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Hypothalamic Glial Cells in Diet-Induced Obesity

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General Abstract:

Hypothalamus is the key region in regulating energy and glucose homeostasis. Now more and more evidence shows that not only neurons, but also glial cells represent important components in this regulatory center. In the current study, we investigated how microglia and astrocytes contribute to the control of energy homeostasis and the pathologies of diet-induced obesity. Microglia are resident macrophages in the brain. Microglia induced innate immune responses have been linked with hypothalamic inflammation and metabolic syndromes. Here, we used genetic and pharmacological approaches to analyze the metabolic causes of hypothalamic microglial activation in metabolic disorders, demonstrating that microglia respond to nutrients, adipokines and gut hormones but not to body weight changes per se. We then analyzed dietary effects on microglia. After comparing a standard chow diet, a high carbohydrate high-fat diet, a low carbohydrate, high fat diet and a ketogenic diet, we found that only the high carbohydrate high fat diet resulted in reactive microglia in hypothalamus. We further discovered that the presence of advanced glycation end products (AGEs) in hypothalamus on high carbohydrate high fat diet might be an important mediator of hypothalamic microgliosis. Mice lacking receptors for AGEs had significant less hypothalamic microgliosis and improved metabolic phenotypes when exposed to high carbohydrate high fat diet. Astrocytes are the other predominant glia population in the brain. Their contributions in neuroendocrine control by responding to hormones and nutrients were underestimated. In this study, we investigated the role of astrocytes in nutrient sensing by generating an
inducible and astrocytes-specific and loss of function model for lipoprotein lipase. We found that lipoprotein lipase controls lipid content in astrocytes and contributes to maintaining systemic glucose and energy homeostasis. When animals were exposed to high-fat diet, mice lacking lipoprotein lipase on astrocytes exhibited accelerated weight gain and impaired glucose homeostasis. Together, these data show essential roles of hypothalamic glial cells in diet-induced obesity and systemic metabolic regulation, which shed light on understanding pathologies of metabolic syndromes.
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Abbreviations:

AGEs advanced glycation end products
AgRP agouti-related peptide
ALCAM activated leukocyte cell adhesion molecule
ANS autonomic nerve system
ANG1 angiopoietin 1
ARC arcuate nucleus
BBB blood-brain-barrier
bFGF basic fibroblast growth factor
BMDPs bone marrow derived macrophages
BrdU bromodeoxyuridine
CD68 cluster of differentiation 68
CNS central nervous system
CPT-1 carnitine palmitoyltransferase I
DIO diet induced obesity
GDNF glial-derived neurotrophic factor
GFAP glial fibrillary acidic protein
GLAST glutamate aspartate transporter
GLUT1 glucose transporter 1
GPIHBP1 glycosylphosphatidylinositol (GPI) - anchored glycoprotein
GTT glucose tolerance test
HFD High fat diet
HCHF High carbohydrate high fat
HPRT hypoxanthine guanine phosphoribosyl transferase
iba1 ionized calcium binding adaptor molecule 1
ICV intracerebral ventricular
IL-6 interleukin-6
LASS (CERS) ceramide synthase
LCFA long chain fatty acids
LCHF low carbohydrate high fat
LPS lipopolysaccharides
LPL lipoprotein lipase
MBH mediobasal hypothalamus
MCR4 type-4 melanocortin receptor
TG triglyceride
TGFβ transforming growth factor-β
TNFa tumor necrosis factor-alpha
POMC proopiomelanocortin
RAGE AGEs receptor
S100b s100 calcium-binding protein B
SC standard chow
Sphk sphingosine kinase
VMH ventral medial hypothalamus
WT wild type
CHAPTER 1

General Introduction
Prevalence of obesity and type 2 diabetes

The prevalence of obesity is increasing year by year in the United States. Nearly 2/3 of the population is considered overweight or obese (Ogden et al, 2006). Similarly, the incidence of cardiovascular disease, stroke and type II diabetes is also rising at an alarming rate (James, 2008). Obesity and its comorbidities put a larger burden on health care system and economy (Ariza et al, 2010). So it is imperative to gain a deep understanding of fundamental metabolism mechanism. This may lead to novel prevention strategies and therapeutics in future.

CNS regulated energy and glucose homeostasis

Energy balance is governed by the energy equation that energy intake is equal to energy expenditure. Although the equation is simple, the energy balance is controlled by a very complex central nervous system (CNS) regulatory network (Langhans & Geary, 2010). CNS integrates gastrointestinal signals, metabolic signals, flavor signals, adipose tissue signals as well as many other signals and then controls the energy balance by affecting our behavior, the autonomic nervous system (ANS) activity and the neuroendocrine system (Spiegelman & Flier, 2001). Thus, obesity could be considered as a CNS disorder if this energy balance is disrupted. The hypothalamus is the key region in the CNS that regulates metabolism. In the medial basal hypothalamus (MBH), the arcuate nucleus (ARC) and the adjacent median eminence (ME) with the fenestrated blood-brain-barrier (BBB) represent a window for direct communication between the CNS and the circulating nutrients and hormones. Glucose, fatty acids, leptin,
insulin, ghrelin and many other metabolically relevant factors in the circulation target the two major neuron populations located in the ARC: agouti-related peptide (AgRP) neurons and proopiomelanocortin (POMC) neurons. These neurons then send signals to the second-order neurons in the hypothalamus like the paraventricular nucleus and the lateral hypothalamus in order to modulate food intake, energy expenditure and glucose homeostasis. The activation of POMC neurons will cause an anorexigenic effect while the activation of AgRP neurons will have effects in the opposite direction. This “hot” area attracts tremendous interest in the metabolic research. Many features of the metabolic syndrome are associated with impairments in the MBH, including signaling molecules transport, receptor mediated signaling cascades, intracellular metabolism, mitochondria functions, inflammatory responses, synaptic plasticity etc. (Balland et al, 2014; Joly-Amado et al, 2012; Williams et al, 2014; Zhang et al, 2008). In the recent years, the contribution of other components in addition to neurons, such as vessels and glial cells has been increasingly recognized. Glial cells, including astrocytes, microglia, tanycytes and oligodendrocytes, are actively involved in a lot of MBH pathological changes. The new concept of a neural-glial-vascular network has emerged in the last few years and has quickly become a hot topic in the field. Therapeutic strategies for obesity will benefit from understanding the interaction between neurons, glial cells and vasculature under physiological conditions and during obesity development.
Microglia

Microglia are the resident macrophages in the CNS. Though sharing most of surface markers and functions with macrophages, microglia are distinct population. Microglia are derived from myeloid progenitors in the yolk sac that arise before embryonic day 8, long before the hematopoietic progenitors appeared in spleen and bone marrow, from where tissue macrophages arise (Ginhoux et al, 2010; Schulz et al, 2012). Unlike these tissue macrophages that have lifespan of a few days, microglia are long-lived cells, reside in the brain for much of the animal’s lifetime, and are different from the circulating monocytes. Microglia are health monitors in the CNS (Kettenmann et al, 2011). The primary function of microglia is to detect and respond to pathogens and injuries in the CNS. Once the pathological changes are detected, activated microglia will quickly proliferate and migrate to the injury site and undergo ramified morphological changes. At the injury site, microglia will engulf offending pathogens and induce the innate immune defense by releasing various cytokines and signaling to nearby cells. Microglia have been reported as being involved in many CNS diseases like sepsis, stroke, multiple sclerosis and Alzheimer disease. Understanding of the microglia reactivity and their interaction with surrounding neurons, vessels and other glial cells will help to learn more about the pathology behind these diseases.
**Microglia and innate immune response**

The brain is under surveillance of microglia all the time. Even when microglia is assumed to be in “resting” state, their ramified processes are highly mobile and under constant remodeling to scan their microenvironment (Nimmerjahn et al, 2005). This enables microglia to respond quickly to any micro injuries or disturbance signals. A wide range of factors could be recognized by microglia. Viral, bacterial and fungal surface structures could be recognized by pattern recognition receptors. Classic example is the Lipopolysaccharides (LPS)-stimulated microglia activation through toll like receptors (Lee et al, 1993, Lehnardt et al, 2003; Qin et al, 2005). Cytokines and chemokines could be sensed through corresponding receptors expressed on microglia like CX3CR1 (Harrison et al, 1998; Lindia et al, 2005). Abnormally formed proteins like β-amyloid could be detected by microglia, which is of great interest in the Alzheimer disease research (Takata et al, 2007). Blood-born factors like albumin (Ibrahim et al, 2011), fibronectin (Nasu-Tada et al, 2006), and thrombin (Stukas Moller et al, 2000) could be caught by peri-vascular microglia, which are important in the study of stroke. Microglia could also receive messages from neurons and glial cells via neuropeptides like a-MSH (Delgado et al, 1998), neurotransmitters like glutamate (Persson et al, 2005; Shaked et al, 2005), glial transmitters like ATP(Davalos et al, 2005) and S100 calcium-binding protein B (S100b) (Bianchi et al, 2010; Petrova et al, 2000). Thus, microglia could guard the health of the CNS.
Activated microglia could initiate either protective or adverse effects. A large body of evidence proves that over-activation of microglia leads to neurotoxicity. LPS has been reported to be neurotoxic only in the presence of microglia. LPS-induced activation of microglia results in cumulative loss of dopaminergic neurons (Gao et al, 2002; Gibbons & Dragunow, 2006). In an experimental autoimmune encephalomyelitis mouse model, the inhibition of microglia activation could attenuate demyelination and therefore reduce the disease development (Heppner et al, 2005). However, in the similar demyelination model, microglia also show their significance in removing debris from demyelination, and releasing trophic factors which are important for remyelination (Bartnik et al, 2000; Filbin, 2003; Reichert & Rotshenker, 2003). Similar situations have been reported in the Alzheimer disease as well. Microglia and macrophages show great ability to phagocytose β-amyloid (Fiala et al, 2005). This ability is attenuated in Alzheimer disease, maybe due to the overload of β-amyloid. The depletion of peri-vascular macrophages results in more severe angiopathy caused by β-amyloid (Hawkes & McLaurin, 2009). On the other hand, a study using minocycline to inhibit microglia activity could significantly improve the behavior of transgenic mice expressing the precursor of human β-amyloid (Fan et al, 2007). The mechanisms of Alzheimer disease and the role of microglia in β-amyloid clearance still require further studies. These studies indicate the complexity of the microglia study. Whether microglia activation is beneficial or detrimental is dependent on the type and extend of the stimulus and the stages of the diseases.
Microglia induced immune response is not only essential upon injury, but also indispensable for some physiological processes. During postnatal development in mice, microglia actively participate in synaptic pruning by engulfing synapses during synaptic maturation, which is important for normal brain development (Paolicelli et al, 2011). Recently, the early deficit of synaptic pruning by transient inhibition of microglia has been associated with impaired brain connectivity and autism-like behaviors (Zhan et al, 2014).

**Microglia in metabolic control and diet induced obesity**

In different brain regions, Microglia has region-specific features (Lawson et al, 1990; Vela et al, 1995). In the field of metabolic research, the microglia reactivity in MBH was of great interest. In the MBH, fenestrated BBB enable neurons to directly detect circulating nutrient and hormonal signals, but also put these cells at risk due to the lack of BBB protection. Thus, the microglia function is essential for maintaining the health of the neural microenvironment. In diet-induced obesity, microgliosis and increased cytokine productions have been observed in the MBH. These responses are attenuated after the first week of the diet but come back on chronic hypercaloric feeding (Thaler et al, 2012). Such an observation suggests a protective effect of microglia at an early stage of HFD consumption but a detrimental effect at a later stage. Chronic microgliosis and increased cytokines levels in MBH (referenced as hypothalamic inflammation) could be toxic to surrounding neurons responsible for metabolic control like Agrp neurons and Pomc neurons. In long-term high-fat, high-sugar feeding-induced obesity,
decreased Pomc neurons number has been observed and apoptosis has been detected (Thaler et al, 2012, Moraes et al, 2009). Cytokines (especially TNFa)-induced NF-KB inflammatory pathway receives a lot of attention in the recent years. It has been linked to disruptions of energy balance, glucose homeostasis and blood pressure regulated by the hypothalamus. (De Souza et al, 2005; Meng & Cai, 2011; Purkayastha et al, 2011; Zhang et al, 2008). The inhibition of IKKb/NF-kB specific in Agrp neurons could protect mice from DIO and glucose intolerance. IKKb/NF-kB pathway in Pomc neurons has been shown to be involved in obesity-related hypertension. Loss of function of IKKb/NF-kB in the MBH had beneficial effects for such phenotypes (Purkayastha et al, 2011). In addition, hypothalamic inflammation has been reported to disrupt mitochondria function and neurogenesis, which are proposed as mechanistic links to DIO (Hotamisligil, 2010; Li et al, 2012; Li et al, 2014; Ozcan et al, 2009; Schneeberger et al, 2013).

Several approaches were applied to inhibit or deplete microglia. When minocycline (a drug known to inhibit microglia activity) was delivered into third ventricle, ghrelin mediated food intake was attenuated (Reis et al, 2015). CD11b-DTR mice were used to deplete CD11b positive microglia. These mice show an enhanced leptin-mediated pSTAT3 signaling, which suggests a function of microglia to inhibit leptin response (Valdearcos et al, 2014). These studies suggest that microglia may also play a role in the physiological sensing process.
It should be pointed out that neither approach could specifically target microglia population only in MBH. Off-site effects could not be ruled out.

Another controversial issue about microgliosis in MBH, as well as in other brain regions, is the relation of brain-derived microglia and infiltrated bone marrow-derived macrophages. Microglia are self-renewable cells (Ajami et al, 2007). They could proliferate from local progenitors. Also, it is known that macrophages could infiltrate into the CNS, and such a process is enhanced when there is BBB dysfunction. MBH per se is a BBB leaky area, and high-fat high-sugar diet could damage the BBB, leading to angiopathy (Yi et al, 2012). This raises the question of whether DIO-associated hypothalamic microgliosis is the result of macrophages infiltration or proliferation of resident microglia. The most direct way to investigate this issue is by using bone marrow transplantation with tagged donor cells easily distinguishable from host cells (Mildner et al, 2007). However, the method itself requires irradiation, which could also damage the BBB. In some improved protocols, bone marrow cells recruitment into the CNS was relatively low (Kierdorf et al, 2013). Also, it is difficult to trace the destination of infiltrated macrophages. Whether these infiltrated macrophages have the same features as resident microglia (like long life span) is still unknown.

Which peripheral signal causes DIO-associated hypothalamic microgliosis? As mentioned above, lots of blood borne factors could stimulate microglia. Also, microglia respond to signals like gliatransmiters and chemokines from astrocytes.
and neurons. In DIO, microglia quickly respond to a high-fat high-sugar diet, and after 16 weeks of hypercarloric diet, microgliosis and weight gain are reversible by switching back to standard chow diet (Berkseth et al, 2014). This suggests that dietary input may be one of the primary driving forces of hypothalamic microgliosis. Saturated fatty acids elevated by a high-fat high-sugar diet have been shown to stimulate microglia via toll-like receptor 4 and myeloid differentiation factor 88 pathway (Milanski et al, 2009). Another important nutrient, glucose, may also be sensed by microglia through glucose transporter 5, which is exclusively expressed in microglia (& Ferraris, 2008; Vannucci et al, 1997). In addition, astrocytes and microglia are both activated in DIO. Recently a study on astrocytes suggested that activated astrocytes in the MBH could release ATP and convert it to adenosine to inhibit Agrp neurons (Yang et al, 2015). ATP could activate microglia via purinergic receptors as well (Inoue, 2002; James & Butt, 2002). This indicates a potential crosstalk between hypothalamic glial cells in DIO.

Together, these data highlight the link between microglia in MBH and metabolic control. The causes of microgliosis in DIO, the mechanistic pathways involved in microgliosis, the consequences of hypothalamic inflammation, and the crosstalk between microglia and surrounding glia and neurons still require further studies.

