcription factors that once bound to specific DNA sequences control the expression of genes involved in growth, development, metabolism, reproduction, and inflammation\(^1^9, 20\). There are 48 known nuclear receptors in the human genome\(^2^1\) which can be subdivided into three general classes. The members of the first class are ligand-activated and include the steroid hormone receptors. Estrogen and glucocorticoid receptors are some examples. Under basal conditions these proteins reside in the cytoplasm, but become activated upon ligand binding. The activated receptors then translocate to the nucleus where they induce the expression of different genes. The second class includes receptors whose endogenous ligands remain unidentified or may simply not be required\(^2^2\). These proteins are known as orphan receptors. The final group encompasses receptors that form complexes with the retinoid X receptor (RXR). Members of this group include the liver X receptors (LXR), retinoic acid receptors (RARs) and the peroxisome proliferator activated receptors (PPARs). PPARs in particular have emerged as important regulators of metabolic and inflammatory signaling specifically in metabolic disease and immunity\(^2^3\). This group of nuclear receptor proteins are present in the nucleus in the form of a complex with nuclear corepressors and histone deacetylases (HDACs). The corepressors serve to inhibit PPARs
by maintaining their inactive state; the HDACs keep the chromatin in a condensed state, effectively preventing gene expression (reviewed in Sundararajan et al, 2006)\textsuperscript{24}.

**PPARs: Isotypes, Structure and Mechanism of Action**

**Isotypes**

The PPAR family consists of three isotypes: PPAR\(\alpha\) (NR1C1), PPAR\(\delta\) or \(\beta\) (NR1C2) and PPAR\(\gamma\) (NR1C3), which will be reviewed in greater detail below. These transcription factors were called peroxisome proliferator activated receptors due to the fact that the first discovered member, now known as PPAR\(\alpha\), was able to cause increased formation of peroxisomes in rodent liver\textsuperscript{25}. The other PPAR group members do not possess this ability and have instead been established as critical regulators of lipid and glucose metabolism\textsuperscript{26}.

**Structure**

PPARs, similarly to other nuclear receptors consist of four domains (reviewed in Faige et al., 2006\textsuperscript{27}). The A/B domain is located at the N-terminus and is an important mediator of PPAR subtype-specificity. It contains a weak ligand-independent transactivation sequence of variable size called AF-1. This region is recognized by co-activations and/or other transcription factors. The A/B domain is followed by the highly conserved C domain also referred to as DNA binding domain (DBD), which interacts with the DNA via a two zinc-finger motif\textsuperscript{27}. Next is the hinged D domain followed by the E/F domain also known as the ligand binding domain (LBD)\textsuperscript{27}. The LBD consists of 12 \(\alpha\) helices and 4 \(\beta\) sheets that form a hydrophobic pocket where ligands bind\textsuperscript{27}. This portion of the LBD is referred to as AF-2 and allows for a ligand-dependent transactivation function of the receptors\textsuperscript{28}. The E/F domain is mainly where interaction with cofactors occurs\textsuperscript{27}.
Mechanism of Action

PPARs form obligate heterodimers with RXR and bind to specific DNA sequence elements called PPAR response elements or PPREs where they regulate gene expression.\textsuperscript{29, 30} The PPRE consensus sequence is an imperfect direct repeat of the hexamer AGGTCA separated by one (DR1) or two nucleotides (DR2). All RXR isotypes (\(\alpha\), \(\beta\), \(\gamma\)) can dimerize with PPARs but it appears that the specificity of association with each PPAR isotype dictates the recognition of target promoters.\textsuperscript{31}

Feige and colleagues have proposed a “genome-scanning model”\textsuperscript{27} of PPAR-mediated activation. In basal conditions the PPARs form constitutive heterodimers with RXRs and translocate to the nucleus. There other proteins, including corepressors, histone deacetylases, and various corepressor exchange factors bind to the PPAR-RXR complex.\textsuperscript{27} These multiplexes appear to transiently interact with the chromatin and “scan” the genome until they encounter a PPRE where they are temporarily stabilized.\textsuperscript{27} Binding of a repressive complex to the PPRE leads to suppression of gene expression, while an activating complex may induce a basal transcription of gene targets. The presence of a ligand changes the confirmation of the PPAR-RXR heterodimer which results in the release of corepressors and binding of coactivators. These new complexes move along the genome rapidly and stochastically, scanning for PPREs. When they reach a consensus sequence, the underlying chromatin undergoes remodeling, including acetylation, which in turn leads to induction of target gene expression.\textsuperscript{27}

Post-translational Modification

PPARs transcriptional activity is modulated by multiple mechanisms including phosphorylation, sumoylation, and ubiquitination of PPARs themselves or their interactors such as RXRs and cofactors.\textsuperscript{33} This characteristic could be successfully manipulated for therapeutic purposes. To this extent the Spiegelman laboratory has demonstrated that in a state of obesity adipose PPAR\(\gamma\) is phosphorylated at serine 273 by the protein kinase Cdk5,
which is also activated by high-fat feeding\textsuperscript{34}. Choi and colleagues\textsuperscript{34} show that this modification interferes with PPARγ’s ability to induce the expression of a number of genes involved in insulin signaling. Furthermore, the authors demonstrate that phosphorylation of serine 273 is successfully blocked by PPARγ ligands such as rosiglitazone, a fact that correlates strongly with the anti-diabetic effects of this medication\textsuperscript{34}. In an attempt to elucidate the mechanism of Cdk5-mediated phosphorylation of PPARγ, the Spiegelman laboratory genetically deleted Cdk5 from adipose tissue of mice\textsuperscript{35}. Surprisingly, the resulting animals had not only increased phosphorylation at serine 273 but also exhibited augmented insulin resistance\textsuperscript{35}. Banks and colleagues show that the observed effect is due to robust activation of the extracellular signal–regulated kinase (ERK). It turns out that Cdk5 suppresses ERK by acting on a novel site in MAP kinase/ERK kinase pathway or MEK\textsuperscript{35}. The authors show that antagonist-mediated inhibition of either ERK or MEK reverses insulin resistance in obese mice\textsuperscript{35}. This most recent evidence suggests that posttranslational modification by ERK/Cdk5 is a critical regulator of PPARγ function and that pharmacological targeting of these modulators could be a potential treatment for type 2 diabetes\textsuperscript{35}.

\textit{The Emerging Concept of a PPAR Triad}

The three PPAR isoforms seem to form a strong interconnection or a functional triad particularly in the brain (reviewed in Aleshin and Reiser, 2013)\textsuperscript{36}. This was first observed in C6 glioma cell line by Leisewitz and colleagues\textsuperscript{37} where overexpression of PPARγ upregulated PPARβ/δ levels with the PPARγ agonist Rosiglitazone strongly potentiating the effect. In turn, overexpression of PPARβ/δ strongly upregulated PPARγ, and to a lesser extent PPARα\textsuperscript{37}. In a primary rat astrocytes study, Alesin et al., 2009\textsuperscript{38} similarly showed that PPARγ exerts a positive influence on PPARβ/δ expression level and activity. The study also demonstrated that in this context PPARα has a negative influence. The authors suggest the formation of a positive-feedback loop by the PPAR-β/δ-dependent
increase in PPARγ expression level. This is in turn counterbalanced by the inhibitory effect of PPARα activation on PPARβ/δ expression level\textsuperscript{38}.

**PPARα**

The human *PPARA* gene is located on the long arm of chromosome 22 at position 22q12-q13.1\textsuperscript{39}; in the mouse this gene is mapped to chromosome 15\textsuperscript{40}. PPARα is characteristically expressed in the liver, brown fat and heart where it plays a role in regulation of β-oxidation of fatty acids particularly in organelles such as mitochondria and peroxisomes\textsuperscript{41,42}. During a prolonged or overnight fast adipose tissue undergoes lipolysis and fatty acids are released. When these reach the liver they cause a strong upregulation of PPARα expression\textsuperscript{43}. PPARα activation in turn promotes liver fatty acid oxidation resulting in improvement of plasma lipid profiles. In rodent models agonist-mediated activation of PPARα has been shown to lower plasma triglycerides, decrease fat mass, including steatosis of liver and skeletal muscle, with the ultimate result of an overall improvement in insulin sensitivity\textsuperscript{44,45}. Synthetic agonists of PPARα are the fibrate class of hypolipidemic drugs which are used to treat dyslipidemia in humans\textsuperscript{46}. PPARα is activated by a number of natural ligands. Examples include polyunsaturated fatty acids such a linoleic acid, leukotriene derivatives and very-low-density lipoproteins (VLDLs) that are in the presence of the activated enzyme lipoprotein lipase (reviewed in Castrillo and Tontonoz, 2004\textsuperscript{23}).

PPARα activation has been demonstrated to play a role in regulating behavior by mediating oleylethanolamide-induced satiety and weight stabilization\textsuperscript{47}. It is believed that the mechanism involves increased production and release of gut NO which has been shown to have an orexigenic function\textsuperscript{47}. This is a rather unexpected role for a nuclear receptor to regulate behavior but it raises the possibility for the potential role of PPARα in treatment of eating disorders.
PPARα is also found in the CNS with the following regions showing the highest level of expression: basal ganglia, some thalamic, mesencephalic and cranial motor nuclei, and the reticular formation\(^48\). In the brain PPARα plays a role in lipid metabolism\(^49,50\), control of reactive oxygen species metabolism\(^51,52\), involvement in neuronal cell proliferation, differentiation and apoptosis\(^53,54\), as well as modulation of fasting and satiety responses through the regulation of the hypothalamo-pituitary axis and peripheral glucose homeostasis\(^55\). Finally, activation of PPARα in the hypothalamus corrects hypophagia in a murine model of disrupted fatty acid metabolism\(^47,55\) supporting an important role of this protein in energy homeostasis regulation.

**PPARδ**

The human *PPARD* gene has been mapped to the short arm of chromosome 6 at position 6p21.1-p21.2\(^56\); in the mouse this gene is located in chromosome 17\(^40\). PPARδ is also known as PPARβ and was identified in *Xenopus laevis* by Wahli and colleagues\(^57\). PPARδ is most ubiquitously expressed\(^58\), which suggests a critical role for PPARδ signaling in many tissues. Consistent with this, global PPARδ knockout mice are characterized by the presence of placental defects with subsequent increased embryonic lethality\(^59\), myelination defects\(^60\), decreased formation of adipose tissue\(^60\) and abnormal wound healing as well as induction of skin inflammatory responses\(^61\). In the periphery PPARδ regulates processes such as fatty-acid metabolism, mitochondrial respiration\(^62\), and reprogramming of muscle fibers\(^63\), with effects leading to improved metabolic profiles and overall resistance to obesity.

Similarly to the other PPARs, PPARδ’s endogenous ligands include unsaturated and saturated fatty acids, carbaprostacyclin and VLDLs (reviewed in Besinger and Tontonoz, 2008\(^64\)). The potent and selective synthetic agonist GW501516 was initially developed by GlaxoSmithKline for the treatment of dyslipidemia. This compound has captured popular interest\(^65\) due to studies by Ronald Evens\(^63,66\) showing that GW501516-
mediated activation of PPARδ enhances endurance, improves metabolic profiles and protects against obesity even in sedentary mice. Despite its great promise as a metabolism modulator, the drug was found to cause cancer in rats and phase II trials were subsequently suspended.

In the CNS PPARδ is the most highly expressed PPAR isoform. It is highly enriched in the mediobasal hypothalamus, among other regions, further suggesting a role in regulation of energy homeostasis. To explore this, the Niswender laboratory deleted neuronal PPARδ using a conditional allele of PPARδ and a Nestin-Cre driver line. These animals are characterized by increased fat mass and lower lean mass on low-fat diet despite the fact that their body weight is lower compared to fl/fl controls. When placed on high fat diet PPARδ knockout mice show increased susceptibility for diet-induced obesity due to profound accumulation of fat mass. Interestingly, the authors demonstrate that brain deletion of PPARδ leads to increased PPARγ and PPARα expression in the hypothalamus. This finding provides an additional evidence for the functional connection between the three PPAR isoforms discussed in the Emerging Concept of a PPAR Triad section above. PPARγ is the focus of this dissertation work and a detailed description of its structure and function follows.

**PPARγ**

*Isoforms: Genomic Characterization, Tissue Distribution, and Target Genes*

The human PPARG gene is located on chromosome 3 at position 3p25, while in mouse the gene has been assigned to position E3-F1 on chromosome 6. Interestingly, in humans PPARG is within 1.5 million bases of D3S1263, a polymorphic marker for lipid-metabolism related diseases such as obesity, lipoatrophy, insulin resistance and diabetes.

The mouse and human PPARγ genes are composed of 9 exons and extend over more than 100 kb of genomic DNA. They are highly conserved (95% identical and 99% similar) between the two species. PPARG is transcribed to three mRNAs: PPARγ1,
PPARγ2, and PPARγ3, as a consequence of alternate use of three promoters and differential splicing of three 5′ exons, designated as A1, A2, and B1 (Figure 1.1). PPARγ1 is encoded by eight exons, of which A1 and A2 are γ1-specific, leading to the formation of 477 amino acids translated product. PPARγ2 is encoded by 7 exons with exon B encoding additional N-terminal amino acids, specific of γ2 and resulting in 505 amino acids protein. A third mRNA, PPARγ3, is controlled by a promoter located between exons A1 and A2 and encodes the same protein as PPARγ1. Interestingly, in the human gene the AUG resulting from splicing of exons A2 and 1 is six nucleotides upstream compared to the rodent AUG. Thus, the human PPARγ1 protein is two amino acids longer at its N terminus than the rodent PPARγ1.

During rodent development, similarly to PPARα, PPARγ mRNA expression is first detected on E13.5 but is restricted mainly to the hindbrain. This level of expression is also transient and by E15.5 it is greatly reduced. Interestingly no other tissues show PPARγ expression at this stage. At E18.5 PPARγ mRNA level is increased again but this time is detected only in brown adipose tissue. In the adult rodent brain PPARγ was detected at very low levels in the hippocampus, cerebellum as well as the retina. In addition, both PPARγ mRNA and protein are detected in the hypothalamus, which is consistent with the recently described role of brain PPARγ in control of energy homeostasis and metabolism. It appears that neurons account for the majority of PPARγ expression in the CNS. A detailed discussion on the existing evidence regarding the role of brain PPARγ in modulating energy balance will be presented in a later section.

In the periphery PPARγ1 is ubiquitously expressed but at low levels. PPARγ2, on the other hand, is characterized by high levels of expression limited primarily to adipose tissue (both white and brown). Other tissues in which expression of PPARγ was detected include: kidney, liver, intestine, placenta, differentiated macrophages and others. In addition, PPARγ expression has been demonstrated in adult rodent brain at low levels predominantly in the hippocampus, cerebellum and the retina.
**Ligands and Mechanism of Transcriptional Regulation by PPARγ**

Similarly to other members of the nuclear receptor family, PPARγ functions as a ligand-activated transcription factor\(^2\). There are multiple natural ligands that can bind PPARγ. Some examples include unsaturated fatty acids and oxidized LDLs metabolites\(^3\). The first endogenous, and perhaps the most potent, PPARγ ligand discovered is 15-deoxy-delta (12,14)-prostaglandinJ2 (15d-PGJ2)\(^4\), a derivative of arachidonic acid. In general PPARγ tends to bind with low affinity to its endogenous ligands, hence the biological relevance in terms of PPARγ activation of some ligands has not been determined\(^5\). In contrast, PPARγ is potently activated by synthetic ligands such as the thiazolidinediones (TZDs), which have very strong insulin-sensitizing properties\(^6\).

PPARγ forms an obligate heterodimer with RXR and as a complex interacts with PPAR response elements (PPREs) in the promoter regions of a target gene (reviewed in Powell et al., 2007\(^7\)). When no ligand is available, the PPARγ-RXR heterodimer recruits multiple cofactors. These include corepressors such as the nuclear receptor corepressor (N-COR) and the silencing mediator of retinoic acid and thyroid hormone (SMRT). Histone deacetylases (HDACs) are also recruited to the PPARγ-RXR multiplex and together with corepressors function as transcriptional inhibitors (Figure 1.2, A). Binding of a ligand to the C-terminal activation function or AF-2 domain of PPARγ induces a change in the confirmation of the PPARγ-RXR heterodimer leading to release of the corepressors from the complex\(^8\). Ligand binding also increases interaction forces between coactivators and PPARγ. This in turn leads to “opening” of condensed chromatin and recruitment of other transcriptional proteins to initiate transactivation of PPARγ target genes\(^9\) (Figure 1.2, B). Coactivators of the nuclear receptor family of proteins also regulate PPARγ activity and include: CREB-binding protein (CBP)/p300, the steroid receptor coactivator-1 (SRC-1), PPARγ coactivator-1α (PGC-1α)\(^7\).

PPARγ target genes are not regulated uniformly, such that some promoters may be actively repressed while others induced. For example, aP2 and GyK both involve binding
of PPARγ/RXR to PPREs in their promoter regions. However, in the case of GyK heterodimer binding is associated with recruitment of corepressors, whereas coactivators are recruited to aP2. Stimulation with TZD leads to marked upregulation of GyK through the induction of PGC-1α, which in turn facilitates the exchange of corepressors with coactivators at the PPRE \(^90\).

PPARγ can also inhibit gene expression in a process known as transrepression. This type of transcriptional regulation is common in inflammatory signaling cascades. The presence of a ligand is a requirement for this process, which uniquely does not depend on the binding of PPARγ-RXR complexes to target PPREs. Christopher Glass and colleagues have proposed a model in which PPARγ-mediated transrepression involves inhibition of inflammatory gene expression by interfering with the release of the corepressor complex\(^91\). The mechanisms elucidated so far suggest that ligand-dependent activation of PPARγ leads to SUMOylation of the receptor. The SUMOylated PPARγ in turn binds to N-COR/HDAC-containing corepressor complex on the target inflammatory gene (examples include inducible nitric oxide synthase (iNOS) and the chemokine CC-chemokine ligand 2 (CCL2) among others) and prevents its [corepressor complex] degradation via proteosomal mechanism and in turn preserving the repressed state\(^91\) (Figure 1.2, C).

**Role of PPARγ in Peripheral Control of Metabolism: Adipogenesis, Lipid Storage, and Insulin Sensitivity**

*Regulation of Adipogenesis and Lipid Metabolism*

PPARγ’s role as a “master regulator” of adipocyte differentiation\(^17, 18, 23, 26, 92-94\) has been firmly established over the past two decades. *In vitro* studies demonstrated that ectopic expression of PPARγ in non-adipogenic embryonic fibroblasts\(^18\), 3T3-L1 preadipocytes\(^95\), and determined myoblasts\(^96\), stimulates the adipocyte gene transcription program and drives adipogenesis. The critical role of PPARγ in adipocyte differentiation is underscored by the fact that no single other factor has yet been identified to drive this
process in the absence of PPARγ\textsuperscript{97}. Elegant studies from the Mortensen as well as the Evans laboratories provided the \textit{in vitro} and \textit{in vivo} confirmation of PPARγ’s obligatory role in fat formation. Rosen and colleagues\textsuperscript{93} demonstrated that embryonic stem cells derived from PPARγ-null mice failed to differentiate into adipocytes in chimeric mice, but were able to contribute to the development of a variety of other cell types. Barak and colleagues\textsuperscript{98} were able to rescue embryonic lethality of global PPARγ deletion in mice by using homologous recombination. In this model the resultant single PPARγ-null mouse was characterized by a complete absence of all types of adipose tissue formation, consistent with other models of lipoatrophy. In addition, this mouse had marked hepatic steatosis, while the mutant placenta was consistently devoid of lipid accumulation\textsuperscript{98}. To circumvent the problem of embryonic lethality in PPARγ- null mice, the Mortensen group used a Mox2Cre-recombinase to preserve PPARγ expression in the extraembryonic material\textsuperscript{99}. Once again, PPARγ inactivation led to severe lipodystrophy and in addition, impaired insulin sensitivity, glucose tolerance and marked dyslipidemia\textsuperscript{99}.

PPARγ’s pivotal role in adipogenesis extends also to the maintenance of the fully differentiated state of adipocytes both \textit{in vivo} and \textit{in vitro}\textsuperscript{100,101}. Imai and colleagues used aP2 promoter-driven tamoxifen-induced Cre-ER\textsuperscript{T2} recombination system to delete PPARγ from mature mouse adipocytes. The group was able to show that mature PPARγ knockout white and brown adipocytes die within 2-3 weeks post PPARγ ablation and are replaced by new PPARγ-positive adipocytes\textsuperscript{100}. Consistent with these findings Schupp et al.\textsuperscript{101} showed that knockdown of PPARγ in differentiated 3T3-L1 adipocytes led to reversal of most adipocyte gene expression to preadipocyte levels. The genes that were down-regulated characteristically play a role in lipid and glucose metabolisms, two core properties of adipocytes, and showed strong enrichment of PPARγ binding sites\textsuperscript{101}.

During the processes of adipocyte differentiation, PPARγ is at the center of the adipogenic signaling cascade by forming a positive feedback loop with a member of the CAAT/enhancer binding protein (C/EBP) family - C/EBP-α (reviewed in Tontonoz and
Spiegelman, 200826). Other members of this family, C/EBP-β and –δ act upstream and induce PPARγ by binding to its promoter. Ligand-activated PPARγ will then act to induce many target genes, including the expression of C/EBP-α. This is the basis for the positive regulatory cycle, in which C/EBP-α binds to the PPARγ promoter to ensure PPARγ’s [and thus its own] stable expression26.

Integral to its role in promoting terminal differentiation of adipocytes, PPARγ induces the transcription of a variety of genes important for triglyceride uptake and storage, fatty acid oxidation, transport or fatty acid synthesis. Examples of such target genes include: adipose-specific fatty acid binding protein (FABP4 also known as aP2)102, lipoprotein lipase (LPL)103, phosphoenolpyruvate carboxykinase (PEPCK)104,105, the fatty acid transporter CD36106, as well as the adipose-specific glycerol channel AQP4107.

**PPARγ and TZDs: Mechanisms of Insulin Sensitization**

The fact that the synthetic thiazolidinedione (TZD)108 class of anti-diabetic medications is a potent activator of PPARγ rendered this receptor of critical importance for clinical medicine. The TZD class includes the following members109: Ciglitazone, the prototypical TZD which was never in clinical use, Rosiglitazone (Avandia), Pioglitazone (Actos), and Troglitazone (Rezulin). Compared to other hypoglycemic medications, TZDs show superior improvement of glycemic control and unparalleled ability to prevent type 2 diabetes mellitus (T2DM)110, 111. However, reports of severe side effects such as myocardial infarction, increased risk of congestive heart failure, bladder cancer and all-cause mortality112-114, have led to tightly restricted clinical use in the United States and a recommendation for a market withdrawal in Europe115. Troglitazone has been withdrawn from the US market since 2000 due to associated hepatotoxicity116. Germany and France have suspended the use of Pioglitazone since 2011 due to serious risk of bladder cancer.117

The level of activation of PPARγ by different TZDs correlates strongly with the insulin sensitizing effects of these agonists118, implicating activation of PPARγ-response
genes as the means of action of TZDs. Consistent with that, mutations in PPARγ, described in the section below, are associated with the development of early and severe insulin resistance.

The prevalent theory of the mechanism by which PPARγ activation leads to lower blood glucose and increased sensitivity to insulin is referred to as the “lipid steal” hypothesis. Increased plasma free fatty acids (FFAs), characteristic of obesity, leads to inappropriate deposition of lipids in liver and muscle where they inhibit insulin-stimulated glucose uptake, causing a state of generalized peripheral insulin resistance\(^{119}\). In patients the resulting hyperglycemia, in combination with insulin resistance, eventually results in a diagnosis of type 2 diabetes mellitus. The “lipid steal” hypothesis postulates that TZD-mediated activation of PPARγ gene response network enhances the ability of adipose depots to store excess lipids away from insulin-sensitive sensitive organs such as muscle and liver\(^{120,121}\). For example, upregulation of the PPARγ target gene PEPCK, leads to inhibition of fatty acid release from adipocytes and ultimately to a lower level of FFAs in plasma\(^{103}\). Consistent with that, animal models\(^{122}\) as well as clinical trials\(^{123}\) have confirmed the ability of TZDs to lower plasma FFAs.

Several genetic mouse models, with tissue specific deletion of PPARγ, provide insights into the role of different metabolic organs as sites of TZD-mediated insulin sensitization. He and colleagues\(^{124}\) used an aP2-promoter driven Cre-loxP system to effectively delete PPARγ from mature adipocytes. Metabolic assessment of these animals revealed marked hyperlipidemia with an increase in the plasma FFAs and triglyceride levels, which resulted in development of hepatic steatosis. When challenged with a high fat diet, the mice were characterized by the presence of adipose and hepatic insulin resistance\(^{124}\). Consistently, TZD treatment failed to decrease plasma FFAs in the adipocyte-specific PPARγ knockout mice, suggesting that PPARγ function in intact adipose tissue is critical for the TZD-mediated regulation of plasma FFAs. Zhang and colleagues\(^{125}\) used a different approach to disrupt the development of adipose tissue in mice. They selectively altered
the PPAR\(_\gamma2\) isoform by using red fluorescence reporter and neomycin sequence to replace exon B. The resulting PPAR\(_\gamma2\) knockout mice were characterized by a significant decrease in white adipose mass, while the brown fat remained similar to controls. Metabolic analysis revealed that these mice exhibited severely impaired insulin sensitivity, which correlated with reduced level of insulin receptor expression in several tissues including: skeletal muscle, white fat, and liver\(^{125}\). The circulating triglycerides and cholesterol levels were surprisingly not elevated and there was absence of lipid accumulation in nonadipose tissue such as the liver. Treatment of these mice with pioglitazone normalized the insulin resistance, suggesting that in this model PPAR\(_\gamma2\) is not required for the TZD-mediated effect. Most recently the Lazar laboratory\(^{126}\), arguing for a more specific approach, utilized adiponectin promoter driven Cre-recombinase (Adipoq-Cre) to ablate PPAR\(_\gamma\) in fat. Compared to the other models described, adiponectin-driven deletion of PPAR\(_\gamma\) led to complete lipoatrophy with marked hepatosteatosis with profound insulin resistance and hyperglycemia in these mice\(^{126}\). Once again these findings stress the in vivo requirement for adipose tissue PPAR\(_\gamma\) for the maintenance of systemic metabolic homeostasis.

