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Qi Wu · Ruimao Zheng *Editors*

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Dr. Ruimao Zheng joined Peking University Health Science Center as a Principal Investigator in 2013. He obtained his Ph.D. in Physiology from Peking University, China, and received his postdoctoral training first at the Department of Pharmacology, University of Washington School of Medicine, and then at Harvard Medical School and the Stem Cell Institute of Harvard University. His lab now utilizes genetically engineered mouse models in conjunction with optogenetic and chemogenetic approaches, recombinant viral vectors, viral tracing techniques, single-cell transcriptomics, and electrophysiology to decipher the neural nuclei and circuits controlling metabolic homeostasis. Dr. Zheng is an investigator in Peking University's 100 Talent Program. His research focuses on neuroscience and neural regulation of metabolic and cardiovascular function, and he has published several research articles in these fields. He is a member of several national and international academic neuroscience and metabolism societies. Dr. Zheng is also engaged in teaching and training graduate and postgraduate students at Peking University.

Chapter 1
Functional Interrogation of the AgRP Neural Circuits in Control of Appetite, Body Weight, and Behaviors

Yong Han, Guobin Xia, and Qi Wu

Abstract Neurons expressing agouti-related protein (AgRP), the so-called hunger neurons, protect mammals from starvation by promoting food-seeking behaviors (Trends Neurosci 36:504–512, 2013). Now an increasing amount of evidence show that these hunger-sensing neurons not only motivate animals to forage and ingest food but also help conserve energy by inhibiting innate processes that demand large amounts of energy such as growth, reproduction, and stress response. It has further been perceived that AgRP neurons transmit signals with negative valence to reward and cognitive centers so as to engage the motivational behavior toward seeking and obtaining foods (Physiol Behav 190:34–42, 2017). Recent advancement in genome editing and neurotechniques unleashed an escalated research of uniquely defined neuronal populations and neural circuits underlying the behavioral regulation of body weight and food responses (Nat Biotechnol 32:347–355, 2014; Proc Natl Acad Sci 113, 2016). In this chapter we will review literatures describing the functional organization of the AgRP circuit and its correlative signaling components that influence ingestive, foraging, motivational, and cognitive responses, a framework that reshaped our thinking toward the new hope and challenges in treatment of obesity and eating disorders.

Keywords Neural circuit mapping · Hypothalamus · Feeding behavior · Energy balance · Food intake · Melanocortin · NPY · MC4R · GABA

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1.1 Introduction

Neurons expressing agouti-related protein (AgRP), the so-called hunger neurons, protect mammals from starvation by promoting food-seeking behaviors [1]. Now an increasing amount of evidence show that these hunger-sensing neurons not only motivate animals to forage and ingest food but also help conserve energy by inhibiting innate processes that demand large amounts of energy such as growth, reproduction, and stress response. It has further been perceived that AgRP neurons transmit signals with negative valence to reward and cognitive centers so as to engage the motivational behavior toward seeking and obtaining foods [2]. Recent advancement in genome editing and neurotechniques unleashed an escalated research of uniquely defined neuronal populations and neural circuits underlying the behavioral regulation of body weight and food responses [3, 4]. In this chapter we will review literatures describing the functional organization of the AgRP circuit and its correlative signaling components that influence ingestive, foraging, motivational, and cognitive responses, a framework that reshaped our thinking toward the new hope and challenges in treatment of obesity and eating disorders.

1.2 Afferent Hormonal and Nutritional Signaling

AgRP neurons, which are exclusively located in the arcuate nucleus (ARC) of the hypothalamus, were initially discovered with a tight correlative role in control of foraging and food-ingestive behaviors [5–8]. Activation of AgRP neurons causes potent hyperphagia and enhances foraging behaviors [9, 10], while acute ablation of AgRP neurons in adult animals abrogates feeding and induces starvation within a week [11, 12].

AgRP neurons are established as a crucial target of a variety of circulating metabolism-related hormones such as ghrelin, leptin, and insulin, through which diversifying actions upon the control of feeding and body weight are achieved [13, 14]. For instance, ghrelin increases feeding by stimulating the co-release of neuropeptide Y (NPY) and AgRP from AgRP neurons onto postsynaptic targets, whereas leptin and insulin inhibit food intake and body weight by decreasing their expression and neuronal activity [15–20]. Ghrelin is identified as an endogenous ligand of the growth hormone secretagogue receptor (GHSR) in 1999 and primarily produced by endocrine cells in the gastrointestinal tract [21, 22]. GHSR is distributed in several hypothalamic nuclei including the ARC, where a predominantly high expression level is found in AgRP neurons with much lesser in the nearby anorexigenic proopiomelanocortin (POMC) neurons [23, 24]. Ghrelin activates AgRP neurons by increasing their firing activity and c-fos immunoreactivity [23, 25]. Ghrelin is proved to indirectly inhibit POMC neurons by activating AgRP neurons [26–28]. So GHSR is more essential for ghrelin-induced excitation of AgRP neurons than that of POMC neurons [29]. Loss of AgRP or AgRP neurons abolishes feeding response

triggered by ghrelin, indicating that AgRP neurons are obligatory mediators of the orexigenic effect of ghrelin [12, 15, 30]. Genetic deletion of GHSR in AgRP neurons using the *Cre/loxP* approach causes a moderate decrease of body weight in the diet-induced obesity (DIO) model but does not affect energy expenditure or insulin sensitivity in mice fed with regular diet [31]. Considering that phenotypes in traditional Cre/loxP model may be masked by developmental compensation and that GHSR expression neurons are widely distributed in the brain, we suggest that using more robust genetic mouse models and mapping a more complete neural circuit orchestrated by GHSR would significantly enhance our understanding of the physiological effects of ghrelin on the control of body weight and metabolism.

As opposed to ghrelin, leptin is a hormone secreted by white adipose tissue that negatively regulates energy balance and food intake [32, 33]. Global inactivation of leptin (*ob/ob* mice) or its cognate receptors (*db/db* mice) causes massive obesity and diabetes, along with other metabolic symptoms [34]. Notably, leptin receptor (LepR) is highly expressed in the hypothalamus particularly within AgRP and POMC neurons [35]. Evidence suggests that leptin decreases food intake and promotes energy expenditure by suppressing the activity of AgRP neurons and depolarizing POMC neurons [36–38]. Genetic deletion of LepR in AgRP neurons using a classic Cre/loxP system gives rise to a mild obese phenotype [39]. Moreover, leptin induces a slow, age-progressive, modulation of AgRP and POMC neurons that is required for its ability to inhibit feeding [40]. Although GHSR and LepR are co-expressed in many neurons within the ARC, the anorexigenic effects of exogenously administrated leptin are similar in wild-type and GHSR knockout mice which indicates the function of LepR is likely to be independent of GHSR [41]. In summary, central leptin signaling plays a key integrative role in regulation of hormonal and nutritional control of appetite. It is speculative that functional mapping of the LepR-organized neural circuit and discovery of novel transmitter signaling components within would greatly transform our current view of central control of body weight as well as facilitating the development of highly efficacious treatment of obesity.

Insulin is a peptide hormone produced and secreted by pancreatic β-cells following a rise in blood glucose and acts through a conserved insulin receptor signaling cascade in the liver, muscle, and fat to lower the blood glucose level [42]. Intracerebroventricular infusion of insulin was shown to decrease body weight and food intake in mammals, acting as an anorexigenic hormone [43]. Like ghrelin and leptin, insulin is perceived to act, at least in part, upon AgRP neurons and POMC neurons to regulate blood glucose metabolism and elicit anorectic responses [44, 45]. Further studies suggest that insulin acts on AgRP neurons to exert this anorexigenic role through activation of phosphatidylinositol-3-kinases and the nuclear export of the forkhead transcription factor FoxO1 [46]. In conclusion, insulin signaling in the brain plays distinct roles in regulating feeding behavior and glucose metabolism.

1.3 Neural Circuit of AgRP ↔ PVN

Recent studies show that ARC contains several genetically identifiable neuronal populations, among which AgRP and POMC neurons exhibit opposing effects toward feeding and metabolism regulation [1]. AgRP neurons release AgRP, an endogenous melanocortin 4 receptor (MC4R) inverse agonist, as well as the inhibitory transmitters GABA and NPY to promote positive energy balance, whereas POMC neurons by releasing the MC4R agonist alpha-melanocyte-stimulating hormone (α-MSH) and a few other transmitters push energy balance to the negative side. Some studies reveal a robust, bidirectional, control of feeding by AgRP neurons. Ablation of AgRP neurons causes rapid starvation [12], while opto- or chemogenetic stimulation of AgRP neurons drives intense feeding [9, 10].

Mice with AgRP neuron lesions exhibit abundant c-fos expression in many postsynaptic targets including the paraventricular hypothalamic nucleus (PVN), the dorsomedial hypothalamic (DMH) nucleus, the bed nucleus of the stria terminalis (BNST), and the parabrachial nucleus (PBN) [11, 27]. PVN is an important brain nucleus for hunger and satiety regulation by receiving dense projections from AgRP neurons [47, 48]. Injection of MC4R ligands into the PVN potently affects feeding [48, 49]. MC4Rs in the PVN are mainly expressed on glutamatergic neurons but not GABAergic neurons. A recent study indicates that AgRP hyperpolarizes PVN^{MC4R} neurons through ligand-induced coupling of MC4R to the closure of an inwardly rectifying potassium channel, Kir7.1, but not through the classical $G\alpha_s$-coupled GPCR signaling [50]. The MC4R-expressing neurons exert suppressive effects on appetite by direct projection to the lateral part of PBN [51, 52]. In addition, optogenetic stimulation of AgRP axonal terminals in the PVN dramatically stimulates feeding within minutes [26, 51]. Photostimulation of these postsynaptic neurons in the PVN markedly blunts the ability of increasing AgRP-triggered feeding response [26, 51]. Retrograde transsynaptic mapping shows the PVN is the main input to AgRP neurons, which indicates a reciprocal connection between AgRP neurons and the PVN. Activation of thyrotropin-releasing hormone (TRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) neurons in PVN in sated mice markedly activates AgRP neurons and induces intense feeding [53]. Together, the AgRP-PVN neural circuit is important for the bidirectional control of hunger and satiety through an MC4R-dependent manner. Key information regarding the connectivity and key regulatory components of the AgRP neural circuit are summarized in Table 1.1.

1.4 Neural Circuit of AgRP → PBN

The PBN is a brain structure located in the dorsolateral pons that surrounds the superior cerebellar peduncle, which is a major relay for gustatory and visceral information and mediates appetite suppression both after a meal and in conditions such

Table 1.1 The AgRP neural circuit and key signaling components in control of appetite and related behaviors

Functions	Neural circuits and key signaling components	Brief descriptions	References
Food ingestion	AgRP → $PVN^{MC4R::Sim1}$ → PBN^{Glu}	AgRP neurons increase feeding by inhibiting MC4R neurons in PVN; activation of PVN SIM1 neurons overcomes the feeding behaviors from AGRP neuron activation; MC4R neurons in PVN provide excitatory glutamatergic input to PBN neurons that suppress feeding	Atasoy et al. [26], Betley et al. [54], Krashes et al. [53], Garfield et al. [51], and Chen et al. [55]
	$PVN^{Glu::TRH::PACAP}$ → AgRP	Thyrotropin-releasing hormone (TRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) neurons provide excitatory input to AgRP neurons; activation of TRH and PACAP neurons in PVN in sated mice significantly enhances activities of AgRP neurons and increases feeding	Krashes et al. [53]
	AgRP → $PBN^{CGRP::Glu}$ → CeA^{Glu}	Ablation of the neural circuit from AgRP neurons to the PBN suppresses feeding and induces starvation; activation of glutamatergic projection PBN^{CGRP} → CeA suppresses feeding; activation of projection AgRP → PBN increases feeding following administration of anorexigenic compounds	Wu et al. [11], Carter et al. [56], Campos et al. [57], and Essner et al. (58)

(continued)

Table 1.1 (continued)

Functions	Neural circuits and key signaling components	Brief descriptions	References
	RMg/ROb$^{5\text{-HT}}$ → NTS$^{5HT3R::Glu}$ → PBN^{Glu} ← AgRP^{GABA}	Ablation of the AgRP^{GABA} → PBN^{Glu} suppresses feeding; PBN neurons mediate inhibition of appetite as induced by exciting the serotonergic neurons within the RMg and ROb; these raphe nuclei send 5-HT signaling to the NTS glutamatergic neurons, which in turn innervate the PBN glutamatergic neurons	Wu et al. [11] and Wu et al. [59]
	NTS$^{CCK::DBH}$ → PBN^{Glu}	Activation of the projection from the CCK- or DBH-expressing neurons in the NTS to the PBN neurons inhibits feeding	Roman et al. [60]
	AgRP^{GABA} → LH^{GABA} → PVN	Activation of GABAergic projection AGRP→LH rapidly increases feeding; activation of GABAergic projection LH → PVN increases feeding	Betley et al. [54], Garfield et al. [51], Wu et al. [61], Steculorum et al. [62], and Chen et al. [55]
	AgRP^{GABA} → aBNST	AGRP neurons provide inhibitory GABAergic input to neurons in aBNST to increase feeding	Betley et al. [54], Steculorum et al. [62], and Chen et al. [55]
	AgRP^{GABA} → PVT	Activation of AGRP→PVT projections significantly increases food intake by releasing GABA	Betley et al. [54] and Wang et al. [63]

(continued)

Table 1.1 (continued)

Functions	Neural circuits and key signaling components	Brief descriptions	References
Foraging	AgRP → MeA^{NPYR1}	AGRP neurons project to and inhibit neurons expressing NPYR1 in MeA to increase foraging and feeding	Padilla et al. [64]
Motivation	$AgRP^{GABA}$ → VTA^{DA}	AgRP neurons influence the development of VTA dopamine neurons and motivational behaviors	Wu et al. [27] and Dietrich et al. [65]
	AgRP → PVN^{MC4R} → PBN	Activation of PVN MC4R → PBN terminals promotes conditional place preference	Garfield et al. [51]
Cognition	AgRP → PVT → BLA → InsCtx	Mapping neural circuit from AgRP neurons to insular cortex via the PVT and BLA to study the role of hunger in InsCtx processing of learned food-predicting cues	Livneh et al. [66]

as illness or exposure to toxins [11]. Recently, PBN is identified as an important hub that integrates signals from several brain regions including AgRP neurons and other brainstem regions to modulate feeding and body weight. These studies of the AgRP-PBN neural circuit reveal the critical importance of PBN heterogeneous population of neurons integrating signals from a variety of brain areas [67]. Enhancement of the GABAergic signaling afferent to the PBN can rescue starvation in AgRP neuron-ablated mice [11]. Glutamatergic neurons in the nucleus tractus solitarius (NTS), as relaying serotonergic signaling from caudal raphe nuclei, are the major excitatory inputs to the PBN in control of the excitability of PBN neurons and inhibit feeding [59]. Furthermore, aphagia, induced by AgRP neuron ablation, resulted from accumulation of the NMDA receptor NR2B subunits in the PBN [68]. Calcitonin gene-related peptide (CGRP)-expressing neurons in the external lateral subdivision of PBN (elPBN) are another subpopulation of PBN that play an inhibitory role on feeding. Optogenetic activation of $elPBN^{CGRP}$ neurons is sufficient to induce conditioned taste aversion in the absence of anorexigenic substances [56]. Functional inactivation of these neurons markedly decreases the perception of meal-induced satiety signals. Another functional mapping study shows that hunger-activated AgRP neurons are connected to and inhibit PBN^{CGRP} neurons [57]. Activation of pathway from AgRP neurons to the PBN increases feeding following administration of anorexigenic compounds such as amylin, cholecystokinin (CCK), and LiCl [69].

Meanwhile, the NTS^{CCK} and dopamine beta-hydroxylase (DBH)-expressing neurons provide direct, excitatory, synapses to the PBN^{CGRP} neurons. Activation of projection from the NTS^{CCK} or NTS^{DBH} neurons to the PBN^{CGRP} neurons inhibits feeding [60]. Another study demonstrates that LepR- and CCK-expressing neurons of the PBN represent a crucial component of the insulin counter-regulatory response [70]. With growing efforts focusing on the AgRP-PBN circuit, we postulate that genetic-defined subpopulations of the PBN neurons each may be identified as playing a unique role in modulation of distinguishable aspects of feeding and energy homeostasis.

1.5 Neural Circuit of AgRP → BNST

The BNST is considered to be a critical hub connecting with various stress regulatory center structures, such as the anterior part of basolateral amygdaloid (BLA) nucleus, anterior part of the central amygdaloid (CeA) nucleus, medial amygdala (MeA), PVN, as well as the reward controlling regions, such as the ventral tegmental area (VTA) and nucleus accumbens (NAc) [71]. Recent mapping studies demonstrate that the BNST receives massive AgRP projections, hinting a role of the AgRP-BNST neural circuit in appetite and metabolic control [63]. Optogenetic stimulation of AgRP terminals in the anterior part of BNST (aBNST) induces a significant increase of food intake [54, 72]. Photostimulation of AgRP-dorsomedial part of aBNST ($aBNST_{dm}$) projections evokes a marked increase in feeding [62]. In contrast, activation of AgRP-ventrolateral part of aBNST ($aBNST_{vl}$) projections does not alter feeding but mediate the effect on brown adipose tissue and insulin sensitivity [62]. However, the AgRP-aBNST neural circuit appears to regulate food intake by an MC4R-independent manner; chemogenetic and optogenetic activations of the $MC4R^{aBNST}$ neurons exert no effect on food intake, indicating that these cells are not involved into the rapid control of appetite [51]. Therefore, it's a critical task to address the identity of the aBNST neuronal population and associated signaling pathways in the regulation of appetite and energy metabolism.

1.6 Neural Circuit of AgRP → LH, VMH, PVT, Etc.

Besides well-defined projections mentioned above, AgRP neurons project to the intra-hypothalamic nuclei such as the median preoptic area (MPA), the median preoptic (MPO) nucleus, the lateral preoptic (LPO) nucleus, the anterior hypothalamus (AH), the lateral hypothalamic area (LHA), the posterior hypothalamus (PH), the dorsomedial hypothalamic (DMH) nucleus, and the ventromedial hypothalamic (VMH) nucleus [63]. The key functional studies about hypothalamic targets of AgRP neurons mainly include the LHA, DMH, and VMH [51, 54, 75, 76]. Disruption of connections between ARC and these hypothalamic nuclei has loss of

function in regulating energy balance [77]. Photostimulation of AgRP projections/AgRP-LH pathway results in a significant increase in food intake [54, 62, 74]. Although the AgRP-LH circuit is a potential orexigenic pathway, LH^{MC4R} neurons are not a downstream target for control of AgRP-driven hunger. There are no light-evoked IPSCs recorded in postsynaptic LH-MC4R neurons in reaction to the optogenetic stimulation of AgRP axonal terminals in the LH [51]. On the other hand, the GABAergic transmission from AgRP neurons plays a role in regulation of feeding by inhibiting the LH^{Glu} neurons [78].

It has been established that AGRP inhibits VMH neurons in naturally overweight rats but has the opposite effect in normal-weight rats [79]. It is reported that administration of AgRP into the VMH causes an increase in meal size in both light and dark phases [80]. Using whole cell recording reveals a depression of glutamatergic VMH neurons in response to α-MSH and MC4R agonist melanotan II (MTII) [81]. Interestingly, AgRP has the same effect as these agonists and also inhibited VMH^{Glu} neurons. And AgRP may act on receptors independent of the known melanocortin system [81].

Recent studies indicate the feeding regulatory actions of AgRP may extend beyond the hypothalamus. AgRP neurons also project to the extrahypothalamic nuclei such as the paraventricular thalamic (PVT) nucleus, CeA, the dorsal raphe nucleus (DRN), the periaqueductal gray (PAG), and dorsal vagal complex (DVC) [61]. Optogenetic activation of AgRP downstream targets AgRP-PVT but not AgRP-CeA, and AgRP-PAG results in a significant increase in food intake [54]. In contrast, photostimulation of the AgRP-DRN and AgRP-DVC projections had no effect on feeding [62]. A recent study using combined mono- and polysynaptic circuit tracing and microprism-based cellular imaging approaches unveils a new pathway from AgRP neurons to the insular cortex (InsCtx) via the PVT and BLA, leading to a role of the InsCtx in hunger perception [68]. In summary, these recent findings strongly indicate that AgRP neurons project to broad brain regions, forming a sophisticated neural circuit that gives rise to multilevel control of feeding and other behavioral responses.

1.7 Neural Circuit of AgRP → Amygdala, VTA, Etc

Feeding behavior is not just an innate physiological activities but also is rewarding and reinforcing. There exists a cross talk between the neural circuit of appetite and motivation. Uncontrolled consumption of palatable food, an addictive-like behavior, is one of the main causal factors of hyperphagia and obesity, indicating that the reward system associated with appetite and energy metabolism has evolutionarily developed to facilitate feeding response. The reward-driven feeding response allows individuals to better accommodate energy change dynamically, to obtain feedback, and to reduce starvation risk.

AgRP signaling regulates a wide variety of physiological activities such as appetite, foraging, anxiety, and stereotypic behaviors [64, 82]. AgRP neurons also have

a sensory role in motivation [83, 84]. In contrast, other appetite-inhibiting neural circuits are demonstrated to transmit aversive response to food stimuli [73, 85]. Activation of AgRP neurons before food is available to animals enhances appetitive response, a positively reinforcing effect that is long-lasting after the presence of food [55]. We suggest that these diverse behavioral responses may be attributed by the differential actions of AgRP neurons upon postsynaptic targets in distinct brain nuclei. The exact role of AgRP neurons affecting reward circuits is incompletely defined.

The motivational circuits encompass parts of the cerebral cortex, hippocampus, amygdala, VTA and substantia nigra in the midbrain, and striatum and NAc in the forebrain. One of the major neurotransmitters in the reward circuits is dopamine [86, 87]. Using optogenetics in combination with electrophysiology, dopaminergic neurons in the VTA could be excited by reward and thought to encode reward prediction error [88]. However, dopaminergic VTA^{DA} neurons are not homogeneous, varying greatly on their electrophysiology and connectivity properties [88, 89]. In a Pavlovian procedure with appetitive and aversive outcomes, recording results show that some dopamine neurons are excited by reward-predicting stimuli and inhibited by aversion-predicting stimuli. However, a greater number of dopamine neurons are excited by both stimuli [89]. These suggest that different groups of dopamine neurons convey motivational signals in distinct manners. On the other hand, activation of dopaminergic neurons facilitates positive reinforcement during reward-seeking [90]. Activation of the reward circuits increases food intake and promotes preference for calorie-dense foods. Although AgRP fibers directly innervate the VTA in the midbrain [75], the melanocortin signaling is not involved into the connection between AgRP neurons and VTA dopamine neurons [27, 65]. Nevertheless, it has been shown that GABA is the major transmitter of the AgRP-VTA neural circuit in regulating VTA dopamine functions, an inhibitory connection which may be involved in the control of motivated behaviors driven by hunger [65].

VTA dopamine pathways are also indirectly regulated by AgRP neurons through output to hypothalamic nuclei involved in homeostatic energy balance including the PVN and LH. It is postulated that such indirect pathways further reinforce motivational food-seeking behaviors by numerous endocrine and metabolic signals such as ghrelin, leptin, and insulin [91–93]. Recent findings reveal that AgRP neurons modify VTA dopaminergic plasticity and dopamine-dependent behaviors during development [65].

Another reward nucleus amygdala is integrated within networks that signal to the brain areas involved in appetite control, which is involved in conditioned taste aversion. There are AgRP axonal projections from the ARC to the amygdala [94], and MC4Rs are also expressed in the amygdala [75, 95, 96]. Expression level of brain-derived neurotrophic factor (BDNF) in the CeA is increased by AgRP in ad-lib-fed rats [97]. The CeA is the major output of adaptive fear response [98]. PBN neurons have axonal projections that activate CeA neurons, which suppresses appetite in conditions when it is unfavorable to eat [56], [99]. The neural pathway from PBN neurons to CeA neurons are also critical for relaying aversive signals like pain, which has a pivotal role of establising a threat memory [100]. When in starvation,

AgRP signaling to the medial nucleus of the amygdala affects normal response to chemosensory cues, regulates territorial aggression, contextual fear and food-seeking behavior [64]. These studies emphasize the importance of a previously ignored contribution of the spino-parabrachial-amygdaloid neural circuit as an important aversive signaling pathway regulating eating disorders.

In summary, the midbrain VTA dopamine system has a primary role in food motivation by integrating information from the environment like visual food and odor cues and from internal energy balance like hunger and satiety which can be sensed by AgRP neurons. Although the importance of AgRP-VTA^{DA} neural circuit in motivated feeding behaviors has been established and a translational potential treatment of anoxia and obesity is highly plausible, there is little progress in defining a specific dopaminergic feeding circuit dissociable from others in mediation of hedonic feeding. Undoubtedly, genetic and functional interrogation of the AgRP-VTA circuit and other reward circuits in regard to the motivational control of feeding would be a territory demanding extensive efforts in the near future.

1.8 Conclusion

The recent endeavor in promoting structural and functional interrogation of the AgRP neural circuit tremendously advances our knowledge of the neural mechanisms underlying the control of foraging and food-ingestive behavior as well as the integrative significance among appetite, rewards, and cognitive functions. Previous studies have identified the PBN as a critical target of AgRP neurons, which bridges the gustatory and visceral signals from peripheral and the homeostatic feeding system. Functional mapping of the afferent and efferent connectivity of the PBN circuit with reward and cognitive systems will greatly promote our understanding of the neural regulation of obesity and eating disorders. Furthermore, the motivational characteristics of AgRP neuron-mediated appetite have also perceived that AgRP neurons transmit positively reinforcing signals to selectively enhance food reward. Although the AgRP-VTA^{DA} neural circuit is studied for motivational behaviors, a defined neural circuit and key mechanisms underlying the innate drive for preference of high- and low-calorie density foods have never been well understood.

It's revealing that activation of AgRP neurons decreases the anxiety level indicating a functional cross talk between feeding and stress circuits [82, 102]. In order to survive in a harsh natural environment, it is a key for an individual to coordinate food-seeking behavior with other competing motivational drives, such as anxiety, innate fear, and social interactions. So it is plausible that the AgRP neural circuit may be differentially structured to modulate survival-driven emergent responses through a dynamic balance with feeding behavior. We suggest that deciphering the neural circuit for the joint control of feeding and mental functions would illuminate the biological and translational significance of a coordinate and dynamic control of feeding behavior.