Astrocytes

Astrocytes, also known as astroglia, are the predominant glial population in the CNS. Astrocytes are derived from neuroepithelial cells in the cerebral ventricular
zone, the same linage as neurons and oligodendrocytes (Malatesta et al, 2000; Noctor et al, 2001). In the adult murine brain, the heterogeneity of astrocytes is more complex than any other glial cell types. To classify astrocytes, based on morphology, there are protoplasmic astrocytes with many fine branches enveloping synapses, and also fibrous astrocytes with few but long and thin processes which usually can be found in white matter (Casper et al, 2007; Emsley & Macklis, 2006; Lee et al, 2006). Besides that, two specialized astrocytes - Bergmann glia and Muller glia - were also identified in the cerebellum and the retina respectively. Based on functional markers, S100b, glutamate aspartate transporter (GLAST), aquaporin 4 (AQP4) and glial fibrillary acidic protein (GFAP) are all typical astrocytes markers representing distinguished astrocytes populations (Emsley & Macklis, 2006; Nagelhus et al, 2004; Rothstein et al, 1994; Storck et al, 1992). Due to the high plasticity of astrocytes, the classifications listed above always overlapped with each other to different extents depending on the brain region or the pathological status. When astrocytes were discovered, they initially were assumed to be scaffold supporting cells for neurons. Later, astrocytes were found to widely participate in processes of synaptic modulation, energy supply, neurotransmitters uptake, brain injury response and BBB formation. Recent studies suggested the role of astrocytes in hormonal sensing, which is of great interest in the metabolic study field.

**Astrocyte - neuron interaction**

One of the well-known functions of astrocytes is their role in the “tripartite” synapses. In addition to traditional elements of pre- and post-synaptic neurons,
adjacent astrocyte is the third element in the synapse (Araque et al, 1999). This “tripartite” structure is essential for most of the excitatory synapse since astrocytes could take up the neurotransmitter - glutamate, and covert to glutamine inside astrocytes, which later could be recycled back to neurons (Anderson & Swanson, 2000). Without astrocytes, a delayed glutamate clearance in synapses could cause neurotoxicity. The glutamate uptake rate has been associated with the synaptic coverage degree by astrocytes processes (Oliet et al, 2001), thus, the synaptic transmission could be modulated by astrocyte morphology. Besides that, astrocytes could also modulate synaptic activity by releasing gliatransmitters. Glutamate could also be released from astrocytes dependent on the cytosolic Ca\(^{2+}\) level, for instance (Agulhon et al, 2010; Santello & Volterra, 2009). In some brain regions, D-serine from astrocytes could serve as a co-agonist (with glycine) of NMDA receptors (Mothet et al, 2005; Panatier et al, 2006; Shleper et al, 2005; Yang et al, 2003). ATP was also identified as a gliatransmitter to activate purinergic receptors on either astrocytes or neurons, resulting in synaptic remodeling (Gordon et al, 2005; Guthrie et al, 1999; Newman, 2003; Zhang et al, 2007). Together, peri-synaptic astrocytes were essential for synaptic homeostasis and could scale synaptic transmission from various aspects.

Astrocytes and neurons are tightly coupled metabolic units. Energy resource of neurons is almost exclusively dependent on glucose. Under some circumstances like hypoglycemia or prolonged starvation, neurons could also use lactate and
ketone bodies as alternative energy substrates (Edmond et al, 1985; Robinson & Williamson, 1980; Tsacopoulos & Magistretti, 1996). In the CNS, there is an astrocytes-neuron lactate shuttle. Lactate derived from astrocytes could fuel neurons transported by monocarboxylate transporters. Besides, astrocytes are the only neural cells able to store glycogen (Brown, 2004). Cultured astrocytes could incorporate lactate into glycogen, or break glycogen into lactate or glucose without consuming ATP (Dringen et al, 1993; Wender et al, 2000). This is an efficient way to fuel neurons upon high-energy demand. During prolonged fasting, ketone bodies could replace glucose as the predominant fuel for neurons (Owen et al, 1967). Ketone bodies in the circulation were mainly produced by the liver during lipolysis. In the CNS, astrocytes are also ketogenic cells. Fatty acids oxidation in the brain primarily happened in astrocytes, though the global rate was low (Auestad et al, 1991; Edmond, 1992; Edmond et al, 1987; Escartin et al, 2007). Astrocytes could use fatty acids as energy substrates and convert them to ketone bodies to fuel neurons. Though there are still arguments about the ratio between circulation derived and astrocytes derived lactate and ketone bodies used by neurons, recent studies show some evidence that astrocytes derived lactate and ketone bodies may not only serve as fuel but also as metabolic signaling molecules in microenvironment (Barros, 2013; Le Foll et al, 2014).

**Astrocytes and blood-brain barrier**

The brain is almost an immune-privileged organ. Blood-brain barrier (BBB) integrity is very important for CNS health. BBB was formed by tight junctions
between endothelial cells, surrounded by basal lamina, perivascular endfeet from astrocytes, and pericytes in some regions. Astrocytes are able to secret BBB inducing factors like transforming growth factor-β (TGFβ), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and angiopoetin 1 (ANG1) (Igarashi et al, 1999; Lee et al, 2003) to help with BBB formation. Astrocytes could also modulate BBB functions by regulating local blood flow (Iadecola, 2004), changing BBB permeability (Dehouck et al, 1990; Deli et al, 1995; Didier et al, 2003), or adjusting transporting receptors (Boado & Pardridge, 2002). Such a function could synchronize the circulating input and neuronal demand. Thus, astrocytes become of great interest in metabolic research due to the special position and close metabolic relation with neurons.

**Astrocytes and CNS injury**

Like microglia, astrocytes play a role in the immune response. Upon CNS injury, astrocytes become reactive, accompanied by morphological changes and cytokine production (Aschner, 1998a; Aschner, 1998b). Moderate astrogliosis was featured with increased GFAP expression and elongated processes. On more severe conditions like tissue lesions, activated astrocytes could form glial scars along the board of the lesion site. The essential protective function of the glial scar was to separate the lesion site from healthy tissue to prevent the spread of infection or inflammation (Sofroniew, 2005; Sofroniew, 2009). However, there are also reports of inhibitory effects of the astroglial scar on axon regeneration (Silver & Miller, 2004). Thus, like microglia, the immune response of
astrocytes could be a double-edged sword for CNS. The detailed molecular mechanism under different pathological states needs to be carefully investigated.

**Astrocytes in metabolic control and diet induced obesity**

As mentioned above, astrocytes couple neurons as a metabolic unit and send endfeet to the BBB, thus they build a bridge for communication between circulating factors and neurons. This attracts great interest in the metabolic study field since a lot of metabolic related enzymes, transporters and hormonal receptors have been found expressed in astrocytes (Diano et al, 1998; Garcia-Caceres et al, 2011; Garcia-Caceres et al, 2014; Hsuchou et al, 2009; Morgello et al, 1995; Vannucci et al, 1997; Zhu et al, 1990). A series of studies unveiled the function of the leptin receptor on the hypothalamic astrocytes. Pan’s group shows that leptin receptors on the astrocytes might participate in the leptin transport on the BBB, and the lack of leptin receptors on astrocytes contributes to the central leptin resistance by attenuating hypothalamic pSTAT3 signaling (Pan et al, 2008; Wang et al, 2015). Chowen group reported that leptin could directly regulate glucose and glutamate transporters in astrocytes, which may ultimately affect glucose sensing and synaptic activity (Fuente-Martin et al, 2012). Recently, Horvath group reported that the lacking of leptin receptor on mouse astrocyte in an adult stage results in morphology changes of astrocytes and the modulation of hypothalamic circuits regulating feeding (Kim et al, 2014). The insulin receptor is also expressed in astrocytes. Our ongoing work suggests that the astrocytic insulin receptor is involved in glucose transport on the BBB to regulate systemic
glucose homeostasis (unpublished data). Besides, astrocytes could also respond to ghrelin (Garcia-Caceres et al, 2014), estrogen (Morselli et al, 2014) and glucocorticoids (Vielkind et al, 1990), but detailed physiological significance of these receptors on astrocytes and their role in metabolic disorders have not been fully described.

In addition to hormones, astrocytes could also sense circulating nutrients like glucose and fatty acids. Glucose transporter 1 (GLUT1) (Morgello et al, 1995) and glucose transporter 2 (GLUT2) (Marty et al, 2005) are both expressed by astrocytes. GLUT1 was the primary glucose transporter in the CNS. During hypoglycemia and hypoxia, GLUT1 was upregulated to enhance the glucose import (Boado & Pardridge, 2002). GLUT2, though much less abundant than GLUT1, was reported as a glucose sensor. Dysfunction of GLUT2 could cause interrupted feeding control and hypoglycemia induced glucagon secretion (Stolarczyk et al, 2010). The glucagon secretion phenotype could be rescued by transgenic expression of GLUT2 in astrocytes but not in neurons. These studies emphasized the essential role of astrocytes in the CNS glucose sensing scenario.

On the other hand, lipid sensing in the brain is less understood than glucose but gains increasing attention during the past decade. Lipids could be sensed by the brain to inhibit food intake and glucose production (Clement et al, 2002; Cruciani-Guglielmacci et al, 2004; Lam et al, 2005a; Lopez et al, 2007; Obici et al, 2002; Oomura et al, 1975). The mechanisms suggested in these studies mainly focused on the process of fatty acids oxidation. The key enzyme and
intermediate metabolites on this pathway like long chain fatty acids-CoA (LCFA-CoA), Malonyl-CoA and carnitine palmitoyltransferase I (CPT-1) are identified as central lipid sensors (Lam et al, 2005a; Obici et al, 2003). As mentioned earlier, astrocytes are important fatty acids consumers in the brain. Therefore, astrocytes may also serve as the lipid sensors in the CNS. Recently, a microdialysis study showed that astrocyte derived ketone bodies were elevated in the ventral medial hypothalamus (VMH) during a HFD meal and resulted in inhibition of food intake. Such a phenomenon could be reversed by a centrally given ketogenic inhibitor (Le Foll et al, 2014). Thus, ketone bodies produced from astrocytes might be used not only as the energy supply, but also as signaling molecules to hypothalamic neurons.

HFD induced obesity was associated with astrogliosis in ARC (Thaler et al, 2012). In the early stage of high-fat feeding, astrocytes in ARC had an increased GFAP expression and branched, elongated processes shown by GFAP immunostaining. This moderate astrogliosis was attenuated somehow after the early stage, but became more severe on the chronic HFD feeding. In 8 months HFD mouse, astrocytes in ARC formed a dense fibrous network and lost the boundaries of cell territory, which is a typical astrocytic response to CNS injury. The triggers of astrogliosis could be the detrimental factors from circulation. Saturated fatty acids could activate the inflammatory pathway in both microglia and astrocytes (Gupta et al, 2012; Milanski et al, 2009). In ARC, fenestrated BBB makes glial cells serve as the first defending barrier. Endfeet from astrocytes on the blood vessel could
be directly affected by elevated saturated fatty acids level by HFD feeding. Also, astrocytes may crosstalk with microglia to get immune response signals. The study of the mechanism on how hypothalamic astrogliosis contribute to DIO is still ongoing. One hypothesis is that astrogliosis increased glial coverage of neurons, like the glial scar tissue, would decrease the circulating nutrients availability to neurons. This could be a protective effect for neurons against overload nutrients, but could also result in an inappropriate transport of the signaling molecules and the less synaptic input (Horvath et al, 2010). There is also evidence showing that gliatransmitters released from activated astrocytes were related with metabolic control. In the HFD induced innate immune response, ARC had gliosis accompanied with increased cytokine production. In an astrocyte specific IL-6 knockout study, mice without IL-6 in GFAP positive cells have increased body weight. Meanwhile, when IL-6 is over expressed in astrocytes, mice become resistant to HFD but with impaired glucose homeostasis (Hidalgo et al, 2010; Quintana et al, 2013). Recently, it has been reported that activated hypothalamic astrocytes could regulate feeding by the release of adenosine to inhibit AgRP neuron activity (Yang et al, 2015). It should be noted that all these studies are still more or less limited by approaches and genetic tools. In different stages of DIO, causes, consequences, intracellular pathway and crosstalk with neighboring cells of hypothalamic astrocytes still need to be understood.

**Dissertation aim**

Collectively, current literature highlights the significance of glial cells in the regulation of metabolism. In this dissertation, we are aiming to explore the
behaviors of microglia and astroglia in diet-induced obesity. Previously, we and others have shown that DIO is associated with hypothalamic gliosis. In chapter 2, we first examine what triggers hypothalamic microgliosis by comparing several obese mouse models in conjugation with pharmacological approaches. We conclude that hormones and diet but not body weight determine the microglia reactivity. In chapter 3 we then analyzed the dietary effect on microglia and found that carbohydrates are required and lipids alone are not sufficient to trigger hypothalamic microgliosis. Our data also suggests that advanced glycation end products might be the essential mediator in such a carbohydrate and fat combination induced microgliosis. In chapter 3, we studied the role of the other glial cell - astrocytes in CNS lipid metabolism and lipid sensing. We demonstrated lipoprotein lipase in astrocytes is required to regulate astrocytes lipid metabolism, which is essential for CNS controlled energy homeostasis. Mice lacking of LPL in astrocytes have ceramide accumulation in the brain and develop increased body weight gain and glucose intolerance on HFD feeding.
CHAPTER 2:

Dietary Nutrients and Hormones, but not Body Weight, Control

Hypothalamic Microgliosis
Abstract

The arcuate nucleus (ARC) of the hypothalamus plays a key role in sensing metabolic feedback and regulating energy homeostasis. Recent studies revealed activation of microglia in mice with high-fat diet (HFD)-induced obesity (DIO), suggesting a potential pathophysiological role for inflammatory processes within the hypothalamus. To further investigate the metabolic causes and molecular underpinnings of such glial activation, we analyzed the microglial activity in wild-type (WT), monogenic obese ob/ob (leptin deficient), db/db (leptin-receptor mutation) and type-4 melanocortin receptor knockout (MC4R-KO) mice on either a HFD or on standardized chow (SC) diet. Following HFD exposure, we observed a significant increase in the total numbers of ARC microglia, immunoreactivity of ionized calcium binding adaptor molecule 1 (iba1-ir), cluster of differentiation 68 (CD68-ir) and ramification of microglial processes. The ob/ob mice had significantly less iba1-ir and ramification. Leptin replacement rescued these phenomena. The db/db mice had similar iba1-ir comparable to WT mice, but had significant lower CD68-ir and more ramification than WT mice. After 2 weeks of HFD, ob/ob mice showed an increase of iba1-ir, and db/db mice showed increase of CD68-ir. Obese MC4R-KO mice fed a SC diet have comparable iba1-ir and CD68-ir with WT mice but have significantly more ramifications than WT mice. Intriguingly, treatment of DIO mice with glucagon like peptide-1 receptor agonists reduced microglial activation independent of body weight. Our results show that diet type, adipokines, and gut signals, but not body weight, affect the presence and activity levels of hypothalamic microglia in obesity.
Introduction

Excessive energy intake is a major cause of obesity, which is recognized as one of the greatest threats to human health worldwide. Disproportionate hunger and food intake signals are generated as a result of an imbalance in the metabolic sensing and regulation networks of the central nervous system (CNS). Studies of the interaction between these networks and metabolism have suggested that malfunction of hypothalamic neurons is the leading candidate to explain the pathology of metabolic disease (Schwartz et al, 2000; Velloso & Schwartz, 2011; Yi et al, 2011). However, the specific causes of neuronal malfunction in these brain regions remain largely unknown, particularly regarding the influence of glia, which are important in maintaining a homeostatic microenvironment for neuronal survival.

Among the different types of glia, the resident macrophages—known as microglia—are responsible for clearance of cell debris and release of cytokines to recruit other immune-responsive cells to the CNS (Neumann et al, 2009). In a recent study of metabolic syndrome induced by consumption of a high-fat diet (HFD), we showed that microglia in the hypothalamic arcuate nucleus (ARC) adopt a pro-inflammatory state. This hyper-activation is linked to an abnormal increase in the production of pro-inflammatory cytokines, which may be toxic to neurons, and results in loss of POMC neurons and decreased function of the neuronal network involved in maintaining energy balance (Smith et al., 2012; Thaler et al., 2012). Thus, in addition to neurons, microglia in the ARC could play
an important role in maintaining energy homeostasis. The metabolic causes of hypothalamic microglial activation in obesity, however, remain to be elucidated.