The Olefsky and Kahn laboratories independently generated mouse models with skeletal muscle-specific deletion of PPAR\(_\gamma\). Both resulting models were characterized by the development of systemic insulin resistance. Hevener and colleagues\(^{127}\), however, reported that TZD treatment failed to normalize the insulin-stimulated glucose disposal rate, which prompted the conclusion that deletion of PPAR\(_\gamma\) leads to skeletal muscle insulin resistance resulting in impaired insulin sensitivity in both adipose tissue and liver. The muscle-specific PPAR\(_\gamma\) knockout generated by Norris and colleagues\(^{128}\) had normal insulin-stimulated glucose disposal rate in the muscle, however, the hepatic insulin sensitivity was impaired. In this model TZD treatment ameliorated the observed insulin action impairments to control levels. The authors also noted that these mice had excess adiposity, which could perhaps explain the effectiveness of the TZD treatment in this but not in Hevener’s model.
Matsusue and colleagues\textsuperscript{129} ablated PPAR\(\gamma\) in the liver of leptin-deficient (\(ob/ob\)) mice. Interestingly, these animals exhibited a marked improvement in hepatic steatosis. This, however, was accompanied by an exacerbation in insulin resistance in both adipose tissue and muscle\textsuperscript{129}, underscoring the critical role of hepatic PPAR\(\gamma\) in maintenance of peripheral insulin sensitivity and glucose balance.

Other mechanisms (reviewed in Tontonoz and Spiegelman\textsuperscript{26}) that should be considered in the PPAR\(\gamma\)-induced insulin sensitivity include the stimulation of adipose tissue production and release of signaling molecules such as adiponectin and leptin. These have been shown to have insulin-sensitizing effects\textsuperscript{130, 131}. In addition, PPAR\(\gamma\) activation leads to the decreased secretion of resistin, as well as pro-inflammatory molecules such as TNF\(\alpha\), both of which are associated with insulin resistance\textsuperscript{132, 133}.

**PPAR\(\gamma\) Polymorphisms**

The importance of PPAR\(\gamma\) in regulation of systemic metabolism is evidenced by the clinical abnormalities (insulin-resistance\textsuperscript{134}, metabolic syndrome\textsuperscript{135}, hypertension\textsuperscript{136}, lipodystrophy\textsuperscript{136-138}, dislipidemia\textsuperscript{120}) that occur in patients carrying mutations in the \textit{PPARG} gene. Currently Pro12Ala is identified as the most common in addition to 19 rare missense and nonsense mutations in the coding regions of \textit{PPARG} have been identified and their function analyzed (reviewed in Jeninga et al., 2008\textsuperscript{139}). To date the \textit{PPARG2} Pro12Ala polymorphism (rs1801282) is the most common genetic variant of PPAR\(\gamma\) and thus has been studied the most. Depending on ethnicity the frequency of occurrence is between 2 and 25\%\textsuperscript{83}. The C to G substitution results in a Proline (Pro) to Alanine (Ala) variant and affects only the unique PPAR\(\gamma\)2 amino-terminal. The mutant protein is characterized by reduced DNA-binding affinity and impaired transcriptional activity\textsuperscript{140, 141}. There is a controversy in the literature regarding the physiological effects of the Pro12Ala mutation. In some studies, carriers of the 12Ala allele have been described to have lower body mass index (BMI) and improved insulin sensitivity compared to Pro/Pro carriers\textsuperscript{140, 141}. 

A Pro12Ala knock-in mouse model showed that the effects of this genetic variant are strongly dependent on metabolic context and involve changes in cofactor recruitment and target gene signaling. On regular chow diet mice carrying two Ala alleles are characterized by improved glycemic and lipid plasma profiles, as well as increased longevity, compared to mice homozygous for the Pro allele. High fat diet feeding, however, completely abolishes the beneficial metabolic effects of the Ala allele. In other studies, the Pro12Ala mutation has been associated with increased BMI, obesity, type II diabetes mellitus, breast cancer susceptibility, as well as, cognitive impairment and neurodegenerative disease risk. Very recently it was reported that PPARG Pro12Ala is associated with lower cognitive, language and motor development outcomes in the offspring of mothers who are carriers of the mutation. The authors speculate that Pro12Ala may lead to interference with proper nutrient transfer through the placenta thus having negative consequences on fetal brain development.

The critical role of PPARγ in adipose tissue development is underscored by the fact that the majority of the rare, heterozygous, loss-of-function mutations lead to lipodystrophic phenotype in patients. To date 19 such mutations, characterized by autosomal dominant mode of inheritance, have been reported to occur either in the DBD or the LBD of PPARγ. Based on functional analysis PPARγ polymorphisms can be divided into two broad categories: mutations leading to decreased or abolished DNA binding and dominant-negative acting mutations which are also characterized by altered cofactor interaction. Missense mutations in the highly conserved DBD zinc fingers (C142R, C159Y, and C190W) interfere with the DNA-binding capacity of the domain as shown by in vitro studies. Premature stop frameshift mutation (F388L also referred to as FSX), leads to deletion of part of the DBD and ultimately complete loss of DNA binding capacity. Examples of dominant-negative (DN) acting mutations include V318M and P495L which are located in the LBD of PPARγ. These mutations are characterized by impaired transactivation and ability to inhibit wild-type PPARγ activity (Figure 1.2, D).
Data from our laboratory and others demonstrate that DN PPARγ induces repression of experimentally validated PPARγ target genes, which show increased expression in response to PPARγ agonists\(^\text{154-156}\). In addition, it has been shown that the P495L mutant exhibits a reduced rate of cycling at PPREs compared to a wild-type protein, leading over time to a greater occupancy of target promoters by mutant receptors, an effect that is overcome at high ligand concentrations\(^\text{153}\). A mouse knock-in model of the P495L mutation was generated by the Maeda group. Interestingly, despite the fact that these animals present with lipodystrophy, their glucose handling and insulin sensitivity are intact even in the presence of high fat challenge\(^\text{157}\). The insulin resistance phenotype of humans with the mutation (see below) was recapitulated in the mice only when the mutation was expressed on a hyperphagic/leptin-deficient background\(^\text{158}\).

Patients carrying mutations in PPARγ DBD and LBD are diagnosed with a familial partial lipodystrophy type 3 (FPLD3)\(^\text{134-137, 152}\), which is characterized by a decreased subcutaneous fat deposition particularly in the extremities and the gluteal region with sparing of the abdominal and visceral regions. The fat depot redistribution is accompanied by clinical findings typical of serious metabolic complications, which include insulin resistance; early onset of type 2 diabetes mellitus; hypertriglyceridemia and low levels of high density lipoprotein or HDL, collectively referred to as dislipidemia; severe hypertension; fatty liver; and affected females also present with polycystic ovarian syndrome.

\textit{Role of PPARγ in Central Nervous System (CNS) Diseases}

\textit{Role of PPARγ Activation in CNS Inflammation}

Neurodegenerative diseases such as Multiple Sclerosis, Alzheimer’s and Parkinson’s disease, among others, are characterized by the presence of chronic inflammation of the CNS\(^\text{159}\). Currently no effective treatments directed at reducing this inflammation exist\(^\text{160}\). PPARγ holds a well-recognized role in the regulation of
inflammatory responses, which makes this receptor a potential target for amelioration of neurodegenerative pathophysiology. Consistent with this, evidence exists to support the role of a variety of distinct PPARγ agonists as effective inhibitors of microglia-mediated release of pro-inflammatory cytokines both in vitro and in vivo\textsuperscript{161-163}. Heneka and colleagues\textsuperscript{164} demonstrated that TZD-mediated PPARγ activation prevents cytokine-induced neuronal inducible nitric oxide synthase (iNOS) expression in vitro, thereby preventing NO-mediated cell death of neurons. Consistent with this, cerebellum targeted administration of the pro-inflammatory stimulus lipopolysaccharide (LPS) stimulates iNOS expression and results in neuronal cell death. Importantly, concomitant administration of PPARγ agonists ameliorated loss of neurons likely through the inhibition of iNOS and other inflammatory genes\textsuperscript{165}. Luna-Medina and colleagues\textsuperscript{163} demonstrated that rosiglitazone significantly reduced expression of TNF-α, IL-6, iNOS, and COX-2 by stimulated astrocytes through PPARγ activation. Importantly, while complete suppression of COX-2 may cause undesirable CNS effects, such adverse activity has not been reported with rosiglitazone treatment\textsuperscript{166}. Discussion on the role of PPARγ and the effects of its agonist-mediated activation in several neurodegenerative conditions follows.

\textit{Role of PPARγ Activation in Multiple Sclerosis}

Multiple sclerosis (MS) is a neurodegenerative disease characterized by autoimmune destruction of CNS myelin that leads to substantial disability in most patients\textsuperscript{167}. Experimental allergic encephalomyelitis (EAE) is an animal model used to study demyelinating diseases such as multiple sclerosis\textsuperscript{168}. EAE reproduces many of the aspects of the human disease including neuronal inflammation with associated infiltration of lymphocytes, demyelination and gliosis with resulting focal neurological deficits as well as paralysis\textsuperscript{169}. Independent studies by Diab and colleagues\textsuperscript{170,171} demonstrated that the course of EAE correlates strongly with specific levels of PPARγ expression in the spinal cord. In naïve mice PPARγ was low but soon after induction of EAE the level of PPARγ
was found to increase and correlate closely with the phases of the disease such that it expression reached a peak in the active phase and decreased to baseline during the remitting phase\textsuperscript{171}.

Furthermore, either antagonist-mediated functional inhibition or genetic reduction of PPARγ expression worsens EAE. For example, PPARγ deficient heterozygous mice are characterized by increased clinical severity and pathology of EAE progression\textsuperscript{172}, a result replicated in wild-type mice by the use of PPARγ antagonists\textsuperscript{173}. Interestingly, patients with MS who are also carriers of the Pro12Ala mutation in the PPARG gene show delayed onset of clinical symptoms\textsuperscript{174}. In addition, it has recently been demonstrated that patients with MS are characterized by a notable elevation of PPARγ in the cerebrospinal fluid (CSF) which correlates with clinical severity\textsuperscript{175}. The authors speculate that perhaps injured neurons, oligodendrocytes and glia release PPARγ into the CSF\textsuperscript{175}. If this is experimentally confirmed then levels of PPARγ in the CSF may indeed serve as a biomarker of MS. Finally two small clinical trials have been conducted with pioglitazone, the results of which suggest that synthetic PPARγ agonists may ameliorate MS pathophysiology. To this extent, 12 month treatment resulted in a significant reduction of gray matter atrophy\textsuperscript{176} as well as delay in the conversion of normal appearing white matter to lesions in MS patients\textsuperscript{177}.

\textit{Role of PPARγ Activation in Alzheimer’s disease}

Alzheimer’s disease (AD) is the most common cause of memory loss in adults with an incidence of over 50% in those 85 years of age and older\textsuperscript{178}. AD is characterized by the accumulation of Aβ peptide in neuritic plaques and hyperphosphorylated tau protein forming neurofibrillary tangles\textsuperscript{179}. This leads to neuronal cell death and ultimately cognitive dysfunction, the quintessential feature of the disease that underscores the significant public health burden of AD in the aging population\textsuperscript{180}. Interestingly, the expression of PPARγ is increased in brains of patients with AD\textsuperscript{181}, suggesting that PPARγ
levels may change in the course of the disease. Consistent with this, agonist-mediated activation of PPARγ has been shown to ameliorate AD-related pathology in amyloid precursor protein (APP) overexpressing transgenic mouse models of the disease. Heneka and colleagues\textsuperscript{182} report that pioglitazone results in 20% reduction of Aβ plaque burden in this model. The mechanisms behind this effect may involve PPARγ-induced suppression of BACE1, the enzyme responsible for processing APP and the release of Aβ\textsuperscript{183}. In addition, overexpression of PPARγ has been shown to increase neuronal and glial uptake of Aβ from the extracellular milieu\textsuperscript{184}. Finally, it is now well established that agonist-mediated activation of PPARγ improves cognition in animal models of AD\textsuperscript{185, 186, 187, 188}. Unfortunately, many wide-scale clinical trials failed to show such effects in humans. It is now considered that this was likely due to testing during the late-stages of the disease\textsuperscript{189}. In contrast, clinical trials involving patients with mild cognitive impairments (early disease stages) showed benefits using PPARγ agonists\textsuperscript{190, 191}. Therefore, before overt neurodegeneration, PPARγ activation may affect signaling cascades that modulate memory in early AD\textsuperscript{192}, thus emerging as a promising method to slow down progression of the disease.

\textit{Role of PPARγ Activation in Parkinson’s Disease}

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease, affecting nearly 1% of the population over 60\textsuperscript{193}. It is characterized by a progressive and extensive loss of dopaminergic neurons in the substantia nigra leading to the classic symptoms of the disorder: tremor, rigidity and impaired gait, balance and coordination. In addition, neuronal cell death is accompanied by robust gliosis, with a concurrent activation of microglia and release of inflammatory cytokines\textsuperscript{194}. In dopaminergic areas PPARγ colocalizes with tyrosine hydroxylase (TH)-positive neurons in the substantia nigra pars compacta\textsuperscript{195} and in the ventral tegmental area\textsuperscript{78}. The efficacy of PPARγ agonists as neuroprotectants in PD has been addressed at first in several
rodent models, the success in which was further investigated in non-human primate models and recently in a phase II clinical trial. Breidert and colleagues\textsuperscript{196} reported that in a MPTP mouse model of PD, treatment with pioglitazone abolished the death of dopaminergic neurons. In addition, there was a significant decrease in the numbers of reactive astrocytes and microglia in the substantia niagra\textsuperscript{196}. The mechanism was found to involve inhibition of NFκB activity\textsuperscript{197}, consistent with ligand-induced PPARγ transrepression\textsuperscript{91}. In MPTP-treated monkeys, pioglitazone prevented the deterioration of motor performance induced by the neurotoxin, while inhibiting the loss of TH-positive neurons\textsuperscript{198}. Interestingly, a recent study identified the PPARγ co-activator (PGC)1-α as an important potential therapeutic target in PD\textsuperscript{199}. PGC1-α-responsive genes were found to be under-expressed in early PD patients. Consistent with this, overexpression of PGC1-α blocked the loss of dopaminergic neurons in a cellular model of α-synucleinopathy, a typical finding in PD histopathology. Currently there are no data on the neuroprotective effects of TZDs in patients with PD. Based on the encouraging preclinical data, a clinical trial in PD (NCT01280123)\textsuperscript{200} was initiated with the goal to assess the impact of pioglitazone on the progression of PD. The study was completed in 2014\textsuperscript{201}, however, the results are yet to appear in publication.

\textit{Role of PPARγ in the Central Nervous System Control of Metabolism}

The unparalleled ability of TZDs to improve glycemic control\textsuperscript{110, 111} comes at a price: in both patients and in rodent models chronic treatment with TZDs leads to increased food intake, body weight and body fat gain\textsuperscript{202-206}. Consistent with the critical role of PPARγ in adipogenesis (detailed in the sections above), the prevailing thinking has been that the TZD-associated weight gain is largely the result of increased deposition of lipids and enhanced adipogenic capacity of white adipose tissue following PPARγ activation\textsuperscript{207-209}. However, findings from Sugii et al.\textsuperscript{210} suggest that constitutive activation of PPARγ in
adipocytes is sufficient for systemic insulin sensitization and occurs without the concomitant weight gain. This suggests that TZDs target tissues other than adipose where both insulin-sensitizing and undesired effects can occur. In support of this hypothesis TZDs have been demonstrated to improve insulin sensitivity in mice completely lacking white and brown adipose tissue, as well as in mice with adipose-specific deletion of PPARγ. Interestingly, Shimizu et al. noted that 60% of the patients in their study complained of increased appetite 4 weeks after the administration of troglitazone. The authors speculated that the hunger sensations were due to the observed reduction of systemic levels of leptin, a critical anorexigenic signal to the hypothalamus. Even though it was not recognized at the time, these reports of “increased hunger” constitute perhaps the first clue that TZD-mediated target activation in the CNS may account for the undesirable secondary responses such as weight gain. Consistent with this, TZDs are well-established to cross the blood-brain barrier and as outlined in the section above, TZDs have been successfully used to ameliorate the pathophysiology of several neurodegenerative diseases. In addition, PPARγ is expressed in the regions of the hypothalamus critical for energy balance, particularly the neurons of the arcuate nucleus. In 2011 several groups provided strong evidence that the activation of brain PPARγ leads to changes in energy balance and feeding behavior. In a diet-induced obesity mouse model, the expression of hypothalamic PPARγ mRNA, but not PPARα and PPARβ/δ, was found to be significantly upregulated compared to lean controls. Work from the Seeley laboratory showed that both acute and chronic activation of brain PPARγ by either TZD microinjection or overexpression of a constitutively active form of PPARγ in the ventral hypothalamus led to increased food intake and body fat gain in rats. In an opposite experiment the authors infused GW9662, a pharmacological antagonist, or shRNA against the receptor to block hypothalamic PPARγ activity and tested the acute hyperphagic effect of peripherally administered rosiglitazone. Blocking PPARγ in the brain completely attenuated the rosiglitazone-induced food intake. Interestingly, Ryan and colleagues also
show that blocking brain PPARγ has an effect only in certain dietary conditions. That is, in *ad libitum* regular chow-fed rats inhibition of PPARγ had no effect\(^{81}\), a finding supported by others\(^{78,218}\). On the other hand, in conditions of fasting or high fat diet (HFD) feeding suppression of PPARγ led to a significant decrease in caloric intake compared to vehicle control\(^{81}\). The conclusion from this work is that modulation of hypothalamic PPARγ leads to changes in feeding behavior and results in overall energy imbalance. The laboratories of Olefsky and Schwartz took advantage of the Cre-*loxP* system driven by synapsin1 promoter to ablate PPARγ from CNS neurons\(^{80}\). The resulting animals were characterized by a reduced food intake and increased energy expenditure (both resting metabolic rate as well as ambulatory activity were increased), which resulted in overall protection against HFD-induced weight gain\(^{80}\). When Lu et al.\(^{80}\) subjected the brain PPARγ knockouts (BKO) to HFD supplemented with rosiglitazone, they did not develop the characteristic hyperphagia and weight gain. In addition, the rosiglitazone–induced hepatic insulin receptor transduction was completely abolished in the BKO\(^{80}\), suggesting that brain PPARγ plays a critical role in liver-mediated insulin sensitivity. Interestingly, the BKO were also characterized by increased sensitivity to the anorexigenic adipokine leptin\(^{219}\) when fed normal chow but this effect was only a trend under HFD treatment despite evidence of increased downstream signaling of the leptin receptor\(^{80}\). These findings are corroborated by results from Ryan et al.\(^{81}\), where pharmacological inhibition of hypothalamic PPARγ restores leptin sensitivity in HFD-fed rats. Ryan et al\(^{81}\) and Lu et al\(^{80}\) were the first to effectively show that activation of brain PPARγ promotes TZD-associated weight gain by depressing energy expenditure and by increasing food intake as well as adipose mass accumulation. The finding that brain PPARγ is required in part for the TZD-mediated hepatic insulin sensitization\(^{80}\) potentially has significant therapeutic implications. The present “gold standard” for treatment of metabolic conditions continues to be direct targeting of PPARγ despite the multiple limitations associated with this therapeutic approach\(^{220}\). This is evidenced by the recent FDA announcement that it is
removing restrictions on the use of Avandia (rosiglitazone)\textsuperscript{221}. As pointed out by Myers and Burant\textsuperscript{222}, amelioration of weight gain effects of TZD cannot be achieved by simply restricting differential access to the brain of PPAR\(\gamma\) formulations, since this may interfere with the anti-diabetic properties of these drugs.

Whereas these studies addressed the global or regional function of PPAR\(\gamma\) in the brain, they failed to reveal the molecular mechanisms as well as the neuronal subtypes sufficient to mediate the central effects of PPAR\(\gamma\) on energy balance. In a series of elegant experiments the Horvath group provided some of the answers. The pro-opiomelanocortin (POMC) and agouti-related peptide/neuropeptide Y (AgRP/NPY) expressing neurons are arcuate nucleus substrates of the central melanocortin system\textsuperscript{223-225} and play a critical role in the maintenance of energy balance\textsuperscript{226, 227}. Diano and colleagues\textsuperscript{79} showed that activation of PPAR\(\gamma\) via intracerebroventricular (icv) injection of rosiglitazone decreased the formation of reactive oxygen species (ROS) in the arcuate nucleus of mice. Importantly, and perhaps surprisingly, this led to suppression of POMC and promotion of AgRP/NPY firing rate, implicating PPAR\(\gamma\) as a critical regulator of the activity of these two subtypes of neurons\textsuperscript{79}. Suppression of peroxisome proliferation by PPAR\(\gamma\) antagonist GW9662 restored ROS levels in POMC and AgRP/NPY neurons. This in turn reversed HFD-induced elevation of AgRP and reduction of POMC neuronal activity. The ultimate result was a decreased caloric intake in DIO mice\textsuperscript{79}. This study provides support for the idea that hypothalamic PPAR\(\gamma\) acts as a nutritional sensor which modulates the firing pattern of POMC and AgRP/NPY neurons via ROS levels to affect feeding behavior. More recently the Horvath and Diano groups were able to show that POMC neurons are sufficient in mediating brain PPAR\(\gamma\)’s effect on systemic energy homeostasis. Long and colleagues\textsuperscript{82} utilized a POMC promoter driven Cre-recombinase to ablate PPAR\(\gamma\) specifically in mouse POMC neurons. Similarly to the global CNS PPAR\(\gamma\) ablation, the POMC-specific deletion of PPAR\(\gamma\) had phenotypic effect only under conditions of HFD\textsuperscript{78, 82}. The animals were characterized by resistance to weight gain as a result of reduced caloric intake and adipose
accumulation with simultaneous increase in energy expenditure (including locomotor activity). Interestingly, peripheral administration of rosiglitazone did not affect food intake or body weight in the null mice\textsuperscript{82}, suggesting the importance of POMC neurons in mediating the weight gain effect of TZD. The authors, however, did not test whether POMC PPAR\(\gamma\) is required for the insulin sensitizing effects of TZDs in the liver. Perhaps this is because the POMC-specific PPAR\(\gamma\) ablation led to a significantly greater baseline insulin sensitivity\textsuperscript{82}. Long et al., demonstrated a critical role for POMC PPAR\(\gamma\) in hepatic gluconeogenesis regulation as its deletion led to significantly lower levels of PEPCK and G6pase expression\textsuperscript{82}. There also was a marked increase in insulin-induced inhibition of endogenous glucose production in the liver\textsuperscript{82}. The question remains, however, whether the increased insulin sensitivity at baseline is still intact in conditions of HFD. Consistent with results from their previous study\textsuperscript{79}, the Diano group now showed that the mechanisms underlying PPAR\(\gamma\)’s effect on systemic metabolism involve modulation of ROS levels through the decrease in peroxisome density in POMC neurons\textsuperscript{82}.

The role of PPAR\(\gamma\) in AgRP/NPY neurons has not been extensively investigated yet. A very recent study by the Bartness laboratory, however, provides some interesting insights. Garretson and colleagues\textsuperscript{218} show that in a state of starvation there is a significant increase in the expression of AgRP mRNA in the arcuate nucleus with a concomitant increase in PPAR\(\gamma\) expression specifically in the AgRP/NPY neurons. The authors speculate that perhaps the upregulation of AgRP is a PPAR\(\gamma\)-dependent process since peripheral administration of the PPAR\(\gamma\) antagonist GW9662 blocks starvation-induced increases in both PPAR\(\gamma\) and AgRP mRNA\textsuperscript{218}. The authors also corroborate the findings of others\textsuperscript{79, 81, 218} that central agonist-mediated activation of PPAR\(\gamma\) triggers increase in food intake in various rodent models.

In addition to its role in the brain-mediated control of energy balance PPAR\(\gamma\) has been shown to act as a peripheral relay to the CNS of information regarding lipid levels. A study from the Gautron and Elmquist laboratories highlights PPAR\(\gamma\) in the vagus nerve
as a critical modulator of energy homeostasis. Visceral organs such as the liver and the GI tract are innervated by vagal sensory neurons which synapse in the nodose ganglion (NG). Postganglionic projections from NG terminate in the nucleus tractus solitarius of the brainstem where peripheral information is processed and communicated to higher brain centers. Interestingly, Liu et al. found that chronic exposure to HFD reduces the expression of PPARγ and several of its classical targets in the cell bodies of the NG. To investigate the role of PPARγ in the visceral nervous system the authors ablated the receptor from both central and peripheral vagal afferents by using Phox2b-driven Cre-recombinase. The null mice were characterized with a decreased fat mass accumulation and increased energy expenditure on either regular chow or HFD. In addition, deletion of PPARγ from vagal afferents led to browning of white adipose tissue and increase in thermogenesis signified by the marked upregulation of UCP1 expression.

