THE ROLE OF ABI3 IN OBESITY AND METABOLIC REGULATION

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DEDICATION

In honor of Nancy and Larry Curtis, thank you for your example of leading with kindness, extending grace instead of judgment, and facing failure with humility and vulnerability.

In honor of my family and friends, thank you for helping me find the light in the dark moments and fanning the flame during the bright moments.

In honor of my mentors, thank you for pushing me when needed but constantly reminding me to center myself around the passion and joy in my work.

I wouldn't have made it to this point without your collective effort on my behalf. In honor of the vital impact you have all had on my life, this work is dedicated to you.

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Daniel Curtis Smith

THE ROLE OF ABI3 IN OBESITY AND METABOLIC REGULATION

Abelson Interactor Protein 3 is an adaptor protein involved in cytoskeletal remodeling. *ABI3* is predominantly expressed within mononuclear phagocytotic immune cells within the brain, such as macrophages, peripherally, and microglia. Until recently, little was known about the function of the ABI3 protein, and even less was known regarding its role in disease. Following the identification of a rare mutation within *ABI3* that increases the risk of developing Alzheimer's disease, our laboratory began to investigate the impact of deleting *Abi3* in mouse models. While we initially set out to investigate ABI3 in the context of neurodegeneration, we unexpectedly discovered that loss of *Abi3* led to obesity in mice. This discovery and the subsequent efforts to uncover the mechanisms by which loss of Abi3 induces obesity are the subject of this dissertation.

First, we demonstrate that deletion of *Abi3* leads to severe obesity in aged mice. We identified significant *Abi3*-dependent transcriptomic changes within the hypothalamus, but not adipose tissue, of these mice. These changes occurred within pathways related to immune function, and subsequent immunostaining revealed decreased microglia number and area within the mediobasal hypothalamus of $Abi3^{-/-}$ mice.

Next, we performed a longitudinal high-fat diet study to explore the impact of loss of *Abi3* on mouse body weight and metabolic regulation during chronic nutrient excess and control conditions. Intriguingly, we found that only female $Abi3^{-/-}$ mice exhibited increased body weight during high-fat diet feeding. Subsequent transcriptomics from the hypothalamus of female $Abi3^{+/+}$ and $Abi3^{-/-}$ mice from both high-fat and control diet

groups revealed cytoskeletal-related changes only in the obese, high-fat diet-fed female *Abi3*$^{-/-}$ mice. Follow-up immunostaining revealed decreased microglia coverage within the mediobasal hypothalamus of the obese, high-fat diet-fed female *Abi3*$^{-/-}$ mice.

While much remains to be explored regarding the precise role of ABI3 in the setting of energy balance regulation and obesity, our investigations revealed that loss of ABI3 is sufficient to induce obesity and appears to occur through altered microglia function within the hypothalamus. This dissertation represents a critical first step in the investigation of a novel regulator of obesity pathology.

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1.1 Introduction

In this chapter, we explore the intricate relationships between microglia, inflammation, obesity, and the ABI3 protein and consider the complex biological underpinnings of these interconnected domains through an extensive review of the literature. Obesity, a pervasive and multifaceted health challenge, will be considered from historical and modern perspectives, including the first studies linking the hypothalamus to obesity to the current understanding of the cellular and molecular pathology underlying this condition. Throughout, we will consider the role of the immune system in metabolic regulation, including concepts such as systemic energy balance regulation and obesity development. Central to this narrative is the role of the central nervous system (CNS), particularly the hypothalamus, in obesity. This narrative will illuminate the evolution of our understanding of the hypothalamus's critical role in regulating energy balance, including the role of inflammation and microglial activity within this region. Overall, the review of the literature provided in this chapter underscores the complexity and significance of CNS involvement in obesity, as well as the centrality of microglia to that involvement.

1.2 An Overview of Obesity

1.2.1 *A General Background on Obesity*

Obesity represents a significant healthcare challenge faced by modern society. This is, however, also an immensely complicated area of study. This is not only because the complex, interrelated biological, psychosocial, and systems-level factors that lead to

obesity are multifactorial and incompletely understood but also because the definition of obesity or whether it should even be considered a disease state are active subjects of debate. That said, it is clear that humans with markedly elevated body fat composition and body weight have substantially worse outcomes than those with less elevated weights.[1] For example, individuals who meet the definition of obesity as described by the World Health Organization as having a BMI greater than 30 have a dramatically increased risk of developing several of the most lethal and debilitating diseases, such as cardiovascular disease, type 2 diabetes, and fatty liver disease.[2] Additionally, the classification of obesity as a unique disease state is bolstered by the unique pathological responses to severely elevated adipose tissue and body weight that subsequently drive further weight gain and prevent effective and maintainable weight loss. This classification is also critical, as it allows modern society to collectively recognize obesity as a condition of metabolic dysfunction and not a condition related to an individual's willpower or moral failings, which is a critical stigma for those in the medical and obesity research community to combat.

At its core, obesity can appear to be a fairly simple issue in which individuals continually intake more energy than they expend, ultimately leading to excess energy storage. This relationship between energy intake and energy expenditure is critical to the regulation of body weight and is often referred to as energy balance. In reality, while this fundamental energy balance is central to the development of obesity, the full scope of the driving factors that underlie obesity are complex and interconnected. Furthermore, the prevention and treatment of obesity will likely require both individual treatment and public health approaches. Given this broad scope, this review will focus on the cellular

and molecular aspects of obesity pathology and explore potential mechanisms that drive this controversial yet undeniably important disease state. Furthermore, this overall work is focused on the immune-related ABI3 protein and, more specifically, ABI3-related functions within microglia; therefore, particular focus will be given to the central nervous system and immune function as they apply to the maintenance of systemic energy balance and the development of obesity.

1.2.2 *The Central Nervous System and Obesity*: *A Historical Perspective*

While both the causes and consequences of obesity have been studied across nearly all physiological systems, the brain and spinal cord, collectively known as the central nervous system (CNS), may be among the most consequential physiological systems involved in the development and progression of obesity. This may initially seem counterintuitive, as the effects of obesity are more immediately apparent in peripheral tissues. These effects include the accumulation of excess adipose tissue, cardiovascular impairment, musculoskeletal dysfunction, and systemic metabolite and hormonal imbalances.[2,3] However, the central nervous system is the physiologic system that integrates all peripheral signals and subsequently modulates the physiologic and behavioral processes that contribute to overall metabolic homeostasis and energy balance. It is not surprising, therefore, that the obesity and metabolic disease field has increasingly focused on the CNS over the last three decades, culminating in a burgeoning, highly sophisticated, interdisciplinary field, especially over the last decade in particular.[4,5]

Undoubtedly, one region of the CNS, known as the hypothalamus, plays the most central role in the regulation of energy balance and systemic metabolism. Long before the more sophisticated CNS–Obesity research field mentioned above began, a small number

of pioneering experiments focused on the hypothalamus laid the stage for an entirely new view of energy balance regulation. Even before these experiments, however, autopsy reports dated back to as early as 1840 revealed that a patient with severe obesity exhibited a large mass on their "hypophysis" or pituitary.[6] Importantly, causation was not suggested in this report. However, this was the first reported observation of a pituitary/hypothalamic adjacent mass in a severely obese patient. Intriguingly, as autopsies became more prominent in the mid- to late 1800s, additional pituitary and hypothalamic lesions began to be reported in conjunction with obesity; among the various lesions observed, perhaps the most central to this dissertation, were the earliest observations of inflammatory lesions within this brain region.[7]

The next major step toward identifying the centrality of the hypothalamus in individuals with obesity came in 1912 from Cushing and colleagues at Harvard Medical School. Due to the aforementioned observations of pituitary masses and lesions being associated with a wide range of health conditions, including obesity, Cushing developed a novel surgical technique for (what he believed to be) targeted lesioning of the pituitary region in canines and observed that this procedure was sufficient to induce obesity and wide-ranging endocrine abnormalities.[8] This key finding was followed by perhaps the most critical study on the origin of CNS obesity. In this study, which was reported in 1921, Bremer and Barley from Harvard Medical School reported similar lesions to Cushing but focused on a region of the brain that had only been recently identified, the hypothalamus. They reported that "even minute" surgical damage to the lower region of the hypothalamus and pituitary could result in polyuria, endocrine dysfunction, and obesity but that the exact location and extent of the injury dictated the extent and severity

of these pathologies.[9] This was the first study to directly report that hypothalamic damage, in and of itself, can induce obesity.

The next major leap in the hypothalamic obesity research field was in 1943, when Hetherington and colleagues from Northwestern University Medical School reported that damage to the hypothalamus, independent of the pituitary gland, could induce obesity.[10,11] Hetherington's study was the first to demonstrate that lesions to the "ventromedial" aspect of the hypothalamus in rats result in hyperphagia and subsequent obesity, thus indicating a central role for the hypothalamus in energy balance regulation.[10] Furthermore, in 1951, Anand and Brobeck from Yale University School of Medicine showed a related but converse finding in which they observed that lesions to the lateral portion of the hypothalamus resulted in cessation of food intake and subsequent starvation in both rats and cats.[12] Over two decades later, in 1975, Keesey and Powley from the University of Wisconsin and Yale University, respectively, presented an integrated view of the hypothalamus as the region of the brain that determines body weight and body fat set point.[13] In this seminal work, the authors provide a model for body weight regulation as being jointly regulated by both the ventromedial region of the hypothalamus, which results in hyperphagia when lesioned, and the lateral hypothalamus, which results in aphagia when lesioned.[13] While this may seem to be common sense to modern obesity or CNS researchers, at the time, it was only accepted that the hypothalamus impacted feeding behavior. The concept that the hypothalamus was responsible for the body weight "set point" of an organism was entirely novel at the time, and this work set the stage for years to come of hypothalamic energy balance research.

Following these seminal works, there were many critical developments in our understanding of the neurobiology of obesity that arose alongside the development of novel neuroscience techniques, such as positron emission tomography (PET) and magnetic resonance imaging (fMRI) neuroimaging modalities. However, perhaps the most influential finding in the CNS obesity research field was the identification of leptin in 1994 by Friedman and colleagues at the Howard Hughes Medical Institute.[14] The discovery of leptin closed the chapter on a remarkable but incomplete discovery over two decades prior, which revealed that parabiosis (shared blood circulation) in ob/ob mice caused by "obese gene" deletion (ob/ob) and "diabetes gene" deletion (db/db) led to hypoglycemia and loss of body weight.[15] It is important to note that these mouse models still serve as key assets for obesity and diabetes research. Friedman's work demonstrated that this loss of body weight in ob/ob mice was due to the absence of leptin (the "obese gene") in these mice and that the diabetic mice expressed leptin but lacked the leptin receptor (the "diabetes gene"). Together, these studies definitively demonstrated that leptin, a circulating factor prominently expressed in adipose tissue, could regulate energy balance and body weight by binding receptors within the CNS, specifically the hypothalamus. This finding resulted in a dramatic shift in the overall focus of the obesity field on the CNS. Following the identification of leptin and the identification of human patients with leptin deficiencies who exhibit severe obesity, there was initially substantial excitement about the potential for leptin to be used as a treatment for obesity.[16] This excitement was sadly not warranted, as future studies could reveal that typical obese patients are not responsive to exogenous leptin; therefore, its utility as a treatment is unfortunately limited.[17] However, the increased attention on how the hypothalamus and

CNS at large contribute to obesity and the role that leptin plays within these regions has formed the springboard for a large portion of today's sophisticated obesity neuroscience research.

1.2.2 *The Central Nervous System and Obesity*: *Mechanisms of Energy Balance Regulation*

The mechanisms that lead to the development and progression of obesity are far more complex than the reductionist "calories in, calories out" framework that is sometimes espoused when discussing this metabolic disease. This point is strikingly clear when we consider the CNS mechanisms underlying obesity. Throughout this section, substantial strides have been made in understanding how the brain, and the hypothalamus in particular, contributes to the pathology of obesity. However, it is also clear that much remains to be discovered as to the exact mechanisms by which these regions drive obesity.

As demonstrated by the various historical studies outlined in the previous section, the hypothalamus is a key region of the brain involved in energy balance regulation. Furthermore, these studies revealed that damage to this region results in metabolic dysfunction and can drive the development of obesity. As additional research has progressed toward understanding the role of the hypothalamus in metabolic disease, the centrality and importance of the hypothalamus in energy balance regulation, obesity, and metabolic disease overall have only been further solidified. Importantly, other regions of the brain and CNS are critical components of the energy balance regulation system, and dysfunction of any of these components can lead to metabolic disease.[5] A truly comprehensive review of how the CNS regulates energy balance and contributes to

obesity pathology could be the subject of an entire textbook; therefore, the information provided here is intended to provide a zoomed-out overview of how the hypothalamus and several other selected brain regions maintain appropriate body weight. Furthermore, given the relevance of the mediobasal aspect of the hypothalamus to energy balance regulation and the findings of this dissertation, the majority of this section will focus on that portion of the hypothalamus.

First, the hypothalamus is a small region located deep within the brain that, despite its small size, is often colloquially referred to as the master control center for homeostasis.[18] This small brain region contains various groupings of neurons, referred to as nuclei, that act to regulate distinct but essential homeostatic functions, such as the regulation of the endocrine system, maintenance of appropriate internal temperature, regulation of the sleep cycle and circadian rhythm, and, critically for this dissertation, the regulation of energy balance through feeding behavior and energy expenditure modulation.[5,18,19] One of the most essential hypothalamic nuclei for the regulation of appropriate energy balance is a region located in the mediobasal aspect known as the arcuate nucleus. The arcuate nucleus lies next to the median eminence of the brain and is a circumventricular organ; moreover, the capillaries in this region allow the passage of far more peripheral signals than do capillaries in other regions where the blood–brain barrier is more restricted.[20] This facilitates the ability of the arcuate nucleus to regulate energy balance, as it enables this region to readily sense the levels of circulating metabolites, hormone signals, and possibly even signals from entering peripheral immune cells, although the latter point is contested by some.[20,21]

The arcuate nucleus regulates energy balance through the coordination of neuronal signals that lead to increased food intake and decreased energy expenditure during times of energy deprivation and decreased food intake and increased energy expenditure during times of excess energy.[22] The full scope of the mechanisms by which the arcuate nucleus regulates energy balance is extensive and still an area of active investigation. However, the mechanisms by which the arcuate nucleus regulates energy balance and energy intake in particular are among the most studied topics in the CNS obesity research field.[20] The arcuate nucleus is established to regulate food intake, at least in part, by signaling conducted via appetite-suppressing (anorexigenic) or appetite-stimulating (orexigenic) neurons.[20] These first-order neurons sense overall metabolite and metabolic signaling hormone levels and subsequently send appropriate response signals to different nuclei of the hypothalamus and other brain regions to maintain proper energy balance.[23]

Specifically, the anorexigenic neurons of the arcuate nucleus reduce energy intake through the release of neuropeptide signals known as cocaine-and-amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC).[4] POMC is further broken down into several shorter transcripts, including adrenocorticotropic hormone (ACTH), which is further processed into α-MSH, a key anorexigenic signaling molecule.[24] The POMC/CART-expressing neurons transmit signals via projections to various regions of the hypothalamus and brain, including the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), lateral hypothalamus (LH), parabrachial nuclei (PBN), and bed nucleus of the stria terminalis (BNST).[20,25] Via these projections, POMC/CART neurons signal to second-order neurons through the release of α-MSH, which activates melanocortin

receptor-expressing neurons (MC3R and MC4R).[26] Among these projections, those terminating in the PVN are especially critical for energy intake regulation. When MC3/4R neurons are activated, a coordinated response in which food intake decreases, sympathetic outflow/energy expenditure, and catabolic metabolic processes increase.[22,26] Taken together, the anorexigenic neurons of the arcuate nucleus drive organisms toward a negative energy balance during times of energy excess, prominently through signaling to melanocortin neurons within the hypothalamus and other brain regions.

Conversely, the orexigenic neurons of the arcuate nucleus activate during periods of negative energy balance through the release of the neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY). First, AgRP acts as an antagonist of melanocortin receptors, thus preventing activation of the anorexigenic melanocortin pathway described above. The signaling pathway of NPY is complex and acts across multiple receptors across multiple hypothalamic and other brain regions but overall acts to stimulate energy intake and decrease energy expenditure.[27] Furthermore, the orexigenic effect of AgRP signaling is absent without concurrent NPY signaling, indicating the importance of concurrent AgRP and NPY signaling.[28] Taken together, these findings suggest that the orexigenic neurons of the arcuate nucleus drive an organism toward a state of positive energy balance during an energy deficit, partially through the inhibition of the melanocortin signaling pathway, among other pathways.

While the above descriptions of the signaling pathways occurring within the arcuate nucleus paint a useful picture of how this region regulates energy balance, it is critical to note that the whole scope of energy balance regulation by this region is far more complex and involves the integration of a diverse array of peripheral and central

signals. For example, AgRP neurons are strongly inhibited by leptin and insulin, both of which are signals of a positive energy balance.[20] In the following section, we will discuss how inflammatory signaling can dramatically impact the ability of this region to regulate energy balance. Altogether, the arcuate nucleus contains a variety of neurons and nonneuronal cells that collectively enable this region to respond to metabolic signaling hormones such as leptin, insulin, and incretins, as well as peripheral metabolites such as glucose and fatty acids, which coordinate across multiple brain regions.[5] Overall, the above discussion is intended to provide an overview of well-established mechanisms by which the arcuate nucleus regulates energy balance while garnering an appreciation of its complexity, which extends beyond the scope of this review.

In addition to the arcuate nucleus, the mediobasal aspect of the hypothalamus, often referred to simply as the mediobasal hypothalamus (MBH), also contains the ventromedial nucleus (VMN). The VMN is another key region for proper regulation of energy balance; therefore, it is not surprising that this region receives extensive projections from the arcuate nucleus.[5] In fact, the VMN was one of the first nuclei of the hypothalamus to be linked to proper body weight regulation. Specifically, lesion studies demonstrated that damage to this region eliminates satiety in animals and leads to massive obesity, as discussed in the prior section. The VMN is therefore considered to be a critical satiety center; however, this region has also been demonstrated to modulate sympathetic outflow and induce brown adipose tissue thermogenesis (a form of energy expenditure that is present in humans and the dominant form of energy expenditure in mice).[29] Additionally, neurons within the VMN produce BDNF, a critical neurotrophic factor that has been demonstrated to drive anorexigenic melanocortin signaling.[30] This

region also contains neurons that are responsive to metabolic and sex hormone signals such as insulin, estrogen, and leptin; furthermore, subsequent downstream signaling by these neurons can modulate glucose levels, metabolic rate, and food intake.[19,31] Together, the arcuate and ventromedial nuclei of the mediobasal hypothalamus respond to a diverse array of circulating and central signals to coordinate the intricate, dynamic responses necessary for the maintenance energy balance.

In addition to the above descriptions of some of the mechanisms by which the mediobasal hypothalamus regulates energy balance, it is critical to note that there are other critical systems that will not be covered in depth here. For example, highly palatable foods are capable of inducing a reward response, similar to that observed in mammals exposed to drugs of abuse. This addictive, pleasurable eating is often referred to as "hedonic feeding". Hedonic feeding stimulates reward centers of the brain, such as the ventral tegmental area and nucleus accumbens.[32] Additionally, numerous complex neuronal pathways participate in the regulation of sympathetic outflow, food intake, and systemic metabolism; however, the information covered above provides a general overview of how the CNS participates in the maintenance of proper body weight, with particular emphasis on a brain region critical to the work of this dissertation, the mediobasal hypothalamus. While the above information outlines how the CNS works to properly maintain energy balance, dysfunction within this system can induce obesity. One prominent source of such dysfunction, which also represents the area of study of this dissertation, is inflammation and impaired immune cell function.

1.3 Inflammation and Obesity

1.3.1 *An Overview of Inflammation in Obesity*

The proper functioning of the immune system is essential for proper maintenance of homeostasis, including the regulation of systemic metabolism. In fact, dysfunction driven by immune cells has been extensively linked to obesity pathology over the past three decades.[33] One of the earliest connections between the immune system and obesity came from a series of studies in the 1960s that evaluated the bloodwork of obese patients. These studies were the first to identify the elevation of systemic inflammatory markers during obesity, but not much was known about this association.[34] This connection was more strongly considered following the discovery of elevated levels of tumor necrosis factor-α (TNFα) within the adipose tissue of multiple mouse models of obesity. This finding was critical because TNFα, a potent proinflammatory cytokine, inhibits multiple pathways downstream of insulin signaling; therefore, this study connected obesity-derived inflammation to type 2 diabetes pathology.[35] Following this study, it was discovered that macrophages infiltrate and expand within adipose tissue during obesity and that these cells drive the proinflammatory response within adipose tissue.[36] These major findings laid the groundwork for more extensive research into the inflammatory mechanisms occurring during obesity and how modulating those immune pathways impacts the pathophysiology of obesity.

Multiple studies have shown that obesity and nutrient excess can lead to the development of widespread chronic, low-level inflammation. This low-level inflammation can lead to dramatic repercussions if not resolved, including the development of insulin resistance, type 2 diabetes, atherosclerosis, cardiovascular

disease, and nonalcoholic fatty liver disease.[37,38] The mechanisms underlying this obesity-driven inflammation are still a matter of active debate. Its origins are likely multifactorial, as a vast array of immune pathways, such as those involving NF-κB, JNK, and Toll-like receptors, are altered during obesity.[39] Recently, single-cell sequencing approaches have expanded our understanding of the mechanisms underlying obesity-driven inflammation. Specifically, recent findings revealed that macrophages within adipose tissue drive the development of inflammation. Initially, adipose tissue macrophages (ATMs) reside in adipose tissue to support beneficial homeostatic functions, but as obesity progresses, these cells undergo a shift in the transcriptional state toward an initially beneficial but ultimately pathological proinflammatory state.[40-43] These studies revealed that the populations of immune cells in adipose tissue are diverse, dynamic, and capable of both beneficial and pathological responses. Taken together, the discoveries outlined above help establish inflammation as a key component of obesity pathology and suggest that the immune system participates in proper metabolic regulation. Various factors can disrupt the proper functioning of the immune system and contribute to the development of obesity, including prominently for this dissertation, the consumption of a high-fat diet.

1.3.2 *High-fat Diet and Inflammation*

The consumption of a high-fat diet, especially one high in saturated fatty acids, induces a robust inflammatory response.[34,44] In fact, this proinflammatory response is considered to be a driving factor in the development of metabolic syndrome during diet-induced obesity.[34,37,39,45] Of the various immune pathways impacted by a high-fat diet, the Toll-like receptor pathway appears to be especially critical.[46] Specifically, innate immune

cells, such as macrophages, express Toll-like receptor 4 (TLR4). TLR4 is a well-known receptor that responds to bacterial lipopolysaccharides (LPS) and other infectious disease related proteins. However, it has been shown that saturated fatty acids, a common component of high fat diet, can also activate TLR4. In this way, saturated fatty acids can induce the activation of the NF-κB pathway and the release of proinflammatory signals, including IL-6, TNF-α, and IL-1β.[46-48] During chronic consumption of high fat diet, this inflammation cannot be resolved, and downstream complications such as insulin resistance begin to surface.[34,44,45] Furthermore, deletion of various immune-related genes (including NFκB pathway members, TNF-α, and IL-6) has been shown to impact body weight and insulin sensitivity during high-fat diet feeding, suggesting that immune function plays a key role in the pathology of diet-induced obesity.[44]

The fact that fatty acids, a common component of the diet of many mammals, can induce an inflammatory response might seem perplexing at first. The idea that a common nutrient drives an inflammatory response that can exacerbate obesity pathology and downstream consequences of metabolic syndrome seems to be an evolutionary oversight. This incongruence between immune function and metabolic regulation stems from assuming that the immune system is relevant only to the pathology. However, this incongruence can be remedied by expanding the view of immune function beyond pathology and considering it a component of the regulation of energy balance and system metabolism. The role of the immune system as a mediator between diet and systemic metabolic regulation has been supported by various studies, particularly those involving macrophages and, intriguingly, microglia within the CNS. Specifically, macrophages have been reported to modulate energy expenditure within brown adipose tissue and

regulate insulin sensitivity within adipose tissue; furthermore, both of these functions are modulated by a high-fat diet.[49-54] These findings demonstrate the interplay between diet, immune function, and the regulation of systemic energy balance. While the role of high-fat diet-driven inflammation in peripheral tissues has yielded important insights for obesity research, the role of this inflammation within the CNS appears to be especially critical.[55,56] The interplay between consumption of a high-fat diet, immune function, and obesity within the CNS will be reviewed in greater depth in the subsequent section on microglia. Together, the above information demonstrates that a high-fat diet is a response by the immune system and that this response can alter the regulation of energy balance.

1.3.3 *Neuroinflammation in Obesity*

In recent years, the intricate relationship between immune activity and inflammation within the CNS, referred to as neuroinflammation, and obesity has become a prominent area of study in the obesity research field. In this section, it will become clear that this complex relationship is multidirectional: obesity can induce neuroinflammation, and neuroinflammation can contribute to the pathogenesis of obesity. First, the connection between inflammation within the CNS and obesity was first reported in 2005.[57] In this seminal study, De Souza and colleagues demonstrated that rats fed a high-fat diet for 13 or 16 weeks exhibited elevations in the mRNA levels of multiple proinflammatory transcripts.[57] These findings not only established the occurrence of inflammation during high-fat diet-induced obesity but also provided a suggestive mechanism for hypothalamic dysfunction during high-fat diet feeding. Specifically, the authors provided evidence of impaired insulin and leptin sensitivity within the hypothalamus and suggested that this impairment was driven by NFκB-mediated JNK

signaling. The authors suggested that this impaired insulin and leptin signaling subsequently impairs anorexigenic signaling within the hypothalamus, which drives increased energy intake and eventual obesity[57.] Following these findings, it was discovered that saturated fatty acids, a component of a high-fat diet, produce a proinflammatory response within the hypothalamus that is driven by TLR4-mediated signaling. The authors provided evidence that pharmacologic and genetic inhibition of TLR4 protects rodents from high-fat diet-induced obesity and restores central leptin and insulin sensitivity.[58] Soon after this study, an investigation demonstrated that delivery of the cytokine IL-4 into the CNS exacerbated inflammation and led to increased body weight during high-fat diet feeding; furthermore, the authors showed that pharmacological inhibition of IKKß blocked these IL-4-mediated effects.[59] In combination, the above findings simultaneously increase the understanding of the potential of modulating immune function as a treatment for obesity and demonstrate the complexity and variety of factors underlying the response of the immune system to obesity and a high-fat diet.