In the present study, we investigated microglial activity under diet induced or monogenic obese conditions, by using immunoreactivity of ionized calcium binding adaptor molecule 1 (iba1-ir), which is up-regulated during microglial activation (Imai et al, 1996; Ito et al, 2001; Postler et al, 2000), and cluster of differentiation 68 (CD68-ir), which is associated with phagocytosis (Betjes et al, 1991). We also analyzed the ramification of the microglial processes under different obese conditions. We found that a high-calorie diet not only increases the number of iba1-ir and CD68-ir positive microglia, but also increases non-iba1 microglia presence in the ARC of mice engineered to express green fluorescent protein (GFP) in microglia. Comparison of microglial activity between wild-type (WT), monogenic obese ob/ob (leptin deficient) (Halaas et al, 1995), db/db (leptin receptor mutation) (Chen et al, 1996), and type-4 melanocortin receptor knockout (MC4R KO) mice (Gantz et al, 1993) revealed that microglial activity is not controlled by obesity per se but by HFD associated factors or leptin. Treating primary cultured hypothalamic microglia with serum from standard chow (SC) diet or HFD fed mice showed HFD—but not SC--serum stimulates microglial activity and production of cytokines. Furthermore, ob/ob and db/db mice hypothalami display different levels of microglial functional related genes expression. Treating DIO mice with a glucagon-like peptide-1 receptor agonist was associated with decrease of microglial iba1-ir and ramification. These data suggest that metabolic
hormones and diet, but not body weight, are major players of controlling the hypothalamic microglia activity under obese conditions.

Materials and Methods

Animals
All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati. Wild type, Lep$^{ob/+}$ (for breeding of ob/ob mice), Lep$^{ob/ob}$ (ob/ob), Lepr$^{db/db}$ (db/db), MC4R KO and CX3CR1-enhanced green fluorescent protein (eGFP) mice (expresses eGFP in monocytes, dendritic cells, NK cells, and brain microglia under the control of the endogenous Cx3cr1 locus) were all obtained from the Jackson Laboratory with C57BL/6 background. Lep$^{ob/ob}$ mice with microglial eGFP expression were generated by crossing Lep$^{ob/+}$ mice with CX3CR1-eGFP mice. All mice were group housed on a 12-h light, 12-h dark cycle (6 a.m.-6 p.m.) at 22°C, with free access to food and water.

Measuring Cell Proliferation Activity in the ARC
To investigate if iba1-ir, CD68-ir, or GFP positive microglia increased due to microglial proliferation inside the ARC, we placed intracerebral ventricular (ICV) infusion probes into the lateral intracerebral ventricle in SC diet and HFD fed mice and injected bromodeoxyuridine (BrdU) for 5 days (10 mg/mL, 5 mL/day). In a parallel group, we also injected BrdU intraperitoneal (i.p.) (10 mg/mL, 50 mL/10 g body weight/day, 5 days). Cell proliferation was also studied by measuring Ki67...
expression in separate groups of SC diet and HFD fed mice 1 week after mechanical injury by inserting a needle into mediobasal hypothalamus area next to the ARC.

**Leptin Treatment of ob/ob Mice and Exendin-4 Treatment of DIO Mice**

For leptin treatment, 10-week-old ob/ob mice and age-matched WT mice were divided into three subgroups (n=5-7): vehicle treatment, leptin treatment and vehicle-treated animals pair-fed to the leptin treatment group. In addition, 16 DIO mice with body weight matched to ob/ob mice were divided into two subgroups: vehicle treatment and leptin treatment. Each group of mice was matched for body weight, body fat mass and food intake at baseline. Subcutaneous injections of leptin (1 mg/kg) or vehicle were administered daily for 5 days; food intake and body weight were monitored daily.

For exendin-4 treatment, 8-month-old DIO mice were divided into three groups (n=5-7): vehicle treatment, exendin-4 treatment and mice pair-fed to the exendin-4 treatment group. Exendin-4 (0.25 mg/kg) or vehicle subcutaneous injections were administered daily for 5 days; food intake and body weight were monitored daily.

**Immunohistochemistry and Immunofluorescence of Mouse Brain Tissue**

Mice used for immunohistochemistry were deeply anesthetized with sodium pentobarbital and perfused transcardially with saline followed by a solution of 4%
paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. The brains were removed and kept at 4°C for overnight post-fixation, equilibrated 48 h with 30% sucrose in 0.1 M Tris-buffered saline (TBS; pH 7.6). Coronal sections (30 µm) were cut on a cryostat and sections were rinsed in 0.1 M TBS. For iba1, CD68, Ki67 and BrdU immunohistochemistry, brain sections at the level between bregma -1.70 and -1.94 (Paxinos & Franklin, 2008) were divided into two groups by selecting alternative sections from 8-9 continuing sections. Every 3-4 sections were incubated with rabbit anti-iba1 primary antibody (Synaptic Systems, Germany) at a 1:1000 dilution, or rabbit anti-CD68 primary antibody (Abcam, USA) at a 1:300 dilution. After primary antibodies incubated overnight at 4°C, sections were rinsed and incubated in biotinylated secondary goat anti-rabbit IgG. For single iba1 or CD68 immunohistochemistry, sections were rinsed and incubated in avidin-biotin complex (ABC method, Vector Laboratories, Inc., Burlingame, CA) for 1 h, and the reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide for 5–7 min. Sections were mounted on gelatin-coated glass slides, dried, dehydrated through a graded ethanol series, cleared in xylene, and cover-slipped for light microscopy. For colocalization of GFP and iba1, sections were incubated with streptavidin-conjugated Cy3 (Jackson ImmunoResearch, USA) for 1 h. All sections were rinsed and mounted on gelatin-coated glass slides, dried, covered with polyvinyl alcohol mounting medium containing DABCO® (Sigma, USA) and observed by confocal microscopy (Zeiss-LSM710, Germany). Cell counting was manually
performed within a frame outlining inside the ARC. Iba1-ir and CD68-ir cells are counted when cell nucleus is immunoreactive.

**Leptin or Serum Treatment in Primary Cultured Hypothalamic Microglia**

Primary cultured hypothalamic microglia obtained from dissected hypothalamus on postnatal day 2 were cultured by a method adapted from (Saura et al, 2003) 17 to 18 days afterward, cultured microglia were treated either with leptin (100ng/ml) and vehicle for 120 min, or 1% serum collected from 6 months HFD or SC diet fed mice for 120 min. Total RNA was isolated and real-time PCR was applied with Tagman® probes (Applied Biosystems).

**Low Density Array Real-Time PCR**

To broadly compare the microglial function and the associated changes in hypothalamus of HFD induced obese mice or mice with leptin deficiency/leptin receptor mutation (body weight matched to diet induced obese mice), we applied low density array real-time PCR (Applied Biosystems, customized microfluidic card) in mice hypothalamic tissue. Hypothalamic tissues from SC diet fed WT, *ob/ob*, *db/db* mice and HFD diet fed WT mice were isolated and fast snap frozen in liquid nitrogen till using. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as house keeping gene. Data were analyzed by target genes relative expression/HPRT.

**Statistical Analysis**
All results are expressed as mean ± SEM. Statistical comparisons were performed using one-way or two-way ANOVA. A $p$ value below 0.05 was considered statistically significant.

Results

Activity levels as well as total numbers of microglia are increased in the ARC of mice fed a HFD diet

In a previous study, we showed that the numbers of iba1-immunoreactive microglia were increased in WT mice fed a HFD diet (Thaler et al, 2012). To determine whether this increase in the numbers of activated microglia is due solely to the activation of resident microglial cells or accompanied by an increase in the total microglia population, we quantified and compared the numbers of activated microglia to the total microglial population after 6 weeks of HFD or SC diet (started at age 6 weeks). In the ARC of mice fed a SC diet for 12 weeks, the average number of detectable iba1-positive, activated microglia was $20.83 ± 1.03$ per 0.06-mm$^2$ field (Fig 2.1A) The total number of microglia (revealed by eGFP expression under the control of the endogenous Cx3cr1 locus) was $27.33 ± 1.76$ per 0.06-mm$^2$ field (Fig 2.1B). Thus, the total number of microglia was significantly higher than the detectable iba1-positive microglia ($p = 0.013$). When mice were exposed to 6 weeks of HFD diet, the total number of microglia increased to $50.50 ± 3.0/0.06$mm$^2$, while the number of detectable iba1-positive cells was $31.92 ± 1.87$ per 0.06-mm$^2$ field ($p < 0.001$ vs total eGFP and $p < 0.001$ vs iba1-positive microglia in 10-week SC-fed mice, Fig 2.1E, F). Increases
occurring in the ARC were not observed in other areas of the hypothalamus. These data indicate that HFD increases the overall number of microglia in the ARC in addition to stimulating microglial activity.

**Microglial Activation Is Mainly Induced by HFD but Not by Increased Fat Mass**

To determine whether microglial activation is caused by HFD or by obesity per se, we quantified and compared iba1-ir, iba1-ir ramified processes and CD68-ir between WT mice fed with SC or HFD and ob/ob, db/db, and MC4R KO mice fed with SC diet (otherwise matched), as well as ob/ob and db/db mice fed with HFD diet. The number of iba1-ir microglia was significantly lower in the ARC of SC-fed obese ob/ob mice than in age-matched SC-fed WT controls (Fig 2.2A, B). Furthermore, the intensity of the iba1-ir in the soma of microglia from ob/ob mice was less than in WT mice. CD68-ir tended to be less in ob/ob mice than WT mice, but the levels did not reach significance (Fig 2.3A, B). Also, there was less extensive ramification of iba1-positive microglial processes in ob/ob than in WT mice (Fig 2.2I). Interestingly, the total microglial number did not differ between WT and ob/ob mice (Fig 2.4), indicating that only microglia activity (demonstrated by iba1-ir) was influenced by leptin deficiency. Obese db/db fed a SC diet had similar numbers of iba1-ir microglia (Fig 2.2E) but significantly less CD68-ir compared to WT control (Fig 2.3F). Moreover, there were significantly more iba1-ir ramified processes in db/db than in WT mice, indicating that leptin deficiency and leptin receptor mutations have diverse influences on microglial activity and
phagocytic capacities. When ob/ob mice were exposed to 2 weeks of HFD, the number of iba1-ir ramified processes and CD68-ir in the ARC became greater than that of WT, SC-fed mice (Fig 2.2C and Fig 2.3C), suggesting that HFD diet alone can change microglial activation in ob/ob mice in the absence of leptin. In db/db mice fed with 2 weeks of HFD, the CD68-ir increased significantly compared to that of fed with SC diet (Fig 2.3G), however, the values were still lower than in WT, in which no clear change in iba1-ir ramified processes was observed (Fig 2.2F). In SC-fed, obese MC4R KO mice, iba1-ir microglial numbers in the ARC were similar to those of WT SC-fed controls (Fig 2.2G) and significantly less than those of body-weight-matched, 10-week HFD-fed WT DIO mice, however, the ramification of MC4R KO mice is similar to 10-week HFD-fed WT DIO mice; furthermore, CD68-ir was comparable to WT mice (Fig 2.3D). These findings were particularly intriguing as the body weight of the MC4R KO mice matched the ob/ob, db/db and 10 weeks HFD DIO mice. Taken together, these data demonstrate that microglial activation is mainly a consequence of HFD diet but not obesity per se. Furthermore, leptin and leptin receptor deficiency can cause different types of microglial under-activation.

**Total Cell Proliferation Activity is Low in the ARC**

After 5 days of ICV or i.p. administration, the BrdU positive nuclei are expressed abundantly around the area damaged by ICV probes; however, there are very few BrdU labeled nuclei in the ARC of SC diet and HFD fed mice. Similarly, Ki67 immunohistochemistry staining shows clear, positive immunoreactivity in the
nuclei of cells in the brain region injured by mechanical injury, but not in the ARC of SC diet or HFD fed mice (data not shown). This indicates that there are very few cell proliferation events taking place in the ARC under either diet. The increased iba1 and CD68-ir microglia may be due to higher expression of these proteins, migration of microglia from other brain regions, or infiltration of peripheral macrophages into the ARC.

**Leptin and GLP-1 Receptor Agonist Normalize Microglial Activity in the ARC**

Given the under-activation of microglia observed in *ob/ob* mice, we examined whether leptin replacement can rescue microglial activation. After 5 days of leptin treatment, injection of leptin in both age-matched WT (Fig 2.5A-E) and body-weight-matched DIO groups had no effect on either number or morphology of the microglia. The *ob/ob* mice treated by leptin had significantly reduced food intake and body weight (data not shown), as shown previously (Halaas et al, 1995). The numbers of iba1-ir microglia and iba1-ir ramified processes in the ARC were significantly increased compared with that of vehicle controls (Fig 2.5F-J). The number of iba1-positive microglia and iba1-ir ramified processes in the pair-fed group (restricted food intake matched to leptin-treated group) was slightly increased but less than in the leptin treated group. These data indicate that leptin is important for maintaining normal microglial activity.
As the rescued microglial activity in leptin-treated ob/ob mice suggests that abnormal microglia activity can be normalized. We therefore tested whether hyperactivation of microglia also can be normalized with the glucagon-like peptide-1 agonist exendin-4 (Flint et al, 1998; Gutniak et al, 1992; Thorens et al, 1993) in DIO mice fed HFD for 8 months. Five days of exendin-4 treatment in DIO mice significantly reduced food intake and body weight (data not shown). As expected, the pair-fed group also showed significantly reduced body weight. However, compared to the vehicle group, the number of iba1-positive microglia and their ramification in the ARC was only significantly reduced in the exendin-4 treated group and not in the pair-fed control group (Fig 2.5L-P). These data indicate that normalization of microglial activation occurs in parallel with improvement of the metabolic syndrome following exendin-4 treatment but not reduction of HFD intake.

**Leptin or High Fat Diet Fed Mice Derived-Serum Stimulates Cytokines Production in Primary Cultured Hypothalamic Microglia**

In primary cultured hypothalamic microglia, to mimic in vivo situation, we choose the leptin dose at 100ng/ml, which is two folds of the plasma leptin level of a 50g mice feed ad libitum on HFD. After 120 min of leptin treatment, the gene expression level of two cytokines that have been shown to increase in the hypothalamus upon HFD (Thaler et al, 2012), TNFα and IL-1β, both increased significantly. The expression level of iba1 did not show significant difference (Fig
2.6A). This suggests that leptin is one of the factors that can stimulate microglial activation and cytokine production. We then treated primary cultured hypothalamic microglia with serum collected from SC diet or HFD fed mice, to check whether and to what extent HFD derived circulating factors influence microglial activity. With 1% concentration, HFD-serum significantly increased iba1, TNFα and IL-1β gene expression (Fig 2.6B), suggesting HFD derived circulating factors can stimulate hypothalamic microglial activity and cytokine production.

**Lack of Leptin Signaling Affects Microglial Function in Hypothalamus**

In homogenized whole hypothalamic tissue, ob/ob mice have significantly less Emr1 (F4/80) (Kwakkenbos et al, 2002) (Fig 2.7), nucleotide binding and oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) (Heneka et al, 2013), integrin-alpha X (Itgax) (Akiyama & McGeer, 1990), ras-related protein Rab-4A (Rab4a) and IL-1β gene expressing. The db/db mice have significantly lower activation and transcription factor-3 (ATF-3), Itgax, Rab4a, IL-1β and TNFα gene expression, while cyclin-dependent kinase inhibitor 1A involved in microglial proliferation (Yamamoto et al, 2012) is higher in both ob/ob and db/db mice. In addition, HFD fed mice have significant higher IL-1β gene expression than in SC diet fed mice. This indicates that lack of leptin signaling can affect different aspects of microglial function that could be involved in abnormality of hypothalamic network in control of metabolic homeostasis.
Discussion

We have shown that in addition to enhanced microglial activation, there is an increase in the total number of microglia in the ARC of mice following induction of obesity through exposure to a HFD. This effect on hypothalamic microglia is induced by HFD itself rather than obesity or adiposity per se. We have also shown that leptin is important for maintaining normal levels of microglial activity and is involved in cytokine production. Lack of leptin signaling in ob/ob and db/db mice causes deficiencies in various aspects of microglia function in the hypothalamus. Furthermore, we have demonstrated that both under- and over-activation of microglia in ob/ob and DIO mice can be normalized by administration of leptin and a GLP-1 receptor agonist. Taken together, these data provide important insights into the control of hypothalamic microglial activation in obesity and highlight a potential new target for the treatment of obesity and related diseases.