**Summary**

The idea that brain PPARγ modulates metabolic functions previously ascribed largely to adipocytes has led to a significant shift in our understanding of PPARγ biology. We now know that TZDs as well as high fat diet lead to activation of brain PPARγ and results in increased caloric intake and body weight gain. This has important implications, in the face of escalating global obesity, as targeting PPARγ remains the standard in treating metabolic diseases. Improved understanding of the mechanisms by which brain PPARγ affects feeding and energy balance is especially critical in providing opportunities to develop newer classes of PPARγ-targeting agents that reduce or eliminate side effects such as increased food intake and associated body fat gain, which counteract the ability of TZDs to prevent type 2 diabetes mellitus. To determine the specific role of brain PPARγ in the neuronal substrates of the energy balance center of the brain, we generated transgenic mouse models expressing wild type and dominant negative mutant form of PPARγ.
specifically targeted to POMC and AgRP/NPY neurons. Results from molecular and physiologic characterization of these models will be presented in Chapter III.
Figure 1.1. Human PPARγ Isoforms. A. Genomic organization of the 5’ end of human PPARG. B. Alternative promoter use leads to three distinct mRNA products. Exons 1 through 6 are common to all isoforms. Exons A1 and A2 are untranslated leading to 477 aa product in the cases of PPARγ 1 and 3 isoforms. Exon B is translated resulting in an additional 28 aa at the N-terminus of PPARγ2. aa, amino acids.
Figure 1.2. Schematic of PPARγ’s Mechanisms of Action. A. In the absence of a ligand PPARγ forms an obligatory heterodimer with RXR. The complex binds PPRE in the promoter region of target genes and recruits corepressors resulting in a ligand-independent repression. B. When a ligand binds to PPARγ, a conformational change of the receptor occurs leading to substitution of the corepressors with coactivators. Downstream target promoters are transactivated in a ligand-dependent manner. C. Certain target promoters, particularly of inflammatory genes (iNOS, CCL2), are controlled without the direct binding of PPARγ to a regulatory sequence. This mechanism is referred to as a ligand-dependent transrepression. D. A transcriptionally impaired dominant negative (DN) PPARγ sequesters RXR away from the wild type PPARγ leading to transcriptional repression.
CHAPTER II.

GENERATION AND VALIDATION OF TRANSGENIC MOUSE MODELS

Introduction

Recently it was discovered that PPAR\(\gamma\) affects feeding, energy balance, and leptin sensitivity via actions in the brain rather than in adipose tissue alone\(^{80,81}\). Injecting TZDs, well-established to cross the blood-brain barrier\(^{213-217}\), or overexpressing PPAR\(\gamma\) in the hypothalamus of rats leads to an acute increase in food intake and body weight\(^{81}\). Blocking endogenous PPAR\(\gamma\) by either shRNA, pharmacological inhibitors, or genetic ablation of PPAR\(\gamma\) from brain neurons, leads to a decrease in feeding and increase in energy expenditure\(^{80}\). The pro-opiomelanocortin (POMC) and agouti-related peptide/neuropeptide Y (AgRP/NPY) expressing neurons are arcuate nucleus substrates of the central melanocortin system\(^{223-225}\) and play a critical role in the maintenance of energy balance\(^{226,227}\). Activation of PPAR\(\gamma\) via intracerebroventricular (i.c.v.) injection of the PPAR\(\gamma\)-agonist rosiglitazone was recently shown to decrease the formation of reactive oxygen species in the arcuate nucleus of mice\(^{79}\). Importantly, and perhaps surprisingly, this led to suppression of POMC and promotion of AgRP/NPY firing rate, implicating PPAR\(\gamma\) as a critical regulator of the activity of these two subtypes of neurons. Most recently, the Diano laboratory\(^{82}\) showed that POMC neurons are sufficient in mediating brain PPAR\(\gamma\)’s effect on energy balance. Whereas these studies addressed the global or even neuron-specific function of PPAR\(\gamma\) in the brain, they failed to reveal the molecular mechanisms through which brain PPAR\(\gamma\)’s affects energy balance. Overexpression of PPAR\(\gamma\) in specific neuronal populations can help elucidate the function of this receptor by correlating molecular and cellular changes to compliment information obtained from studies using knockout animals. To this extent we have generated two transgenic (Tg)
mouse models, a PPARγ-P467L dominant negative (DN) and a wild type (WT) by using an inducible transgene expression system. The transgene was designed to express both human PPARγ and the reporter gene tdTomato after its selective activation by Cre-recombinase (Figure 2.1). These animals were crossed with mice transgenic for Cre-recombinase under POMC, AgRP or Nestin-Cre promoters to generate the double transgenic mice used in the experiments as part of this dissertation. We chose a transgenic instead of a PPARγ-DN knock-in approach, because the latter has previously failed to recapitulate the phenotype of patients carrying the DN mutation even when exposed to high-fat-diet (HFD), and required expression on a hyperphagic/leptin-deficient background to recapitulate the phenotype. Leptin deficiency is a confounding factor for studying the effects of brain PPARγ on leptin sensitivity (an important goal of this dissertation work). Finally, pragmatic reasons such as low targeting efficiency and much longer model generation time, further discouraged us from using a knock-in approach.

**Materials and Methods**

**Construction of Inducible Transgene Expression System**

Human (h) PPARγ1 cDNA (either wild type (WT) or containing P467L dominant-negative (DN) mutation) was cloned into pLVX-IRES-tdTomato vector (Clontech, catalog No. 631238) (SpeI). CAG promoter followed by LoxP-STOP-LoxP sequence was PCR amplified from Ai3 vector (Addgene, plasmid 22797) using the following oligos: 5’-GCA TGC GTT ATT AAT AGT AAT CAA TTA-3’ and 5’-TTG GGC TGC AGG TCG AGG GA-3’ and cloned into SphI/PstI restriction sites of pSTEC1-M3-PolyA. The hPPARγ-IRES-tdTomato-WPRE sequence was PCR amplified using the following primers: 5’-GCG ACC GGC CGC TCA CGC GTA CTA GTA TGA CCA TGG TTG ACA CA-3’ and 5’-GCG ACC CGG CGG CCG CTC ACG CGT TGA ATT AGC CCT TCC AGT CCC-3’ and was then cloned into NotI linearized pSTEC1-M3-CAGLSL-PolyA to create the final
constructs, designated as either CAG-PPAR-WT-T or CAG-PPAR-P467L-T (Figure 2.2). All cloning junctions were confirmed by sequencing.

**Cell Culture Validation Experiments**

The function of the CAG-PPAR-WT-T and CAG-PPAR-P467L-T constructs was tested in either HEK293T or 3T3-L1 cell lines. The cells were seeded to 80% confluence 12-16 hours prior to transfection. The 3T3-L1 cells were starved for 5 additional hours prior to transfection. Transfection was carried using Lipofectamine LTX (Lifetechnologies) according to manufacturers’ protocol. Total protein or RNA lysates were collected and stored at -80°C until further analysis.

**Generation of Transgenic Mice**

The transgene constructs were submitted to the University of Iowa Transgenic Animal Facility for generation of transgenic mice. The transgene (Figure 1.1) was excised using the restriction enzymes SphI (5’ end) and XhoI (3’end), purified and microinjected into pronuclei of B6SJL mice (C57BL/6J x SJL/J) to generate founders. Identification of transgene-positive mice was done by PCR analysis of tail genomic DNA using the following primers: 5’-TGA TCA AGA AGA CGG AGA CAG-3’ and 5’-GCG CAT GAA CTC TTT GAT GAC-3’.

**Breeding and Maintenance of Transgenic Lines**

All mice were fed standard mouse chow (7013, Teklad Premier Laboratory Diets) and water *ad libitum*. The care of mice used in the experiments met the standards set forth by the NIH in their guidelines for the care and use of experimental animals. All procedures were approved by the University Animal Care and Use Committee at the University of Iowa. Four DN and 10 WT transgenic founders (F0) were identified. All founder lines produced positive offspring (F1). Two founder lines (a **main one** and a backup) per
transgenic construct were kept for propagation: 66534/4 and 66549/1 for CAG-PPAR-WT-T and 66293/1 and 66293/2 for CAG-PPAR-P467L-T. Transgenic mice were normal in size and appearance, and were not distinguishable for wild-type littermates. Transgene transmission was as follows: 66534/4 - 53.2% (n=62), 66549/1 - 39.4% (n=213), 66293/1 - 40.3% (n=315), and 66293/2 - 21.0% (n=81). Transgene-positive mice were maintained by backcross breeding to C57BL6/J.

Liver-Specific Transgene Activation Experiments

Six to 8 week old transgenic F1 and non-transgenic littermate controls were injected with either Ad5-CMV-Cre (6x10^{10} pfu/mouse) or Ad5-CMV-eGFP (2x10^{10} pfu/mouse) in the right jugular vein. Mice were anesthetized with ketamine/xylazine and were affixed supine to a metal platform maintained at 37° C. The ventral surface of the neck was shaved, area sterilized with betadine solution and a 0.5 – 1.0 cm incision was made just lateral to the sternoclavicular joint. The jugular vein was exposed as a pulsating area just ventral to the pectoralis muscle, which was held aside with forceps. Using a 1-mL syringe, the tip of 27-gauge needle was inserted into the jugular vein and 80 microliters of the virus was injected. Immediately after the injection, the pectorals muscle was the released serving as a natural compression force for concomitant bleeding. The skin was glued shut using Vetbond (3M). Mice were allowed to recover from the anesthesia in home cages over a warming pad until fully ambulatory at which point they were transferred to a special room in the vivarium for 1 week for shedding the virus. Mice were euthanized 7 days post viral delivery. Liver tissue was extracted and processed accordingly for the following assays: western blotting and qPCR.

Brain-Specific Transgene Activation: Intracerebral Injection Experiments

Six, 6 to 8 week old, DN-PPARγ F1 transgenic and non-transgenic littermate controls were subjected to intracerebral microinjection of Ad5-CMV-Cre (6x10^{7}}
pfu/mouse). Mice were anesthetized using 5% isoflurane in O₂ and maintained with 2 ± 1% isoflurane in O₂. Mice were placed in a stereotaxic apparatus and the skin covering the skull was shaved, sterilized with betadine and a 1.0-2.0 cm incision was made to uncover the skull. An area just anterior to the lateral ventricle was determined using the following coordinates: AP: 0.2 mm; ML: 1.0 mm; DV: 3.0 mm. The skull was drilled to expose the brain surface. One microliter adenovirus was injected via a Hamilton syringe over 5 minute period. The skin was sewed shut and the animal allowed to recover in home cage on a warming pad. Once ambulatory shut the mice were transferred to a designated room in the vivarium for shedding of the virus. Mice were euthanized 7 days post viral delivery. The brains were dissected and immersion fixed in 4% paraformaldehyde solution for 24-48 h. The brains were sectioned to 30 µm slices using a vibratome (Leica), mounted with Vectashield (Vector Laboratories) and imaged for the presence of red fluorescence on Olympus IX-71 fluorescent microscope.

Skeletal Muscle-Specific Transgene Activation: Electroporation Experiments

Two, 8 weeks old, DN-PPAR-γ F1 transgenic and non-transgenic (littermate) mice were subjected to tibialis anterior (TA) muscle electroporation of Cre-recombinase containing plasmids. The mice were anesthetized with ketamine-xylazine, hindlimbs were shaved, and the TAs were injected with 30 µl of 0.4 U/µl bovine placental hyaluronidase (Sigma) resuspended in sterile 0.9% saline. Two hours later, mice were reanesthetized. The TAs were then injected with 30 µl plasmid DNA in sterile saline, coated with ultrasound jelly, and subjected to ten, 20-ms pulses of 175 V/cm using an ECM-830 electroporator (BTX Harvard Apparatus)231. Seven days post electroporation, the TAs were dissected and ½ of a TA was frozen for immunoblotting and ½ was immersion fixed in 4% paraformaldehyde for histology. TAs were sectioned on a CryoStar NX70 (Thermo Scientific) into 10-µm sections, washed three times with PBS, mounted with Vectashield (Vector Laboratories), and then imaged on an Olympus IX-71 fluorescent microscope.
**Plasmids used:** *pBS185* was described previously \(^{232}\) and encodes modified Cre-recombinase under CMV promoter. *pBS185-ΔCre* was used as a control and was generated via BamHI enzymatic digestion removing 392 bp of 3’end of Cre-recombinase cDNA, rendering the product non-functional. *p-eGFP* encodes enhanced green fluorescent protein (eGFP) under the control of CMV promoter.

**Western Blotting**

HEK293T transfected cells, liver, brain, or skeletal muscle tissue was homogenized in a lysis buffer containing: 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA (pH 7.5), and 0.1% v/v SDS, with protease inhibitors (Thermo Scientific). Protein concentration was determined using the BCA assay (Pierce Protein Research Products). Protein lysates were separated by SDS-PAGE gel and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked for 30 min at room temperature with 5% BSA in TBS-T (tris-buffered saline, 0.1% Tween-20). PPARγ expression was determined using monoclonal anti-PPARγ C26H12, Cell Signaling Technology). Rabbit anti-β-actin was used as a loading control (ab8229, AbCam). Primary antibodies were diluted in 1% BSA/TBS-T to a final concentration of 1:1000 for anti-PPARγ and 1:10,000 for anti-β-actin, and incubated overnight at 4°C. Secondary antibodies were diluted in 5% milk/TBS to final concentration of 1:10,000 and applied for 1 hour at room temperature. Blots were treated with Pierce SuperSignal Western Pico chemilumi-nescence reagent for visualization.

**Quantitative Real Time PCR (qRT-PCR) Analysis**

One hundred microgram of liver tissue from CAG-PPAR-WT or DN single transgenic mice injected with either Ad5-CMV-Cre or Ad5-CMV-eGFP was suspended in ice-cold Trizol (Invitrogen). Similar procedure was followed with brain tissue from Nestin-PPAR-WT or P467L double transgenic mice. Total RNA was isolated using
RNeasy spin columns (RNeasy Mini Plus Kit, QIAGEN) following the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop ND-1000. cDNA was generated using SuperScript (Invitrogen). qRT-PCR was performed using the TaqMan (applied Biosystems) gene expression assay from 10.0 nanogram cDNA in a total volume of 10.0 microliters following the manufacturer’s recommendations. The assay numbers for TaqMan (Applied Biosystems) probes were the following: Mm99999915_g1 (mouse GAPDH), Mm00445878_m1 (mouse FABP4), and Mm01135198_m1 (mouse CD36). The TaqMan probe used for detection of human PPARγ was custom designed and targets sequences that differed between the mouse and human PPARγ (Hs01115513_m1). The expression of tdTomato (Forward: 5’- CAC CAT CGT GGA ACA GTA CG-3’ and Reverse: 5’-GCG CAT GAA CTC TTT GAT GA-3’) and Cre-recombinase (Forward: 5’-CGT ACT GAC GGT GGG AGA AT-3’ and Reverse: 5’-CCC GGC AAA CAG GTA GTT A-3’) were determined using SYBR green (Bio-Rad) according to the manufacturer’s protocol. Mouse GAPDH was used as an internal control.

**Immunohistochemistry**

Brains from CAG-PPAR-DN single transgenic and littermate control mice injected with Ad5-CMV-Cre were sectioned to 30µm thickness using vibratome. The sections were mounted on slides with Vectashield with DAPI (Vector Labs) and imaged on fluorescent microscope (Olympus XL60). The region of the Arcuate nucleus (medial basal hypothalamus) from brains of POMC-PPAR-DN double transgenic mice were sectioned to 30 µm via vibratome. For antigen retrieval the sections were incubated sequentially in the following solutions: 1% NAOH and 1% H2O2 in distilled water for 20 min, 0.3% glycine in PBS for 10 min, 0.03% SDS in PBS for 10 minutes, and finally blocked in in PBS for 30 minutes. All incubations were performed at room temperature. Following the incubations the sections were rinsed with PBS 3 times for 10 min. PPARγ expression was determined using monoclonal anti-PPARγ (C26H12, Cell Signaling Technology) diluted
in 3% normal goat serum with 0.1% Triton X-100 to a final concentration of 1:100 and incubated at 4°C overnight. ACTH expression was determined using antiserum to rabbit ACTH (A.F. Parlow, National Hormone and Peptide Program) diluted in 3% goat serum, 0.3% Tween20 in TBS to a final concentration of 1:1000 and incubated at 4°C overnight. The sections were then rinsed in TBS-T (TBS with 0.3%Tween20) 3 times for 10 min. The secondary antibody Alexa488 (Abcam) was diluted in 5% goat serum, 0.1% Triton X-100 in TBS to a final concentration of 1:200 and incubated at room temperature for 1h. The sections were as describes above. All incubation and rinsing steps were performed under constant agitation. The sections were mounted on glass slides with Vectashield (Vector Laboratories) and imaged for the presence of red fluorescence on Olympus XL60 fluorescent microscope.

**Confocal Imaging**

The brain sections were images using Zeiss LSM710 confocal microscope. Single-plane images were collected. When comparing detection of transgene expression between samples, the microscope settings including laser power, gain and offset were kept constant throughout image collection. Final images were processed using ImageJ software to make adjustments to image size or linear parameters such as brightness and contrast. All adjustments were kept consistent across samples.

**Results**

**Expression of Transgenic Proteins in Heterologous Cells**

Initial validation of CAG-PPAR-WT and P467L-T vectors’ function was achieved though testing in heterologous cell systems. Either construct was transfected into HEK293T cells with or without Cre-recombinase (pBS185 plasmid was used as a Cre-recombinase source). Red fluorescence, indicative of tdTomato expression, was observed only in cells co-transfected with either of the CAG-PPAR-T constructs and pBS185 (Figure
2.3, C and D). This suggests that the LoxP-STOP-LoxP sequence was successfully excised by the recombinase leading to expression of the transgenic proteins. No fluorescence was observed in cells transfected only with the constructs (Figure 2.3, A and B), demonstrating the absence of leaky expression prior to appropriate induction.

Transgenic PPARγ protein expression was confirmed via immunoblot (Figure 2.4). Strong intensity bands above 50kDa (the expected molecular weight of PPARγ1 protein is 53kDa) were detected only in cells co-transfected with either CAG-PPAR-P467L-T or CAG-PPAR-WT-T construct and pBS185. In addition, expression of hPPARγ and tdTomato mRNA was confirmed via qPCR.

To validate the function of the hPPARγ-WT or P467L proteins in the context of the transgene constructs 3T3L1 fibroblasts were utilized. These are immortalized murine cells that have been widely used to study adipogenesis\textsuperscript{233} as well as PPARγ function\textsuperscript{18}. For the experiments CAG-PPAR-WT and P467L-T as well as an “empty” control vector (does not contain hPPARγ cDNA) were modified by deleting the LoxP-STOP-LoxP sequence to ensure continuous expression of the transgenic proteins. To achieve this the constructs were transformed into competent BS591 bacteria that express Cre-recombinase constitutively as previously described\textsuperscript{234, 235}. The modified plasmids were purified and excision of the desired region confirmed by sequencing. The new constructs, designated as ΔE (empty control), ΔWT and ΔP467L, were transfected into undifferentiated 3T3L1 mouse fibroblasts and the effect on expression of aP2/FABP4, a classic PPARγ target gene\textsuperscript{102}, was evaluated (Figure 2.5). Cells transfected with the ΔWT plasmid showed 16.14 fold increase (range: 1.17 upper to 0.85 lower) in the expression of aP2 (average CT values: ΔWT 25.4), p <0.001 compared to ΔE and ΔP467L, n=4 experiments. This suggests that overexpression of hPPARγ-WT alone is sufficient to initiate adipose differentiation in these cells by inducing the expression of the adipocyte-specific genes, such as aP2\textsuperscript{236}. This finding recapitulates early work by Tontonoz, Hu and Spiegelman, 1994\textsuperscript{18} and importantly demonstrates that the hPPARγ-WT cDNA functions as expected in the context of the
transgene construct. The 3T3-L1 cells transfected with either ΔE or ΔP467L showed no increase in the expression of aP2; (average CT values: ΔP467L 29.3; ΔE 29.2), p=1.00 for ΔE vs. ΔP467L. This result is consistent with the previously described impairment in transcriptional activity of the PPARγ-P467L mutant\textsuperscript{134, 156} and confirms that the transgenic mutant protein functions as expected.

**Expression of Transgenic Proteins in Mice**

To test transgene activation in the different founder lines, Adenovirus-Cre (Ad-Cre) was injected in the jugular vein of the mice to induce liver-specific recombination. As shown in Figure 2.6, several founder lines showed expression of PPARγ transgenic protein following injection of Ad-Cre but not with Ad-eGFP, which was used as a negative control. Nontransgenic littermate controls tested with Ad-Cre did not express transgenic protein as expected. For line 66293/2 (CAG-PPAR-P467L-T) the fact that no PPARγ expression was observed in the liver of a transgenic mouse post Ad-eGFP injection confirmed that there was no leaky expression of the transgene (Figure 2.6, last lane). This line was subsequently identified as one of the final two kept. Portions of the liver were also cryosectioned and examined for the presence of red fluorescence indicative of tdTomato expression. However, no evidence of tdTomato was observed. This may reflect problematic tissue preparation. Transgene activation was next tested in the skeletal muscle by electroporation of pBS185 (plasmid containing Cre-recombinase cDNA) or pBS185-ΔCre (plasmid lacking Cre-recombinase) into the tibialis anterior (TA) of transgenic mice. Cross-sections of TA receiving pBS185 exhibited strong red fluorescence signal, indicating expression of tdTomato protein post TA-specific transgene recombination (Figure 2.7, E). Administration of pBS185-ΔCre plasmid did not induce tdTomato expression in the transgenic animals as expected (Figure 2.7, F). This confirms the absence of unregulated transgene expression in skeletal muscle tissue in addition to liver.

Expression of PPARγ protein was evaluated by immunoblot. As expected only TAs from
transgenic mice receiving pBS185 plasmid demonstrated PPARγ expression (Figure 2.8). Finally, transgene activation was induced in the brains of transgenic mice by intracerebral microinjection of Ad-Cre. Red fluorescence was observed only in the injected subcortical areas of transgenic mice (Figure 2.9, A), while littermate controls showed no evidence of tdTomato expression (Figure 2.9, B). Positive detection of both PPARγ and tdTomato in multiple tissues confirms that the transgenic proteins are being made in a tissue-specific, Cre-recombinase-dependent manner.

To test chronic expression of the transgene in the brain we crossed CAG-PPAR-WT-T or CAG-PPAR-P467L-T and Nestin-Cre mice. The brain tissue of the resulting Nestin-PPAR- P467L double transgenic (DT) mice demonstrated 215 fold increase in the level of hPPARγ mRNA (range: 491 upper to 94 lower), p=0.036  (n=3 DT and n=5 single or nontransgenic (S/NT) littermates) (Figure 2.10, A). The average CT values were 24.3 for DT and 33.44 for S/NT mice. Expression of tdTomato mRNA followed a similar pattern and was 149 fold higher in DT mice (range: 205 upper to 109 lower) compared to the control littermates, p=0.002 with average CT values of 22.7 (DT) and 31.3 (S/NT) (Figure 2.10, B). Brain tissue from the same animals was analyzed for expression of PPARγ protein via immunoblot (Figure 2.11). As expected strong intensity bands of above 50 kDa, characteristic of PPARγ, were detected only in DT mice and not in NT or single transgenic littermate controls. These data are consistent with the acute in vivo models of Ad-Cre-mediated transgene activation in several somatic tissues and confirm that the model is working as designed.

**Induction of hPPARγ-WT Expression in the Brain Upregulates Classic Gene Targets**

To test the transgene function in the brain, expression of classic PPARγ gene targets was investigated in Nestin-PPAR DT mice. Mice with activated CAG-PPARγ-WT-T transgene had 54.2 (± 9.3 SEM) fold increase in the expression of aP2/FABP4 mRNA compared to those with CAG-PPARγ-P467L-T (1.8 ± 0.7 SEM) or littermate controls (1.5
± 1.0 SEM) (p=0.008, One-way ANOVA, Tukey’s post hoc analysis) (Figure 2.12, A). The average aP2 mRNA CT values were as follows: all controls 30.5, Nestin-PPAR-P467L 31.6, and Nestin-PPAR-WT 24.0. Similarly, CD36 was significantly upregulated only in the brains of Nestin-PPAR-WT mice (14.6 ±2.4 SEM fold induction; average CT value of 26.0, p=0.0137 vs. controls, One-way ANOVA, Tukey’s post hoc analysis), while controls and Nestin-PPAR-P467L mice exhibited equally undetectable levels of CD36 mRNA (the average CT value for all controls was 30.0 and for Nestin-PPAR-P467L was 30.2) (Figure 2.12, B).