The connection between neuroinflammation and obesity was further strengthened by a landmark study in 2012 by Thaler and colleagues. First, using MRI, the authors identified significantly increased T2 signaling within the mediobasal hypothalamus of obese patients.[60] Increased T2 signaling is a hallmark of inflammatory processes; therefore, the authors concluded that this enhanced signaling was indicative of injury and gliosis. This was the first report demonstrating hypothalamic gliosis and inflammation within the brains of obese patients, which was a seminal finding that established a clear connection between neuroinflammation and obesity in humans.[60] The occurrence of

reactive gliosis was confirmed soon after by a separate investigation, providing further support for the occurrence of neuroinflammation within the hypothalamus in obese humans.[61] However, perhaps the most compelling piece of evidence from the Thaler report came not from a human study but from their investigation of rats fed a high-fat diet.

Remarkably, the authors demonstrated, for the first time, that a high-fat diet immediately induces changes in inflammatory signaling within the hypothalamus well before the development of diet-induced obesity. Specifically, they showed changes in the mRNA levels of inflammatory markers (including IL-6, TNF-α, and multiple NFκB pathway members) within the hypothalamus of rats after just one or three days on a high-fat diet. This immediate neuroinflammatory response to a high-fat diet was soon after confirmation by an independent investigation, further confirming this somewhat unexpected phenomenon.[62] Intriguingly, a Thaler investigation revealed that proinflammatory marker levels returned to baseline levels after one and two weeks of feeding on a high-fat diet but increased again after four weeks of feeding.[60] These findings suggest that the inflammatory response to a high-fat diet occurs prior to the development of obesity and that this response varies over the course of chronic high-fat diet feeding. Critically, these findings help contextualize the studies covered in the prior paragraph, which showed that the inhibition of immune signaling could impact susceptibility to diet-induced obesity.[57-59] Furthermore, these findings might suggest that if the immune system immediately responds to high fat intake, disrupting this immune activity may impact the ability of the hypothalamus to properly regulate energy balance. These findings laid some of the initial groundwork for considering neuroimmune

function as a homeostatic component of energy regulation, not just as a pathologic agent driving metabolic disease. This concept is foundational to the work performed in this dissertation, as the following chapters will reveal.

Following the findings discussed above, numerous studies have confirmed that consumption of a high-fat diet induces both rapid and chronic changes in neuroinflammatory signaling.[63-72] However, these studies have reported variable results regarding the level of neuroinflammatory signaling during high-fat diet feeding. Overall, a high-fat diet induces an initial proinflammatory or activated immune state beginning as soon as one hour and ending within one week, followed by a brief anti-inflammatory or quiescent stage, until approximately one month, when chronic inflammation occurs.[56,73,74] Regardless of the exact timeline, it is clear that the immune response exhibits a distinct response to high-fat diet consumption. Furthermore, perturbation of this immune response via genetic or pharmacologic inhibition has been repeatedly demonstrated to impact susceptibility to diet-induced obesity.[55,73,74] As research on the neuroimmune mechanisms of obesity has continued, the centrality of microglia to these processes has become evident. Based on the evidence, microglia act as central regulators of the hypothalamic inflammatory response and participate in the regulation of energy balance; these topics are discussed in the following section.

1.4 Microglia in the Context of Obesity and Energy Balance Regulation

1.4.1 *An Overview of Microglia in Obesity*

As research on obesity and diet-driven neuroinflammation has increased, interest has also increased in uncovering the cellular mechanisms underlying this phenomenon. One of the first major breakthroughs toward this goal came from a 2012 report that

pointed to microglia, the myeloid-derived, highly dynamic resident immune cells of the CNS, as a key component of diet-induced neuroinflammation. In this report, the authors demonstrated that a high-fat diet induced the accumulation of IgG within the arcuate nucleus of the hypothalamus and that the IgG was predominantly localized to microglia within this region.[75] This study opened the door to a decade of compelling, novel research aimed at understanding how microglia participate in the neuroimmune responses that have been discussed above.

Throughout this section, we will gain an understanding of discoveries made regarding the role of microglia in obesity and discuss how those discoveries have revealed critical insights into the neuroimmunological aspects of metabolic disorders and energy balance regulation. Critically, we will also discuss how these discoveries helped shift the field toward a more nuanced understanding of microglia as an integral component of the energy balance and systemic energy balance regulatory system. Specifically, this section provides a comprehensive overview of the involvement of microglia in obesity, encompassing their response to high-fat diets, role in energy balance regulation, potential for therapeutic interventions, and impact of sex-specific differences.

1.4.2 *Microglia During High-Fat Diet and Obesity Pathology*

The conceptual connection between microglia and obesity was initially drawn from discoveries related to how these cells respond to a high-fat diet. Soon after the initial identification of microglia as possible mediators of high-fat diet-induced neuroinflammation, as outlined above, a report was published that demonstrated that the saturated fatty acid palmitate, a common component of high-fat diets, induces an inflammatory response in BV2 cells, an immortalized mouse microglia-like cell line.[72]

These findings were followed by a seminal study published in 2014 by Valdearcos and colleagues at the University of California San Francisco, which demonstrated that the inflammatory response to high-fat diet consumption was regulated primarily by microglia.[76] This study provides evidence of saturated fatty acid accumulation within the mediobasal hypothalamus during high-fat diet-induced obesity, which the authors suggest drives the inflammatory response observed during high-fat diet feeding. The authors observed that a high-fat diet and direct injection of saturated fatty acids into the brain induced an increase in the number of microglia within the mediobasal hypothalamus, as did an increase in the expression of proinflammatory markers. Perhaps most compellingly, the authors provided evidence that removing microglia, which they attempted via pharmacologic CSF1R inhibition and a CD11b-Diptheria Toxin Receptor model, abrogates the inflammatory response to saturated fatty acids.[76] These findings further solidified that the neuroimmune response to a high-fat diet precedes the development of obesity and established microglia as the key regulators of this response. Since then, multiple reports have confirmed that the number of microglia within the mediobasal hypothalamus can increase during high-fat diet-induced obesity.[62,70,71,77-79]

While the finding that microglia respond to dietary fat and undergo reactive gliosis during high-fat diet-induced obesity is intriguing, the functional impact of these findings, as well as the molecular mechanisms underlying these findings, still remain relatively unclear. Although this topic of research is extremely active, several investigations have helped shed light on the functional importance of microglia not only in high-fat diet-induced obesity but also in overall energy regulation. Chief among these was the seminal discovery that targeted deletion/modulation of genes specifically within

microglia could lead to obesity even in the absence of a high-fat diet.[80] This investigation performed targeted deletion of the *Tnfaip3* gene, an NFκB pathway inhibitor, within microglia specifically by using the *Cx3cr-Cre* mouse model, along with irradiation and bone marrow transplantation to eliminate the contribution of peripheral macrophages. Remarkably, they demonstrated that microglial-specific deletion of *Tnfaip3* led to a robust obesity phenotype; these findings indicated that altered microglial function alone could drive the development of obesity. This critical finding relates to the findings of this dissertation, as we demonstrated that deletion of the *Abi3* gene, which is expressed only within microglia within the CNS, leads to the development of obesity in mice.[81] These findings suggest that microglial functions can directly contribute to the development of obesity.

In addition to these discoveries, multiple reports have demonstrated that genetic modification of microglia can exacerbate or protect against high-fat diet-induced obesity.[80,82-87] Taken together, these findings suggest that the mechanisms by which microglia contribute to energy balance are complex and varied. For example, altered lipid processing, prostaglandin signaling, phagocytosis, chemokine and cytokine signaling, and mitochondrial function within microglia are among several of those reported mechanisms.[77,80,82-89] While the exact mechanisms driving the ability of microglia to regulate energy balance are varied and require further study, the repeated observation that genetic modulation of microglia could both exacerbate and directly induce obesity provides compelling evidence for the necessity of properly functioning microglia for proper regulation of body weight.

In addition to these genetic approaches, multiple pharmacologic and environmental interventions have connected alterations to hypothalamic microglia to altered susceptibility to obesity. One such approach is the use of CSF1R inhibition, a receptor that is essential for microglial survival, to eliminate microglia. Multiple investigations have explored the impact of the absence of microglia on metabolic regulation, and differing findings have been reported depending upon when microglia are removed. To elaborate, one investigation applied CSF1R inhibition to embryonic mice and reported increased food intake as well as reduced POMC neuron numbers within the arcuate nucleus.[90] These findings suggest that the presence of microglia can alter the neuronal environment of the mediobasal hypothalamus; this finding has substantial implications for microglial neuron interactions in the setting of energy balance regulation, which will be discussed in more detail in the following subsection. These findings also suggest that the presence of microglia is critical for proper food intake regulation. Additionally, a separate study revealed that CSF1R inhibition in adult mice led to resistance to high-fat diet-induced obesity, with decreased food intake. Furthermore, the authors reported that removal of microglia did not impact the energy balance in mice fed a control diet, which is a finding that challenges the necessity of microglia in regulating body weight in the absence of a high-fat diet.[80] A similar study was performed, but in place of CSF1R inhibition, intraventricular infusions of arabinofuranosyl cytidine, an antimitotic drug, were utilized to prevent high-fat diet-induced proliferation of microglia within the mediobasal hypothalamus. These authors found that, compared with nontreated mice, arabinofuranosyl cytidine-treated mice had fewer microglia within the arcuate nucleus during high-fat diet feeding. Additionally, the treated mice consumed less

food when fed a high-fat diet, but not when fed a control diet.[91] As with the preceding study, these findings also suggested that microglia can play a harmful role during high-fat diet feeding but that they may be expendable under more standard, balanced diet conditions. However, these findings are challenged by the findings of previous genetic studies, which suggested that altered microglial function can drive obesity regardless of diet.[80,81] CSF1R inhibition in mice with pancreatic ductal adenocarcinoma (PDAC) worsened PDAC-associated anorexia. Interestingly, the authors also reported that PDAC-associated anorexia is accompanied by an increase in microglia within the mediobasal hypothalamus. Taken together, these findings could indicate that the initial increase in microglia is protective in nature, as removing microglia via CSF1R inhibition exacerbates anorexia. Nonetheless, additional evidence indicates that microglia are necessary for the regulation of energy balance beyond the importance of high-fat diet feeding.

Along with the growing multitude of studies focused on hypothalamic microglia, the number, morphology, or activation status (often determined by the presence of CD68 expression) of these cells are commonly evaluated parameters. Therefore, general trends in how these parameters change during high-fat diet feeding and obesity have been identified. Additionally, multiple investigations have provided evidence that the number or morphology of microglia within the hypothalamus changes, as does the regulation of energy balance.[70,71,76-78,80,82-84,86,90-103] Many of these studies have reported that increases in the number or activation of microglia within the mediobasal hypothalamus are associated with obesity and, further, that decreases in the number or activation of hypothalamic microglia either have no effect or are protective against obesity. However, a few key studies have demonstrated that a reduced microglia number or activation

within the mediobasal hypothalamus can also drive obesity.[81,86,104] While the directionality of these changes is not consistent, the findings consistently suggest that hypothalamic microglia are capable of altering, and potentially necessary for, energy balance. These findings paint a more complex image of the role of hypothalamic microglia in energy expenditure, where an appropriate level of activation may be necessary to maintain proper body weight. In this view, both too much and too little activation of microglia within the mediobasal hypothalamus can disrupt energy balance. Therefore, microglia are necessary for the regulation of food intake and energy expenditure.[55] Considering this, it is important to consider the specific mechanisms that microglia employ to modulate energy balance, a topic that will be discussed in the following subsection.

1.4.3 *Mechanisms of Microglial Regulation of Energy Balance*

While the connection between neuroinflammation, microglia, and obesity has been made apparent by the studies discussed above, much remains to be discovered about the specific molecular and cellular mechanisms by which microglia induce pronounced changes in energy balance. One of the simpler mechanisms by which microglia alter energy balance is through the release of inflammatory signals. For example, microglia within the hypothalamus can produce TNF-α, IL-1β, and IL-6, all of which have been demonstrated to induce changes in body weight.[105] These changes are dependent on cytokines, as IL-6 appears to be protective against obesity, while TNF-α appears to drive increases in body weight. Intriguingly, the evidence suggests that both of these immune signals regulate body weight through modulation of the neurons of the mediobasal hypothalamus. TNF-α has been shown to alter the firing of POMC neurons, while IL-6

has been shown to decrease NPY neuronal signaling.[106-108] Overall, it appears that one way microglia regulate energy balance is through the release of cytokines or chemokines, which directly bind to receptors on the anorexigenic or orexigenic neurons of the mediobasal hypothalamus to modulate their signaling. This concept is further supported by findings suggesting that the loss of appetite observed in sickness behavior is driven by NFκB-related cytokines that increase anorexigenic neuronal activity within the hypothalamus.[109] Together, the above findings conveniently connect hypothalamic neuroinflammation and energy balance signaling; however, several investigations have reported findings that suggest a more complex role for microglia in the regulation of the neuronal environment of the arcuate nucleus.

In addition to direct signaling through immune molecules, multiple studies have provided evidence suggesting that microglia may alter energy balance through the modification of synaptic organization and neuronal numbers within the arcuate nucleus. At minimum, this concept applies to development, where the pruning and remodeling of synapses by microglia are well established. Specifically, if a mouse brain develops without microglia, the number of POMC neurons decreases, and subsequently, food intake increases.[90] In addition to development, it has been suggested that during high-fat diet feeding and obesity, microglia can alter synaptic organization and neuron number within the arcuate nucleus.[71,76,80,83,84,86,104,110-115] Furthermore, multiple reports have shown that high-fat diet feeding leads to decreases in synaptic density or neuronal number/area.[84,86,112,113,115] These studies collectively led to a growing consensus that microglia may exert part of their regulation of energy balance through remodeling of the neuronal architecture within the arcuate nucleus. This concept is further supported by the

findings of a very recent study in which deletion of the prostaglandin PGE2 receptor EP4 within microglia resulted in impaired phagocytosis, decreased microglial contact with POMC neurons, and resistance to high-fat diet-induced obesity. The authors provided evidence that the loss of EP4 expression within microglia prevents the loss of POMC neurons and preserves POMC neuronal projections within the mediobasal hypothalamus.[83] Furthermore, it has been shown that the consumption of a high-fat diet can induce changes in the proportion of excitatory and inhibitory POMC synapses.[84,115] Recently, a study reported that these high-fat diet-driven changes in synaptic proportion are significantly altered by deletion of the *Ucp2* gene within the microglia of mice. Taken together, the above studies indicate that microglia directly contribute to the maintenance of synaptic architecture within the hypothalamus during high-fat diet feeding.[83,84,115] Overall, microglia regulate energy expenditure, in part, via microglia–neuron interactions that alter synapses within the arcuate nucleus.

Despite the progress that has been made, much has yet to be learned about the exact mechanisms that microglia employ to regulate energy balance. Several studies have shown associations between changes in microglial number, morphology, and activation status and changes in energy balance, as outlined above; however, the exact manner in which microglia induce changes in the neuronal environment to alter energy balance has not been determined. This is critical because by learning the exact processes that microglia use to alter energy balance, the ability to therapeutically leverage these processes for the treatment or prevention of obesity becomes possible. This concept will be discussed in the following section.

1.4.4 *Therapeutic Interventions Targeting Microglia*

Given their central role in hypothalamic inflammation and energy homeostasis, interest in microglia as potential therapeutic targets for obesity has increased. Conceptually, this would entail the modulation of microglial activity to restore or maintain healthy metabolic functions and subsequently treat or prevent obesity. Some proposed therapeutic approaches that could be used will be discussed throughout this section. First, given the reported importance of neuroinflammation during and preceding obesity pathology, as discussed previously, the use of anti-inflammatory agents for obesity treatment has been explored. For example, minocycline, a compound with robust anti-inflammatory properties, has shown efficacy in reducing microglial activation and attenuating obesity-induced hypothalamic inflammation.[95] Specifically, a recent investigation treated high-fat diet-fed mice with minocycline or a control compound and revealed that the minocycline-treated mice had less microglial reactivity within the hypothalamus, reduced neuroinflammation, and less resistance to diet-induced obesity.[95] These findings suggest that anti-inflammatory agents could reverse or prevent high-fat diet-driven changes within hypothalamic neurons that subsequently impair the regulation of energy balance; however, further research must be performed to validate this claim. Additionally, dietary intervention represents another possible therapeutic approach for modulating microglia for the treatment and prevention of obesity. As established extensively above, microglia respond to dietary components such as saturated fatty acids, which is partly how these cells regulate energy balance. Therefore, the use of dietary modifications aimed at limiting the inflammatory response of microglia is considered a potential obesity treatment. For example, diets rich in omega-3 fatty acids have been

reported to exert anti-inflammatory effects on microglia, thereby ameliorating the hypothalamic inflammation associated with high-fat diets.[116] Conversely, dietary saturated fatty acids and carbohydrates have been demonstrated to exacerbate neuroinflammation within the hypothalamus and exacerbate diet-induced obesity.[76,117] Taken together, these findings suggest that dietary modifications could be a viable strategy for managing obesity through microglial modulation. Additionally, direct pharmacological modulation of specific microglial functions could eventually be employed in the treatment of obesity. While this approach is largely conceptual at this point, future understanding of microglia could enable the development of specific drugs targeting microglial-specific receptors or signaling pathways to treat or prevent obesity. For example, it has been speculated that purinergic or fractalkine signaling could be targeted in the context of obesity.[88,118-120] Specifically, both pathways are involved in microglial-neuronal interactions, such as migration and other cytoskeletal remodeling-related functions, and alterations of these functions could enhance beneficial microglial-neuronal interactions while inhibiting detrimental interactions. However, this type of treatment approach requires substantially more research, as we cannot modulate disease-relevant mechanisms until we identify and better understand them.

While therapeutic targeting of microglia in obesity holds promise, there are significant challenges. One major concern is the specificity of the interventions. Beyond the setting of obesity and body weight, microglia play diverse roles in brain health, and indiscriminate modulation of their activity could have unintended consequences.[121] Therefore, therapies need to be fine-tuned to affect only the pathological aspects of microglial function without compromising their normal physiological roles. This is

further supported by findings that link lower microglial activity with impaired energy balance, demonstrating that effective treatments will require more than just completely inhibiting microglial functions.[81,86,104] Additionally, the complexity of microglial responses to different stimuli and their interactions with neurons and other glial cells in the context of obesity are not fully understood. Future research should aim to unravel these intricate relationships to facilitate the development of more effective and safer therapeutic strategies. Overall, targeting microglial activation and function has potential as an innovative approach for the treatment and prevention of obesity. However, realizing this potential requires a deeper understanding of microglial biology in the context of metabolic health, alongside careful consideration of the challenges in modulating immune responses within the brain. As research in this field progresses, it holds the potential to positively impact the management of obesity and related metabolic disorders.

1.4.5 *Sex Differences in the Microglial Response to Obesity*

While much has been learned about how microglia contribute to the regulation of energy balance and to the development of hypothalamic neuroinflammation, the vast majority of these studies have been performed on male animals only. Due to this limitation, the field currently has limited knowledge of the full impact of sex on hypothalamic microglial function. However, a small number of important insights have been made in this regard. First, it has been demonstrated that female mice are less susceptible to diet-induced obesity than both male mice and ovariectomized female mice.[122] This finding suggested that females with intact sex organs, presumably due to signaling derived from these organs, are resistant to the mechanisms that drive diet-induced obesity. Interestingly, it has also been shown that female mice exhibit differences

in high-fat diet-driven gliosis within the mediobasal hypothalamus.[87,111,123,124] Taken together, these findings suggest that sex-specific differences in how microglia respond to a high-fat diet may, in part, explain the sex-specific differences in susceptibility to obesity.

Among the variety of sex-specific mechanisms underlying these differences, the role of fractalkine has been particularly well studied. As a result, the intricate role of fractalkine signaling, which involves predominantly the neuron-produced ligand CX3CL1 and its predominantly microglia-localized receptor CX3CR1, has emerged as a critical axis in understanding the sex-specific differences observed during diet-induced obesity. While the role of microglia in obesity, particularly in relation to hypothalamic neuroinflammation, has been extensively covered, the specific nuances of how fractalkine signaling modulates these microglial functions in a sex-dependent manner offer a new dimension to this field. Within the CNS, fractalkine signaling is largely known for its roles in neuron–microglia communication.[125] While the CX3CL1-CX3CR1 axis has been implicated in various neuroinflammatory conditions, such as neurodegeneration, its specific influence on obesity, especially concerning sex differences, has garnered attention only recently.[118] Specifically, a recent investigation explored differences in fractalkine signaling between male and female mice with and without deletion of *Cx3cr1* fed a high-fat diet.[87] This study demonstrated that female mice were resistant to diet-induced obesity, as reported previously, but that this resistance was eliminated in the absence of CX3CR1. Furthermore, they reported that, compared with male mice, wild-type, high-fat diet-fed female mice had significantly elevated CX3CL1 levels. They further provided evidence that AAV-driven overexpression of CX3CL1 within the brains

of male mice led to “female-like” protection from high-fat diet-induced obesity. Taken together, these findings provide compelling evidence that increased fractalkine signaling is a significant contributor to the resistance of female mice to obesity.[87] Additional investigations, albeit without considering sex effects, have demonstrated the same protective effect of fractalkine signaling. Specifically, recent investigations have provided evidence suggesting that increased fractalkine signaling reduces high-fat diet-associated neuroinflammation within the hypothalamus and confers resistance to diet-induced obesity.[88,89] Furthermore, one study provided evidence that fractalkine reduces microglial activity and subsequently prevents the reduction in POMC neuron excitability induced by a high-fat diet.[89] Taken together, these findings clearly reveal that fractalkine plays a critical role in enabling microglia–neuron interactions that subsequently enable proper regulation of energy balance; furthermore, it appears that the resistance to diet-induced obesity exhibited by female mice is at least partially due to increased fractalkine signaling to microglia.

In addition, the impact of sex hormones, particularly estrogen, on microglial function is considered a potential cause of sex-specific differences in susceptibility to obesity. Estrogen signaling plays a pivotal role in modulating microglial activity and is intricately connected with the hypothalamic control of energy homeostasis and body weight.[126-129] For example, it has been demonstrated that estrogen deficiency is associated with both increased hypothalamic inflammation and the development of obesity.[129] Treatment with estrogen or estrogen analogs can reduce hypothalamic inflammation and the severity of diet-induced obesity.[128,130] This finding suggested that part of the resistance to obesity exhibited by female mice may be due to the impact of estrogen-

mediated modulation of microglia; however, additional mechanistic investigations are necessary to support this claim. Therefore, estrogen can directly and robustly modulate microglial functions, including reducing the extent of the proinflammatory response to saturated fatty acids.[126-128] Overall, these findings suggest that estrogen is an important factor that dictates sex-specific differences in the microglial response to obesity and a high-fat diet. Furthermore, the fact that sex drives such distinct responses by microglia underscores the complexity of microglial involvement in obesity. While it is clear that sex modulates the role microglia play in hypothalamic inflammation and metabolic dysfunction, the mechanisms underlying sex-specific responses require substantially more investigation. Future research should focus on the interplay between hormones, genetic factors, and environmental influences in shaping microglial function in the context of obesity. Such insights could pave the way for sex-specific therapeutic strategies targeting microglial function to combat obesity and related metabolic disorders.

1.5 An Overview of ABI3 Biology

Abelson Interactor Protein Family Member 3 (ABI3) is an interactor protein thought to be involved primarily in actin remodeling-related functions. This gene is expressed primarily in monocytes and macrophages peripherally and in microglia within the central nervous system.[131,132] The actin cytoskeleton is a dynamic structure vital for various cellular functions but is especially relevant to immune cell functions, including motility, membrane morphology, phagocytosis, and intracellular transport. ABI3 is thought to impact actin dynamics primarily through its participation in the Wiskott-Aldrich Syndrome Protein Family Verprolin-homologous Protein Complex 2, or WAVE2 complex, a key regulator of actin polymerization.[133-138] WAVE2 is a pentameric protein

complex that regulates actin remodeling-dependent cellular functions, and one of the interacting members is ABI3, although the complex can contain ABI1 or ABI2.[133,139] Interestingly, it has been demonstrated that the WAVE complex can perform different functions dependent on the participating ABI protein; for example, it was demonstrated that the WAVE2-ABI3 complex does not participate in the phosphorylation of c-abl tyrosine kinase but does drive the induction of specific membrane protrusions.[140] In the immune system, the WAVE2 complex has been demonstrated to participate in the actin remodeling necessary for immune cell activation, such as the formation of an immunological synapse (a specialized cell–cell connection regulating immune activity).[141]

Given that immune cells such as microglia require dynamic actin remodeling and that WAVE2 regulates this remodeling, it is not unreasonable to expect that deletion of ABI3 could lead to impairment of microglial and immune cell functions. Additionally, it was later demonstrated that ABI3 overexpression within the CNS can alter neuronal dendritic spines and synapses, suggesting that ABI3-dependent actin remodeling may be important for the formation and maintenance of these structures.[142] This study is limited by the fact that ABI3 is overexpressed in all cell types across the brain, even though ABI3 is expressed only by microglia within the brain.[142] In addition to these findings, ABI3 may be regulated in part through phosphorylation mediated by the PI3K/AKT pathway, a pathway that is central to the regulation of metabolism, including the insulin response.[138] Furthermore, it was suggested that this phosphorylation impacts the ability of WAVE2 to participate in the WAVE2 protein complex.[138] However, these findings have

yet to be explored further, so additional research will be necessary to uncover the mechanisms that regulate ABI3.

Until recently, the extent of our knowledge about the specific functions of ABI3 has been largely limited. However, after a seminal genome-wide association study revealed that rare coding variants in *ABI3* increase the risk of developing Alzheimer's disease, a series of investigations aimed at understanding the role of ABI3 in microglia were initiated.[143] Our laboratory and others have demonstrated that ABI3 is involved in actin-remodeling functions of microglia, such as migration, phagocytosis, and surveillance.[81,144-146] Furthermore, these investigations demonstrated that the absence of ABI3 in microglia can lead to dramatic differences in the progression of neurodegenerative pathology, as well as in measures of neuroinflammation.[144,145,147,148] The relevance of neuroinflammation and microglial function to obesity provides context for the central findings of this dissertation; that is, deletion of the *Abi3* gene locus in mice leads to the induction of obesity.[81] In this dissertation, we explore these exciting findings as well as the impact of the deletion of *Abi3* on high-fat diet-induced neuroinflammation and obesity.