We observed that the total number of microglia in the ARC is significantly increased in HFD-fed DIO mice, likely due to migration of microglia from other brain regions, or infiltration of macrophages from periphery (Nelson et al, 2002) as no clear cell proliferation profiles can be detected in the ARC of HFD fed mice. This possibility of infiltration is supported by our recent observation that during HFD feeding there is extensive deposition of IgG inside the ARC microglia (Yi et al, 2012b), indicating increased permeability of the blood-brain barrier (BBB). This functional change in the BBB is consistent with the observation that hyper-
vascularization and angiopathy, along with endothelial damage, are induced in the hypothalamus by exposure to HFD (Yi et al, 2012b). It is possible that malfunction of the BBB during HFD feeding leads to increased recruitment of macrophages from the general circulation to assist the resident microglia with maintaining an adequate immune response. Whether or not these pathological changes in the vascular system are solely responsible for the observed effects on hypothalamic microglia, it seems clear that calorie-rich dietary conditions have deleterious effects on the microenvironments that are required to support neuronal survival. Interestingly, in ob/ob and/or db/db mice, although most of the microglial activity markers in the total hypothalamic homogenized tissue are lower than in the WT mice, one of the cell proliferation genes, cdkn1a, has a higher expression level, suggesting there are factors stimulating cdkn1a but further cell proliferation processes are blocked by other factors associated with lack of leptin signaling. The exact mechanism under this phenomenon requires further study.

In contrast to the effect of HFD on the accumulation and activation of microglia in the ARC of WT mice, the absence of leptin signaling in obese ob/ob mice obstructs normal microglial activity and production of cytokines such as IL-1β. Interestingly, the expression of another microglial derived cytokine, TNFα, is lower in db/db but not in ob/ob mice compared to WT mice. Furthermore, db/db mice do not show lower iba1-ir in the ARC, revealing another phenotypic distinction, in addition to persistent hyperglycemia, between ob/ob and db/db
mice. Whether hyperglycemia in the db/db mice is involved in maintaining some aspects of microglial activity, or normal iba1-ir is unknown. Regardless, the different microglial phenotypes between db/db and ob/ob mice raise the possibility that the abnormal activity of microglia is one of the causes of the ob/ob and db/db phenotype. Mixed cultures of macrophages from ob/ob and db/db mice have been shown to have deficient phagocytic activity due to lack of leptin signaling (Lafrance et al, 2010; Loffreda et al, 1998). This deficient phagocytic activity probably also applies to microglia in the CNS. The ob/ob and db/db mice have lower IL-1β and/or TNFα in the hypothalamus, which could be the direct cause of the inability of these mice to maintain or recruit other microglia or macrophages into the hypothalamus for endocytic clearance of debris and maintainence of a healthy microenvironment (Napoli & Neumann, 2009). Leptin is able to stimulate TNFα and IL-1β production in primary cultured hypothalamic microglia, providing evidence that leptin is one of the essential triggers of cytokine production in microglia. Whether reanimate cytokine production in ob/ob and db/db mice can rescue the obese and/or diabetic phenotypes remains unanswered. However, though leptin is required for the normal microglia activity, excess leptin can not further trigger microgliosis. MC4R KO mice have higher circulating leptin levels due to obesity, but microgliosis has not been detected.

Microglia activation has different stages depending on the level of the injury. Morphology in 6 stages during microglia activation has been described before,
from small soma, ramified process in resting stage to large round soma, less ramifications in phagocytic stage (Jonas et al, 2012). Compared with physical injury and virus infection (Kalin et al, 2015), microglia haven’t reached their maximal activation capacity by HFD feeding. The largest microglia soma is observed in long term HFD group. Therefore in the current study, we quantify the ramification of each microglia and assume the ramification level is positively correlated with activation level since the extreme phagocytic morphology of microglia has not been observed in any of these obese models.

The GLP-1 receptor agonist exendin-4 is a well-characterized anti-obesity and anti-diabetic agent (Barrera et al, 2011). It acts through the GLP-1 receptor, which is reported expressed by microglia (Iwai et al, 2006) to deactivate microglial hyperactivity induced by consumption of a HFD. In other animal models of neurodegenerative diseases, it has been shown that GLP-1 treatment can protect neurons from degeneration via the reduction of pro-inflammatory molecules and cytokines arising from suppression of microglial activity (Kim et al, 2009; McClean et al, 2011). Thus, in the present study, by suppressing microglial hyperactivity, exendin-4 could also protect ARC neurons from the effects of inflammatory cytokines.

Overall, our results reveal that hypothalamic microglial activation is induced by HFD independent of body weight. This activation can be suppressed by GLP-1 receptor agonism while improving other metabolic abnormalities. Thus, microglial
activation in the hypothalamus is an important cellular readout of the metabolic syndrome. As a major player in controlling immunity and homeostasis in the CNS, microglia may represent a relevant target for the prevention and treatment of metabolic disorders. Thus, combinational pharmacotherapy aimed at not only modifying neuronal function but also normalizing microglia and other components of the neuronal “supporting unit” may represent a promising therapeutic approach.
Figure 2.1 High fat diet (HFD) increases both the total number of microglia and the number of activated iba1-positive microglia in the arcuate nucleus of the hypothalamus. Wild-type mice fed a standard chow diet have a modest number of microglia (expressing eGFP (green) under the control of the endogenous Cx3cr1 locus) (B), including some iba1-positive cells (red, arrows in A & C). After 6 weeks of HFD diet, the total number of microglia (E) and the number of iba1-positive microglia (D) are both increased. Under these conditions, still only a proportion of the total microglial population is iba1-positive (F). III indicates third ventricle; scale bar = 25 µm.
Figure 2.2 Iba1-ir microglia in the hypothalamic arcuate nucleus (ARC) of diet induced and monogenic obese mice. The numbers of iba1-positive microglia in the ARC of ob/ob mice (B) is reduced compared to wild-type (WT) mice (A) on standard chow (SC) diet as well as to WT mice on high fat diet (HFD) (D, H), and the microglial processes are less extensively branched (high-magnification images for the cells pointed by white arrows are in the upper right panel) (I). The ARC of ob/ob mice fed a high fat diet (HFD) contains increased numbers of iba1-ir positive microglia, which also have more extensively branched processes (C). When body weight is matched to the ob/ob group (D), similar numbers of iba1-positive microglia are observed in db/db (E) and WT mice, but significantly lower than 10 weeks HFD group. The ramification of microglia in db/db mice is significantly lower than in SC diet fed WT mice and higher than in HFD fed WT mice. After feeding with HFD two weeks (F), number of iba1-ir
positive microglia are increased in the ARC, while ramification were not changed. In obese MC4R KO mice (G) with body weight matched to ob/ob and db/db mice and 10 weeks HFD-fed mice, the numbers and morphology of iba1-positive microglia are similar to WT HFD-fed controls. WT mice fed for 10 weeks with HFD have the largest number of iba1-positive microglia with the most extensive ramification (D). $p < 0.05$ for *: vs WT SC group, ^^: vs ob/ob SC group, §: vs db/db SC group, #: vs MC4 KO group, ^: vs db/db HFD group, by one way ANOVA and subsequent Bonferroni's post hoc test; Data homogeneity is checked by Bartlett's test. Scale bar = 100 µm.
Figure 2.3 CD68-ir microglia in the hypothalamic arcuate nucleus of diet induced and monogenic obese mice. In comparison to Iba1-ir, which stains both soma and processes of microglia, CD68-ir is mainly visible inside the soma of the microglia. $p < 0.05$ for $\ast$: vs WT SC group, $^\wedge \wedge$: vs ob/ob SC group, $\S$: vs db/db SC group, $^\wedge$: vs db/db HFD group, determined by one way ANOVA and subsequent Bonferroni’s post hoc test; Data homogeneity is checked by Bartlett’s test. Scale bar = 100 μm
Figure 2.4 Total microglial population in the hypothalamic arcuate nucleus (visualized by eGFP expression under the control of the endogenous Cx3cr1 locus) does not differ between WT mice (A) and ob/ob (B) mice. III indicates third ventricle; scale bar = 100 μm.
Figure 2.5 Effect of anti-obesity pharmacotherapy on hypothalamic microglial activation in monogenic obese mice. Compared to vehicle control (A), no effects on iba1-ir and ramification were observed in WT mice treated with 5 days of leptin or pair-fed (high-magnification images for the cells pointed by white arrows are in the upper right panel) (A-E); 5 days of leptin treatment significantly increased the iba1-ir microglial number and ramification in the arcuate nucleus (ARC) of ob/ob mice in comparison to their vehicle group. The pair-fed (to leptin) group also had significantly increased microglial activation but with less-extensive ramification (F-J). Exendin-4 treatment in mice with diet-induced obesity significantly reduced the number of iba1-positive microglia number and ramification in the ARC compared to vehicle control and pair-fed control groups (L-P). $p < 0.05$ for #: vs vehicle in I, J, O and P; *: vs leptin group in I and J and vs exendin-4 group in O and P, determined by one way ANOVA.
and subsequent Bonferroni’s post hoc test; Data homogeneity is checked by Bartlett’s test. Scale bar = 100 µm.
Figure 2.6 Cultured primary hypothalamic microglia treated with leptin or serum. Leptin treatment stimulate IL-1β and TNFα, but not iba1 genes expression (A); while HFD fed mice serum treatment stimulate iba1, IL-1β and TNFα genes expression, in comparison to chow diet fed mice serum (B). \( p < 0.05 \) for * vs Vehicle or Chow by student’s t-test. Data variances are checked by F test.
Figure 2.7 Microglial functional related genes expression profile in the hypothalamus of diet-induce obese and monogenic obese mice. *, p < 0.05 vs WT SC, # p<0.05 vs both WT SC and WT HFD groups, determined by one way ANOVA and subsequent Bonferroni's post hoc test; Data homogeneity is checked by Bartlett's test.
CHAPTER 3:

Dietary Carbohydrate Triggers Hypothalamic Microgliosis via Advanced Glycation End Products
Abstract

Hypothalamic neurocircuits help maintain energy homeostasis by controlling behavior and systemic metabolism in response to nutrient input. We and others have previously reported that hypothalamic microglia rapidly respond to hypercaloric diets by entering a reactive stage which potentially contributes to the metabolic syndrome. Here we report that consumption of a high-carbohydrate, high-fat (HCHF) diet but not of a low-carbohydrate, high-fat diet, triggers hypothalamic microgliosis in mice. We conclude that dietary carbohydrates are required and that the intake of excess dietary lipids alone is not sufficient to drive a hypothalamic innate immune response (“hypothalamic inflammation”). Advanced glycation end products (AGEs) were significantly increased in the mediobasal hypothalamus (MBH) of HCHF-fed mice, suggesting that elevated AGEs might mediate the HCHF-diet effects on the innate immune response in the hypothalamus and subsequent microglial reactivity. To assess this, we fed HCHF diet to wild-type mice and mice lacking receptors for AGEs (RAGE KO, ALCAM KO, RAGE-ALCAM double KO). Mice lacking one AGEs-relevant receptor and fed the HCHF diet had significantly lower microglial reactivity in the MBH and significant metabolic improvements compared to controls. This effect was even more pronounced in mice lacking both AGE-relevant receptors. We conclude that the hypercaloric consumption of a diet high in fat per se is not sufficient to induce hypothalamic inflammation, and that rather the excessive consumption of high fat together with consumption of high carbohydrates is the driver for hypothalamic inflammatory processes. Our data suggests that the
hypothalamic changes induced by overconsumption of a HCHF diet are mediated
by the hypothalamic impact of AGE products, thus potentially contributing to the
development of the metabolic syndrome.

**Introduction**

The hypothalamus is a key brain region involved in metabolic sensing and
regulation. It contains highly heterogeneous neuronal and glial populations that
receive and integrate diverse metabolic feedback signals from the periphery.
Accumulating evidence points to disrupted hypothalamic functioning as a major
player in hypercaloric environment-induced obesity and associated metabolic
disorders (De Souza et al, 2005; Kälin et al, 2015). Maintenance on a
hypercaloric diet activates an innate immune response in the hypothalamus. This
response is accompanied by astrocytosis, de novo angiogenesis, and the loss of
anorexigenic POMC neurons (Yi et al, 2012, Thaler et al, 2012) Importantly,
reactive microglia are not observed in monogenic obese ob/ob or MC4R KO
mice, nor in hyperglycemic/obese db/db mice that are fed a chow diet. The
syndrome does appear rapidly, however, when these mouse models are fed a
diet enriched with fat and carbohydrates (Gao et al, 2014). Thus, enhanced
hypothalamic microglial activity is associated with fat- and carbohydrate-rich diets
but not with body weight or hyperglycemia *per se.*

In high-fat, high-carbohydrate diet-induced obesity, the fat in the diet, and
especially saturated fatty acids (SFAs), is considered to be the essential
component activating pro-inflammatory responses in the hypothalamus (Milanski et al, 2009) as well as the detrimental health effects associated with consumption of SFAs such as the risk of type-2 diabetes (Eyre et al, 2004). What is not known is whether or how dietary carbohydrates, in the context of a hypercaloric diet, contribute to this hypothalamic response. A recent clinical study found that a reduction of carbohydrates in the diet caused subjects to lose more body weight than a reduction of fat, an effect that was not associated with total caloric intake (Bazzano et al, 2014). Such studies raise questions about the role of dietary carbohydrates in a hypercaloric environment with respect to the hypothalamic innate immune response and the consequent impact on hypothalamic control of energy homeostasis.

To understand these issues, we fed “wild-type” (WT) control mice with a non-ketogenic Atkins-style diet (LCHF1) as is often used for dieting purposes and which was very low in its carbohydrate content but high in protein and fat (Bielohuby et al, 2011; Mobbs et al, 2007), or alternatively with a ketogenic low-carbohydrate, high-fat diet (LCHF2), which had low dietary protein (Bielohuby et al, 2011; Klein et al, 2014; Shai et al, 2008) as used for treatment of pediatric epilepsy. We then compared the effects of these low-carb diets to those of a diet consisting of high carbohydrates (sucrose), high fat and ample protein (HCHF). We found that hypothalamic microglial reactivity was induced by the HCHF diet, but not by the LCHF1 or LCHF2 diets lacking carbohydrates.
It is known that proteins and lipids become non-enzymatically glycated and oxidized after contact with glucose, generating so-called advanced glycation end products (AGEs) (Singh et al, 2001) which contribute to vascular complications in diabetes (Goldin et al, 2006). We therefore assessed AGE levels in the MBH under different dietary conditions and discovered that AGEs were only significantly increased in the HCHF group. Mouse mutants displaying a knockout of the AGEs receptor (RAGE), or the RAGE analogue - the activated leukocyte cell adhesion molecule (ALCAM) - or a double knockout of both RAGE and ALCAM, were then challenged with the HCHF diet. A HCHF diet-induced hypothalamic innate immune response and associated metabolic disorders were improved in mice lacking RAGE or ALCAM, and more so in mice lacking both RAGE and ALCAM.

Materials and Methods

Animals

All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Helmholtz Center Munich, Bavaria, Germany. All mice were group housed on a 12-h light dark cycle at 23°C, with free access to food and water.

Body composition

Whole-body composition (fat and lean mass) was measured using nuclear magnetic resonance technology (EchoMRI-100; Echo Medical Systems,
Glucose Tolerance Test

Intraperitoneal glucose tolerance test (ipGTT) was performed by injection of glucose (2 g/kg, 25% wt/vol. d-glucose (Sigma, Germany) in 0.9% wt/vol. NaCl after a 5-h fast. Tail blood glucose levels (mg/dl) were measured with glucometer TheraSense Freestyle (Abbott Diabetes Care, Inc., Alameda, CA) before (0 min) and at 15, 30, 60 and 120 min after injection.

Primary Microglia Culture

Primary hypothalamic microglia are isolated as described in chapter 2, based on the method adapted from (Li et al, 2011c). When microglia are ready for experiments, cells were incubated with 50μM AGEs (EMD Milipore, Germnay) or PBS for 72 h. Total RNA was harvested for gene expression analysis.

Immunohistochemistry

Immunohistochemistry was performed as described in chapter 2. Briefly, Mice used for immunohistochemistry were perfused and fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. After equilibrated 48 h with 30% sucrose in TBS, Coronal sections (30 μm) were cut on a cryostat and sections were rinsed in 0.1 M TBS. For iba1, POMC and AGEs staining, 3 sections per mice from MBH were incubated with rabbit anti-iba1 primary antibody (Synaptic Systems, Germany) at a 1:1000 dilution, rabbit anti-POMC antibody (Phoenix,
Germany) at 1:1000 and rabbit anti-AGEs (ab23722 abcam) at 1:1000 at 4°C overnight. Sections were rinsed and incubated in biotinylated secondary goat anti-mouse IgG and avidin-biotin complex (ABC method, Vector Laboratories, Inc., Burlingame, CA). The reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide. For immunofluorescent, RAGE is visualized by either endogenous GFP or goat anti-GFP antibody (Abcam, Germany) at 1:250, followed by alexa 594 anti-goat (Jackson ImmunoResearch, Germany). Iba1 and AGEs are stained by same primary antibody and followed with alexa 594 or alexa 647 anti rabbit (Jackson ImmunoResearch, Germany).