Transgenic PPARγ and tdTomato Proteins Express in POMC Neurons of DT Mice

A main question that this dissertation work is addressing is whether modulation of brain PPARγ function, specifically in the neurons of the melanocortin system, will have an effect on energy balance. For this purpose we conditionally expressed hPPARγ-P467L or hPPARγ-WT in either POMC or AgRP neurons via crosses with mice transgenic for Cre-recombinase under the control of POMC or AgRP promoter. Immunohistochemical analysis of coronal brain sections at the level of the medial basal hypothalamus of POMC-PPAR-P467L adult mice show that the ACTH-labeled POMC neurons co-express the reporter tdTomato (Figure 2.13, A). PPARγ immunolabeling co-localizes with tdTomato endogenous expression (Figure 2.13, B). PPARγ staining is characterized by predominantly nuclear distribution as expected and previously described\(^7\). Due to antibody incompatibility (both anti-ACTH and anti-PPAR are rabbit), co-staining with ACTH and PPARγ was not attempted and instead inference regarding expression of transgenic hPPARγ protein in POMC neurons was based on the localization pattern of the reporter tdTomato.
Discussion

CAG-PPAR-WT and P467L-T transgene constructs were generated and validated in heterologous cell systems. PPARγ and tdTomato protein detection was confirmed prior to submission to the Transgenic Animal Facility. Single transgenic animals survived to adulthood and were observed to have no obvious morphological differences from their wild-type littermates. Transgenic protein expression was detected in transgenic mice following either acute (Adenovirus-Cre) or chronic (genetic cross) transgene activation. Since this dissertation work seeks to investigate the role of brain PPARγ in modulation of energy balance, validation of transgene activation and function specifically in the brain was thoroughly conducted. For this purpose expression throughout the nervous system was achieved by using the pan-neuronal nestin promoter. PPARγ and tdTomato mRNA and protein expression was detected only in double transgenic (DT) and never in single or nontransgenic littermate controls. In addition, as expected, overexpression of the transgenic hPPARγ led to modulation of classic PPARγ targets such as aP2\textsuperscript{102} and CD36\textsuperscript{106}. aP2/FABP and CD36 levels were upregulated only in brains from Nestin-PPAR-WT DT mice but not in controls nor, importantly, in Nestin-PPAR-P467L mice. This serves as a functional confirmation that the transgenic PPAR-P467L is indeed transcriptionally impaired. The dominant-negative function of the PPAR-P467L mutant has been previously demonstrated by Li and Leff\textsuperscript{153}. By using a competitive immunoprecipitation assay the authors were able to show that the mutant PPARγ is able to outcompete the wild type protein for binding to a PPRE-containing promoter\textsuperscript{153}. Interestingly, global CNS overexpression of hPPARγ-WT but not hPPARγ-P467L leads to severe brain abnormality and early post neonatal lethality phenotype which will be discussed in detail in Chapter 5.

A specific question addressed in this work is whether the neurons of the melanocortin system, namely POMC and AgRP, are sufficient in mediating the effects of brain PPARγ on energy homeostasis. Immunohistochemical analysis of brain sections from POMC-PPAR-P467L DT mice demonstrate co-localization of transgenic tdTomato
expression and POMC-specific ACTH staining. This confirms that the generation of this double transgenic model has been successful. To our knowledge this is the first model of neuron-specific overexpression of PPARγ. The system that we have generated can help elucidate the metabolic effects of this transcription factor by modulating its function specifically in POMC or AgRP neurons. Overexpression of the dominant-negative mutant form of the receptor (PPARγ-P467L) provides an additional tool for dissecting the molecular mechanisms of brain PPARγ function. As previously demonstrated by our laboratory, expression of PPARγ-P467L leads to higher levels of transcriptional repression\textsuperscript{154, 155} than possible by genetic deletion\textsuperscript{237}, which is currently the only described model used to study the role of brain PPARγ in energy metabolism\textsuperscript{81}. 
Figure 2.1. Inducible Transgene Expression System. A. Driven by a CAG promoter, the construct contains floxed STOP cassette upstream of the gene of interest (hPPARγ) and followed by the red reporter tdTomato. B. Only in the presence of Cre-recombinase the LoxP-STOP-LoxP sequence is excised allowing for constitutive expression of hPPARγ and tdTomato.
Figure 2.2. Schematic Representation of the CAG-PPAR-WT (P467L)-T Transgene vector.
Figure 2.3. The Red Reported TdTomato Is Expressed Only in the Presence of Cre-Recombinase, Demonstrating a Functional LoxP-STOP-LoxP Sequence. Fluorescence imaging showing expression of tdTomato in HEK293T cells 24h post transfection (Lipofectamine LTX, Invitrogen) with either CAG-PPAR-WT (A and C) or P467L-T (B and D) final constructs. TdTomato s expressed only in wells co-transfected with Cre-recombinase carrying plasmid pBS185 (2µg total plasmid DNA/well) (C and D).
Figure 2.4. Expression of hPPARγ Protein. Immunoblotting of hPPARγ protein 24h post HEK293T cell transfection with final constructs w/out Cre-recombinase, 10 µg protein/lane. A plasmid previously demonstrated to express PPARγ only in the presence of Cre-recombinase is used as control. The transfection efficiency with Lipofectamine, LTX (Invitrogen) was 90-100% and validated by red fluorescence microscopy.
Figure 2.5. Functional Validation of CAG-hPPARγ-WT (P467L) Constructs.

Only hPPARγ-WT leads to upregulation of classic target aP2/FABP4 mRNA in 3T3-L1 fibroblasts, suggesting hPPARγ-P467L is likely transcriptionally inactive. hPPARγ-WT leads to 16.14 fold increase vs. control or hPPARγ-P467L. Total RNA isolated 24 h post transfection. SYBR to aP2, normalized to HPRT internal control. One-Way ANOVA, Bonferroni post-hoc analysis. Data are means ± SE from 4 experiments. dE2, vector plasmid without hPPARγ, dP467, plasmid containing hPPARγ-P467L, dWT, plasmid containing hPPARγ-WT.
Figure 2.6. Transgene Induction in Liver. PPARγ is expressed only in transgenic mice that received Ad-Cre (10 microgram of protein loaded per lane). Positive control: HEK293T cells transfected with plasmid carrying PPARγ-WT transgene. β-Actin serves as loading control. Fo, founder.
Figure 2.7. Transgene Induction in Skeletal Muscle.  A. Cross-section of tibialis anterior (TA) (bright field).  B. eGFP expression, control for the electroporation conditions.  C. pBS185 in TA of nontransgenic control (NT) results in lack of tdTomato expression as expected.  D. pBS185-ΔCre in TA of NT.  E. tdTomato expression in TA of CAG-PPAR-P467L-T transgenic (TG) mouse injected with pBS185.  F. No fluorescence with pBS185-ΔCre in TA of TG.
**Figure 2.8. Transgene Induction in Skeletal Muscle.** PPARγ is expressed in tibialis anterior muscle protein lysate only in transgenic (TG) mice electroporated with Cre-recombinase-containing plasmid (30 micrograms of protein loaded per lane). GFP lanes indicate muscle samples from a nontransgenic mouse injected with eGFP-containing plasmid (positive control for the electroporation procedure). Positive control: HEK293T cells transfected with plasmid carrying PPARγ-P467L vector (2.5 microgram of protein loaded). α-Actin and GAPDH serve as loading controls.
Figure 2.9. Transgene Induction in Brain. A. tdTomato expression (red fluorescence) is evident in the brain of a CAG-PPAR-P467L-T transgenic mouse 7 days post Ad5-CMV-Cre intracerebral microinjection. B. No red fluorescence is observed in the brain of a non-transgenic littermate control. Consecutive sections pictured. White punctate lines mark needle track.
Figure 2.10. Transgene Expression in Brains of Nestin-PPAR-P467L Double Transgenic Mice.  A. PPARγ and B. tdTomato mRNA expression is detected in whole brain tissue lysates from double transgenic (DT) but not nontransgenic (NT) littermate control mice. Average CT values for PPARγ: 24.3 (DT) and 33.4 (NT); tdTomato: 22.7 (DT) and 31.3 (NT). TaqMan to hPPARγ, normalized to GAPDH; SYBR to tdTomato, normalized to HPRT. Student t-test. Data are means ± SE from 3 DT and 5 NT controls.
Figure 2.11. Transgene Induction in Brains of Nestin-PPAR-P467L Double Transgenic Mice. PPARγ is expressed in whole brain protein lysate only in double transgenic mice (50 micrograms of protein loaded per lane). Positive control: HEK293T cells transfected with plasmid carrying PPARγ-P467L vector. β-Actin serves as loading control.
Figure 2.12. Global Overexpression of PPARγ-WT in the Brain Upregulates Target Genes. A. aP2/FABP4 and B. CD36 mRNA expression is increased in whole brain tissue lysates from Nestin-PPAR-WT but not Nestin-PPAR-P467L or littermate control mice. Average CT values for aP2: 24.0 (Nestin-PPAR-WT), 31.6 (Nestin-PPAR-P467L), and 30.5 (all controls); CD36: 26.0 (Nestin-PPAR-WT), 30.2 (Nestin-PPAR-P467L) and 30.0 (all controls). TaqMan to aP2 and CD36, normalized to GAPDH. One-way ANOVA, Tukey post hoc analysis. Data are means ± SE.
Figure 2.13. Transgene Expression in POMC-PPAR-P467L Double Transgenic Mice. A. POMC neurons immunolabeled with antibody against ACTH (left upper and lower panels). Right lower panel is a merged image showing co-localization of ACTH and endogenous tdTomato red fluorescence (white arrows). No tdTomato is detected in the control brain (middle upper panel). B. Immunofluorescence using antibody against PPARγ (green, left lower panel) and tdTomato endogenous fluorescence (red, middle lower panel) detected in coronal sections at the level of the mediobasal hypothalamus. Right lower panel is a superimposed image showing co-localization of PPAR and tdTomato (white arrows). Upper panels show absence of transgene expression in a transgene positive but Cre-negative control. Immunohistochemistry and images by Deng Fu Guo, PhD.
CHAPTER III.

EFFECTS OF INTERFERECE WITH BRAIN PPARγ FUNCTION ON ENERGY BALANCE AND GLUCOSE REGULATION

Introduction

Over two decades ago, the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) was identified as the master regulator of adipocyte function\textsuperscript{17, 18}. Shortly thereafter, PPARγ was recognized as the highly specific cellular target of the antidiabetic thiazolidinedione (TZD) drugs, implicating this transcription factor as a key player in the maintenance of metabolic homeostasis\textsuperscript{108}. Consistent with its critical role in peripheral glucose and lipid metabolism\textsuperscript{26}, humans with dominant-negative (DN) mutations in a single allele of PPARG have partial lipodystrophy and insulin resistance but interestingly normal body weight\textsuperscript{134, 135, 238, 239}. In contrast, a gain of function mutation in PPARγ has been reported to lead to a severe obesity in the carriers\textsuperscript{240}.

Recently it was discovered that PPARγ affects feeding, energy balance, and leptin sensitivity via actions in the brain, rather than in adipose tissue alone\textsuperscript{80, 81}. Injecting TZDs, which can cross the blood-brain barrier\textsuperscript{213-217}, leads to increased food intake and weight gain both in patients and in rodent models\textsuperscript{202-205}. Similarly, overexpression of PPARγ in the hypothalamus of rats results in an acute increase in food intake and body weight\textsuperscript{81}. Blocking endogenous PPARγ by either shRNA, pharmacological inhibitors, or genetic ablation of PPARγ from brain neurons, leads to a decrease in feeding and increase in energy expenditure and thus overall protection form the effects of high fat diet (HFD) treatment\textsuperscript{80}. Different mechanisms have been proposed to mediate the effects of PPARγ blockade including increased activation of intracellular leptin receptor signaling. This is evidenced by the enhanced phosphorylation of leptin receptor’s classic downstream mediator - the
signal transducer and activator of transcription-3 (STAT3) in the hypothalamus following PPARγ deletion.

As a transcription factor PPARγ is characterized by a complex mechanism of action. Without a ligand, PPARγ/Retinoid X receptor heterodimers bind to target genes and inhibit transcription by recruiting corepressors. With a ligand, coactivators replace the corepressors, leading to increased transcription rates. The discovery of the rare DN mutation in the ligand-binding domain of PPARγ in patients with early onset hypertension and insulin resistance revealed impaired transactivation by the mutant along with an ability to inhibit wild-type PPARγ activity. Surprisingly, studies utilizing tissue-specific genetic deletion of PPARγ failed to recapitulate these hypertension phenotypes. Gene deficiency and PPARγ agonists both result in hypotension, an effect which could be explained by the lack of corepressor recruitment in the case of gene deficiency. Indeed, genetic ablation of PPARγ in smooth muscle leads to increased expression of β2-adrenergic receptor, a PPARγ target gene. Unlike gene deficiency, mouse models with either a global or tissue-specific DN-PPARγ mutation have recapitulated the hypertensive phenotype observed in patients. Data from our laboratory demonstrate that DN-PPARγ induces repression of experimentally validated PPARγ target genes, which show increased expression in response to PPARγ agonists.

To further study the mechanisms through which PPARγ in the brain regulates energy balance, we generated a transgenic mouse model in which the human dominant-negative (DN) mutant (P467L) form of PPARγ is conditionally expressed in the CNS via the pan-neuronal promoter nestin. Herein we demonstrate that global interference with PPARγ function in the CNS leads to impaired glucose regulation and altered energy balance. The NestinCre/PPARγ-P467L mice are characterized with resistance to weight gain in response to 60% high fat diet (HFD). Interestingly, when treated with isocaloric low fat control diet these mice remain smaller than their littermate controls, although the difference is not as pronounced as under HFD conditions. The smaller body weight in
Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice is due to significantly lower lean mass. In addition, these mice exhibit extremely low plasma insulin levels even when fed and in conditions of HFD, which correlate strongly with impaired glucose tolerance. We conclude that interference with brain PPAR\textgamma results in impaired insulin and glucose regulation, which then leads to reduced growth rate and altered metabolic homeostasis.

**Material and Methods**

**Generation of Conditional hPPAR\textgamma Transgenic Mice**

Conditional transgenic mice expressing a human (h) dominant – negative (DN) mutant (P467L) form of PPAR\textgamma were generated at the University of Iowa Transgenic Facility as described in Chapter 2. The CAG-PPAR-P467L mice carry a transgene designed to express both hPPAR\gamma (P467L) and the tdTomato reporter gene after its selective activation by Cre-recombinase. Neural overexpression of the mutant (P467L) form of hPPAR\gamma was achieved by crossing CAG-PPAR-P467L-T mice (described in Chapter 2) with Nestin-Cre mice [B6.Cg-Tg(Nes-cre)1Kln/J], purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Iowa.

The following mice were used for the experiments described herein: Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L\textsuperscript{+} and their littermate controls (Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L\textsuperscript{+}, Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L\textsuperscript{−} or Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L\textsuperscript{−})

All animal procedures described below have been approved by the University of Iowa Institutional Animal Care and Use Committee. Mice were kept under standard laboratory conditions, with free access to food and water.

**Western Blotting for Transgenic PPAR\textgamma Protein**

One hundred mg of tissue (brain, liver, spleen, pancreas or adipose) was homogenized in a lysis buffer containing: 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA,
0.1 mmol/L EGTA (pH 7.5), and 0.1% v/v SDS, with protease inhibitors (Thermo Scientific). Protein concentration was determined using the BCA assay (Pierce Protein Research Products). Protein lysates were separated by SDS-PAGE gel and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked for 30 min at room temperature with 5% BSA in TBS-T (tris-buffered saline, 0.1% Tween-20). PPARγ expression was determined using monoclonal anti-PPARγ C26H12, Cell Signaling Technology). Rabbit anti-β-actin was used as a loading control (ab8229, AbCam). Primary antibodies were diluted in 1% BSA/TBS-T to a final concentration of 1:1000 for anti-PPARγ and 1:10,000 for anti-β-actin, and incubated overnight at 4°C. Secondary antibodies were diluted in 5% milk/TBS to final concentration of 1:10,000 and applied for 1 hour at room temperature. Blots were treated with Pierce SuperSignal Western Pico chemiluminescence reagent for visualization.

**Quantitative Real Time PCR (qRT-PCR) Analysis**

One hundred microgram of brain tissue from Nestin^{Cre}/PPARγ-P467L double transgenic mice was suspended in ice-cold Trizol (Invitrogen). Total RNA was isolated using RNeasy spin columns (RNeasy Mini Plus Kit, QIAGEN) following the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop ND-1000. cDNA was generated using SuperScript (Invitrogen). qRT-PCR was performed using the TaqMan (applied Biosystems) gene expression assay from 10.0 nanogram cDNA in a total volume of 10.0 microliters following the manufacturer’s recommendations. The assay numbers for TaqMan (Applied Biosystems) probes were the following: Mm99999915_g1 (mouse GAPDH) and human PPARγ (Hs01115513_m1). The expression of tdTomato (Forward: 5’- CAC CAT CGT GGA ACA GTA CG-3’ and Reverse: 5’-GCG CAT GAA CTC TTT GAT GA-3’) was determined using SYBR green
(Bio-Rad) according to the manufacturer’s protocol. Mouse GAPDH was used as an internal control.

**Mice and Diet**

All animal procedures described below were conductance in accordance with the guidelines and regulations of the University of Iowa Institutional Animal Care and Use Committee. All mice were fed either HFD, isocaloric-match control diet or regular lab chow diet starting at 5 weeks of age for 15 weeks. The HFD was 60% (D12492, Research Diets, New Brunswick, NJ). The isocaloric-match control diet was 10% fat (D12450J, Research Diets). Regular diet was 7013 (Teklad Premier Laboratory Diets). Bodyweights from group-housed mice were measured weekly at 8-9 am until 20 weeks of age. All experiments were performed at 20 weeks of HFD, unless otherwise reported. Daily food intake and body weight were assessed in individually housed mice.

**Measurement of Body Composition**

Body composition was measured at the end of diet treatment period (25 weeks) in vivo by Nuclear Magnetic Resonance (Bruker Minispec LF-90). Male and female Nestin^{Cre}/PPARγ-P467L and control mice (on either HFD or control diet) were evaluated.

**Measurement of Energy Expenditure**

Male Nestin^{Cre}/PPARγ-P467L and control mice, 20 weeks of age, on HFD were individually housed 1 week before measurements. Mice were placed into temperature-controlled, insulated chambers for estimation of heat production by respirometry. First, the CO₂ (model CD-3A, AEI) and O₂ analyzers (model S-3A/II, AEI) were calibrated to standardized air containing 5,000 ppm CO₂ and 20.50% O₂ (Praxair), respectively. Mice were then placed into water-jacketed, temperature-controlled, and air-sealed chambers, and
the change in effluent $O_2$ and $CO_2$ concentrations were recorded using a PowerLab and Chart software (AD Instruments). Flow was determined by mass flow meters (EM1; Sensiron) to STP-correct flow values. Heat production was estimated using the equation based on Lusk (1928): Heat = $\dot{V}O_2 \cdot [1.232 \text{ respiratory exchange ratio (RER)} + 3.815]$, where $\dot{V}O_2 = [\Delta O_2\%] \cdot \text{[STP-corrected flow]}$ and RER = $\Delta CO_2\% / \Delta O_2\%$.

Male Nestin$^{C57/PPAR\gamma-P467L}$ mice were also subjected to CLAMS (Comprehensive Lab Animal Monitoring System, Columbus Instruments, Columbus, OH). Metabolic parameters were analyzed over a 3-day period using the Oxymax Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Briefly, mice were acclimatized to the cages for 12 h before measurements began. The volumes of oxygen consumption ($\dot{V}O_2$) and carbon dioxide consumption ($\dot{V}CO_2$) were measured through indirect calorimetry. Respiratory exchange ratio (RER) was calculated as the ratio of $\dot{V}O_2$ into $\dot{V}CO_2$. Energy expenditure (EE) was calculated using the Lusk equation: $3.815 \times \dot{V}O_2 + 1.232 \times \text{RER}$. Activity levels were counted as infrared beam breaks along the $x$-axis of the cage.

**Fasting Glucose Measurements**

Mice were individually housed and fasted overnight (16-18h). Fasting blood glucose levels were measured via Accu-Chek Aviva Blood Glucose Meter (Roche) using tail blood.

**Glucose and Insulin Tolerance Tests**

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as previously described$^{248}$. Four to five month old mice were fasted overnight (GTT) or for 5 h (ITT) in a clean cage. Body weight and basal glucose levels were obtained prior to intraperitoneal glucose (2.0g/kg) or insulin (1U/kg) administration. During the test blood glucose levels
were determined at the following time points: 15, 30, 60 and 120 min using a glucometer (Accu-Chek Aviva Glucose Meter, Roche) and tail-blood.

**Plasma Measurements**

Blood was extracted from the left ventricle of Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L and control mice at the time of sacrifice. Plasma from each blood sample was isolated using 0.5 M EDTA and by centrifugation at 7,000 rpm for 5 min at 4°C. Plasma levels of leptin, insulin, thyroid hormone (free T3), and growth hormone were measured by commercially available ELISA kits (BioRad for leptin, Crystal Chem for insulin, Cusabio for free T3, and Cloudclone for growth hormone).

**Statistics**

All data are expressed as means ± SEM. The means between two groups were analyzed by 2-tailed Student’s t test. The means of 2 or more groups and 2 genotypes were analyzed by a 2-way ANOVA with Bonferroni post-hoc tests unless otherwise stated. ANCOVA was used to analyze the effect of both genotype and body weight on EE, and RER. Significance was recorded at p<0.05.

**Results**

**Generation and Validation of Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L Mice**

To evaluate the effect of interference with PPAR\textgamma function in CNS neurons, CAG-PPAR-P467L mice (described in Chapter 2) were crossed with mice transgenic for \textit{Cre} under the pan-neuronal promoter nestin. The expression of the transgenic PPAR\textgamma and tdTomato proteins in Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice was validated via both immunoblot (Figure 2.13, Chapter 2 and Figure 3.1) and RT-qPCR analysis (Figure 2.12, Chapter 2) of brain lysates from Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice. Double transgenic mice showed
increased expression of PPARγ protein either in whole brain (Figure 2.13) or dissected regions - hypothalamus, cortex, hippocampus and brain stem, while no PPARγ was detected in the single transgenic littermate controls as expected (Figure 3.1, A). Also, there is no evidence of transgene leakage in peripheral tissues of Nestin\textsuperscript{Cre}/PPARγ-P467L double transgenic mice as no PPARγ protein expression was detected in liver, spleen, or subcutaneous adipose tissue. Transgenic PPARγ mRNA expression was also evaluated in perigenital adipose tissue (33.2 ± 0.15 CT value in Nestin\textsuperscript{Cre}/PPARγ-P467L vs. 33.1 ± 0.22 in controls) and in brown adipose tissue (30.3 ± 0.25 CT value in Nestin\textsuperscript{Cre}/PPARγ-P467L vs. 32.3 ± 0.23 in controls). This data confirm the CNS-specific activation of the transgene.

The function of the transgenic DN PPARγ was tested in these mice by evaluating the level of expression of two canonical PPARγ targets - aP2 and CD36. The mRNA levels of both aP2 and CD36 were significantly upregulated in the brains of Nestin\textsuperscript{Cre}/PPARγ-WT double transgenic mice but not in brains from littermate controls or Nestin\textsuperscript{Cre}/PPARγ-P467L double transgenic mice (Figure 2.14, Chapter2). This confirms that the transgenic DN PPARγ is transcriptionally impaired.

**Overexpression of DN PPARγ in the Nervous System Alters Energy Balance**

**Response to HFD.** Since ablation of neuronal PPARγ leads to resistance to HFD\textsuperscript{80}, we hypothesized that overexpression of the DN PPARγ will result in similar body weight phenotype with perhaps greater protection against HFD due to the strong transcriptional repression exerted by the mutant protein. When treated with 60% high fat diet (HFD) for 15 weeks, male Nestin\textsuperscript{Cre}/PPARγ-P467L mice exhibit significantly lower body weight 42.7±1.71g compared to their littermate controls 48.8 ± 1.47g (p=0.0005, Two-way RM ANOVA) (Figure 3.2). Interestingly, it was noted that the double transgenic mice weigh less at baseline (15.1 ±0.78g vs. 17.6 ± 0.60 for controls, p=0.0235, Unpaired t test). Therefore the change in body weight was also calculated (27.46 ± 1.96g vs. 31.2 ± 1.17g
in controls, \( p = 0.0009 \), Two-way RM ANOVA) (Figure 3.2, B). These data demonstrate the significantly decreased rate of growth in the Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L double transgenic mice. Interestingly, this effect is sex-dependent as it is observed only in the male mice. There is no difference in body weight in female Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice compared to controls in response to HFD (37.47 ± 2.919g in Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L vs. 35.72 ± 2.656g in controls, \( p = 0.6684 \), Unpaired T test, \( n=7\)-10/group).

The reduced growth rate of the Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L male mice under conditions of HFD is reflected in their significantly decreased lean mass (16.21 ± 0.5023g vs. 23.34 ± 0.3393g in controls, \( p < 0.0001 \), Unpaired T test, \( n\geq8\)/group) (Figure 3.3, C) as well as in the smaller organ weights of these mice (liver: 1.326 ± 0.1213g vs. 2.895 ± 0.1628g in controls, \( p<0.0001 \); heart: 0.1486 ± 0.0071g vs. 0.1927 ± 0.0045g in controls, \( p<0.0001 \); kidney 0.2571 ± 0.0106g vs. 0.3891 ± 0.0158g in controls; and spleen: 0.0829 ± 0.0081g vs. 0.1273 ± 0.0081g in controls, \( p = 0.0031 \), Unpaired T test) (Figure 3.5).

The low lean mass in the Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice is not due to presence of muscle atrophy. Histological examination of tibialis anterior (TA) muscle cross sections revealed no difference between genotypes in the muscle fiber morphology (data not shown). In addition, the expression levels of several previously described markers of muscle atrophy\textsuperscript{231} were examined in the TA muscles as well. These also revealed no differences between genotypes (Gadd45 CT value: 30.2 ± 0.2897 vs. 30.2 ± 0.3445 in controls; Fbxo32 CT value: 24.7 ± 0.4439 vs. 23.8 ± 0.4593 in controls; Cdkn1a CT value: 28.9 ± 0.1532 vs. 29.0 ± 0.2432 in controls; Smox CT value: 23.6 ± 0.9939 vs. 24.8 ± 0.3599, for all genes \( n\geq2\)/group) (data not shown).