1.6 Conclusion

This chapter delves deeply into the roles of microglia, inflammation, obesity, and the ABI3 protein. This exploration has offered an extensive overview of the historic and modern findings connecting these concepts and identified areas for future research. First, the historical perspective on obesity was covered, highlighting the centrality of the hypothalamus and CNS in energy balance regulation. Furthermore, we underscore that obesity is not merely a result of energy imbalance but is also profoundly influenced by

complex neurobiological mechanisms. We reviewed the pivotal role of leptin and how the evolution of our understanding of its interaction with the CNS reflected a paradigm shift in obesity research. We then reviewed the importance of inflammation in obese individuals, both systemically and in the context of the CNS. This section outlines how our understanding of the immune system has expanded beyond its involvement in disease pathology to encompass its complex participation in metabolic regulation. Relatedly, we discussed the impact of a high-fat diet on inflammation, especially the role of microglia in directing hypothalamic inflammation, which helps highlight the multidirectional relationships among diet, the neuroimmune response, and metabolic health. Subsequently, we discussed how microglial function may bridge the gap between neuroinflammation and metabolic dysregulation. We reviewed how this concept was substantially supported by the revelation that alterations in microglial function can lead to obesity, even in the absence of a high-fat diet. The extensive research discussed here, ranging from genetic studies to pharmacological interventions, establishes the complexity of microglial functions in obesity as well as the uncertainty regarding the centrality of these functions to energy balance regulation.

The exploration of sex differences in microglial response to obesity added another layer of complexity to this topic. Furthermore, sex-specific research is needed, especially given the unique responses driven by factors such as estrogen and fractalkine signaling. Finally, we reviewed the connection between ABI3 and obesity, which was revealed in recent research, suggesting that the ABI3-mediated functions of microglia may play a critical role in metabolic regulation and neuroinflammation. In conclusion, this chapter provides a rich and multifaceted perspective on obesity, highlighting the intricately

interconnected roles of the hypothalamus, inflammation, and microglia. These findings emphasize the complexity of metabolic regulation and the importance of the CNS in this process. Ultimately, our hope is that the discoveries connecting microglial function to energy balance regulation will help pave the way for novel research and, eventually, therapeutic strategies to help us better understand and treat the pervasive and challenging health issue obesity.

Chapter Two: Deletion of the *Abi3* gene locus results in obesity and systemic metabolic disruption in aged, male mice.

2.1 Introduction

As we outlined in the previous chapter, perturbations in immune function have been demonstrated to exacerbate neurodegeneration and metabolic diseases [149-151]. Additionally, it has been reported that metabolic diseases, such as obesity, increase the risk of developing neurodegenerative disease such as Alzheimer's disease (**AD**) [152,153]. Intriguingly, it has been suggested that disrupted immune function is a potential link between these disease states [154,155]. However, the field currently has limited knowledge regarding the specific molecular drivers that alter the immune system to jointly modulate metabolic and neurodegenerative disease states. In this report, we describe how an unexpected discovery revealed that an AD-related gene, Abelson interactor family member 3 (*Abi3)*, is involved in both immune and metabolic regulation. In combination with our previous report demonstrating the importance of *Abi3* in AD-like pathology,[145] we have provided evidence to suggest that *Abi3* may serve important roles at the intersection of metabolic, neurodegenerative, and immune regulation.

As discussed in Chapter One, our initial interest in the *ABI3* gene was inspired by a human genetics study that identified a rare coding variant within *ABI3* gene locus that is associated with increased risk of AD [156]. Subsequently, we set about investigating the impact of *Abi3* deletion on AD neuropathology in the 5xFAD amyloid-β (Aβ) amyloidosis mouse model. In a recent report, our laboratory demonstrated that deletion of the *Abi3* gene locus significantly exacerbated Aβ pathology and neuroinflammation in these 5xFAD-*Abi3*$^{-/-}$ mice [145]. In the course of that study, we unexpectedly discovered

that *Abi3* knock-out (*Abi3*$^{-/-}$) mice, the non-transgenic littermates of the experimental mice, develop obesity.

As discussed in the preceding chapter, *Abi3* is predominately expressed in myeloid derived monocytic and microglial immune cells [131,132]. As we have established, this is relevant in the context of metabolism because recent studies have demonstrated that microglia, in particular microglia within the hypothalamus, impact the progression and onset of obesity [41,157-159]. However, the underlying molecular mechanisms by which these cells contribute to metabolic dysfunction and obesity require further exploration. While much remains to be discovered regarding the cellular functions of *Abi3,* it is suggested to exert its function through its participation in the WASP Family Verprolin-Homologous Protein Complex 2 (WAVE2 complex), a key regulator of cytoskeletal remodeling.[138,160] However, the role of *Abi3* in the context of metabolic regulation and metabolic disease was completely unknown prior to the findings reported herein.

In this chapter, we aim to address this knowledge gap by investigating the impact of *Abi3* deletion on measures of systemic metabolism and obesity. Specifically, we demonstrate that aged, male *Abi3* knock-out (*Abi3*$^{-/-}$) mice exhibit an obese phenotype characterized by increased body weight and body fat, as well as impaired glucose tolerance and insulin sensitivity. Further, we determined that aged, male *Abi3*$^{-/-}$ mice exhibited significantly reduced energy expenditure but exhibited no significant differences in food intake. Additionally, through RNA-seq analysis, we found that deletion of the *Abi3* gene locus altered gene expression, when controlling for differences in body weight, in the hypothalamus but not adipose tissue. Additionally, microglia number and area were decreased specifically within the mediobasal hypothalamus of

Abi3$^{-/-}$ mice. Together, these findings suggest that the absence of *Abi3* may drive the induction of obesity due to altered microglia function within the hypothalamus. Altogether, this study is the first to determine the functional importance of the *Abi3* gene locus in the regulation of systemic metabolism and maintenance of healthy weight.

2.2 Material & Methods

2.2.1 *Animals*

The mice utilized in this study were initially generated from a cross between 5XFAD and *Abi3*$^{-/-}$ mice; our study utilized the non-transgenic littermates from this cross. The 5xFAD mice were acquired from Jackson Laboratory [MMRRC 34840, B6SJL-Tg (APPSwFlLon, PSEN1* M146L* L286V) 6799Vas/Mmjax)]. The *Abi3*$^{-/-}$ mice were acquired from the University of California-Davis MMRRC Mouse Biology Program [C57BL/6N-Abi3tm1.1(KOMP)Vlcg]. The velocigene vector targeted the *Abi3* gene locus on chromosome 11 from 95,842,143 to 95,832,627. The non-transgenic (no 5xFAD) mice produced from the initial cross were used in this study. Our experiment utilized male mice at 15 months of age. The mice were provided standard housing conditions with ad libitum access to food and water.

Ethics Statement- The present study was performed in accordance with our reviewed and approved animal protocol from the Institutional Animal Care and Use Committee of Indiana University School of Medicine.

2.2.2 *Tissue Preparation*

Mice were given Avertin (250 mg/kg) intraperitoneally to induce anesthesia. Mice were then perfused transcardially with PBS. The brains and brown adipose tissue (BAT) were then rapidly removed. For RNA samples, the brains were dissected immediately

frozen in dry ice, along with the brown adipose tissue. For immunofluorescence, brain samples were fixed with 4% paraformaldehyde in PBS for 24 hours at 4 °C. The immunofluorescence samples were then passed through a sucrose gradient with 15% sucrose in PBS overnight, followed by 30% sucrose in PBS overnight, followed by embedding and freezing in Fisher Healthcare OCT compound (cat # 23-730-571). All samples were then stored in -80 °C prior to their final processing.

2.2.3 *Body Weight and Composition*

The mice were weighed on an Ohaus-SCOUT™-SPX622 Portable Precision Balance. Body composition was measured via EchoMRI™ 500. For EchoMRI, mice were guided into a containment tube which restricts movement, and imaging was acquired over a 1-4 minute period using a 0.05 T electromagnetic field. Lean mass and fat mass were then calculated from T_1 and T_2 relaxing curves via standard algorithms.

2.2.4 *Glucose and Insulin Tolerance Tests*

For the glucose tolerance test (GTT), mice were fasted for 16 hours prior to the start of the experiment. The mice then received 1g/kg glucose by intraperitoneal injection. The tails of the mice were snipped, and blood was then collected at 0, 10, 20, 30, 60, 90, and 120 minutes. Blood glucose was measured via glucometer at each time point.

For the insulin tolerance test (ITT), mice were fasted for 2 hours prior to the start of the experiment. The mice then received 0.75 units/kg insulin via intraperitoneal injection. The protocol for measuring blood glucose was identical to GTT, except readings were recorded only at 0, 15, 30, 45, 60 minutes.

2.2.5 *Metabolic Cage Analysis*

PhenoMaster/LabMaster System (TSE Systems) calorimetry unit was used to measure food and water intake, gas consumption and production, respiratory exchange rate and locomotor activity of the mice. From these recordings, additional measures of metabolism such as energy expenditure and respiratory exchange ratio were generated. Mice were transferred and singly housed in the Phenomaster system, where they were given three days to acclimate. Following acclimation, recordings were collected over 84 hours.

2.2.6 *Total Cholesterol Assay*

Total cholesterol levels were measured in plasma samples using a total cholesterol assay kit (Cell Biolabs, STA-384). Briefly, plasma was added to a reaction mixture that produces a colorimetric probe proportional to the amount of total cholesterol in the sample. The absorbance of the samples and standardized controls were then measured at 562 nm to determine the total amount of cholesterol in each sample, using a BioTek Synergy HTX Multi-Mode Plate Reader. The samples were run at a 1:50 dilution.

2.2.7 Leptin ELISA

Leptin concentration was measured in plasma samples using the U-Plex Mouse Leptin ELISA (Meso Scale Discovery, K1525ZK). Electrochemiluminescence signal was read on a MESO QuickPlex SQ 120. The samples were run at a 1:2 dilution.

2.2.8 *RNA Extraction*

RNA was extracted from the hypothalami and brown adipose tissue via a standard phenol/chloroform extraction using TRIzol reagent (MRC). The concentration and purity

of the RNA samples were then assessed via Nanodrop 2000 spectrophotometer. The isolated RNA was then stored at -80 °C until further processing.

2.2.9 *QuantSeq 3' mRNA-Seq*

Isolated RNA was shipped to Lexogen for library preparation and sequencing. Libraries were prepared manually according to the manufacturer's instructions using QuantSeq 3' mRNA-Seq FWD Library Prep Kit as outlined previously [145]. Sequencing was performed on a NextSeq 500 instrument with SR75 High Output Kit (Illumina). Seventy-six-base-pair single-end reads were generated. FastQ files were processed based on the workflow outlined in the Lexogen's user guide for QuantSeq 3' mRNA-Seq Integrated Data Analysis (Version 015UG108V0310).0

2.2.10 *Differential Gene Expression Analysis*

Gene read count files were imported into RStudio (R v.4.2.0). Differential expression analysis was performed using DESeq2 v.1.36.0 [161]. When body weight was used as a covariate, the weights were first scaled with the scale function from the base R package. Then, the DESeq data set was created using the design argument ~ Weight + Condition where Weight is the scaled body weight and Condition is the genotype as indicated before. Only genes with total read counts greater than or equal to 5 were included in the model fitting. Log-fold change shrinkage was employed using the lfcShrink function as previously described [162]. Differential expressed genes were considered to be genes with an FDR-adjusted (Benjamini-Hochberg) p-value ≤ 0.05.

2.2.11 *Immunofluorescence*

Frozen brains embedded in OCT were coronally sectioned in 30 um increments, rinsed with PBS, and mounted onto glass slides. Slides were blocked with 2.5% normal

goat serum in PBS for 1 hour, followed by overnight incubation with Iba1 antibody (1:1000, Abcam, ab178846) at 4 °C. Slides were then incubated with goat anti-rabbit secondary (1:500, Alexa Fluor 568, A11036) for 1.5 hours. Slides were then mounted with coverslips using Vectashield Mounting Medium with DAPI (H-1200).

Images were then acquired with a Leica DMi8 fluorescent microscope. Images were analyzed by in-house pipelines developed using CellProfiler (Broad Institute) and ImageJ [163,164]. For each mouse, three sections from differing anatomical coordinates (separated by 720 μm) were analyzed and the average value from these sections was used. Iba1+ signal and DAPI signal were measured and normalized by the total area of the section. Using CellProfiler, both Iba1+ cell number and Iba1+ signal area were quantified.

2.2.12 *Statistical Analysis*

Statistics were performed with GraphPad Prism 9 software. comparisons between *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$were performed using unpaired t-tests. For GTT and ITT, two-way ANOVA with Tukey's post hoc test was utilized for the time-course graphs. The energy expenditure versus weight graphs were analysed using simple linear regression. Data were represented as means ± SEM.

2.3 Results

2.3.1 *Increase in body weight and fat mass and impairment of glucose and insulin tolerance in Abi3*$^{-/-}$ *mice.*

In the course of our AD project, we unexpectedly found that deletion of the *Abi3* gene locus resulted in a dramatic obese phenotype in non-transgenic mice. Because of this unexpected nature, our investigation into the role of ABI3 in metabolism began with

already aged mice (15 months). First, we measured the body weight of the *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice (Figure 1A). Deletion of the *Abi3* gene locus significantly increased body weight, with *Abi3*$^{-/-}$ mice weighing an average of 10.70 g, or 32.4%, more than *Abi3*$^{+/+}$ mice (Figure 1A). Next, to determine whether this increase in weight was due to increased fat and/or lean mass, we performed EchoMRI body composition analysis on these mice (Figure 1B-C). Similar to body weight, fat mass was significantly increased in *Abi3*$^{-/-}$ mice. Specifically, *Abi3*$^{-/-}$ mice had more fat mass than *Abi3*$^{+/+}$ mice by an average of 7.11 g, or 208.4% (Figure 1B). Lean mass, however, was not significantly altered in *Abi3*$^{-/-}$ mice (Figure 1C).

To further understand the impact of the loss of ABI3 function on systemic glucose regulation, we performed glucose tolerance (GTT) and insulin tolerance (ITT) tests (Figure 1D-G). In the GTT, *Abi3*$^{-/-}$ mice exhibited significantly elevated blood glucose levels as compared to *Abi3*$^{+/+}$ mice at 60, 90, and 120 minutes following glucose administration (Figure 1D). Similarly, area under the curve analysis of GTT data revealed that *Abi3*$^{-/-}$ mice had significantly greater area than *Abi3*$^{+/+}$ mice (Figure 1E). These data indicate that *Abi3*$^{-/-}$ mice had impaired glucose tolerance relative to control. In the ITT, *Abi3*$^{-/-}$ mice had significantly elevated blood glucose levels at 30, 45, and 60 minutes, indicative of insulin resistance, as compared to *Abi3*$^{+/+}$ mice (Figure 1F).
Area under the curve analysis of ITT data revealed that *Abi3*$^{-/-}$ mice had significantly greater area than *Abi3*$^{+/+}$ mice, indicating that *Abi3*$^{-/-}$ mice had impaired insulin response relative to control (Figure 1G).

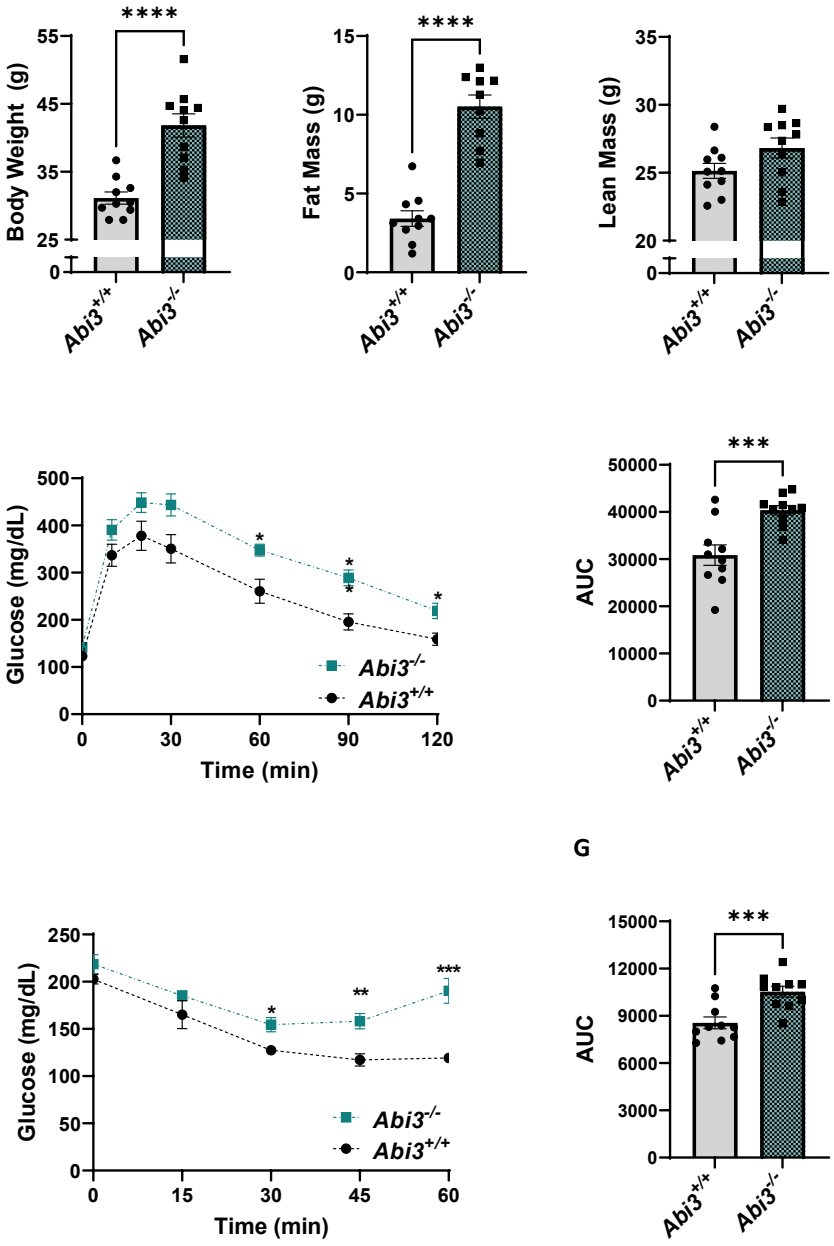


Figure 1. Aged *Abi3*$^{-/-}$ mice exhibit increased body weight and fat mass and impaired glucose tolerance and insulin sensitivity. (A) Body weight was measured in 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10). (B-C) Body composition was measured in these mice using EchoMRI. (D-G) Glucose (GTT) and Insulin tolerance tests (ITT) were performed, and the glucose levels of 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10) were recorded over time. (D) In the GTT, mice were injected with 1 g/kg glucose following a 16 hour fast. Blood glucose (mg/dL) was measured via glucometer at 30, 60, 90, and 120 minutes post injection. (E) The area under the curve for the GTT was calculated. (F) In the ITT, mice were injected with 0.75 units/kg insulin following a 2 hour fast. Blood glucose (mg/dL) was measured via glucometer at 15, 30, 45, and 60 minutes post injection. (G) The area under the curve for ITT was calculated. Data represent mean ± SEM. For GTT and ITT time-course, 2-way ANOVA with Tukey's post hoc test was used. For all other comparisons, unpaired t-test was used; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

2.3.2 *Energy expenditure during light cycle is impaired, while food intake is unchanged, in Abi3$^{-/-}$ mice.*

To determine if the obese phenotype was secondary to differences in food intake, energy expenditure, or a combination of these factors, we performed comprehensive metabolic phenotyping and indirect calorimetry, via metabolic cage, on the *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice (Figure 2).

First, we measured the food consumed during the 84-hr testing period within the Phenomaster system. The deletion of *Abi3* gene locus did not alter food intake between groups (Figure 2A-B). Next, we evaluated energy expenditure from the calorimetry experiment during the light, dark, and combined full day cycles. There were no gross changes in average energy expenditure between the genotypes (Figure 2C-D). However, further regression analysis comparing the relationship of energy expenditure versus weight revealed that *Abi3*$^{-/-}$ mice had significantly lower slope than *Abi3*$^{+/+}$ mice during the light cycle, indicative of impaired energy expenditure (Figure 2E). No significant differences in slope were detected between *Abi3*$^{-/-}$ and *Abi3*$^{+/+}$ mice for the dark cycle or full day regression analyses (Figure 2F-G).

Additionally, no differences in respiratory exchange ratio, a measure of carbohydrate versus lipid fuel utilization, were observed between the *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice (Figure 3A-B). Finally, to determine if differences in energy expenditure were secondary to changes in overall movement, we evaluated the total and average movement of the *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice during the calorimetry experiment (Figure 4). *Abi3* genotype status had no impact on total or average movement counts (Figure 4A-C).

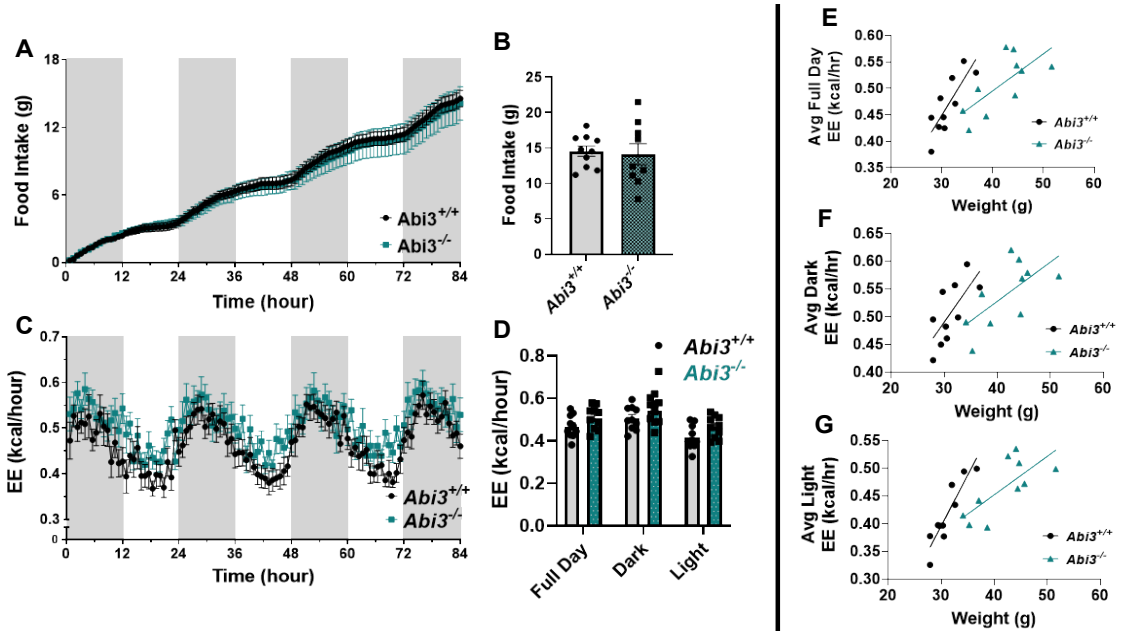


Figure 2. Aged *Abi3*$^{-/-}$ mice exhibit impaired energy expenditure and unaltered food intake. 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10) were singly housed in metabolic cages and **(A-B)** food intake and **(C-D)** energy expenditure were recorded over 84 hours. For time course graphs (A, C), the shaded portions represent night hours (dark cycle) and the unshaded portions represent daytime (light cycle). (A) Food intake over time and (B) overall total food intake were recorded. (C) Energy expenditure over time and (D) average energy expenditure were measured. (D) Average energy expenditure was calculated for the daytime/light hours (Light Cycle), night/dark hours (Dark Cycle), and the entire 84 hours (full day). **(E-G)** Subsequent linear regression analysis of average energy expenditure versus weight was performed to assess the relationship between lean mass and energy expenditure. Regressions were performed for (E) full day cycle, (F) dark cycle, and (G) light cycle. Data represent mean ± SEM. Unpaired t-test, simple linear regression; *p < 0.05.

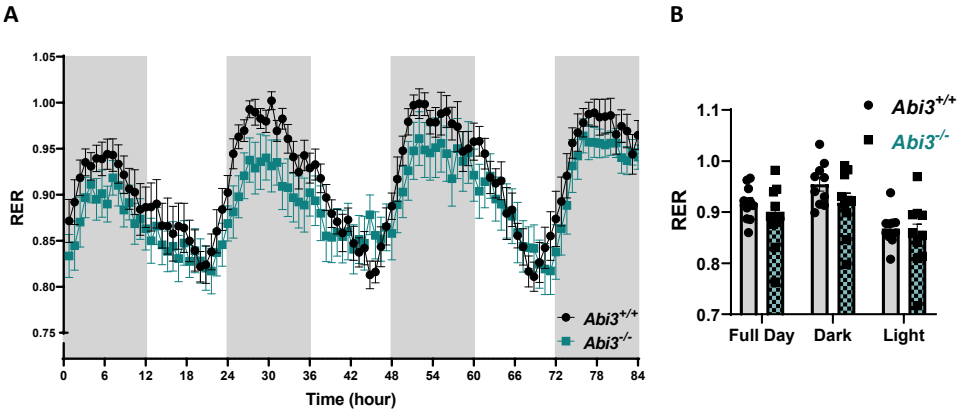


Figure 3. Deletion of *Abi3* gene locus does not alter respiratory exchange ratio (RER) in mice. 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10) were singly housed in metabolic cages and indirect calorimetry recordings were collected over 84 hours. For the time course graph (**A**), the shaded portions represent dark cycle, and the unshaded portions represent light cycle. **(A)** RER was measured over time. **(B)** The overall RER for each mouse was calculated for the full days, dark cycles only, and light cycles only. Data represent mean ± SEM. For time-course data, 2-way ANOVA with Tukey's post hoc test was used. For all other comparisons, unpaired t-test was used.

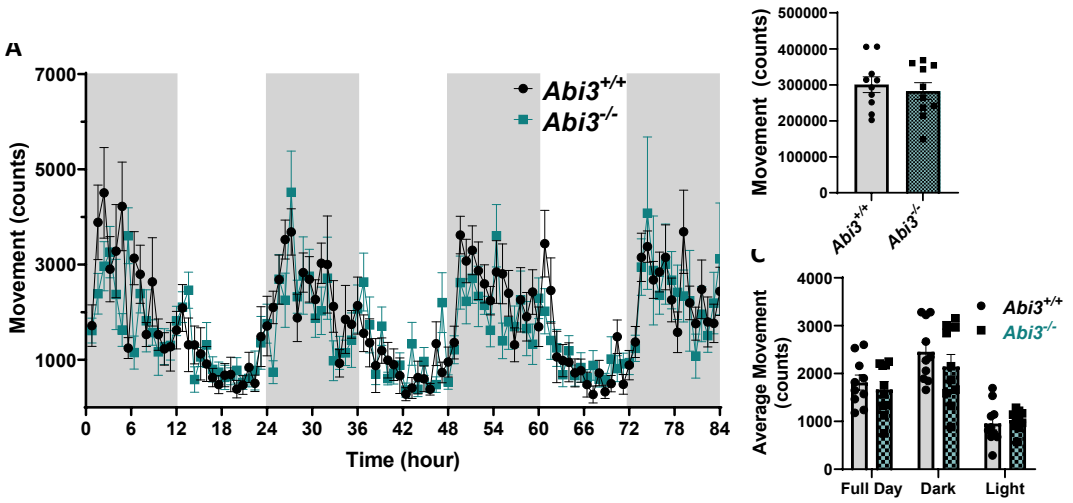


Figure 4. *Abi3* genotype does not impact the total movement of mice. 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10) were singly housed in metabolic cages and movement recordings were collected over 84 hours. For the time course graph (**A**), the shaded portions represent dark cycle and the unshaded portions represent light cycle. Movement was measured by software that recorded a count for each distinct movement in the XY plane**. (A)** Movement was measured over time. **(B)** Total movement was calculated along with **(C)** the overall average movement for each mouse during the full days, dark cycle only, and light cycle only. Data represent mean ± SEM. For time-course data, 2-way ANOVA with Tukey's post hoc test was used. For all other comparisons, unpaired t-test was used.