**Western Blot**

Tissues from MBH of each experimental group were snapped frozen. The same amount of protein (about 10 mg) from tissue lysates was placed in 1.5-mL tubes and brought up to equal volumes with lysis buffer. 4XNuPAGE LDS Sample Buffer (invitrogen, cat.NP0007) was added to each and incubated at 95°C for 5 min. After heating, the mixture was kept on ice for 20 min. The 40ug protein lysates form each sample were then separated by 10% precast gel (Bio-Rad cat.400096180), and transferred to nitrocellulose membranes (Bio-Rad cat.170-4159). After the transfer, the membranes were blocked in 5% milk for 1 h. Primary antibodies were diluted in 5% milk and incubated with the membrane overnight at 4°C.(Rabbit anti AGEs, Abcam, cat. no. 23722; Rabbit anti-CD166, Abcam, cat.109215; Rabbit anti-beta-actin, cell signaling, cat.4970); On the following day, membranes were washed by Tris-buffered saline with
Tween (TBST) three times for 10 min and incubated with the HRP conjugated secondary antibody for 1 h at room temperature and washed again in TBST (three times for 10 min). Membranes were then developed by ECL (Bio-Rad, cat.170-5060) and imaged with Odyssey imaging system (LI-COR Bioscience).

**Far Western Blot**

Far-Western blotting was executed as described before (Wu et al, 2007). Briefly, 3µg recombinant CD166/ALCAM protein (Recombinant Mouse ALCAM/CD166 Fc Chimera; R&D Systems) were separated on 7.5% SDS gels (Bio-rad) and transferred to PVDF membranes. 0.5ug BSA-AGE was loaded as positive control. Membranes were then incubated with AC buffer (100 mM NaCl, 20 mM Tris [pH 7.6], 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder, and 1 mM DTT) containing guanidine-HCl 6M, 3M, 1M for 30 min at room temperature, then followed by AC-buffer (guanidine-HCL 0.1M and 0M) at 4°C for 30min and 1h respectively and finally blocked with 5% milk for 1 h at room temperature. Membranes were then incubated with 400 µg of AGE-BSA (Advanced Glycation Endproduct-BSA, EMD MILLIPORE, cat. no. 121800) in protein-binding buffer (100 mM NaCl, 20 mM Tris [pH 7.6], 10% glycerol, 0.1% Tween-20, 2% skim milk powder, 1 mM DTT, 4 mM CaCl2) overnight at 4°C. On the following day, membranes were washed four times with PBS with Tween 20(0.1%), and then incubated with anti-AGE Ab (Rabbit anti AGEs; Abcam, cat. no. 23722) diluted 1:500 in 5% milk containing 4mM CaCl2 overnight at 4°C. After washing, secondary Ab (goat anti rabbit IgG, Santa Cruz, sc-2004) were
added at 1:2000 in 5% milk and incubated with membranes for 1h at room temperature. Membranes were developed by same methods as Western Blotting.

**PCR**

For gene expression analysis, hypothalamic tissue or primary cultured microglia were harvest and total RNA was isolated by RNeasy lipid tissue kit (Qiagen, Germany). After reverse transcription by QuantiTect Rev. Transcription Kit (Qiagen, Germany), gene expression was analyzed by real-time PCR with Taqman probes (Applied Biosystems). Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as housekeeping gene.

**Statistical Analysis**

All data are expressed as means ± SEM, P value of less than 0.05 was considered significant. Student t-test and one-way or two-way analysis of variance (ANOVA) were used to test for differences between individual experimental groups. Group size estimations were based upon a power calculation to minimally yield an 80% chance to detect a significant difference in body weight of $P < 0.05$ between the treatment groups.

**Results and Discussion**

To dissect the effects of each dietary component on hypothalamic microglial reactivity and the innate immune response, we fed WT mice (C57BL/6, 8 week old) with standard chow, HCHF, LCHF1 or LCHF2 diets (Table 3.1) ad-libitum for
4 week. In comparison to the chow group, all three HF diet groups had a significantly higher daily caloric intake, being highest in the LCHF1 group (Fig 3.1A). The HCHF group gained the most body weight (BW) in each of the four weeks, followed by the LCHF1 group, the difference reaching statistical significance vs. chow at week 4. In contrast, mice fed with LCHF2 lost BW in all four weeks (Fig 3.1B&C). In comparison to the chow group, all three HF diet groups gained significantly more fat mass (EchoMRI), consistent with significantly higher plasma leptin concentrations (data not shown) and previous studies using LCHF diets in rats (Bielohuby et al, 2011). Consistent with previous studies, mice fed the HCHF diet had a high immunoreactivity of the microglial activity marker, ionized calcium-binding adapter molecule 1, in the MBH (iba1-ir); and the gene expression of iba1 in MBH per se was significantly increased in the HCHF diet group when compared to expression in mice fed with chow. In sharp contrast, this was not observed in the LCHF1 or LCHF2 diet groups (Fig 3.1E&F). Furthermore, tumor necrosis factor-alpha (TNFα), which is known to be produced mainly by microglia in the brain, was also increased only in the HCHF diet group. Other cytokines, including IL-1β and IL-6, were also increased, but these did not reach statistical significance (Fig 3.1E). Thus, although mice fed with LCHF1 or LCHF2 comparably consumed a HF diet as HCHF mice, the lack of carbohydrates in the diets was associated with hypothalamic microglia remaining in a quiescent state. Protein contents were comparable among standard chow, HCHF and LCHF1, thus protein impact on microglia activation has been excluded.
In the early phase of diet-induced obesity in mice, the anorexigenic hypothalamic peptide proopiomelanocortin (POMC) is elevated (Ziotopoulou et al, 2000). In the present study, among the four diets, the number of POMC-ir neurons was significantly increased only in the HCHF diet fed group (Fig 3.1G). Furthermore, the impact of HCHF diet on microglial and POMC neuronal activity was not associated with plasma fatty acid levels, since the concentrations of free fatty acids and free glycerol were increased in all three HF diet groups, and total triglycerides were increased only in LCHF2 group (data not shown).

All of these data imply that the presence of a high percentage of carbohydrates in the diet is essential for inducing hypothalamic microglial reactivity under conditions of concomitant high-fat content. We consequently measured the level of AGEs in the MBH under each dietary condition and found that AGEs is significantly increased only in the HCHF group (Fig 3.2A), and a strong increase of AGEs-ir on vessels in the MBH, again only in HCHF mice (Fig 3.2B). These AGEs cumulate on the vessel walls and bind with their receptors which are expressed on the endothelial cells and peri-vascular microglia (Sup.Fig 3.1).

To determine if AGEs are an important mediator of HCHF-induced metabolic disorders via stimulation of hypothalamic innate immune responses, we fed 8-week old WT mice and mice displaying a knockout of the receptor for AGEs (RAGE<sup>−/−</sup>) with chow or the HCHF diet for 16 weeks. When maintained on chow, food intake and BW were the same for RAGE<sup>−/−</sup> and WT controls. However, the
HCHF diet significantly increased BW in WT but not RAGE $^{-/-}$ mice. Also, in the HCHF condition, RAGE $^{-/-}$ mice had a decreasing trend of food intake and significantly lower BW gain at week of 15 and 16 compared to WT (RAGE $^{+/+}$) mice (Fig 3.2B). Genetically, RAGE is derived from an adhesive molecule. One of the adhesive molecules, the activated leukocyte cell adhesion molecule (ALCAM), shares the same genomic organization as RAGE in the gene fragment coding for the cytoplasmic domain (Sessa et al, 2014). RAGE and ALCAM have similar quaternary structures; both can bind to pro-inflammatory cytokines and show compensatory reciprocal up regulation in animals devoid of either receptor (von Bauer et al, 2013). Interestingly, there was up regulation of ALCAM in the hypothalamus of HCHF-fed mice (Fig 3.2C), suggesting that increased AGEs induced by the HCHF diet might stimulate ALCAM expression in the hypothalamus, leading to a compensatory action from ALCAM compromising the effects of the lack of RAGE. To determine whether AGEs can still bind to ALCAM in RAGE $^{-/-}$ mice, we performed a far western blotting experiment and found that AGEs can in fact still bind to ALCAM (Fig 3.2D). We then challenged 8-weeks old ALCAM $^{-/-}$ mice with chow and HCHF diets. Similar to RAGE $^{-/-}$ mice, when fed chow, ALCAM $^{-/-}$ mice were not different from the WT controls with respect to food intake and BW. In contrast, when fed HCHF, ALCAM $^{-/-}$ mice had a significant decrease in food intake and lower BW gain from week 14 to 16 in comparison to WT (ALCAM $^{+/+}$) mice (Fig 3.2E).
To completely block the effect of AGEs, we challenged 8 weeks old mice with a double knockout of both RAGE and ALCAM (RAGE-ALCAM −/−) with chow or HCHF. On chow diet, there was no difference in food intake or BW changes in RAGE-ALCAM −/− compared to WT mice (Fig 3.2F). Thus, the lack of AGEs action under normal caloric chow diet conditions does not appear to influence metabolic homeostasis. In contrast, on the HCHF diet, food intake in RAGE-ALCAM −/− mice was significantly decreased. The change in BW of RAGE-ALCAM −/− mice was already lower than that of RAGE-ALCAM +/+ mice after one week of HCHF diet. This BW difference continued for four weeks, at which time, the RAGE-ALCAM −/− mice began to regain BW to close to the level of RAGE-ALCAM +/+ mice. This regain of BW was alleviated at week 12 of HCHF diet and the BW of RAGE-ALCAM −/− mice was significantly lower compared to WT mice again until week 16 (Fig 3.2F). To further evaluate the impact of loss of RAGE-ALCAM on metabolism, we performed an intraperitoneal glucose tolerance test (i.p.GTT) after 16 weeks of chow or HCHF. Interestingly, although neither caloric intake nor BW differed between WT and RAGE-ALCAM −/− mice fed chow, a significantly improved glucose tolerance was detected in RAGE-ALCAM −/− mice. Furthermore, the improved glucose tolerance in RAGE-ALCAM −/− mice was also observed under HCHF conditions (Fig 3.2G).

We next asked whether the improvement of metabolic disorders in RAGE-ALCAM −/− mice is associated with interference of AGEs action in the hypothalamus. As expected, WT mice fed HCHF for 16 week had significantly
increased iba1-ir microglial number compared to chow-fed mice. Although the iba1-ir microglial number was not different between WT and RAGE−/− or ALCAM−/− mice (Fig 3.3A), it was significantly lower in RAGE-ALCAM −/− on chow diet conditions in comparison to levels in WT mice (Fig 3.3B). On the other hand, after 16 weeks of HCHF diet feeding, the iba1-ir microglia number in both ALCAM−/− and RAGE-ALCAM −/− mice was significantly lower than that in WT mice.

After 16 weeks of HCHF diet, the POMC-ir neurons in the MBH of WT mice were slightly but not significantly decreased on the HCHF diet (data not shown). This trend likely reflects the loss of POMC neurons that occurs in the chronic HCHF-diet induced obese model as we have reported previously; in that study, HCHF-diet mice had significantly fewer POMC-ir neurons compared to chow-fed mice (Thaler et al, 2012). There was no difference in POMC-ir between WT and RAGE-ALCAM −/− mice on the HCHF diet. In Alzheimer's disease, RAGE-dependent signaling in microglia contributes to the inflammatory mechanism (Fang et al, 2010; Yan et al, 1996). To test the hypothesis that the diet-derived AGEs in the MBH can stimulate microglial activity, we treated primary cultured microglia with AGEs for 72 h and detected increased TNFa and IL1b gene expression (Fig 3.3C).

To summarize, in attempting to understand the relative contributions of high levels of dietary carbohydrates and fats for metabolic disorders via hypothalamic
mechanisms, we found that a high percentage of dietary carbohydrates produces AGEs, which are important players of inducing hypothalamic innate immune responses, when there is also a high dietary fat component (Fig 3.3D). The crucial role of hypothalamic AGEs in diet-induced metabolic disorders was verified by the improvement of metabolic disorders in mice fed HCHF diet but lacking the AGEs receptor and/or the AGEs receptors analog. Therefore, simply restricting dietary fat is not the only factor that needs to be considered when controlling for body weight. Furthermore, it is known that inflammatory mechanisms play a crucial role in neurodegenerative disorders, and it is also known that elimination of the dietary carbohydrates exerts neuro-protective effects by alleviating symptoms in Alzheimer’s disease and Parkinson’s disease (Jabre & Bejjani, 2006; Reger et al, 2004). The current study is based on a global knockout mice model of AGEs receptors, whether the neuro-protective effects of low carbohydrate diets (LCHF diets in the present study) are mediated by microglial mechanisms needs to be explored in future studies.
Table 3.1 Diets components and the metabolizable energy.

<table>
<thead>
<tr>
<th>Diet composition</th>
<th>Fat (%)</th>
<th>Protein (%)</th>
<th>Carbohydrate (%)</th>
<th>Metabolizable Energy (Mcal/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Chow with protein Chow</td>
<td>16.7</td>
<td>19</td>
<td>64.3</td>
<td>3.84</td>
</tr>
<tr>
<td>High carbohydrate high fat with protein HCHF</td>
<td>58</td>
<td>16.4</td>
<td>25.5</td>
<td>5.56</td>
</tr>
<tr>
<td>Low carbohydrate high fat with protein LCHF1</td>
<td>78.7</td>
<td>19.1</td>
<td>2.2</td>
<td>6.17</td>
</tr>
<tr>
<td>Low carbohydrate high fat low protein LCHF2</td>
<td>92.8</td>
<td>5.5</td>
<td>1.7</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Figure 3.1 Diverse impacts of different hypercaloric diets on energy metabolism and hypothalamic innate immune response. (A-D) Daily caloric intake, changes in body weight and fat mass of chow, HCHF, LCHF1 and LCHF2 diet fed mice. (E) Gene expression of iba1 and cytokines in the mediobasal hypothalamus in chow, HCHF, LCHF1 and LCHF2 diet fed mice. (F-G) Iba1-ir microglia and POMC-ir neurons in the MBH in chow, HCHF, LCHF1 and LCHF2 diet fed mice. III: third ventricle; p < 0.05 for *: vs chow group, #: vs HCHF group, ^: vs LCHF1 group, determined by one way ANOVA and subsequent Bonferroni’s post hoc test; Data homogeneity is checked by Bartlett’s test. Scale bar = 100 µm.
Figure 3.2 Mice lacking RAGE, ALCAM or RAGE-ALCAM are resistant to HCHF induced obesity to different extents. (A&B) AGEs level (by western blotting in A, by immunoreactivity in B) in MBH is significantly higher in HCHF diet fed mice. (C) Daily caloric intake and BW of RAGE\(^{-/-}\) mice fed chow or HCHF diet. (D) AGEs reactive to ALCAM by far western blotting (≈122KD). (E) Daily caloric intake and BW of ALCAM\(^{-/-}\) mice were reduced under HCHF diet. (F) RAGE-ALCAM \(^{-/-}\) mice showed the most profound decrease in daily caloric intake and BW under HCHF diet. (G) RAGE-ALCAM \(^{-/-}\) mice had improved glucose tolerance under chow and HCHF diets. (H) Hypothalamic microglial cytokine production with AGEs treatment. \(p < 0.05\) for \(*\): vs chow group, \#: vs HCHF in A, \(*\) vs RAGE\(^{+/+}\), ALCAM\(^{+/+}\) or RAGE-ALCAM \(^{+/+}\) groups in C,E,F left.
panels, by one way ANOVA and subsequent Bonferroni’s post hoc test; \( p < 0.05 \) for \( \ast: \) vs RAGE-ALCAM \(+/-\) groups in G at indicated time point by student’s t-test or Welch’s t-test with unequal variances. \( p < 0.05 \) for \( \ast: \) vs RAGE\(^{+/-}\), ALCAM\(^{+/-}\) or RAGE-ALCAM \(^{+/-}\) groups in C,E,F right panels by two way ANOVA ans subsequent Bonferroni’s post hoc test. Scale bar = 50 µm.
Figure 3.3 Hypothalamic innate immune reactivity response to HCHF diet is differentially improved in RAGE−/−, ALCAM−/− or RAGE-ALCAM−/− mice. (A-B) Hypothalamic iba1-ir microglia numbers under chow or HCHF diets in RAGE−/−, ALCAM−/− or RAGE-ALCAM−/− mice. (C) Hypothalamic microglial activity and cytokine reaction to AGEs. (D) A schematic illustration of the impact of AGEs derived from diets on hypothalamic innate immune reactivity and metabolic disorders. III: third ventricle; p < 0.05 for *: vs RAGE+/+, ALCAM+/+ or RAGE-ALCAM+/+ groups, #: vs HCHF group in A,B, by one way ANOVA and subsequent Bonferroni’s post hoc test Data homogeneity is checked by Bartlett's test. p < 0.05 for *: vs VEH in C by student’s t-test.
Supplemental Figure 3.1 RAGE is expressed on endothelial cells and microglia, and co-localizes with AGEs. RAGE is expressed in the endothelial cells of vessels and microglia (A,D,E,F). AGEs deposit on the vessel wall where RAGE is expressed (A-C). Scale bar = 20um.
CHAPTER 4:
Disruption of Lipid Uptake in Astroglia Exacerbates Diet Induced Obesity
Abstract

Neuronal circuits in the brain help to control feeding behaviors and systemic metabolism in response to afferent nutrient and hormone signals. Although astrocytes are assumed less relevant for such a neuroendocrine control, we investigated if lipid uptake in astrocytes is required for the central regulation of energy homeostasis. In ex vivo hypothalamic-derived astrocytes, the lipoprotein lipase (LPL) expression was upregulated by oleic acids but decreased in response to palmitic acids or serum from mice fed on high fat/high sugar diet compared with standard chow diet. The deletion of LPL from astrocytes reduced the astrocytic lipid content. The astrocyte-specific postnatal disruption of LPL in mice (GFAP Cre\textsuperscript{ERT2}/LPL flox mice + ip tamoxifen injection) did not initially lead to marked changes in the metabolic phenotype. However, when exposed to a high fat/high sugar diet, astrocyte specific LPL-knockout mice exhibited exaggerated body weight gain as well as glucose intolerance. In the hypothalamus, ceramide contents were increased in neurons, which may contribute to the hypothalamic insulin resistance. These data suggest that LPL in astrocytes might be responsible for local lipid partitioning, which is required for appropriate nutrient sensing and contributes to the central control of systemic energy homeostasis and glucose metabolism.