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Chapter 2
POMC Neurons: Feeding, Energy Metabolism, and Beyond

Cheng Zhan

Abstract The central melanocortin system is a well-established neuronal pathway involved in regulating energy metabolism. Pro-opiomelanocortin (POMC) neurons, agouti gene-related protein (AgRP) neurons, and their downstream cells expressing the melanocortin-3 (MC3R) and melanocortin-4 receptors (MC4R) are three key components of the central melanocortin pathway. This chapter focuses on the *Pomc* gene and the POMC neural system. First, I summarize the established role of this system in inhibiting food intake. Second, in light of new cutting-edge techniques, our understanding of how POMC neurons function to regulate energy homeostasis has been refined during the last few years. I describe some recent advances and discuss bidirectional effects of POMC neurons on feeding. Finally, the physiological significance beyond energy metabolism, in particular for reward and sex, is also discussed.

Keywords Feeding behavior · POMC neurons · Arcuate nucleus · Nucleus tractus solitarius · α-MSH · β-Endorphin · Reward

2.1 The *Pomc* Gene and Energy Metabolism

The *pomc* gene in mammals is expressed in multiple organs including the pituitary, the brain, and several other peripheral tissues. *pomc* encodes a precursor peptide, which is tissue specifically cleaved to give rise to tens of peptides including the adrenocorticotropic hormone (ACTH); α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, γ-MSH); corticotropin-like intermediate peptide (CLIP); and the endogenous opioid β-endorphin, γ-lipotropin, and met-enkephalin, among others. These peptides are packaged in large vesicles and are released to function in an array of biological activities, including pigmentation, adrenocortical function, and regulation of energy metabolism, sexual behavior, reward, and immunity. The

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important roles of *Pomc* in energy metabolism are the best known among these diverse physiological functions.

The *Pomc* gene controls energy metabolism by producing melanocortin peptides such as ACTH and α-MSH. *Pomc-KO* mice lacking ACTH and α-MSH are hyperphagia, are obese, and exhibit altered pigmentation [49]. ACTH deficiency also results in defective adrenal development. Similarly, human POMC-deficient patients exhibit severe obesity, adrenal insufficiency, and red hair pigmentation [26].

Pomc-derived α-MSH and ACTH control energy metabolism via the central nervous system. Intracerebroventricular (ICV) injection of either α-MSH or melanotan II (MT-II), a cyclic analog of α-MSH, or $ACTH_{1–24}$ suppressed food intake and increased energy expenditure in rodents [37, 44]. In contrast, selectively restored peripheral melanocortin and corticosterone secretion in *Pomc* null mice did not reduce food intake but exacerbated obesity [41].

Melanocortin-4-receptor (MC4R), a seven-transmembrane G protein-coupled receptor, is the receptor of α-MSH and plays a key role in preventing obesity. The MC4R is widely expressed throughout the CNS, as well as the peripheral nervous system and in intestinal L cells. The essential role of MC4R in controlling energy metabolism is evident in both gene knockout rodents and humans with naturally occurring mutations. Lack of MC4R increases food intake and decreases energy expenditure, ultimately resulting in severe obesity [6, 21]. The inhibitory effect of α-MSH on food intake can be blocked by co-injection of SHU9119, a cyclic peptide antagonist of MC4R. ICV injection of SHU9119 alone increased food intake in mice [15].

Interestingly, restoration of MC4R in the hypothalamic paraventricular nucleus (PVN) and a subpopulation of amygdala neurons completely blocked overeating but has no effect on energy expenditure [6], suggesting the divergence of MC4Rs in controlling energy metabolism. In summary, POMC-derived melanocortin peptides and the receptor MC4R control food intake and energy balance via the central nervous system.

2.2 The POMC Neural System

POMC neurons are defined by their expression of the *pomc* gene that is transiently expressed in many brain areas during development, but most such expression programs do not persist into adulthood [36]. POMC neurons in the adult mouse brain are restricted in the hypothalamic arcuate nucleus (ARC) and the nucleus tractus solitarius (NTS) of the brainstem. The ARC of the hypothalamus and the NTS of the brainstem are both adjacent to circumventricular organs, facilitating POMC neurons to sense and integrate signals from adipostatic factors and satiety factors in the circulation system, respectively [12]. In mice, there are approximately 3000 POMC neurons in the ARC and 300 POMC neurons in the NTS [13, 20]. Alternatively, a recent study, quantifying POMC neurons using an antibody specifically against the POMC precursor, estimated around 9000 immunoreactive neurons in the ARC [28].

In addition to classifying POMC neuron populations according to their anatomical locations, there are also subpopulations of POMC neurons in the ARC that are distinguished by their amino acid neurotransmitter phenotypes. Using in situ hybridization, the presence of mRNA of the transporters responsible for packaging glutamate (vesicular glutamate transporter) was detected, suggesting that the ARC POMC neurons can use glutamate as a neurotransmitter. Approximately 7% of POMC neurons express vesicular glutamate transporter 2 (vGlut2), and the highest percentage of vGlut2-positive POMC cells are located in the rostral ARC [22]. Although mRNA of vesicular γ- aminobutyric acid (GABA) transporter (vGat) was not detected in POMC neurons [22], the enzymes responsible for GABA synthesis, glutamic acid decarboxylase (GAD), are present in ~40% of ARC POMC neurons [19]. Whole-cell recordings in primary cultured POMC neurons confirmed the release of GABA or glutamate [19]. Thus, the ARC POMC neurons can use either GABA or glutamate as neurotransmitters (Fig. 2.1, left panel). Given the opposing actions of these two transmitters, it is inferred that GABAergic and glutamatergic POMC neurons may have distinct physiological functions.

Heterogeneity of POMC neurons can also be defined based on the receptors expressed on cell surface. POMC neurons express various receptors, including leptin receptor, insulin receptor, and serotonin 2C receptor ($5HT_{2C}R$), glucagon-like peptide 1 receptor (GLP1R), and neuropeptide Y receptors, among others (Fig. 2.1, right panel). POMC neurons expressing $5HT_{2C}Rs$ are distinct from those expressing leptin receptors [42], and POMC neurons expressing leptin receptors are distinct from those expressing insulin receptors [47]. Recent single-cell RNA sequencing studies systemically examined the transcripts of individual murine hypothalamic POMC neurons and revealed a striking heterogeneity [10] [27]. Notably, leptin receptor and $5HT_{2C}R$ are predominantly expressed in two distinct subpopulations of POMC neurons [10]. Only 12% of POMC neurons express leptin receptor, and less

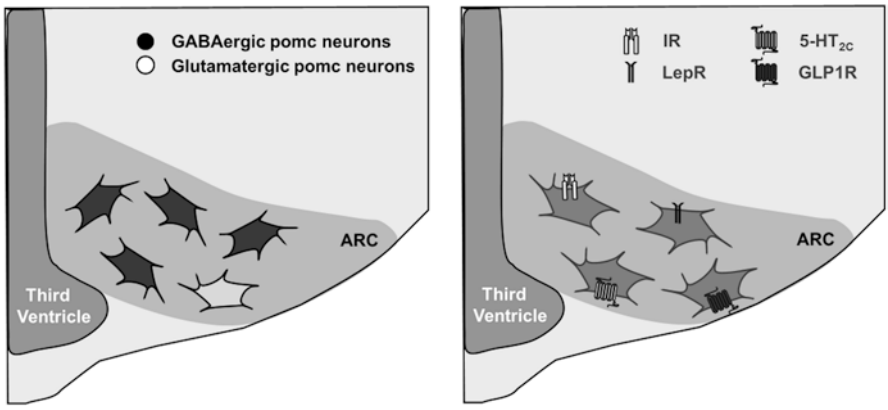

Fig. 2.1 Heterogeneity of POMC neurons (Figures are designed by Jiarui Liu, Peking University Health Science Center)

than 10% of POMC neurons express the GLP1R. Very few POMC neurons express both $5HT_{2C}R$ and GLP1R receptors.

Overall, hypothalamic POMC neurons can be divided into multiple distinct groups based on its expression profile of GPCRs. The great gene expression heterogeneity is also evident in their functional heterogeneity. Transcriptionally defined POMC subtypes show greater distinct responses to the alteration of nutrition status. For example, fasting significantly altered expression of tens of genes only in particular subtypes of POMC neurons [10]. However, the functional heterogeneity of the various subpopulations of POMC neurons is still poorly understood.

2.3 Anatomical Connections of POMC Neurons

2.3.1 Neural Inputs to POMC Neurons

The activity and excitability of POMC neurons are regulated not only by numerous neuropeptides from peripheral organs but also by innervated nerve fibers from upstream neurons. Traditional retrograde tract-tracing studies in rodents have revealed that the ARC receives inputs from broad brain areas including the brainstem, midbrain, intrahypothalamus, and preoptic area [11, 31]. Specifically, a recent study using the viral-based cell-type-specific retrograde transsynaptic tracing technique mapped the direct inputs of POMC neurons through the whole brain areas [45]. ARC POMC neurons receive inputs from more than 50 brain nuclei, which are distributed in the cortex, the septum, the striatum, the amygdala, the pallidum, the hypothalamus, the thalamus, the hippocampus, the midbrain, the pons, and the medulla. Overall, the hypothalamic areas and several other forebrain nuclei represent the major input sources for ARC POMC neurons.

The NTS is capable of integrating signals from both peripheral vagal afferents [40] and central neural inputs [43]. Vagal afferents deriving input from the gastrointestinal tract respond to three basic stimuli: gastric and duodenal distension or contraction, chemical contents of the lumen and gut peptides, and neurotransmitters released from the stomach and duodenum in response to nutrients. Anterograde-transport studies by direct injections of tracer either into upper gastrointestinal sites (esophagus, stomach) or into individual subdiaphragmatic vagal branches have demonstrated NTS termination fields [1, 34]. However, there have been very few studies examining the inputs of NTS POMC neurons from gut splanchnic afferent structure. In contrast, the central inputs of NTS POMC neurons have been well examined [11, 39, 45]. Unlike ARC POMC neurons that mainly receive inputs from the hypothalamus and other forebrain nuclei, NTS POMC neurons predominantly receive their inputs from the pons and medulla (Fig. 2.2). Only scattered inputs are found in a few forebrain areas. Notably, although the input patterns for POMC neurons in the ARC and NTS are very different, there exist several brain areas that project to both sets of POMC neurons, suggesting their activity may be coordinately controlled by the same upstream regulators.

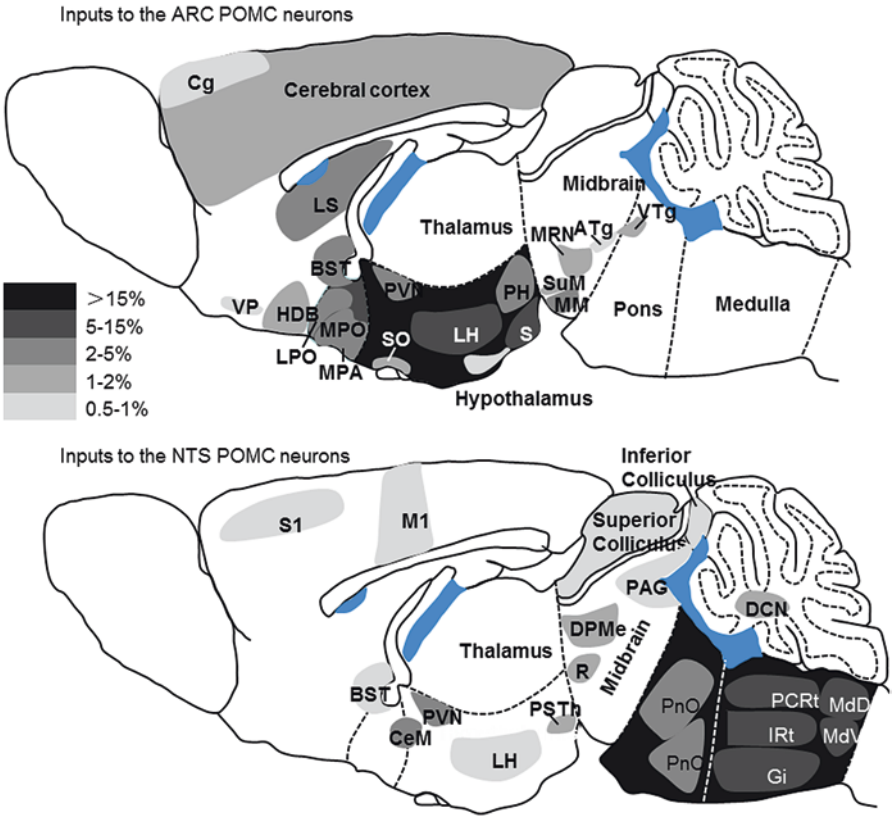

Fig. 2.2 Summary of the major inputs to POMC neurons in the ARC (top panel) and NTS (bottom panel). Abbreviations: *AHi* amygdalohippocampal area, *ATg* anterior tegmental nucleus, *BST* bed nucleus of the stria terminalis, *CeM* medial part of the central amygdaloid nucleus, *Cg* cingulate cortex, *DCN* deep nuclei of cerebellum, *DpMe* deep mesencephalic nucleus, *Gi* gigantocellular reticular nucleus, *HDB* horizontal diagonal band of Broca, *IRt* intermediate reticular nucleus, *LH* lateral hypothalamus, *LPO* lateral preoptic area, *LS* lateral septum, *M1* primary motor cortex, *MdD* dorsal parts of medullary reticular nucleus, *MdV* ventral parts of medullary reticular nucleus, *MM* medial mammillary nucleus, *MPA* medial preoptic area, *MPO* medial preoptic nucleus, *PAG* periaqueductal gray, *PCRt* parvicellular reticular nucleus, *PH* posterior hypothalamus, *PnC* caudal parts of the pontine reticular nucleus, *PnO* oral parts of the pontine reticular nucleus, *PSTh* parasubthalamic nucleus, *PVN* paraventricular hypothalamic nucleus, *R* red nucleus, *SuM* supramammillary nucleus, *VP* ventral pallidum, *VTg* ventral tegmental nucleus (Modified from a published paper [45])

2.3.2 *Outputs of POMC Neurons*

ARC POMC neurons send projections widely throughout the brain to affect an array of physiological functions. A number of anterograde tracing studies [11, 24, 45] have revealed that ARC POMC neurons send the heavy projections to the anterior hypothalamus, medial preoptic area, lateral hypothalamus, dorsomedial

hypothalamus, ventromedial hypothalamus, PVN, para-subthalamic nucleus, and posterior hypothalamus; send the moderate projections to the bed nucleus of the stria terminalis, the lateral septum, the diagonal band of Broca, and the accumbens nucleus in the forebrain; and issue the light projections to the periaqueductal gray, the deep gray layer of the superior colliculus, and the deep mesencephalic nucleus in the midbrain.

Unlike ARC POMC neurons, which generally target forebrain centers, NTS POMC neurons mainly send their axons into the brainstem. NTS POMC neurons send heavy projections to discrete brainstem areas, including the parvicellular reticular nucleus, the dorsal and ventral parts of medullary reticular nucleus, the subcoeruleus nucleus, the gigantocellular reticular nucleus, the oral parts of the pontine reticular nucleus, the intermediate reticular nucleus, the supra-trigeminal nucleus, and the lateral parabrachial nucleus. In the hypothalamus, the moderate levels of axonal terminals from NTS POMC neurons were found.

Overall, both ARC and NTS POMC neurons have extensive inputs from and outputs to numerous brain regions, and the regions to which POMC neurons projects generally have projections to POMC neurons. Thus, POMC neurons may serve as a key area for receiving and integrating signals from numerous brain areas and may regulate both behavioral and physiological functions by controlling downstream targets. However, this does not imply that all POMC neurons have such a broad array of anatomical connections or that they affect multiple physiological functions. On the contrary, it seems that subpopulations of POMC neurons have distinct anatomical connections. For example, only a small (~10%) population of POMC neurons terminate in any individual target site, and most POMC neurons seem to innervate specific target sites and mediate selective actions [24]. In the future, it will be interesting and important to study the inputs and outputs of subpopulations or even single POMC neurons.

2.4 ARC POMC Neurons and Food Intake

The ARC is regarded as a primary location for the integration of circulating signals of hunger and satiety. POMC neurons and AgRP neurons are intermingled in the ARC and thought as “first-order neurons” that respond to the circulating signals of hunger and satiety and then drive physiological and behavioral responses. POMC neurons play key roles in maintaining normal feeding behavior and energy metabolism. Mice with global ablation of POMC neurons exhibit a progressive adult-onset obesity with overeating and increased weight gain, similar to what occurs in *pomc* null mice [48]. Furthermore, selective ablation of POMC neurons in the hypothalamus increases food intake and decreases energy expenditure and causes a broad spectrum of metabolic and endocrine disorders associated with obesity [50]. These behavioral effects of ablating ARC POMC neurons resemble the metabolic syndromes produced by removing POMC neurons in the entire brain.

The development of optogenetics and designer receptors exclusively activated by designer drugs (DREADDs) has enabled selective manipulation of POMC neurons and provides insight for understanding the critical role of ARC POMC neurons in feeding behavior. Acute inhibition of ARC POMC neurons does not significantly alter food intake, while chronic POMC neuron suppression does reduce food intake and cause weight loss [2, 50]. Consistently, chronic but not acute DREADDs-mediated inhibition of ARC POMC neurons induces hyperphagia [25]. It was therefore concluded that ARC POMC neurons are mainly responsive to long-term regulation of food intake.

The chronic inhibitory effect of ARC POMC neuron on feeding requires melanocortin receptor signaling. Activation of POMC neuron in A_y mice, in which the ectopically expressed agouti protein constitutively blocks melanocortin receptors such as MC4R, does not inhibit food intake [2]. Notably, this requirement for melanocortin receptor signaling indicates that other POMC neurons co-express neuropeptides such as the anorexigenic peptide cocaine and amphetamine-related transcript may not be sufficient to reduce food intake.

2.5 NTS POMC Neurons and Food Intake

The vagal nervous system is an important bridge between the brain and peripheral organs. The brainstem NTS is the major portal through which visceral afferent information for homeostatic reflexes enters the brain. Several lines of evidence suggest important roles of neurons in the NTS in the satiety. Firstly, acute pharmacological administration of melanocortin peptides into the fourth ventricle in rodents reduces food intake as potently as that in the hypothalamus [18]. Secondly, POMC neurons in the NTS are activated by cholecystokinin (CCK), a gut-released peptide that inhibits food intake, and also by satiety induced by feeding [16]. The activation effects of CCK on NTS POMC neurons are mediated by increasing vagal excitatory inputs [3]. Last but certainly not least, direct activation of NTS POMC neurons rapidly decreases meal number and meal size and eventually inhibits feeding [50]. The inhibitory effects mediated by NTS POMC neurons on feeding are acute and do not persist. However, and differently from ARC POMC neurons, ablating NTS POMC neurons does not affect food intake, body weight, body composition, or lipid and glucose metabolism [50].

In summary, hypothalamic POMC neurons are involved in the long-term regulation of food intake, whereas the NTS POMC neurons receive input afferents carrying short-term information on satiety. This functional distinction between these two populations of POMC neurons endows the POMC neural system with the potential to integrate two different feedback pathways involved in energy homeostasis. However, the mechanism that underlies the functional difference between ARC and NTS POMC neurons remains unknown.

2.6 POMC Neural Circuitry in Feeding Behavior

AgRP and POMC neurons lie in close proximity within the ARC but have opposing effects on feeding. AgRP functions as an inverse agonist of MC4R by decreasing cAMP levels produced by the constitutive activity of wild-type or mutant receptors [35]. Selective ablation of AgRP neurons in adults causes a rapid starvation and loss of body weight [30], whereas acute optogenetic activation of AgRP neurons rapidly increases food intake [2].

AgRP neurons are established as regulators of POMC neurons. In the ARC, nearly all POMC neurons receive direct inhibitory inputs from GABAergic AGRP neurons [13]. Optogenetic activation of AgRP neurons strongly inhibits ARC POMC neuron activity by releasing GABA, although AgRP neurons do not significantly contribute to the strong spontaneous GABA input to POMC neurons [38]. However, suppression of POMC neuron activity by AgRP neurons is not required for acute feeding inductions. Co-activation of AgRP and POMC neurons does not blunt increased feeding responses evoked by solely activation of AgRP [5, 8].

Outside of the ARC, AgRP neurons and POMC neurons send overlapping projections to many intra-hypothalamic and extra-hypothalamic nuclei [8, 45], although POMC neurons appear to innervate multiple regions not receiving AgRP innervations. Selective activation of AgRP fibers in the bed nucleus of the stria terminalis, the lateral hypothalamus, the PVN, and the paraventricular thalamic nucleus increases food intake, whereas a number of AgRP neurons' projection fields are not sufficient to evoke feeding [5, 8]. It is thought that AgRP neurons and POMC neurons can antagonize each other in their downstream targets. Indeed, a single ICV injection of AgRP increases food intake for up to a week, and co-injection of α-MSH does blunt the orexigenic effects of the former. However, unlike AGRP neurons, efferent projection stimulation strategy has not been employed for systemically studying the functions of POMC neurons in feeding behavior. The functional roles of diverse POMC projections in feeding behavior remain poorly understood.

The PVN receives heavy projections from POMC neurons and displays the highest MC4R expression. The PVN is considered to host the predominant energy-intake-regulating MC4R population within the CNS. Reactivation of MC4Rs in the PVN and the central amygdaloid nucleus of MC4R null mice completely prevented hyperphagia but failed to rescue the reduced expenditure [6]. Thus, the PVN was the downstream controlling food intake, whereas energy expenditure is regulated by MC4Rs elsewhere, suggesting distinct functional roles of POMC neural circuitry in energy metabolism.

Given the heterogeneity and complex anatomical connections of POMC neurons, it is difficult to assume that all POMC neurons and associated neural circuitry have the same physiological effects on the food intake and energy metabolism. In the future, dissecting the functional roles of specific POMC neural circuitry will be important for comprehensively understanding the POMC neural system.

2.7 Emerging Challenges for Traditional Theory of POMC Neurons in Regulating Food Intake

POMC neurons may play opposite roles in regulating feeding behavior via releasing different peptides. There are multiple POMC-derived peptides, including both melanocortins like α-MSH and opioids like beta-endorphins. α-MSH has inhibitory effects on appetite and body weight, whereas β-endorphin likely exhibits dose-related biphasic effects on food intake. Pharmacological studies have generally indicated that opioids stimulate food intake [23] and microinjection agonist of mu-opioid receptors into the striatal stimulates food intake [51]. β-Endorphin is able to antagonize the inhibitory effects of α-MSH on food intake and body weight gain. On the contrary, chronic ICV injections of high doses of beta-endorphin were shown to decrease food intake directly [14]. Male mice that selectively lack β-endorphin but retain normal melanocortin signaling are hyperphagia and obese [4], and re-expression of beta-endorphin fully rescues the obese phenotype.

One recent study argued that hypothalamic POMC neurons may also promote feeding [25]. It was found that the stimulation effect of CB1R on feeding may be achieved by releasing β-endorphin but not α-MSH from POMC neurons. Acute inhibition of ARC POMC neurons reduces cannabinoid-induced feeding.

2.8 POMC Neurons Can Be Rapidly Excited by Food Cues

Considering POMC neurons express both leptin receptors and insulin receptors, it is thought that ARC POMC neurons contribute to regulate long-term food intake and energy balance by integrating long-term nutrition signals. According to the traditional model, POMC neurons are excited by gradual changes in the concentrations of hormones that develop during food intake such as leptin, CCK, and glucose. This generates a "satiety" signal to cease feeding and persists until these hormones return to their previous level, thereby inhibiting POMC neurons and evoking the desire to eat. However, this textbook view is being challenged by several recent independent studies that recorded POMC neuronal activity in freely behavior animals using cutting-edge in vivo optical recording/imaging techniques. Unexpectedly, POMC neurons can be activated within seconds by the sensory detection of food. This rapid activation can persist during food consumption. Consistently, rapid inhibitory responses also happen in AgRP neurons. These responses were much too fast to be mediated by a hormonal signal, implying that they may arise from changes in neural input. This finding also suggests an important role for POMC neurons in sensory detection and the control of appetitive behaviors, such as foraging. More importantly, this observation seems to contradict the fact that (i) the activity of POMC neurons is necessary to inhibit food intake and (ii) loss of POMC neurons causes hyperphagia. These new discoveries have driven a major shift in our understanding

of the POMC neural system and raise a critical question of how POMC neurons are able to prevent feeding at all.

2.9 Additional Functional Roles of POMC Neurons

2.9.1 Reward

POMC neurons have been implicated in reward by several lines of evidence. First, POMC-derived beta-endorphin can create general states of well-being and pleasure. For example, elevation of circulating β-endorphin released from *pomc*-expressing skin cells under chronic UV exposure results in an opioid receptor-mediated addiction. A skin-specific knockout prevents UV exposure-induced β-endorphin elevation and the behavioral responses [17]. Secondly, there is a strong functional link between the melanocortin and dopamine systems. In vivo microdialysis analysis has shown that injection of α-MSH into the ventral tegmental area induced a significant increase in dopamine levels in the nucleus accumbens. This increase was completely blocked by pre-treatment with the MC4R antagonist, indicating that the effects of α-MSH on stimulating dopamine release may be mediated by the MC4R [29]. Thirdly, activity of AgRP neurons likely encodes a negative-valence signal. Activation of AGRP neuron results in place avoidance, whereas inhibition of AGRP neuron in energy-deficit conditions could flavor place preference [9]. Since POMC neurons receive direct inhibitory GABAergic inputs from AGRP neurons, it is possible that the negative-valence signal transmitted by AGRP neurons may be mediated by its inhibition effects on POMC neurons, which reduce the releasing of β-endorphin. These studies suggest a positive reinforcing property of POMC neurons, although the neural circuitry mechanisms are unknown.

2.9.2 Sexual Behavior

Converging lines of evidence underscore the importance of central pathways, in particular the POMC neural system in the control of sexual function. Direct central administration of α-MSH and ACTH induces sexual excitement in a range of experimental species including dogs, rabbits, monkeys, and cats [7]. Melanocortins have been tested in clinical trials for the treatment of erectile dysfunction. Actually, melanotan II is a synthetic analog of α-MSH that was ever under development as drug candidates for female sexual dysfunction and erectile dysfunction, but this work has now ceased due to side effects such as nausea, vomiting, and decreased appetite. Selective activation of MC4R improved sexual function in wild-type mice, whereas block of MC4R abolished MT-II-induced increase in intracavernosal pressure [33]. Injection of pseudorabies virus into the corpus cavernosum retrograde

transsynaptically labeled neurons in the PVN [32], a major target of POMC neural terminals. ICV injection of MC3/4R antagonist SHU-9119 inhibited penile erections [46], suggesting central melanocortin receptors can modulate penile erection.