Interestingly the total fat mass was not different between genotypes in the male Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice (23.26 ± 1.697g in Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L vs. 20.58 ± 0.7509g in controls) (Figure 3.3, B). However, dissection of the various adipose depots revealed the following differences between Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L and their littermate controls: decreased brown adipose tissue (0.1529 ± 0.0169g vs. 0.2745 ± 0.0118g in
controls, p<0.0001, Unpaired T test) (Figure 3.6, A) but increased perigenital adipose tissue mass (1.834 ± 0.1084g vs. 1.460 ± 0.0717g in controls, p=0.0114, Unpaired T test) (Figure 3.6, B). Subcutaneous white adipose tissue mass did not differ between genotypes (2.864 ± 0.2845g in Nestin^{Cre}/PPARγ-P467L vs. 2.638 ± 0.1507g in controls, p = 0.4524, Unpaired T test) (Figure 3.6, C).

Female Nestin^{Cre}/PPARγ-P467L mice show no difference in body composition compared to controls in response to HFD treatment (fat mass: 23.23 ± 2.340g vs. 20.02 ± 2.368g in controls, p=0.3669; lean mass: 14.77 ± 0.7819g vs. 15.80 ± 0.6050g in controls, p=0.3077; fluid mass: 6.086 ± 0.3700 vs. 5.510 ± 0.371g in controls, p=0.2577, Unpaired T test for all measures, n≥7/group) (Figure 3.4).

**Energy Expenditure (EE) in Response to HFD:** To test whether the decreased body weight in response to HFD in Nestin^{Cre}/PPARγ-P467L males is due to changes in energy expenditure or activity level, the mice were subjected to respirometry as well as CLAMS. Despite the fact that heat production, as an absolute value, was lower in the Nestin^{Cre}/PPARγ-P467L males (0.1890 ± 0.0148 kcal/hr. vs. 0.2588 ± 0.0178 kcal/hr. in controls), when analyzed with ANCOVA, no statistical difference in EE was found between genotypes (p=0.2572) (Figure 3.7, A). Heat production was also normalized by lean body mass which similarly revealed no statistical difference between genotypes (11.82 ± 0.1609 kcal/kg x hr. in Nestin^{Cre}/PPARγ-P467L vs. 11.32 ± 0.7310 kcal/kg x hr. in controls, p=0.6399, Unpaired T test) (Figure 3.7, B).

Neuronal ablation of PPARγ has been shown to increase energy expenditure through upregulation of thermogenic genes such as UCP1 in brown adipose tissue (BAT)\textsuperscript{80}. Despite the fact that Nestin^{Cre}/PPARγ-P467L male mice have significantly smaller amounts of BAT, they showed similar levels of expression of UCP1 in BAT as the controls (average CT value for Nestin^{Cre}/PPARγ-P467L: 16.7 ± 0.2273 vs. 16.8 ± 0.6562,
n=6/group) (graph not shown). This is consistent with similar levels of energy expenditure between the genotypes.

Activity measurements showed a trend toward decreased movement in the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L males compared to controls. Due to the low number of mice tested there is no statistical difference between genotypes (light phase: 68.30 ± 17.63 beam breaks in Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L, n =5 vs. 99.73 ± 23.47 beam breaks in controls, n=6, p=0.3286, Unpaired T test; dark phase: 130.1 ± 14.37 beam breaks vs. 256.7 ± 56.48 beam breaks in controls, p=0.1253, Unpaired T test) (data not shown).

**Food Intake in Response to HFD.** To understand the cause of the reduced body weight gain in Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L male mice food intake was measured. Analysis of 24-hour food intake, averaged over 5 days, showed no difference between genotypes were found in male Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice (2.417 ± 0.3092g/day vs. 2.513 ± 0.0854g/day in controls, n≥6/group, p=0.7401, Unpaired T test) (Figure 3.8, A).

**Other Metabolic Measures in Response to HFD.** Leptin is an adipokine with a well-established role in increasing energy expenditure and reducing energy consumption via central effects\textsuperscript{249}. In addition, HFD is known as inducer of leptin resistance\textsuperscript{250}. As Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L male mice gain significantly less weight on HFD, we hypothesized that interference with neuronal PPAR\textgreek{y} protects against leptin resistance. The basal circulating levels of leptin were similar between Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L and control mice (62.13 ± 11.573ng/mL vs. 74.38 ± 6.874ng/mL in controls, p=0.3806, n=7/group, Unpaired T test) (Figure 3.9). This is consistent with the similar total adipose mass in Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L males and their littermate controls.

As thyroid hormone plays a major role in regulating metabolic function, the level of free T3 was evaluated in Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice and did not differ between
Effect of DN PPARγ on Glucose Homeostasis. Brain PPARγ has been shown to play a critical role in hepatic insulin sensitivity. To evaluate the effect of interference with brain PPARγ function on glucose homeostasis, the levels of fasting glucose were measured in Nestin^Cre/PPARγ-P467L mice post 15 weeks of HFD treatment. Double transgenic mice showed significantly lower fasting glucose levels (85.57 ± 3.02 mg/dl vs. 143.3 ± 12.0 mg/dl in controls) (p=0.0018, Unpaired t test) (Figure 3.10). This may be an overall reflection of the reduced body weight in Nestin^Cre/PPARγ-P467L.

Surprisingly, the Nestin^Cre/PPARγ-P467L male mice treated with HFD are characterized by severe hypoglycemia compared to controls in fed state (1.183 ± 0.1623ng/ml vs. 6.575 ± 0.2270ng/ml in controls, p<0.0001, n=7/group, Unpaired T test) (Figure 3.11). The same trend is observed after 6 hour fast (1.2 ± 0.61 ng/ml vs. 2.2 ± 1.00ng/ml in controls, p=0.3414, n=6/group, Unpaired T test) (graph not shown).

Based on the hypoinsulinemia exhibited by these mice, we hypothesized that interference with brain PPARγ leads to impaired glucose handling. To test this a glucose tolerance test (GTT) was performed. The Nestin^Cre/PPARγ-P467L male mice were significantly glucose intolerant compared to their littermate controls (at 60 min post glucose bolus blood glucose levels in Nestin^Cre/PPARγ-P467L were 491.2 ± 24.39mg/dl vs. 416.5 ± 37.14mg/dl in controls, p=0.0260, Two-way RM ANOVA) (Figure 3.12). At the end of the test, however the Nestin^Cre/PPARγ-P467L mice were able to clear the glucose
to levels similar to controls, suggesting that despite the significant hypoinsulinemia these mice may be more sensitive to insulin than the controls. Consistent with this a trend for higher levels of insulin receptor mRNA expression in the livers of Nestin<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L mice (fold change: 4.5 ± 2.4 arbitrary units vs. 2.1 ± 1.0 arbitrary units in controls, n=6/group) (graph not shown).

**Response to Isocaloric Low Fat Control Diet.** A cohort of Nestin-PPAR-P467L mice has been placed on 10% fat matched-control diet. Taking into account baseline differences in body weight between genotypes, Nestin<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L double transgenic male mice remain significantly smaller after 7 weeks on the diet 23.14 ± 0.8214g vs. 26.77 ± 1.063g in controls (p=0.0380, Two-way RM ANOVA) (Figure 3.13, A). Again, as seen with HFD treatment, the body weight phenotype in response to control diet is sex-dependent. The female Nestin<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L mice show no difference in body weight in response to 15 week treatment with control diet (24.76 ± 0.6305g vs. 25.55 ± 1.083g in controls, p=07367, Two-way RM ANOVA) (Figure 3.13, B).

**Discussion**

The critical role of brain PPARγ in the regulation of energy balance has been recently demonstrated through several models involving genetic deletion of PPARγ. Whole brain ablation of PPARγ results in protection against diet-induced obesity through mechanisms involving increase in energy expenditure and decrease in energy intake<sup>80</sup>. POMC neuron-specific deletion of PPARγ recapitulates the whole brain phenotype in inducing resistance to HFD and elucidates additional mechanisms underlying these protective effects, i.e. increase in the levels of reactive oxygen species, which are required for the proper functioning of the anorexigenic POMC neurons<sup>82</sup>.
PPARγ null mutations do not exist in vivo as they lead to embryonic lethality due to impaired placental development\textsuperscript{98}. In contrast, even though rare, dominant negative (DN) loss-of-function mutations in PPARγ have been described in humans to lead to severe metabolic disturbances and early onset of hypertension\textsuperscript{134, 135}. One such mutation (Pro467Leu) occurs in the ligand-binding domain of PPARγ and has been shown to interfere with the wild type form of the receptor by competing for target gene promoter binding sites\textsuperscript{153}. In addition, the P467L mutant is transcriptionally defective\textsuperscript{134, 153}. Our laboratory has previously demonstrated the utility of the PPARγ-P467L mutant as a potent repressor of experimentally validated PPARγ target genes which otherwise show increased expression levels in response to PPARγ agonists\textsuperscript{154, 155}. Herein we generated a transgenic mouse model with CNS-specific overexpression of a DN PPARγ-P467L mutation under the pan-neuronal promoter nestin. We show that the model is functioning as expected based on upregulation of canonical target genes in a control mouse model overexpressing the wild type form of PPARγ in the CNS but not in mice with the transcriptionally defective PPARγ-P467L mutation. The Cre recombinase-mediated CNS-specific activation of the transgene is demonstrated through the expression of transgenic PPARγ and the reporter tdTomato (both protein and mRNA) only in CNS structures without leakage in peripheral tissues. The dominant negative function of the transgenic brain PPARγ-P467L has not been evaluated in vivo in this model. A potential experiment to address this could involve administration of rosiglitazone to Nestin\textsuperscript{Cre}/PPARγ-P467L and littermate control mice and measuring change in body weight. The expectation is that since rosiglitazone crosses the blood-brain barrier\textsuperscript{213, 214, 217} and brain PPARγ is required for the weight gain effect of the drug\textsuperscript{80}, the Nestin\textsuperscript{Cre}/PPARγ-P467L mice will be resistant to the effects of rosiglitazone and weigh less than the controls. A caveat remains, however, as saturating doses of rosiglitazone have been demonstrated to overcome the DN effect of PPARγ\textsuperscript{153}, thus careful titration of the dose is required.
Since genetic ablation of neuronal PPARγ leads to negative energy balance in response to HFD treatment\textsuperscript{80, 82}, we expected that the Nestin\textsuperscript{Cre}/PPARγ-P467L mice would show similar body weight phenotype under conditions of high fat feeding. Indeed, when treated with 60% HFD for 15 weeks these mice gain significantly less body weight compared to their littermate controls. Genetic deletion of global brain PPARγ has been reported to result in significantly lower body mass of knockouts compared to flox/flox control mice at young age and when fed regular chow diet\textsuperscript{80}. We observed the same in the Nestin\textsuperscript{Cre}/PPARγ-P467L at baseline, prior to treatment with HFD, and also during feeding with low fat isocaloric control diet. The differences in body mass reported in the brain PPARγ knockouts disappear by 13 weeks\textsuperscript{80}. In our model these differences are not transient and persist through adulthood. The divergence in body weight is most pronounced under conditions of high fat feeding but remains significant even with low fat diet, suggesting the presence of a growth rate impairment. Indeed, at 20 weeks of age the male Nestin\textsuperscript{Cre}/PPARγ-P467L mice exhibit significantly decreased lean body mass, which is due to smaller individual organ weights. There is no evidence, however, of impaired skeletal muscle morphology or presence of muscle atrophy in this model. We hypothesized that these mice are protected from the effects of HFD through mechanisms of altered energy intake and/or output. This, however, was not the case as the Nestin\textsuperscript{Cre}/PPARγ-P467L double transgenics show no difference in energy expenditure or energy intake. In addition, since the decrease in body weight and lean mass in the Nestin\textsuperscript{Cre}/PPARγ-P467L mice is also evident during control low fat feeding, it is likely that the metabolic alterations observed during HFD are a secondary effect of impaired growth rate resulting from interference with brain PPARγ function. We were able to show that the reduced growth in these mice is not due to pituitary impairment, which has been previously described to be a problem in Nestin-cre mice\textsuperscript{251}, as our model is characterized by normal levels of growth hormone. Interestingly, the growth alteration appears to be a sex-dependent effect as
female Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L do not show this phenotype regardless of feeding conditions.

Global neuronal ablation of PPAR\textgreek{y} leads to impaired glucose tolerance and reduced insulin sensitivity\textsuperscript{80}. We hypothesized that the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice will also demonstrate impaired glucose homeostasis. We observed that the fasting glucose levels in the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L after 15 weeks of HFD were significantly lower than those of controls. Initially this effect was attributed to the lower body weight of the animals. We were surprised, however, to find out that the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L males are characterized by a significant fed state hypoinsulinemia. Similarly lower plasma levels of insulin are observed during fasted state. Consistent with this, the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice show impaired glucose tolerance at 60 min post glucose bolus injection. Interestingly, at 120 min post bolus administration, the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice were able to clear the glucose to control levels, suggesting increased insulin sensitivity. This is also consistent with the trend for increased insulin receptor expression in the liver of the Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L mice.

The role of insulin as a growth factor has been well-established\textsuperscript{252} as impaired growth is a significant complication of uncontrolled diabetes\textsuperscript{253, 254}. Winter and colleagues\textsuperscript{255} described the stunted growth of a 7-year old diabetic patient with Mauriac syndrome. The child had normal circulating levels of growth hormone (GH) but his insulin-like growth factor-1 (IGF-1) levels were in the hypopituitary range. Interestingly, administration of exogenous GH failed to affect IGF-1 levels and linear growth in Mauriac syndrome; the serum IGF-1 and growth rate increased only with improved diabetes control\textsuperscript{255}. The growth effects of insulin have been replicated in animal models as well, where infusion of insulin in diabetic rats leads to improvement in linear growth\textsuperscript{256}. In our model interference with neuronal PPAR\textgreek{y} leads to significant hypoinsulinemia, which is particularly obvious during fed state and under conditions of HFD, but the circulating
levels of GH are normal. Considering the importance of insulin as a growth regulator it is likely that the decreased overall and lean body mass is due to impaired insulin production in these mice. Circulating IGF-1 levels have not been measured in the plasma of Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L mice but we expect those to be low as well. Our model stands in contrast with the insulin resistance phenotype of humans carrying the DN PPAR\textgamma mutations. These patients are characterized by severe fasting hyperinsulinemia as well as hyperglycemia consistent with the presence of significant insulin resistance\textsuperscript{135}. The human DN PPAR\textgamma mutations are heterozygous and affect every tissue in the body. Our model is neuron-specific and we have confirmed the absence of transgene activation in peripheral tissues. This gives us confidence to conclude that interference with the function of brain, and not extra-CNS, PPAR\textgamma leads to impairment in insulin production.

The hypothalamus regulates energy and glucose balance by maintaining reciprocal neural communications with the preganglionic motor neurons of the autonomic nervous system\textsuperscript{257}. It has been recently shown that abnormal insulin signaling in POMC neurons, in response to HFD exposure, leads to altered POMC projections to the preautonomic paraventricular nucleus of the hypothalamus (PVH) and thus impaired pancreatic parasympathetic innervation, demonstrated by decreased glucose-stimulated insulin secretion\textsuperscript{258}. Activation of PPAR\textgamma in POMC neurons has been shown to lead to decreased activity pattern of these neurons, which is abolished by treatment with PPAR\textgamma antagonist\textsuperscript{79}. One possibility for the hypoinsulinemia observed in our model is that interference with neuronal PPAR\textgamma leads to impaired parasympathetic output to the pancreas as a result of altered input to parasympathetic preganglionic neurons. This in turn leads to decreased hormone release by the pancreas. Changes in the parasympathetic innervation of the pancreatic \( \beta \) cells can be evaluated by staining for vesicular acetylcholine transporter (v AChT)\textsuperscript{259}. 
In summary, our data showed that interference with brain PPARγ results in impaired insulin and glucose regulation. This in turn has significant implications in altering the growth rate and metabolic homeostasis. In light of the well-established role of PPARγ in regulating insulin sensitivity, this is the first report implicating brain PPARγ in controlling peripheral insulin levels.
Figure 3.1. Transgene Induction in Brains of Nestin^{Cre}/PPAR-P467L Double Transgenic Mice. A. Transgenic PPARγ protein is expressed in the following brain regions: hypothalamus, cortex, hippocampus and brainstem of double transgenic mouse (30 μg of protein loaded per lane) but not a littermate control. B. No transgenic PPARγ expression is detected in peripheral tissues (liver, pancreas and subcutaneous adipose tissue) of either double transgenic or littermate control mice. Positive control: HEK293T cells transfected with plasmid carrying PPARγ-P467L vector (1.5 μg loaded in A and 30.0 μg loaded in B. β-Actin serves as loading control.
Figure 3.2. Male Nestin\textsuperscript{Cre}PPAR\textgamma-P467L Mice Resistant to Weight Gain in Response to 60\% High Fat Diet (HFD) Treatment. A. Body weight B. Change in body weight in Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L\textsuperscript{male} mice treated with HFD for 15 weeks. Data in the graphs are expressed as the mean ± SEM.
Figure 3.3. Body Composition Characteristics of Male Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L Mice after 15 Weeks of 60\% (HFD) Treatment. A. Body weight B. Fat mass C. Lean mass and D. Fluid mass. Data in the graphs are expressed as the mean ± SEM.
Figure 3.4. Female Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L Show No Difference in Body Composition after 15 Weeks of 60\% HFD Treatment. A. Body weight B. Fat mass C. Lean mass and D. Fluid mass. Data in the graphs are expressed as the mean ± SEM.
Figure 3.5. Organ Weights in Nestin$^{Cre}$/PPAR$\gamma$-P467L Male Mice after 15 Weeks of 60% HFD Treatment. A. Liver B. Heart C. Kidney and D. Spleen weight. Data in the graphs are expressed as the mean ± SEM.
Figure 3.6. Adipose Depots Weights in Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L Male Mice after 15 Weeks of 60% HFD Treatment. A. Brown adipose tissue (BAT) B. Perigonadal and C. Subcutaneous adipose tissue weight. Data in the graphs are expressed as the mean ± SEM.
Figure 3.7. Energy Expenditure in Response to HFD Treatment in Nestin\textsuperscript{Cre}/PPAR\textgamma-P467L Male Mice. A. Heat production analyzed by ANCOVA. B. Heat production normalized by lean body mass. Data in the graphs are expressed as the mean ± SEM.
Figure 3.8. No Effect of 15 Weeks of 60% HFD Treatment in Nestin^{Cre}/PPAR_{γ-}P467L Male Mice in A. Food intake and B. Body weight. Data in the graphs are expressed as the mean ± SEM.
Figure 3.9. No Difference in Plasma Leptin Levels in Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L Male Mice and Controls After 15 Weeks of HFD Treatment. Data in the graph are expressed as the mean ± SEM.
Figure 3.10. Fasting Glucose Levels in Nestin<sup>Cre</sup>/PPARγ-P467L Male Mice and Controls After 15 Weeks of HFD Treatment. Data in the graph are expressed as the mean ± SEM.
Figure 3.11. Severe Fed State Hypoinsulinemia in Nestin^{Cre}/PPARγ-P467L
Male Mice after HFD Treatment. Data in the graphs are expressed as the mean ± SEM.
Figure 3.12. Impaired Glucose Handling in Nestin\textsuperscript{Cre}/PPAR\textgreek{y}-P467L Male Mice after HFD Treatment. A. Blood glucose levels B. Change in blood glucose levels relative to baseline. Data in the graphs are expressed as the mean ± SEM.
Figure 3.13. Body Weight Response to Isocaloric 10% fat Control Diet in Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L Mice. 

A. Male double transgenic mice maintain lower body weight and exhibit a decreased growth rate. 

B. Female Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L mice show no differences in body weight between genotypes after 15 weeks on the diet. 

Data in the graphs are expressed as the mean ± SEM.
CHAPTER IV.

THE ROLE OF PPARγ IN THE CONTROL OF ENERGY BALANCE BY THE MELANOCORTIN SYSTEM NEURONS

Introduction

The increasing prevalence of obesity\textsuperscript{260, 261} is strongly linked to the consumption of high fat diet (HFD)\textsuperscript{262, 263} and is associated with resistance to insulin\textsuperscript{264}, leptin\textsuperscript{265}, and other CNS regulatory signals\textsuperscript{266}. The nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) is a transcription factor\textsuperscript{267} highly expressed in adipose tissue\textsuperscript{94}, where it is essential for normal adipogenesis\textsuperscript{93} and regulation of lipid and glucose metabolism\textsuperscript{267}. Recently it was discovered that PPARγ affects feeding, energy balance and leptin sensitivity through its actions in the brain rather than in the adipose tissue alone\textsuperscript{80, 81}. Injecting TZDs, synthetic agonists of PPARγ which are well-established to cross the blood-brain barrier\textsuperscript{213-217}, or overexpressing PPARγ in the hypothalamus of rats leads to an acute increase in food intake and body weight\textsuperscript{81}. Blocking endogenous PPARγ by either shRNA, pharmacological inhibitors, or genetic ablation of PPARγ from brain neurons, leads to a decrease in feeding and increase in energy expenditure\textsuperscript{80}. A variety of mechanisms have been proposed to mediate the effects of PPARγ blockade including increased activation of intracellular leptin receptor signaling. This is evidenced by the enhanced phosphorylation of leptin receptor’s classic downstream mediator - the signal transducer and activator of transcription-3 (STAT3) in the hypothalamus following PPARγ deletion\textsuperscript{80}.

The pro-opiomelanocortin (POMC) and agouti-related peptide/neuropeptide Y (AgRP/NPY) expressing neurons are arcuate nucleus substrates of the central melanocortin system\textsuperscript{223-225} and play a critical role in the maintenance of energy balance\textsuperscript{226, 227}. Activation
of PPARγ via intracerebroventricular (i.c.v.) injection of the PPARγ-agonist Rosiglitazone was recently shown to decrease the formation of reactive oxygen species in the arcuate nucleus of mice. Importantly, and perhaps surprisingly, this leads to suppression of POMC and promotion of AgRP/NPY firing rate, implicating PPARγ as a critical regulator of the activity of these two subtypes of neurons. Most recently a study by Long and colleagues established that selective ablation of PPARγ in POMC neurons alone protects mice against effects of HFD and leads to improved leptin sensitivity. While the current evidence suggests that POMC may be the neuronal subtypes critical in mediating PPARγ’s effect on energy balance, the molecular mechanisms of these effects remain to be fully elucidated.

The role of PPARγ in AgRP/NPY neurons has not been extensively investigated yet. A recent study, however, provides some interesting insights. Garretson and colleagues show that in a state of starvation there is a significant increase in the expression of AgRP mRNA in the arcuate nucleus with a concomitant increase in PPARγ expression specifically in the AgRP/NPY neurons. The authors speculate that perhaps the upregulation of AgRP is a PPARγ-dependent process since peripheral administration of the PPARγ antagonist GW9662 blocks starvation-induced increases in both PPARγ and AgRP mRNA. The authors also corroborate the findings of others that central agonist-mediated activation of PPARγ triggers increase in food intake in various rodent models.

To study the mechanisms by which PPARγ in POMC and AgRP neurons controls energy balance, we generated transgenic mouse models in which a dominant-negative (DN) mutant (P467L) or a wild type form of PPARγ is conditionally expressed in either POMC or AgRP neurons. The PPARγ-P467L is a rare mutation in the ligand-binding domain of the receptor and in humans causes early onset severe hypertension, insulin resistance, along with lipodystrophy, and metabolic syndrome. The experimental approach detailed in this study capitalizes on the novel use of the DN PPARγ which has
been previously shown by our laboratory to effectively interfere with PPARγ’s transcriptional activity154, 155, 243-246.

We report herein that interference with the function of PPARγ in POMC but not AgRP neurons plays a role in energy balance under certain dietary conditions. We found that POMC\textsuperscript{Cre}/PPARγ-P467L double transgenic mice gained significant weight on low fat diet. Furthermore, this increase in body mass was driven by a significant accumulation of adipose tissue. Interestingly, these mice did not differ from littermate controls in either body weight or composition when treated with 60% high fat diet. We conclude that interference with the function of PPARγ in POMC but not AgRP neurons affects energy balance only under certain dietary conditions.

**Material and Methods**

**Mice and Diet**

All animal procedures described below were conducted in accordance with the guidelines and regulations of the University of Iowa Institutional Animal Care and Use Committee. All mice were fed either HFD, isocaloric-match control diet or regular lab chow diet starting at 5 weeks of age for 25 weeks. The HFD was either 60% (D12492, Research Diets, New Brunswick, NJ) or 45% HFD (D12451). The isocaloric-match control diet was 10% fat (D12450J, Research Diets). Regular diet was 7013 (Teklad Premier Laboratory Diets). Bodyweights from group-housed mice were measured weekly at 8-9 am until 30 weeks of age. All experiments were performed at 25 to 27 weeks of HFD, unless otherwise reported. Daily food intake and body weight were assessed in individually housed mice.