Introduction

The metabolic homeostasis is regulated by complex CNS network. This network senses and integrates nutrient and hormonal signals from the periphery to
regulate feeding behavior, energy expenditure and glucose homeostasis. Metabolic sensing neurons like POMC and AgRP neurons have been under intensive studies. Meanwhile, glial cells constitute around 80% of total cells in the whole brain, but receive less attention in metabolic research. In the recent years, more and more studies uncover the important roles of glial cells in systemic metabolic regulation.

Astrocytes are the most abundant and diverse glial cells in the brain. They play a key role in maintaining the homeostasis in the local microenvironment (Sofroniew & Vinters, 2010). Astrocytes project their endfeet towards the BBB and function as a buffer between blood, extracellular space and neurons (Abbott et al, 2006). This feature enables astrocytes to build a “bridge” for neurons and circulating factor to communicate with each other. In addition, a wide range of metabolic related enzymes, transporters and hormonal receptors have been found expressed in astrocytes (Diano et al, 1998; Garcia-Caceres et al, 2011; Garcia-Caceres et al, 2014; Hsuchou et al, 2009; Morgello et al, 1995; Vannucci et al, 1997; Zhu et al, 1990), which suggests the potential role of astrocytes in regulating these communication processes. In MBH, this bridge has more significance due to the fenestrated BBB and metabolic sensing neurons sitting behind.

Many evidences demonstrated that the brain could sense lipids to regulate energy and glucose homeostasis (Clement et al, 2002; Cruciani-Guglielmacci et
Astrocytes, as the primary cell population in the brain to synthesis and metabolize lipids (Edmond, 1992; Le Foll et al, 2014), might be the major players in the scenario. In the brain, fatty acids oxidation, though at a very low rate compared to glycolysis, happens primarily in astrocytes (Edmond, 1992; Edmond et al, 1987). Key components of the fatty acids oxidation machinery like Malonyl-CoA and CPT-1 have been reported as lipid sensor in the brain (Lam et al, 2005a; Obici et al, 2003). Chronic consumption of HFD results in hyperlipidemia and hypothalamic astrogliosis (Thaler et al, 2012). Whether there is a casual link or not has not been answered yet. Also, a recent study reported that the ketone bodies level in VMH slightly increased after a HFD meal and exerted an inhibitory effect on food intake. The resource of the ketone bodies is very likely astrocytes (Le Foll et al, 2014). Thus, whether astrocytes are functional in the lipid transport or metabolism, and if so, how such function contributes to metabolic regulation if there is overload of lipid upon HFD feeding, are worthwhile questions.

Though long chain fatty acids (LCFAs) sensing in the brain attracted a lot of attention, the resource of LCFAs in the CNS is still under debate. In the circulation, the LCFAs level was increased during prolonged fasting, which are derived from adipose lipolysis (Ruge et al, 2009). This is in conflict with the proposed hypothesis that lipids serve as satiety signals. Whereas, postprandial increased triglyceride-rich particles like chylomicrons and very low-density lipoproteins, should be considered as more physiological forms of lipids. However,
triglyceride (TG) transport, metabolism, and functional significance in the brain are much less understood. Recently, central lipoprotein lipase (LPL) - the key enzyme to hydrolyze triglyceride-, has been highlighted in the metabolic research (Wang et al, 2011). LPL is the lipid “gate-keeper” in peripheral tissues. It determines the distribution of fatty acids derived from triglyceride-rich lipoproteins (Fielding & Frayn, 1998). This lipase could be regulated by nutrients and hormones in a tissue specific manner (Wang & Eckel, 2009). LPL expression and enzyme activity have also been detected in the brain on both neurons and glia cells (Huey et al, 1998; Nishitsuji et al, 2011; Wang et al, 2011; Wang & Eckel, 2012). In the current study, we aim to investigate the function of LPL on the astrocytes in the brain lipid metabolism and potential contributions of astrocytic LPL to diet induced obesity. We found that in astrocytes, LPL was responsible for controlling the cellular lipid storage. In a loss-of-function study, mice lacking LPL in astrocytes resulted in glucose intolerance and accelerated bodyweight gain on HFD feeding. Furthermore, we found an enhanced ceramide de novo synthesis and increased ceramide content in the hypothalamus of astrocytic LPL deficit mice. An accumulation of ceramides could lead to insulin resistance. Together, our data suggest that LPL in astrocytes mediates the lipid partitioning in the brain, which is required for appropriate CNS nutrient sensing and the regulation of the energy homeostasis.

Materials and Methods

Animals
All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati and Institutional Animal Care and Use Committee of the Helmholtz Center Munich, Bavaria, Germany. All mice were group housed on a 12-h light dark cycle at 23°C, with free access to food and water. Mice were fed either on a standard chow diet, or HFD comprising 58% kcal fat (D12331; Research Diets, New Brunswick, NJ).

Astrocyte specific postnatal LPL knockout mice (GFAP-LPL-/-) were generated by crossing the LPL loxP mice (Augustus et al, 2004) with transgenic mice harboring the tamoxifen inducible Cre recombinase driven by a human glial fibrillary acidic protein promoter (hGFAP-Cre-ERT2) (Ganat et al, 2006). At the age of 6 weeks, tamoxifen was given by IP injection for 5 consecutive days with a dose of 100ug per day. LPL loxP homozygous and Cre positive mice were used as knockout mice (GFAP-LPL-/-). Their littermates with LPL loxP homozygous but Cre negative were used as WT controls (GFAP-LPL+/+).

For the flow cytometry study and the in-situ hybridization study, GFAP-LPL+/+ and GFAP-LPL-/- mice were crossed with hGFAP-GFP to label the Cre targeted cells with an eGFP tag. GFAP-eGFP-LPL+/+ and GFAP-eGFP-LPL-/- mice were used in the study.

*Primary culture*
Hypothalami were isolated from 2-day-old LPL loxP homozygous mice and triturated in MEM (Life Technologies, CA) containing 1% penicillin-streptomycin, 10% fetal calf serum (FCS; life Technologies) and 5.5mM glucose. The cell suspension was centrifuged for 7 min at 1000 rpm and pellet was re-suspended and seeded in a 175-cm\(^3\) cell culture flask. Cells were incubated at 37°C and 5% CO\(_2\) for 9 days with a regular medium changed every 3 days. Prior to detachment, the flasks were placed in a 37°C shaking incubator at 240 rpm overnight to remove microglia. The cells were then washed and incubated for 2 min at 37°C with a 0.05% trypsin/EDTA solution (Biochrom AG, Berlin, Germany). Tripsinisation was blocked with MEM + 10% FCS + 1% antibiotics. After centrifugation for 5 min at 1000 rpm, the cell pellet was re-suspended in MEM + 10% FCS + 1% antibiotics and seeded for experiments. For primary neurons, the hypothalami were isolated from 14 days embryos in PBS. After 5 min tripsinisation, the tissue pellets were washed two times with neurobasal medium (21103049, Invitrogen, Germany) and homogenized by gently pipetting up and down. Tissue lysates were passed through a 40um filter. After cell counting, cells were seeded in 6 well cell culture plates with 2500k cells per well in neurobasal + 1 x B27 (17504044, Invitrogen, Germany) + 0.5mM Glutamax (35050-061, Invitrogen, Germany) +1% antibiotics. Neurons were maintained in the same medium for 6 days and were ready for experiments.

To obtain astrocytes with and without LPL, primary astrocytes isolated from hypothalamus of LPL loxP neonatal pups were seeded in 6 well cell culture
plates as described above. Once they reached 90% confluent, astrocytes were transfected with adeno-Cre and adeno-control virus (Vector biolabs, USA) plus 1% adenoboost (AD1012, ATCGbio Life Technology, BC, Canada) for 4 hours to generate Astro-LPL+/+ and Astro-LPL-/ astrocytes.

**Seahorse analysis**

Astrocytes were seeded in the XF24 plate with 80,000 cells per well (Seahorse Bioscience, North Billerica, MA, USA). 24 hours after adeno-cre or control virus transfection, cells were washed with PBS and incubated with XF assay medium containing 5.5mM glucose for 1 h in a 37°C air incubator. The XF24 plate was then transferred to a temperature controlled (37°C) Seahorse (extracellular flux) analyzer (Seahorse Bioscience) and subjected to an equilibration period. Each assay cycle consisted of a 1-min mix, 2-min wait, and 3-min measure period. After 4 basal assay cycles, oligomycin (20ug/ml) was added by automatic pneumatic injection to inhibit the ATP synthase to determine the proportion of respiration used to drive ATP synthesis. For the analysis of extracellular acidification rates (ECARs) derived from glycolysis, the measurements were ended by the addition of 2-deoxy-glucose (2DG; 100 mM). ECARs were converted into proton production rates (PPRs) by taking into account the buffer capacity. 2DG-sensitive PPR estimates the ATP production from glycolysis with a 1:1 ratio. To normalize respirometry readings to cell number per well, cells were stained with crystal violet after the flux experiment in the seahorse XF Analyzer. Briefly, cells were fixed with 4% paraformaldehyde for 30 minutes at room
temperature. After washing 3 times with PBS, 200ul of 0.1% crystal violet was added into each well and incubated for 10 min. After incubation time, the crystal violet was removed and the plate was rinsed with distilled water. When the plate was completely dry, 500ul of 10% acetic acid was added into each well. Absorbance was read at 590nm.

**Lipase activity assay**

Cells were collected in 20mM Tris 150mM NaCl, PH=7.5. 15ug/ml heparin (cat.10429693, Fisher Scientific, Germany) was added. After sonication, cell lysates were kept on 37°C for 45 min, following by 10000g centrifuge at 4°C for 10min. The lipase activity was determined by a fluorometric lipoprotein lipase activity assay kit (STA-610-CB, cell biolabs, Germany). The protein level was determined by BCA assay. Lipase activity was normalized to equal protein content.

**PCR**

For the gene expression analysis, hypothalamic tissue was harvested and total RNA was isolated by RNeasy lipid tissue kit (Qiagen, Germany). After reverse transcription by QuantiTect Rev. Transcription Kit (Qiagen, Germany), the gene expression was analyzed by a real-time PCR with Taqman probes (Applied Biosystems) and primers set for ceramide pathway. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as housekeeping gene. For PCR to detect LPL KO after Cre-loxP deletion, sequences of primers set are as follows:
Forward 5'-CGCCCTGGAACATCACTAAT-3'; Reverse: 5'-CTTCTCAATTGTGGCAGGT-3'.

Flow cytometry
GFAP-eGFP-LPL+/+ and GFAP-eGFP-LPL-/- mice were used to isolate GFP tagged astrocytes with or without LPL. We followed the protocol generated by the Gotz lab published before (You et al, 2011). Briefly, the forebrain was dissected and dissociated by enzymatic and mechanical approaches. Single cell was separated by sucrose gradient. Sorting was done by flow cytometry cell sorter: FACS Aria I with FACS Diva software (BD Biosciences) based on GFP signals. Sorted astrocytes were used for the LPL expression analysis by real-time PCR as described above.

Glucose Tolerance Test
An intraperitoneal glucose tolerance test (ipGTT) was performed by injection of glucose (2 g/kg, 25% wt/vol. d-glucose (Sigma, Germany) in 0.9% wt/vol. NaCl after a 5-h fast. Tail blood glucose levels (mg/dl) were measured with glucometer TheraSense Freestyle (Abbott Diabetes Care, Inc., Alameda, CA) before (0 min) and at 15, 30, 60 and 120 min after injection.

Indirect calorimetry
Measurements of food intake, energy expenditure and physical activity were performed by a customized indirect calorimetric system (TSE Systems Gmbh,
Bad Homburg, Germany). Mice were adapted in the system for 24 hours before data collection for 4 continues days.

**Immunohistochemistry & In-situ hybridization**

Immunohistochemistry was carried out as described in chapter 2. Briefly, mice used for immunohistochemistry were perfused and fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. After equilibrated 48 h with 30% sucrose in TBS, coronal sections (30 µm) were cut on a cryostat and sections were rinsed in 0.1 M TBS. For ceramide staining, 3 sections per mice from MBH were incubated with mouse monoclonal ceramide primary antibody (MID15B4, Enzo life science) at 4°C overnight. Sections were rinsed and incubated in biotinylated secondary goat anti-mouse IgG and avidin-biotin complex (ABC method, Vector Laboratories, Inc., Burlingame, CA). The reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide.

For in-situ hybridization, 30um coronal sections treated by 0.1 M PBS-buffered methanol and H2O2 for 10 min, 0.2 N HCl for 10 min, 2ug/ml proteinase K 30min at 37°C, 0.2% glycine buffer 30 s and 0.1% Triton X-100 for 10 min. Brain sections were hybridized in hybridization buffer (Sigma-Aldrich, USA) containing 200 nM locked nucleic acid (LNA) – modified cDNA probes labelled with digoxigenin (5’-TGGAACTTTCTCTCTAATAT-3’) (Exiqon, Denmark) at 52 °C for 8 h. After stringent washing in SSC buffer in 52 °C and final washing in 0.1 M PBS, the sections were then coincubated with goat anti-digoxigenin antibody.
(1:200, Abcam) and chicken anti-GFP (1:500, AP3179, Acris Germany) at 4 °C overnight. Sections were incubated with biotinylated anti-chicken secondary antibody for an hour and then incubated with streptavidin conjugated with DyLight 488 (1:200, for GFP) and rabbit anti-chicken antibody conjugated by Dylight 647 (1:200, for digoxigenin) (all from Jackson ImmunoResearch Laboratories). After thorough rinsing, the sections were mounted with mounting medium and visualized by confocal microscopy (Zeiss-LSM710, Germany).

**Statistical Analysis**

All results are expressed as mean ± SEM. Statistical comparisons were performed using one-way or two-way ANOVA by Graph Pad Prism (GraphPad Software, San Diego, California, USA). A p value below 0.05 was considered statistically significant.