Over the last several decades, POMC neurons have been implicated in multiple physiological functions such as energy metabolism, reward, and sexual behavior discussed in this chapter. New data have come to light suggesting that the roles of the POMC neural system in feeding are more complex than previously thought. One direction for further study is revealing the circuitry mechanism(s) through which this extremely heterogeneous system controls different functions via its broad anatomical connections.

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Chapter 3
Neuronal cAMP/PKA Signaling and Energy Homeostasis

Linghai Yang

Abstract The brain plays a key role in the regulation of body weight and glucose metabolism. Peripheral signals including hormones, metabolites, and neural afferent signals are received and processed by the brain which in turn elicits proper behavioral and metabolic responses for maintaining energy and glucose homeostasis. The cAMP/protein kinase A (PKA) pathway acts downstream G-protein-coupled receptors (GPCR) to mediate the physiological effects of many hormones and neurotransmitters. Activated PKA phosphorylates various proteins including ion channels, enzymes, and transcription factors and regulates their activity. Recent studies have shown that neuronal cAMP/PKA activity in multiple brain regions are involved in the regulation of feeding, energy expenditure, and glucose homeostasis. In this chapter I summarize recent genetic and pharmacological studies concerning the regulation of body weight and glucose homeostasis by cAMP/PKA signaling in the brain.

Keywords Brain · GPCR · Metabolism · Body weight

3.1 Introduction

The brain regulates energy balance and glucose homeostasis through cooperation with peripheral tissues including fat, gut, pancreatic islets, skeletal muscle, liver, etc. [1–5]. Signals from the body including hormones, metabolites, and neural signals communicate with the brain to report on the body energy status [6]. By coordinating these inputs, the brain regulates energy intake and expenditure to adapt to environmental changes and maintain energy and glucose homeostasis. Many of these peripheral signals are sensed by neurons in the hindbrain and hypothalamus, which communicate with other brain regions such as the striatum to regulate

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metabolic homeostasis, including food intake, energy expenditure, and glucose homeostasis [7–9].

Cyclic adenosine monophosphate (cAMP) was initially discovered in studying the effects of hormones glucagon and epinephrine on glucose metabolism in the liver [10]. It was later revealed in skeletal muscle that the glycogenolytic effect of cAMP depends on a cascade of protein phosphorylation, cAMP/PKA → phosphorylase kinase → glycogen phosphorylase → glycogen degradation, which was a breakthrough discovery in biochemistry revealing that protein phosphorylation is an important regulatory mechanism for enzyme activity [11]. The 1992 Nobel Prize in Physiology or Medicine was awarded to Edwin Krebs and Edmond Fischer for their discovery of this phosphorylation cascade. PKA is the major receptor for cAMP in mammals, and the physiological role of PKA in metabolic regulation is increasingly appreciated with the availability of genetically modified mice and pharmacological evidence [12].

In cells, cAMP is generated from adenosine triphosphate (ATP) catalyzed by adenylyl cyclase (AC), which is activated by stimulatory Gα protein (Gαs) following the activation of Gs-coupled receptors (Fig. 3.1). The termination of cAMP signaling is mediated by phosphodiesterase (PDE)-catalyzed conversion of cAMP to AMP [13]. PKA is a serine/threonine kinase, and the holoenzyme is inactive and formed by a dimer of two regulatory (R) subunits that each binds a catalytic (C) subunit [14]. Each R subunit contains two cAMP binding sites, and cAMP occupation leads to conformational changes of the R subunits and release of the active C subunits. Four types of R subunits (RIα, RIβ, RIIα, and RIIβ) and two types of C subunits (Cα and Cβ) have been identified in mice to be encoded by different genes. In general the α isoforms of RI, RII, and C subunits are expressed in all tissues, whereas β isoforms have more restricted expression. The subcellular localization of PKA is dependent on the binding of R subunits to A kinase anchoring proteins (AKAPs) which generally show higher affinity for type II holoenzymes [15].

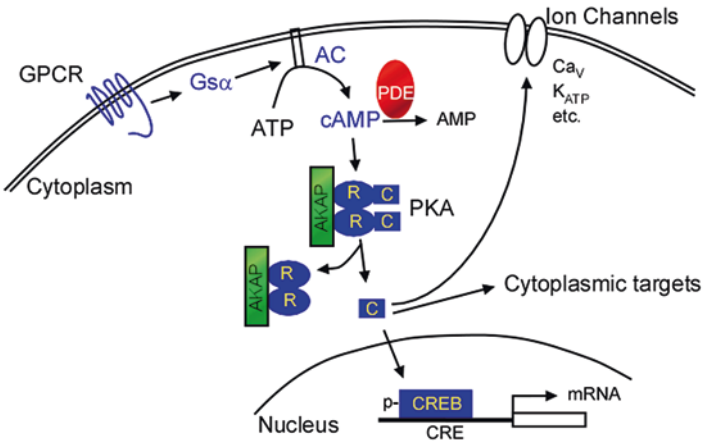

Fig. 3.1 cAMP/PKA signaling in mammalian cells

Binding to AKAPs is essential for the spatial and temporal regulation of cAMP/PKA signaling [16, 17]. Mice with global or conditional mutants of different R subunits, C subunits, or AKAPs have been generated and greatly enhanced our understanding of the role of cAMP/PKA in different physiological and pathological processes, such as neural synaptic plasticity, cardiac hypertrophy, tumor progression, and glucose homeostasis [18–20]. Moreover, different PKA subtypes have distinct expression and distribution patterns in mouse brain [21, 22], suggesting their involvement in different physiological processes.

3.2 Neuronal cAMP/PKA and Food Intake

3.2.1 Melanocortin System

The brain melanocortin system is involved in regulating energy homeostasis and mainly consists of melanocortin receptors MC3R and MC4R, the antagonist/inverse agonist agouti-related peptide (AgRP), and agonistic melanocortins including proopiomelanocortin (POMC)-derived peptides ACTH and α-, β-, and γ-melanocyte-stimulating hormone (MSH) [23]. Both MC3R and MC4R are Gs-coupled receptors and activate AC/cAMP/PKA pathway upon agonists binding. MC3R deletion has complex effects on energy balance by altering nutrient partitioning and leading to obesity, however, in the absence of increased body weight or food intake [24, 25]. Specifically, MC3R is expressed in hypothalamic POMC neurons where it acts as an inhibitory autoreceptor, and the MC3R-specific agonist D-Trp^{8}-γ-MSH inhibits the firing rate of POMC neurons and increases food intake [26]. Another possible mechanism for this MC3R-stimulated food intake is that AgRP can act as an inverse agonist for MC3R to suppress cAMP/PKA signaling in POMC neurons [27]. These results showed that MC3R has complex regulatory roles in energy balance and the cell type-specific roles of MC3R signaling through cAMP/PKA pathway in regulation of food intake remain to be determined [28, 29].

In contrast to MC3R, MC4R has been well documented to be involved in regulation of both food intake and energy expenditure [30]. Haploinsufficiency of MC4R in humans has been identified as the most common monogenic cause of severe obesity accounting for up to 5% of all cases [31, 32]. Genetic deletion of MC4R in mouse leads to hyperphagia, hypometabolism, hyperinsulinemia, and increased linear growth [33]. Mice with heterozygous deficiency of *MC4R* show intermediate phenotypes, suggesting the existence of gene dosage effects and consistent with human studies. MC4Rs in hypothalamic paraventricular nucleus (PVN) neurons regulate food intake but not energy expenditure [34, 35]. Studies have suggested that MC4Rs in PVN neurons suppress food intake through activation of cAMP/PKA pathway since mutations in MC4R that destroy cAMP production abolish the satiety effect of α-MSH and lead to hyperphagia and obesity [36]. A recent study found that a bone-derived protein hormone lipocalin 2 can inhibit food intake by

binding to PVN MC4Rs and stimulating cAMP production in PVN neurons [37]. In brainstem parasympathetic preganglionic neurons, MC4R activation suppresses neuronal activity by cAMP/PKA-dependent activation of K_{ATP} channels and hyperpolarization [38]. In the NTS, presynaptic MC4Rs on vagal afferent endings mediate inhibition of food intake through a cAMP/PKA-dependent manner [39]. These results suggested that MC4Rs in multiple brain sites can regulate food intake via the cAMP/PKA intracellular pathway. However, studies using genetic depletion approaches suggested that PVN MC4R activation inhibits food intake mainly through $G_{q/11}$/PLC/Ca^{2+} signaling rather that the classical G_s/cAMP signaling [40], consistent with findings that mice with PVN-specific Gαs deficiency have subtle changes in food intake and body weight [41]. In contrast, PVN MC4R signaling seems to regulate sympathetic activity and blood pressure via the cAMP/PKA pathway [40, 42]. Another study also found that MC4R-mediated depolarization of PVN neurons is independent of Gs/cAMP/PKA pathway and instead dependent on direct coupling of MC4R to the inwardly rectifying potassium channel Kir7.1 [43]. These results suggest that cAMP/PKA signaling is not required for PVN MC4R-dependent regulation of food intake, but it remains to be determined whether cAMP/PKA activation in PVN MC4R neurons is sufficient to suppress food intake. Thus it is still ambiguous if cAMP/PKA signaling in PVN MC4R neurons is physiologically involved in the regulation of food intake.

AgRP neurons in hypothalamic arcuate nucleus (ARC) are an essential part of the melanocortin system by releasing antagonistic peptide AgRP for MC4R [23]. In addition to inhibiting α-MSH binding to MC4R, AgRP has also been identified as an inverse agonist for MC4R, and its binding promotes $G_{i/o}$-coupled signaling and suppresses cAMP/PKA signaling in MC4R-expressing cells [44]. AgRP neurons are required for normal food intake, and acute depletion of these neurons in adult mice leads to starvation [45]. Optogenetic [46, 47] or chemogenetic [48] activation of AgRP neurons is sufficient to induce food intake in satiated mice. Recently, it was shown using AAV-mediated Gs-coupled DREADD (GsD) expression that activation of cAMP/PKA/CREB pathway in AgRP neurons induced prolonged overeating by increasing AgRP expression [49]. Endogenously, it was proposed that PACAP receptors (PAC_1R, Gs-coupled GPCR) on AgRP neurons might mediate the increased cAMP/PKA signaling and AgRP expression during fasting [49]. Consistently, another study has found that PACAP-containing glutamatergic neurons in the paraventricular nucleus of the hypothalamus (PVN) are synaptically connected to AgRP neurons and stimulate feeding upon activation [50].

Another metabolic factor that stimulates food intake potentially by increasing AgRP neuronal cAMP/PKA signaling during fasting is the hunger hormone ghrelin. AgRP neurons express ghrelin receptors which are coupled to both Gq/PLC/Ca^{2+} and Gs/cAMP/PKA signaling pathways, and activation of the cAMP/PKA signaling is essential for neuronal activation and increased feeding [51–53]. On the other hand, the anorexigenic effect of leptin has been shown depending on its activation of hypothalamic PI3K/PDE3B signaling and subsequent decrease of cAMP level [54], suggesting the existence of crosstalk between leptin signaling and the cAMP/PKA pathway for the regulation of food intake.

The central cannabinoid system is another important regulator for food intake potentially through modulation of the cAMP/PKA signaling and melanocortin system. Cannabinoid receptor CB1Rs are primarily Gi/o-coupled and presynaptically localized, to which endocannabinoid binding leads to decreased cAMP/PKA signaling and reduced release of either GABA or glutamate [55]. Typically, CB1R activation is associated with increased food intake, and CB1R antagonists and inverse agonists have been developed to aid weight loss in humans. Recent studies suggested that CB1R activation promotes food intake at least partially through suppressing GABAergic innervation (disinhibition) of AgRP neurons [56] and, unexpectedly, activation of POMC neurons for β-endorphin release [57]. It remains to be determined, however, where these afferent CB1R axonal terminals arise from and if suppression of presynaptic cAMP/PKA signaling is required for the neuronal effects of CB1R activation on feeding behaviors.

Recently it was shown that AgRP neurons are quickly inhibited by gastrointestinal hormones CCK, serotonin, and PYY after food digestion [58]. While both CCK1 receptor and CCK2 receptor are stimulatory G-protein (Gq or Gs)-coupled, PYY receptor NPY2Rs are inhibitory G-protein (Gi)-coupled and suppress cAMP/PKA pathway. It remains unknown if CCK receptors are expressed in AgRP neurons, but previous studies have suggested an indirect regulatory effect of CCK on AgRP neurons [59]. NPY2Rs are highly expressed in AgRP neurons [60] and have been suggested to exert a tonic inhibition on AgRP neurons potentially through suppression of the cAMP/PKA pathway [58]. Serotonin receptors are either stimulatory or inhibitory on neuronal activity, depending on the subtypes. The Gi-coupled 5HT1BRs are expressed in AgRP neurons and may mediate the inhibitory effects of serotonin on AgRP neurons and food intake [61]. Taken together, these results showed that cAMP/PKA signaling plays an essential role in the regulation of AgRP neurons activity and feeding behavior.

3.2.2 Glucagon and GLP-1

Glucagon is a peptide hormone, produced by α cells of the pancreas under conditions of hypoglycemia to increase endogenous glucose production [12]. Glucagon receptor is Gs-coupled and mainly expressed in the liver and kidney since they are the major sites for glucose production under fasting conditions. Glucagon receptor is also expressed in the mediobasal hypothalamus including the ARC and VMH [62, 63]. Glucagon signaling in the ARC has been shown to inhibit food intake and decrease the expression of AgRP, in a dependent manner on activation of cAMP/PKA and inhibition of CaMKKβ/AMPK pathway in the ARC [62]. The results are consistent with previous finding that PKA acts upstream AMPK to suppress its activation [64]. However, it remains unknown if glucagon acts directly on AgRP neurons or on adjacent neurons (likely GABAergic neurons) to indirectly suppress AgRP neurons to exert the anorexigenic effect. If glucagon acts directly on AgRP neurons, as implicated in previous studies showing that glucagon receptors are

expressed in AgRP neurons [63], it seems controversial that glucagon-induced activation of cAMP/PKA in AgRP neurons suppresses the expression of AgRP, whereas GsD and PACAP-induced PKA activation increases AgRP expression as mentioned above [49]. It is also puzzling why glucagon, a hunger hormone stimulated by hypoglycemia, acts in the brain to inhibit feeding in a state requiring food intake. These questions merit future studies.

Glucagon-like peptide-1 (GLP-1) is a neuropeptide produced from L cells in the intestines and from neurons in the nucleus tractus solitarius (NTS) [65]. GLP-1 system is currently one of the most promising targets for the development of effective obesity pharmacotherapies, and the GLP-1 analog liraglutide has been approved by FDA for weight loss treatment in obese individuals. GPL-1 receptor is Gs-coupled and expressed in multiple CNS sites relevant to energy balance regulation, such as NTS, parabrachial nucleus (PBN), hippocampus, PVN, lateral hypothalamus (LH), bed nucleus of stria terminalis (BNST), central nucleus of the amygdala (CeA), etc. [66–68]. It has been well documented that GLP-1R activation leads to suppression of food intake [69, 70]. In the NTS, it was shown that GLP-1R activation-induced suppression of food intake is dependent on cAMP/PKA activation and subsequent suppression of AMP kinase and activation of MAP kinase [71]. In the PVN, GLP-1R is expressed on corticotropin-releasing hormone (CRH) neurons, and its activation leads to cAMP/PKA-dependent phosphorylation of GluA1 AMPA receptors and enhanced excitation of CRH neurons and suppression of feeding [72]. Selective depletion of GLP-1R on PVN neurons leads to increased food intake and obesity, suggesting its physiological significance [72]. GLP-1Rs in other brain sites, such as the hippocampus [73], PBN [74, 75], lateral septum [76], and PVT [77], are also involved in the regulation of food intake, but whether cAMP/PKA signaling is required for the anorexigenic effects of GLP-1R activation in these sites remains to be determined.

In a previous study, we showed that fasting induces significant increases in PKA activation and CREB phosphorylation in multiple sites in the hypothalamus [78]. This fasting-induced PKA and CREB activation is reversed by refeeding and greatly diminished by RIIβ-PKA depletion in the hypothalamus, suggesting that hypothalamic RIIβ-PKA is physiologically involved in the regulation of food intake. Our results are consistent with the finding that NPY and food deprivation increase CREB phosphorylation and DNA binding in rat hypothalamus while PACAP or refeeding antagonize this increased CREB activity [79]. The neuronal types showing changes in PKA activity remain to be determined; the major nutritional signals that mediate these effects are also unknown, although the hormones mentioned above could be promising candidates.

3.2.3 Striatal Dopamine Signaling

The regulation of food intake by striatum is largely revealed by studies on the midbrain-striatum dopamine system. Dopamine-deficient (DD) mice lack feeding responses to intrinsic orexigenic signals and are severely aphagic and starve by

4 weeks of age [80]. Restoration of dopamine signaling in the dorsal striatum is sufficient to rescue the food intake of dopamine-deficient mice, suggesting that dorsal striatum provides a permissive signal allowing feeding and consumption in response to metabolic demand [81, 82]. In humans, dopamine signaling in the dorsal striatum is also involved in food motivation [83]. In contrast, restoration of dopamine signaling in the ventral striatum, the nucleus accumbens (NAc), could not restore feeding of DD mice but restored the preference for sugar and a palatable diet [81]. It has been suggested that NAc is involved in rewarding processes underlying food intake [82, 84]. Inhibition of NAc dopamine signaling by selective neuronal ablation or intra-NAc injection of dopamine receptor antagonist disrupts effort-related food seeking [85], while elevating NAc dopamine level increases effortful food seeking [86]. Thus dopamine signaling in the dorsal and ventral striatum may mediate different aspects of food intake-related behaviors.

Most of striatal neurons (>90%) are GABAergic medium spiny neurons (MSNs) which express either dopamine D1R or D2R receptors, with a small subpopulation expressing both receptors [87]. D1R is Gs/olf-coupled and stimulates adenylyl cyclase and cAMP production, while D2R is Gi-coupled and inhibits adenylyl cyclase activity and cAMP production [88]. Both D1R and D2R signaling in the striatum have been shown to be involved in regulation of food intake [89]. We have shown previously that selective expression of a dominant-negative RIα PKA subunit (dnPKA) in striatal MSNs (including D1R and D2R neurons) leads to reduced food intake and a lean phenotype in mice [90]. These mice are hypophagic on either regular chow diet or high-fat diet (HFD) compared to WT control mice. In addition, the HFD-induced overeating is absent in these mice suggesting that hedonic responses are impaired by inhibition of PKA in MSNs [90]. However, selective expression of the dnPKA in either D1R or D2R neurons had marginal effects on feeding behaviors and body weight (L. Yang et al., unpublished data), suggesting that D1R and D2R neurons can functionally compensate each other for feeding control when PKA is inhibited in either type of neurons. This is in contrast with the prevailing view that D1R and D2R neurons have an opposing role on striatum-mediated behaviors but consistent with recent findings that both types of striatal MSNs promote goal-directed behaviors including food intake [91, 92].

There are several other regulatory systems that exert their effects on food intake by modulation of striatal neurons such as endocannabinoid [93], serotonin [94], and the melanin-concentrating hormone (MCH) system [95], in which cAMP/PKA signaling is involved. For example, activation of 5-HT4R in the NAc inhibits food intake by increasing the expression of anorexic peptide CART which is dependent on activation of the cAMP/PKA pathway in MSNs [94]. The NAc contains high levels of receptor for MCH, a lateral hypothalamic peptide critical for feeding and metabolism. MCH receptor (MCHR1) activation in the NAc increases food intake, while its blockade reduces feeding. MCHR1 activation leads to Gi/o-coupled suppression of cAMP/PKA pathway, decreased PKA-dependent phosphorylation of GluR1 and other PKA substrates, and inhibition of neuronal firing [95]. This is consistent with the general model that inhibition of NAc neurons increases feeding-related behaviors [96].

3.3 Neuronal cAMP/PKA and Energy Expenditure

To maintain energy homeostasis, food intake must be balanced by energy expenditure, which includes basal metabolic rate, adaptive thermogenesis, and physical activity. Neuronal cAMP signaling has been shown to be involved in the regulation of all three processes.

3.3.1 Basal Metabolic Rate

Basal metabolic rate (BMR) contributes to the majority (60–75%) of energy expenditure in humans, and reductions in BMR can result in weight gain and obesity [97]. BMR is the amount of energy that is required to keep the body functioning at rest, such as processes of breathing, heart beating, cell growth, brain and nerve function, etc. Most of these activities are regulated by autonomic nervous system. As mentioned above, MC4Rs in multiple brain nuclei are involved in the regulation of sympathetic nervous system activity and are important regulators for BMR. Mutation of MC4R leads to reduced BMR in both human [98] and rodent [99, 100]. Agonists for MC4R have been shown to increase BMR [101, 102]. Specifically, MC4R activation in the PVN increases sympathetic nerve activity to the cardiovascular system and increases blood pressure and heart rate via the Gs/cAMP/PKA pathway [40, 42], thus potentially contributing to BMR. It remains to be determined if MC4Rs on other neuronal groups regulate BMR via the cAMP/PKA pathway.

Thyroid hormone is an essential humoral regulator of metabolism and BMR. Its production is regulated by the hypothalamic-pituitary-thyroid (HPT) axis, in which the activation of thyrotropin-releasing hormone (TRH) neurons within the hypothalamus ultimately leads to increased thyroid hormone (T4/T3) signaling at peripheral tissues [103]. In addition to the well-characterized T3-mediated regulation of TRH transcription, hypothalamic TRH expression is also regulated by nutritional signals such as leptin and the melanocortin receptors [104]. It has been shown that MC4R activation increases TRH transcription by enhancing CREB phosphorylation and binding to TRH promotor, potentially through activating the Gs/cAMP/PKA/CREB pathway [104]. It has been shown that the stress hormone norepinephrine also stimulates TRH expression in the PVN through β-adrenergic receptor/cAMP/PKA/CREB pathway and increases BMR [105].

3.3.2 Adaptive Thermogenesis

Adaptive thermogenesis is non-shivering heat production in response to changes in environmental or physiological settings such as cold, fever, diet, and stress. In rodents, brown adipose tissue (BAT) is the major contributor to adaptive

thermogenesis. Several hypothalamic and brainstem nuclei play key roles in driving the sympathetic innervation of BAT thermogenesis [106]. As mentioned above, MC4R on PVN neurons regulates food intake but not energy expenditure [34, 35]. Further genetic dissections have identified that MC4Rs on cholinergic preganglionic sympathetic neurons of the intermediolateral nucleus of the spinal cord (IML) are the critical regulator for energy expenditure [107, 108]. In addition to IML, MC4Rs expressed in the median preoptic nucleus [109] and DMH [110] also contribute to sympathetic regulation of BAT thermogenesis. Specifically, it has been shown that selective MC4R depletion in DMH neurons leads to reduced energy expenditure and obesity without significant effect on food intake in mice [111]. Similar phenotypes were seen in mice with DMH-specific deletion of Gsα, suggesting that MC4Rs in DMH neurons promote energy expenditure by the Gsα/cAMP/PKA pathway [111]. The reduced energy expenditure is mainly due to a reduced sympathetic activation of brown fat tissue (BAT) thermogenesis at ambient temperature because this defect is absent at thermoneutral temperature 30 °C [111]. However, it remains unknown if cAMP/PKA signaling is required for MC4R-mediated activation of IML cholinergic neurons and increase in energy expenditure [38].

BAT thermogenesis is mediated by the mitochondrial uncoupling protein UCP-1, which uncouples substrate oxidation from ATP production and instead dissipates chemical energy as heat. In RIIβ-PKA knockout mice, we have found increased mitochondrial density and increased UCP-1 protein level in brown adipocytes, suggesting enhanced BAT thermogenesis [112]. We have shown that the lean phenotype of RIIβ-PKA knockout mice is caused by neuronal PKA deficiency rather than adipose PKA deficiency [113], potentially through enhancing hypothalamic leptin sensitivity in multiple nuclei, including the ARC, VMH, and DMH [78]. Leptin signaling in these hypothalamic nuclei is a key regulator for sympathetic drive to BAT [114, 115]. Therefore, we speculate that the enhanced BAT thermogenesis in RIIβ KO mice is caused by neuronal PKA deficiency in multiple hypothalamic nuclei, although further studies using BAT-specific RIIβ knockout are needed to validate the hypothesis.

3.3.3 Physical Activity

Physical activity is the most variable component of energy expenditure in humans and constitutes 15–30% of daily energy expenditure [116]. Physical exercise can also influence BMR and has beneficial effect on energy homeostasis [116]. Spontaneous physical activity is strongly influenced by metabolic signals such as leptin through mechanisms involving dopamine signaling in the brain [117, 118]. The dopamine reward system is essential for regulation of physical activity, and in the extreme case, animals with dopamine deficiency are extremely hypoactive [80]. Neurons that express dopamine receptor D1R or D2R in the striatum are key components of the direct and indirect pathway, respectively, that regulate motor activity

[91]. As mentioned above, the physiological effects of D1R and D2R activation are largely mediated by the cAMP/PKA pathway [88]. PKA activity in D1R and D2R neurons is shown to be positively and negatively associated, respectively, with reward-related locomotor activity and behavior [119].

RIIβ-PKA is the major subtype of PKA in the striatum, and RIIβ deficiency leads to impaired gene expression in MSNs and defective motor behaviors [120], with increased locomotor activity [121]. Genetic studies using Cre-mediated RIIβ re-expression in either D1R or D2R neurons showed that the increased running activity is reversed by D2R-specific RIIβ expression but not by D1R-specific RIIβ expression [113], suggesting that PKA deficiency in D2R neurons (the indirect pathway) plays a dominant role in mediating the increased locomotion of RIIβ KO mice. However, selective PKA inhibition in striatal MSNs by expression of a dominant-negative PKA RIα subunit leads to reduced locomotor activity [90], suggesting that different PKA subtypes in the striatum have different regulatory effects on motor activity.

The melanocortin receptor MC4R is also important for the regulation of physical activity. Locomotor activity is reduced in young Mc4r-null male mice before obesity development [100] and in rats lacking functional MC4R [122]. The agouti lethal yellow (A^y) mice with ectopic expression of the MC4R antagonist/inverse agonist agouti protein are also hypoactive, and concomitant RIIβ-PKA deficiency in A^y mice can largely restore their locomotor activity [123]. Interestingly, co-expression of MC4R with D1R and D2R dopamine receptors in striatum and the hypothalamus has been observed and suggested to play a role in the regulation of locomotor activity as well as food intake [124, 125]. These results suggest that both MC4Rs and dopamine receptors regulate physical activity via the cAMP/PKA pathway.