Conditional transgenic mice expressing either a human (h) wild type (WT) or a dominant–negative (DN) mutant (P467L) form of PPARγ were generated at the University of Iowa Transgenic Facility as described in Chapter 2. The GAG-PPAR-WT and CAG-
PPAR-P467L mice carry a transgene designed to express both hPPARγ (P467L or WT) and the tdTomato reporter gene after its selective activation by Cre-recombinase. The transgenic mice were then crossed with either POMC-Cre [Tg(Pomc1-Cre16Lowl/J)] or AgRP-Cre [Tg(tm1(cre)Lowl/J), both purchased from The Jackson Laboratory and bred at the University of Iowa. The following mice were used for the experiments described herein: POMC<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>+/−</sup> and their littermate controls (POMC<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>+/+</sup>, POMC<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>−/−</sup> or POMC<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>−/+</sup>); POMC<sup>Crc</sup>/PPARγ<sup>−</sup>-WT<sup>+/−</sup> and their littermate controls (POMC<sup>Crc</sup>/PPARγ<sup>−</sup>-WT<sup>+/+</sup>, POMC<sup>Crc</sup>/PPARγ<sup>−</sup>-WT<sup>−/−</sup> or POMC<sup>Crc</sup>/PPARγ<sup>−</sup>-WT<sup>−/+</sup>); AgRP<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>+/−</sup> and their littermate controls (AgRP<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>+/+</sup>, AgRP<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>−/−</sup> or AgRP<sup>Crc</sup>/PPARγ<sup>−</sup>P467L<sup>−/+</sup>). In addition male and female mice of each genotype were tested.

**Hypothalamic Immunohistochemistry**

The region of the Arcuate nucleus (medial basal hypothalamus) from brains of POMC<sup>Crc</sup>/PPARγ<sup>−</sup>P467L double transgenic mice and littermate control mice were sectioned to 30 µm via vibratome. For antigen retrieval the sections were incubated sequentially in the following solutions: 1% NAOH and 1% H<sub>2</sub>O<sub>2</sub> in distilled water for 20 min, 0.3% glycine in PBS for 10 min, 0.03% SDS in PBS for 10 minutes, and finally blocked in in PBS for 30 minutes. All incubations were performed at room temperature. Following the incubations the sections were rinsed with PBS 3 times for 10 min. PPARγ expression was determined using monoclonal anti-PPARγ (C26H12, Cell Signaling Technology) diluted in 3% normal goat serum with 0.1% Triton X-100 to a final concentration of 1:100 and incubated at 4°C overnight. ACTH expression was determined using antiserum to rabbit ACTH (A.F. Parlow, National Hormone and Peptide Program) diluted in 3% goat serum, 0.3% Tween20 in TBS to a final concentration of 1:1000 and incubated at 4°C overnight. The sections were then rinsed in TBS-T (TBS with
0.3% Tween20) 3 times for 10 min. The secondary antibody Alexa488 (Abcam) was diluted in 5% goat serum, 0.1% Triton X-100 in TBS to a final concentration of 1:200 and incubated at room temperature for 1 h. The sections were as described above. All incubation and rinsing steps were performed under constant agitation. The sections were mounted on glass slides with Vectashield (Vector Laboratories) and imaged for the presence of red fluorescence on Zeiss LSM710 confocal microscope. Single-plane images were collected. When comparing detection of transgene expression between samples, the microscope settings including laser power, gain and offset were kept constant throughout image collection. Final images were processed using ImageJ software to make adjustments to image size or linear parameters such as brightness and contrast. All adjustments were kept consistent across samples.

Quantitative Real Time PCR (qRT-PCR) Analysis

Mediobasal hypothalamus (MBH) or 100.0 mg of liver tissue, kidney, heart, spleen, subcutaneous adipose tissue from POMCCre/PPARγ-P467L mice injected was suspended in 1.0 mL of ice-cold Trizol (Invitrogen). To evaluate transgene expression in POMC neurons, punches were taken at the region of the arcuate nucleus from POMCCre/PPARγ-P467L and littermate control mice. Total RNA was isolated using RNeasy spin columns (RNeasy Mini Plus Kit or RNeasy Micro Kit, QIAGEN) following the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop ND-1000. cDNA was generated using SuperScript (Invitrogen). qRT-PCR was performed using the TaqMan (applied Biosystems) gene expression assay from 10.0 nanogram cDNA in a total volume of 10.0 microliters following the manufacturer’s recommendations. The assay numbers for TaqMan (Applied Biosystems) probes were the following: Mm99999915_g1 (mouse GAPDH) and human PPARγ (Hs01115513_m1). The expression of tdTomato (Forward: 5’-CAC CAT CGT GGA ACA GTA CG-3’ and Reverse: 5’-GCG CAT GAA CTC TTT
GAT GA-3)’ and Cre-recombinase (Forward: 5’-CGT ACT GAC GGT GGG AGA AT-3’ and Reverse: 5’-CCC GGC AAA CAG GTA GTT A-3’) were determined using SYBR green (Bio-Rad) according to the manufacturer’s protocol. Mouse GAPDH was used as an internal control.

The expression of the following energy balance regulating genes was tested in MBH lysates from POMC\textsuperscript{Cre}/PPAR\textgreek{g}-P467L males post 25 weeks of isocaloric low fat control diet: S18 (Forward 5’-ACT GCC ATT AAG GGC GTG G-3’ and Reverse: 5’-CCA TCC TTC ACA TCC TTC TG-3’), POMC (Forward: 5’-CTG CTT CAG ACC TCC ATA GAT GTG-3’ and Reverse 5’-CAG CGA GAG GTC GAG TTT GC-3’), AgRP (Forward: 5’-CAG AAC TTT TGG CGG AGG T-3’ and Reverse: 5’-AGG ACT CGT GCA GCC TTA CAC-3’), NPY ( Forward: 5’-TCA GAC CTC TTA ATG AAG GAA AGC A-3’ and Reverse: 5’-GAG AAC AAG TTT CAT TTC CCA TCA-3’), Sox3 (Forward: 5’-ACC AGC GCC ACT TCT TCA CG-3’ and Reverse: 5’-GTG GAG CAT CAT ACT GAT CC-3’), PTP1B (Forward: 5’-GAC TCG TCA GTG CAG GAT CA-3’ and Reverse: 5’-GAC TCG TCA GTG CAG GAT CA-3’), ObRb (Forward: 5’-TGT TTT GGG ACG ATG TTC CA-3’ and Reverse: 5’-GCT TGG TAA AAA GAT GCT CAA ATG-3’) and CART (Forward: 5’-GAG CCT GGC TTT AGC AAC AAT AA-3’ and Reverse: 5’-GCA CAC ACA CCA ACA CCA TCC-3’). Mouse S18 was used as internal control.

**Measurement of Body Composition**

Body composition was measured at the end of diet treatment period (25 weeks) in vivo by Nuclear Magnetic Resonance (Bruker Minispec LF-90). Male and female POMC\textsuperscript{Cre}/PPAR\textgreek{g}-P467L, POMC\textsuperscript{Cre}/PPAR\textgreek{g}-WT, AgRP\textsuperscript{Cre}/PPAR\textgreek{g}-P467L and control mice (on either HFD or control diet) were evaluated.
**Measurement of Energy Expenditure**

Male POMC\textsuperscript{Cre}/PPAR\textgamma-P467L and control mice, 18 weeks of age, on control diet were individually housed 1 week before measurements. Animals were then transferred to CLAMS (Comprehensive Lab Animal Monitoring System, Columbus Instruments, Columbus, OH). Metabolic parameters were analyzed over a 3-day period using the Oxymax Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Briefly, mice were acclimatized to the cages for 12 h before measurements began. The volumes of oxygen consumption (\(\dot{V}_{O_2}\)) and carbon dioxide consumption (\(\dot{V}_{CO_2}\)) were measured through indirect calorimetry. Respiratory exchange ratio (RER) was calculated as the ratio of \(\dot{V}_{O_2}\) into \(\dot{V}_{CO_2}\). Energy expenditure (EE) was calculated using the Lusk equation: 3.815 x \(\dot{V}_{O_2}\) + 1.232\*RER. Activity levels were counted as infrared beam breaks along the \(x\)-axis of the cage.

**Fasting Glucose Measurements**

Mice were individually housed and fasted overnight (16-18h). Fasting blood glucose levels were measured via Accu-Chek Aviva Blood Glucose Meter (Roche) using tail blood.

**Leptin Sensitivity**

Male and female POMC\textsuperscript{Cre}/PPAR\textgamma-P467L, POMC\textsuperscript{Cre}/PPAR\textgamma-WT, AgRP\textsuperscript{Cre}/PPAR\textgamma-P467L and control mice on HFD; and male POMC\textsuperscript{Cre}/PPAR\textgamma-P467L and controls on low fat diet were individually housed. Sham (vehicle) was injected at 8 am and 4 pm for 4 days (1\(\mu\)L/g body weight) prior to leptin treatment. Recombinant murine leptin (1.0 \(\mu\)g/g body weight, Peprotech) was injected i.p. twice daily (at 8 and 4 pm) for 4 days. Body weight and food intake were measured daily at 8 am.
Peripheral Injection of Rosiglitazone

Fourteen to 16 week-old female POMC<sup>Cre</sup>/PPARγ-P467L and POMC<sup>Cre</sup>/PPARγ-WT and their respective littermate controls (n=7-11/group) were used for this experiment as previous described<sup>82</sup>. The mice were single housed one day prior to the experiment, switched from regular laboratory chow to 45% HFD and were injected i.p. with vehicle (10% DMSO in saline) for 5 days at 3:00 pm. Rosiglitazone (Cayman Chemicals, Ann Arbor, MI) was dissolved in 10% DMSO/saline and injected i.p. at a dose of 28mg/kg for 5 days at 3:00 pm. In addition, the mice were fasted for 6 hours (12:00pm to 6:00 pm) prior to the onset of the dark cycle. Food intake was measured daily at 8:00 am. Body weight was measured at the start and the end of the treatment.

Statistics

All data are expressed as means ± SEM. The means between two groups were analyzed by 2-tailed Student’s t test. The means of 2 or more groups and 2 genotypes were analyzed by a 2-way ANOVA with Bonferroni post-hoc tests unless otherwise stated. ANCOVA was used to analyze the effect of both genotype and body weight on EE, and RER. Significance was recorded at p<0.05.

Results

Generation and Validation of POMC<sup>Cre</sup>/PPARγ-P467L and WT Mice

To evaluate the effect of interference with PPARγ function specifically in POMC neurons, CAG-PPAR-P467L or CAG-PPAR-WT mice were crossed with mice transgenic for Cre recombinase under the POMC promoter. Immunocytochemistry at the level of the arcuate nucleus in POMC<sup>Cre</sup>/PPARγ-P467L mice showed colocalization of POMC-labeling and transgenic tdTomato signal. PPARγ staining and tdTomato expression were evident only in brains of POMC<sup>Cre</sup>/PPARγ-P467L but not in littermate control mice (Figure
2.15, Chapter 2). In addition, RT-qPCR analysis of punches from POMC^{Cre}/PPARγ-P467L and POMC^{Cre}/PPARγ-WT mice showed increased expression of transgenic PPARγ in Arcuate nucleus, but not in cortex, or liver of double transgenic mice (Figure 4.1). Human PPARγ levels were undetectable in single transgenic littermate controls as well as Nestin-PPAR-P467L /- or CAG-PPAR-P467L – mice as expected (Figure 5.1, Chapter 5). Transgenic tdTomato mRNA levels were also examined and their pattern of expression correlates strongly with hPPAR mRNA levels in all of the samples evaluated (data not shown). A detailed analysis of transgene levels was conducted in the peripheral tissues from POMC^{Cre}/PPARγ-P467L and control mice. No difference in the levels of transgenic hPPARγ or tdTomato mRNA was found between double transgenic mice and littermate controls in the following tissues: heart, kidney, spleen, subcutaneous white adipose tissue (WAT), perigenital WAT and interscapular brown adipose tissue (BAT) (Figure 4.2).

**Overexpression of PPARγ-WT in POMC Neurons Leads to Increased Sensitivity to Rosiglitazone-Induced Weight Gain**

To evaluate the physiological effect of overexpression of transgenic hPPARγ in POMC neurons, the response to peripheral administration of Rosiglitazone was examined in POMC^{Cre}/PPARγ-P467L and POMC^{Cre}/PPARγ-WT mice. Since POMC-specific deletion of PPARγ has been shown to lead to protection against the hyperphagic and body-weight increasing effects of Rosiglitazone\(^8\), we hypothesized that overexpression of PPARγ-WT in POMC neurons will have the opposite effects, i.e. will result in increased sensitivity to the drug. In addition, this experiment served as a functional evaluation of our POMC-specific transgenic models. Intraperitoneal Rosiglitazone administration to lean POMC^{Cre}/PPARγ-WT female mice exposed to 45% HFD resulted in a significant increase in body weight compared to littermate control mice (1.675 ± 0.3127g vs. 0.600 ± 0.2045g in controls, \(p=0.0080\), Unpaired \(t\) test) (Figure 4.3, A). In contrast, under the same
conditions as above, Rosiglitazone failed to induce significant body weight accumulation in POMC<sup>Cre</sup>/PPARγ-P467L female mice compared to their littermate controls (1.086 ± 0.3706g vs. 0.6714 ± 0.1570g in controls, p=0.3209, Unpaired t test) (Figure 4.3, B). Both transgenic lines showed no significant changes in body weight gain in response to saline compared to controls (0.6 ±0.5809g in POMC<sup>Cre</sup>/PPARγ-WT vs. 0.6 ± 0.3493g in controls, p=0.9390; 0.5 ± 0.4364g in POMC<sup>Cre</sup>/PPARγ-P467L vs. -0.5 ± 0.4550g in controls, p=0.1666) (graph not shown).

**Overexpression of PPARγ-P467L Specifically in POMC Neurons Alters Energy Balance Only under Certain Dietary Conditions**

**Response to HFD.** At 25 weeks post treatment with 60% HFD POMC<sup>Cre</sup>/PPARγ-P467L male mice gain similar amounts of body weight as their littermate controls (55.7±1.01g vs. 57.1±1.18g in controls, p=0.1436, Two-way RM ANOVA) (Figure 4.4, A). The 60% HFD treatment results in similar lack of difference in body weight between genotypes in female POMC<sup>Cre</sup>/PPARγ-P467L mice (48.9±0.51g vs. 47.6±0.36g in controls, p=0.3044, Two-way RM ANOVA) (Figure 4.4, B).

Ablation of PPARγ specifically in the POMC neurons is sufficient to induce resistance to diet-induced obesity<sup>82</sup>. One possible explanation for the lack of effect of the P467L DN transgenic mutant in the current treatment conditions is that 60% HFD provides sufficient ligands to activate the endogenous PPARγ and overcome the effect of the mutant. To evaluate this, I placed a cohort of male POMC<sup>Cre</sup>/PPARγ-P467L mice on 45% HFD. There was no difference in body weights compared to controls (48.1±0.80g vs. 48.0±0.97g in controls, p=0.9989, Two-way RM ANOVA) in response to this treatment (data not shown).

**Response to Leptin after HFD.** Deletion of global brain PPARγ<sup>80</sup> or antagonist-mediated inhibition of hypothalamic PPARγ leads to increased sensitivity to leptin<sup>81</sup>. Importantly,
ablation of PPARγ in POMC neurons alone is sufficient to increase sensitivity to leptin. Therefore, I hypothesized that similar interference with PPARγ function in POMC neurons alone will be able to alleviate leptin resistance caused by HFD treatment. At the end of 60% HFD treatment POMC<sup>Cre</sup>/PPARγ<sub>P467L</sub> mice were subjected to twice daily IP injections of vehicle (PBS) for 4 days with a consecutive 4 day course of leptin injections at 1mg/kg. There was no difference between genotypes in male POMC<sup>Cre</sup>/PPARγ<sub>P467L</sub> mice in food intake in response to PBS (2.7±0.23g/day vs. 2.8±0.12g/day in controls) (Figure 4.5, A) or leptin (2.7±0.11g/day vs. 2.7±0.23g/day in controls) (Figure 4.5, B). Body weight also showed no changes between genotypes in response to leptin in these mice (-0.1±0.06g/day vs. -0.1±0.08g/day in controls) (Figure 4.5, D). Similarly, female POMC<sup>Cre</sup>/PPARγ-P467L mice did not show difference between genotypes in response to leptin (data not shown).

**Other Metabolic Outcomes after HFD Treatment.** Following HFD no differences between genotypes and within sexes were observed in POMC<sup>Cre</sup>/PPARγ-P467L mice in the following metabolic measures: body composition (Figure 4.6), fasting glucose (Figure 4.7), organ and adipose depots weights (Figure 4.8).

**Response to 10% Fat Isocaloric-Match Control Diet.** When the POMC<sup>Cre</sup>/PPARγ-P467L males mice were placed on 10% fat isocaloric-match control diet, they gained significantly more weight 36.0±0.90g than the controls 31.7±0.47g (p<0.0001, Two-way RM ANOVA) (Figure 4.9, A). Similar trend was observed when POMC<sup>Cre</sup>/PPARγ-P467L males were placed on a regular laboratory chow diet 7013, containing 18% fat (41.1±2.62g vs. 39.2±2.26g in controls, p=0.9996, Two-way RM ANOVA) (data not shown). This effect is sex-dependent and observed only in male POMC<sup>Cre</sup>/PPARγ-P467L, as the females show no difference in body weight compared to controls when treated with low fat diet (24.8 ± 1.21g vs. 25.5 ± 0.80g in controls, p=0.9991, Two-way RM ANOVA) (Figure 4.9, B).
Body Composition after Control Diet. The difference in body weight in the male POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L mice is strongly correlated with significantly increased fat mass (8.9±1.21g vs. 5.4±0.69g in controls, p=0.0216, Unpaired t test) but no difference in lean mass (21.51±0.2976g vs. 21.03±0.5273g in controls, p=0.4382, Unpaired t test) or % fluid content (12.66±0.2241g vs. 12.51±0.1882g in controls, p=0.6324, Unpaired t test) (Figure 4.10). Female POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L mice show no difference in body composition in response to low fat control diet (Figure 4.11).

Other Metabolic Outcomes after Control Diet. No difference in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L and littermate control mice regardless of sex was found in the following outcome measures after treatment with control diet: fasting glucose (80.9±6.7mg/dL in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L male mice vs. 78.1±2.5mg/dL in controls, n=10/group, p=0.6915; 77.3±6.4mg/dL in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L female mice vs. 72.6±3.6mg/dL in controls, n=6-12/group, p=0.4964) (graph not shown) and food intake (2.6±0.1g/day in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L male mice vs. 2.6±0.1g/day in controls, n=6-11/group, p=0.7356; 2.0±0.3g/day in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L female mice vs. 2.2±0.1g/day in controls, n=6-12/group, p=0.3278) (graph not shown).

Response to Leptin after Control Diet Treatment. The leptin sensitivity testing was conducted in the POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L male mice that were treated with low fat isocaloric control diet for 25 weeks. Leptin administration significantly lowered the food intake (-0.4900±0.1803g, p=0.0085, One-way ANOVA, Tukey’s multiple comparisons test) and body weight (-3.070±1.027g, p=0.0059, One-way ANOVA, Tukey’s multiple comparisons test) in control mice confirming that the drug is working. There is no statistical difference in the response to leptin in POMC\textsuperscript{Cre}/PPAR\textgreek{a}-P467L males (-4.478±0.6851g
for body weight and 2.230 ± 0.1230g for food intake) compared with littermate controls (-4.400 ± 1.003g for body weight and 2.111 ± 0.1086g for food intake (Figure 4.12).

In male POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L treated with low fat control diet, there was a trend toward elevated weights in all adipose depots, which is consistent with the increased body fat composition in these mice. One week following leptin treatment, however, it was noted that the POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L males had experienced greater cumulative body weight loss of 3.5 ± 0.5836 g vs. 1.9 ± 0.5818 g in the controls. This weight loss has inevitably affected the adipose tissue weights. There was no difference in organ weights between genotypes (data not shown) and a trend toward increased mass in subcutaneous (0.6400 ± 0.07812g vs. 0.4780 ± 0.05042g in controls, p=0.0932, Unpaired t test,) and brown adipose tissue depots (0.1678 ± 0.1839g vs. 0.1280 ± 0.0120g in controls, p=0.0821, Unpaired t test) but not in perigenital adipose depots (graphs not shown). The measurements were obtained on 9-10 animals per group.

Levels of expression of the following energy balance regulating genes we examined in the mediobasal hypothalamus of POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L males and littermate controls: POMC, CART, AgRP, NPY, SOX3, ObRb, and PTP1B. No difference between genotypes was found in mRNA levels (Figure 4.13).

**Energy Expenditure (EE) and Locomotor Activity during Low Fat Diet.** To evaluate whether the increased body mass observed in POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L males is due to changes in EE and locomotor activity, a new cohort of POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L males was treated with control diet for 13 weeks. At this time point the divergence in body weight had not occurred in the previous experiment and we hypothesized that if interference with PPAR<sub>γ</sub> in POMC neurons leads to changes in metabolism or activity, which in turn induce the weight gain, such changes would be observable before any difference in weight becomes obvious. Male POMC<sup>C<sub>re</sub></sup>/PPAR<sub>γ</sub>-P467L mice showed no difference in EE compared with littermate controls (0.34 ± 0.0082 kcal/hr vs. 0.34 ± 0.0081 kcal/hr in
controls, p=0.7322, ANCOVA). There was no difference in locomotor activity between genotypes (450.46 ± 87.98 beam breaks per 24 hours in POMC\textsubscript{Cre}/PPARγ-P467L vs. 440.88 ± 57.09 beam breaks in controls, p=0.9250, Unpaired t test) (Figure 4.14).

**Overexpression of PPARγ-WT Specifically in POMC Neurons Does Not Alter Energy Balance under Various Dietary Conditions**

Administration of Rosiglitazone into the 3\textsuperscript{rd} ventricle or expression of constitutively active PPARγ specifically in the hypothalamus of rats leads to significant increase in hyperphagia and weight gain\textsuperscript{81}. To test the hypothesis that chronic overexpression of PPARγ only in the POMC neurons affects energy balance in the manner previously described, we generated POMC\textsubscript{Cre}/PPARγ-WT double transgenic mice.

**Effect of 60% HFD Treatment.** At 25 weeks post treatment with 60% HFD POMC\textsubscript{Cre}/PPARγ-WT male mice gain similar amounts of body weight as their littermate controls (55.32 ± 1.006g vs. 53.99 ± 0.7881g in controls, p=0.9997, Two-way RM ANOVA) (Figure 4.15, A). The 60% HFD treatment results in similar lack of difference in body weight between genotypes in female POMC\textsubscript{Cre}/PPARγ-WT mice (48.24 ± 3.972g vs. 51.99 ± 1.55g in controls, p=0.5988, Two-way RM ANOVA) (Figure 4.15 B).

**Other Metabolic Outcomes after HFD Treatment.** Following 25 weeks of HFD treatment no differences between genotypes and within sexes were observed in POMC\textsubscript{Cre}/PPARγ-WT mice in the following metabolic measures: body composition, fasting glucose, food intake, leptin sensitivity, organ and adipose depots weights (data not shown).

**Effect of Low Fat Control Diet.** There is no difference between genotypes in body weight in POMC\textsubscript{Cre}/PPARγ-WT male mice in response to control diet (data not shown). Female
POMC\textsuperscript{Cre}/PPAR\textgamma-WTs were not placed on control diet due to low number of double transgenic mice.

**Overexpression of PPAR\textgamma-P467L Specifically in AgRP Neurons Does Not Alter Energy Balance under Various Dietary Conditions**

To examine the effects of chronic interference with PPAR\textgamma specifically in AgRP neurons, we generated the AgRP\textsuperscript{Cre}/PPAR\textgamma-P467L mice.

**Effect of 60\% HFD Treatment.** At 25 weeks post treatment with 60\% HFD AgRP\textsuperscript{Cre}/PPAR\textgamma-P467L male mice gain similar amounts of body weight as their littermate controls (52.75 ± 1.292g vs. 55.17 ± 1.185g in controls, p=0.3598, Two-way RM ANOVA) (Figure 4.16, A). The 60\% HFD treatment results in similar lack of difference in body weight between genotypes in female AgRP\textsuperscript{Cre}/PPAR\textgamma-P467L mice (49.37 ± 1.347g vs. 49.73 ± 2.349 g in controls, p=0.9992, Two-way RM ANOVA) (Figure 4.16 B).

**Other Metabolic Outcomes after HFD Treatment.** Following 25 weeks of HFD treatment no differences between genotypes and within sexes were observed in AgRP\textsuperscript{Cre}/PPAR\textgamma-WT mice in the following metabolic measures: body composition, fasting glucose, food intake, leptin sensitivity, organ and adipose depots weights (data not shown).

**Effect of Low Fat Control Diet.** There is no difference between genotypes and within sexes in body weight in AgRP\textsuperscript{Cre}/PPAR\textgamma-P467L mice in response to control diet (data not shown).

**Discussion**

The importance of brain PPAR\textgamma in regulation of energy homeostasis has been demonstrated through the use of genetic deletion of the receptor in the whole brain\textsuperscript{80} as
well as specifically in the POMC neurons\textsuperscript{82}. Deletion mutations in PPAR\(\gamma\) have not been described in humans because of the lethality of the germline knockout of PPAR\(\gamma\)\textsuperscript{98, 268}. The discovery of a rare dominant-negative (DN) mutation in the ligand-binding domain of PPAR\(\gamma\) in patients with early onset hypertension and insulin resistance\textsuperscript{134} revealed impaired transactivation by the mutant along with an ability to inhibit wild-type PPAR\(\gamma\) activity\textsuperscript{153}. Surprisingly, studies utilizing tissue-specific genetic deletion of PPAR\(\gamma\) failed to recapitulate these hypertension phenotypes\textsuperscript{237, 241, 242}. Gene deficiency and PPAR\(\gamma\) agonists both result in hypotension, an effect which could be explained by the lack of corepressor recruitment in the case of gene deficiency\textsuperscript{99}. Indeed, genetic ablation of PPAR\(\gamma\) in smooth muscle leads to increased expression of \(\beta_2\)-adrenergic receptor, a PPAR\(\gamma\) target gene\textsuperscript{237}. Unlike gene deficiency, mouse models with either a global\textsuperscript{157, 158} or tissue-specific\textsuperscript{243-246} DN-PPAR\(\gamma\) mutation have recapitulated the hypertensive phenotype observed in patients.