**Results**

*LPL was Expressed by Astrocytes and Regulated by Nutrients Input*

Lipoprotein lipase in peripheral was mainly produced by heart and adipose tissue, and regulated by energy requirements and hormonal levels. In the brain, it was previously reported that LPL activity in the hypothalamus responded to prolonged fasting (Eckel & Robbins, 1984). To further determine the LPL function in hypothalamus and astrocytes, we first compared the LPL expression level in the hypothalamus and the primary isolated hypothalamic astrocytes under different nutrient factors stimulus. In 8 months HFD fed mice, LPL was decreased in the
hypothalamus compared to those chow diet fed mice (Fig 4.1A). Consistent with previous reports, LPL was expressed by both neurons and glial cells (Nimmerjahn et al, 2005; Wang et al, 2011). Here we further compared the relative abundance of LPL in primary isolated hypothalamic neurons and astrocytes, and found that mRNA of LPL was more abundant in astrocytes than in neurons (Fig 4.1B). To mimic the situation in vivo that astrocytes chronically exposed to HFD derived circulating factors, we treated primary cultured astrocytes with medium containing 10% serum collected from chow diet or HFD fed mice for 6 days. LPL expression was decreased by HFD serum treatment compared to Chow (Fig 4.1C)—a trend same as hypothalamic LPL level in response to HFD feeding (Fig 4.1A). To further dissect how astrocytic LPL responded to different lipid species, we incubated astrocytes with 0.02% intralipid (a triglyceride rich lipid emulsion), 50uM oleic acids and 50uM palmitic acids overnight. BSA was used as vehicle. We found that Triglyceride (TG) and palmitic acids had similar effects as HFD serum on astrocytes, while oleic acids had opposite effects (Fig 4.1D&E). Interleukine-6 (IL-6) is an important cytokine involved in an inflammatory process in the hypothalamus upon HFD (Thaler et al, 2012), and astrocytes are the major source of IL-6 upon injury and inflammation. Therefore, we examined IL-6 production in astrocytes under above treatments. Fig 4.1C-E showed that HFD serum, TG and palmitic acids stimulated the astrocytes IL-6 production while oleic acids reduced it. These data suggests that LPL in astrocytes also responds to HFD related factors and could be regulated
differently dependent on the lipid species. IL-6 production in astrocytes always went to an opposite direction to LPL changes.

**LPL KO in Hypothalamic Primary Astrocytes Changed Lipid content and Glycolysis Capacity.**

To further study the function of LPL in astrocytes, we treated primary hypothalamic astrocytes isolated from LPL-flox mice, and treated them with either adeno-cre or control virus to obtain astrocytes with and without LPL (Astro-LPL+/+ and Astro-LPL-/-). Transfection was confirmed by qPCR (Fig 4.2C) and the lipase activity assay (Fig 4.2D). We observed that, by Oil red O staining, lipid droplets existed in astrocytes under normal culture condition but significantly reduced in astrocytes without LPL (Fig 4.2A&B). In Astro-LPL-/- cell, lipogenesis related genes Cd36 and Fasn increased. In ceramides pathway, ceramide synthase 3 (CERS3 or LASS3) was significantly downregulated while the other 5 subtypes of ceramide synthases were not altered much. Sphingosine kinase 1(Sphk1) was increased and sphingosine kinase 2 (Sphk2) was not changed. To analyze the metabolic status of astrocytes, we ran the seahorse measurements. Extracellular acidification rate (ECAR), an indicator of glycolysis was shown in Fig 4.2E. Astro-LPL-/- cells had increased the maximal glycolytic capacity (Fig 4.2 F&H) and more ATP was produced from enhanced glycolysis compared with Astro-LPL+/+ (Fig 4.2G). In addition, the lactate secretion was increased in the medium from Astro-LPL-/- (Fig 4.2I), which is in line with the increased glycolysis.
These data indicate that LPL participates in regulating lipid content in astrocytes. Lack of LPL changes lipid and glucose metabolism in astrocytes.

**Astrocyte Specific LPL KO Mice Exhibited Exaggerated Body Weight Gain and Glucose Intolerance Fed on HFD**

To assess the astrocytic LPL functional significance in vivo, we generated postnatal astrocyte specific LPL knockout mice. GFAP-Cre$^{ERT2}$-LPL flox+/- mice and LPL flox+/- littermates were injected with tamoxifen at age of 6 weeks. WT mice were presented by GFAP-LPL+/+ and KO mice were presented by GFAP-LPL-/-.. Animal model was confirmed by multiple approaches (Fig 4.3 A-H). GFAP-LPL+/+ and GFAP-LPL-/- mice were crossed with GFAP-eGFP mice to label GFAP promoter targeted astrocytes. Knockdown was demonstrated by in-situ hybridization (Fig 4.3 A-F) and qPCR on astrocytes sorted by flow cytometry from hypothalamus of GFAP-eGFP-LPL+/+ or GFAP-eGFP-LPL-/- mice (Fig 4.3H). Primers were designed based on genetic construction of LPL flox sequences to detect a specific band after Cre induced recombination (Fig 4.3G). Primers design was validated in LPL KO tissue samples (data not shown). Since GFAP was also expressed in peripheral tissues like stellate cells in liver and pancreas, we checked the potential off target effects in this animal model. LPL expression was not altered in the liver (Fig 4.3I). The insulin content was not significantly different between GFAP-LPL+/+ and GFAP-LPL-/- (Fig 4.3J). In isolated islet, glucose induced insulin secretion was not changed neither (Fig
4.3K). Thus, liver and pancreas were not influenced much in this animal model and the metabolic phenotypes were most likely from the CNS.

Initially, moderate metabolic phenotypes were observed when animals were fed on chow diet (data not shown). Briefly, GFAP-LPL-/- mice had no changes on BW, fat or lean mass and slightly higher food intake at age of 24 weeks compared with GFAP-LPL+/+. However, they had glucose intolerance. When these mice were exposed to HFD, compared with controls, GFAP-LPL-/- mice had accelerated body weight gain (Fig 4.3M) and glucose intolerance (Fig 4.3L), which is not a secondary effect of increased body mass considering the phenotypes when fed on chow diet. Calorimetric measurements after 10 weeks HFD showed GFAP-LPL-/- mice had increased food intake (Fig 4.3N), lower locomotor activity (Fig 4.3O) and thus may contribute to the increased body weight gain. The energy expenditure, however, was higher in GFAP-LPL-/- mice instead of lower (Fig 4.3P). Such inconsistency has been reported in calorimetric studies quite often (Tschop et al, 2012). We speculated that increased energy expenditure might be an adaptive change to the increased BW gain. Taken together, our data indicate that GFAP-LPL-/- mice had glucose intolerance and exaggerated body weight on HFD, which may be due to increased food intake and less locomotor activity. Phenotypes were mainly due to the LPL manipulation on astrocytes in the CNS. Thus, LPL on astrocytes played an important role in regulating the metabolic homeostasis.
Astrocyte Specific LPL KO Increased Ceramide Content in Hypothalamus

To further understand how LPL in astrocytes regulate lipid metabolism in vivo and potential mechanisms contribute to metabolic regulation, we looked at the ceramide content in the hypothalamus. Recently the LPL knockdown in hippocampus has been linked to an increased de novo ceramide synthesis and ceramide content (Picard et al, 2014). Ceramide is an important multi-functional intracellular signaling molecule. The link between ceramide accumulation and insulin resistance has been documented very well by the Summers group (Chavez & Summers, 2012; Holland et al, 2011). Here we speculate a similar process happened in GFAP-LPL-/ mice. Indeed, by immunohistochemistry staining, we found that in the ventral medial hypothalamus, ceramide immunoreactive (−ir) positive cells were markedly increased in GFAP-LPL-/ mice fed on either chow diet (Fig 4.4A, B, E) or HFD (Fig 4.4C,D, E). This MID 15B4 ceramide antibody is specific for C16 and C24 ceramide under physiological conditions. Antibody has been used in previous publications for immunohistochemistry, immune blot and flow cytometry, and validated in the neutralization study (Grassme et al, 2002; Kim et al, 2012). In the current study, mouse IgG has been used to replace the primary antibody as the negative control. No significant signal has been detected, shown by Fig 4.4A2. Gene expression profiles of the ceramide metabolic pathway (Fig 4.4F) were in line with the staining results, which suggested the enhanced ceramide de novo synthesis in the GFAP-LPL-/ mice hypothalamus. In addition, although the ceramide immunoreactivity could be detected in astrocytes, the majority of
ceramide-ir positive cells were neurons (data not shown). Thus, these data suggested that in vivo, astrocytes derived LPL not only affected astrocytes, but also initiated the ceramide de novo synthesis and resulted in the ceramide accumulation in neurons which may contribute to the hypothalamic insulin resistance and metabolic disorders.

**Discussion**

In this study, we investigated the function of LPL in astrocytes and its role in the systemic metabolic control. We showed that LPL controls the lipid storage of astrocytes in response to various nutrient inputs. A lack of LPL in astrocytes resulted in exaggerated body weight gain and glucose intolerance when fed on HFD. Such phenotypes were associated with increased the ceramide de novo synthesis and the ceramide content in the hypothalamus. Our results suggested that astrocytes could directly participate in nutrient sensing and contribute to the CNS energy balance control.

Lipid sensing in the brain is essential for the CNS controlling energy homeostasis (Lam et al, 2005b; Levin et al, 2011; Migrenne et al, 2011; Moulle et al, 2014; Rasmussen et al, 2012; Yue & Lam, 2012). Fatty acids excitatory and inhibitory neurons have been proven to shown exist in hypothalamus (Le Foll et al, 2009). A central infusion of oleic acids inhibited food intake and glucose production indicates oleic acids as satiety signals for the brain (Obici et al, 2002). So far, two
major mechanisms are proposed: one thinks that the fatty acids transporter (CD36) itself is responsible for sensing (Le Foll et al, 2013; Le Foll et al, 2015; Moulle et al, 2013), while the other suggests that the fatty acids oxidation and the related metabolites are involved (Lane et al, 2008; Moulle et al, 2013; Obici et al, 2003; Wolfgang et al, 2006). In both scenarios, there are some gaps which have not been filled. As mentioned in the beginning, the resource of the lipids is not clear yet. The circulating fatty acids level increases significantly during prolonged fasting, which should not be a satiety signal to the CNS. Such paradox raises the focus on triglyceride-rich lipoproteins and their hydrolyzing enzyme—lipoprotein lipase in the brain lipid sensing in recent years. The TG-rich lipoproteins level increased after a meal and may serve better as the satiety signal. Recent studies demonstrated that TG-rich lipoproteins could be taken up and sensed in the hypothalamus, the hippocampus and the mesolimbic regions to regulate energy homeostasis and reward seeking behavior. Such process is regulated by lipoprotein lipase (Cansell et al, 2014; Picard et al, 2014; Wang et al, 2011). In our study, we first showed that in the hypothalamus of long term HFD fed mice, the LPL expression was decreased, suggesting a potential role of LPL in the DIO pathology. Also, we found that the LPL abundance is higher in astrocytes than in neurons. This is in line with the classic view that astrocytes are active lipid consumers in the brain. However, so far most studies about lipid sensing and fatty acid oxidations in the brain do not clarify the cell specificity. Herein, we are the first to specify the role of the astrocytes in TG sensing and energy homeostasis control.
We showed that the LPL level in astrocytes could be adjusted according to extracellular lipid load and species. LPL was down-regulated upon serum from HFD fed mice, TGs and palmitic acids, but up regulated upon oleic acids. These data suggest that LPL may protect astrocytes from detrimental lipid overload and such a regulatory function maybe essential for the astrocytes proper function (like IL-6 secretion) during HFD induced hyperlipidemia. Studies using the same GFAP promoter to investigate the IL-6 function in astrocytes reported that mice lack of IL-6 from astrocytes resulted in increased body weight than WT (Quintana et al, 2013), while mice showing an overexpression of IL-6 in astrocytes were resistant to DIO but with impaired glucose tolerance (Hidalgo et al, 2010). If LPL was knocked out from astrocytes, the loss of such a protective function lead to disrupted lipid metabolism in astrocytes. CD36 and fatty acids syntheses were both reported before as physiological energy sensors (Cassolla et al, 2013; Kim et al, 2004; Le Foll et al, 2015; Lopez et al, 2008). Besides, as a tightly coupled energy unit, astrocytes may send wrong “hunger” signals to neighboring neurons.

We found an increased ceramide content, accompanied with an increased de novo ceramide synthesis in the hypothalamus of HFD fed mice lack of LPL from astrocytes. This is consistent with another study which reported that LPL in the hippocampus is regulating the energy balance via ceramide pathway (Picard et al, 2014). We observed a similar trend of increased de novo ceramide synthesis but mediated via different enzyme subtypes. Such a difference implies either the
region specificity or the cell specificity of the ceramide synthase and serine palmitoyltransferase. In the peripheral, it has been established that an increased ceramide synthesis is required for TLR4 dependent insulin resistance. The down-regulation of the ceramide synthase or serine palmitoyltransferase could help mice to improve insulin sensitivity, glucose tolerance and bodyweight gain on HFD feeding (Chavez & Summers, 2012; Holland et al, 2007; Turpin et al, 2014). A similar mechanism has been also reported in the hypothalamus. Lipid infusion and HFD feeding cause the ceramide accumulation in the hypothalamus, suggesting the ceramide mediated insulin resistance may contribute to obesity pathology (Holland et al, 2011). Thus, in our study, we speculate that the lack of LPL in astrocytes may accelerate such a process during HFD feeding and finally contribute to the exaggerated body weight gain and glucose intolerance.

It should be pointed out that, in classic view, LPL is synthesized by parenchymal cells and then translocated across the endothelial cell to become active and functional on the lumen of capillaries. Such a translocation is mediated by glycosylphosphatidylinositol (GPI) - anchored glycoprotein (GPIHBP1) (Davies et al, 2010). However, the GPIHBP1 level in the brain is not appreciable. Such a phenomenon suggests a possibility that LPL produced in the brain may not be translocated to the capillaries lumen but instead stay on the surface of the cell generating it. Thus, LPL maybe involved in lipid partitioning in local microenvironment. The blockade of lipid uptake into astrocytes may cause elevated ceramide level and be toxic to neurons.
There are also some limitations of current study. The animal model used to target astrocytes in the study is a GFAP-Cre\textsuperscript{ERT2} transgenic mouse line. We employed this tamoxifen inducible model to avoid disturbance of LPL during development since LPL is known as highly expressed in the brain during lactating. However, due to the complex heterogeneity of astrocytes, GFAP promoter cannot target all the astrocytes population. When we crossed this line with tdTomato reporter line, after tamoxifen injection, the GFP signal could be visualized mainly in the hypothalamus, the thalamus and less in the cortex and the cerebellum. The hippocampus was almost not affected. In addition, some stellate cells in liver and pancreas were also targeted. In the liver, LPL was not expressed at an appreciable level during adulthood; instead, the immunoreactivity of LPL was strong in the liver, indicating the liver as the major place for circulating LPL degradation (Neuger et al, 2004; Vilaro et al, 1988). In the pancreas, LPL was detected in alpha and beta cells but not altered by HFD or fast/re-feeding (Nyren et al, 2012). Thus, our model did not influence the LPL function relevant cells in liver and pancreas. Phenotypes were mainly contributed by a subpopulation of astrocytes in the CNS.