3.4 Neuronal cAMP/PKA and Glucose Homeostasis

The brain plays a key role in maintaining glucose homeostasis such as modulation of pancreas insulin secretion and hepatic glucose production [5]. Leptin regulates glucose homeostasis mainly by its actions on hypothalamic neurons [4]. Insulin also acts in the brain to regulate food intake and hepatic glucose production [2, 126]. In hypothalamic neurons, cAMP signaling has been shown to be modulated by several hormones such as insulin, leptin, glucagon, and GLP-1 [127]. As mentioned above, GLP-1Rs are expressed throughout the brain. In addition to its suppression on feeding, neural GLP-1R activation also affects glucose metabolism through enhancing insulin secretion, skeletal muscle glucose uptake, and suppressing hepatic glucose production [128, 129]. Specifically, GLP-1R signaling in the arcuate hypothalamic nucleus is required for these effects of centrally administered GLP-1 [130]. It remains unclear if cAMP/PKA signaling is involved in this arcuate GLP-1R signaling in a similar manner as seen in the PVN CRH neurons [72]. A recent study revealed that glucagon acts in the hypothalamus to inhibit hepatic glucose

production through activation of hypothalamic cAMP/PKA pathway [63]. Inhibition of PKA activity in the mediobasal hypothalamus (MBH) abolishes the suppressive effect of glucagon on HGP and leads to increased i.v. glucagon injection-induced HGP. On the contrary, activation of PKA in MBH inhibits HGP and resembles the effects of glucagon injection to the hypothalamus. The glucagon-induced activation of hypothalamic PKA and inhibition of HGP are both attenuated in rats fed a high-fat diet, whereas direct activation of MBH PKA by Sp-cAMPS infusion can still efficiently inhibit HGP in these rats [63]. These findings suggest that hypothalamic glucagon resistance contributes to increased HGP and hyperglycemia in diabetes and obesity and that PKA activation in MBH could be a strategy to bypass the glucagon resistance.

The ubiquitously expressed G-protein α subunit Gsα is coupled to AC activation and cAMP production. The gene encoding for Gsα (GNAS) shows parental specific genomic imprinting and patients with maternal inactive mutations exhibit obesity and insulin resistance [131]. Animal studies have shown that brain-specific maternal Gsα mutation leads to obesity, severe insulin resistance, and diabetes [132]. Moreover, these mice developed insulin-resistant diabetes prior to the development of obesity, indicating that central Gsα signaling directly regulates peripheral glucose metabolism. The specific neuronal populations that mediate the effects of Gsα mutations on glucose homeostasis remain to be determined, and it is likely that the effects are mediated through the central melanocortin system [131, 132]. As discussed above, Gsα deficiency in glucagon and GLP-1-responsive neurons in the hypothalamus may lead to central glucagon/GLP-1 resistance and contribute to the development of hyperglycemia and diabetes in these mice.

In mouse hypothalamus, multiple PKA R and C subunits are expressed, and RIIβ-PKA is one of the major subtypes [78]. Mice with global knockout of RIIβ subunit exhibit elevated insulin and leptin sensitivity and resistance to diet-induced obesity and diabetes [78, 133, 134]. The lean phenotype and likely the improved glucose metabolism as well are caused by RIIβ-PKA deficiency in the hypothalamus [113]. Similar leanness and improved insulin sensitivity have also been observed in mice lacking the PKA Cβ subunits which are highly expressed in brain regions including the hypothalamus [135, 136]. Cβ deficiency has no significant effects on total PKA activity in the brain due to a compensatory increase of Cα subunits. However, Cβ deficiency significantly reduces basal PKA activity [137], suggesting that Cα-PKA is less sensitive to basal levels of cAMP compared to Cβ-PKA. It remains unknown how PKA activity is affected by Cβ deficiency in specific brain regions and neuronal types that are essential for the regulation of energy balance. Generation of conditional mutant mice lacking Cβ in selective neuronal types will provide more mechanistic insights. RIIβ deficiency leads to reduced PKA Cα and Cβ subunits and decreased total PKA activity in multiple brain regions [78, 120]. A compensatory increase of type I PKA was observed, and this shift from Type II to Type I PKA leads to increased sensitivity to activation by cAMP [120] and likely altered intracellular localization of PKA as well. Although the neuronal populations and molecular mechanisms responsible for the improved glucose metabolism in RIIβ KO and Cβ KO mice

remain largely unknown, these findings suggest that PKA manipulations in the brain, most likely in hypothalamic neurons, have the potential to improve glycemic control in diabetes.

3.5 Summary

In summary, cAMP/PKA signaling in different neuronal types has pleiotropic effects on energy balance and glucose homeostasis. Anorexigenic factors can either inhibit (insulin, leptin) or activate (glucagon, GLP-1) cAMP/PKA signaling in different neuronal types, so are orexigenic factors (ghrelin, cannabinoids, NPY, AgRP, etc.). In the hypothalamus, cAMP/PKA pathway has profound effects on energy balance and glucose homeostasis that merit further studies. Although it is clinically challenging to yield tissue-specific manipulation of cAMP/PKA pathway, the findings that metformin exerts its antidiabetic effect by inhibiting hepatic cAMP/PKA/CREB pathway [138, 139] strongly support this signaling cascade as a target for the development of new therapeutics for metabolic diseases. New insights into the neural systems underlying cAMP/PKA-regulated energy and glucose homeostasis may guide the development of small-molecule or other innovative pharmacological or viral therapies targeting unique phenotypes of different brain nuclei.

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Chapter 4
Synaptic Regulation of Metabolism

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Abstract Neurons in the brain, particularly those in the hypothalamus, are essential for the maintenance of whole-body metabolic homeostasis. Dysfunctions or dysregulations of them can result in various metabolic diseases, including eating disorders, obesity, and diabetes. In addition to hormonal and peptidergic regulation, accumulating evidence has shown that these neurons are subject to synaptic regulation, which has been largely overlooked. In this chapter, we focus on synaptic neurotransmission of hypothalamic neurons and summarize current knowledge of synaptic plasticity in the maintenance of energy balance. Synaptic modulation engaged by circulating hormones is also discussed.

Keywords Glutamate · GABA · Synaptic plasticity · Neurotransmission · Metabolism · Hypothalamus · Leptin · Ghrelin

4.1 Introduction

Accumulating studies have demonstrated that neurotransmission is an essential component for the brain to regulate metabolic homeostasis [27, 56, 157]. Early studies have reported that intracerebroventricular (icv) injection of glutamate or muscimol, a $GABA_A$ receptor antagonist, stimulated feeding in rats [6, 125], suggesting a critical role for synaptic transmission in regulating energy balance. The synapse is a highly specialized intercellular structure that permits neurotransmission in the nervous system. Since Santiago Ramón y Cajal proposed that the relationship between nerve cells was not continuous, but contiguous [156], the past century has witnessed remarkable progress in our understanding of the "neuron

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doctrine." In the area of modern neuroscience, our understanding of synaptic mechanisms has expanded through studies in synaptic function, neurotransmitter release and reception, synaptic modulation and its contribution to neural circuit physiology and function, and synaptic regulation as it pertains to physiological behaviors. The output of a neuronal population is influenced by different organizations of synaptic input, known as synaptic plasticity.

Hypothalamic neurons have been known to retain dynamic plasticity throughout life [95, 122, 131]. Indeed, synaptic plasticity can be further modulated by a variety of hormones in the periphery. Nevertheless, synaptic plasticity was not considered as a key component for the regulation of metabolism until a pioneering study showed that systemic administration of leptin in leptin-deficient *ob/ob* mice could elicit a rapid rearrangement of both excitatory and inhibitory synapses [105]. Shortly after this discovery, leptin was found to regulate the synaptic organization of hypocretin/orexin neurons in the lateral hypothalamus [57]. Of interest, a gut hormone and orexigenic neuropeptide, ghrelin, was also shown to control synapse density in the hippocampus [26]. Ghrelin can also modulate the excitatory synaptic input of neurons in various brain regions, including the midbrain dopamine neurons [1] and hypothalamic AgRP neurons [154]. Of note, in a more recent study, a considerable amount of dendritic spines, on which glutamatergic synapses reside, were observed on AgRP neurons but not on adjacent, intermingled POMC neurons, a subpopulation of neurons known to have opposing regulations in energy balance [84]. AgRP neurons also exhibited dynamic, hunger-induced changes in spine density, glutamatergic neurotransmission, and firing activities, in an NMDAR-dependent manner. Therefore, metabolism-regulating neurons can be largely plastic, and their synaptic plasticity can contribute to both metabolic regulation and metabolism-related disorders.

In this chapter, we will summarize recent findings focusing on neurotransmission and synaptic plasticity of the metabolism-regulating neurons in the brain. Metabolic stress and their induced synaptic dysregulation in other neurological disorders will also be discussed.

4.2 A Brief Introduction of Synapse

The word "synapse," derived from Greek etiology meaning "conjunction," was first introduced by English neurophysiologist Charles Sherrington in 1897. A synapse is typically defined by the presence of a presynaptic compartment with synaptic vesicles, a postsynaptic density (PSD), and a well-defined synaptic cleft mediating neurotransmission between these two parts. In the adult brain, neuronal processes contain a high density of synapses, integrating many inputs from various brain regions. One of the most fascinating properties of the synapse is its plasticity, a capacity to modify the strength or efficacy of synaptic transmission at preexisting synapses driven by neuronal activity. In turn, synaptic plasticity contributes to functional changes in the brain, thereby modifying subsequent thoughts, feelings, and behaviors.

4.2.1 Synaptic Plasticity and Neurotransmission

Plasticity, a fundamental property of the neuron, allows the brain to learn and memorize patterns in response to experience, to recover function from injury, and to maintain responsiveness across a broad range of conditions. The ability to alter synaptic strength to different stimuli, also called synaptic plasticity, is an essential feature of neural circuits that adapt in an experience-dependent manner. Synaptic transmission can be either enhanced or depressed by activity, persisting from milliseconds to hours, or even days. The transient forms of plasticity can be linked to short-term sensory inputs, as well as transient changes in behavior states and short-term memory. On the other hand, long-term plasticity is thought to contribute to long-term information storage, such as learning and memory. Given these diverse functions, elucidating the mechanisms underlying synaptic plasticity in different brain regions is critical in understanding the neurobiological basis of both physiological and pathological brain states.

Two fundamentally different types of synapses, chemical and electrical, can be distinguished by the basis of their mechanisms of transmission. In a chemical synapse, electrical activity in presynaptic terminals drives the release of chemicals known as neurotransmitters, which bind to receptors located on the postsynaptic membrane and initiate secondary messenger pathways that can either excite or inhibit postsynaptic neurons. In an electrical synapse, electrical current mediates the communication between the presynaptic and postsynaptic membrane through specialized channels called gap junctions. Since chemical synapses are the predominant form of neurotransmission in the brain, we will focus solely on this form of synaptic transmission for the remainder of this chapter.

Neurotransmitter receptors located on the postsynaptic membrane fall into two major families: ionotropic and metabotropic. Ionotropic receptors, also called ligand-gated ion channels, contain two functional domains: (1) the extracellular domain that binds neurotransmitters and (2) the membrane-spanning domain that forms an ion channel. Binding of the neurotransmitter results in an immediate conformational change that opens the channel portion of the receptor, allowing ions to pass through the membrane, thus altering the membrane potential within 0.1–2 milliseconds. The property of the receptor, excitatory or inhibitory, leads to differential responses at the postsynaptic cell. The binding of excitatory neurotransmitters, such as glutamate, to postsynaptic NMDA and AMPA receptors opens cation channels and permits Na^+ ion influx, leading to rapid depolarization of the postsynaptic membrane. In contrast, binding of inhibitory neurotransmitters, like GABA, to postsynaptic $GABA_A$ receptors causes an influx of chloride and leads to membrane hyperpolarization of the postsynaptic membrane.

The second family of neurotransmitter receptors consists of metabotropic receptors, which couple to G proteins to permit the flow of ions through one or more metabolic steps. The coupled G proteins are activated after neurotransmitter binding, dissociate from the receptor, and interact directly with ion channels to trigger effector proteins to regulate ion conductance. Compared to ionotropic receptors, the

postsynaptic responses of metabotropic receptors are much slower and longer-lasting. Additionally, while ionotropic receptors exert effects in localized regions, metabotropic receptors can have effects spreading from the local binding region throughout the whole cell.

This inherent diversity of receptors endows neurons with the ability to produce postsynaptic actions that range from less than a millisecond to minutes, hours, or even days and respond to a single neurotransmitter in various groups of neurons. For example, acetylcholine, the first neurotransmitter to be identified with multifunctional roles in different nerve and muscle cells, can either boost an excitatory response lasting only milliseconds when binding to nicotinic acetylcholine receptors, or induce hyperpolarizing effects lasting seconds when binding to muscarinic acetylcholine receptors. In the following sections, we will focus on ionotropic receptors and discuss their functional role in regulating metabolism.

4.2.2 Excitatory Synapses: Glutamatergic Neurotransmission

Glutamate is the most abundant neurotransmitter in the central nervous system and elicits excitatory neurotransmission. Extracellular glutamate levels are tightly regulated, due to the neurotoxicity associated with high glutamate concentrations [17]. Therefore, several mechanisms exist to recycle glutamate from the extracellular space. Excitatory amino acid transporters, which are heavily expressed on astrocytes, clear glutamate from the extracellular space by forming tripartite synapses. Then, the recycled glutamate is metabolized to glutamine or used in the tricarboxylic acid (TCA) cycle. Following this event, glutamine is taken up by neurons and reconverted to glutamate by glutaminase. These sequential events are critical for the tight regulation of extracellular glutamate levels in order to prevent neurotoxicity and permit efficient excitatory synaptic transmission.

In order for presynaptic glutamate release to occur, glutamate must be packaged into synaptic vesicles at high concentrations. The synaptic vesicles that have been identified in the CNS are vesicular glutamate transporters 1–3 (VGLUT1, VGLUT2, and VGLUT3) [39]. Upon release into the synaptic cleft as a result of an action potential, glutamate binds to its postsynaptic receptors, including both ionotropic N-methyl-D-aspartate receptor (NMDAR) and D,L-alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid receptor (AMPAR), as well as metabotropic receptors (mGluRs). The binding of glutamate to its receptors opens cation channels and allows the passage of Na^+ and K^+ ions, thus leading to rapid depolarization of the postsynaptic membrane.

Ionotropic glutamate receptors, like other ligand-gated channel receptors, form from a variety of protein subunits that combine to produce receptor isoforms. For example, AMPARs are composed of a combination of four types of subunits (GluA1, GluA2, GluA3, GluA4), while NMDARs are composed of a heterotetramer between two GluN1 and two GluN2 subunits. NMDARs allow the entry of Ca^{2+} ions, as well as Na^+ and K^+ ions. Thus, the excitatory postsynaptic potentials elicited by NMDA

receptors can increase the calcium concentrations within the postsynaptic neurons and activate signaling cascades. Furthermore, NMDARs exhibit a voltage-dependent activation, which is blocked by extracellular magnesium at negative membrane potential [93, 100]. As a result, NMDARs contribute little to the postsynaptic response during basal synaptic activity. However, magnesium dissociates from NMDARs when the cell is depolarized and allows sodium and calcium to enter the dendritic spine. Thus, these mechanisms exist to permit efficient glutamatergic neurotransmission, which is critical for synaptic plasticity.

4.2.3 Inhibitory Synapses: GABAergic Neurotransmission

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. GABA is synthesized by mature isoforms of glutamate decarboxylase (GAD), GAD65 and GAD67, which convert glutamate into GABA [15]. The uptake of GABA into synaptic vesicles is mediated by vesicular GABA transporters (VGAT) [91]. Of note, although VGAT used to be the exclusive vesicular transporter for GABA, vesicular monoamine transporters (VMAT), specifically VMAT2, has also been shown to package GABA into synaptic vesicles in dopaminergic neurons [135]. Upon release, GABA activates two different classes of membrane receptors, the ionotropic $GABA_A$ receptors or metabotropic $GABA_B$ receptors, and binding of GABA to its receptors leads to hyperpolarization due to the opening of Cl^- channels.

$GABA_A$ receptors are heteropentameric ligand-gated ion channels that are heterogeneous with at least 19 known subunits. $GABA_A$ receptors are composed of two alpha subunits (α1–α6), two beta subunits (β1–β3), and one auxiliary subunit (γ1–γ3, δ, ε, π, θ, ρ1–ρ3). These isoform differences of $GABA_A$ receptors affect their properties, including agonist affinity, probability of opening, and conductance. For example, receptors containing α1, α2, α3, or α5 subunits with β and γ subunits are largely benzodiazepine-sensitive, are synaptically located, and mediate phasic inhibition in the CNS. In contrast, α4 and α6 subunits with β and δ subunits are extrasynaptic, mediate tonic inhibition, and are largely insensitive to benzodiazepine [62]. $GABA_A$ receptors are ubiquitously expressed in the CNS, with different isoform compositions being more expressed in different anatomical locations. Deficits in the expression of $GABA_A$Rs have been linked to a variety of neurological disorders, including epilepsy, anxiety disorders, depression, schizophrenia, and substance abuse [62].

$GABA_A$Rs are assembled in the ER and trafficked to the Golgi apparatus for packaging into vesicles for transportation and insertion into the plasma membrane. Notably, the trafficking of these receptors is dependent on various cellular processes, including ubiquitylation, palmitoylation, and phosphorylation events. Depending on the subunit composition, $GABA_A$Rs are clustered at synaptic or extrasynaptic sites and have either phasic inhibition or tonic inhibition, respectively. Furthermore, the clustering of $GABA_A$Rs at inhibitory synapses is also dependent

on various proteins such as gephyrin, which binds directly to receptor subtypes containing α2 to regulate synaptic targeting.

GABAergic neurotransmission in the CNS is an important therapeutic target for various neurological and psychiatric disorders, including epilepsy, anxiety disorders, schizophrenia, and substance abuse. For instance, in status epilepticus (SE) and temporal lobe epilepsy patients, a significant imbalance in excitatory/inhibitory activity has been observed with unique alterations in $GABA_AR$ trafficking [98, 130]. In schizophrenia, significant reductions in mRNA levels of GAD67, an important enzyme for GABA synthesis, have been observed in the prefrontal cortex of schizophrenia patients [141]. Notably, disruptions in GABAergic transmission have also been shown to cause dysfunctions in metabolism. Thus, studies on fast inhibitory GABAergic neurotransmission will provide key insights on how fine-tuning synaptic strength can be used as a therapeutic approach for both neurological and metabolic diseases and lead to the development of novel therapeutic drug targets.

4.3 Synaptic Dysfunction: A Hallmark for Metabolic Diseases

Approximately one hundred billion neurons within the human brain act in precise arrangements to generate and organize our every thought, memory, emotion, and behavior. Given the critical role synapses play in maintaining normal neurophysiology as we discussed above, it is not surprising that loss of synapse integrity may contribute to many neurodegenerative diseases. Synaptic dysfunction and/or loss is a common feature of neurological disorders, such as Alzheimer's disease [113], motor neuron disease [38, 40], Huntington's disease [83], and Parkinson's disease [10, 25]. However, accumulating evidence has indicated that metabolic disorders, such as diabetes, insulin resistance, and obesity, are potential risk factors linked to neurodegenerative diseases [102, 120]. Intriguingly, meta-analysis of BMI GWAS studies provides strong support for a role of synaptic function in obesity susceptibility [86], suggesting that synaptic features are not only potential therapeutic targets for neurological disorders but also contribute to metabolic dysfunctions.

4.3.1 Synaptic Dysfunctions in Metabolic Disorders: Obesity

Obesity is an imbalanced metabolic state in which excess body fat has accumulated to the extent that has profound consequences on both quality and length of one's life. During the past decades, compelling evidence suggests that the CNS, at least in part, induces fat accumulation [33, 41, 65, 110]. Of the various brain regions, the hypothalamus emerged as a critical site responsible for energy homeostasis, given the discovery in 1994 of a satiety hormone, leptin, which is produced in adipose

tissue and acts exclusively in the CNS to regulate energy balance by inhibiting food intake and promoting energy expenditure [16, 53, 104, 159]. Mice homozygous for the obese spontaneous mutations exhibit obesity, hyperphagia, hyperglycemia, and glucose intolerance, thus recapitulating human morbid obesity. Additionally, exogenous administration of leptin in *ob/ob* mice rapidly decreased food intake and rescued body weight [16, 53, 104]. Thus, *ob/ob* mice and their wild-type littermates provide an excellent model to investigate the synaptic dysfunctions in hypothalamic circuits and their contribution to obesity.

Early studies focused on two distinct populations of neurons, the agouti-related peptide (AgRP) neurons and proopiomelanocortin (POMC) neurons, which are part of the central melanocortin system and localized in the arcuate nucleus (ARC). The ARC is situated near the third ventricle and median eminence, allowing ARC-containing neurons to sense peripheral signals from hormones such as ghrelin, insulin, and leptin. AgRP and POMC neurons have been shown to control energy balance and glucose homeostasis, in that elevated AgRP/NPY activity and reduced POMC activity increased feeding and fat deposition. Consistently, leptin was found to increase the firing rate of POMC neurons [21] while inhibiting the firing rate of AgRP neurons during fasting [9]. In *ob/ob* mice, compared to wild-type littermates, *Npy* and *Agrp* mRNA expression levels were increased, whereas *Pomc* mRNA levels were decreased. Indeed, leptin treatment normalized these mRNA expression levels [51]. To more easily and precisely identify the two populations of neurons, two lines of transgenic mice expressing GFP under the transcriptional control of either POMC or NPY genomic sequence were generated.

In 2004, a seminal study was performed, in which synaptic dysfunctions in AgRP and POMC neurons were shown to contribute to obesity in *ob/ob* mice. Intriguingly, patch-clamp electrophysiology recordings in slice preparations showed that *ob/ob* mice differed from wild-type mice in the number of excitatory and inhibitory synapses, as well as postsynaptic currents onto AgRP and POMC neurons [105]. In addition, the synaptic density was rapidly normalized within 6 h to several hours after leptin was delivered systemically to *ob/ob* mice. This study suggested that (1) synaptic organization is required for maintaining normal energy balance and synaptic dysfunction could potentially contribute to obesity pathologies and (2) peripheral signals from hormones such as leptin can modulate synaptic plasticity.

Indeed, stable leptin expression levels are not only required for synaptic organization in adult mammals but also critical for the formation of hypothalamic circuits during development. In *ob/ob* mice, the neural projections from the ARC to the paraventricular nucleus of the hypothalamus (PVH) were found permanently disrupted, and leptin treatment in adulthood failed to reverse these effects. However, treatment of neonates with exogenous leptin rescues the development of ARC projections [14]. These data provide evidence that leptin functions as an essential factor for brain development, promoting the formation of hypothalamic circuits that later convey leptin signals to brain regions regulating food intake and energy consumption.

In addition to the aforementioned finding that synaptic dysfunction in the *ob/ob* mouse model is due in part by leptin deficiencies during development, a positive

energy balance caused by overeating in adulthood has also been linked to changes in synaptic coverage of POMC and AgRP neurons. Animals maintained on a high-fat diet (HFD) develop obesity with increased accumulation of fat in the adipose tissue. Diet-induced obesity in rodents recapitulates several features of human obesity, including the fact that obesity-prone (DIO) and -resistant (DR) types are inherited as polygenic traits that can arise from a single population [78–81]. These animals present suitable models for studying the pathogenesis of human obesity and the synaptic roles in regulating body adiposity. When comparing the DIO and DR animals, a distinct difference in the quantitative and qualitative synaptology of POMC cells was identified, with a significant increase in the number of inhibitory inputs in POMC neurons of DIO rats compared to DR rats [58]. In addition, when exposed to a HFD, a significant loss in synapse number was observed in POMC neurons of DIO rats. Furthermore, the neighboring NPY/AgRP neurons from mice fed a HFD exhibited decreased excitatory inputs without changes in inhibitory inputs. Another recent study looked at synaptic plasticity in the ARC of naive animals treated with a short-term HFD by assessing its molecular and functional signature [11]. In this study, they found that HFD for 3 days rewired the hypothalamic arcuate nucleus, increasing the anorexigenic tone due to activated POMC neurons. Notably, polysialic acid molecule (PSA) was identified as a mediator of this effect, and disruption of PSA signaling limited metabolic adaptation to HFD, as treated mice failed to normalize energy intake and showed increased body weight gain after a HFD challenge. These findings reveal that defects in the hypothalamic plasticity-driven adaptive response to HFD are obesogenic and could be involved in the development of metabolic diseases.

4.3.2 Synaptic Neurotransmission Is Essential for Regulation of Energy Balance

Over the past decade, several studies have shown that glutamatergic and GABAergic transmission play crucial roles for the maintenance of energy balance. Both glutamate and GABA are fine-tuned and have complex mechanisms to regulate neuronal activity during appetite regulation. These neurotransmitters control synaptic transmission, and application of their receptor antagonists into discrete hypothalamic nuclei dynamically regulates feeding. Indeed, intracerebroventricular (icv) injection of glutamate promoted food intake in rats [125]. Furthermore, icv injection of muscimol, a $GABA_A$ receptor antagonist, stimulated feeding [6]. Selective antagonists for GABA and glutamate receptors eliminated all fast, miniature, and evoked synaptic activity in hypothalamic slices [139]. These early studies suggest that both glutamatergic and GABAergic neurotransmission regulate metabolism. In this section, we highlight the current knowledge of glutamatergic and GABAergic transmission in the hypothalamus and their dysfunctions leading to the development of metabolic diseases.

4.3.3 Glutamatergic Transmission: Regulation of Energy Balance

Within the past decade, many studies have been performed to assess the importance of glutamatergic transmission in regulating energy balance. These studies focused on hypothalamic orexigenic NPY/AgRP neurons and anorexigenic POMC neurons in the arcuate nucleus. It is well known that AgRP neurons drive voracious feeding, whereas POMC neurons drive satiety. However, the role of excitatory glutamatergic input on these neurons was not yet determined. NMDAR disruption in AgRP neurons was performed by disrupting *Grin1*, a critical subunit of NMDARs. *Grin1* knockout in AgRP neurons significantly reduced body weight and food intake, with consistent reductions in fasting-induced c-Fos protein levels and decreases in *Agrp* and *Npy* mRNA expression levels. Interestingly, after 24 h fasting, a markedly increased spine number was observed on the dendrites of AgRP neurons, and disruption of NMDAR signaling abolished this increase [84]. Thus, fasting induces increased glutamatergic input on AgRP neurons, with significant increases in dendritic spine number. These findings suggest that glutamatergic synaptic transmission and its modulation by NMDARs play essential roles in controlling AgRP neurons in response to fed and fasted conditions.