Data from our laboratory demonstrate that DN-PPAR\(\gamma\) induces repression of experimentally validated PPAR\(\gamma\) target genes, which show increased expression in response to PPAR\(\gamma\) agonists\textsuperscript{154, 155}. Herein we utilize the same DN mutation occurring in humans (PPAR\(\gamma\)-P467L) as a novel method to interfere with function of endogenous brain PPAR\(\gamma\). We generated transgenic mice overexpressing either the wild type PPAR\(\gamma\) (PPAR\(\gamma\)-WT) or the mutant form of the receptor in specific neuronal populations and assessed the responses of the resultant mice to different dietary stressors.

The results of our study demonstrate that overexpressing the transgenic PPAR\(\gamma\)-WT in POMC neurons, acutely and significantly increases sensitivity to the body weight accumulating effects of Rosiglitazone and HFD. This is consistent with a previous study where administration of Rosiglitazone in the 3\textsuperscript{rd} ventricle of rats leads to acute induction of hyperphagia and results in weight gain\textsuperscript{81}. In addition, our findings corroborate those of Long et al\textsuperscript{82}, where POMC-specific deletion of PPAR\(\gamma\) renders the mice resistant to the combined effects of Rosiglitazone and HFD. Interestingly, when we utilize a chronic model of HFD treatment, mice overexpressing PPAR\(\gamma\)-WT in POMC neurons show no
difference in weight gain or body composition compared to littermate controls. Perhaps simply saturating these neurons with PPARγ is not sufficient and the presence of a ligand such as a TZD provides the critical activation of the receptor. In the study by Lu et al\textsuperscript{80}, the addition of Rosiglitazone to the HFD treatment led to significant difference in the body weights between global brain PPARγ knockouts and controls. This suggests that the body weight changes induced by manipulation of brain PPARγ are subtle and to be elicited may require experimental paradigms manipulating not only the dietary variables but also the levels of agonists present.

The mice overexpressing PPARγ-P467L in POMC neurons gained similar amount of weight as their controls when treated with a combination of Rosiglitazone and HFD, confirming that the mutant receptor is transcriptionally impaired and also interferes with the ability of the endogenous wild type form to be activated by the drug. Chronic exposure of the POMC\textsuperscript{Cre}/PPARγ-P467L to HFD, however, led to no difference in weight gain compared to littermate controls. Saturating doses of a TZD agonist have been shown to overcome the transcriptional deficiencies in the mutant PPARγ-P467L\textsuperscript{153}. Perhaps chronic treatment with HFD leads to build up of ligands in the brain that activate the mutant PPARγ resulting in similar phenotype as the littermate controls. This is supported by the observation that reducing the fat content of the diet (from 60% to 45%) does not influence the outcome of the body weight phenotype. Alternatively, treatment with HFD leads to inactivation of PPARγ via Cdk5-mediated phosphorylation at Ser273\textsuperscript{34}. It is possible that this HFD-induced phosphorylation causes equal level of impairment in both the endogenous and the transgenic PPARγ-P467L protein, thus in effect neutralizing the DN effect we sought to achieve through overexpression.

Interestingly, when these mice are subjected to low fat isocaloric control diet, they gain significantly more weight compared to controls. Furthermore, overexpression of PPARγ-P467L in the POMC neurons appears to induce significant accumulation of adipose
tissue under the conditions of control diet. These effects are sex-dependent as interference with brain PPARγ in female mice does not lead to changes in energy balance. Since the control diet is an iso-caloric match to the HFD, carbohydrates are used to compensate for the decrease in fat calories. In this diet 70% of calories are derived from cornstarch. One possibility is that interference with PPARγ in POMC neurons sensitizes these animals to the presence of increased glucose.

POMC-specific deletion of PPARγ leads to improved sensitivity to leptin in conditions of HFD treatment. In our model overexpression of PPARγ-P467L does not lead to changes in response to leptin administration neither during HFD nor control diet treatment. It is likely that after 25 weeks of HFD exposure the mice have developed significant hyperleptinemia and secondary leptin resistance such that a dose of 1.0 mg/g is not sufficient to induce a decrease in food intake. In control diet-fed mice the same dose is sufficient to inhibit food intake and lead to decrease in body weight compared to vehicle, however, the response to leptin in mice with POMC-specific overexpression of PPARγ-P467L does not differ from that in controls. This suggests that perhaps interference with PPARγ in POMC neurons may lead to altered response of these neurons to adipokines other than leptin, which may in turn also account for the increased weight gain observed in these mice. Furthermore, since POMCs are only a subset of leptin-responsive neurons in the brain, it is possible that there is a compensatory signaling from other neurons where the DN PPARγ is not being expressed. To further test the effect of DN PPARγ on leptin signaling in the brain, one possibility is to use double transgenic mice in which the transgene is driven by the leptin receptor (ObRb) promoter.

Currently the role of PPARγ in AgRP neurons has been tested indirectly through the use of pharmacological means in vivo or ex vivo brain slice preparations. At this time no studies have addressed the AgRP-specific role of PPARγ by direct manipulation of the receptor’s expression level. We generated a mouse model overexpressing the DN
PPARγ-P467L specifically in AgRP neurons. These animals showed no difference in metabolic outcome measures such as body weight and composition, food intake, fasting glucose, and sensitivity to leptin when exposed to either HFD or control diet. These results suggest that perhaps the role of PPARγ in AgRP is not critical for the brain-mediated regulation of metabolism. This, however, contradicts previous findings where acute agonist-mediated stimulation of PPARγ in the brain leads to decrease in the firing rate of AgRP neurons. Evidence suggests that PPARγ expression is tightly regulated during early development, suggesting the possibility for induction of compensatory mechanisms to counteract the effects of the mutant receptor. To this extent, deletion of the Agrp gene or ablation of AgRP neurons in young mice has little or no effect on food intake, body weight regulation, and adiposity. In contrast, ablation of AgRP neurons in adult mice inhibits feeding and results in starvation. This suggests that Agrp inactivation during development may promote compensatory mechanisms that mask the crucial functions conferred by this gene. Thus, it is possible that chronic interference with PPARγ function in AgRP neurons, more so than POMC neurons, would fail to induce changes in energy balance phenotype. To overcome this potential limitation, one possibility is to use tamoxifen-inducible cre:ER<sup>T2</sup> mice controlled by AGRP regulatory elements. This will allow for the precise regulation of spatial and temporal control of PPARγ expression only in AgRP neurons upon tamoxifen administration.

In summary, the herein presented data show that chronic interference with the function of brain PPARγ affects energy balance only under certain dietary conditions and through specific neuronal populations. We show that POMC neurons are particularly sensitive to modulation of PPARγ activity. These observations give support to the notion that cellular adaptations in POMC neurons, driven by PPARγ, represent critical components in the regulation of metabolic homeostasis.
Figure 4.1. RT-qPCR Analysis of Brain Punches from POMC^{Cre}/PPARγ-P467L and POMC^{Cre}/PPARγ-WT Mice. Expression of transgenic hPPARγ is detectable only in double transgenic mice POMC^{Cre}/PPARγ-P467L or WT mice in the arcuate nucleus but not in any other punch samples (red circles). Nestin-PPAR-P467L+/- punches serve as positive and Nestin-PPAR-P467L-/- as well as single transgenic GAC-PPAR-P467L-serve as negative controls.
Figure 4.2. RT-qPCR Analysis of Transgene Expression in Peripheral Tissues from POMC<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L Mice. Levels of expression of transgenic hPPAR<sub>γ</sub> mRNA in POMC<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L and littermate control mice, n=3 per group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>hPPAR&lt;sub&gt;γ&lt;/sub&gt; in POMC-P467L (CT values ± SEM)</th>
<th>hPPAR&lt;sub&gt;γ&lt;/sub&gt; in Littermate Controls (CT values ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>31.2 ± 0.7</td>
<td>33.1 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>29.4 ± 1.2</td>
<td>34.0 ± 2.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>28.5 ± 0.2</td>
<td>34.5 ± 3.0</td>
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<tr>
<td>Spleen</td>
<td>27.8 ± 0.8</td>
<td>28.3 ± 2.9</td>
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<tr>
<td>Subcutaneous Adipose</td>
<td>25.3 ± 1.4</td>
<td>27.9 ± 2.8</td>
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<tr>
<td>Perigenital Adipose</td>
<td>26.9 ± 0.9</td>
<td>27.5 ± 2.8</td>
</tr>
<tr>
<td>Brown Adipose</td>
<td>27.4 ± 2.7</td>
<td>30.0 ± 2.3</td>
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Figure 4.3. Peripheral Administration of Rosiglitazone Leads to Increased Body Weight in POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-WT but not in POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-P467L Mice. A. The effect of rosiglitazone administration (28 mg/kg, i.p. for 5 days) on body weight (BW) in lean POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-WT female mice exposed to 45% HFD. The change in BW before (vehicle treated) and after rosiglitazone treatment reveals that POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-WT are significantly more sensitive to the effects of the drug than their littermate controls. B. The effect of rosiglitazone administration (28 mg/kg, i.p. for 5 days) on BW in lean POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-P467L female mice exposed to 45% HFD. The change in BW before (vehicle treated) and after rosiglitazone treatment shows that rosiglitazone failed to increase BW in POMC\textsuperscript{Cre}/PPAR\textsubscript{\gamma}-P467L mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.4. Twenty-five Weeks of 60% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in POMC<sup>Cre</sup>/PPARγ-P467L Mice. Neither male (A) nor female (B) POMC<sup>Cre</sup>/PPARγ-P467L mice show difference in body weight accumulation compared to littermate controls. Data in the graphs are expressed as the mean ± SEM.
Figure 4.5. POMC<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L Male Mice Show No Difference in Leptin Sensitivity after 25 Weeks of 60% HFD Treatment. Effect of vehicle (A and C) or leptin (1.0mg/kg, twice daily) (B and D) on food intake (A and B) and body weight change (A and D) in POMC<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L male mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.6. Twenty-five Weeks of 60% HFD Treatment Results in no Difference in Body Composition in A. Male and B. Female POMC\textsuperscript{Cre}/PPAR\textgamma-P467L mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.7. Twenty-five Weeks of 60% HFD Treatment Results in no Difference in Fasting Glucose Levels in A. Male and B. Female POMC$^{Cre}$/PPAR$\gamma$-P467L mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.8. Twenty-five Weeks of 60% HFD Treatment Results in no Difference in Organ and Adipose Tissue Weights in A. Male (n=11-14/group) and B. Female POMC\textsuperscript{Cre}/PPAR\textgamma-P467L (n=7-12/group) mice. Data in the graphs are expressed as the mean ± SEM. WAT, white adipose tissue; BAT, brown adipose tissue.

### A.

<table>
<thead>
<tr>
<th>Organ Masses</th>
<th>Littermate Controls</th>
<th>POMC\textsuperscript{Cre}/PPAR\textgamma-P467L</th>
<th>T Test</th>
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</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3.167 ± 0.1854</td>
<td>3.219 ± 0.1944</td>
<td>p = 0.8495</td>
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<tr>
<td>Heart</td>
<td>0.2140 ± 0.0064</td>
<td>0.2236 ± 0.0092</td>
<td>p = 0.3844</td>
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<td>Kidney</td>
<td>0.4693 ± 0.0139</td>
<td>0.4891 ± 0.0218</td>
<td>p = 0.4233</td>
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<tr>
<td>Spleen</td>
<td>0.1387 ± 0.0080</td>
<td>0.1327 ± 0.0071</td>
<td>p = 0.6035</td>
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### B.

<table>
<thead>
<tr>
<th>Organ Masses</th>
<th>Littermate Controls</th>
<th>POMC\textsuperscript{Cre}/PPAR\textgamma-P467L</th>
<th>T Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.473 ± 0.1260</td>
<td>1.800 ± 0.2480</td>
<td>p = 0.2050</td>
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<tr>
<td>Heart</td>
<td>0.1758 ± 0.012</td>
<td>0.1629 ± 0.004</td>
<td>p = 0.3654</td>
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<tr>
<td>Kidney</td>
<td>0.3458 ± 0.012</td>
<td>0.3543 ± 0.018</td>
<td>p = 0.7012</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.1175 ± 0.010</td>
<td>0.1286 ± 0.021</td>
<td>p = 0.5957</td>
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</table>

### Adipose Masses

<table>
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<tr>
<th>Adipose Masses</th>
<th>Littermate Controls</th>
<th>POMC\textsuperscript{Cre}/PPAR\textgamma-P467L</th>
<th>T Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous WAT</td>
<td>2.773 ± 0.1981</td>
<td>3.209 ± 0.2842</td>
<td>p = 0.2139</td>
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<tr>
<td>Perigenital WAT</td>
<td>2.888 ± 0.3202</td>
<td>3.384 ± 0.3198</td>
<td>p = 0.3228</td>
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<tr>
<td>Interscapular BAT</td>
<td>0.2408 ± 0.305</td>
<td>0.2743 ± 0.046</td>
<td>p = 0.5706</td>
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Figure 4.9. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Treatment Leads to Increased Body Weight (BW) in POMC<sup>Cre</sup>/PPARγ-P467L Male but not Female Mice. A. Male POMC<sup>Cre</sup>/PPARγ-P467L mice show significant increase in BW in response to low fat control diet compared to littermate controls. B. The effect is sex-dependent and not observed in female POMC<sup>Cre</sup>/PPARγ-P467L mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.10. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Treatment Leads to Increased Fat Mass in POMC\textsuperscript{Cre}/PPAR\textgamma-P467L Male Mice. 
A. BW post 25 weeks of control diet treatment. B. Fat mass. C. Lean mass and D. % fluid mass in POMC\textsuperscript{Cre}/PPAR\textgamma-P467L males as measured by Nuclear Magnetic Resonance. Data in the graphs are expressed as the mean ± SEM.
Figure 4.11. Twenty-five Weeks of 10% Fat, Isocaloric-Match Control Diet Results in No Difference in Body Composition of POMC<sup>Cre</sup>/PPAR<sub>γ</sub>-P467L Female Mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.12. POMC<sup>Cre</sup>/PPARγ-P467L Male Mice Show No Difference in Leptin Sensitivity after 25 Weeks of 10% Fat Isocaloric-Match Control Diet. Change in food intake (A) or body weight (B) in response to vehicle or leptin (1.0 mg/kg) administration in POMC<sup>Cre</sup>/PPARγ-P467L male mice. Data in the graphs are expressed as the mean ± SEM.
Figure 4.13. No Difference in the Level of Expression of Energy Balance Regulating Genes in the Mediobasal Hypothalamus of POMC$^{Cre}$/PPARγ-P467L Males and Littermate Control Mice after 25 Weeks of 10% Fat Isocaloric-Match Control Diet. Data in the graphs are expressed as the mean ± SEM.
Figure 4.14. Interference with PPARγ in POMC Neurons Does Not Lead to Changes in Energy Expenditure or Locomotor Activity. Neither EE (A) nor locomotor activity (B) differ between POMC\textsuperscript{Cre}/PPARγ-P467L male mice and their littermate controls after 13 weeks of 10% fat diet treatment. Data in the graphs are expressed as the mean ± SEM.
Figure 4.15. Twenty-five Weeks of 60% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in POMC<sup>Cre</sup>/PPARγ-WT Mice. Neither male (A) nor female (B) POMC<sup>Cre</sup>/PPARγ-WT mice show difference in body weight accumulation compared to littermate controls. Data in the graphs are expressed as the mean ± SEM.
Figure 4.16. Twenty-five Weeks of 60% High Fat Diet (HFD) Treatment Fails to Induce Changes in Body Weight in AgRP<sup>C<sub>re</sub></sup>/PPARγ-P467L Mice. Neither male (A) nor female (B) AgRP<sup>C<sub>re</sub></sup>/PPARγ-P467L mice show difference in body weight accumulation compared to littermate controls. Data in the graphs are expressed as the mean ± SEM.
CHAPTER V.

OVEREXPRESSION OF PPARγ IN THE BRAIN LEADS TO SEVERE MICROCEPHALY, GROWTH RETARDATION AND EARLY DEATH

Introduction

The peroxisome proliferator activated receptors (PPARs) belong to the nuclear receptor family of ligand-activated transcription factors. Three PPAR isotypes, PPARα, PPARβ/δ and PPARγ, have been identified in vertebrates and are highly conserved among species. These receptors play important roles in various aspects of development such as differentiation of adipose tissue, brain, placenta and the skin. PPARγ in particular is referred to as “a master regulator” of adipogenesis, driving the switch from undifferentiated to differentiated fat cell phenotype. Braissant and Wahli describe the unique nervous system expression patterns of the three isotypes during rodent development. PPARα is characterized by a transient expression peak in the entire central nervous system (CNS) around E13.5 stage. In the adult CNS PPARα expression diminishes to very low levels and becomes restricted to olfactory bulbs, hippocampus, cerebellum, and retina. In contrast, a high level of expression of PPARβ/δ is detected as early as E8.5 in the embryonic ectoderm and at E11.5 in the rat CNS. In adult rodents PPARβ/δ is ubiquitously and abundantly expressed, with tissues such as brain, adipose, and skin exhibiting particularly high levels of mRNA. PPARβ/δ appears to be highly expressed in oligodendrocytes both in culture and in vivo. Interestingly, stimulation of primary glial cultures with PPARβ/δ agonist induces the formation of processes and huge membrane sheets typical of differentiated oligodendrocytes. Consistent with a role for PPARβ/δ in the myelination process, PPARβ/δ-null mice exhibit alteration in the myelination of the corpus callosum. In addition, these mice are characterized by impaired
development evidenced by the significantly lower fetal and postnatal weights of the null compared to wild-type controls\textsuperscript{60}.

During rodent development, similarly to PPAR\textalpha{}, PPAR\textgamma{} mRNA expression is first detected on E13.5 but is restricted mainly to the hindbrain. This level of expression is also transient and by E15.5 it is greatly reduced. Interestingly no other tissues show PPAR\textgamma{} expression at this stage. At E18.5 PPAR\textgamma{} mRNA level is increased again but this time is detected only in brown adipose tissue\textsuperscript{76}. In the adult rodent brain PPAR\textgamma{} was detected at very low levels in the hippocampus, cerebellum as well as the retina\textsuperscript{77}. In addition, both PPAR\textgamma{} mRNA and protein are detected in the hypothalamus\textsuperscript{78}, which is consistent with the recently described role of brain PPAR\textgamma{} in control of energy homeostasis and metabolism\textsuperscript{79-82}. It appears that neurons account for the majority of PPAR\textgamma{} expression in the CNS\textsuperscript{78}.

Accumulating evidence suggests an important functional role for PPAR\textgamma{} in the differentiation of neuronal cells. Activation of PPAR\textgamma{} has been demonstrated to induce the expression of neurogenic differentiation factor (Neurod1), a basic helix-loop-helix transcription factor that plays a role in the development of the nervous system\textsuperscript{281}. Wada and colleagues\textsuperscript{77} demonstrated a critical role for PPAR\textgamma{} in the development of murine CNS during early embryogenesis via the control of proliferation and differentiation of embryonic neural stem cells (NSC). Stem cells derived from mice heterozygous for PPAR\textgamma{} (PPAR\textgamma{}+/−) fail to form neurospheres compared to wild-type clones. This is consistent with the observation that complete deficiency of PPAR\textgamma{} leads to disordered embryonic development and lethality at E10.0\textsuperscript{98} and suggests that PPAR\textgamma{} is critical for proper growth and normal neural development. Interestingly, PPAR\textgamma{} exhibits a biphasic action in NSC such that moderate activation of PPAR\textgamma{} pathway leads to proliferation and concurrent inhibition of NSC differentiation into neurons. Excessive activation of PPAR\textgamma{}, however, induces NSC death. Thus, PPAR\textgamma{} appears to tightly regulate the expansion and contraction of NSC mass in the developing CNS\textsuperscript{77}. However, Morales-Garcia and
colleagues$^{282}$ demonstrate that Pioglitazone-induced PPARγ activation increases neuronal differentiation of NSCs and inhibition of PPARγ abrogates this process. Perhaps the difference in experimental conditions and isolation of NSCs may account for the inconsistency in the outcomes achieved by the two groups. Further evidence supporting a role for PPARγ in enhancing terminal differentiation of NSC is found in the work of Ghoochani and colleagues$^{283}$ who demonstrated that PPARγ inhibition led to a decrease in the expression of terminal differentiation markers in differentiated neural cells. In addition, this group was able to show that PPARγ1, rather than PPARγ2, levels increase during induced neuronal differentiation of mouse embryonic stem cells. This increase in turn dropped in mature neurons$^{283}$. *In vivo* administration of PPARγ agonists such as pioglitazone and rosiglitazone, have been shown to increase cellular proliferation and differentiation in the subventricular zone (SVZ)$^{282}$, a major CNS source of neural stem cells in the adult mammalian brain$^{284}$. Finally, a most recent work by Bernal and colleagues$^{285}$ showed the presence of PPARγ in the mouse neural precursor cells from the SVZ. *In vitro* results demonstrate that PPARγ regulates proliferation and maintenance of undifferentiated precursor phenotype by modulation of Epidermal growth factor receptor levels in the adult NSCs. This work complements the function of PPARγ in embryonic mouse NSCs$^{77}$.

Consistent with its role in regulating neural precursor cell differentiation, PPARγ has also been demonstrated to play an important role in increasing dendritic spine densities$^{286}$ and inducing neuronal polarity$^{287}$. Brodbeck el al.$^{286}$, established that rosiglitazone treatment significantly increases dendritic spine densities in a dose-dependent manner in cultured cortical neurons from wild-type mice. This effect was completely abolished by GW9662, a PPARγ-selective antagonist, suggesting that the ability of rosiglitazone to increase spine density is due to activation of PPARγ. Most importantly, the authors show that rosiglitazone treatment rescues the significantly decreased dendritic spine density in cultured cortical neurons from a mouse model of Alzheimer’s disease$^{286}$. 
Quintanilla and colleagues show that troglitazone-induced PPARγ activation leads to enhanced axonal growth in hippocampal neuron culture. Addition of GW9662 completely blocked axon elongation, suggesting that this effect was mediated through the induction of PPARγ. Interestingly, the increase in axonal growth was also prevented by SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), indicating that the effect of PPARγ on neuronal polarity involves the activation of JNK pathway\textsuperscript{287}.

The aim of the present study is to evaluate the effects of conditional overexpression of PPARγ in the mouse nervous system. Transgene expression is achieved by utilizing Nestin promoter driven Cre-Lox technology. We show that mice overexpressing PPARγ have severe growth retardation and brain malformation compared to their littermate controls.

**Materials and Methods**

**Generation and Characterization of Conditional hPPARγ Transgenic Mice**

Neural overexpression of either wild type (WT) or mutant (P467L) form of hPPARγ was achieved by crossing CAG-PPAR-WT-T mice (described in Chapter2) with Nestin-Cre mice [B6.Cg-Tg(Nes-cre)1Kln/J], purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the University of Iowa. All animal procedures described below have been approved by the University of Iowa Institutional Animal Care and Use Committee. Mice were kept under standard laboratory conditions, with free access to food and water.

**Determination of Cre-Mediated Recombination in Brains of Nestin\textsuperscript{Cre}/PPARγ\textsuperscript{WT} Double Transgenic Mice by PCR**

DNA was extracted from whole brain samples using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted with 30.0 µL
water and the concentration determined using NanoDrop 2000c spectrophotometer. The following oligonucleotides were used in the PCR reaction: Forward (5’-GCA TGC ACA TAA CTT ACG GTA AAT GGC CC-3’), which anneals upstream from the left loxP site (Figure A) and Reverse (3’-ACA CCT GTG GAG AGA AAG GC-3’), which anneals downstream form the right loxP site. The expected sizes of the products were 1339bp for transgene product and 432 bp for recombined fragment. The amplification conditions were as follows: 94˚C for 5 min, 50˚C for 30 sec, followed by a final 7 min elongation step at 72˚C in an Applied Biosystems 2400 PCR system.