2.3.3 *Total Cholesterol and leptin concentration are increased in the plasma of Abi3*$^{-/-}$ *mice.*

In order to further evaluate the metabolic consequences observed in *Abi3*$^{-/-}$ mice, we performed cholesterol and leptin measurements on the plasma from *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice. Using a colorimetric total cholesterol assay, we found that *Abi3*$^{-/-}$ mice had significantly elevated cholesterol levels (Figure 5A). Then, we performed a leptin ELISA on the plasma from these mice and similarly observed that *Abi3*$^{-/-}$ mice had significantly elevated leptin concentration (Figure 5). Elevated leptin and cholesterol levels are characteristic of obesity, and therefore these results further demonstrate the extent of the obese phenotype observed in *Abi3*$^{-/-}$ mice [165,166].

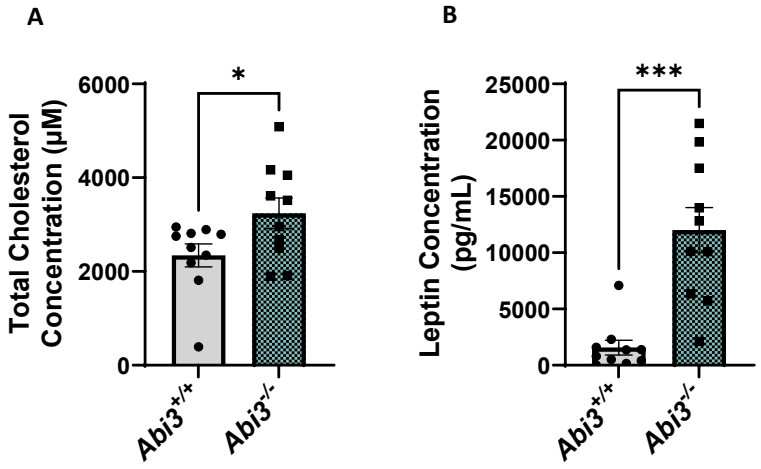


Figure 5. *Abi3*$^{-/-}$ mice exhibit increased plasma cholesterol and leptin concentration. (**A-B**) Total cholesterol and leptin concentration were measured in the plasma of 15-month-old male *Abi3*$^{+/+}$ (n=10) and *Abi3*$^{-/-}$ mice (n=10). (A) The cholesterol level in plasma was quantified via a colorimetric total cholesterol assay. (B) The leptin concentration was quantified via an electrochemiluminescence ELISA for mouse leptin. Data represent mean ± SEM. Unpaired t-test; *p < 0.05, ***p < 0.001.

2.3.4 *Abi3 locus deletion alters the transcriptome, independent of differences in weight, in the hypothalamus but not in brown adipose tissue.*

To gain insight into the potential pathways that are regulated by *Abi3* gene locus deletion, we evaluated the gene expression profile of tissues central to body weight and energy expenditure regulation by performing bulk RNA-seq on the hypothalami and brown adipose tissue of *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice. To isolate changes that were due to the absence of *Abi3* itself, rather than secondary to *Abi3* dependent differences in body weight, we performed weight-covariate controlled analysis of the RNA-seq data. For our analysis, differentially expressed genes (DEGs) were defined by a cut off of adjusted p-values less than 0.05. Within the brown adipose tissue, no DEGs were identified between *Abi3*$^{-/-}$ versus *Abi3*$^{+/+}$ mice (Figure 6). Within the hypothalamus, however, 31 weight covariate controlled DEGs were identified, including *Shank1*and *Cartpt*. (Figure 7A). Next, to determine the potential biologic relevance of these DEGs identified within the

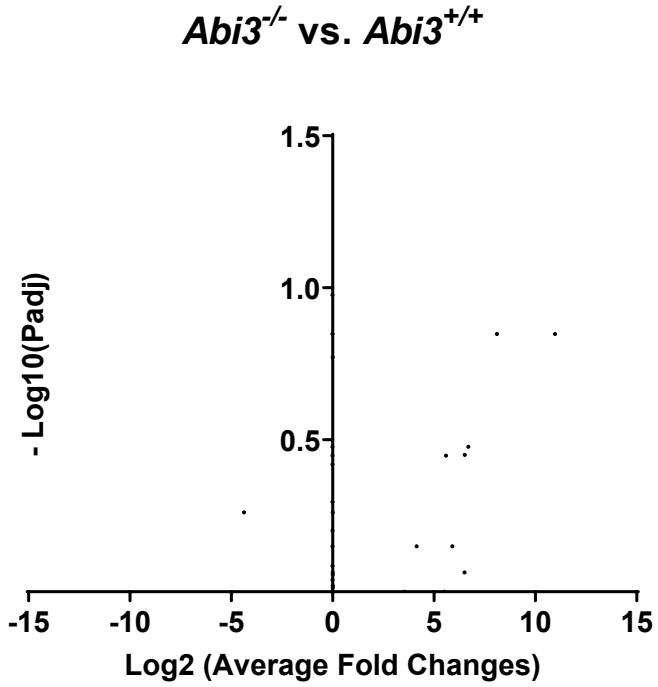


Figure 6. No alteration in the transcriptome of the brown adipose tissue between genotypes. Bulk RNA sequencing was performed with the brown adipose tissue of 15-month-old male *Abi3*$^{+/+}$ (n=4) and *Abi3*$^{-/-}$ mice (n=4). Weight co-variate controlled analysis was performed to identify differentially expressed genes (DEGs), defined by an adjusted-p value < 0.05. (A) Volcano plot representing the transcriptomic analysis of the BAT for *Abi3*$^{+/+}$ vs *Abi3*$^{-/-}$. No differentially expressed genes were identified.

hypothalamus, we performed secondary process network and pathway analyses using the Metacore pathway analysis software. The DEGs identified in *Abi3*$^{-/-}$ mice were significantly enriched in biologic processes including cytoskeletal regulation, synaptic cellular adhesion, and neuropeptide signalling, among others (Figure 7B). Further, these DEGs were significantly enriched in pathways including G-protein/Rac1 signalling and leptin regulation, among others (Figure 7C).

2.3.5 *Microglia number and area were decreased in the mediobasal hypothalamus, but not other brain areas in Abi3*$^{-/-}$ *mice.*

Because only the hypothalamus showed changes in gene expression due to the deletion of *Abi3* gene locus, and because *Abi3* is predominantly expressed by microglia

within the CNS, we performed Iba1 immunofluorescence on brain sections from *Abi3*$^{-/-}$ and *Abi3*$^{+/+}$ mice. Specifically, we assessed the changes in microglia number and area throughout multiple CNS regions (Figure 8). Across the cortex and hippocampus the (Figure 8A-D). Further, there was no significant difference in Iba1+ cell number or area when evaluating the entire hypothalamus (Figure 8E-F). However, within the mediobasal subregion of the hypothalamus, a region critical to the regulation of energy balance, *Abi3*$^{-/-}$ mice showed significantly lower number and area of Iba1+ cells (Figure 8G-H).

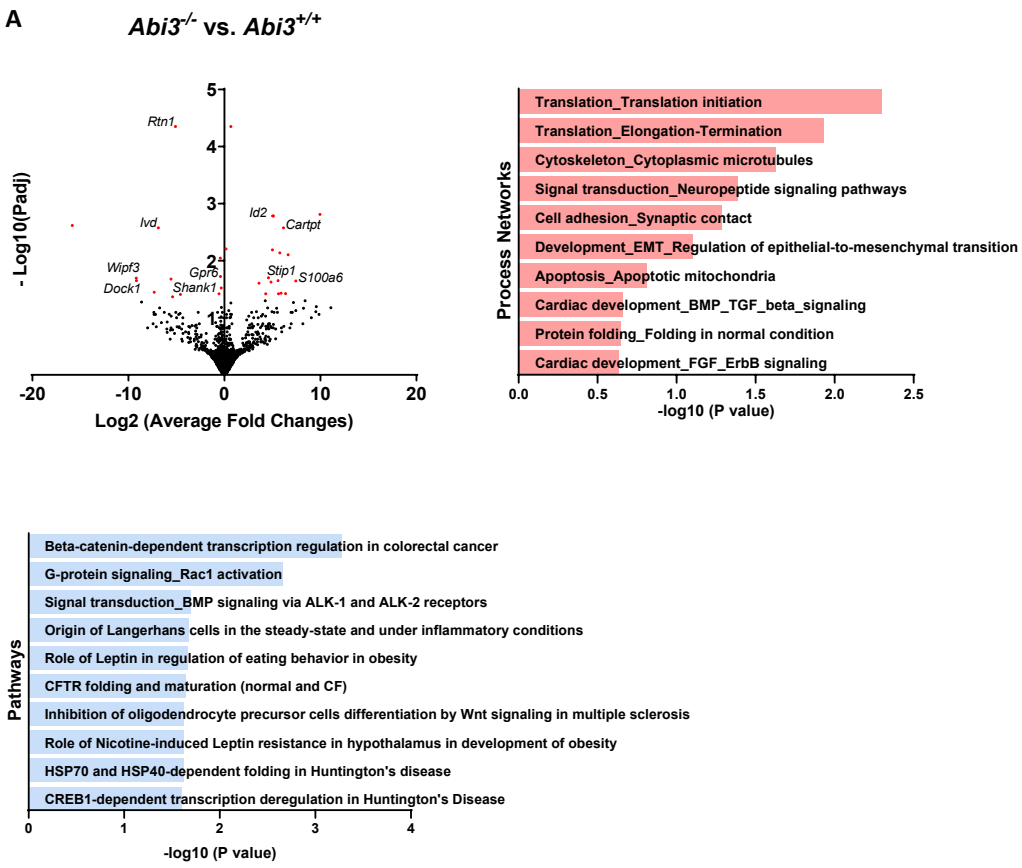


Figure 7. Deletion of *Abi3* gene locus, independent of differences in body weight, alters the transcriptome within the hypothalami. Bulk RNA sequencing was performed with the hypothalami of 15-month-old male *Abi3*$^{+/+}$ (n=4) and *Abi3*$^{-/-}$ mice (n=4). Differentially expressed genes (DEGs), defined by an adjusted-p value < 0.05, were then identified. (A) Volcano plot demonstrating the DEGs from the hypothalami of *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$mice. The red points represent differentially expressed genes. (B-C) The DEGs were used to perform (B) process network and (C) pathway analysis using the Metacore software. The significantly enriched (B) process networks and (C) pathways are listed.

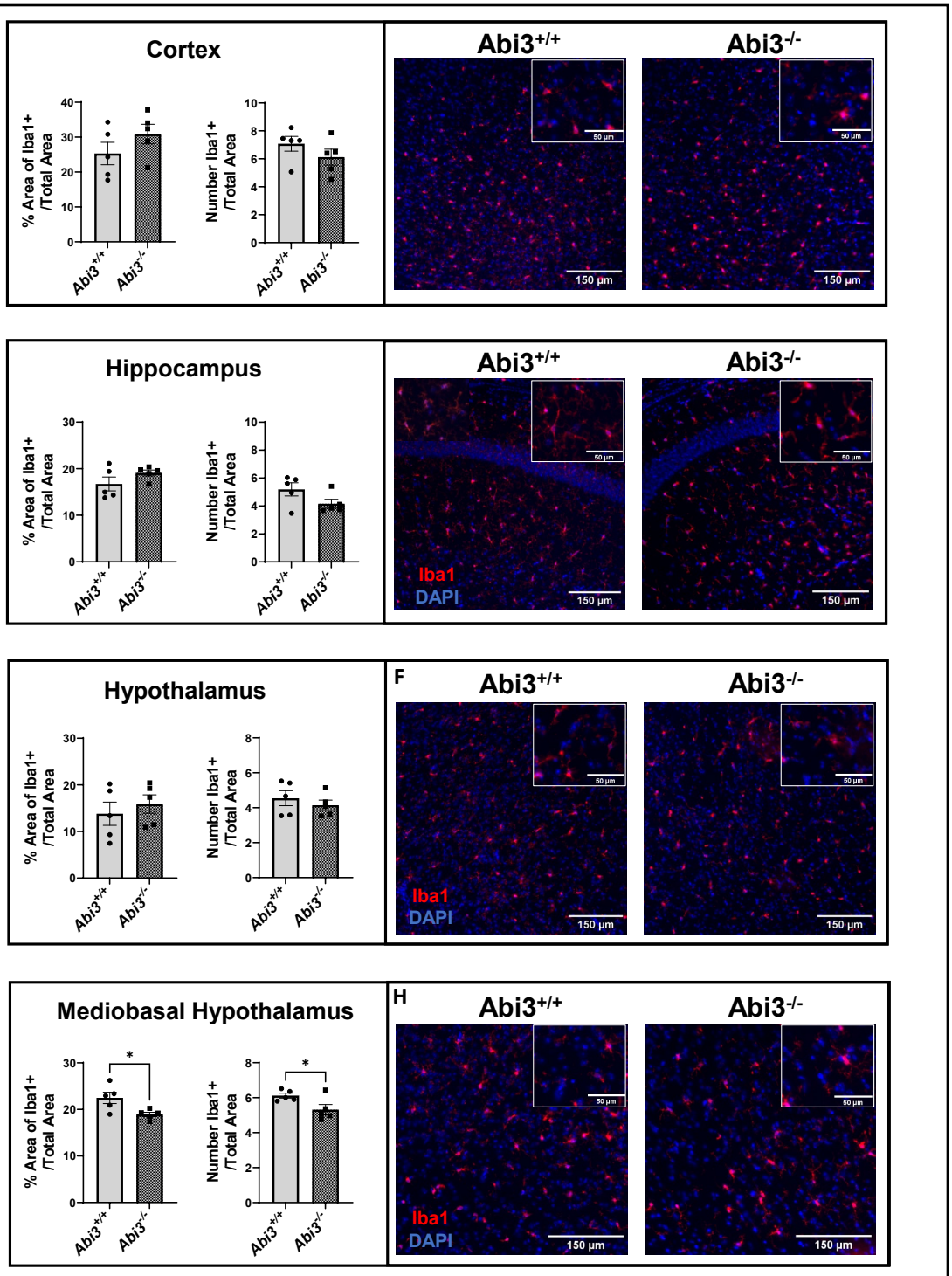


Figure 8. Deletion of *Abi3* gene locus reduces microglia number and area within the mediobasal hypothalamus, but not other brain regions. Coronal brain sections from 15-month-old male *Abi3*$^{+/+}$ (n=5) and *Abi3*$^{-/-}$ mice (n=5) were stained with Iba1+ (microglia marker) and DAPI (nuclear counterstain). (A, C, E, G) Representative images, for each brain region, show Iba1+ signal in red and DAPI in blue. (B, D, F, H) The number of microglia and area occupied by microglia were quantified across these brain regions. The area and number of Iba1+ signal was measured in the (A,B) cortex, (C,D) hippocampus, (E,F) whole hypothalamus, and (G,H) mediobasal hypothalamus. Data represent mean ± SEM. Unpaired t-test; *p < 0.05.

2.4 Discussion

In this chapter, we reported our discover that aged, male *Abi3*$^{-/-}$ mice had significantly elevated body and fat mass, and reduced energy expenditure. Importantly, these mice had reduced microglia number and area within the mediobasal hypothalamus, the brain region critical to the regulation of energy balance. Altogether, our data suggest that the functions of ABI3 are not only important in the response to neurodegenerative pathology as reported previously,[145] but also in the regulation of systemic metabolism.

Due to the unexpected nature of our initial discovery regarding the impact of *Abi3* deletion on body weight, our original observation of the obese phenotype did not occur until the mice were 15 months old. In these mice, we found that body weight and fat mass were significantly increased in the *Abi3*$^{-/-}$ mice, while lean mass was not altered. These findings indicated that the increased weight was driven predominantly by increased fat mass, which is indicative of an obese phenotype. Furthermore, we found that *Abi3*$^{-/-}$ mice had impairments in both glucose tolerance and insulin sensitivity. This likely suggests that the obesity in *Abi3*$^{-/-}$ mice induced impairments to systemic glucose regulation, as is typically observed in obese mice.[167] Additionally, as these findings were observed in aged mice, it is possible that the obese phenotype is an age dependent phenomenon. This is a finding that will be addressed during the next chapter, where the body weight of mice with and without *Abi3* was recorded weekly over multiple months. Additionally, only male mice were available for this investigation because female mice were already utilized for another project by the time that we unexpectedly observed the metabolic consequences of *Abi3* deletion. The impact of sex specific differences will also be addressed in the following chapter, where both male and female mice, with and without

ABI3 are investigated in the context of high fat diet induced obesity. However, future studies will be necessary to determine the full extent to which sex specific differences in microglia functions drive differential susceptibility to obesity.

Next, we investigated whether the difference in weight was driven by increased food intake, decreased energy expenditure, or a combination of both. We determined that the food intake and the overall movement of the mice did not change between the genotypes. However, *Abi3*$^{-/-}$ mice had significantly reduced slope on the regression of energy expenditure versus body weight during the light cycle but not the dark cycle. This indicates that *Abi3*$^{-/-}$ mice have reduced energy expenditure during their more inactive hours relative to *Abi3*$^{+/+}$ mice. Overall, these findings suggest that the loss of ABI3 function may drive obesity through impairments to energy expenditure. It is important, however, to note that these findings were observed in mice that were already obese. Therefore, it is possible that there might be additional mechanisms driving the obese phenotype in *Abi3*$^{-/-}$ mice. This may provide context for some of the conflicting results that will be reported and discussed in the following chapter. Critically, however, the regression analysis addressed the potential confounding effects of differing body weight; this enabled identification the direct genotype effect of the deletion of *Abi3* on energy expenditure, at least for those aged, male mice that already exhibited obesity.[168]

Energy expenditure in mice is predominantly regulated by the hypothalamus, centrally, and largely by thermogenic brown adipose tissue (BAT), peripherally [25,169,170]. Since we observed that *Abi3*$^{-/-}$mice exhibited impaired energy expenditure, we performed bulk RNA-seq on these tissues from *Abi3*$^{+/+}$ and *Abi3*$^{-/-}$ mice. In an attempt to isolate the direct effect of *Abi3* locus deletion on gene expression within these tissues, we performed

a weight-covariate controlled analysis. This approach did not identify any DEGs within the BAT but did identify DEGs within the hypothalamus. This suggests that the deletion of *Abi3* gene locus itself, as opposed to *Abi3* dependent differences in body weight, induced changes to gene expression within the hypothalamus but not within BAT. This could suggest that hypothalamus, not BAT, triggered the initial dysfunction that subsequently drives the impairment to energy expenditure. Furthermore, our pathway analysis indicated that the hypothalamic DEGs were enriched in biologic processes including cellular adhesion at the synapse, energy balance related neuropeptide signaling (*Cartpt*) and cytoskeletal remodeling (*Shank1*). The enrichment of the cytoskeletal related processes matches with the previously reported functions of ABI3. Specifically, ABI3 has been reported to be participant in cellular processes that require remodeling of the actin cytoskeleton, including cellular migration and membrane protrusion [140,145]. As *Abi3* is predominantly expressed by microglia within the CNS, our transcriptomic analyses suggests that the deletion of *Abi3* is impacting the function of microglia within the hypothalamus. Further, microglia are highly dynamic cells that are constantly surveying their microenvironment and communicating with other CNS cells, both of which are functions that require cytoskeletal remodeling [121,135]. Therefore, these sequencing data might point towards impaired cytoskeletal remodeling, and subsequently an impaired ability of microglia to perform their homeostatic functions, as a contributing factor to the induction of the obese phenotype. However, future investigation will be required to comprehensively test this hypothesis.

As previously discussed, altered microglia function within the hypothalamus has been connected to the concepts of maintenance of body weight and obesity [90,157,171-176].

Multiple studies have demonstrated that microglia number increases within the mediobasal hypothalamus during diet induced obesity.[60,70,175,177-180] Further, it has been demonstrated that interventions that reduce dietary induced microgliosis can also protect against diet induced obesity. [84,157,176] It has also been reported that genetic or pharmacologic treatments that increase microgliosis within the mediobasal hypothalamus are capable of inducing obesity even in the absence of dietary challenge [157,171]. Conversely, a recent study demonstrated that by deleting a gene specifically within microglia, the number of microglia within the mediobasal hypothalamus decreased but diet induced obesity was exacerbated [173]. Given the importance of microglia within the mediobasal hypothalamus to obesity, in conjunction with our sequencing analysis, we evaluated whether *Abi3*$^{-/-}$ mice had altered microglia number within the mediobasal hypothalamus or any other CNS region. Intriguingly, we found that microglia number and area were decreased specifically within the mediobasal hypothalamus, but not in other areas, of *Abi3*$^{-/-}$ mice compared to *Abi3*$^{+/+}$. We are not the first to report a decrease in the number of mediobasal hypothalamic microglia in the setting of impaired body weight regulation; however, to the best of our knowledge, we are the first to report such findings in the absence of dietary intervention.

Our data contribute to the growing concept that an appropriate level of microglia activity within the hypothalamus is necessary to maintain proper control of energy balance [158]. It appears that both hyperreactivity and the reduced reactivity of hypothalamic microglia may lead to dysfunctional regulation of systemic metabolism. Intriguingly, a similar paradigm regarding microglia activity has become well established in the neurodegenerative disease field [181]. Currently, however, little is known about the

exact cellular mechanisms by which hypothalamic microglia modulate energy balance. It has been postulated that microglia regulate control of energy balance via the modulation of neuronal signaling within the hypothalamus through microglia-neuron interactions [182]. Recent studies have shown that both increased and decreased microglial inflammatory activity within the mediobasal hypothalamus can disrupt leptin/melanocortin related neuronal signaling, a pathway that is central to the regulation of energy balance [84,157,173]. Further, it has been suggested that microglia may directly alter the organization of melanocortin system neuronal synapses [84,115].

In the context of evidence reported in this chapter, it is possible that the deletion of the *Abi3* gene locus impaired the ability of microglia to perform the complex functions that are necessary to regulate energy balance [158,183]. This possibility seems feasible when considering the importance of dynamic cytoskeletal remodeling for microglia to perform their basic functions (migration, surveillance, phagocytosis, etc.), along with the reported importance of ABI3 in processes that require cytoskeletal remodeling such as migration and phagocytosis [140,145]. However, future study will be necessary to uncover the specific functional impairment in microglia that led to impaired regulation of energy balance in *Abi3*$^{-/-}$ mice. Additionally, while our data are suggestive of a CNS driven disruption of energy balance, it remains possible that peripherally driven dysfunction could partly underly the obese phenotype. Future studies could utilize microglial specific inducible knock-out of *Abi3* to address this limitation more definitely, however, this resource was not available during the course of the research performed for this dissertation.

Overall, this chapter provides a novel, initial exploration into the role of *Abi3* in the regulation of systemic metabolism, opening the door to future investigation aimed at

uncovering how the manipulation of ABI3-related function can modulate metabolic disease states. In combination with the findings of our previous neurodegeneration-focused ABI3 investigation, our laboratory has now demonstrated how deletion of *Abi3* gene locus can have dramatic impacts on the seemingly distinct disease states of obesity and neurodegeneration, presumably due to disruptions in microglia functions.[145] Together, the findings reported in this chapter help to address the critical knowledge gap regarding the specific molecular drivers that may jointly underly the microglia dysfunction observed in metabolic disease.

Chapter Three: Deletion of the *Abi3* immune gene locus modulates the metabolic and neuroinflammatory response to a high-fat diet in a sex-dependent manner.

3.1 Introduction

The burgeoning obesity epidemic, with its vast array of associated metabolic disorders, has become a significant public health concern, underscoring the need to further elucidate the underlying genetic and molecular mechanisms that drive this disease state, as outlined in Chapter 1.[1,2] To address this need, the CNS-obesity field has expanded its research efforts to include the exploration of neuroimmune mechanisms as contributors to the development and progression of obesity. Most recently, these investigations have been directed at uncovering how microglia, the resident immune cells within the CNS, direct the metabolic and neuroinflammatory processes associated with energy balance regulation in order to modulate susceptibility to diet-driven obesity.[55,105,184,185] As outlined previously, the role of microglia within the hypothalamus, and in particular the mediobasal aspect of the hypothalamus, seems to be particularly important in metabolic regulation. To elaborate, the hypothalamus plays a pivotal role among all brain regions in regulating energy homeostasis, and alterations in microglial function within this brain region have been connected to disruption of this energy homeostasis, as discussed extensively in Chapter One.

The *Abi3* gene, an immune cell gene that initially garnered attention due to its implications in neurodegeneration, has also recently emerged as a necessary component in metabolic regulation. Importantly, within the CNS, *Abi3* expression is localized to microglia, as discussed in the preceding chapters. Further, we recently found that mice without *Abi3* became obese, seemingly due to altered microglia function, although

additional mechanisms cannot be ruled out, as discussed in Chapter Two.[81] However, the report in Chapter Two was limited by the fact that only 15 month old, male mice were investigated. Therefore, the present study aims to build upon these previous findings, extending the exploration of *Abi3* to determine its role in mediating the susceptibility to high fat diet-induced obesity. To address some of the limitations of the previous study, this report utilized both male and female mice, across multiple time points, during both high-fat and control diet conditions. Additionally, we aimed to determine the functional impact of the absence of *Abi3* on the neuroinflammatory response to the high fat diet. U

Understanding the molecular determinants that regulate diet associated neuroinflammation is valuable, as multiple investigations have demonstrated that this neuroinflammation is connected to the energy balance dysfunction observed during obesity.[76,77,80,98,112,186,187] Further, by examining both male and female mice, this study provides additional evidence to support the growing consensus that sex can dramatically impact both microglia responses and susceptibility to obesity.[87,89,123,124,126-129,188,189] Recent studies have highlighted the importance of considering sex differences in metabolic research, as males and females exhibit distinct susceptibilities to diet-induced obesity and related metabolic disorders, as reviewed in Chapter One. This study's focus on sex-dependent responses is, therefore, particularly relevant.