Given the aforementioned finding showing that excitatory input onto AgRP neurons is important in caloric-deficiency-induced activation and is incredibly plastic depending on its caloric state, another recent study focused on identifying the source of excitatory inputs onto AgRP neurons. Intriguingly, both thyrotropin-releasing hormone (TRH) neurons and pituitary adenylate cyclase-activating polypeptide (PACAP) provide a robust excitatory drive onto AgRP neurons, and chemogenetic stimulation of these neurons in fed mice promoted intense feeding through activation of AgRP neurons [74]. These data suggest that a strong excitatory drive onto AgRP neurons exists from TRH and PACAP neurons in the paraventricular nucleus of the hypothalamus (PVH) to regulate energy balance.

Moreover, LTP and LTD studies have been performed on AgRP and POMC neurons in response to changes in fed or fasted conditions. Indeed, after tetanic stimulation in ad libitum fed mice, LTP is induced in AgRP neurons, which converts to LTD expression in food-deprived mice [107]. In contrast, the same induction protocol induces LTD in POMC neurons of fed mice. Furthermore, genetic deletion of GluN2B from AgRP neurons reduces body weight, fat mass, and food intake [137]. Taken together, a clear diagram can be put forth from these approaches regarding the feedback loop formed by AgRP neurons: hunger → hunger signals → increased spinogenesis → increased excitatory inputs → activation of AgRP neurons → feeding behaviors → energy refill → satiety signals → decreased spinogenesis → decreased excitatory inputs → inhibition of AgRP → satiety. This model is insufficient to elucidate the complexity of hunger-driving behavior but provides a path for future studies regarding the synaptic regulation of metabolic functions.

Glutamatergic neurotransmission has also been studied in the VMH and PVH, given their abundant expression of VGLUT2. Of note, VGLUT2 deletion in the

VMH, specifically in SF1 neurons, induced no changes in body weight in mice fed with chow diet, but caused mild increases in body weight in mice fed with HFD, suggesting that VMH glutamatergic neurotransmission is involved in the regulation of energy balance [133]. Additionally, MC4R re-expression in Sim1 neurons in the PVH of Mc4r-null mice drastically reduced the obese effect in Mc4r-null mice. Intriguingly, this anti-obese effect was reversed with disruptions in glutamate release in Sim1 neurons [152]. These data suggest that glutamatergic transmission in these key anatomical regions of the hypothalamus can dynamically regulate energy balance.

Glutamatergic transmission has also been studied on leptin action, given leptin's crucial role in maintaining energy balance. Notably, disruption of glutamate release using a *Vglut2*$^{fl/fl}$ line crossed with *Lepr-IRES-Cre* mice induced mild obesity due to reduced energy expenditure [151]. Indeed, these mice exhibited bouts of rapidly reduced energy expenditure as measured through oxygen consumption, as well as reduced body temperature and reduced locomotive behaviors. Altogether, these results suggest that glutamate release is required for leptin action in the CNS to regulate energy expenditure.

4.3.4 GABAergic Transmission: Regulation of Energy Balance

Given the importance of GABAergic transmission in the CNS, understanding the function of GABA release from hypothalamic neurons will likely reveal important information regarding the synaptic regulations of energy balance. Indeed, for GABA to be released from synaptic terminals, they must first be packaged into vesicular transporters. For GABA release, there is only one vesicular GABA transporter (VGAT), although vesicular monoamine transporter 2 (VMAT2) has also been shown to be required for GABA release in other brain regions [135]. Transgenic mice have been created to study the effects of VGAT disruption in various hypothalamic neurons in the CNS [134]. Of note, in the hypothalamus, a majority of ARC neurons are GABAergic, while the DMH contains both glutamatergic and GABAergic neurons. Using deletion of VGAT to disrupt GABA release, studies are beginning to highlight the role of GABA in regulating energy balance [133, 134].

Studies on GABA release have been performed in AgRP neurons, given that GABA is co-released with AgRP and NPY. To elucidate the role of GABA release in AgRP neurons, mice with genetic disruption of GABA release were generated by disrupting VGAT in these mice [134]. Interestingly, disruption of GABA release in AgRP neurons induced leanness on both chow and HFD, suggesting that GABA in AgRP neurons is required for maintaining energy balance. Furthermore, ablation of AgRP neurons and application of bretazenil (a $GABA_AR$ partial agonist) to the parabrachial nucleus (PBN) were able to sufficiently restore feeding [147]. Additionally, disruptions in GABA biosynthesis (GAD 65 and GAD67) in AgRP

neurons induced dramatic loss of body weight due to reduced food intake and elevated energy expenditure in young mice [92]. Of note, genetic inactivation of GABA signaling in AgRP neurons of older mice had transient reductions in feeding and body weight. These results suggest that GABA release is required from AgRP neurons to regulate feeding and that age-dependent mechanisms exist for GABAergic signaling in AgRP neurons.

Studies were also performed highlighting the role of GABA release mediating leptin action in the CNS to regulate energy balance. Of note, mice with LEPR deletion in all GABAergic neurons displayed a severely obese phenotype, suggesting that GABAergic neurons mediate a majority of leptin action to regulate energy balance [142]. Future studies are necessary to determine the specific GABAergic neuronal population that mediates this effect.

Additional effects of GABAergic neurons mediating leptin action were observed through the genetic identification of GABAergic RIP neurons in the ARC that selectively regulate energy expenditure [72]. Indeed, disruption of GABAergic signaling in these neurons reduced energy expenditure as measured through oxygen consumption and iBAT temperature, severe obesity, and blunted responses to leptin regulation of body weight, food intake, and thermogenesis. Furthermore, ARC RIP neurons monosynaptically innervate PVH neurons that project to the NTS to mediate its effects on energy expenditure, but not feeding. These results suggest that GABAergic RIP neurons in the ARC selectively drive energy expenditure, contribute to leptin action on energy balance and thermogenesis, and protect against diet-induced obesity.

4.4 Peripheral Signals and Synaptic Plasticity

The central nervous system and peripheral tissues are in constant crosstalk to coordinate adaptive changes in food intake and energy expenditure in response to unpredictable, altered metabolic conditions. Metabolic signals from peripheral tissues, such as the liver, pancreas, adipose tissue, gut, and muscle, are integrated into the brain and ensure that appropriate energy balance is maintained. This peripheral modulation is reflected by the ability of neurons to modulate synaptic strength in response to frequent changes in short- and long-term signals of nutrient availability. Our understanding of synaptic modulation has lagged behind that of neural circuitry, as historically more efforts have been put into dissecting distinct groups of neural circuits and delineating their regulation of relevant metabolic functions. Nevertheless, recent studies support the idea that synaptic remodeling occurs in response to altered metabolic states. Intensive studies have revealed different signaling pathways from various hormones and nutrients such as leptin, ghrelin, insulin, and glucose. However, due to the heterogeneity and complexity of neural circuitry, these hormones and nutrients use different mechanisms to act on different neuron populations in response to a given signal. Additionally, determining whether signaling is required at pre- and/or postsynaptic sites in a given group of neurons adds

another layer of complexity. Thus, more precise and systematic identification of signal-induced synaptic plasticity underlying synaptic regulation of metabolic behaviors is essential. Here, we briefly discuss the key findings and their implications.

4.4.1 Leptin

Since the adipocyte-derived hormone leptin was first identified [159], the brain was thought to be the main orchestrator of metabolic functions. Indeed, the central melanocortin system in the arcuate nucleus of the hypothalamus has been shown to be critical in maintaining energy balance [34, 59]. The neuropeptides αMSH [118, 155] and AgRP [101, 117] and the neurons that express them (POMC and AgRP neurons) in the arcuate [13, 48, 89, 149] were subsequently found to play key roles in regulating energy balance, thus raising the possibility that these two neuronal populations might serve as first-order, leptin-responsive neurons. This idea was mainly supported by the following: (1) αMSH and AgRP are the sole endogenous ligands of MC4R [96, 101]. (2) LEPRs are abundantly expressed in AgRP and POMC neurons [8, 31, 144, 145]. (3) Leptin can inhibit AgRP neurons and excite POMC neurons, respectively [21, 129, 140]. Unfortunately, after deletion of LEPR in both POMC and AgRP neurons [7, 138], the mice showed no effects on body weight and food intake, suggesting that there are likely other important first-order, leptin-responding neurons. However, given the broad expression pattern of LEPRs within the hypothalamus [32, 37, 42, 55, 77, 97, 111], to determine the "key neurons" mediating leptin's anti-obesity actions will be challenging. The realization that a distributed network of leptin-responsive neurons might account for leptin's effect [76] provided a deeper logic underlying the mode of neural communication regarding energy balance. Thus, new approaches or different angles are needed for narrowing in on these leptin neural networks.

Since glutamate and GABA are the two primary neurotransmitters adjusting the excitatory/inhibitory action of a neural circuit, it is of interest to determine if disturbed neurotransmission correlates with changed metabolic states. After depleting LEPR from glutamatergic and GABAergic neurons, separately, leptin's anti-obesity effect was found to be mediated predominantly by $VGAT^{+}$ neurons rather than $VGLUT2^{+}$ neurons [142]. Moreover, GABA transmission was proved to play an important role in mediating leptin's action by conditional knockout of vesicular GABA transporter in LEPR expressing neurons [150]. More studies are needed to determine the subcellular site of action and the mechanism in which leptin modulates GABA transmission.

The earliest evidence of plasticity in hypothalamic circuits came from whole-cell patch recordings that showed a rapid synaptic transmission in the hypothalamus in response to leptin [44]. POMC and AgRP neurons in the ARC of the hypothalamus [52] are both principle sites of LEPR expression and the source of potent neuropeptide modulators. Indeed, melanocortins and neuropeptide Y exert opposing effects

on feeding and metabolism [19, 65]. Interestingly, after acute leptin treatment, Socs3 mRNA levels were increased in both POMC and AgRP neurons, but c-Fos protein was increased only in POMC neurons, suggesting that leptin has distinct actions on these neurons by activating POMC neurons, but not AgRP neurons [31]. Leptin activated POMC neurons through a nonspecific cation channel and reduced inhibition by local NPY/GABAergic neurons [21], suggesting both pre- and postsynaptic roles for leptin action on POMC neurons.

The excitatory and inhibitory synapses, as well as postsynaptic currents on AgRP/POMC neurons, were later quantified by using fluorescent-tagged transgenic mice [105]. *ob/ob* mice differed from wild-type mice in the number of excitatory and inhibitory synapses and postsynaptic currents onto NPY and POMC neurons. In addition, leptin treatment rapidly normalized the changed synaptic density, suggesting that leptin can rapidly modulate synaptic inputs. Interestingly, a marked reduction in the number of excitatory synapses on POMC neurons of *ob/ob* mice did not translate to substantially decreased sEPSCs, suggesting disruptions in synaptic connectivity onto POMC neurons. Further evidence supporting this idea was observed when shorter dendritic spines were found in POMC neurons compared with AgRP [84]. In addition, these inhibitory inputs mediate the effects of leptin in POMC neurons indirectly, in that genetic disruption of leptin receptors in POMC neurons does not affect inhibitory currents, nor does it impair leptin's ability to suppress fasting-induced increases in sIPSCs [142]. In contrast, deletion of LEPR in all GABAergic neurons leads to hyperpolarization of POMC neurons at baseline conditions and a failure of leptin to suppress fasting-induced inhibitory currents [142]. These observations indicate that GABAergic inputs play a fundamental role in modulating postsynaptic currents on POMC neurons.

On the other hand, increased excitatory and decreased inhibitory inputs were observed in AgRP neurons of *ob/ob* mice, and leptin could normalize these changes. AgRP neurons are inhibited by leptin [140], and the activation of ATP-sensitive potassium channels (K_{ATP}) was suggested to mediate this inhibition [121]. To support this hypothesis, recent studies have shown that leptin potentiates GABAergic transmission in the developing hippocampus, likely via an increase in the number of functional synapses, thus providing evidence that leptin's action on GABAergic transmission occurs both pre- and postsynaptically through distinct mechanisms [49]. A question worth exploring is whether the same mechanism exists in hypothalamic neurons to regulate energy balance.

4.4.2 Ghrelin

Ghrelin, the endogenous ligand of growth hormone secretagogue receptors (GHSR), regulates energy balance through modulation of hypothalamic circuitry [22]. Indeed, the firing rate of AgRP neurons is elevated in food-deprived mice [129], and consequently, serum ghrelin concentrations are increased by fasting and normalized by re-feeding [136]. Moreover, in slice preparations, AgRP and POMC neurons are

activated and inhibited by ghrelin, respectively [22]. These studies highlight that ghrelin might serve as a bridge linking food deprivation and synaptic plasticity in AgRP neurons. In support of this idea, recent studies showed that either food deprivation or ghrelin treatment in fed mice could increase sEPSCs in AgRP neurons, which can be occluded by pretreatment with a Ghsr1 antagonist [154]. These results suggest that ghrelin mediates deprivation-induced synaptic plasticity. To gain further insight into the signaling mechanisms involved, a presynaptic AMPK signaling was described to mediate ghrelin's effect on synaptic upregulation and AgRP neuron activation. In contrast, genetic disruption of NMDARs on AgRP neurons caused a reduction in fasting-induced firing rate and dendritic spine number [84], indicating a postsynaptic effect from ghrelin. Furthermore, a postsynaptic AMPK→p21 pathway was elucidated to drive fasting-induced synaptic plasticity in AgRP neurons [73]. Taken together, these data suggest that ghrelin plays an important role in mediating glutamatergic transmission in AgRP neurons. Future studies are needed to (1) uncover the relationship between GHSR signaling and NMDAR activation and to (2) identify whether NMDAR activation is required for AMPK activity.

Ghrelin is also known to play a role in the brainstem to mediate changes in synaptic function. Indeed, brainstem catecholamine (CA) neurons regulate food intake and act through NTS CA neurons [23]. Ghrelin was shown to inhibit the frequency of spontaneous EPSCs on these neurons, an effect that was blocked when applying the GHSR antagonist, d-Lys-3-GHRP-6. Consistently, application of d-Lys-3-GHRP-6 decreased the basal firing rate of NTS CA neurons and decreased the frequency of mEPSCs, suggesting that ghrelin's actions are presynaptic to regulate glutamate release. These data suggest that ghrelin can inhibit NTS CA neurons and regulate energy homeostasis by modulating glutamatergic neurotransmission.

4.4.3 GLP-1

Glucagon-like peptide 1 (GLP-1) is an incretin hormone and a posttranslational cleavage product of preproglucagon, encoded by the *Gcg* gene. GLP-1 is produced in L-cells in the intestine, as well as in the hindbrain by NTS neurons. GLP-1 is composed of two alpha helices from amino acid position 13–20 and 24–35. Interestingly, GLP-1 is the only incretin known to decrease blood glucose levels in a glucose-dependent manner through enhancement of insulin secretion. Therefore, GLP-1 is an important therapeutic target for patients with type 2 diabetes and is also associated with weight loss and lower risks of hypoglycemia.

In the CNS, GLP-1 plays an essential role in regulating food intake and body weight, in that application of a GLP-1R agonist suppresses feeding and attenuates body weight gain [94, 112]. Interestingly, a recent study has shown that stimulation of GLP-1 expressing neurons in the hindbrain that project to the PVH suppresses food intake independent of glutamate release [85]. Furthermore, GLP-1R activation strengthens the excitatory synaptic strength in PVH corticotropin-releasing hormone (CRH) neurons. Additionally, GLP1-R activation was shown to promote

GluA1 membrane trafficking by phosphorylation of S845, suggesting that GluA1 S845 is likely a specific target of GLP1-R signaling in the brain. Intriguingly, depletion of GLP1-R in the PVH induced obesity due to increases in food intake and reduced locomotor activity. Thus, these data unravel the cellular mechanisms underlying GLP-1 action from NTS neurons projecting to the PVH, as well as its ability to modulate glutamatergic transmission and, in turn, regulate energy balance.

4.5 Synaptic Pathology as a Shared Mechanism in Neurologic Disorders

While various neurodegenerative diseases exhibit distinct causative factors and late-phase pathologies, investigating the early-stage pathogenesis reveals a coalescent point: synaptic dysfunction. Synaptic accumulation of disease-related proteins is found to induce synaptic loss and cause disruptions in synaptic plasticity by altering various synaptic machineries, including pre- and postsynaptic receptor trafficking, synaptic transmission, and neurotransmitter release and uptake.

Although current therapeutic programs are in place to treat these various neurodegenerative diseases with varying levels of success, novel approaches include combined efforts in attempt to explore common mechanisms for treatment of early-stage neuropathologies. Since less than 5% of cases of neurological diseases are traceable to genetic causes, identification of other factors linking neuropathological processes is critical. Epidemiological research supports the hypothesis that vascular- and lifestyle-related factors, such as obesity, diabetes, and hypertension, are associated with the development of neurological disorders, including dementia and cognitive decline [119, 120].

4.5.1 Adiposity and Cognitive Decline

Adiposity refers to the amount of adipose tissue in the body, which is associated with higher risk of type 2 diabetes (T2DM) and hypertension. BMI is normally used to indicate the adiposity of the whole body, and both high and low BMI have been associated with increased risk of cognitive decline [46, 50, 88, 99, 103, 123]. As the largest endocrine organ, adipose tissue stores various secreting hormones, cytokines, and growth factors [50], which are linked to various dysfunctions in metabolism, such as obesity, insulin resistance, inflammation, and dementia [75]. Leptin, an important adipokine stored in adipose tissue, is known to regulate appetite, energy balance, and neuroendocrine functions. A growing body of evidence suggests that leptin may also play a role in learning and cognition. Firstly, leptin receptors (LEPRs) are ubiquitously expressed in the brain, including the hippocampus and neocortex [43]. In addition, direct administration of leptin into the CA1 region

has been shown to improve learning and memory in mice [35], and impairment of leptin signaling in hippocampus reduces LTP, LTD, and spatial memory [82]. Moreover, at a cellular level, leptin enhances the function of NMDARs [115] and reduces the production of Aβ in AD transgenic mice [36].

4.5.2 Insulin Resistance and AD

Insulin resistance, which usually occurs in type 2 diabetes mellitus, has been found to increase the risk of the AD and promote cognitive dysfunctions [87, 143]. Insulin can accelerate Aβ peptide trafficking to the plasma membrane from trans-Golgi network. Moreover, the secretion and degradation of Aβ can be increased and inhibited by insulin, respectively [126]. On the other hand, Aβ can compete with and inhibit insulin binding to insulin-degrading enzyme (IDE), which could potentially mediate brain insulin resistance in AD [148]. In support of this idea, studies in situ demonstrated an increased IDE immunoreactivity surrounding Aβ and reduced IDE expression in AD hippocampi [12, 20]. Insulin resistance also promotes lipolysis, which then generates toxic lipids, such as ceramides. Since ceramides are lipid soluble, they can readily cross the blood-brain barrier (BBB), and recent evidence suggests that ceramide accumulation might lead to brain insulin resistance and increased oxidative stress, DNA damage, and lipid peroxidation.

4.5.3 Hypertension and Cognitive Dysfunction

Hypertension and elevated blood pressure are the major players for cerebrovascular disease (CVD) and are prevalent among people aged 65 years and over. Long-term population-based follow-up studies have suggested that high blood pressure contributes to an increased AD risk later in life [68, 108]. The involved mechanism has not been clearly elucidated. A possible idea is that hypertension leads to AD through vascular damage, which makes the brain more sensitive to ischemic injury even with normal pressure. Evidence from animal models have shown that deposition of Aβ increases with hypertension and, in turn, induces vascular dysfunction that impairs functional hyperemia [60].

4.6 Technologies in Modern Neuroscience

Our understanding of the CNS at the cellular, molecular, and circuit levels has been propelled by the advent of technological capabilities. A number of exciting innovations have recently emerged, which are well-suited to help neuroscientists investigate neural complexity at each hierarchical level. These innovations include

advances in genetic manipulation, high-resolution microscopy, and in vivo recordings. Here, we highlight these developments and discuss areas for improvement that could further expand capabilities for neuroscience research.

4.6.1 Applications of CRISPR Systems in Neuroscience

Precise genetic modifications have paved a way for studying the nervous system at a cellular and molecular level. Recently developed novel technologies to study gene function with high efficiency and temporospatial specificity have been established. Of note, the clustered regularly interspaced short palindromic repeat (CRISPR) systems have been developed to facilitate site-specific genomic modifications [90]. The benefits of using the CRISPR-Cas9 system to study the nervous system are highlighted by several successful applications in different animal species and cell types to study synaptic and circuit function [61, 124, 128], neurodevelopment [5, 63, 114, 116], and disease pathologies [162].

In vivo gene editing with CRISPR allows the systematic genetic dissection of neural circuits with no further need to engineer germline-modified mutant strains. This experimental approach is fast, efficient, and independent of genetic background while avoiding developmental compensations. In addition, using Cas proteins, functional domains of DNA methylation, de-methylation enzymes, or histone modifiers can be easily targeted to specific DNA sequences to edit the epigenome with high spatiotemporal specificity in vivo [54, 66, 70, 71]. Moreover, the vast number of established Cre-driver mouse lines and inducible Cas9 systems [29, 106, 158], combined with powerful viral tools, such as adeno-associated viruses (AAVs), provide enormous combinatorial power to decipher the logic of complex neuronal networks.

However, to extend the advances of CRISPR system, several limitations need to be addressed. First, methods for delivering Cas proteins and RNA guides must be optimized; second, more efforts should be put on balancing the specificity and efficiency; and lastly, safety concerns have to be carefully addressed when applying to the development of human therapeutics. Addressing these issues will allow us to use CRISPR therapeutics in humans to produce novel gene therapy approaches to treat both neurological and metabolic disorders.

4.6.2 Two-Photon and Super-Resolution Microscopy

Two-photon excitation (2PE) laser scanning microscopy allows for high-resolution and high-sensitivity fluorescence microscopy in vivo and can be applied to neuroscience-related studies at a synaptic level [127]. For example, 2PE microscopy has contributed greatly to our understanding of the dynamics of single channels in individual synapses, as well as detailed studies of dendritic spines. Compared

to confocal microscopy and other systems, 2PE microscopy allows for higher-resolution and high-contrast fluorescence microscopy and penetrates deeper in the brain with less photobleaching and phototoxicity by greatly restricting light scattering.

Two-photon microscopy has several advantages: (1) the excitation wavelength (deep red and near IR) penetrates more optimally compared to visible wavelengths, thus reducing light-scattering properties; (2) excitation is limited to a small, precise space, whereas scattered light will not cause considerable fluorescence; and (3) the localized excitation (i.e., all fluorescent photons) will have robust signal detection. These specifications allow 2PE to be used for neurobiological studies to image the structure and function of dendritic spines in brain slices.

2PE allows for several applications in modern neuroscience. Indeed, 2PE microscopy has been combined with Ca^{2+} imaging to reveal spine function in a single synapse. Furthermore, electrophysiology can be performed, in which calcium imaging in single spines can be used to study excitatory transmission at a single synapse. Additionally, the use of viral transduction methods to deliver GFP and other fluorescent proteins into the neurons allows for imaging of dendritic spines with high resolution, as well as other synaptic structures. Lastly, 2PE can be combined with FRET and fluorescence lifetime imaging (2P-FLIM) to study protein dynamics in real time in vivo. Thus, 2PE laser scanning microscopy will reveal many more details of synaptic structure and function that were previously undetectable using confocal microscopy systems.

4.6.3 In Vivo Recording and Manipulation

The connectivity, function, and neurophysiological dynamics of precise neural circuits during or following discrete behavioral states can be systematically characterized by optogenetic techniques, in vivo measurement strategies, and combinatorial approaches.

In order to assess the functional properties of genetically defined neuronal populations, a Cre recombinase-dependent viral vector encoding the light-activated cation channel, channelrhodopsin (ChR2), is most widely used to genetically manipulate neurons [3, 4, 146, 160]. However, the functional role of particular neurons depends on how they are integrated within a circuit. Indeed, specific neuronal populations that project to different postsynaptic target regions can display various firing patterns. Therefore, extracellular recordings of genetically defined neurons in a projection-specific fashion can further refine the computational role within a complex circuit. To accomplish this, multielectrode recording devices combined with ChR2 are used to identify the projection-specific spikes [18, 64]. Furthermore, improvements in recording equipment as well as light sources have significantly facilitated this application. For instance, the design of wireless micro-LEDs that are incorporated with electrophysiological sensors permits the ability to control and monitor the activity of circuit elements [67]. Furthermore, the construction of

optrode devices that allow for simultaneous recording and optical stimulation has also extended the application [2].

While in vivo extracellular recordings can produce valuable neurophysiological informations, the poor spatial resolution (~100 um) of electrode arrays [47] and the diminished long-term performance and instability of the recording device in tissue [30, 132] are major limitations to hinder this investigation. Two-photon microscopy combined with calcium imaging in head-fixed, awake behaving rodents permits the detection of Ca^{2+} associated activity in neural circuits [69, 109] and allows for long-time imaging with the application of noninvasive, thinned-skull cranial window techniques [153]. As the behavioral readouts from such experiments are limited due to the fixed position during imaging, a virtual-reality system that uses linear or spherical treadmills and stimulated environment in head-restrained mice helps increase the complexity of behavioral tasks [28, 161].

Optical-fiber-based approaches are better suited for more complicated, freely moving behavioral experiments that require in vivo imaging [24]. Indeed, microendoscopes are used for this task and are composed of a relay lens with a gradient-index (GRIN) lens attached at the bottom [45]. High-speed, miniature epi-fluorescence microscopes equipped with a complementary metal oxide semiconductor (CMOS) image sensor and interfaced with a microendoscope are able to resolve simultaneous Ca^{2+} signals in approximately 1,000 neurons in the hippocampus [161]. Further technical developments, including the improvement of indicators (brighter and larger signal calcium dyes) and hardware performance, can enhance imaging techniques to probe the nervous system.