**Western Blotting for Transgenic PPARγ Protein**

Brain tissue was homogenized in a lysis buffer containing: 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA (pH 7.5), and 0.1% v/v SDS, with protease inhibitors (Thermo Scientific). Protein concentration was determined using the BCA assay (Pierce Protein Research Products). Protein lysates were separated by SDS-PAGE gel and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked for 30 min at room temperature with 5% BSA in TBS-T (tris-buffered saline, 0.1% Tween-20). PPARγ expression was determined using monoclonal anti-PPARγ C26H12, Cell Signaling Technology). Rabbit anti-β-actin was used as a loading control (ab8229, AbCam). Primary antibodies were diluted in 1% BSA/TBS-T to a final concentration of 1:1000 for anti-PPARγ and 1:10,000 for anti-β-actin, and incubated overnight at 4°C. Secondary antibodies were diluted in 5% milk/TBS to final concentration of 1:10,000 and applied for 1 hour at room temperature. Blots were treated with Pierce SuperSignal Western Pico chemiluminescence reagent for visualization.
**Quantitative Real Time PCR (qRT-PCR) Analysis**

One hundred microgram of brain tissue from Nestin\textsuperscript{Cre}/PPAR\textgamma-WT double transgenic mice was suspended in ice-cold Trizol (Invitrogen). Total RNA was isolated using RNeasy spin columns (RNeasy Mini Plus Kit, QIAGEN) following the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop ND-1000. cDNA was generated using SuperScript (Invitrogen). qRT-PCR was performed using the TaqMan (applied Biosystems) gene expression assay from 10.0 nanogram cDNA in a total volume of 10.0 microliters following the manufacturer’s recommendations. The assay numbers for TaqMan (Applied Biosystems) probes were the following: Mm99999915_g1 (mouse GAPDH) and human PPAR\textgamma (Hs01115513_m1). The expression of tdTomato (Forward: 5’- CAC CAT CGT GGA ACA GTA CG-3’ and Reverse: 5’-GCG CAT GAA CTC TTT GAT GA-3’) was determined using SYBR green (Bio-Rad) according to the manufacturer’s protocol. Mouse GAPDH was used as an internal control.

**Histological Analysis of Brain Tissue**

Following extraction brains were immersion fixed in 4% paraformaldehyde in PBS for 48h at 4°C. The tissue was then cryosectioned to 14 μm thick coronal sections and mounted on slides. Nissl staining was performed by incubating the slides consecutively in the following solutions: PBS 1X, distilled deionized (dd) H\textsubscript{2}O, 70 % EtOH, 80% EtOH, 95% EtOH and 100% EtOH for 5 min. The slides were then rinsed in 100% EtOH for 10 min and incubated two times in xylene for 20 min. After two rinses in 100% EtOH for 1 min each, the slides were air dried. Cresyl Violet solution was prepared as follows: 0.5g of Cresyl Violate Acetate (Sigma) was dissolved in 80 mL dd H\textsubscript{2}O and 20 mL 100% EtOH, ripened for 48 hours and activated with of glacial acetic acid [15 drops per 45 mL of solution] for final pH of 3.5 to 3.7 before use. Slides were incubated in Cresyl Violet for
1 hour at room temperature and differentiated twice in 95% EtOH as well as 100% EtOH. Following a brief rinse in xylene (3 times 1 min each) the slides were coverslipped with Permount (Fisher Scientific) and dried overnight. The slides were then imaged on an Olympus BX-61 light microscope.

**Results**

*Nestin*<sup>Cre</sup>/PPARγ-WT Mouse Model Validation Studies

PCR analysis was performed on brain genomic DNA to validate the occurrence of transgene recombination in Nestin<sup>Cre</sup>/PPARγ-WT double transgenic mice. Following Cre-mediated excision of the LoxP-Stop-LoxP signal, the expected 432 bp fragment was observed only in the brain from a Cre+/PPARγ+ mouse (Figure 5.1). Expression of transgenic PPARγ-WT protein was then assessed in the brains of Nestin<sup>Cre</sup>/PPARγ-WT and littermate control mice. PPARγ protein is detected only in mice double positive for Cre-recombinase and PPARγ-WT transgene. This also confirms the absence of leaky expression of the transgene in PPARγ-WT-positive but Cre-negative control mice (Figure 5.2), corroborating the PCR results.

The function of the transgene was evaluated by assessing the level of expression of several canonical PPARγ targets - aP2/FABP4 and CD36 in the brains of Nestin<sup>Cre</sup>/PPARγ-WT mice, described in Chapter 2. As seen in Figure 2.14, overexpression of PPARγ-WT in the brain leads to significant upregulation of aP2 and CD 36 mRNA. This is not observed in brains from littermate controls or in mice expressing the dominant-negative mutant PPARγ-P467L. These results confirm the generation of a functional model with global nervous system expression of the PPAR-WT transgene.
Overexpression of PPARγ-WT in the Nervous System Leads to Significant Growth Retardation and Structural Brain Abnormalities

When born Nestin^{Cre}/PPARγ-WT are indistinguishable from the rest of the litter. Between P7 and P10 they weigh 1.0 to 1.5 g less than their littermate control mice. They seem to have difficulty feeding as a relative lack of milk in their stomachs, compared to littermates, was noted at P1-P5. At P21 the double transgenic mice are severely growth restricted (4.512 ± 0.1152 g vs. 8.397 ± 0.1973g in controls, p<0.0001, Unpaired Ttest) (Figure 5.3). On average, these animals do not survive past P21. The growth restriction is caused by severe microcephaly (Figure 5.4) and abnormal brain structural development. Histological analysis of Nestin-PPAR-WT brains reveals significant distortion of the brain architecture (Figure 5.5). The striatum and the hippocampi are severely aplastic and are replaced by prominent ventricular spaces with accentuated posterior horns. Thinning of the cortical layers accentuates the ventricular enlargement. Agenesis of corpus callosum (ACC) is present uniformly in all samples examined by histology so far with cingulate cortex also absent (n=10). There does not appear to be a myelination defect in these mice as white matter presence was confirmed with OsO₄ staining. The striatal and hippocampal hypoplasia seem to vary in severity, such that two distinct phenotypes can be distinguished. Of the 10 examined sections 6 had a severe phenotype with significant hippocampal and striatal hypoplasia and prominent colpocephaly (Figure 5.6 A). Four samples had the milder phenotype characterized with development of the striatum and absence of ventricular enlargement (Figure 5.6 B).

Discussion

Overexpression of PPARγ-WT in the mouse nervous system during early development leads to severe brain malformation, impaired postnatal growth and early death. Interestingly, similar body weight phenotype has been previously reported in
response to maternal administration of TZDs in a rat model. These drugs decrease triacylglycerolemia by activating PPARγ in adipose tissue and inducing the expression of target genes such as fatty acid transporters, adipocyte-binding protein, phosphoenolpyruvate carboxykinase and LPL. The presence of maternal hyperlipidemia is strongly correlated with normal accumulation of fetal adiposity and growth. The TZDs cross the placenta and have been shown to lead to retarded fetal development likely through their hypolipidimic effects on maternal metabolism. Interestingly, despite exposure to the drug only in utero, suppression of postnatal growth in the offspring is also observed. Perhaps this is related to the fact that activation of PPARγ has been implicated in the inhibition of tissue growth, a property that has incited interest in the use of these drugs as anti-cancer therapies.

However, none of the studies examining the effect of activation of PPARγ by TZDs on fetal and neonatal outcomes, investigated their consequences on nervous system development. To my knowledge, the model I have developed is the first report of growth retardation and brain abnormalities caused by the conditional overexpression of PPARγ-WT in the brain. This phenotype is consistent with the existing, but rather sparse, evidence of the important role PPARγ plays in differentiation of neuronal cells. To this extent physiological concentrations of TZDs, between 100nM and 3.0 µM, were found to stimulate neuronal stem cell (NSC) growth. This is associated with activation of the ERK pathway by phosphorylation and up-regulation of epidermal growth factor receptor and cyclin B protein levels. However, at concentrations above 10.0µM, there was a marked inhibition of NSC growth as well as differentiation into neurons. The latter was found to be mediated by activation of STAT3, which has been previously reported to be sufficient in maintaining mouse embryonic stem cells in undifferentiated state.

When PPARγ is overexpressed in the mouse nervous system, in the majority of cases examined in this study, the brain structure is severely abnormal and is notable for
agenesis of the corpus callosum (ACC), hippocampal and striatal hypoplasia, cortical layer thinning and prominent enlargement of the lateral ventricles. The lack of corpus callosum is associated with absence of the cingulate cortex, which gives rise to the neurons sending the first axons to cross the midline\textsuperscript{295}. But the myelination of these brains appears intact, suggesting that if neurons develop, they form projections and their axons get myelinated. Therefore the ACC defect is largely due to aberrant neuronal cell development in the cingulate cortex. This, along with the hypoplasia observed in other structures such as the hippocampus and striatum, could be explained with the timing of accumulation of PPARγ in neurons. The transgene is “activated” by the pan-neuronal promoter nestin very early in embryonic development at E10.5 when few neuronal structure have started to develop. For example, the start of neurogenesis for the transient fetal subplate zone is E10\textsuperscript{296}. This structure along with the marginal zone and the cortical plate form the precursors of the mammalian cerebral cortex. The neurogenesis of the other structures that appear grossly malformed in the Nestin\textsuperscript{Cre}/PPARγ-WT mice, consistently starts or peaks at or after E11\textsuperscript{296}. In addition, the first evidence of PPARγ mRNA expression in the embryonic rodent brain was reported to occur at E13.5 and to be restricted mainly to the hindbrain. Furthermore, its expression appears to be transient and greatly reduced by E 15.5\textsuperscript{76}. Thus, this mouse model and the existing evidence point toward a very tight regulation of the levels of PPARγ in the developing nervous system and insults such as premature overload with the protein result in failure of precursor neurons to differentiate normally.
Figure 5.1. Representative PCR Analysis for Recombination (Deletion) Event in Brains of Nestin\textsuperscript{Cre}PPAR\textgamma-WT Mice. A. Schematic diagram for the location of primers used to detect recombination. LoxP-Stop-LoxP sequence is 907 bp, the unrecombined amplicon is 1339 bp. B. Only in the presence of Cre-recombinase the LoxP-STOP-LoxP sequence is excised allowing for detection of a 432 bp fragment as seen in mouse 8 (red circle). Mice 1 and 6 are PPAR\textgamma-positive and Cre-negative, and thus demonstrate presence of 1339 bp fragments. Tg, purified transgene serves as a positive control.
Figure 5.2. Representative Western Blot Demonstrating Overexpression of Transgenic PPARγ-WT Protein in the Brain of Nestin$^{\text{Cre}}$/PPARγ-WT Mouse. PPARγ is expressed only in Nestin-PPAR-WT double transgenic mice (30.0 microgram of protein loaded per lane). Positive control: HEK293T cells transfected with plasmid carrying PPARγ-WT transgene. β-Actin serves as loading control.
Figure 5.3. Overexpression of PPARγ-WT in the Brain Leads to Severe Postnatal Growth Restriction. A. Representative image showing size difference between Nestin$^{\text{Cre/PPAR-WT}}$ and littermate control mice as 21 days of age. B. The body weight of the double transgenic mice (4.512 ± 0.1152g) is significantly lower compared to controls (8.397 ± 0.1973g).
Figure 5.4. Overexpression of PPARγ-WT in the Brain Leads to Severe Microcephaly. Representative images of whole-mount brains from Nestin\textsuperscript{Cre}/PPAR-WT and littermate control mice at 21 days of age. Significant microcephaly is noted in the double transgenic brains. Thinning of the cortex and accompanying enlargement of the lateral ventricles (black arrow) is also visible.
Figure 5.5. Overexpression of PPARγ-WT in the Brain Leads to Agenesis of the Corpus Callosum. A. Paxinos Mouse Brain Atlas image demonstrating typical brain organization at the level of the hippocampus. B. Nissl staining of a coronal section of a littermate control brain at the level of the hippocampus. The corpus callosum is seen as a lightly stained structure connecting the two hemispheres just ventral to the longitudinal cerebral fissure. C. Representative photomicrograph of a Nestin-CrePPAR-WT brain coronal section demonstrating severe disruption of brain morphology. There is a complete absence of the corpus callosum (asterisk), with significant hippocampal hypoplasia (triangle). In addition, there is thinning of the cortical layers as well as hypoplasia of the striatum leading to significant enlargement of the lateral ventricles. D. Coronal section (196.0µm caudal to section in C.) showing severe colpocephaly (arrows), absence of the corpus callosum (asterisk) and hippocampal hypoplasia (triangle). All sections are from mice 21 days of age. Brains were immersion fixed in 4% PFA, cryosectioned at 14.0µm thickness and stained with Nissl stain.
Figure 5.6. Abnormal Brain Phenotype Following Overexpression of PPAR-WT Is Characterized by Different Levels of Severity. Ten coronal sections were examined from P21 Nestin\textsuperscript{Cre}\textsuperscript{-}/PPAR-WT mice. A. Six sections were characterized by severe hippocampal and striatal hypoplasia as well as significant ventricular enlargement. B. Four sections demonstrated relatively mild phenotype. All sections were characterized by the absence of the corpus callosum.
GENERAL DISCUSSION

Interference with PPARγ in the Brain: Lessons from Transgenic Mouse Models

The discovery of the dominant-negative (DN) mutation in the PPARγ gene in patients with severe and early onset of insulin resistance, type 2 diabetes mellitus and hypertension\textsuperscript{134}, highlights the critical role of this transcription factor in the regulation of metabolic, glucose and cardiovascular homeostasis. This mutation is the result of a single nucleotide substitution which leads to a Proline (P) to Leucine (L) variant at codon 467 located in the ligand binding domain of PPARγ. Loss of ligand-binding ability, impaired transactivation, as well as antagonism of the wild type PPARγ at the regulatory regions of target genes\textsuperscript{134, 153}, lead to the overall loss-of-function phenotype in carriers of the P467L mutation\textsuperscript{134, 135}. Since in humans the mutant protein is expressed in all tissues of the body, it is difficult to define a particular organ system that is the critical mediator of the concurrently occurring metabolic and cardiovascular disturbances. Maeda and colleagues generated knock-in mice with an equivalent DN mutation (P465L) in PPARγ. Similarly to humans, mice heterozygous for DN PPARγ (P465L/+) develop hypertension and abnormal adipose tissue distribution\textsuperscript{157}. Interestingly, however, the mutant animals fail to develop insulin resistance even when exposed to high fat diet (HFD)\textsuperscript{157}, and instead required expression on a hyperphagic/leptin-deficient background to recapitulate the phenotype\textsuperscript{158}. These observations provide evidence that the hemodynamic effects of impaired PPARγ activity are independent of the reduced glycemic control and likely constitute a direct effect of the PPARγ mutation in cardiovascular tissues. Several groups tested the hypothesis that smooth muscle cell (SMC) PPARγ regulates blood pressure using a SMC-specific deletion of the receptor. The phenotypes observed, however, ranged from mild elevation of blood
pressure\textsuperscript{242} to hypotension\textsuperscript{237}. Using an alternative approach, namely introducing the disease-causing P467L mutation specifically in vascular smooth muscle cells, our laboratory has been able to recapitulate the hypertensive phenotype observed in human carriers\textsuperscript{244-246}. The discrepancy in the phenotypes resulting from genetic deletion versus direct interference with the function of PPAR\(\gamma\) in mice may be due to changes in the transcriptional regulation of PPAR\(\gamma\) target genes. Mechanistically, complete knockout of PPAR\(\gamma\), which does not occur naturally, would be expected to abrogate PPAR\(\gamma\)-mediated active repression. This is because, in the absence of a ligand, PPAR/RXR heterodimers, bound to corepressors, actively silence transcription at the PPAR Response Elements (PPREs)\textsuperscript{22}. Thus deletion of PPAR\(\gamma\) would be expected to result in higher basal expression of certain target genes due to loss of such active repression, as has been reported\textsuperscript{21, 64, 297}. Consequently, in certain contexts, genetic ablation of PPAR\(\gamma\) would mimic PPAR\(\gamma\) activation, although the induction of gene expression would not be expected to be as robust as ligand-mediated transactivation.

Early development of severe insulin resistance is another hallmark phenotype in patients with the P467L mutation in PPAR\(\gamma\). The circulating levels of insulin in these patients ranges from 127 to 703 mmol/l with a reference range of 0 ± 60 mmol/l\textsuperscript{134}. In addition, their sensitivity to insulin is severely impaired to as low as 5% of normal\textsuperscript{134}. Several genetic mouse models, with tissue specific deletion of PPAR\(\gamma\), provide insights into the role of different metabolic organs as mediating insulin sensitivity. Deletion of PPAR\(\gamma\) from mature adipocytes results in the presence of adipose and liver insulin resistance in mice when challenged with HFD\textsuperscript{124}. Ablation of adipocyte-specific PPAR\(\gamma\) during development results in severe impairment in insulin sensitivity in skeletal muscle, liver as well as white adipose tissue\textsuperscript{125}. Most recently the Lazar laboratory\textsuperscript{126}, arguing for a more specific approach, utilized adiponectin promoter driven Cre-recombinase (Adipoq-Cre) to ablate PPAR\(\gamma\) in fat. Compared to the other models described, adiponectin-driven deletion of PPAR\(\gamma\) leads to profound insulin resistance and hyperglycemia in these mice\textsuperscript{126}. 

142
Deletion of PPARγ in the muscle was accomplished by two separate groups with both models resulting in development of systemic insulin resistance\textsuperscript{127, 128}. Finally, liver-specific deletion of PPARγ has been reported in leptin-deficient (\textit{ob/ob}) mice\textsuperscript{129}. This model is characterized by exacerbation of insulin resistance in both adipose tissue and muscle. The described studies highlight the critical requirement of PPARγ in any of the insulin-sensitive tissues for maintenance of systemic glucose homeostasis. Interestingly, genetic ablation of PPARγ in this context recapitulates the severe insulin resistance characteristic of human carriers of the P467L mutation. Mechanistically, this could be due to the fact that PPARγ target genes are not regulated uniformly, such that some promoters may be actively repressed while others induced. For example, aP2 (adipocyte protein 2) and GyK (glycerol kinase) both involve binding of PPARγ/RXR to PPREs in their promoter regions. However, in the case of GyK, heterodimer binding is associated with recruitment of corepressors, whereas coactivators are recruited to aP2. Stimulation with TZD leads to marked upregulation of GyK through the induction of PGC-1α, which in turn facilitates the exchange of corepressors with coactivators at the PPRE\textsuperscript{90}.

To date there have been no reports of animal models expressing the PPARγ-P467L mutation in tissue-specific manner and only in insulin-sensitive organs. We generated a transgenic mouse model that overexpresses either the P467L mutant or wild type (WT) form of PPARγ in the CNS under the pan-neuronal promoter nestin. Based on recent evidence that brain PPARγ plays a critical role in control of energy balance\textsuperscript{80, 82}, we hypothesized that interference with PPARγ in the brain will have similar protective effects against diet-induced obesity (DIO). Indeed, when challenged with 60\% HFD the Nestin\textsuperscript{Cre}/PPARγ-P467L mice gain significantly less weight compared to their littermate controls. This is consistent with the phenotype observed in the brain PPARγ knockout mice. Interestingly, however, the lean mass is significantly decreased in Nestin\textsuperscript{Cre}/PPARγ-P467L, while their fat mass remained unchanged between genotypes. In addition, consistent with this, all major organs (liver, heart, spleen, and kidney) had significantly
reduced mass in Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L compared to their littermate controls, suggesting an overall growth development problem. Interestingly, the circulating levels of GH are normal in these mice. In our model, interference with neuronal PPAR$\gamma$ leads to significant hypoinsulinemia, which is particularly obvious during fed state and under conditions of HFD. Considering the importance of insulin as a growth regulator, it is likely that the decreased overall and lean body mass is due to impaired insulin production in these mice. Consistent with the low level of circulating insulin, the Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L are characterized by impaired glucose tolerance for up to 60 min after glucose bolus administration. However, the mice are able to clear the exogenous glucose load to levels similar to controls by 120 min post injection. It is notable that, the mice exhibit a significantly improved insulin tolerance test compared to controls. Thus, it is possible that the Nestin$^{\text{Cre}}$/PPAR$\gamma$-P467L have an impairment in the production or release of insulin from the pancreas, a hypothesis currently under investigation.

Our model stands in contrast with the insulin resistance phenotype of humans carrying the DN PPAR$\gamma$ mutations. These patients are characterized by severe fasting hyperinsulinemia as well as hyperglycemia consistent with the presence of significant insulin resistance. Of course the human DN PPAR$\gamma$ mutations affect every tissue in the body. Our model is neuron-specific and we have confirmed the absence of transgene activation in peripheral tissues. This gives us confidence to conclude that interference with the function of brain, and not extra-CNS, PPAR$\gamma$ leads to impairment in insulin production. In addition, it suggests the possibility that in patients the effect of the P467L mutation expressed in neurons may in fact be beneficial for insulin sensitization in the periphery. The extent of such a beneficial effect, however, is completely counteracted by the overwhelming insulin resistance induced by the expression of the mutant PPAR$\gamma$ protein in insulin-sensitive tissues. This, however, would have to be confirmed experimentally.

We also generated a mouse model in which we overexpressed the WT PPAR$\gamma$ in the CNS using nestin promoter-driven Cre recombinase. We hypothesized that these mice
will be significantly more sensitive to DIO and develop insulin resistance compared to the mice expressing the mutant PPARγ. We were surprised to find out that the NestinCre/PPARγ-WT mice are also significantly smaller in mass compared to their littermate controls. In addition, these mice tend to die shortly after weening (about 3 weeks of age). The profound disturbance in growth and early death phenotype in the NestinCre/PPARγ-WT mice are due to severe microcephaly present in this model. The brain architecture is characterized by significant distortion and hypoplasia of multiple regions, including hippocampus, striatum, cortex, leading to substantial enlargement of the lateral ventricles. Most strikingly, in all mice the agenesis of the corpus callosum was evident.

Summarizing the findings from the two mouse models, it is evident that the levels of PPARγ in the brain are very tightly regulated. Either “too little”, or “too much”, PPARγ in the CNS leads to failure to thrive. The mechanisms accounting for this effect in each case, however, are very different. The overexpression of the P467L mutant PPARγ in the brain leads to decreased levels of circulating insulin and impaired growth; while the saturation of the CNS with the WT PPARγ causes severe microcephaly and distortion of brain architecture incompatible with normal growth and development.

**New Therapeutic Interventions Targeting PPARγ**

Proper function of PPARγ is critical for the maintenance of metabolic homeostasis. In patients this is well demonstrated by the fact that loss or gain-of-function mutations in PPARγ are associated with the development of Metabolic Syndrome (MetS)-like phenotypes. Currently TZDs offer the most effective treatment for metabolic dysfunction. The ubiquitous expression of PPARγ, however, leads to a number of serious off-target effects associated with the use of these medications such as weight gain, fluid retention and edema, bone fractures, and bladder cancer, which have led to a significant decrease in prescription of these drugs. This has fueled interest in the
development of selective PPARγ modulators (SPPARMs) which lower glucose levels and improve insulin resistance without the above-mentioned adverse effects \(^{301}\).

Spiegelman’s group first reported that the inflammatory cytokine and free fatty acids (FFAs)-mediated phosphorylation of PPARγ at Ser273 in adipocytes is blocked by TZDs. This results in upregulation of specific genes, such as adiponectin, which promote insulin sensitization \(^{34}\). The group developed a new compound, SR1664, which selectively inhibits Ser273 phosphorylation \(^{302}\), with a resulting improvement in insulin resistance in DIO mice. Interestingly, there are minimal to no side effects in the SR1664-treated mice compared to a rosiglitazone-treated group. Two additional compounds, GQ-16 \(^{303}\) and F12016 \(^{304}\), have been reported to have similar properties, supporting the hypothesis that selective inhibition of Ser273 phosphorylation eliminates side effects but retains insulin-sensitizing characteristics. The molecular mechanism of PPARγ-Ser273 phosphorylation has also attracted attention over the years. Initially, it was proposed that the inflammatory cytokines or FFA-mediated phosphorylation at Ser273 in the adipose tissue occurs through a cyclin-dependent kinase 5 (CDK5)-conditional mechanism \(^{34}\). Surprisingly, fat-specific deletion of CDK5 exacerbates phosphorylation at Ser273 and increases obesity-mediated insulin resistance \(^{35}\). Proteomic studies reveal that the activity of extracellular mitogen-activated protein kinase (MAP) kinase (MEK) is increased in adipocytes from mice with fat-specific ablation of CDK5. In vitro data suggest that MEK phosphorylates PPARγ-Ser273 to a greater extent than CDK5. Treatment with MEK-specific inhibitor improves insulin resistance in both mice with adipocyte-specific CDK5 deletion and ob/ob mice.

PPARγ’s ubiquitous expression places the receptor at a unique position to exert direct biological effects on multiple organ systems, including cardiovascular, nervous, immune, and gastrointestinal. This, however, also accounts for the undesired effects observed with TZD-mediated activation. Modulation of PPARγ’s activity through targeted protein modifications raises the exciting possibility of tissue-selective activation and elimination of such off-target effects. Activation of PPARγ can induce “browning” of
white adipose tissue. Qiang et al., recently reported that one of the mechanisms accounting for this effect is the posttranslational modulation of PPARγ at Lys268 and Lys293 (PPARγ2). Deacetylation of these sites by the NAD-dependent deacetylase, SirT1 leads to an increase in selective brown adipose tissue genes, while decreasing white adipocyte gene markers associated with insulin resistance. The deacetylation of Lys268 and Lys293 by TZDs results in enhancement of energy expenditure and insulin sensitivity in adipocyte tissue. The processes of posttranslational modification of PPARγ have been most extensively studied in adipose tissue. Little is currently known whether similar alterations affect PPARγ’s function in other tissues including the nervous systems. Identification of mechanisms leading to tissue-specific changes in PPARγ activity can lead to the development of targeted treatments that are characterized by little or no deleterious effects.

The following important clinical consideration has emerged from my work: it is imperative to apply special care when considering PPARγ modulation therapy in vulnerable populations, particularly pregnant patients. The research findings presented in this dissertation show that early activation of PPARγ, specifically in the nervous system, may have deleterious effects on normal brain development and lead to growth retardation. Similarly, blocking the function of brain PPARγ may interfere with the establishment of normal metabolic homeostasis regulatory mechanisms.
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