Considering the established role of *Abi3* in immune cell function, and our own findings connecting *Abi3* to energy balance regulation, we set about determining how the absence of this gene impacts hypothalamic microglial activity and neuroinflammatory response to dietary fatty acids, as well as energy balance regulation. To accomplish this, our investigation began with an *in vitro* approach that utilized BV2 microglia-like cells in

order to evaluate the impact of lowering *Abi3* expression level via siRNA on the inflammatory response to the saturated fatty acid, palmitic acid. Saturated fatty acids were employed as an "*in vitro* high fat diet", because they are a common element of high-fat diets and are thought to be a key inciting factor in high fat diet induced neuroinflammation.[76,182] Our findings revealed that the absence of *Abi3* does in fact modulate the inflammatory response of microglia to saturated fatty acids. Subsequently, this inspired us to investigate whether this response would be recapitulated *in vivo*, concomitant with the energy balance dysfunction. Towards that end, we performed a long-term high fat diet investigation on mice, with and without *Abi3*, as discussed above. In this investigation, we discovered that *Abi3* deletion led to increased body weight, elevated body fat mass, and increased food intake, but only in high fat diet fed female mice. Male mice, however, exhibited no differences during high fat diet feeding. These differing results observed across experimental groups underscore the complex interplay between genetic factors, dietary influences, and sex in metabolic regulation.

Furthermore, we performed a targeted neuroinflammation transcriptomic assay on the hypothalamus of the female mice. We utilized this transcriptomic analysis in order to determine the impact of *Abi3* deletion, high fat diet, and the combined impact of both *Abi3* deletion and high fat diet, on neuroimmune related gene expression within the hypothalamus. Downstream analysis revealed that the differentially expressed genes were enriched in processes and networks related to cytoskeletal remodeling (such as synaptic pruning and chemotaxis), but only in the presence of both high fat diet and *Abi3* deletion. Intriguingly, the combined presence of high fat diet and *Abi3* deletion was also the only condition that led to significant elevations in body weight, in female mice. To investigate

this potential connection further, we performed immunostaining and discovered that the area covered by microglia was decreased within the mediobasal hypothalamus but, again only, in the high fat diet fed, *Abi3*$^{-/-}$ female mice. Taken together, the findings suggest that *Abi3* related microglial functions may be necessary for the regulation of energy balance only in certain diet and sex-dependent contexts.

In conclusion, this research represents a significant advancement in understanding the complex interactions between immune function, diet, and metabolic health. By investigating the role of the *Abi3* gene in the context of high-fat diet-induced obesity, this report increased our understanding of the role of *Abi3* related functions in the regulation of energy balance, and that the importance of those functions is dependent on multiple factors such as diet and sex. The insights gained from this research hold the potential to inform future studies aimed at uncovering the exact cellular mechanisms by which microglia modulate energy balance and susceptibility to obesity.

3.2 Materials & Methods

3.2.1 *Cell Culture*

BV2 cells, derived from immortalized mouse microglia, were initially thawed and cultured in a humidified incubator with 5% CO2 at 37°C. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin to prevent bacterial contamination and 2% fetal bovine serum (FBS) to provide essential growth factors. The medium was refreshed every 48 hours, and cell confluency was closely monitored. Upon reaching optimal confluency of approximately 80%, the BV2 cells were gently detached using 0.25% trypsin-EDTA solution and subsequently plated for experimental treatment.

At the time of plating, the cells were transfected in order to knock down *Abi3* expression. Specifically, BV2 cells were treated with either a non-targeting siRNA negative control or a specific siRNA targeting the Abi3 gene for 24 hours, to allow for effective gene knockdown. Post transfection, BV2 subsequently were treated with either 100 μM of bovine-serum albumin (BSA) conjugated palmitic acid, which is a saturated fatty acid, or BSA control for an additional 24 hours. Immediately following this treatment, the cells were processed with RNA extraction, as described below.

3.2.2 *Animals & Diet Conditions*

The details of the *Abi3*$^{-/-}$ animals were described in the methods section of the preceding chapter, but this investigation had a few key differences. In this chapter, we utilized both male and female mice and employed the use of a high fat diet. Specifically, male and female mice, with and without deletion of *Abi3*, were fed either a high fat diet or control diet for 18 weeks, starting at 12 weeks of age. The diets utilized were high fat diet (45% calories from fat, Research Diets, D12451) and the sucrose and nutrient matched control diet (10% calories from fat, D12450). The mice were otherwise given standard housing conditions with access to food and water ad libitum. As with the animals utilized in Chapter Two, this study was performed with approval from the Institutional Animal Care and Use Committee of Indiana University School of Medicine and animals were housed within the vivarium of the Stark Neuroscience Research Institute.

3.2.3 *Tissue Preparation*

The tissues utilized in this investigation were collected and processed using the same method described in Chapter Two. In brief, we first administered Avertin to the

mice at a dosage of 250 mg/kg via intraperitoneal injection to achieve anesthesia. Following this, we performed a transcardial perfusion using ice-cold phosphate-buffered saline (PBS). Subsequently, both the brain and peripheral tissues were extracted promptly. For the purpose of RNA analysis, these tissues were dissected, then immediately preserved on dry ice. The procedures employed to prepare brain samples for immunofluorescence experiments are covered in the immunofluorescence methods section below. Prior to the final stages of processing, all the samples were stored at a temperature of -80 °C.

3.2.4 *Body Weight and Composition*

The methods utilized to measure body weight and composition in Chapter Two were also used for this study. Briefly, the body weight of the mice was determined using the Ohaus-SCOUT™-SPX622, a portable precision scale. For this study, the body weight of all mice was measured weekly, over the course of the 18 weeks of high fat diet or control feeding. Body composition was measured on the 12th week of high fat diet or control feeding. The body composition of the mice was measured by the EchoMRI^{TM} 500 system, at the Indiana Biological Research Institute. During the EchoMRI process, the mice were placed in a specialized tube designed to limit their movement. This setup facilitated the acquisition of imaging data over a duration of 1 to 4 minutes, employing an electromagnetic field with a strength of 0.05 Tesla. Subsequently, the images were analyzed in order to ascertain the lean and fat mass of the mice. As with the EchoMRI analysis employed in chapter two, this analysis was performed by interpreting the T1 and T2 relaxation curves using the established algorithms.

3.2.5 *Glucose Tolerance Test*

For the Glucose Tolerance Test (GTT), we initiated the procedure by fasting the mice for a period of 16 hours. Following this fasting period, each mouse was administered a glucose dose of 1 gram per kilogram body weight through an intraperitoneal injection. To facilitate blood collection, a minor incision was made in the tail of each mouse. Blood samples were subsequently collected at specific intervals: immediately before the glucose administration (0 minutes) and then at 10, 20, 30, 60, 90, and 120 minutes post-injection. The glucose levels in these samples were measured using a glucometer at each of the aforementioned time points. This experiment was performed during the 10^{th} week of experimental feeding.

3.2.6 *Insulin Tolerance Test*

The Insulin Tolerance Test (ITT) is similar to the protocol of the GTT, but with a few key differences. First, mice were only fasted for 2 hours prior to the assay. Following the fast, insulin was administered intraperitoneally at a dose of 0.75 units per kilogram of body weight. The procedure for blood glucose measurement in ITT mirrored that of the GTT, with the exception of the time points for sampling. In the ITT, blood glucose levels were recorded just before the insulin injection (0 minutes) and at intervals of 15, 30, 45, and 60 minutes following the injection. This experiment was performed during the 14^{th} week of experimental feeding.

3.2.7 *Calorimetry Cage Analysis*

As with the metabolic cage experiments performed in the preceding chapter, this investigation also utilized the PhenoMaster System from TSE Systems. This system records multiple metabolic relevant parameters including food and water consumption,

respiratory gas concentrations, and locomotor activities. In this study, only the high fat diet fed female mice were analyzed using this system. Further, the mice underwent calorimetry cage analysis on the 12th week of high fat diet feeding. The mice were individually housed within the calorimetry cages of the PhenoMaster system. Following entry, the mice were given a three-day acclimatization period to adapt to the experimental environment. Following acclimation, the experimental period began, and the metabolic and behavioral parameters outlined above were recorded over 72 hours. From these collected data, we then extrapolate critical metabolic indices, such as energy expenditure and the respiratory exchange ratio.

3.2.8 *RNA Extraction*

In the preceding chapter, we detailed our methodological approach for RNA extraction and analysis. The same approach was utilized to extract RNA from the hippocampus of mice, and from BV2 cells. Briefly, RNAs were obtained via a standard extraction with phenol and chloroform. Tissues and cells were initialized lysed using TRIzol reagent. Subsequently, we evaluated the concentration and purity of the extracted RNA using a Nanodrop 2000 spectrophotometer. Post-assessment, the RNA samples were meticulously preserved at a temperature of -80 °C to maintain their integrity for subsequent processing steps.

3.2.9 *qPCR*

Quantitative PCR (qPCR) was employed to quantify the mRNA expression levels of immune related transcripts in BV2 cells subjected to treatment with saturated fatty acids. Immediately following RNA extraction as described above, reverse transcription was performed on the RNA samples using the high-capacity Applied Biosciences cDNA

Reverse Transcription Kit in order to generate complementary DNA (cDNA) for qPCR analysis. The qPCR reaction was performed on Quantstudio 3 qPCR instrument, and the detection reagent used was Fast SYBR green. The experimental primers used in this study were Abi3, C1qa, IL-6, Nos1, Tnfα, and IL-1ß, while GAPDH served as the endogenous control. The relative quantification of mRNA levels was calculated using the comparative cycle threshold (ΔΔCt) method. This approach allowed for the comparison of gene expression levels in siRNA-treated and control cells, both with and without palmitic acid exposure.

3.2.10 *nCounter Nanostring Neuroinflammation Transcriptomic Panel*

The NanoString Mouse Neuroinflammation Panel was employed to profile gene expression in these samples. Specifically, the Neuroinflammation panel is designed to detect 592 neuroimmune related genes. This panel was performed using the nCounter Analysis System, as directed by the manufacturer's protocols. For this targeted transcriptomic experiment, we utilized the hypothalami from high-fat diet and control diet fed, $Abi3^{+/+}$ and $Abi3^{-/-}$ female mice. For these samples, RNA extraction was performed as described above, and the experimental samples were then diluted to 15 ng/µL.

The transcriptomic data were then evaluated using the Limma package (version 3.44.3) in RStudio (version 1.3.959), utilizing R (version 4.0). Additionally, the NanoString panel gene expression data were log-normalized. This analysis enabled the identification of neuroimmune genes that are differentially expressed due to the impact of *Abi3* deletion and/or high fat diet consumption. To culminate our analysis, we conducted

pathway and network analyses using MetaCore software. This approach enabled us to determine what pathways and functions are enriched among the identified DEGs.

3.2.11 *Immunofluorescence*

Following perfusion with ice cold PBS, mouse brains were collected and stored in 4% PFA overnight for 48 hours. Following this, the brain samples were stored in 30% sucrose in PBS for 48 hours. Then, the brain samples were embedded in frozen OCT, coronally sliced, and then mounted to slides for immunofluorescence staining, as described in the methods of Chapter 2. To review, three 30 μm brain sections were mounted from each mouse, separated by a distance 120 μm between each section. Then, the brain sections were permeabilized in PBS with 0.3% Triton X-100 and were then stained with the following primary antibody overnight: IBA1 (Rb, ab178846), used at a dilution of 1:1000 in PBS with 0.1% Triton X-100 with 2.5% animal serum. The following secondary antibodies then then used: Alexa Fluor® 488 Donkey Anti-Rabbit (712-545-152), at a dilution of 1:400 PBS with 0.1% Triton X-100 with 2.5% animal serum. Sections were subsequently immersed in a DAPI containing solution for 10 minutes and, finally, were then mounted with coverslips using Aqua-Poly/Mount mounting medium. The slides were then imaged with the Leica Thunder DMi8 Fluorescent Microscope. Our image analysis workflow began with segmentation of the regions of interest in ImageJ, followed by deconvolution using Ilastik software, and finally end with the use of CellProfiler to quantify the positive signal.

3.2.12 *Statistical Analysis*

The statistical analysis for this study was performed largely as described in equivalent methods section of Chapter Two. In brief, comparisons between two groups,

such as Abi3$^{+/+}$ and Abi3$^{-/-}$, were performed using unpaired t-tests. Multiple group comparisons, such as the evaluation of body weight between high fat diet fed Abi3$^{+/+}$ and Abi3$^{-/-}$ mice and control diet fed Abi3$^{+/+}$ and Abi3$^{-/-}$ mice, were performed using one-way analysis of variance (ANOVA). For experiments that utilized repeated measures, such as GTT and ITT, two-way ANOVA with Tukey's post hoc test was utilized. In analysing the relationship between energy expenditure and weight, we employed the use of simple linear regression. This statistical method helps in understanding how the change in one variable (weight) affects the other (energy expenditure) and allows for comparing how genotype (*Abi3* status) alters the relationship between those variables. Lastly, all data in this study were presented as means ± standard error of the mean (SEM).

3.3 Results

3.3.1 *Knockdown of Abi3 in BV2 cells alters inflammatory gene expression following saturated fatty acid treatment.*

Our original discovery regarding the metabolic consequences of *Abi3* deletion was observed in aged mice that were fed a standard diet. Because of this, we became interested in exploring the potential impact the absence of *Abi3* would have in the presence of a more direct metabolic challenge. Given the relevance of high fat diet to obesity and metabolic disease, and the fact it is a potent pro-inflammatory stimulus, we became interested in evaluating how the absence of *Abi3* would impact the metabolic and inflammatory effects of high fat diet.

Prior to investigating the effect of high fat diet *in vivo*, we aimed to determine if loss of *Abi3* expression in BV2 microglia-like cells would impact the inflammatory response to saturated fatty acids. Saturated fatty acids (SFAs) are a potent

proinflammatory stimulus and are suggested to underly the inflammatory effects seen during consumption of a high fat diet.[72,76,175,190] Therefore, saturated fatty acid treatment of cell culture can be used as an *in vitro* model of high fat diet.[48,191]

Therefore, we measured the inflammatory response of BV2 microglia cells during treatment with the saturated fatty acid, palmitic acid. Specifically, BV2 cells were first treated with siRNA mediated knockdown of *Abi3* or a control siRNA. The cells then were treated with either palmitic acid (PA) or BSA-conjugated control for 24 hours (Figure 9).

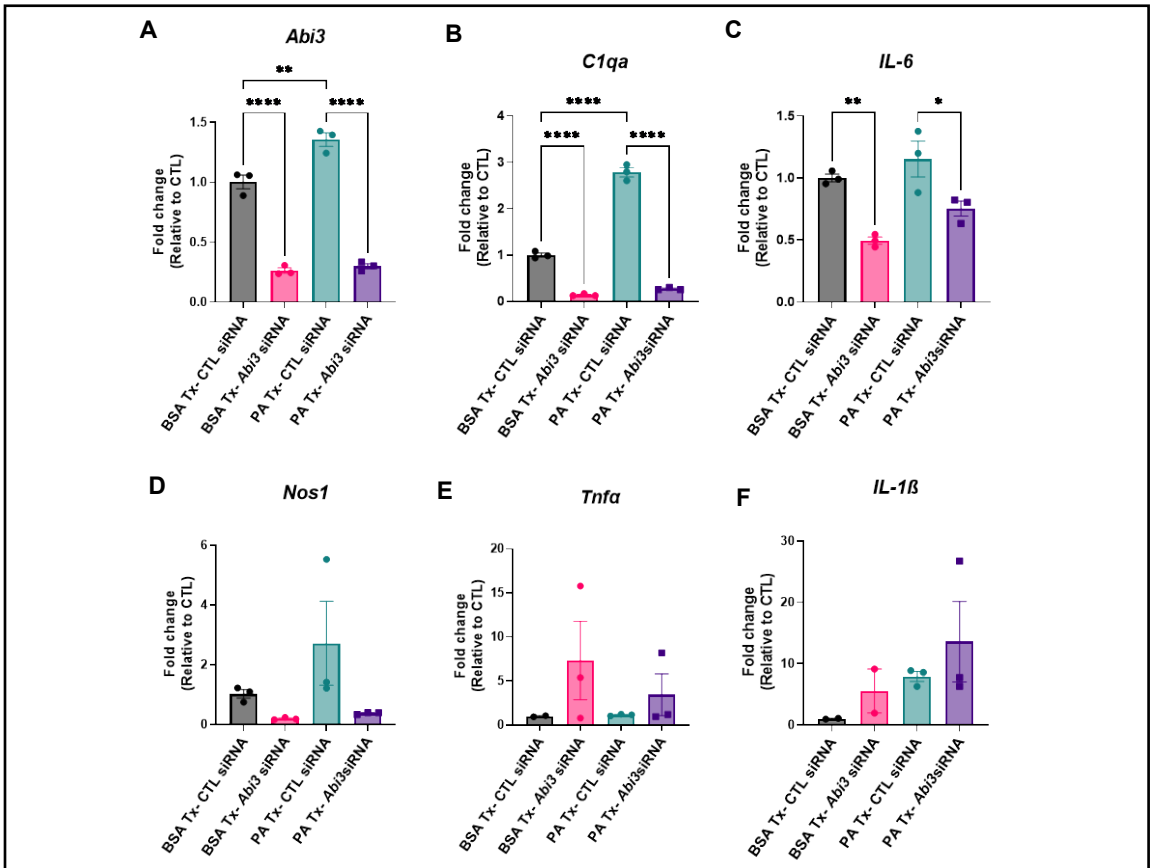


Figure 9. The knockdown of *Abi3* alters the expression of immune proteins in both palmitic acid treated and BSA-control treated BV2 Cells. BV2 cells were treated with 20 nM control (**CTL**) or Abi3 siRNA, and then were treated with 100 µM of palmitic acid (**PA**) or BSA control for 24 hours. The mRNA levels of (A) Abi3, (B) C1qa, (C) Il-6, (D) Nos1, (E) Tnfα, (F) Il-1ß were then measured via qPCR. Data represent mean ± SEM. Unpaired t-test; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

We measured expression level of *Abi3, C1qa, Il-6, Nos1, Tnfà, Il-β* via qPCR, targets reported to be impacted by exposure to fatty acids. First, we confirmed the efficiency of our siRNA mediated knockdown of *Abi3* (Figure 9A). Intriguingly, the treatment with palmitic acid led to a significant elevation *Abi3* (Figure 9A). The expression of C1qa was significantly increased by palmitic acid treatment, but the knockdown of *Abi3* significantly reduced *C1qa* expression in both palmitic acid and control conditions (Figure 9B). While palmitic acid had no effect on expression of *Il-6*, *Abi3* knockdown significantly reduced the expression of *Il-6* in both conditions. No significant changes were observed in the expression of N*os1, Tnfα, or Il-1ß*.

3.3.2 *Deletion of Abi3 in mice impacts their susceptibility to high fat diet induced obesity, in a sex dependent manner.*

Our investigation in Chapter Two revealed that the absence of *Abi3* results leads to the development of obesity. Further, the above the cell culture finding indicate a potential role for *Abi3* in mediating the inflammatory response to high fat diet. Together, these data suggested that an investigation the impact of *Abi3* deletion on susceptibility to high fat diet induced obesity was warranted. Specifically, we provided high fat or control diet to both male and female, $Abi3^{-/-}$ vs $Abi3^{+/+}$ mice for 18 weeks. The experimental feeding began when the mice reached 12 weeks of age, and the body weight of the mice was measured weekly during experimental feeding (Figure 10).

In female mice (Figure 10A), the high fat diet fed (HFD-fed), $Abi3^{-/-}$ mice exhibited highest overall weight throughout the feeding. When evaluating the body weight data over time, the HFD-fed, $Abi3^{-/-}$ female mice exhibited significantly elevated bodyweight as compared to both CTL-fed female groups ($Abi3^{-/-}$ vs $Abi3^{+/+}$) starting at 5

weeks, and this remained until the completion of the experiment. As for the HFD-fed female mice, we observed that HFD-fed *Abi3*$^{-/-}$ mice had elevated average body weight, as compared to HFD-fed *Abi3*$^{+/+}$ mice, throughout the experiment that reached statistical significance at 7, 13, and 18 weeks. Interestingly, the HFD-fed *Abi3*$^{+/+}$ female mice did not exhibit significantly elevated body weight when compared to the CTL-fed female mice at any point. Further, there were no significant differences in body weight between the female, CTL-fed, *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$ mice throughout the experiment.

For male mice (Figure 10B), while there is a trend towards increased body weight of CTL-fed *Abi3*$^{-/-}$ mice as compared to CTL-fed *Abi3*$^{+/+}$ mice, no statistically significant differences in body weight were observed between *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$ mice in either diet condition. When comparing HFD vs CTL-fed, male mice, we observed that HFD-fed mice had significantly elevated weight, as compared to CTL-fed mice, by 4 weeks which remained significant for the remainder of the experiment.

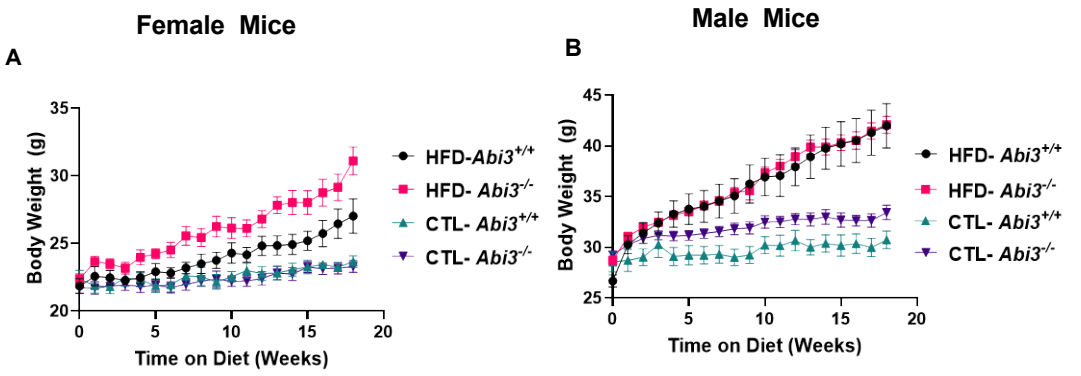


Figure 10. Deletion of *Abi3* increases the body weight of HFD fed female mice. Body weight was recorded weekly for 18 weeks, upon starting CTL or HFD diet at 12 weeks of age, in (**A**) female, HFD-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ mice (n=14) and CTL-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ mice (n=12), as well as their (**B**) male, HFD-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ (n=23) and CTL-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ (n=17) counterparts. Data represent mean ± SEM. 2-way ANOVA with Tukey's post hoc test was utilized.

Because the deletion of *Abi3* only resulted in significant differences in body weight high fat diet fed female mice, we performed body composition analysis on the

HFD-fed, *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$ female mice (Figure 11). Further, the body composition was assessed via EchoMRI, on the 12th week of experimental diet. Female, HFD-fed, *Abi3*$^{-/-}$ mice exhibited significant elevations in body fat mass and percentage (Figure 11A-B), without changes in lean mass and percentage (Figure 11C-D); this indicates that the increased body weight is being primarily driven by increased fat mass. Additionally, HFD-fed, female, *Abi3*$^{-/-}$ mice had significantly elevated body weight at the final weight time point (Figure 12A). Further, tail length was also measured at this time point, and no significant differences were observed (Figure 12B).

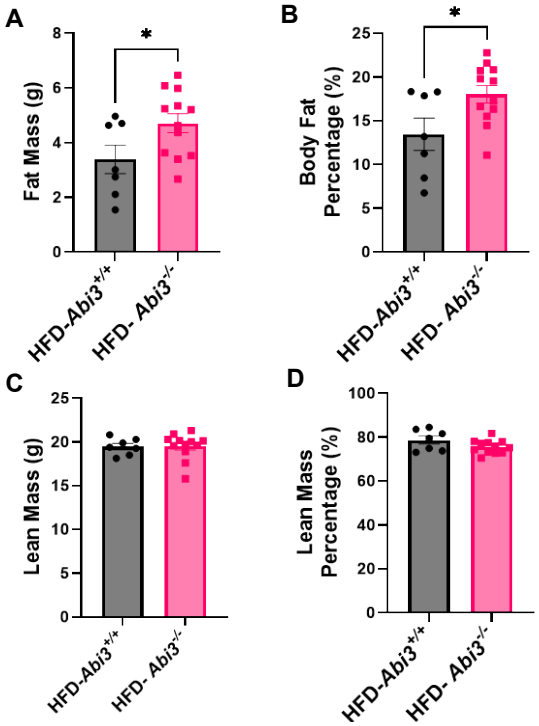


Figure 11. Body weight and body fat are increased, while lean mass is unchanged in female, HFD-fed, *Abi3*$^{-/-}$ mice. (**A-D**) The body composition of female, HFD-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ mice (n=14) was evaluated via EchoMRI on week 12 of feeding (6 months old). The **(A)** fat mass and **(B)** percentage and **(C)** lean mass and **(D)** percentage were recorded. Additionally, the weight (**E**) and **(G)** length, from base of the tail to tip of the nose, of these mice was recorded on the 18th and final week of feeding. Data represent mean ± SEM. Unpaired t-test was used; *p < 0.05.

3.3.3 *Deletion of Abi3 in mice, in combination with high fat diet feeding, leads to impairment in glucose tolerance.*

Our study aimed to elucidate the combined and independent impacts of Abi3 deletion and high-fat diet (HFD) intake on glucose homeostasis. To this end, we conducted glucose tolerance tests (GTT) and insulin tolerance tests (ITT) on female mice across both diet and genotype conditions. Because the deletion of *Abi3* only resulted in significant differences in body weight in female, HFD-fed mice, only female mice were utilized in this experiment.

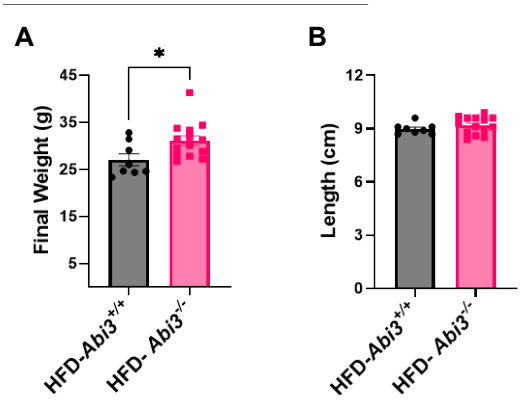


Figure 12. At the final experimental timepoint, body weight was elevated, and body length unchanged in female, HFD-fed, *Abi3*$^{-/-}$ mice. (A) The weight and (B) body length, from base of the tail to tip of the nose, was recorded on the 18th and final week of feeding of the female, HFD-fed *Abi3*$^{+/+}$ (n=8) and *Abi3*$^{-/-}$ mice (n=14). Data represent mean ± SEM. Unpaired t-test was used; *p < 0.05.