4.7 Perspectives

Homeostasis demands an internal network of communication, with sensors capable of identifying deviations from the acceptable ranges and effectors to return those deviations back within acceptable limits. In a given feedback loop, described as peripheral → CNS → peripheral, the central neural circuit is not the endpoint sensing peripheral inputs, but another start gating the outputs to maintain metabolic balance. The ability to modulate synaptic plasticity in response to frequent changes from peripheral signals adds another layer of complexity: How does synaptic plasticity affect central and peripheral functions and, in turn, modulate metabolic function? Several studies on this topic are provided in this section.

Recent GWAS studies have identified a role of the central nervous system in obesity susceptibility and implicate new genes and pathways related to synaptic function [86]. Interestingly, out of the 97 identified genome-wide significance (GWS) loci, 35 were in high linkage disequilibrium (LD) with 1 or more GWS SNPs in the National Human Genome Research Institute GWAS catalog and are associated not only with metabolic traits but also with schizophrenia and Alzheimer's disease, suggesting common links between metabolic dysfunction and neurodegenerative diseases. Furthermore, by applying multiple complementary methods,

potential causal genes at BMI-associated loci were identified using a novel Data-driven Expression Prioritized Integration for Complex Traits (DEPICT) approach. Surprisingly, the study found that the largest category comprised genes involved in neuronal processes, including monogenic obesity genes involved in hypothalamic function and energy homeostasis, and genes involved in neuronal transmission and development. Of note, many of the genes overlap with CNS processes, including synaptic function, cell-cell adhesion, and glutamate signaling, and are known to cause monogenic obesity syndromes and have been shown to induce obesity in mouse models. Overall, these data suggest that the strong enrichment of pathways among genes with associated loci highlights a causal role for these pathways and provides strong evidence for a role of CNS processes in the regulation of human body mass.

Recent technological advances have also emerged to permit detailed studies on synaptic transmission and its importance in the regulation of metabolism. Given recent evidence showing that the neural circuits controlling energy balance are highly sensitive to developmental compensations [48, 89], recent genome editing systems have been developed for knockout of important elements of synaptic transmission in a spatiotemporal-specific manner. Additionally, high-resolution two-photon microscopy has been developed and used for detailed studies in synaptic plasticity, with the capability to image dendritic spines and related synaptic structures. Inevitably, these technological advances will push the field of modern neuroscience forward, allowing for detailed studies of how synaptic plasticity and its dysfunction contribute to metabolic and neurological disorders.

Given the vast technological advances that have been developed in the past decade for the study of modern neuroscience, important questions remain to be answered: do these synaptic organizations impact neuronal activity and therefore change the metabolic phenotype? Even if this synaptic "fine-tuning action" is not strong enough to affect action potentials, the neuron might be able to sense the changing input organization. If true, then the intracellular signaling cascade that senses such rapid changes needs further investigation. Another fundamental question is whether the primary site of the synapse that is responsive to metabolic hormones is localized on the pre- and/or postsynaptic site. A detailed understanding of these neurobiological questions may lead to novel strategies for treating metabolic disorders.

4.8 Concluding Remarks

It is clear that multiple peripheral signals thru various intracellular mechanisms propagate rapid changes in synaptic plasticity. In turn, synaptic plasticity that occurs in a wide variety of neurons contributes to metabolic functions (Fig. 4.1). However, what remains to be determined is how plasticity might contribute to metabolic

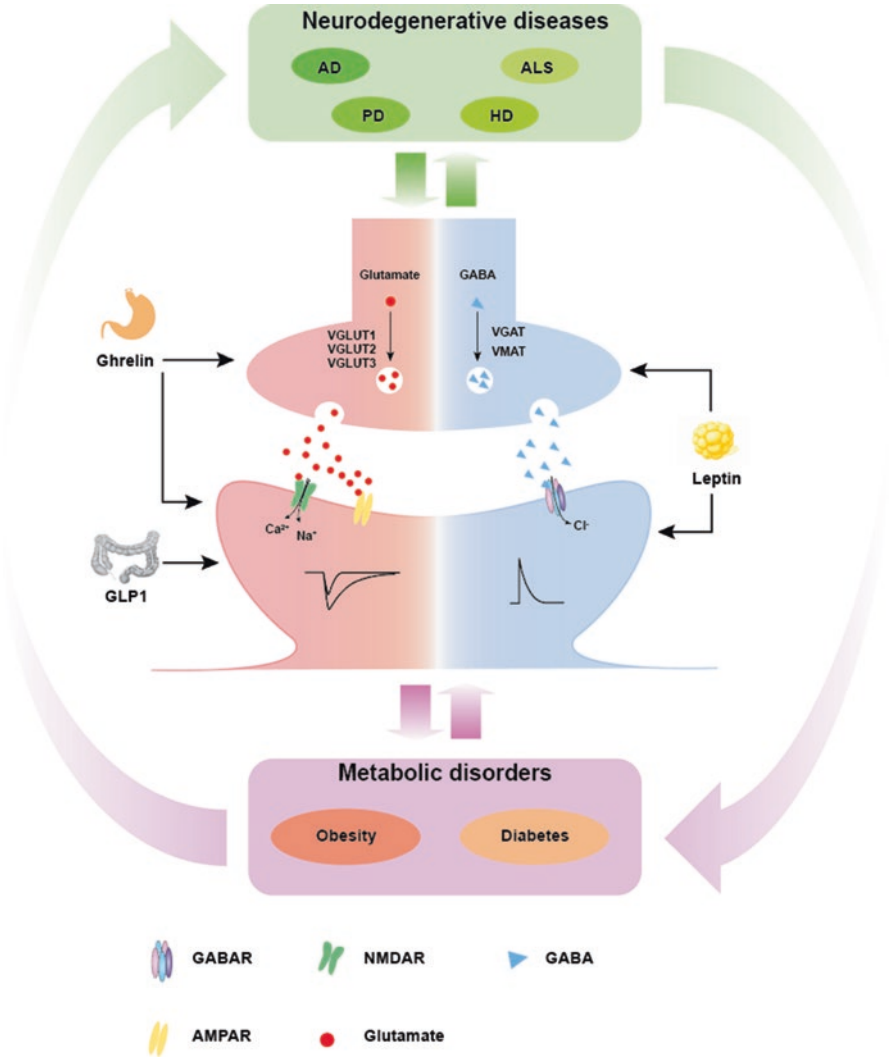

Fig. 4.1 Diagram to show synaptic transmission and the synaptic regulation of neurodegenerative diseases and metabolism

diseases. To address this question, additional tools that allow for site-specific manipulation of key molecular pathways that underlie the regulation of synaptic plasticity, in conjunction with assessment of metabolic endpoints, would provide an easier way to conceptualize the mechanisms linking these processes. Future technological innovation will create possibilities for discoveries that will cumulatively lead to a revolution in understanding the brain.

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Chapter 5
Central Circadian Clock Regulates Energy Metabolism

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Abstract Our body not only responds to environmental changes but also anticipates them. The light and dark cycle with the period of about 24 h is a recurring environmental change that determines the diurnal variation in food availability and safety from predators in nature. As a result, the circadian clock is evolved in most animals to align locomotor behaviors and energy metabolism with the light cue. The central circadian clock in mammals is located at the suprachiasmatic nucleus (SCN) of the hypothalamus in the brain. We here review the molecular and anatomic architecture of the central circadian clock in mammals, describe the experimental and observational evidence that suggests a critical role of the central circadian clock in shaping systemic energy metabolism, and discuss the involvement of endocrine factors, neuropeptides, and the autonomic nervous system in the metabolic functions of the central circadian clock.

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5.1 Introduction

Metabolic disorders, including obesity and type 2 diabetes, have reached pandemic levels in modern human societies. The underlying etiology was assumed to be an imbalance between total calorie intake and total energy expenditure. However, recent studies have suggested that the temporal pattern of calorie intake plays a critical in the pathogenesis of metabolic disorders. Restricting feeding exclusively to the active/dark phase almost eliminated high-fat diet-induced metabolic disruption without changing total calorie intake in mice [72, 170]. Conversely, restricting feeding exclusively to the normal sleep phase disturbed metabolism in animals [20, 21]. In humans, shiftwork has been associated with increased susceptibility to many metabolic disorders [83, 89, 165]. It has also been recognized that patients with cardiovascular diseases have a higher risk of heart attacks in the early morning compared to other times of the day [167] and that many diabetic patients display the dawn phenomenon, abnormal elevation of blood glucose in the early morning [117]. These findings not only demonstrate that time of the day is a critical factor in metabolism but also pose a challenge to the homeostasis concept in physiology. Instead of homeostatic, many biological processes are actually homeodynamic.

The biological system is intrinsically rhythmic rather than trying to stay in a steady state. Robust rhythmic patterns, on a time scale ranging from milliseconds to months, are observed in many biological processes such as neuronal firing, heartbeat, breathing, sleeping, feeding, reproduction, molting, and migration, across a variety of animals in nature. Circadian rhythms, with a period of about 24 h, are fundamental biological rhythms that are essential for normal energy metabolism. Most living animals sleep in a circadian pattern, with the body rotating between anabolism during the active/feeding phase and catabolism during the sleep/fasting phase.

The circadian rhythm is manifested at multiple levels. (1) At the whole-organism behavioral level, both consummatory behaviors and locomotor activities exhibit robust rhythm, which leads to diurnal patterns of calorie intake and energy expenditure. This behavioral rhythm is controlled by the central nervous system and is directly entrained by light that is perceived by the eye. (2) At the tissue and organ level, metabolic organs such as the liver, muscle, and adipose receive or release different metabolites depending on the time of the day. The direction and the rate of metabolic fluxes within the metabolic organs are driven by two sets of mechanisms: anticipatory mechanism governed by the endogenous circadian clock within the metabolic tissue and responsive mechanisms that react to external neuronal, behavioral, or endocrine factors. (3) At the cellular and molecular level, many intracellular signaling pathways are involved. The anticipatory mechanism is controlled by the molecular clock machinery that, through various transcription factors and coregulators, orchestrates rhythmic gene expression of many metabolic enzymes. The responsive mechanisms, on the other hand, are initiated by binding of an external

signaling molecule to its cellular receptor. For example, the insulin receptor at the cell membrane initiates cytosolic kinase cascades in response to elevated blood insulin levels during feeding, while the glucocorticoid receptor in the cell nucleus alters gene expression in response to oscillating glucocorticoid levels that are dictated by the central nervous system [127]. We will review recent findings on these mechanisms, with an emphasis on how the central circadian clock controls systemic energy metabolism.

5.2 Intrinsic Rhythm of Energy Metabolism

Glucose is a major fuel source for many cells, and the body has developed intricate regulatory mechanisms to maintain normal blood glucose levels. Within the normal range, blood glucose levels exhibit a clear daily rhythm in healthy human subjects as well as in animals [82, 91]. The basal fasting glucose levels peak at the onset of locomotor activities during the transition from the sleep phase to the active phase [29, 33, 92]. Interestingly, glucose tolerance also shows rhythm, with the highest glucose tolerance in the morning and the lowest tolerance at evening or night in human [102]. In one study addressing rhythmicity in glucose tolerance, blood insulin and glucose levels were measured continuously for 24 h in normal healthy human subjects under a constant glucose infusion. Blood glucose levels display around 15% elevation at around the middle of the sleep as compared to daytime levels in all subjects. The results are not confounded by the infusion rate or on the time elapsed since the beginning of the infusion because the timing of initiation of the infusion was varied to differentiate effects of the circadian time from effects of the infusion duration [178].

Insulin is the major hormone controlling blood glucose levels. Both secretion of insulin from the pancreas and the systemic insulin sensitivity display circadian rhythm [86]. In one study addressing rhythmicity of insulin throughout the circadian cycle, rats were subjected to a feeding regimen of six identical meals equally distributed over a 24 h period in order to remove the entraining capacity of food [78]. Under these conditions, basal blood glucose levels peak at the onset of the active phase, while basal insulin levels peak at the late sleep phase. Food-induced increase in blood glucose levels is almost identical across different times. Food-induced increase in insulin levels is the most dramatic during the early active phase and clearly diminished during the second half of the sleep phase [78]. These results show that the circadian regulation of basal blood glucose and feeding-induced insulin responses is independent of the feeding schedule.

Insulin lowers blood glucose levels by suppressing endogenous glucose production (EGP) from the liver and promoting glucose rate of disposition (Rd) through uptake by the muscle and adipose tissues. The glucose tolerance reaches the highest point of the day at the activity onset. This diurnal variation in glucose tolerance is unlikely to be caused by variations in insulin secretion because glucose-induced insulin secretion is almost identical across different time points in rats [93]. This suggests that the circadian rhythm of insulin sensitivity underlies the diurnal varia-

tion in glucose tolerance. Hyperinsulinemic-euglycemic clamp analysis, the gold standard assay for systemic insulin sensitivity, has been performed at different times of the day. Zeitgeber time (ZT) is used to indicate the time of the day during the normal 12 h light/12 h dark cycles with ZT0 indicating the onset of light and ZT12 indicating the onset of darkness. Clamp analysis of mice showed that the glucose infusion rate (GIR) at ZT18 is higher than ZT6 in mice. This is mainly contributed by lower EGP under the hyperinsulinemic condition at ZT18 than ZT6 because blood glucose Rd does not show changes between ZT6 and ZT18 [37]. Consistent with this study, a separate study found that fasting glucose at ZT7 is higher than that at ZT1, ZT13, or ZT19 in mice, while GIR at ZT7 is lower than GIR at ZT1, ZT13, or ZT19 [152]. Glucose Rd change is minimal, with a slightly higher Rd at ZT14 than ZT2 in rats. However, in another study, mice displayed higher glucose tolerance at ZT11 than ZT1 [7].

5.3 The Molecular Circadian Clock

At the molecular level, what gives rise to the circadian rhythm is a cell-autonomous transcription-translation feedback loop that exists in most cells of the body [166]. The core molecular clock is composed of multiple transcription factors with short half-lives. In mammals, CLOCK and BMAL1 (brain and muscle ARNT-like 1) form a heterodimeric complex and transcriptionally activate the expression of cryptochromes (CRYs), periods (PERs), and REV-ERBs (nuclear receptor subfamily 1, group D, members 1 and 2) (Fig. 5.1) [3, 11, 25, 63, 90, 149, 150, 155, 161, 168, 179]. Once the PERs and CRYs proteins have reached a critical concentration, they directly bind to the CLOCK/BMAL1 complex and block their transactivation activities. REV-ERBs, on the other hand, bind to the BMAL1 promoter and transcriptionally suppress BMAL1 gene expression [134]. Once the CLOCK/BMAL1 activity is suppressed, CRYs, PERs, and REV-ERBs themselves start to decay because their own gene expression is shut down. Once the decay induces the protein

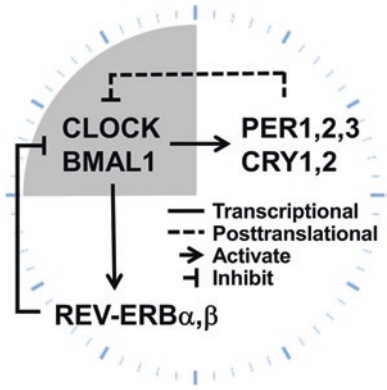

Fig. 5.1 The molecular circadian clock machinery

levels to drop below a certain threshold, their inhibition on CLOCK/BMAL1 is disarmed, allowing CLOCK/BMAL1 to be reactivated. Such negative feedback regulation forms a self-sustainable cycle that repeats itself every 24 h. These genes described above are referred to as core clock genes because they are required for the molecular clock to generate circadian rhythm. Depletion of these genes and their paralogs renders the clock nonfunctional and causes loss of intrinsic behavioral circadian rhythm in the absence of external light cues [166].

Most of the central circadian clock genes in mammals exist as paralogs including PER1/2/3, CRY1/2, and REV-ERBα/β. The transcription factor NPAS2 (neuronal PAS domain protein 2) is able to functionally substitute for CLOCK in the central clock in mice to regulate circadian rhythmicity, suggesting that CLOCK and NPAS2 can independently heterodimerize with BMAL1 to maintain molecular and behavioral rhythmicity [41]. Generally, all paralog genes need to be knocked out to confer arrhythmicity under constant darkness. The only exception is BMAL1, whose single knockout confers arrhythmicity, despite the presence of its paralog BMAL2 [25]. BMAL1 knockout mice also display hypoinsulinemia and glucose intolerance as well as abnormal locomotor activities and feeding behaviors [109]. Interestingly, constitutive expression of BMAL2 rescued the behavioral and metabolic phenotypes of BMAL1 knockout mice [151]. It was postulated that BMAL2 is regulated by BMAL1 and that BMAL1 knockout actually results in dysfunction of both BMAL1 and BMAL2 [151]. REV-ERBα/β belong to the nuclear receptor superfamily of ligand-regulated transcription factors [45, 96, 116]. REV-ERBα/β compete with RORα/β/γ (retinoic acid-related orphan receptors) for DNA binding [134, 144], which further regulates other rhythmic transcription factors including NFIL3 (nuclear factor, interleukin-3 regulated; also known as E4BP4), DBP (D-box binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor) [60, 115].

The phase, period, and amplitude for the oscillation of the core clock genes are subject to modulation by multiple environmental factors. This allows the molecular clock to be aligned with the environment, a process referred to as entrainment. Many signaling molecules can respond to environmental cues and regulate the activity or stability of the proteins encoded by the core clock genes. Currently known regulators include casein kinase 1 (CK1) and phosphoprotein phosphatase [99], FBXL3 (an F-box-type E3 ligase) [27, 64, 156], FBXW7 (another F-box protein E3 ligase) [188], O-GlcNAc transferase (OGT) [76], sirtuins [9, 122], and AMP-activated protein kinases (AMPK) [95, 177].

In addition to the upstream cytosolic signaling pathways, a number of chromatin remodelers and epigenome modifiers work closely with the core clock machinery and serve as output mechanisms for the clock to regulate gene expression [111]. The CLOCK/BMAL1 complex directly associates with various histone acetyltransferases, including p300, CREB-binding protein (CBP), and p300/CBP-associated factor (PCAF), to promote histone acetylation and transcription activation [39, 52]. CLOCK also has histone acetyltransferase activity by itself [44]. CLOCK/BMAL1 also recruits histone methyltransferases and demethylases, such as mixed lineage leukemia 1 (MLL1) and Jumonji/ARID domain-containing protein 1A (JARID1a),

which contributes to rhythmic histone methylation [43, 85]. CRY proteins can recruit enhancer of zeste homolog 2 (EZH2), a histone methyltransferase of the polycomb repressive complex 2 (PRC2), that contributes to transcriptional repression [53]. PER proteins can recruit histone deacetylase 1 (HDAC1) and methyltransferase such as SUV39h [46]. REV-ERBs can recruit nuclear receptor corepressors (NCORs) and HDAC3 that regulates expression of many metabolic enzymes in the liver and muscles [55, 73, 164, 186].

5.4 Neural Anatomy of the Central Circadian Clock

Although the molecular clock machinery operates in almost all cells throughout the body, the master circadian clock that dictates the behavioral rhythm is within the brain. The suprachiasmatic nucleus (SCN) of the hypothalamus is the site of the central circadian clock in mammals. Bilateral SCN lesions cause locomotor arrhythmicity under even the normal light/dark cycle [75]. Grafting of the SCN into arrhythmic animals restores normal circadian rhythmicity that exhibited the period of the donor genotype [100, 136], demonstrating that the SCN is the *bona fide* location of the central clock. Interestingly, grafting SCN even within a semipermeable polymeric capsule is able to restore locomotor rhythm, suggesting that diffusive factors contribute to SCN-originated signals during the control of locomotor rhythm [157]. The SCN is a paired structure with a ventral core region receiving photic input and a dorsal shell region receiving non-photic input. Most SCN neurons are positive with γ-aminobutyric acid (GABA). The core region is enriched with neurons expressing vasoactive intestinal polypeptide (VIP) and the shell region is enriched with neurons expressing arginine vasopressin (AVP) [181]. The core region projects to the shell region as well as the lateral subparaventricular zone (SPZ), while the shell region projects to the dorsomedial hypothalamus (DMH) and medial SPZ [98] (Fig. 5.2).

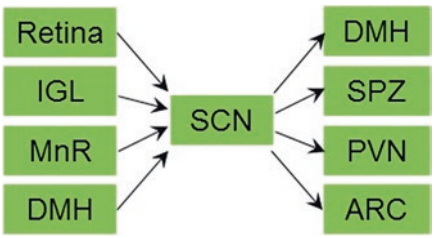

Fig. 5.2 Major inputs and outputs of the SCN
The suprachiasmatic nucleus (*SCN*) receives projections from retina, the thalamic intergeniculate leaflet (*IGL*), the median raphe (*MnR*), and the dorsomedial hypothalamic nucleus (*DMH*). The SCN projects to the DMH, the lateral subparaventricular zone (*SPZ*), the paraventricular nucleus (*PVN*), and arcuate nucleus (*ARC*)

The SCN receives many afferent neural connections [120]. The best-characterized one originates from the retina and targets the SCN with a distinct projection of the optic nerve known as the retinohypothalamic tract (RHT). This projection allows photic information, received by rod or cone photoreceptors or intrinsically photoreceptive retinal ganglion cells (ipRGCs), gains access to the central clock [106, 132]. This photic information influences phase and period of the central circadian clock, a process referred to as photoentrainment. The SCN in both diurnal and nocturnal is the same for the astronomical times [32, 40]. It is therefore believed that the downstream polysynaptic relay mediates the activity difference between diurnal and nocturnal animals [158]. Actually, some animal species can switch between diurnal and nocturnal behaviors [74].

Another robust afferent projections to the SCN is the geniculohypothalamic tract (GHT) from the thalamic intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN), regions that receive projections from retinal ipRGCs [113, 118]. It has been postulated that the GHT mediates non-photic arousal-inducing phase shifts of circadian rhythms [71]. Recently, it was shown that optogenetic activation of GHT neurons suppresses SCN responses to retinal input in a time-dependent manner, suggesting that the GHT allows the thalamic activity to gate retinal input to the SCN according to the time of day [69]. A third well-known afferent projection to the SCN is serotonergic neurons originating from the median raphe (MnR). Disruption of this input causes an earlier onset and later offset of the active phase as well as increased SCN sensitivity to photoentrainment, which demonstrates a critical role of the MnR in modulating the light sensitivity of the SCN central clock [114, 119]. Pharmacological blockade of the serotonin signaling within the SCN renders mice unable to be synchronized by wheel running without changing the overall locomotor activity, suggesting that the serotonin afferents are required for physical activity-mediated entrainment of the SCN central clock [51].

The SCN receives projections from the dorsomedial hypothalamus (DMH), an input that plays a role in food anticipatory activities (FAA). When nocturnal animals anticipate the scheduled food in the light cycle when they would otherwise normally sleep, the DMH neuronal activities increased and inhibited the SCN neuronal activity through the GABAergic inhibitory input into the SCN. DMH lesions diminished FAA, while double lesions of the DMH and SCN restored FAA. These findings suggest that DMH-mediated inhibition of the SCN activity overrides clock-controlled sleep and permits locomotor activity in the sleep phase [1]. In addition to the projections described above, the SCN has been found to be directly innervated by over 35 anatomical routes [120], which allows the central clock to integrate multiple signals received from different brain regions.

The SCN neurons send efferent projections to multiple regions of the brain. The two subdivisions of the SCN project to different hypothalamic areas, with the core projecting to the lateral SPZ and the shell to the DMH and the medial SPZ [97, 98]. The SPZ of the hypothalamus is the main efferent target of neural projections from the SCN and an important relay for the circadian timing system. The ventral SPZ (vSPZ) is critical for rhythms of sleep and locomotor activity. Interestingly, anterograde tracing with biotinylated dextran amine (BDA) showed that the anatomic

architecture from the SCN to the vSPZ are highly conserved between diurnal Nile grass rats and nocturnal lab rats despite distinct oscillation phases of the vSPZ neuronal activities, suggesting that the neural basis for a diurnal or nocturnal phase preference is independent of the anatomic structures [146]. Unlike the vSPZ, the dorsal SPZ (dSPZ) relays signals from the SCN in controlling body temperature rhythms [105].

The SCN projects to the paraventricular nucleus (PVN) of the hypothalamus, which dictates the circadian rhythm of circulating glucocorticoids. Anterograde tracing revealed a direct connection between the SCN and the PVN neurons producing corticotropin-releasing hormones (CRH) [180]. AVP released from the SCN during the light cycle represses CRH releases in the PVN in nocturnal animals. In diurnal animals, AVP stimulates CRH releases, suggesting a neurochemical difference in the PVN interneurons between different chronotypes [127]. In addition to the direct projection to the PVN, diffusive endocrine effects of the AVP, indirect multi-synaptic projections from the SCN to the PVN, as well as neuronal routes connecting the SCN with the adrenal cortex could all play important roles in the glucocorticoid rhythm [23].

The SCN also projects to the arcuate nucleus (ARC), a hypothalamic region that controls feeding and energy expenditure. A robust circadian rhythm was observed in the firing activity of the ARC neurons expressing α-melanocyte-stimulating hormone (α-MSH), with the peak activity in the late dark cycle in rats. Bilateral SCN lesions blocked this rhythm, and a direct projection from the SCN to the ARC was identified by neuronal tracing [66]. This finding suggested a potential time-dependent regulation of the appetite by the central circadian clock. The ARC also projects to the SCN. Surgical micro-cuts that eliminate these reciprocal connections lead to arrhythmicity in locomotor activities, corticosterone levels, and body temperature in the constant darkness condition in rats. Interestingly, the SCN clock gene rhythmicity was not altered by these micro-cuts, while the ARC gene rhythmicity was disrupted [24]. These findings suggest that the autonomous clock in the SCN controls the molecular clock in the ARC and potentially other brain regions through a complex neuronal projection network.

Although neurons have been considered as the major pacemaker for the central clock, astrocytes also contribute to circadian rhythms. All the core clock genes are expressed in astrocytes. Depletion of either BMAL1 or CK1ε specifically in the SCN astrocytes increased the period of both the SCN molecular clock rhythm and the locomotor behavioral rhythm [171]. Pharmacological modulation of a GABA receptor rescued the behavioral phenotype, suggesting the involvement of the GABA signaling [12]. In contrast to the SCN neurons, SCN astrocytes are more active at night as measured by intracellular calcium imaging. The SCN astrocytes suppress SCN neuronal activity by regulating extracellular glutamate levels that are sensed by a presynaptic glutamate receptor complex in the SCN neurons [19].