Overall, our data highlight the role of astrocyte lipid uptake in energy homeostasis. Mice lack of LPL in astrocytes results in the disrupted lipid metabolism in astrocytes and a ceramide accumulation in neurons, which contributes to impaired glucose tolerance and accelerated body weight gain on HFD feeding.
Figure 4.1 Lpl expressions in mice hypothalamus and astrocytes in different nutrients environments. Expression level of Lpl in mice hypothalamus fed on HFD for 8 months (A), and on primary isolated hypothalamic neurons and astrocytes (B). In primary astrocytes, Lpl level and interleukin-6 production were modulated by (C) 6 days incubation with serum from Chow fed or HFD fed mice, 0.02% TG-emulsion (D), and 50uM of oleic acid, palmitic acid or BSA vehicle (E). $p < 0.05$ by student’s t-test or Welch’s t-test with unequal variances, for *: vs chow group in (A&C), *: vs neurons in (B), *: vs Veh or BSA in (D&E).
Figure 4.2 LPL KO in hypothalamic primary astrocytes changed lipid content and glycolysis capacity. Astrocytes were isolated from hypothalami of LPL flox mice and transfected with adeno-GFP or adeno-CRE virus to obtain astrocytes with LPL (Astro-LPL+/+) and without LPL (Astro-LPL−/−). Lipid content in astrocytes stained by Oil Red O was decreased in Astro-LPL−/− (A&B). Gene expression changes were shown in (C). Lipase activities were down regulated in Astro-LPL−/− (D). Seahorse analysis shows Astro-LPL−/− had enhanced glycolysis (E&F), increased glycolytic capacity (G), and increased ATP production from glycolysis (H). Lactate secretion was also higher in Astro-LPL−/−. p <0.05 by student's t-test or Welch's t-test with unequal variances, for *: vs Astro-LPL+/+. Scale bar=50um.
Figure 4.3 Astrocyte specific LPL KO mice exhibited exaggerated body weight gain and glucose intolerance fed on HFD. Astrocyte specific LPL postnatal KO mice were achieved by crossing GFAP-CreERT2 (+/-) with LPL flox(+/+). After tamoxifen injection at 6 weeks old, LPL deletion was confirmed by in-situ hybridization (A-F), PCR (G), and qPCR on astrocytes isolated by FACS from GFAP-LPL+/+ and GFAP-LPL-/-(H). Off side effects of GFAP promoter in liver and pancreas were examined by Lpl expression in liver (I), total islet insulin
content (J) and ex vivo isolated islet function (K). GFAP-LPL-/- mice fed on HFD showed impaired glucose tolerance (L), increased body weight gain (M) and food intake (N) compared with GFAP-LPL+/+ littermates. Calorimetric analysis shows GFAP-LPL-/- mice have lower locomotor activity (O) and higher energy expenditure (P). p<0.05 for *, p<0.01 for **: vs GFAP-LPL+/+, determined by two-way ANOVA in M and student’s t test or Welch’s t-test with unequal variances for the rest of the graph. Scale bar=10um. n=3 for in-situ hybridization and flow cytometry. n= 6-12 for metabolic phenotyping and calorimetric study.
Figure 4.4 Astrocyte specific LPL KO increased ceramide level in hypothalamus. Ceramide-ir positive cells in ventromedial hypothalamus from increased in GFAP-LPL-/− mice fed on either standard Chow (A,B,E) or HFD (C,D,E). Mouse IgG is used as the negative control (A2). Gene expression of enzymes involved in ceramides synthesis pathway in hypothalamus of HFD fed GFAP-LPL+/+ and GFAP-LPL-/− mice (F). p < 0.05 for * determined by one-way ANOWA in E and student’s t-test or Welch’s t-test with unequal variances in F. Scale bar=100um. n=6-12 for ceramides staining. n=4 for qPCR.
CHAPTER 5

General Discussion
**Summary of Dissertation Research**

In this dissertation, we discussed the role of the hypothalamic microglia and astrocytes in diet-induced obesity, particularly focusing on their responses to high-fat high-sugar diet. Our data showed that the driving forces of DIO associated hypothalamic microgliosis are high-fat high-sugar dietary input and hormones like GLP-1 and leptin, instead of body weight changes per se. HFD feeding increased the total number, the reactivity, and also the phagocytosis of microglia in ARC and such phenotypes were not observed in other genetic obese models like ob/ob, db/db and MCR4 KO mice. Ob/ob mice had even lower microglia reactivity than WT, which can be rescued by leptin treatment. This indicates that leptin is required for the normal function of the microglia. Also, we showed that GLP-1 receptor agonists reduced HFD-induced microglial activation independent of body weight decrease. This will shed light on mechanistic studies of GLP-1 based therapies. We next focused on the dietary effect on microglia reactivity. By comparing the microglia reactivity in mice fed with 4 different diets, we found that excess lipids alone are not sufficient to drive microgliosis. Dietary carbohydrates played a key role to activate microglia together with lipids. Furthermore, our data suggest that advanced glycation end products accumulated in ARC upon high-fat high-carbohydrate feeding might be the essential mediators in DIO associated hypothalamic microgliosis. Mice lacking AGEs relevant receptors showed attenuated microgliosis in ARC and improved metabolic disorders. Our data also indicate that lipids metabolism in astrocytes had an impact on the regulation of energy homeostasis. Lipoprotein
lipase deficiency in astrocytes disrupted the lipids metabolism in astrocytes and triggered the ceramide accumulation in the hypothalamus. When challenged with HFD, these mice had impaired glucose homeostasis and became more obese than their littermates control. Taken together, our data highlight the essential roles of hypothalamic glial cells in the systemic metabolic control and the contributions to pathophysiology of DIO.

**Hypothalamic inflammation and obesity**

In obese patients and animal models, neuroinflammation is not only observed in the hypothalamus, but also reported in other brain regions like putamen, amygdale and hippocampus (Drake et al, 2011; Koga et al, 2014; Thaler et al, 2012). In obesity associated disorders, like cognitive disorders and memory disorders, neuroinflammation has been proposed as one of the potential pathological mechanisms. However, the casual link among HCHF diet, inflammation, obesity and associated disorders has not been established very well in these brain regions. Whether the neuroinflammation in these regions is a direct consequence of a HCHF diet, and what is the role of local glial cells in such a progress have not been studied in details. It has been reported that only the gliosis in MBH is correlated with BMI (Thaler et al, 2012). The hypothalamus is the key region to regulate metabolism. Therefore it is reasonable to speculate that inflammation in the hypothalamus has a higher potential than other brain regions to contribute to DIO pathology.
So far many reports offer evidence to support a causal link between hypothalamic inflammation and obesity (Zhang et al, 2008). More detailed mechanisms involving glial cells have been proposed by several groups as well. Besides the direct effect of pro-inflammatory cytokines on neurons, glial cells may contribute to DIO by other ways as well. Leptin resistance has been identified as a characteristic mechanism responsible for DIO. Recently, it has been reported that the leptin transport into MBH requires ERK signaling in tanycytes in the median eminence. Tanycytes ERK signaling is disrupted by the HCHF die feeding, which finally results in less leptin availability of MBH neurons in DIO (Balland et al, 2014). Another mechanism has been proposed that in DIO, gliosis (including microglia, astrocytes and also tanycytes) increases the ensheathment of neurons which makes neurons have less access to blood vessels (Horvath et al, 2010). This will result in inappropriate nutrient and hormonal sensing by hypothalamic neurons.

**Lipids, sugar and hypothalamic inflammation**

Saturated fatty acids are considered as the key components in HFD-induced pro-inflammatory effects in peripheral tissues. Many studies point to a similar role in HFD-induced hypothalamic inflammation. However, our data indicate that that the combination of lipids and sugar is a key point in inducing hypothalamic inflammation. The saturated fatty acids content is similar among all three high-fat diets, however, two high-fat low-carbohydrate diets do not trigger microgliosis, demonstrating that excess lipids alone cannot activate microglia reactivity. On the
other side, compared with standard chow diet fed group, basal blood glucose level in animals is higher, though not significant, in the HCHF diet group, but lower in the LCHF1 and LCHF2 groups (Bielohuby et al, 2011). This suggests that, besides lipids, glucose input is also required in hypothalamic microgliosis. In chapter 2, hypothalamic microglia patterns are different in ob/ob and db/db mice. One of the distinctions between ob/ob and db/db mice is the persistent hyperglycemia in db/db mice. This may explain that in db/db mice, the iba-1 positive microglia number is not as low as in ob/ob mice. Upon a lack of leptin signaling, the hyperglycemia and hyperlipidemia condition in db/db mice may stimulate hypothalamic microglia activity, which to some extend compensates a leptin deficiency induced low microglia activity that was observed in ob/ob mice.

In the diet components study, although the standard chow diet has higher carbohydrates percentage, these carbohydrates contain more fibers and ground wheat instead of sucrose and maltodextrin in HCHF diet. Fibers and ground wheat cannot increase the blood glucose level as efficient as sucrose. In the chronic DIO model it is known that DIO mice have higher basal glucose than mice fed on a standard chow diet, however in the current study, glycemia differences haven’t reached significance between the HCHF group and the chow group after one month feeding. This suggests that not only the glucose level, but also the type of carbohydrates in the diet maybe relevant for AGEs formation. Three key factors affect AGEs formation: the turnover rate of the target molecule for glycation, the sugar availability and the oxidative stress level (Goldin et al, 2006). Thus, a high-fat high-carbohydrate diet provides the best environment for
the AGEs generation. Our data suggests that AGEs is one of the major factors contributing to hypothalamic inflammation. On chronic HCHF diet feeding with an even longer period than the current study, we speculate that the AGEs accumulation will cause more vasculature problems which would result in more infiltrated macrophages to exaggerate inflammation. This is supported by previous findings in our lab that mice on long-term HFD feeding have endothelial damage and hypervascularization in the hypothalamus, accompanied with the IgG deposition in the ARC microglia (Yi et al, 2012b).

**Glial lipid metabolism and DIO**

In recent years a series of study on brain lipoprotein lipase and its endogenous inhibitors Angptl family suggest that fatty acids taken up by LPL could be sensed as a satiety signal to regulate energy homeostasis. So far, this LPL mediated lipid sensing model has never been evaluated under HFD condition. Our data suggests that the lipid uptake in astrocytes may be not that essential for the daily feeding regulation under physiological condition, but more relevant under HFD feeding. We speculate that astrocytes may play an important role in removing extra lipid to prevent lipid toxicity on neurons under lipid overload conditions. One of the LPL functions reported in the brain is to recycle or scavenge the lipid debris during degeneration (Blain et al, 2004; Gong et al, 2013). It is reasonable to expect a similar function of LPL in hypothalamic astrocytes on HFD feeding. Thus, the loss of such a function will cause lipid toxicity to neurons, which is consistent with our findings that the loss of LPL results in ceramides
accumulation in neurons. Comparisons between astrocytic and neuronal LPL deficiency models suggest different functional significance of LPL on each population. Neuronal LPL deficiency in mice generates hyperphagia and obesity even on chow diet, while a lack of LPL on astrocytes exaggerates metabolic phenotypes on HFD. This indicates that neuronal LPL participates in the physiological feeding control, and astrocytic LPL executes a monitoring and protective function. From translational aspects, astrocytic LPL may serve as a better target than neuronal LPL since the manipulation of astrocytic LPL and the related pathway had minor influence physiological food intake but be more specific to pathological changes. Besides neurons and astrocytes, LPL is also expressed in microglia. The functional significance of microglial LPL has not been evaluated. Studies on peripheral macrophages show that LPL regulates macrophage phagocytosis and that activated macrophages have increased LPL secretion (Behr & Kraemer, 1986; Yin et al, 1997). It would be interesting to investigate whether similar functions could be found in hypothalamic microglia. If the LPL on microglia would increase during hypothalamic microgliosis and enhance phagocytosis on HFD feeding, microglial LPL might be a key enzyme in the hypothalamic innate immune response.

**Genetic approaches to manipulate glial cells**

One of the limitations in glial cell study is the correct approach to target specific populations. Astrocytes are very heterogeneous. GFAP is the most widely accepted astrocytes marker. However, when we crossed the transgenic GFAP-
Cre\textsuperscript{ERT} mice with tdTomato reporter mice and injected tamoxifen, not all the targeted astrocytes have immunoreactivity to GFAP antibody. Also, hippocampus has strong immunoreactivity of GFAP antibody but it did not show tdTomato expression in this model. Such mysterious phenotype has been also reported by others but not understood yet (Emsley & Macklis, 2006). Other Cre transgenic lines based on astrocytes specific promoters like Aldh1l1-Cre and Glast-Cre still need comprehensive mapping and characterization, especially in the hypothalamus.

Microglia is less heterogeneous than astrocytes. The difficulty in the microglia study is to distinguish microglia from other myeloid cells in the CNS. As mentioned in the introduction, microglia and bone marrow-derived macrophages (BMDMs) are generated from different precursors during the development. Microglia are long-lived, un-replaceable cells, while BMDMs have a short life and fast turnover rates. BMDMs also exist in the CNS and synchronize with microglia in the CNS immune response. However, it is difficult to separate them from resident microglia by surface marker staining. Iba1 is the most widely used marker to stain microglia. However, iba1 cannot stain all the microglia. Some BMDMs are also positive for iba1. In Fig 2.2 and Fig 2.4, we showed that in ob/ob mice, total microglia number represented by Cx3cr1-GFP was not different from WT but iba1-ir positive cells were significantly less in ob/ob mice. This suggests that if a microglia activity is under a certain threshold, they might be undetectable by iba1 staining. In the median eminence, the iba1 positive cells in the external
zone have macrophages-like morphology. Whether they have distinct functions from parenchymal microglia in ARC in hypothalamic immune response is still unknown (Kalin et al, 2015). Transgenic mouse models used to target microglia in previous studies are usually based on traditional macrophage promoters like Lysm-Cre, CD11b-Cre and Cx3cr1-Cre (Clausen et al, 1999; Ferron & Vacher, 2005; Pfrieger & Slezak, 2012). These models either have a low recombination rate in microglia, or had stronger impact on peripheral macrophages than on microglia. Recently, a Cx3cr1-Cre\textsuperscript{ERT} mice line has been generated and shows good efficiency to target microglia without affecting peripheral macrophages too much. This model takes advantage of the different turnover rate between microglia and BMDMs. After tamoxifen injection, microglia and BMDMs are both influenced. Later, targeted BMDMs could be quickly replaced by newborn unaffected macrophages while the microglia retains the genetic deletion (Parkhurst et al, 2013). This would be a good model for future studies on microglia.

**Glia and obesity: therapeutic aspects**

The ultimate goal of understanding the roles of glial cells in DIO is to find effective therapeutic approaches to treat obesity. Combined with current genetic and pharmacological techniques, glial cells might represent new targets for treating a hypothalamic dysfunction in metabolic disorders. One of the ideas is to target glial cells with anti-inflammatory compounds. This idea could benefit from the conjugated peptide therapy approach. For example, the GLP1 and GLP1R
agonist conjugated peptide gained a lot of attention in recent years. GLP1-glucagon conjugates, GLP1-estrogen conjugates and GLP1R-MC4R co-agonist all succeeded in improving metabolic phenotypes (Clemmensen et al, 2014; Clemmensen et al, 2015; Finan et al, 2012). The rationale of such conjugates is based on targeting specific cell population by one of the conjugates and avoiding off target effects of another conjugate. In chapter 2, we showed that the GLP1R agonist could attenuate hypothalamic microgliosis. This indicates a potential drug design to bring other anti-inflammatory peptides to target microglia. This concept might be applied in astrocytes as well. Several drugs affecting lipid metabolism like CPT-1 inhibitors, LPL inhibitors, FASN inhibitors given in the brain has been shown to affect energy homeostasis. Our data indicate that astrocytes lipids uptake and metabolism may have a protective function for neurons exposed to HFD feeding but without interrupting physiological processes. A rational design to manipulate lipid uptake in astrocytes may be a hopeful field in DIO study. One thing of notice is the gender difference. Most of the studies are done in male mice, however recently it has been pointed out that glial cell responses to HFD also have gender differences (Morselli et al, 2014). Microglia reactivity on HFD feeding is not different between males and females but astrocytosis has not been found in female mice hypothalamus on HFD. Such phenotypes are proposed due to the existence of the estrogen receptor alpha in astrocytes but not in microglia. Therefore, the gender difference should be always taken into consideration from translational aspects. Another idea to treat inflammation is to inactivate microglia by drugs like minocycline, or to delete the entire glial population by drug or drug
combined genetic models like DTR mice (Fan et al, 2007; Valdearcos et al, 2014). Microglia depletion in CD11b-DTR mice shows attenuated inflammatory response and enhanced leptin sensitivity, though mechanism remains unclear. Astrocytes depletion has also been reported in Aldh111-DTR mice, suggesting astrocytes are indispensable in synaptogenesis and repairment in the spinal cord (Tsai et al, 2012). Studies in the hypothalamus have not been reported yet. So far, most of these approaches are still limited on the animal models. How to apply glial cell based therapeutic approaches to treat obesity in humans still needs further studies.

**Overall Conclusion**

Glial cells are important for proper neuronal functions. Hypothalamic glial cells are of special interest in this study since their role in mediating DIO-associated hypothalamic innate immune responses and cause metabolic dysfunction. We demonstrate that the driving force of DIO associated hypothalamic microgliosis are dietary inputs but not increased adiposity per se. Leptin and GLP1 agonists could also affect the microglia reactivity. We also show that lipids alone are not sufficient to drive the hypothalamic microgliosis. The combination of dietary carbohydrates and lipids increases the advanced glycation end products level in MBH, which mediates the microglia activation. Mice lacking AGEs receptors show improved metabolic phenotypes on HFD feeding. Our data also suggest that lipid uptake in astrocytes is a required process for energy homeostasis on HFD feeding. Mice lacking lipoprotein lipase in astrocytes have increased
ceramide content and exaggerated metabolic syndromes on HFD. We speculate a protective effect of astrocyte lipid uptake by attenuating lipid toxicity to neurons during HFD feeding. Together, these data show essential roles of hypothalamic glial cells in diet-induced obesity and systemic metabolic regulation. A deep understanding of neural-glial interaction is still required in future to provide potential glial cell based therapeutic approaches.
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