First, glucose tolerance was evaluated utilizing the GTT on all female mice, on the 12th week of experimental feeding. Notably, only the HFD-fed, *Abi3*$^{-/-}$ mice exhibited significantly higher blood glucose levels as compared to their Abi3$^{+/+}$ counterparts on the same diet, as well as both CTL-fed groups (Figure 13A). This difference was quantitatively determined by the increased area under the curve (AUC) for the HFD-fed Abi3$^{-/-}$ group, signaling a compromised glucose tolerance (Figure 13B). Apart from this

distinct observation, the GTT did not reveal any other significant differences among the groups (Figures 13A).

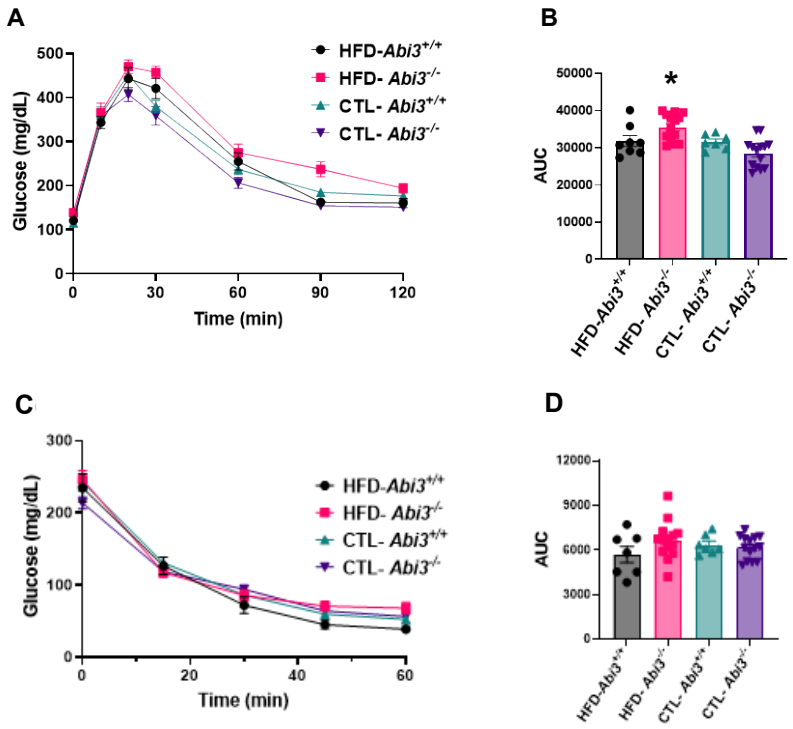


Figure 13. HFD-fed *Abi3*$^{-/-}$ female mice exhibit mild impairment in glucose tolerance. Glucose tolerance test (GTT) and insulin tolerance tests (ITT) were performed on HFD-fed *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) and CTL-fed *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) female mice. GTT and ITT were performed at 10th and 14th week of experimental diet, respectively. For both (**A-B**) GTT and (**C-D**) ITT, mice were fasted prior 16 or 2 hours, and then injected with either 1 g/Kg glucose or 0.75 units insulin, respectively. Blood glucose readings were then collected via glucometer. over 30, 60, 90, and 120 minutes or 15, 30, 45, and 60 minutes following injection, respectively. Data represent mean ± SEM. For GTT and ITT time-course data, 2-way ANOVA with Tukey's post hoc test was used. For all other comparisons, unpaired t-test was used; *$p < 0.05$.

Contrastingly, the ITT results were uniform across all experimental conditions. Across all time points and all experimental groups, no significant differences were identified in the blood glucose levels or in the integrative AUC analyses (Figure 13C-D). This suggests that, within the parameters of this study, insulin sensitivity is unaffected by the absence of *Abi3* at this experimental time point.

3.3.4 *Deletion of Abi3 results in increased food intake, without changes in energy expenditure, in high fat diet fed, female mice.*

In an effort to elucidate the mechanisms underlying the observed body weight discrepancies between high fat diet (HFD) fed Abi3$^{+/+}$ and Abi3$^{-/-}$ mice, we utilized comprehensive metabolic phenotyping with the TSE Phenomaster system. This approach, employed calorimetry cage analysis, allowing for the simultaneous monitoring of multiple metabolic parameters including energy expenditure, food intake, and activity levels, and thereby providing insights into the causative drivers underlying the weight variations.

Initial analyses focused on energy expenditure across various phases of the diurnal cycle—dark, light, and the aggregate 24-hour period. Our findings revealed no significant differences in average energy expenditure between HFD-fed, female, Abi3$^{-/-}$ and Abi3$^{+/+}$ mice (Figure 14A-B). Further regression analysis, aimed at exploring potential alterations in energy expenditure relative to body mass, corroborated these results, showing no deviation in energy efficiency across the diurnal spectrum, irrespective of *Abi3* genotype status (Figure 14C-E). In addition, respiratory exchange ratio (RER), which serves as an indicator of substrate utilization, did not differ significantly between the two groups, suggesting a balanced energy derivation from lipids and carbohydrates across both genotypes under HFD conditions (Figure 15A-B). This was further supported by regression analyses that evaluated the relationship between body weight and RER, which also failed to show significant differences (Figure 15C-E). Contrastingly, the analysis of locomotor activity unveiled a notable increase in total

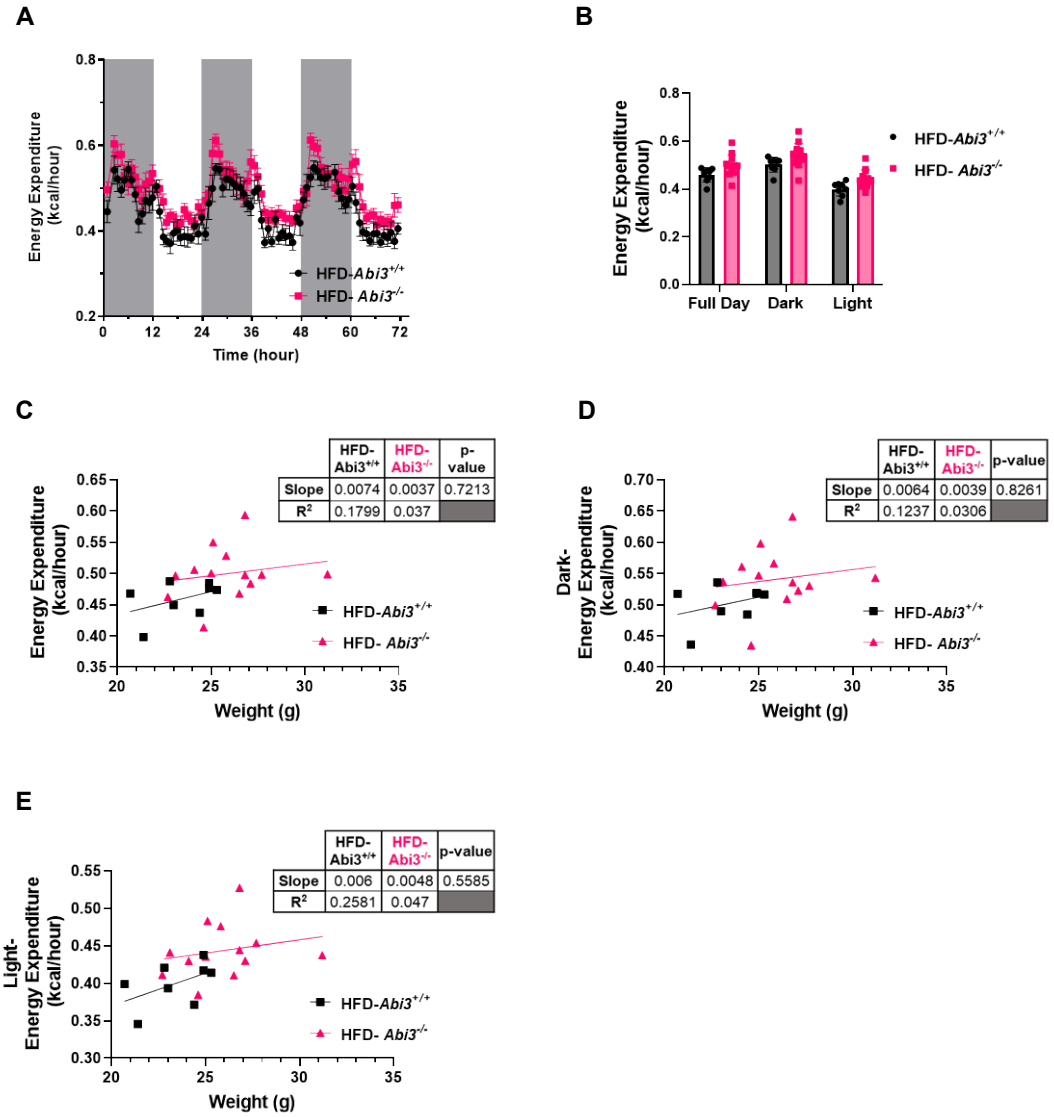


Figure 14. Deletion of *Abi3* does not alter energy expenditure in HFD fed female mice. HFD fed *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) female mice were placed in calorimetry cages and **(A)** energy expenditure was recorded over 72 hours. For the energy expenditure graph (**A**), the dark regions indicate night (dark), and the light regions represent day (light) (**B**) Average energy expenditure was calculated for Light hours, Dark hours, and the entire 72 hours (full day). (**C-D**) Subsequent linear regression analysis of average energy expenditure versus weight was performed to assess the relationship between mass and energy expenditure. Regressions were performed for the (**C**) light, (**D**) dark, and (**G**) full portions of the day. Data represent mean ± SEM. Unpaired t-test, simple linear regression.

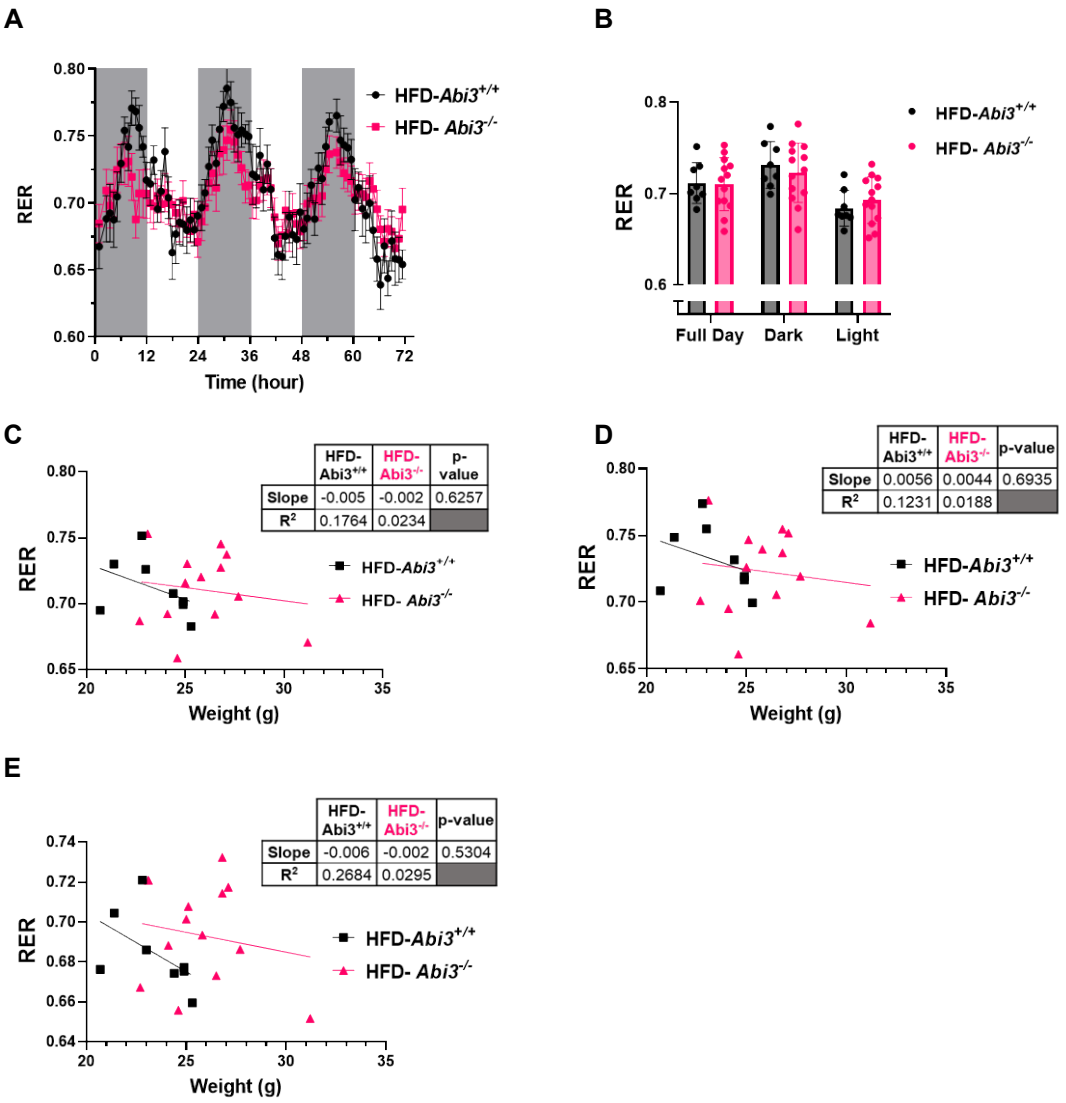


Figure 15. Deletion of *Abi3* does not alter the respiratory exchange ratio (RER) in HFD fed mice. HFD fed *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) female mice were placed in calorimetry cages indirect calorimetry data was collected over 72 hours. For the (**A**) RER graph, the dark regions indicate night (dark) and the light regions represent day (light) (**B**) Average RER was calculated for Light hours, Dark hours, and the entire 72 hours (full day). (**C-D**) Subsequent linear regression analysis of average RER versus weight was performed to assess the relationship between mass and energy expenditure. Regressions were performed for the (**C**) light, (**D**) dark, and (**G**) full portions of the day. Data represent mean ± SEM. Unpaired t-test, simple linear regression.

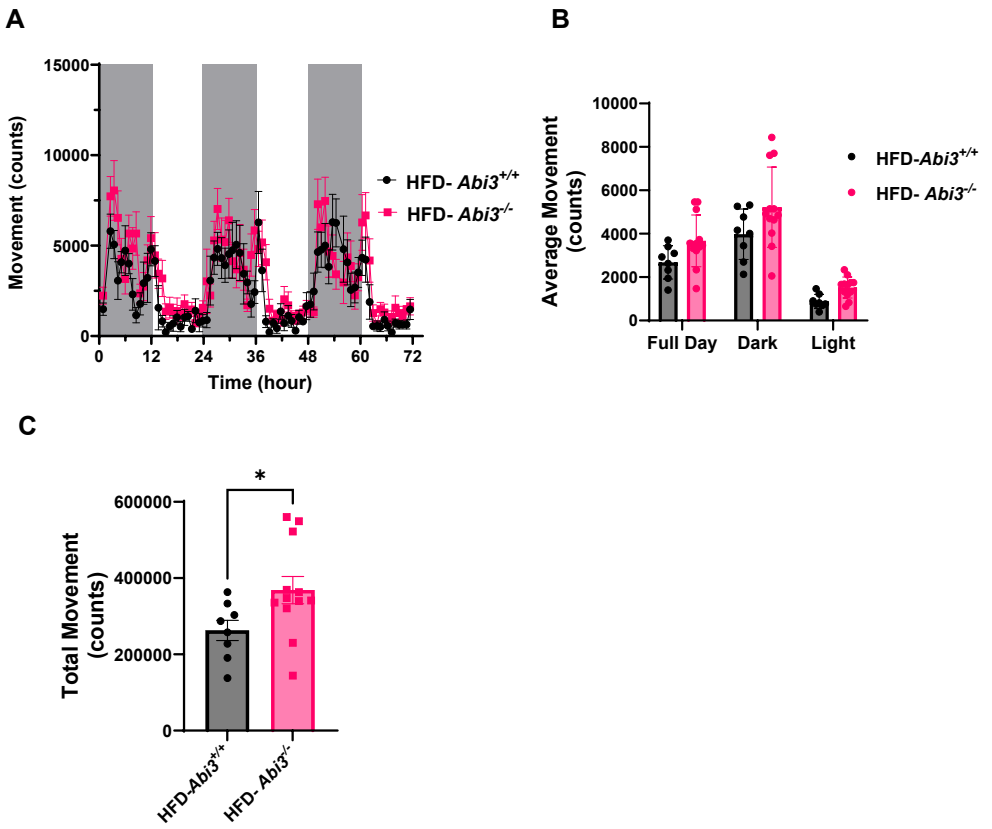


Figure 16. HFD-fed, female *Abi3*$^{-/-}$ mice exhibited increased overall movement. HFD-fed, female *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) mice were placed in calorimetry cages movement was collected over 72 hours. For the (**A**) movement over time graph, the dark regions indicate night (dark), and the light regions represent day (light) (**B**) Average movement was calculated for Light hours, Dark hours, and the entire 72 hours (full day). (**C-D**) Movement was detected by software that tallies a count for each distinct movement in the XY plane**. (A)** Movement was recorded over time. **(B)** Total movement was calculated along with **(C)** the overall average movement for each mouse during the full, dark, and light portions of the day. Data represent mean ± SEM. For time-course data, 2-way ANOVA with Tukey's post hoc test was used. For all other comparisons, unpaired t-test was used. Data represent mean ± SEM. Unpaired t-test, simple linear regression. *p < 0.05.

movement among HFD-fed Abi3$^{-/-}$ female mice compared to their Abi3$^{+/+}$ counterparts, despite no significant change in average movement (Figure 16A-C). Lastly, examination of food intake during the calorimetry cage assessment revealed a significant increase in consumption among the female, HFD-fed Abi3$^{-/-}$ mice (Figure 17). This increase in caloric intake, juxtaposed with the unaltered energy expenditure, may drive the observed weight gain in this group.

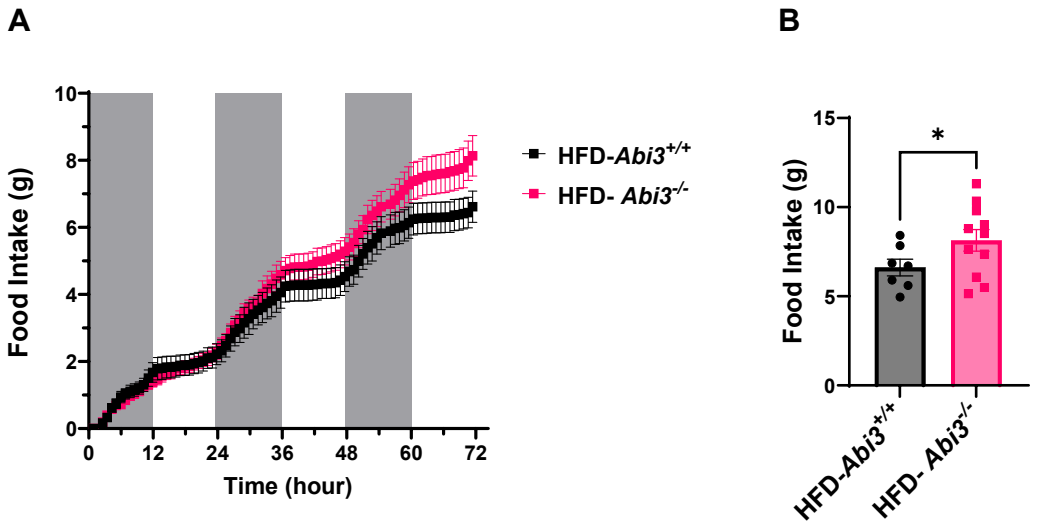
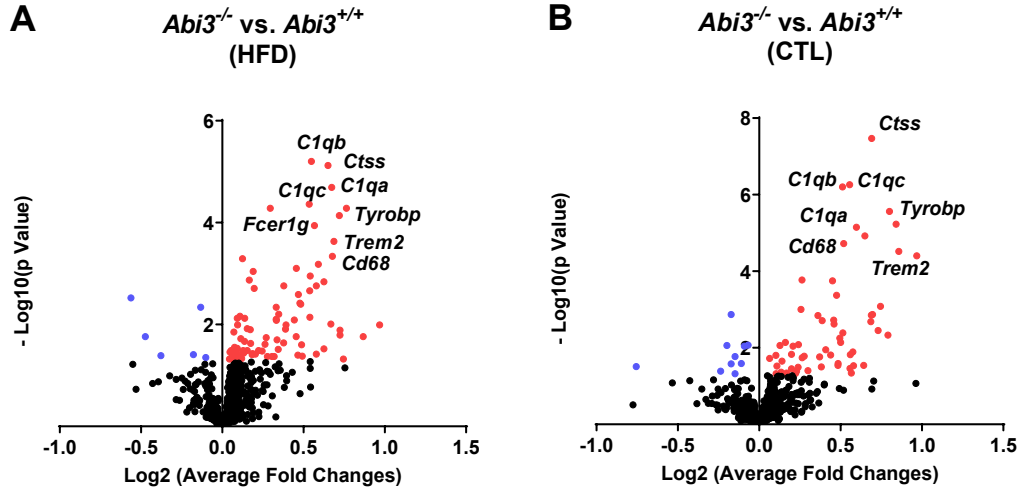
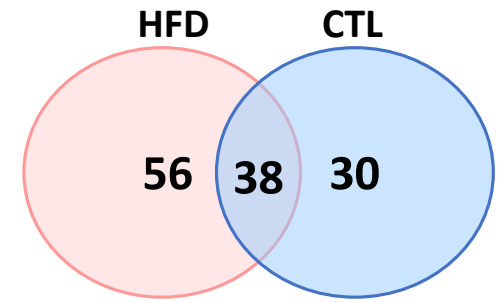


Figure 17. Deletion of *Abi3* led to increased food intake in female, high fat diet fed mice. HFD-fed, female, *Abi3*$^{+/+}$ (n=7) and *Abi3*$^{-/-}$ (n=14) female mice were placed in calorimetry cages and (A) food intake was measured over 72 hours. (A) Food intake over time and (B) overall total food intake consumed during the 72-hour experiment were recorded. For the (A) food intake over time graph, the dark regions indicate night (dark), and the light regions represent day (light). Data represent mean ± SEM. Unpaired t-test. *p<0.05

3.3.5 *The absence of Abi3 and the consumption of high fat diet, both individually and in concert, uniquely alter neuroimmune associated gene expression within the hypothalamus.*

As discussed in Chapter One, the consumption of a high fat diet is established to induce and exacerbate neuroinflammation particularly within the hypothalamus [192-194]. Additionally, the hypothalamic microglia are thought to serve a central role in the modulation of this neuroinflammation.[158] Given our preliminary findings highlighting Abi3's critical role in body weight regulation and microglial functionality, we aimed to determine how deletion of *Abi3* impacted the neuroinflammatory immune response to high fat diet.





D

HFD-specific (56)					CTL diet-specific (30)			Common DEGs (38)		
Anapc15	Cox5b	Irf3	Pcna	Setd1a	Abcc3	Gadd45a	Psmb8	Apc	Cx3cr1	Man2b1
Atg9a	Cx3cl1	Itgam	Pik3cb	Slamf9	Agt	Inpp5d	Ptprc	Bcl2a1a	Dock2	Mpeg1
Axl	Dlg1	Lamp1	Pik3cg	Slc2a5	Bcl2l1	Jam2	Rapgef3	C1qa	Dst	Ncf1
Becn1	Fabp5	Lgmn	Prkaca	Slc6a1	Blnk	Jun	Serpine1	C1qb	Fcer1g	Olfml3
Bnip3l	Fcrls	Lilrb4a	Prkce	Sqstm1	Casp8	Lag3	Sesn1	C1qc	Fcgr3	Plcg2
Calr	Fgd2	Mapk10	Pten	Srgn	Cd83	Lrrc3	Slc17a6	C3ar1	Gna15	Plxdc2
Casp6	Gja1	Mfge8	Ptms	Sumo1	Cdkn1a	Map1lc3a	Tcirg1	C4a	Gpr34	Ptpn6
Ccr5	Grin2a	Nefl	Rab6b	Tmem204	Epg5	Mavs	Tgfb1	Cd68	Gria4	Slco2b1
Cd70	Homer1	Nfkbia	Rad50	Tnfrsf1a	Fcgr1	Nlgn1	Tnfrsf1b	Cd84	Grn	Tmem119
Cideb	Hprt	Nostrin	Rad51c	Tubb3	Fcgr2b	Nthl1	Trp53bp2	Cd86	Hpgds	Trem2
Clic4	Irak4	P2ry12	Rela	Vps4a				Csf1r	Irf8	Tyrobp
Cnn2								Csf3r	Itgb5	Vav1
								Ctss	Mafb	

Figure 18. Deletion of *Abi3* alters neuroinflammation associated gene expression within the hypothalamus, in ways that are both unique to and dependent on diet. Targeted NanoString Neuroinflammation profiling of the hypothalamus enabled the identification of DEGs between *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$mice, in both HFD and CTL fed conditions (n=6/genotype/diet). The volcano plots (**A-B**) visually represent this NanoString analysis, with significance, shown as -log10(pValue), on the y-axis and the log2 fold change on the x-axis. For both (**A**) HFD and (**B**) CTL groups, the red values on the right x-axis indicate an upregulated DEG in *Abi3*$^{-/-}$ as compared to *Abi3*$^{+/+}$ mice, while blue values on the left x-axis indicate downregulation. Our significance threshold for DEGs was set at -log10(pValue) > 1.3. The (**C**) Venn diagram enables visualization the shared and unique DEGs between the HFD and CTL-fed groups. The (**D**) table lists these specific DEGs in each of their respective categories.

To accomplish this, we conducted targeted transcriptomic profiling of the hypothalami from female, *Abi3*$^{-/-}$ and *Abi3*$^{+/+}$ mice subjected to both HFD and control (CTL) diets. This was performed utilizing the NanoString Neuroinflammation panel. The comparative analysis of differentially expressed genes (DEGs) between Abi3 genotypes, across both dietary conditions, revealed a total of 124 genotype-driven DEGs, with 56 DEGs unique to the HFD-fed group, 30 DEGs unique to CTL-fed group, and 38 common DEGs across diets using a significance threshold of $p < 0.05$ (Figure 18).