5.5 Evidence Supporting a Role of Central Clock in Energy Metabolism

5.5.1 Lesion

SCN lesions caused arrhythmicity in sleep and wake behaviors, consummatory behaviors, and energy expenditure [38, 108, 112]. SCN lesions also abolished daily change in plasma free fatty acids, disrupted the circadian rhythm in glucose tolerance, and blocked hyperglycemic effects of GABA-A antagonist at the PVN [80]. Bilateral SCN lesion dramatically reduced GIR and increased EGP without changing glucose Rd [38]. Transplant of the fetal SCN into the 3rd ventricle of the SCN-lesioned animals reinstated locomotor rhythm, although did not restore the rhythm of glucocorticoids or melatonin [100, 101, 157]. Parabiosis between the SCN-lesioned and SCN-intact mice showed that circulating factors or behavioral cues are sufficient to maintain the clock gene expression rhythms in the liver and kidney, but not in the heart, spleen, or skeletal muscle, indicating that the central clock communicates with different peripheral tissues through distinct mechanisms [65]. Bilateral SCN lesion eliminated circadian rhythms of blood glucose and insulin and abolished time-dependent responses to 2-deoxy-D-glucose, an inhibitor of glucose utilization [121]. The SCN-lesioned rats did not show the glucose rhythm even on a scheduled feeding regimen [92]. The rhythm of glucose tolerance was also lost in SCN-lesioned rats [93].

5.5.2 Light and Feeding

Nighttime light, shift work, and social jetlag have become prevalent since industrialization, which could disrupt our central circadian clock and contribute to the pandemic of metabolic disorders [57, 140]. Shift workers who work from 10 pm to 6 am make up about 20% of the work force in modern society [6] and have a higher prevalence of obesity and heart disease [83, 89, 165]. Dim light at night or prolonged daily light exposure promotes obesity and metabolic disorders in animal models [10, 87, 125]. Housing mice under constant light caused arrhythmicity in locomotor activity, increased food intake, reduced energy expenditure, increased body fat mass, and impaired insulin sensitivity [37, 152]. Although blood glucose displays robust diurnal changes in constant darkness in rats, light exposure at any circadian time could increase blood glucose, suggesting that light can directly regulate blood glucose independently of the circadian clock [33].

In addition to light, feeding is an important factor that entrains the peripheral circadian clocks in metabolic tissues. On the one hand, feeding is not required for rhythmic glucose or insulin responses in the normal physiological condition because

a regimen of six identical meals per day did not disrupt rhythmic glucose or insulin responses [78]. On the other hand, food can cause profound remodeling of the molecular clock in metabolic tissues [50, 169]. Scheduled feeding in particular can reverse the changes in the amplitude or phase of the peripheral clocks that were altered by the constant light housing in mice [68]. Restricting feeding only within the active phase almost eliminated high-fat diet-induced metabolic disruption without changing total calorie intake in mice [72, 170]. Conversely, restricting feeding in the normal sleep phase disturbed metabolism in animals [20, 21, 183]. These studies suggest that the feeding schedule and the light schedule need to be aligned with each other to maintain metabolic health. Night-eating syndrome (NES) is characterized by a delayed circadian pattern of food intake and is defined by consumption of 25% or more of the total daily calories after the evening meal [4]. With disrupted rhythmic patterns of sleep and eating, NES is strongly associated with metabolic disorders, with a particularly high prevalence among individuals seeking gastric bypass surgery [61, 124].

5.5.3 Sleep Disturbance

Circadian clock disruption is intertwined with sleep disturbance [174]. Human epidemiology studies have found that lack of sufficient sleep or poor sleep quality is associated with diabetes, metabolic disorders, increased appetite, obesity, and disrupted hormone levels [88]. In addition to the observational studies, experiments have also been performed in human subjects. Restricting sleep to 5 h per day for 5 days caused ~20% reduction in oral and intravenous insulin sensitivity in normal healthy human subjects [48]. In another human study, restricting sleep to 4 h per day for 5 days reduced systemic insulin sensitivity by 25%, which was mostly due to non-hepatic insulin resistance and was associated with increased fasting nonesterified fatty acids (NEFA) levels in the blood [137]. Actually, loss of one-night sleep was sufficient to cause glucose intolerance in human [31]. Elevated sympathetic tone, glucocorticoids levels, and growth hormone levels could all contribute to the sleep restriction-induced metabolic disruption.

5.5.4 Neurectomy and Pharmaceuticals

The SCN innervates both the sympathetic and parasympathetic nervous systems (SNS and PSNS) [13, 22]. Virus-mediated neuronal tracing revealed that the SCN innervated the liver via both the SNS and PSNS. Administration of GABA-A or VAP antagonists at the PVN elevated blood glucose levels in rats, which was blocked by hepatic sympathectomy, but not hepatic parasympathectomy. Silencing neuronal firing by tetrodotoxin administration at the SCN, but not the PVN, elevated blood glucose levels [79]. Consistent with this finding, tetrodotoxin administration

at the SCN, but not at the PVN, increased EGP in the glucose clamp analysis [58]. Considering the direct projection from the SCN to the PVN and the high basal blood glucose levels in antiphase with the SCN neuronal firing activity in rats, these findings support a role of the SCN-PVN-SNS-EGP signaling in governing the rhythmic blood glucose levels under the normal physiological condition. Interestingly, hepatic sympathectomy in combination with a non-circadian feeding regimen disrupted the blood glucose rhythmicity without disrupting the clock gene expression rhythmicity in the liver, suggesting the molecular clock in the liver is not sufficient for generating the blood glucose rhythmicity [29]. Of note, simultaneous hepatic sympathectomy and parasympathectomy in combination with a non-circadian feeding regimen did not abolish the blood glucose rhythmicity, suggesting that unbalanced interplay between the SNS and PSNS accounts for the elevated glucose levels [30].

In addition to the liver, the SCN also innervates white adipose tissues (WAT) and brown adipose tissues (BAT) through sympathetic nerves as shown by retrograde tracing from adipose tissues [13]. BAT is a major thermogenetic tissue critical for maintaining the body temperature that has a clear circadian rhythm culminating in the dark cycle in nocturnal animals. The thermoregulatory median preoptic nucleus (MnPO) is innervated by both VAP-expressing SCN neurons and α-MSH-expressing ARC neurons. Administration of AVP into the MnPO decreased body temperature in the dark phase, while an AVP receptor antagonist increased body temperature in the early light cycle. Conversely, administration of a melanocortin receptor agonist into the MnPO prevented the diurnal drop in body temperature, while a melanocortin receptor antagonist induced a nocturnal decrease in body temperature. These findings suggest that the opposite effects of VAP and α-MSH govern the body temperature rhythm through the MnPO [67]. The ventromedial hypothalamus (VMH) is another brain region that controls BAT activity and body temperature. VMH-specific depletion of BMAL1 in mice increased nocturnal expression of thermogenic genes in the BAT, enhanced BAT lipid oxidation, and increased body temperature especially in the dark cycle, without affecting the clock gene oscillation in the BAT. Administration of a β3 adrenoreceptor antagonist rescued the phenotype, demonstrating a role of the SNS in relaying the circadian signals into the BAT [126].

5.5.5 Genetic Animal Models

Knockout or mutation knock-in of core clock genes in mice altered glucose or lipid metabolism, which has been summarized recently [49]. Genetic deletion of core clock genes, including CLOCK, BMAL1, PERs, CRYs, REV-ERBs, or RORs, led to several metabolic abnormalities and disrupted glucose homeostasis. In addition to the core clock genes, genetic manipulation of clock-controlled output mediators could specifically affect metabolism without altering the clock itself or other clock-controlled biological processes, which has therapeutic implications. REV-ERBs recruit the NCOR/HDAC3 complex to the chromatin on a genome-wide scale in a rhythmic manner in the liver and skeletal muscles, regulating the diurnal metabolic

fluxes in lipid anabolism in the liver and amino acid catabolism in the muscle. Depletion of HDAC3 in the liver or muscle disrupted the rhythmic metabolic flexibility and caused unique metabolic disorders [55, 73, 162, 163].

In addition to the molecular output mediators, the SNS is an important anatomic mediator that connects the central clock with metabolic processes in the liver, BAT, and other peripheral tissues through adrenergic receptors in the target tissue [185, 187]. Recent studies have demonstrated diverse metabolic functions of adrenergic receptors using genetic mouse models (Table 5.1).

5.5.6 Human Genetics

Smith-Magenis syndrome (SMS) is a rare genetic disorder characterized by intellectual disability, sleep disturbance, obesity, metabolic changes, multiple congenital anomalies, and psychiatric behaviors. It is caused by a heterozygous microdeletion on chromosome region 17p11.2 containing the retinoic acid-induced 1 (RAI1) gene or mutations within RAI1. RAI1 is a transcription factor and was shown to regulate expression of CLOCK as well as other circadian clock genes. Dysregulation of the molecular clock is therefore considered as an underlying cause of many abnormalities in SMS patients [35].

Genetic association studies have identified the association of BMAL1 polymorphism with hypertension ($P = 0.0042$ for rs9633835) and diabetes ($P = 0.0036$ for rs7947951) [42, 147, 182]. A series of CLOCK polymorphisms have also been associated with susceptibility to nonalcoholic fatty liver disease, metabolic syndrome ($P = 0.0015$ for rs1801260), weight gain ($P < 0.001$ for combined rs1554483G and rs4864548A), or small dense low-density lipoprotein levels in the circulation [148, 159, 160, 175]. A recent GWAS has identified a missense polymorphism (rs10462020; Gly639Val) of PER3 associated with type 2 diabetes [16]. Another meta-analysis of multiple GWAS studies identified CRY2 associated with fasting glucose levels [47]. Genetic variants in the gene melatonin receptor 1B (MTNR1B) have been consistently shown to have a robust association with diabetes, although how it contributes to diabetes pathophysiology remains unclear [17, 18, 107, 135].

5.6 Hormones and Neuropeptides in Metabolic Functions of the Central Clock

Hormones play pivotal roles in metabolic homeostasis. A common feature for many hormones is the robust circadian rhythm that is not only due to responsiveness to environmental or behavioral variations associated with the sleep/wake cycle but also is regulated by the anticipatory circadian clock under the normal physiological condition [62]. When the normal circadian rhythm is disrupted by irregular light or

Table 5.1 Genetic mouse models of adrenergic receptors (AR)

Model	Metabolic phenotypes compared to control wild-type (WT)	Reference
α1A-AR KO	Lowered glucose uptake and GLUT translocation in the adult heart	[153]
α1A-AR OE	Increased glucose uptake, increased GLUT1 and GLUT4 membrane translocation in the adult heart	[153]
α1B-AR KO	Higher blood glucose and insulin levels during the transition from fed to fasting, higher leptin levels in the fed state, insulin resistance with impaired suppression of EGP, higher susceptibility to diet-induced obesity and glucose intolerance	[26]
α2A-AR KO	Lower basal glucose levels, abolished effects of dexmedetomidine and atipamezole on blood glucose or insulin levels	[54]
α2A-AR KO	Hyperinsulinemia, lower blood glucose levels, improved glucose tolerance	[145]
α2C-AR KO	Impaired glucose tolerance that was reversed by pretreatment with propranolol, higher adrenaline secretion, unaltered insulin secretion	[143]
α2A/α2C-AR DKO	Similar glucose and insulin phenotype as knockout α(2A)-AR alone but more sensitive to the glucose-lowering effect of insulin than WT mice	[143]
α2A, α2B, or α2C-AR KO	Ex vivo experiments demonstrating that α2A and α2C mediate the inhibitory effects of adrenaline on pancreatic insulin release	[130]
β1-AR KO	Hypothermia during cold exposure and reduced BAT thermal response to norepinephrine; more susceptible to diet-induced obesity, hypercholesterolemia, hypertriglyceridemia, glucose intolerance, hyperglycemia, and steatohepatitis; defective diet-induced thermogenesis	[176]
β2-AR KO	Lower hepatic triglyceride content and body weight during aging, lower glucose tolerance in young age and improved glucose tolerance in old age	[154]
β2-AR KO	Fasting hyperinsulinemia; higher PEPCK (PCK1) gene expression in the liver; similar glucose intolerance, body weight gain, and liver lipid content as WT on high-fat diet	[56]
β3-AR KO	Normal BAT thermogenesis, increased susceptibility to diet-induced obesity, elevated inflammation, similar glucose tolerance as WT, ameliorated hypertriglyceridemia and hypercholesterolemia on high-fat diet	[133]
β1,2,3-AR TKO (beta-less mice)	Increased fat mass, glucose intolerance, impaired glucose-induced insulin secretion, higher liver PEPCK gene expression in the fed state, enhanced insulin sensitivity	[8]

KO knockout, *DKO* double knockout, *TKO* triple knockout, *OE* overexpression

feeding schedules, the endocrine system is also altered and can contribute to the metabolic derangement in these conditions [14]. Neuropeptides and neurotransmitters are involved at all levels of the SCN clock functions, including receiving the upstream input from environmental entraining cues, synchronizing different neurons within the SCN, and relaying the SCN efferent information to other parts of the body [5, 139]. Understanding how hormones and neuropeptides contribute to the

clock function is important for successful manipulation of the clock therapeutically.

Melatonin is released from the pineal gland and is unique in that it peaks in the dark phase in both diurnal and nocturnal animals, while other hormones display the opposite circadian phase between nocturnal and diurnal animals. Melatonin release is controlled by the SCN. GABA release from the SCN inhibits the PVN-originated projections to the pineal gland during the light cycle. This rhythmic GABAergic inhibitory signal, in combination with a constant glutamatergic excitatory projection from the SCN, restricts the melatonin release from the pineal gland only during the dark cycle [77]. Suppression of melatonin secretion by pinealectomy abolished the blood glucose circadian rhythm in rats under a non-circadian scheduled feeding regimen without altering blood insulin levels [94]. Pinealectomy impaired glucose tolerance, which could be corrected by exogenous melatonin administration [59]. The chronic melatonin administration was shown to improve insulin sensitivity and ameliorate obesity in some animal models [2, 123], but was also shown to cause glucose intolerance in other studies [28, 142]. Many inbred mouse strains including the C57BL/6J mice have no detectable levels of melatonin due to genetic mutations [84, 141]. However, the depletion of melatonin receptor type 1 or type 2 in mice abolished the blood glucose circadian rhythm without altering the rhythmic expression of clock genes in the skeletal muscle, liver, or adipose tissue [128].

Glucocorticoids are another class of hormones with robust circadian rhythm and pivotal roles in energy metabolism. The blood glucocorticoid level peaks at the activity onset, which is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The corticotrophin-releasing hormone (CRH) from the PVN stimulates adrenocorticotropic hormone (ACTH) from the pituitary [77]. The SCN rhythmically releases vasopressin during the light in rats, which inhibits CRH neurons in the PVN. This control could be through diffusive vasopressin via the cerebrospinal fluid (CSF) or through the SCN to PVN neural projections [180]. PVN neurons in nocturnal animals respond to vasopressin differently from diurnal animals [81]. Glucocorticoid can partially restore the peripheral circadian rhythm due to the SCN lesion, suggesting that it could relay signals from the SCN to peripheral tissues [131, 138]. Within the target tissue, glucocorticoid binds to the glucocorticoid receptor and regulates glucose utilization as well as hepatic gluconeogenesis [127].

Orexin is a neuropeptide secreted by the lateral hypothalamus (LH) with low levels in the CSF during sleep and high levels during wake [36]. Darkness activates orexin neurons in nocturnal rodents [110], while orexin suppresses the SCN neurons either directly or indirectly through augmenting the IGL-mediated suppression of the SCN neurons [15, 129]. Activation of orexin at the onset of the active phase not only facilitates wakefulness but also adapts the peripheral glucose metabolism to the active phase [77]. Orexin deficiency caused sleep disorders, obesity, and glucose intolerance in human and mouse models [34, 70, 104, 172]. Overexpression of orexin protected mice from diet-induced obesity and glucose intolerance, mainly through the orexin receptor 2 (OX2R). Pharmacological activation of the LH neurons or orexin ICV administration increased EGP in rats, an effect that can be blocked by orexin receptor 1 (OX1R) antagonist or hepatic sympathectomy [184].

Although ICV administration of orexin during the day increased blood glucose levels in mice, orexin administration at night decreased blood glucose levels associated with reduced gluconeogenesis gene expression, an effect that can be blocked by hepatic parasympathectomy [173]. Thus, orexin bidirectionally regulates hepatic gluconeogenesis through an OX2R-sympathetic pathway in the day and an OX1R-parasympathetic pathway at night.

Acetylcholine is a major neurotransmitter of the PSNS through activation of muscarinic acetylcholine receptors (mAChRs) in target tissues. The subtype M3 is the only mAChR expressed in the mouse hepatocytes. Interestingly, knockout of the mAChR-M3 did not alter glucose tolerance or hepatic expression of metabolic enzymes in mice fed either normal chow or high-fat diet, suggesting that metabolic effects mediated by hepatic vagal nerves are acetylcholine independent [103].

5.7 Concluding Remarks

Energy metabolism is a biological process with intrinsic circadian rhythms at multiple levels, which is orchestrated by both the anticipatory mechanism from the central circadian clock and the responsive mechanism that reacts to the sleep/wake cycle or fasting/feeding cycle. The molecular clock is composed of a handful of transcription factors in negative feedback loops that exist in the brain as well as in the peripheral tissues. The central clock in the SCN of the hypothalamus is entrained by light and synchronizes with oscillators in other brain regions through neuronal projections or neuropeptides. The central clock regulates systemic metabolism through the autonomous nervous systems and endocrine factors. Disruption of the circadian clock system by irregular light or feeding schedules contributes to metabolic disorders such as obesity and diabetes. Harnessing the circadian clock system with chronotherapy or lifestyle intervention is a promising strategy for combating against these metabolic diseases.

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Chapter 6
Glial Regulation of Energy Metabolism

Yu-Dong Zhou

Abstract The major function of brain glial cells is to maintain a homeostatic milieu for neurons to work properly in response to a variety of environmental alterations. Recent studies have shown that glial cells in the hypothalamus, a brain center controlling homeostatic physiological functions, are essential for regulating energy metabolism in both physiological and pathological conditions. Astrocytes, tanycytes, and NG2-glia shuttle and/or sense key metabolic factors presented to the hypothalamus either directly, by glial metabolic enzymes, receptors, and transporters, or indirectly, by modulating the sensing ability of other types of hypothalamic cells. Astrocytes, tanycytes, and microglia are critically important in the development and maintenance of hypothalamic circuits regulating energy balance. Hypothalamic inflammation commonly associated with diet-induced obesity is manifested via hypothalamic reactive gliosis involving microglia and astrocytes, contributing to the correlated abnormal energy metabolism. Although many glial functions in energy metabolism remain to be fully elucidated, we are at the dawn of targeting glia-neuron interactions in the hypothalamus for therapeutic applications in metabolic disorders.

Keywords Energy metabolism · Astrocyte · Tanycyte · NG2-glia · Microglia

6.1 Introduction

Glial cells are a group of highly heterogeneous populations of non-excitable cells in the brain. They not only play a canonical role in providing structural or trophic support to neurons but also are involved in promoting neurogenesis and synaptogenesis [7, 28, 35], sensing nutrients and metabolic signal molecules crucial for neuronal survival and function [65, 68], and are critically important in mediating immune

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responses in the brain [92]. Essentially, glial cells in the brain function to maintain a homeostatic environment for neuronal circuits to work properly in response to a variety of environmental alterations [110]. These homeostatic regulations provided by the neuroglia range from systemic homeostasis involving bidirectional communications between the brain and the periphery and defensive homeostasis of surveilling the environment and clearing the damage to cellular and molecular homeostasis of establishing and maintaining a proper neuronal network in response to developmental cues and environmental triggers [110]. As a major brain area regulating homeostatic physiological functions, the hypothalamus constantly monitors the environment; integrates central, endocrine, and autonomic inputs; and responds with hormonal and neural regulations of a variety of fundamental behaviors essential for survival [102]. To this end, glial cells are indispensable for hypothalamic control of all major physiological functions, including energy balance regulation.

As an evolutionarily old structure that shows anatomical similarity across all vertebrates, the hypothalamus contains a core circuitry that is comprised of a number of hypothalamic nuclei communicating with other central areas and the autonomic system to regulate energy balance [42, 74, 103]. The paraventricular nucleus of the hypothalamus (PVH) receives inputs from the arcuate hypothalamic nucleus (ARH) and projects to the solitary nucleus (NTS) in the brain stem, regulating food intake and energy expenditure. The ARH and ventromedial hypothalamic nucleus (VMH) are key central targets of multiple peripheral signaling molecules and play a critical role in regulating homeostatic feeding behavior. With the current technical advances of mapping and manipulating the neuronal circuitry involving these interconnected nuclei and their inputs and outputs, we have gained an enormous amount of knowledge of how different types of neurons in these areas integrate multiple visceral sensory inputs, control autonomic outflow to the periphery, regulate the neuroendocrine outputs, and adjust their activities by various central and peripheral signaling molecules [4]. One significant example is the opposing action of the anorexic proopiomelanocortin (POMC) neurons and the orexigenic agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons in the ARH in controlling food intake [25]. However, we cannot overlook the involvement of a large number of glial cells in the hypothalamus in regulating energy metabolism. Indeed**,** glial cells outnumber neurons by a large margin within the hypothalamus [85]. Although the mechanisms by which hypothalamic glial cells regulate energy balance remain to be fully elucidated, they influence energy metabolism mainly through activation of glial receptors and transporters for various metabolic hormones and factors [23, 65]. Gliosis and secretion of inflammatory cytokines in response to high-fat diet (HFD) and other pathological insults are also hallmarks for glial activation in the hypothalamus [50, 80, 108]. Thus, in principle, hypothalamic neuroglia function in both physiological and pathophysiological conditions to regulate the homeostasis of energy balance and feeding.

In the current review, I aim to summarize the central roles of four types of hypothalamic glia in controlling energy metabolism: microglia, astrocytes, tanycytes, and NG2-glia. Emphasizes will be put on (1) sketching how astrocytes and tanycytes assist in transporting key circulating metabolic factors across the blood-brain

barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (BCSFB); (2) recapitulating how astrocytes, tanycytes, and NG2-glia regulate metabolic sensing of various nutrients and metabolic hormones that occur in the hypothalamus; (3) summarizing how microglia and astrocytes function in hypothalamic inflammation that is usually associated with diet-induced obesity; and (4) outlining how these four types of glia maintain hypothalamic circuit homeostasis. Please be advised that the above four glial functions I reviewed in this chapter sometimes are difficult to separate. For example, metabolic transporting and sensing contributed by glial cells may overlap considerably. Hypothalamic gliosis certainly alters hypothalamic circuit homeostasis.

6.2 Glial Cells in the Hypothalamus

It is a general consensus that the number of glial cells is proportional to the volume of the brain. However, the ratio of the number of glial cells to the number of neurons varies across different brain areas. By using the isotropic fractionation method to count the total number of neuronal and non-neuronal cells in human brains, Azevedo et al. were able to show that in brain areas other than the cerebral cortex and the cerebellum, the non-neuronal cells outnumber the neurons by a ratio of ~11:1 [6]. This is in drastic contrast to the ratios of ~1:1 and 0.2:1 in the cerebral cortex and the cerebellum, respectively. Although there is not much information about the ratio of the number of glial cells to the number of neurons in every nucleus of the hypothalamus in humans, careful examination of the numbers of neurons and glial cells using the stereological method has revealed a glial/neuronal ratio of about 5–8:1 in the medial mammillary nucleus (MMN) [11]. A thorough stereological counting of neurons and glial cells in the hypothalamus of the rhesus monkey unravels an overall glial/neuronal ratio of ~3–4:1 [85]. In hypothalamic areas with densely packed cells, such as the VMH and the suprachiasmatic nucleus (SCN), this glial/neuronal ratio was found to be less than 2:1. The ratio increased to ~6.2–8.5:1 in the lateral hypothalamus, where neurons are loosely packed [85]. These studies demonstrate that there are more glial cells than neurons in the hypothalamus, suggesting glial cells are critically important for hypothalamic functions.

As in other brain areas, glial cells in the hypothalamus can be generally divided into two groups: macroglia originated from the ectodermal tissue and microglia derived from the yolk sac. Neuroepithelial cells in the neural ectoderm give rise to radial glia and then differentiate into three major types of macroglia in addition to neurons in the hypothalamus: stellate-shaped astrocytes, myelin-producing oligodendrocytes, and ependymal cells lining the third ventricle. Astrocytes are the most abundant glial cell type in the brain and are heterogeneous with different functions and morphologies (e.g., protoplasmic astrocytes in the gray matter and fibrous astrocytes in the white matter) [67]. In the hypothalamus, protoplasmic astrocytes are the predominant type. Astrocytes express specific markers such as glial fibrillary acidic protein (GFAP, mainly expressed by white matter astrocytes in vivo) and

aldehyde dehydrogenase 1 family member L1 (Aldh1l1, expressed by both gray and white matter astrocytes) [67]. Oligodendrocytes insulate neurons and play a significant role in axonal action potential conduction. Oligodendrocyte precursor cells (OPCs), which express NG2 chondroitin sulfate proteoglycan and thus are also known as NG2-glia, are present in the hypothalamus and are capable of continuously producing new oligodendrocytes throughout adulthood [88]. Tanycytes, a specialized group of hypothalamic ependymal cells lining the floor and ventral side walls of the third ventricle, are commonly considered as radial glia remained in the mediobasal area of the hypothalamus (MBH) [31]. Tanycytes distinguish from other ependymal cells by their long processes extending to the hypothalamus and by expressing specific markers such as the orphan G protein-coupled receptor GPR50. Tanycytes are highly heterogeneous [18] and are commonly subdivided into α- and β-tanycytes based on their location and function. Microglia, on the other hand, originate from monocyte-lineage cells derived from the yolk sac and migrate to the brain mesenchyme during a narrow window of embryonic development. The resting microglia use their ramified processes to monitor the microenvironment for injury- or infection-induced tissue damage [12].