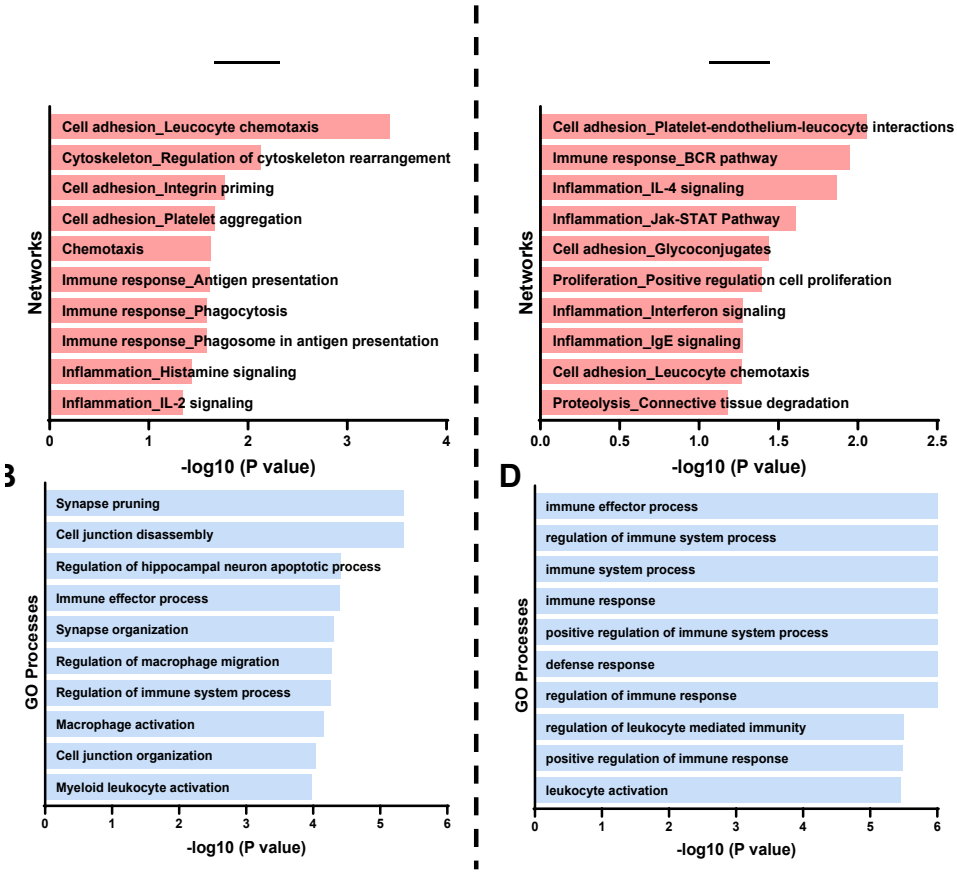
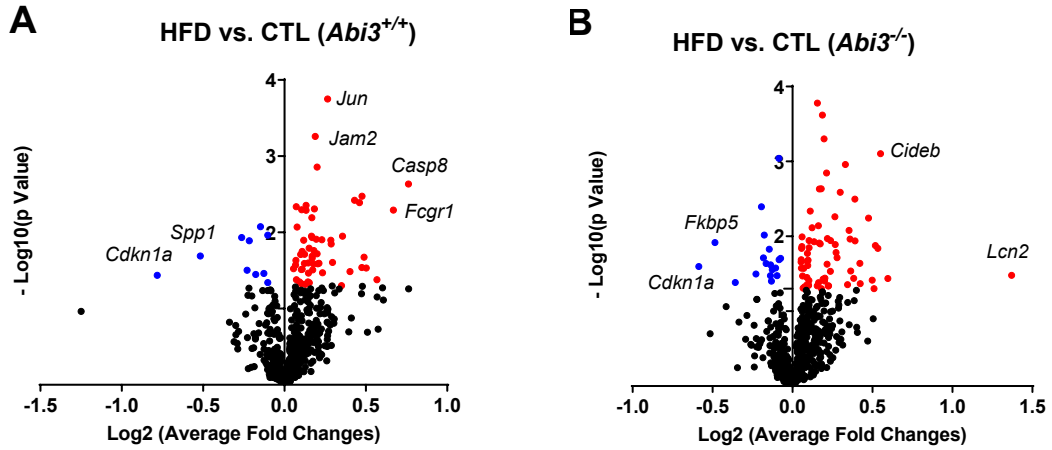
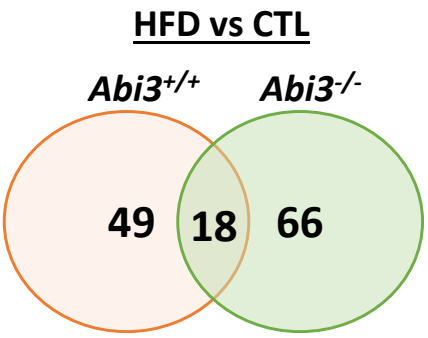


Figure 19. The deletion of *Abi3* results in distinct, diet-dependent patterns of network and process enrichment. Using the DEGs that were identified between *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$ female mice (n=6/genotype/diet), Metacore network, process, and associated disease analyses were performed. The left column shows the enriched (A) networks, (B) Processes for the DEGs observed in HFD-fed mice, while the right column shows the (C, D) equivalent analysis for the CTL-fed mice. The terms are listed in descending order of significance, with the most significantly enriched terms at the top of the lists.





D

Abi3+/+ (49)			Abi3-/- (66)				Common DEGs (18)	
Jun	C4a	Setd2	Map1lc3a	Gstm1	Grm3	Tgm2	Mapk10	Hira
Jam2	Agt	Prkar2b	Fabp5	Fcrls	Serping1	Aldh1l1	Smarca4	Gria4
Mavs	Shank3	Map2k1	Calr	Pak1	Mapt	Reln	Cideb	Prkce
Casp8	Ppfia4	Npnt	Grin2a	Grn	Xiap	Rnf8	Eif1	
Egr1	Atr	Bdnf	Lmna	Atp6v1a	Opalin	Cks1b	Olfml3	
Cdc7	Smc1a	Dock1	Pecam1	Rad51c	Suv39h1	Anapc15	C1qb	
Gria2	Spp1	Psen2	Stmn1	Irf3	C1qa	Ly6a	Gdpd2	
Ldha	Rad50	Ifnar1	Mfge8	Dlg1	Bid	Nrm	Becn1	
Nlgn1	Fos	Inpp5d	Pms2	Sox10	Bad	Coa5	Ugt8a	
Pik3ca	Epg5	Gria1	Slc17a6	Tmem206	Bag4	Vamp7	Plxdc2	
Fcgr1	Kdm1a	Parp1	Srgn	Brd2	Rpl28	Ccni	Dst	
Bmi1	Parp2	Slc6a1	Slco2b1	Optn	Lgmn	Prkcq	Ikbkb	
Dnmt1	Kdm5c	Homer1	Gbp2	Prkar1a	Gsn	Fcer1g	Mcm6	
Lfng	Kmt2c	Chuk	Cx3cl1	Gja1	Mog	Vps4a	Cdkn1a	
Eef2k	Hdac6	Tie1	Fkbp5	Lcn2	Setd1a	Hdac2	Tyrobp	
Tnfrsf1a	Ptprc		Hat1	Man2b1	Ly9			
Kdm2b	Lag3		Csf1r	Cox5b	P2ry12			

Figure 20. Deletion of *Abi3* alters neuroinflammation associated gene expression within the hypothalamus, in ways that are both unique to and dependent on diet. Targeted NanoString Neuroinflammation profiling of the hypothalamus enabled the identification of diet driven DEGs, across both *Abi3*$^{-/-}$ and *Abi3*$^{+/+}$mice (n=6/genotype/diet). The volcano plots (**A-B**) visually represent this NanoString analysis, with significance, shown as -log10(pValue), on the y-axis and the log2 fold change on the x-axis. For both (**A**) *Abi3*$^{+/+}$ and (**B**) *Abi3*$^{-/-}$ groups, the red values on the right x-axis indicate an upregulated DEG in HFD-fed vs CTL-fed mice, while blue values on the left x-axis indicate downregulation. Our significance threshold for DEGs was set at -log10(pValue) > 1.3. The (**C**) Venn diagram enables visualization the shared and unique DEGs between the genotype groups. The (**D**) table lists these specific DEGs in each of their respective categories.

Subsequent pathway enrichment analysis was performed in order to identify the pathways and processes in which the identified DEGs are enriched (Figure 19). Notably, processes such as synaptic pruning, synapse organization, and cytoskeletal dynamics (including cytoskeletal rearrangement and chemotaxis) were prominently enriched only in the HFD-fed, *Abi3*$^{-/-}$ mice (Figure 19A-B); this finding may suggest that *Abi3* related functions become uniquely disrupted during high fat diet, but not under normal conditions.

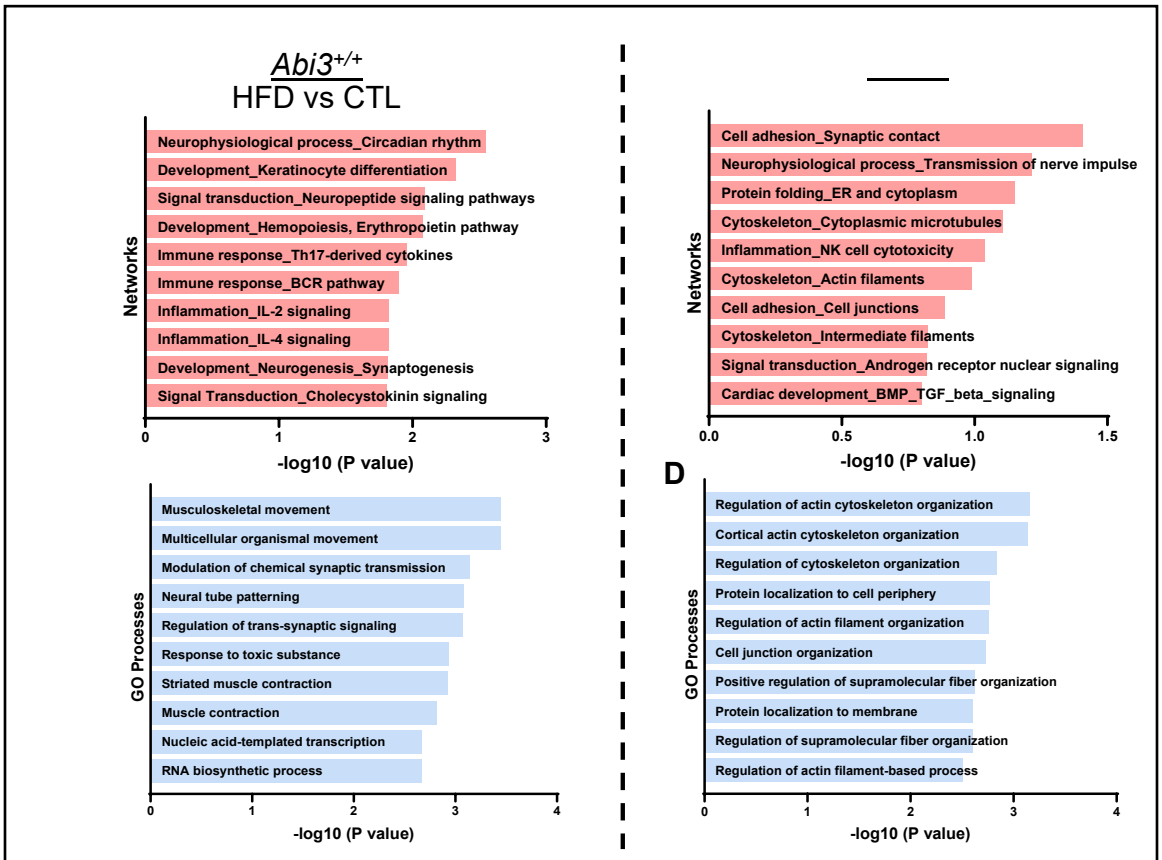


Figure 21. The absence of *Abi3* alters the neuroimmune pathways and processes that are in enriched by exposure to high fat diet. Using the DEGs that were identified between HFD-fed vs CTL-fed, female mice (n=6/genotype/diet), Metacore network and process were analysis were performed. The left column shows the enriched (A) networks, (B) Processes for the DEGs observed in *Abi3*$^{+/+}$ mice, while the right column shows the (C, D) equivalent analysis for the *Abi3*$^{-/-}$ mice. The terms are listed in descending order of significance, with the most significantly enriched terms at the top of the lists.

Furthermore, we were additionally interested in exploring how *Abi3* genotype modulates the neuroinflammatory gene expression changes induced by HFD. Therefore, we identified the DEGs that were induced by high fat diet (HFD-fed vs CTL-fed), across each Abi3 genotype (Figure 20). This analysis unveiled 133 DEGs, with 49 specific to $Abi3^{+/+}$ mice, 66 specific to $Abi3^{-/-}$ mice, and 18 that were shared across both genotypes (Figure 20C). Pathway analysis of the HFD-induced DEGs, performed using Metacore, identified significant enrichment in pathways related to actin remodeling, cytoskeletal organization, and synaptic contact, but only in the $Abi3^{-/-}$ mice (Figure 21). These findings may suggest that *Abi3*-deficient mice exhibit a unique neuroinflammatory response to high fat diet, with significant alterations in pathways integral to the known functions of *Abi3*, such as actin remodeling.

3.3.6 *Deletion of Abi3 results in a reduction in the area covered by microglia within the mediobasal hypothalamus.*

In the investigation described in Chapter Two, we established that obesity in $Abi3^{-/-}$ mice is associated with a reduction in both the number of, and area covered by, Iba1+ microglia within the hypothalamus. Further, through our NanoString analysis, we discovered that the deletion of *Abi3* uniquely alters the neuroinflammatory response to high fat diet, with gene expression changes enriched in processes related cytoskeletal remodeling and synaptic contact. Building on these findings, we extended our investigation to evaluate how *Abi3* deletion and high fat diet, independently and jointly, impact the number and coverage of microglia within the mediobasal hypothalamus, as well as the cortex.

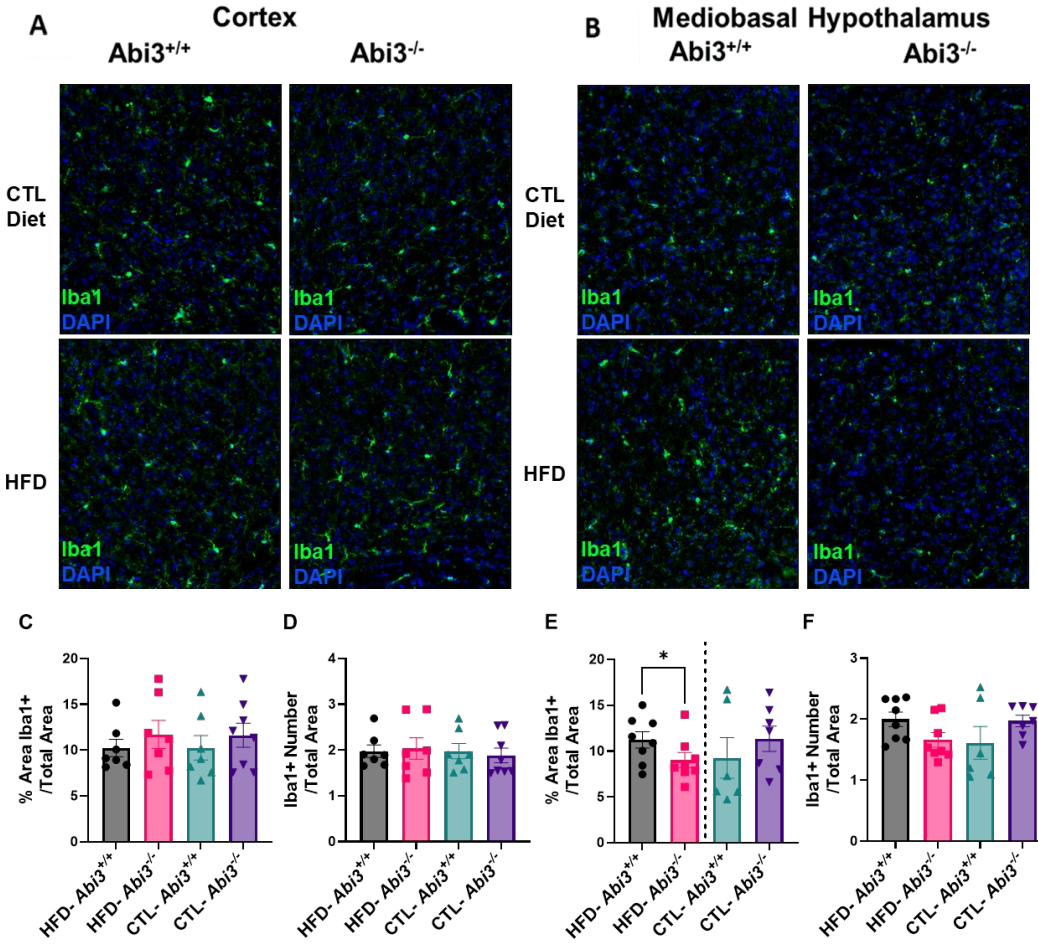


Figure 22. The area of Iba1+ microglia decreased in the mediobasal hypothalamus but did not change in the cortex. Brain sections from female, HFD and CTL-fed, *Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$ mice were stained with Iba1+ (microglia marker) and DAPI (nuclear counterstain). Representative images from the (A) cortex and (B) mediobasal hypothalamus (MBH) are shown illustrating Iba1+ microglia (green) against a DAPI nuclear counterstain (blue). The Iba1+ area and number were quantified for (C-D) cortex and (E-F) mediobasal hypothalamus. Statistical significance was assessed using a unpaired two-tailed Student's t-test. Data represent mean ± SEM, $p<0.05$.

Towards that end, brain sections from female Abi3$^{-/-}$ and Abi3$^{+/+}$ mice underwent immunofluorescent staining to detect Iba1+ microglia and were counterstained with DAPI for nuclear visualization. Analysis of the cortex (Figure 22A) revealed no significant alterations in the Iba1+ area between the two genotypes (Figures 22C-D). Conversely, the mediobasal hypothalamus (MBH) (Figure 22B) exhibited a notable reduction in the Iba1+ area in Abi3$^{-/-}$ mice compared to their Abi3$^{+/+}$ counterparts

(Figures 22E-F). This reduction suggests a decrease in microglial cell coverage of this brain region during concomitant *Abi3* deletion and high fat diet feeding.

3.4 Discussion

In this investigation, we aimed to build upon the findings of our previous investigation, in which we discovered that mice with deletion of the *Abi3* gene develop obesity. In order to build upon these findings, we expanded our experimental approach to include both male and female mice, across multiple time points, and across both control and high-fat diet (HFD) conditions. Prior to this, however, given the reported relevance of high fat diet induced neuroinflammatory responses to the progression of diet induced obesity, we first aimed to determine if the presence of *Abi3* altered neuroinflammatory response to high fat diet. To accomplish this, we initially performed an *in vitro,* proof of concept, experiment where we demonstrated that siRNA-mediated reduction of *Abi3* expression alters the expression of neuroinflammation associated genes following treatment with the saturated fatty acid, palmitic acid. This is pertinent, as palmitic acid is a saturated fatty acid (SAF) that is prominent with most high fat diets.[76] Furthermore, SAFs are a common, pro-inflammatory component within a high fat diet. In fact, SAFs are reported to be key regulators of the neuroinflammatory response to high fat diet. SAFs are primarily reported to regulate neuroinflammation through direct stimulation of TLR-4 on microglia within the hypothalamus.[94] In light of this context, it becomes clear why palmitic acid is frequently used to simulate an *"in vitro* high fat diet*"* of sorts. It is important to note that this experiment is limited by several *in vitro* related limitations. For example, microglial-like cell lines often exhibit distinct responses as compared to both primary and *in vivo* microglia.[195,196] Additionally, the replicability of experimentally

induced inflammatory response is notoriously inconsistent in microglia-like cell culture.[195,196] Despite these and numerous other limitations of cell culture, this study provided experimentally-backed justification for the *in vivo* investigation into the impact of *Abi3* deletion on the neuroinflammatory response induced by HFD.

Through the *in vivo* investigation, we discovered that the extent to which *Abi3* deletion impacts body weight regulation is dependent upon sex and diet condition. We accomplished this by, first, recording body weight weekly, across the 18-week experimental feeding period. Unexpectedly, we observed that only female, HFD-fed, *Abi3*$^{-/-}$ mice exhibited statistically significant increases in body weight as compared to the HFD-fed, wild-type counterparts. Across all combinations of diet and sex, the deletion of *Abi3* did not induce significant in body weight. Critically, this suggests that the absence of *Abi3* drives the development of obesity in a sex (only female) and diet (only HFD-fed) dependent manner, at least for across the timepoints measured in this study. These findings also suggest that the obesity we originally discovered, in the aged, male *Abi3*$^{-/-}$ mice, is a phenomenon driven by advanced age; as evidenced by the absence of any *Abi3* dependent differences in body weight, even latest experimental time point for all male mice, as well as the control-fed female mice. Considering both Chapters Two and Three in combination, the evidence seems to indicate that the absence of *Abi3* can drive the development of obesity under specific, and potentially stress inducing, conditions such as high fat diet and advanced age. However, further investigation would be required to provide experimental support for this claim.

As with the investigation performed in Chapter Two, we next performed metabolic cage analyses in order to determine if the increased weight gain exhibited by

HFD-fed, *Abi3*$^{-/-}$ mice was primarily incited by dysfunction in energy expenditure or food intake. Intriguingly, unlike the study reported in Chapter One, no significant differences in energy expenditure were observed between the female, HFD-fed *Abi3*$^{-/-}$ versus *Abi3*$^{+/+}$ mice, fed a high fat diet. These findings contradict the results of Chapter One, which demonstrated a marked reduction in energy expenditure among the obese, aged, male mice. However, it is important to consider the context of the findings, especially given that the body weight results of Chapter Three suggest that the impact of *Abi3* deletion on body weight differs across sex, diet, and age. Furthermore, it is possible that the initial report of decreased energy expenditure in the aged male mice could have been a finding that was actually secondary to the advanced obesity itself rather than the direct impact of *Abi3* deletion, as can occur during obesity within mice.[197] However, it could also be the case that the difference in energy expenditure is driven by *Abi3* deletion, but that this does not become relevant until later time points.

Additionally, the metabolic cage experiments revealed that the female, HFD-fed, *Abi3*$^{-/-}$ mice exhibited increased food intake over the course of the analysis. This suggests that the obese phenotype observed in these mice is driven by increased food intake, without changes in energy expenditure, in direct contrast to the results of the Chapter One metabolic cage analysis. As discussed above, there are a variety of explanations that can be provided in an attempt to justify this discrepancy. However, these explanations must be validated, potentially through the use of calorimetry cage analysis at multiple time points from early adulthood to advanced aged, to warrant further discussion. Somewhat related to this, was the observation that HFD-fed, *Abi3*$^{-/-}$ exhibited significantly elevated total movement during the calorimetry cage analysis. This is interesting in the context of

previous findings which have demonstrated the removal of microglia, via CSF1R inhibition, during development leads to the induction of hyperphagia and increased movement, as well as reduced POMC neuron number within the hypothalamus, in a sex dependent manner.[90] This finding could be interpreted as potentially supportive to our hypothesis, which posits that main metabolic impacts of *Abi3* deletion are dictated within the CNS. To elaborate, both the CSF1R inhibitor and our HFD studies, employed experimental approaches thought to impact microglia function; further, both studies also occur alongside a similar, sex-dependent, pattern of impaired regulation of food intake, weight gain, and hyperactivity.[90] Together these findings appear to suggest that the increased body weight of HFD-fed, *Abi3*$^{-/-}$ may be secondary to altered microglia function within the CNS. However, the CSF1R inhibitor study reported that this specific pattern of energy balance disruption was secondary to embryonic depletion of microglia; by the same logic above, this finding may indicate that future investigation as to the embryonic impact of *Abi3* deletion should be considered. However, these concerns are mitigated, at least somewhat, by the fact that *Abi3* deletion does not appear to impact energy balance and body weight regulation at 12 weeks of age; therefore, it is unlikely that these processes would be impacted prior to this point. However, this claim requires experimental validation.

Further, to elucidate the connection between *Abi3* and the inflammatory response to high fat diet, we again set about investigating the impact of *Abi3* deletion on neuroinflammatory gene expression, but this time, expression was evaluated *in vivo* within the hypothalamus. Towards that end, we performed targeted transcriptomic profiling using the NanoString Neuroinflammation panel on the hypothalmi of female,

HFD- and CTL-fed, *Abi3*$^{-/-}$ and *Abi3*$^{+/+}$ mice. Subsequently, we found that the HFD-fed, *Abi3*$^{-/-}$ mice exhibit a unique pattern in the expression neuroinflammation-related genes within the hypothalamus. This pattern is marked by the differential expression of genes uniquely enriched in biological processes such as cytoskeletal remodeling and synaptic interactions. This is intriguing to consider as cytoskeletal remodeling, and the related concept of synaptic interactions, are both processes in which *Abi3-dependent* functions may be particularly relevant.[140,145,146] Furthermore, the conditions in which these processes terms were the same conditions that led to significant elevations in weight gain, high fat diet feeding, female sex, and deletion of *Abi3*. Additionally, pathway analysis for the DEGs induced by HFD (HFD vs CTL) and for the DEGs induced by *Abi3* deletion (*Abi3*$^{-/-}$ vs *Abi3*$^{+/+}$), both revealed the unique enrichment patterns of synaptic interaction and cytoskeletal remodeling that were only identified in the presence of both HFD-feeding and *Abi3* deletion.

Finally, we moved to further explore the neuroimmune line of investigation through the use of immunofluorescence image analysis of microglia (as determined by Iba1+ signal). Subsequently, we determined that female, HFD-fed, *Abi3*$^{-/-}$ mice were the only group in which significant changes in Iba1+ immunostaining were detected, a finding consistent with the microglia findings reported in Chapter Two. Specifically, female, HFD-fed, *Abi3*$^{-/-}$ mice exhibited as a reduction in the area covered by microglia within the mediobasal hypothalamus. In summary, this investigation not only identified that the metabolic impact of *Abi3* deletion was sex and diet-dependent of Abi3 deletion, but also pointed to on the presence of distinct neuroinflammatory mechanisms at play in female, HFD-fed, Abi3$^{-/-}$ mice, underscoring the complexity of this genetic modulation in

dictating metabolic dysfunction. Together, our research suggests an important, but context dependent, role of the Abi3 gene in mediating the neuroimmune response to a high-fat diet and the susceptibility to high fat diet induced obesity. These findings suggest that *Abi3*-related functions in microglia maybe connected to maintenance of energy homeostasis under dietary challenges, with disruptions in these functions potentially leading to the observed sex-specific metabolic phenotypes. It is our hope that these insights pave the way for future research aimed at unraveling the specific cellular and molecular events that underpin the observed sex-dependent metabolic outcomes.

Chapter Four: Conclusion

4.1 Summary

This dissertation has embarked on a quest to unravel the role of Abelson Interactor Protein 3 (ABI3) in the previously unexplored setting of obesity and metabolic regulation. The central focus of this work was to explore the impact of deleting *Abi3* in mice on susceptibility to obesity and the hypothalamic neuroimmune response associated with this condition.

Chapter 1 reviews the relevant literature spanning decades of research on the interconnected topics of obesity, the central nervous system, and immune function. Perhaps most foundational to this dissertation, given the focus on *Abi3*, was the extensive review of the reports that connected the functions of microglia within the hypothalamus to the development and progression of obesity. This review highlighted how these cells, once thought to be mere bystanders, are actually active participants in metabolic regulation. Furthermore, it has been established that microglia can apparently accomplish this metabolic regulation through a variety of processes, but this regulation is often ultimately mediated via modulation of the neurons that regulate energy balance within the hypothalamus. This chapter also introduces ABI3 as a potential key player in the regulation of energy balance in microglia, providing insight into its role in the context of obesity.

In Chapter 2, we reported that the deletion of *Abi3* leads to the development of obesity in aged male mice. This unexpected finding was the inspiration for this dissertation, as it linked ABI3 and obesity for the first time. Specifically, we demonstrated that the absence of ABI3 led to significant changes in body weight, energy

balance, and microglial coverage within the mediobasal hypothalamus in older male mice. These key findings indicated that ABI3 may be necessary for the regulation of energy balance and that this regulation may be dictated by the ABI3-dependent functions of microglia. However, further research is necessary to support that claim more definitively.

In addition, Chapter 3 expanded the scope of research to explore the impact of the absence of ABI3 on susceptibility to high-fat diet-induced obesity and high-fat diet-induced neuroinflammation. Notably, this chapter sheds light on the importance of sex in determining the metabolic consequences of the deletion of *Abi3,* potentially suggesting that the necessity of ABI3-dependent functions in regulating energy balance is dictated by a complex interplay between genetics, diet, and sex obesity. Specifically, this investigation revealed that *Abi3* deletion only promoted the development of obesity in female mice fed a high-fat diet, revealing that the importance of *Abi3* in body weight regulation is context dependent. These findings may suggest that *Abi3*-related microglial functions are necessary for the regulation of energy balance only during times of challenge (such as dietary stress in this study or advanced age in the preceding study).

In conclusion, this study revealed, for the first time, a connection between ABI3, hypothalamic microglia, obesity and metabolic regulation. In the absence of ABI3, hypothalamic microglia are altered (including reduced microglial coverage and altered neuroinflammation-related gene expression), and energy balance is disrupted, subsequently driving the development of obesity. However, these findings are only apparent in specific dietary, sex, and age-related contexts, thus emphasizing the complexity underlying the immune-related mechanisms of energy balance regulation.

Ultimately, we hope that this research will pave the way for future studies aimed at more comprehensively elucidating the mechanisms by which microglial functions, including ABI3-related functions, contribute to the dynamic regulation of energy balance.

4.2 Significance

The findings from this dissertation mark an advancement in our understanding of the role of microglia in obesity, garnered through our exploration of the impacts of *Abi3* deletion in mice. Specifically, the investigations described in this dissertation are the first to establish a direct link between the *Abi3* gene and susceptibility to obesity. Furthermore, this link is also significant because it provides further support for the growing concept that disrupting immune-related, especially microglial-related, gene expression can modulate susceptibility to obesity. This discovery enhanced our understanding of the genetic underpinnings of obesity by identifying *Abi3* as a key factor in the regulation of energy balance.

Furthermore, this dissertation is significant in that it contributes knowledge in a relatively young but exciting field of research: the intersection of neuroimmunology and metabolism regulation. Our study demonstrated that the obesity induced by *Abi3* deletion is associated with altered microglial coverage and microglia-driven neuroinflammation. Although further work will be necessary to confirm this, our findings suggest that microglial dysfunction, due to the absence of *Abi3*, may underlie the eventual disruption of the energy balance observed in aged male and HFD-fed female *Abi3*$^{-/-}$ mice.

This dissertation is also significant in that it sets the stage for future research into the cytoskeletal remodeling-dependent mechanisms by which microglia influence the regulation of energy balance. This was accomplished in part by showing that the deletion

of *Abi3*, an immune cell gene that primarily exerts its function through cytoskeletal remodeling, can drive obesity. However, this finding was further supported by the transcriptomic analyses, which revealed that, in both studies, the group of mice that exhibited significantly elevated weight gain also exhibited gene expression changes uniquely enriched in cytoskeletal remodeling-related processes. The connection between the cytoskeletal remodeling functions of microglia and the regulation of energy balance has rarely been explored, despite the acknowledgment that microglia require dynamic remodeling to exert their functions. This research may provide an impetus for further exploration into the specific cytoskeletal remodeling processes that are necessary to maintain proper energy balance. Overall, the significance of this research lies in its innovative approach to understanding obesity through the lens of neuroimmunology and its focus on a novel genetic contributor to this disease state. This study provides a novel perspective on metabolic regulation, emphasizing the potential roles of microglial functions and ABI3-related cellular functions in the development of obesity.

4.3 Limitations

While the findings of this dissertation contribute to the field of CNS-focused obesity research, it is essential to acknowledge certain limitations that present opportunities for further investigation.

Initially, Abi3 was implicated in the development of obesity in mice of advanced age. We attempted to address this concern during the follow-up investigation reported in Chapter Three by measuring body weight weekly for all mice across genotypes and diet conditions. Unfortunately, the investigation in Chapter Three did not recapitulate the findings of the original Chapter Two study; even at the latest time point of 30 weeks (7

months), no significant differences were observed as a result of *Abi3* deletion during control feeding. Due to limitations related to the experimental timeline, this follow-up study was unable to identify the time point at which the absence of *Abi3* drives the development of obesity. Future work could investigate when this shift occurs, along with exploration into the potential factors that dictate this aging-dependent shift. Additionally, the extent to which *Abi3* deletion affects metabolic regulation through changes that occur at younger or developmental stages remains unclear. Understanding the developmental aspects of the influence of Abi3 on metabolism is important considering that our investigation employed a constitutive knockout; therefore, the absence of *Abi3* could already alter immune- and metabolic-related functions during development and childhood. Our data indicate that the absence of *Abi3* does not cause significant differences in body weight at early time points, alleviating some of the concerns about the developmental impact of *Abi3* deletion in the context of this study.

As we thoroughly discussed, the findings of the investigation described in Chapter Three revealed that the extent to which the deletion of *Abi3* modulates susceptibility to a high-fat diet is dependent upon sex. While these findings offer valuable insight into the complex array of factors that mediate the metabolic impact of *Abi3* deletion, they also limit the generalizability of the findings across sexes. Future research should aim to unravel the specific factors and mechanisms underlying sex-specific differences.

Additionally, the investigations described in this dissertation primarily focused on the CNS and microglia to explore the metabolic impact of *Abi3* deletion. Although this approach is innovative, future studies could provide a more comprehensive understanding of the roles of Abi3 in metabolic regulation by investigating peripheral tissues in greater

detail. Given that the regulation of body weight is systemic in nature and given the reported importance of peripheral immune cells such as macrophages in the progression of obesity, it is important that future investigations work to elucidate the potential effects of *Abi3* deletion outside of the CNS.

Each of these limitations offers a direction for future research to expand upon the current understanding of the role that *Abi3* plays in the regulation of energy balance and the neuroinflammatory response to obesity. Additionally, several of these limitations are discussed in the following section. Addressing these gaps will help in developing a more nuanced and comprehensive understanding of the impact of Abi3 on obesity and overall physiology.

4.4 Future Directions

The findings from this dissertation on the role of Abi3 in obesity and metabolic regulation, particularly through altered microglial functions, paves the way for several promising avenues for future research. These directions aim not only to address these limitations, as outlined above, also to deepen our understanding of the intricate relationship between neuroimmunology and metabolic disorders.

The exploration of the role of the ABI3 gene within the complex landscape of metabolic regulation and neuroimmune interactions, as detailed in this dissertation, not only reveals new pathways but also provides further insight. The journey through the multifaceted realm of ABI3's influence on obesity has revealed critical insights, yet it also unveils a vast expanse of uncharted territories awaiting exploration. The pathways we have traversed offer a beacon for future scientific endeavors to follow, each potential direction

promising for enriching our understanding of metabolic disorders and the intricate web of neuroimmunological mechanisms that underpin them.

As discussed in the preceding section, future exploration of the developmental roles of ABI3 is warranted. However, the timeframe over which ABI3 influences metabolic processes, including during development, remains to be mapped. Therefore, future studies could explore the impact of *Abi3* deletion across additional time points to address this knowledge gap. Additionally, extending the recording of body weight in adult mice to include later ages might help identify the critical windows during which ABI3 modulation has the most pronounced impact on energy balance regulation and subsequent obesity pathology.

As previously discussed, the investigation in Chapter Three revealed that the impact of *Abi3* deletion on energy balance and neuroinflammation was dependent upon sex. Although this finding provides valuable insight into the importance of considering sex as a biological variable, the exact mechanisms that drive this sex dependence are currently unclear. Further research dedicated to dissecting these sex-specific pathways, including the measurement of hormones and glucocorticoids, as well as consideration of the stage of the estrous cycle as a biological variable, could help to reveal the mechanisms by which sex modulates ABI3-related functions to subsequently drive unique responses to a high-fat diet.

While our investigations have exclusively explored the impact of *Abi3* deletion, it is likely that it is important to additionally consider the impact of variable levels of *Abi3*. Future studies could employ approaches such as overexpression or targeted genetic approaches to alter the levels of *Abi3* expression but not eliminate it entirely.

Additionally, future studies could generate and evaluate mice with heterozygous deletion of Abi3 to explore whether the metabolic findings exhibit a gene dosage effect. Such studies could help uncover the impact of differing levels of ABI3 on the regulation of energy balance and neuroimmune functions to offer a more comprehensive, nuanced view of ABI3 biology. Relatedly, we initially reported that ABI3 acts not in isolation but rather through interactions with various proteins; chief among these proteins are the other members of the WAVE2 complex, with which ABI3 interacts to regulate actin remodeling. The scope and importance of the interactions between ABI3 and WAVE2-related proteins, as well as the role of these related proteins in the maintenance of healthy body weight, remain to be fully appreciated. Various approaches could be employed toward this end, such as utilizing genetic models to manipulate the levels of various WAVE2 complex members or immunoprecipitation experiments to probe for *Abi3*-interacting proteins, *which* could yield more insight into the specific molecular mechanisms that regulate ABI3.

Additionally, as we discussed above, the studies described herein focused extensively on the CNS and hypothalamic microglia. However, it is probable that the influence of ABI3 extends beyond the confines of the brain, as it is expressed within peripheral immune cells such as monocytes and macrophages. Therefore, additional investigations that explore the impact of *Abi3* deletion in metabolically relevant tissues known to be impacted by immune function, including brown and white adipose tissue, muscle, pancreas, intestines, liver, and muscle, are warranted.

Furthermore, while this dissertation has thoroughly discussed the complex interactions connecting microglial function, neuroinflammatory responses, and metabolic

regulation, understanding of the exact mechanisms by which microglia induce a shift in neuronal regulation of energy balance is warranted. Importantly, given the wide array of factors that have been linked to altered microglial function within the hypothalamus, a comprehensive understanding of these mechanisms will likely be built upon the accumulation of multiple incremental studies that continue to identify, piece by piece, the vast and varied factors that dictate metabolically relevant microglial functions. However, as more sophisticated genetic and experimental tools aimed at targeted gene modulation of microglia, in addition to the availability of highly advanced sequencing approaches such as single-cell spatial transcriptomics, groundbreaking research that can explore the dynamics of microglial activity, their communication with neuronal networks, and their role in energy balance regulation in previously unexplored ways is possible.

Additionally, it is important to consider the similar microglia dysfunction that is observed during both Alzheimer's disease (AD) and obesity. Understanding this dysfunction may potentially shed light on the pathological mechanisms that underpin these seemingly disparate conditions. Microglia are pivotal in maintaining brain homeostasis, engaging in a myriad of functions including synaptic pruning, neuronal support, and immune surveillance. Disruption of these critical microglia functions, perhaps unsurprisingly, can have dramatic effects on the ability of the brain to prevent and respond to disease pathology. Our laboratory has demonstrated how the loss of ABI3-dependent microglial functions, through the deletion of *Abi3,* exacerbates disease pathology in the context of both AD and obesity. In AD, microglia become activated in response to the accumulation of amyloid-beta plaques, tau-based neurofibrillary tangles, and dying neurons. This activation has been suggested to be both potentially beneficial

and necessary to respond to neurodegenerative pathology. However, eventually this activation is thought to become detrimental and leads to exacerbation of AD neuropathology.[198] The importance of neuroinflammation and neuroimmune function in the context of AD shares similarities with the neuroinflammatory processes implicated in obesity, potentially suggesting a common pathological role for microglia across these conditions. Because of the similar inflammatory processes occurring in these distinct disease states, therapeutic approaches aimed modulating microglial activity are under investigation for both AD and obesity. The convergence of microglial dysfunction in AD and obesity underscores the importance of understanding the mechanisms by which microglia contribute to disease pathology. In fact, multiple microglia related genes that have been previously identified as potential risk factors for AD, including Abi3, Lpl, and Trem2, have all been recently also shown to modulate obesity pathology.[81,86,199] This further underscores that potential shared importance of microglia functions across these diseases. Taken together, it is apparent that we must continue to uncover the exact cellular processes that are being disrupted during disease associated neuroinflammation, as our understanding is still significantly limited in both contexts. This understanding could pave the way for novel therapeutic strategies that target microglial function, offering hope for the treatment and prevention of both obesity and AD. For example, the results from our laboratory, including our previously published AD studies as well as the obesity research described throughout this dissertation, suggest that modulation of actin remodeling within microglia can have substantial implications for the progression of obesity and metabolic disease. In conclusion, the exploration of microglia dysfunction in AD and obesity reveals a shared pathological landscape. Future research should continue

to unravel the intricate mechanisms underlying microglial dysfunction in these conditions, with the aim of developing targeted interventions that address the root causes of disease pathology across these similar distinct but interrelated disease processes.

While the therapeutic potential of targeting ABI3 and microglial functions in the treatment of obesity is promising, several limitations exist that may hinder the translatability of these interventions into clinical settings. These limitations stem from the complex nature of microglial functions, the intricate interplay between the immune system and metabolic processes, and the challenges inherent in modulating immune mechanisms without adverse effects. As we have extensively discussed, microglia are involved in a myriad of CNS functions, including synaptic pruning, neuroinflammation, and response to injury. However, the multifaceted roles of microglia also pose a challenge for targeted therapies. Interventions that modulate microglial activity must be finely tuned to avoid disrupting their beneficial functions while mitigating their contribution to obesity. This is challenging, as we currently still are working to when "microglial activation" is beneficial and when it is detrimental in the setting of obesity and also during AD. Furthermore, we also are still unsure of the exact functions that are disrupted during disease associated microglial dysfunction. That said, it still may be possible to employ more broad therapeutic interventions aimed at modulating disease progression by reducing overall microglia number or neuroinflammation. As discussed throughout this document, these types of interventions have already been employed within animal models in the setting of obesity and metabolic disease. Several of these studies demonstrated that it is possible to decrease obesity pathology via pharmacologic or microglia activity is capable of modulating susceptibility to obesity. Similar trends

have been observed in mouse models of AD, where reductions in microglial activity have been shown to be occasionally protective against disease progression.[198,200-202] However, in both obesity and AD, the impact of these broad anti-inflammatory approaches is variable.[198] This again suggests that there is potential for microglia directed therapies for both disease states, but that much further research is warranted before such a therapeutic approach could be deemed viable. Perhaps most importantly, future work should focus on identifying the time point and specific conditions (sex, diet, etc) that are most efficacious for microglial directed therapies.

Targeting ABI3-related or other microglial functions specifically within the hypothalamus without affecting other brain regions or peripheral tissues is a significant challenge. Furthermore, the hypothalamus is a heterogeneous brain regions that serves essential homeostatic functions such as energy balance regulation, hormonal signaling, and arousal. Therefore, it is important to consider how a treatment could exert differential effects within the hypothalamus, even if the therapy was somehow localized exclusively within this brain region.

Frequently, the sophisticated approaches that are employed to target microglia in animal models are not feasible in the clinical setting. For example, rodent studies often use genetic approaches that allow for targeted modulation of microglia, or even microglia subpopulations. Unfortunately, the clinical utility of these types of approaches are currently closer to science fiction rather than reality. However, as genetic and biomedical tools rapidly develop, it may become feasible to employ sophisticated approaches within the clinic. The transition from animal models to human treatments is fraught with difficulties. While deletion of *Abi3* in mice provided valuable insight into some of the

mechanisms driving obesity, human physiology and immune responses can differ significantly from those in rodents. Additionally, the genetic and environmental diversity among human populations may lead to variable responses to treatments targeting ABI3 or microglial functions.

As it stands, however, any available approach that targets microglia will have to consider off-target effects. Because of the various homeostatic roles that microglia serve, off-target effects could potentially lead to unintended severe consequences, such as impairing cognitive functions or exacerbating neurodegenerative or metabolic disease processes. Additionally, most "microglia" targeted approaches will also impact peripheral immune cells, which could lead to a variety of potential complications such as immunodeficiency, allergy, or other immune related effects. Any intervention targeting the immune system must be approached with caution due to the potential for unintended immune activation or suppression. The immune system is intricately connected to almost every bodily process, and dysregulation can lead to a range of adverse effects, from increased susceptibility to infections to autoimmune reactions.

The findings of our laboratory and others have clearly indicated that there are sex-specific differences in the microglial response to obesity and high-fat diets. This suggests that any therapeutic interventions targeting microglia or ABI3-related functions may need to be tailored to account for these differences. The mechanisms underlying these sex-specific responses are not fully understood and require further investigation, which complicates the development of universal treatments. It is possible to envision a future where patients are classified by their neuroinflammatory status, sex, diet composition, in

order to stratify and identify the populations that will be most likely to respond to a microglia directed therapy.

In exploring these directions for future research, it is evident that they have the opportunity to build upon the foundational knowledge regarding the metabolic consequences of *Abi3* deletion laid down by this dissertation. Each approach could yield critical discoveries that will deepen our understanding of the complex and interconnected concepts of neuroimmunology and metabolic regulation.

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Curriculum Vitae
Daniel Curtis Smith

Education

Indiana University School of Medicine — Indianapolis, IN
MD — Anticipated 2026

Indiana University — Indianapolis, IN
PhD, Major in Medical Neuroscience — 2024
Minor in Life Sciences
Dissertation: The Role of ABI3 in Obesity and Metabolic Regulation.

Ball State University — Muncie, IN
BS, Major in Chemistry (Concentration in Biochemistry) — 2017
Minor in Biology (Concentration in Cellular and Molecular Biology)
GPA: 4.0, *summa cum laude*

Research Experience

Indiana University School of Medicine (IUSM) — Indianapolis, IN
Stark Neuroscience Research Institute — 2019-2024
Graduate Research Fellow
Advisor- Jungsu Kim, PhD
Research Focus- Investigating the role of ABI3 and microglia function in metabolic and neurodegenerative disease.
Research Approach- Utilized mouse models to explore the functional impact of loss of ABI3 via advanced microscopy and image analysis, omics data acquisition and analysis, comprehensive metabolic phenotyping, and standard protein and nucleic acid techniques.

Center for Neuroimaging — 2018
Graduate Research Rotation
Advisor- Andrew Saykin, PsyD
Research Focus- Investigating the impact of retirement age on measures of resilience in neurodegenerative disease.
Research Approach- Investigated the Alzheimer's Disease Neuroimaging Initiative (ADNI) database to determine the connection between occupational factors (such as attainment and age of retirement) and neurodegenerative pathology, as determined by human MRI and PET imaging analysis.

Stark Neurosciences Research Institute — 2017
Graduate Research Rotation
Advisor- Gary Landreth, PhD
Research Focus- Investigating the importance of TREM2 isoforms in Alzheimer's disease pathology.
Research Approach- Employed cell culture and molecular biology techniques to determine the effect of differing *TREM2* isoforms on microglial cell function and morphology in the context of Alzheimer's disease.

Yale School of Medicine New Haven, CT
Department of Pathology 2016
National Science Foundation Undergraduate Research Fellow
Advisor- Marcus Bosenberg, MD PhD
Research Focus- Identifying the genetic determinants of malignant melanoma brain metastases.
Research Approach- Generated a melanoma cell line that metastasizes specifically to the brain of mice and employed transcriptomics to identify the genetic signature underlying the brain-specific metastasis pattern. Acquired foundational experience in cellular biology techniques such as cell culture and bioinformatic analysis.

Ball State University | IUSM- Muncie Muncie, IN
Center for Medical Education 2014-2017
Undergraduate Research Assistant
Advisor- Bart Pederson, PhD
Research Focus- Investigated the protective role of glycogen against hypoglycemia-induced cognitive impairment.
Research Approach- Utilized mouse models without glycogen in the central nervous system to determine if the absence of glycogen impacted hypoglycemia-induced cognitive impairment. Gained foundational experience in molecular and biochemical techniques such as western blotting, qPCR and PCR, ELISA, metabolite measurement, and animal behavior assays.

Honors

Predoctoral Fellowship in Diabetes and Obesity Research 2021-2024
NIH T32 Training Grant Fellowship

Poster Presentation Award- 3rd Place Overall 2023
Big10 Neuroscience Annual Meeting
Awarded to the top 3 Poster Presentations in the Big10 Conference

Appointment to the Medical Scientist Training Program at IUSM 2017-2026
NIH Funded MSTP Program
Selected as 1 of 9 trainees nationally for fully funded MD/PhD training program

IUPUI University Fellowship 2017
Graduate Recruitment Fellowship
Described as "the most prestigious award offered by IUPUI to incoming graduate students."

Honorable Mention- The National Barry Goldwater Scholarship 2016
National Scholarship Awarded for Excellence in Sciences, Mathematics, and Engineering

The Mikal L. Sousa Memorial Scholarship for Excellence in Chemistry 2015
Awarded to the top student in the Department of Chemistry
at Ball State University

Aspire Student Research Grant 2015
Internal Grant Awarded for Promising Student Research Projects
at Ball State University

Awarded for "Investigating the Protective Role of Glycogen in Hypoglycemia Induced Cognitive Impairment"

Select Presentations

Smith, DC, Karahan, H, Wijeratne, Al-Amin, M, Tate, M, Mantor, J, Sharify, D, & Kim, J. Deletion of the *Abi3* immune gene locus modulates the metabolic and neuroinflammatory response to a high-fat diet in a sex-dependent manner.
Poster Presentation delivered at the Big10 Neuroscience Annual Meeting. Indianapolis, IN. June 2023.

- *Poster Award- 3rd Place Overall*

Smith, DC, Karahan, H, Wijeratne, Al-Amin, M, Tate, M, Mantor, J, Sharify, D, & Kim, J. Deletion of the *Abi3* immune gene locus modulates the metabolic and neuroinflammatory response to a high-fat diet in a sex-dependent manner.
Poster Presentation delivered at Mechanisms of Metabolic Signaling Conference at Cold Spring Harbor Laboratory. New York City, NY. May 2023.

Smith, DC, Karahan, H, Wijeratne, HRS, Al-Amin, M, McCord, B, Moon, Y & Kim, J. Deletion of the *Abi3* immune gene locus in mice results in obesity and systemic metabolic disruption.
Poster Presentation delivered at 82nd Scientific Sessions of the American Diabetes Association. June 2022.

Smith, DC, Karahan, H, McCord, B, & Kim, J. The Role of ABI3 in Obesity and Metabolic Regulation.

Oral Presentation delivered at the Stark Neuroscience Research Institute Annual Symposium. July 2020.

Conferences Attended

Mechanisms of Metabolic Signaling	New York City, NY
Cold Spring Harbor Laboratory	2023
CDMD Diabetes Symposium	Indianapolis, IN
Center for Diabetes and Metabolic Diseases at IUSM	2023
Big10 Neuroscience Annual Meeting	Indianapolis, IN
Big 10 Conference	2023
82nd Scientific Sessions	New Orleans, LA
American Diabetes Association	2022
Neuroscience 2019	Chicago, Illinois
Society for Neuroscience	2019
Indiana CTSI Annual Meeting	Indianapolis, IN

Indiana Clinical & Translational Sciences Institute 2019

Stark Neuroscience Research Institute Annual Symposium Indianapolis, IN
Stark Neurosciences Research Institute at IUSM Annually 2019-2023

Peer-Reviewed Publications

Smith, DC, Karahan, H, Wijeratne, HRS, Al-Amin, M, McCord, B, Moon, Y & Kim, J. Deletion of the Alzheimer's disease risk gene Abi3 locus results in obesity and systemic metabolic disruption in mice. *Frontiers in Aging Neuroscience* **14**, doi:10.3389/fnagi.2022.1035572 (2022).

Karahan, H, **Smith, DC**, Kim, B, Dabin, LC, Al-Amin, MM, Wijeratne, HRS, Pennington, T, Viana di Prisco, G, McCord, B, Lin, PB, Li, Y, Peng, J, Oblak, AL, Chu, S, Atwood, BK & Kim, J. Deletion of Abi3 gene locus exacerbates neuropathological features of Alzheimer's disease in a mouse model of Aβ amyloidosis. *Sci Adv* **7**, eabe3954, doi:10.1126/sciadv.abe3954 (2021).

Karahan, H, **Smith, DC**, Kim, B, McCord, B, Mantor, J, John, SK, Al-Amin, MM, Dabin, LC & Kim, J. The effect of Abi3 locus deletion on the progression of Alzheimer's disease-related pathologies. *Frontiers in Immunology* **14**, doi:10.3389/fimmu.2023.1102530 (2023).

Acri, DJ, You, Y, Tate, MD, Karahan, H, Martinez, P, McCord, B, Sharify, AD, John, S, Kim, B, Dabin, LC, Philtjens, S, Wijeratne, HRS, McCray, TJ, **Smith, DC**, Bissel, SJ, Lamb, BT, Lasagna-Reeves, CA & Kim, J. Network analysis identifies strain-dependent response to tau and tau seeding-associated genes. *The Journal of Experimental Medicine* **220**, doi:10.1084/jem.20230180 (2023).

Moutinho, M, Coronel, I, Tsai, AP, Di Prisco, GV, Pennington, T, Atwood, BK, Puntambekar, SS, **Smith, DC**, Martinez, P, Han, S, Lee, Y, Lasagna-Reeves, CA, Lamb, BT, Bissel, SJ, Nho, K & Landreth, GE. TREM2 splice isoforms generate soluble TREM2 species that disrupt long-term potentiation. *Genome Medicine* **15**, 11, doi:10.1186/s13073-023-01160-z (2023).