Although the vast majority of cells in the hypothalamus are glial cells, we know little about the molecular mechanisms underlying their development. In the mouse tuberal hypothalamus, which includes energy metabolism-regulating nuclei such as the ARH and VMH, gliogenesis generally follows a first wave of neurogenesis and begins around embryonic day (E) 13.5 [70]. This second wave of generating Sox9- and Olig2-expressing glioblasts and oligodendrocyte precursor cells (OPCs) peaks at E15.5 and followed by a third wave of astrocytogenesis producing astrocyte precursor cells between E15.5 and E17.5 [70]. Several key morphogens that drive the patterning of the hypothalamic primordium during early embryonic development have been shown to also play a significant role in promoting region-specific gliogenesis in the hypothalamus. The morphogen sonic hedgehog (Shh), which is essential for patterning of the hypothalamic area during early embryonic development, expresses at a high level in the posterior end of the hypothalamus in a later stage. Shh-expressing progenitors in the posterior mouse hypothalamus before embryonic day (E) 9.5 give rise to astrocytes in the most caudal areas of the hypothalamus, including the mammillary and posterior tuberal regions, and tanycytes in the median eminence (ME) [2]. There is a rostral shift of astrocytogenesis from Shh-expressing progenitors after E9.5, as they generate astrocytes in the tuberal and preoptic regions of the hypothalamus; but no astrocytes originated from these Shh-expressing progenitors are found in the anterior area of the hypothalamus [2]. The morphogen fibroblast growth factor 10 (Fgf10), which is essential in patterning the hypothalamus [36], controls radial glia differentiation in the cortical ventricular zone [90]. Interestingly, Fgf10-expressing tanycytes in the ME of juvenile mice maintain the neurogenic and gliogenic potential of radial glia [45, 87]. These Fgf10+ tanycytes keep generating neurons, tanycytes, and astrocytes in the tuberal hypothalamus until 2 months after birth [45] and require fibroblast growth factor signaling to maintain their proliferation capabilities [87]. Some transcription factors are also involved in regulating gliogenesis in the hypothalamus. For example, the

basic-helix-loop-helix (bHLH) transcription factor family member achaete-scute homolog 1 (Ascl1) has been shown to suppress oligodendrogenesis in the tuberal hypothalamus, as deleting Ascl1 resulted in an increase in oligodendrocyte cells [70]. Future studies are required to fully understand the developmental regulation of gliogenesis in the hypothalamus.

6.3 Microglia

Hypothalamic Inflammation Microglia in the hypothalamus are the main glial cells that mediate inflammatory processes in response to HFD [5, 39]. The three-dimensional grid-like distributions of microglia in the brain [12] make these brain macrophages perfect sentinels for continuously detecting pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in the brain parenchyma via surface receptors [81]. Indeed, microglia in the MBH can be effectively activated by excess consumption of dietary long-chain saturated fatty acids (SFAs) such as palmitic acid [107]. Direct intraventricular injection of palmitic acid has been shown to cause central leptin resistance and impair peripheral glucose and lipid metabolism [21]. Depletion of microglia in the hypothalamus, on the other hand, reduces SFA-induced inflammation and neuronal stress, leading to enhanced leptin signaling and decreased food intake [107]. Thus, microglia may function as the primary SFA sensor in the hypothalamus. By contrast, both hypothalamic neurons [22] and astrocytes [107] are resistant to SFA-induced inflammation and insulin resistance.

SFA sensing by hypothalamic microglia may depend on toll-like receptors (TLRs) that reside on the surface of these brain sentinels. SFAs such as palmitic and lauric acid have long been regarded as DAMPs that can be recognized by TLR2 and TLR4, mostly TLR4, leading to myeloid differentiation primary response 88 (MyD88)- and Akt-dependent activation of nuclear factor-κB (NF-κB) [63] and leptin and insulin resistance [72]. Knocking out MyD88 in the CNS alleviates HFD-induced weight gain and intraventricular palmitic acid-induced leptin and insulin resistance [56]. Although most TLR4 expression occurs in activated microglia in the hypothalamus [72], it will be interesting to delete TLR-mediated signaling in a specific type of glial cells to establish TLRs, and their associated signaling molecules in the glial cells are essential for SFA-induced leptin and insulin resistance in future studies [96].

SFA-induced microgliosis in the MBH requires activation of microglial NF-κB [106]. Selective attenuation of microglial NF-κB-dependent signaling reduces microglial activation, prevents recruitment of additional bone marrow-derived myeloid cells, and abrogates diet-induced hyperphagia and weight gain [106]. In addition to HFD-induced microglial activation, HFD-elicited recruitment of peripheral myeloid cells is essential for balancing cerebral glucose metabolism [51]. This HFD-induced recruitment of bone marrow-derived cells may result from

hypothalamic inflammation-induced expression of fractalkine CX3CL1 in neurons [73], implicating a critical interplay between central and peripheral innate immune systems.

Activated microglia may regulate the activities of hypothalamic neurons to control energy metabolism. Acute exposure to a microglial TLR4 agonist in the ARH inhibits the firing of AGRP/NPY neurons, whereas the same treatment increases the activity of POMC neurons in the ARH [82]. Inhibiting microglia function or blocking TLR4 restores the activity of AGRP/NPY neurons and increases food intake [82]. In contrast to the neuroprotective role played by short-term microglial activation, sustained ARH inflammation has been shown to cause neuronal injury [104]. Thus, it will be interesting to test whether microglial regulation of hypothalamic neuronal activity is dependent on the time course of microgliosis in future studies. Indeed, proinflammatory cytokines such as tumor necrosis factor (TNF)-α, which can be secreted by activated microglia [58], have been shown to bidirectionally regulate synaptic strength in a state-dependent manner [101]. On the other hand, neuronal α-melanocyte-stimulating hormone (MSH) and NPY can in turn modulate microglial cytokine secretions [27, 33]. In summary, these lines of evidence support the notion that microglia-neuron interactions in the hypothalamus are important in controlling energy balance [108].

Hypothalamic Circuit Homeostasis Microglia not only regulate defense homeostasis in the CNS but also are essential for normal CNS development and circuit maintenance [53, 79, 92]. Microglia are actively involved in synaptogenesis [47, 97] and phagocytic elimination of synapses [93] during development. Notably, high-calorie diets increase the expression CD68, a phagocytic marker of microglia, in the ARH [39], suggesting a modulatory effect of microglial activation on synaptic integrations of hypothalamic circuits. Homeostatic regulation of neural circuits may also involve functional integration of newborn neurons into the circuits. Long-term activation of hypothalamic microglia has been shown to impede neurogenesis in the MBH [66]. Deleting microglial inhibitor of nuclear factor κB (IκB) kinase β (IKKβ) in the MBH prevents the inflammatory paracrine actions of microglia on hypothalamic neural stem cells (NSCs) and promotes hypothalamic NSC differentiation and neurogenesis [66]. These findings indicate that microglial activation induced by chronic HFD consumptions contributes to the regulation of hypothalamic circuits.

6.4 Astrocytes

Metabolic Transporting and Sensing Hypothalamic astrocytes express metabolic enzymes, receptors, and transporters for many important metabolic factors, thus endowing these star-shaped glial cells with effective metabolic signal transporting and sensing properties. Indeed, astrocytes have been reported to sense and shuttle nutrients (e.g., glucose and lipids) and hormones (e.g., insulin and leptin) [65] and thus are critically important for regulating energy metabolism.

Along with endothelial cells, pericytes, tanycytes, and neurons, astrocytes are key members of the neurovascular unit (NVU) that controls the permeability of the BBB [3] and allows the dynamic passage of metabolic factors (e.g., glucose and ketone bodies) from the blood to the hypothalamus [46]. Astrocytes support endothelial function with their end feet and establish communication with local neurons and are thus key regulators of BBB development, maintenance, and regulation [3]. Transporting blood glucose across the BBB into the brain is largely dependent on glucose transporter (GLUT)-1 [1]. GLUT-1 is highly expressed in NVU astrocytes [98, 109], supporting the notion that astrocytes are actively involved in glucose influx through the BBB. Monocarboxylic acid transporter (MCT)-1, which is also predominantly expressed in NVU astrocytes [109], is the key transporter protein for the delivery of ketone bodies from the blood to the brain. Astrocytes not only play a significant role in transporting ketone bodies across the BBB along with endothelial cells in the NVU; they are also the only source of ketone body production in the brain [15]. HFD increases astrocytic ketone body generation in the VMH, contributing to altered food intake [61].

Glucose sensing is mainly performed by two types of neurons in the hypothalamus: glucose-excited (GE) and glucose-inhibited (GI) neurons [65]. Astrocytes facilitate glucose sensing by lactate shuttling from astrocytes to glucose-sensing neurons [77]. Glucose is first taken up by hypothalamic astrocytes through GLUT-2 and then metabolized to lactate. After being transported to the extracellular space by glial MCT-4 and MCT-1, lactate is shuttled into neurons by neuronal MCT-2 [64, 100]. Lactate is a source for ATP which is a key regulator for ATP-sensitive K^+ channels (K_{ATP}) channels [76]. Thus, astrocyte-neuron lactate shuttle modulates the activity of glucose-sensing neurons via K_{ATP} channels.

Astrocytes are also involved in lipid sensing. The fatty acid translocase (FAT) CD36 is expressed in astrocytes and plays an important role in fatty acid sensing [60]. Knocking out CD36 in mice results in a significant reduction in the uptake of monounsaturated fatty acids, whereas the uptake of polyunsaturated fatty acid is unaltered [99]. In addition to FAT, lipid sensing can also be conducted with lipoproteins. Astrocytes express a great amount of apolipoprotein E (ApoE), whose expression level can be upregulated by leptin [94]. Leptin-induced upregulation of ApoE in hypothalamic astrocytes reduces food intake and energy balance [94, 95]. Lipid uptake via lipoprotein lipase (LPL) in astrocytes has also been reported to regulate energy homeostasis [38]. Postnatal ablation of LPL in astrocytes exacerbates HFD-induced body weight gain and glucose intolerance [38].

Nutrient sensing by astrocytes can be further modulated by hormones. As mentioned above, lipid sensing by astrocytic ApoE can be upregulated by leptin, since hypothalamic astrocytes express various isoforms of the leptin receptor (ObR) [54]. Astrocytic glucose transporter GLUT-2, however, is downregulated by intracerebroventricular injection of leptin [37]. This leptin sensing by astrocytic ObR can also exert direct effects on hypothalamic astrocytes per se. For example, chronic leptin treatment increases the length of the processes of hypothalamic astrocytes, indicating a sign of leptin-induced homeostatic regulation [40]. In addition to leptin, insulin signaling in astrocytes is required for efficient glucose uptake into the brain [41].

Impairing astroglial insulin signaling causes a decrease in the efficiency of brain glucose uptake, thereby compromising hypothalamic glucose sensing and consequently disrupting systemic glucose homeostasis [41].

Hypothalamic Inflammation Although microgliosis is the main event of HFD-induced hypothalamic inflammation, reactive astrocytosis in the hypothalamus may also be a primary or secondary response to dietary excess. HFD feeding results in the accumulation and activation of astrocytes in the hypothalamus [17, 69, 104], alluding to the possibility that hypothalamic astrogliosis impairs energy balance. Indeed, reduction of astrocyte inflammatory signaling by astrocyte-specific deletion of IKKβ protects mice from HFD-induced hypothalamic inflammation and reduces metabolic abnormalities [30, 118]. HFD-induced astrocyte activation may represent a secondary effect following the initial microglia activation in the hypothalamus, as pure cultured astrocytes have been shown not to respond to SFA treatment [107], although direct SFA-elicited release of TNF-α and interleukin (IL)-6 in cultured cortical astrocytes has been reported [44]. Since astrocytes express TLR4 [119] that predominantly mediates MyD88-dependent activation of mitogen-activated protein kinases in young animals [96], future research is therefore required to determine whether diet-induced hypothalamic astrogliosis is age-dependent.

How do activated astrocytes regulate energy metabolism? As mentioned above, astrocytes dynamically alter their processes in response to nutritional status. HFD-induced activation of astrocytic IKKβ/NF-κB shortens the processes of hypothalamic astrocytes and reduces extracellular γ-aminobutyric acid (GABA) uptake, thus increasing extracellular GABA levels which in turn impairs brain-derived neurotrophic factor (BDNF) secretion by local neurons and the subsequent energy metabolism [118]. In addition, obesity-induced secretion of transforming growth factor (TGF)-β by astrocytes induces IκBα mRNA decay in POMC neurons, leading to NF-κB activation in the hypothalamus [114]. Thus, astrocyte-neuron interactions in response to dietary excess are fundamental events in hypothalamic inflammation.

Hypothalamic Circuit Homeostasis Astrocytes are the major glial type that function to maintain a proper working environment for neural circuits. They tightly engage with local neurons to regulate ion diffusions and neurotransmitter uptakes. They release gliotransmitters to interact with adjacent neurons and initiate key signaling events. They secrete neurogenic and synaptogenic factors in response to developmental cues and environmental triggers to assemble and remodel a fitting circuitry. All these astrocytic regulations have a common purpose: to achieve and maintain a well-regulated neural circuit homeostasis.

Similar to HFD-induced astrocyte morphological plasticity [118], astrocytes rely on their processes to regulate hypothalamic circuit homeostasis [48, 55]. Rapid remodeling of astrocytic processes affects glial coverage, thus changing synaptic and neuronal functions [105]. Indeed, deleting ObRs in hypothalamic astrocytes alters glial morphology, decreases both excitatory and inhibitory synap-

tic inputs to ARH neurons, and causes increased food intake after fasting or ghrelin administration [54].

Rapid remodeling of astrocytic processes also alters the geometry and diffusion properties of the extracellular space, thus changing extracellular ionic and neurotransmitter homeostasis, gliotransmitter release, and hence neuronal and circuit functions [105]. Hyperglycemia has been reported to downregulate astrocytic Kir4.1 channels, leading to impaired K^+ buffering and glutamate clearance by astrocytes [83]. Activation of astrocytes within the MBH using the chemical-genetic designed receptor exclusively activated by designed drug approach engages an adenosine-mediated inactivation of AGRP neurons in the ARH via adenosine A1 receptors, suppressing both basal- and ghrelin-evoked food intake in mice [115]. Thus, adjusting the astrocytic buffering capability and gliotransmitter release represents an effective way to regulate the hypothalamic energy-balance circuit.

6.5 Tanycytes

Metabolic Transporting and Sensing Tanycytes are an integral part of the BCSFB and have been proposed to play significant roles in the control of energy balance [16]. Tanycytes, mainly ME β2-tanycytes, are endowed with nutrient and metabolic signal transporting and sensing properties. Like astrocytes in the BBB, GLUT-1 [89] is expressed in tanycytes along the BCSFB; thus tanycytes are capable of transporting glucose from the blood to the CSF. Similar to astrocytes, tanycytes assist glucose sensing first by transporting glucose into the glia via GLUT-2 and then by shuttling the converted lactate from tanycytes to adjacent neurons via glial MCT-1/4 and neuronal MCT-2 [32]. Suppression of GLUT-2 expression in tanycytes disrupted the hypothalamic glucose-sensing mechanism, leading to increased food intake and body weight [9]. Tanycytes can also directly respond to glucose by releasing ATP that acts in an autocrine way to activate the purinergic P2Y receptors, evoking robust ATP-mediated Ca^{2+} waves [34, 75]. In addition, tanycytes are capable of transporting lipids. HFD-induced elevated levels of saturated and unsaturated fatty acids are associated with increased synthesis of neutral lipids and higher lipid droplet content in tanycytes [49], indicating HFD triggers an accelerated fatty acid transporting event.

Tanycytes have recently been reported to function as hypothalamic gatekeepers controlling the access of circulating metabolic hormones. Notably, tanycytes express ObR and are responsible for gating the passage of leptin into the hypothalamus [8]. Leptin binds to ObR-b receptors located in tanycyte processes that are in contact with the fenestrated capillaries at the ME, triggering clathrin-mediated internalization of leptin [8]. Leptin is then transported to the cell body and released to the CSF and hypothalamus [8]. In addition to leptin, ghrelin [24] and thyroid hormones [10] have been suggested to take advantage of the same mechanism for their shuttling

between the blood and CSF/hypothalamus. Therefore, tanycytes might represent a common gatekeeper in the ME for gating a variety of metabolic hormones.

Interestingly, the permeability of the ME barrier can be modulated by nutritional status. Low glucose levels during fasting increase the permeability of the ME barrier, allowing easy access of metabolic factors to the ARH [59]. Low level glucose-induced remodeling of the ME barrier is mediated by vascular endothelial growth factor (VEGF)-A secreted from tanycytes, as neutralizing the VEGF-A signaling blocks fasting-induced barrier remodeling and impairs the refeeding behavior [59]. These results indicate that tanycytes control the shuttling of metabolic factors either actively or passively.

Hypothalamic Circuit Homeostasis Adult neurogenesis has long been observed in the mammalian hypothalamus, especially in the ependymal layer of the 3rd ventricle [57, 112]. De novo adult-born neurons and transplanted progenitors in the hypothalamus are known to regulate energy balance [26, 78]. A recent study has identified tanycytes are self-renewing stem cells that can be regulated by nutritional and dietary status [62]. HFD rapidly induces tanycytes in the ME (the major hypothalamic proliferative zone) to proliferate and to consequently differentiate into functional neurons, as inhibiting ME neurogenesis results in decreased weight gain in HFD-fed mice [62]. Chronic HFD, on the other hand, suppresses neurogenesis due mainly to increased apoptosis of newborn neurons in the hypothalamus [71]. Thus, tanycytes regulate energy-balancing circuit by changing the rate of neurogenesis in the hypothalamic proliferative zone.

6.6 NG2-glia

Metabolic Sensing NG2-glia distribute widely in the hypothalamus [88]. A recent seminal work demonstrates that NG2-glia located in the ME play a significant role in maintaining the leptin sensing ability of ObR-expressing neurons in the ARH [29, 86]. Genetically or pharmacologically ablating adult NG2-glia in mice results in a dramatic increase in food intake and develops leptin resistance [29]. This leptin resistance is mainly due to a diminished leptin responsiveness of ObR neurons located in the ARH. Carefully examining the ME reveals that the processes of ARH ObR neurons extending to the ME degenerate when NG2-glia are ablated, causing the ARH ObR neurons devoid of leptin sensing ability [29]. This explains why the obesity risk is associated with cranial radiation therapy, as this therapy may kill NG2-glia in the ME. It will be interesting to explore whether NG2-glia in the ME assist neuronal sensing of other metabolic hormones.

Hypothalamic Circuit Homeostasis The constant self-renewing and differentiating features of NG2-glia make them an exciting glial type to engage in a variety of CNS functions in adult animals. NG2-glia are the precursor cells of myelinating oligodendrocytes and are thus important for neural circuit maintenance [84],

remodeling [116], and repair [117]. One unique feature of NG2-glia is that they listen to neurons by receiving excitatory and inhibitory synaptic inputs and may in turn respond with spiking activities [13, 52], suggesting NG2-glia and neurons have strong reciprocal interactions [14, 43, 91, 111]. Although we know little about how NG2-glia regulates hypothalamic circuit controlling energy metabolism, excess proliferation of NG2-glia in the hypothalamus has been shown to correlate with antipsychotic-induced weight gain [113]. Deleting NG2 proteoglycan in NG2-glia results in a decreased body weight in mice [19], suggesting NG2 secreted from NG2-glia has a regulatory effect on hypothalamic circuitry [86]. More experiments are called for to assess how NG2-glia regulate hypothalamic local circuitry.

6.7 Conclusions

In summary, we now know hypothalamic glial cells are actively involved in every phase of energy metabolism. Their involvement in controlling energy balance starts with metabolic transporting and sensing. Hypothalamic glia (astrocytes and tanycytes) actively assist endothelial cells in the NVU to transport metabolic fuels such as glucose and ketone bodies and function as a gatekeeper to control the entry of a number of metabolic signals. Hypothalamic glia help local neurons to sense nutrients and metabolic hormones by shuttling nutrient metabolites to neurons (astrocytes and tanycytes) and by keeping sensing integrity of neurons (NG2-glia), respectively. Some of the glial metabolic sensing events (e.g., SFA sensing) then activate robust gliosis (microglia and astrocytes) in the hypothalamus, leading to hypothalamic inflammation that alters energy balance. Hypothalamic glia (astrocytes, tanycytes, and microglia) regulate hypothalamic system homeostasis by controlling neurotransmitter levels at the synapse, releasing gliotransmitters capable of altering neuronal circuits, and directly participating in the construction of energy metabolism-regulating circuitry. The knowledge of glial control of energy metabolism greatly advances our understanding of the physiology of energy acquisition and expenditure and the pathology of metabolic disorders.

However, many key questions in the field of glial control of energy metabolism remain to be answered. One central task in the field is establishing the correlation between the glial heterogeneity and central regulation of energy metabolism. We know little about whether chemically and functionally distinct subpopulations of microglia, astrocytes, NG2-glia, and tanycytes exist in different regions in the hypothalamus [20]. We also do not know whether these heterogeneities of multiple types of glia are changing with development and in the context of metabolic conditions. Solving these problems will certainly frame a comprehensive and dynamic scheme of glial control of energy balance.

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Chapter 7
The Leptin Signaling

Jiarui Liu, Xiaoning Yang, Siwang Yu, and Ruimao Zheng

Abstract Leptin plays a critical role in the regulation of energy balance and metabolic homeostasis. Impairment of leptin signaling is closely involved in the pathogenesis of obesity and metabolic diseases, including diabetes, cardiovascular disease, etc. Leptin initiates its intracellular signaling in the leptin-receptor-expressing neurons in the central nervous system to exert physiological function, thereby leading to a suppression of appetite, a reduction of food intake, a promotion of mitochondrial oxidation, an enhancement of thermogenesis, and a decrease in body weight. In this review, the studies on leptin neural and cellular pathways are summarized with an emphasis on the progress made during the last 10 years, for better understanding the molecular mechanism of obesity and other metabolic diseases.

Keywords Leptin · Neural pathways · Cellular pathways

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7.1 Introduction

In 1969, Dr. Coleman and his colleagues performed a series of parabiosis studies on the naturally obese ob/ob and db/db mice. Both mouse models are due to the genetic mutations. These studies led to a discovery of a circulating factor in the bloodstream, which is prominently involved in the regulation of body weight. The ob/ob mice were missing the factor, while db/db mice were unresponsive to it, resulting in their obesity [1]. Subsequently, after 25 years, Friedman from the Rockefeller University confirmed the existence of this circulating factor in 1994 and named it as leptin [2].

As a member in the family of long-chain helical cytokines, leptin is a 167 amino acid peptide with a molecular weight of 16kD, encoded by the *ob* gene and secreted in adipose tissues. The *ob* gene is widely expressed in mammals, amphibians, reptiles, fish, and so on, whose sequence is highly conservative across these species [3, 4]. There are six leptin receptor (LepR) isoforms (LepRa-f) generated by alternative splicing of the *lepr* gene, which are expressed in both central nervous system (CNS) and peripheral organs, including the hypothalamus, hippocampus, brain stem, cerebral cortex, midbrain, as well as islet, liver, thyroid, heart, etc. Among these receptors, LepRb features an extended intracellular signaling domain, playing a key role in actions of leptin on energy homeostasis and the whole neuroendocrine chain. LepRa, LepRc, LepRd, and LepRf are crucial for the transportation of leptin from peripheral region to the CNS, while LepRe is a soluble type binding to circulating leptin to function [5]. In the CNS, leptin acts on neurons expressing LepR and signals via multiple neural and cellular circuits, which maintains energy balance and metabolic homeostasis through the achievement of a series of functions, including suppressing appetite, enhancing energy expenditure, decreasing synthesis of triglyceride, etc. In addition, leptin also participates in the regulation of metabolism, cognition, neuroendocrine, and immune functions [6]. Whereas, the impairment of leptin signaling is closely associated with obesity and obesity-related diseases, containing hyperlipidemia, diabetes mellitus, metabolic syndrome, and so on. Moreover, leptin resistance caused by the dysfunction of leptin signaling has been regarded as a critical contributor to the metabolic disorders mentioned above [7, 8]. Nowadays, with the rapid upsurge of global obesity epidemic, obesity is becoming a challenging public health problem. Thus, further studies of leptin signaling and leptin resistance will be necessary to understand the pathogenesis of obesity and ultimately solve the problem.

7.2 The Neural Mechanism Underlying Leptin Function

In the brain, leptin conveying the information of peripheral lipid and glucose metabolic statues activates its signaling pathways, which induce specific gene expressions, causing the inhibition of food intake, suppression of appetite, promotion of mitochondrial oxidation, enhancement of thermogenesis, and activation of

sympathetic nerves. The abovementioned function depends on the interaction of leptin with its receptors expressed in the specific neurons located in some nervous nuclei or brain areas. Until recently, a number of nuclei expressing LepR have been revealed, which exert the functions closely associated with the metabolic regulation. In the recent years, with the emerging of cutting-edge biotechnology, a group of novel LepR-expressing nuclei are also unveiled, which may greatly extend the understanding of the leptin physiology.

7.2.1 Classic Brain Nuclei Related to Leptin Function

The classic brain nuclei containing LepR-expressing neurons are as follows:

1. Arcuate nucleus (ARC): Hypothalamic ARC is a prominent structure that plays key roles in relaying leptin signals to nuclei of the hypothalamus and other brain regions, maintaining energy balance and metabolic homeostasis. There are a lot of cell types in the ARC, among which, the agouti-related peptide/neuropeptide Y (AgRP/NPY) neuron and the proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neuron mainly participate in relaying leptin signals from the periphery. These two types of neurons are named as their co-expressing of the orexigenic AGRP and NPY or the anorexigenic POMC and CART [9].

In physiological conditions, leptin activates the POMC/CART neurons whereas inhibits the AgRP/NPY neurons [9]. By the mediation of the melanocortin system, leptin signals from the ARC are spreading to the PVN, the LHA, and other brain regions. Generally, POMC neuron-derived peptides (e.g., α-MSH) activate melanocortin-4 receptors (MC4R) in the PVN, whereas AgRP released from the AgRP/NPY neuron counterbalances this process [3]. Intriguingly, at least in part, the control of the melanocortin system by leptin is mediated indirectly, via GABA- and Nos1-containing neurons [10, 11].

Notably, the AgRP neurons cause transient and major effects in the feeding as well as the metabolic regulation, while the POMC neurons lead to long-term changes of the energy homeostasis [12]. The above processes finally achieve the leptin functions, leading to a reduced food intake, an increased energy expenditure, an inhibited hepatic glucose production, as well as a lower blood glucose level [3, 13]. In addition, a recent research also discovered a novel pathway, in which leptin coordinates both melanocortin and kisspeptin to contribute to the pubertal maturation by providing the metabolically supportive regulation [14].

2. Dorsomedial hypothalamus, dorsal hypothalamic area (DMH/DHA), and median preoptic area (mPOA): The DMH/DHA and POA are a functional complex of nuclei in the hypothalamus, controlling feeding, drinking, body-weight regulation, and circadian activity. The POA is also responsible for thermoregulation [15, 16]. Under the physiological condition, leptin induces an activation of the

POA neurons, which further activates the downstream effector neurons in the nucleus raphe pallidus (RPa) partially via a projection derived from POA to DMH/DHA, promoting the sympathetic activity in the brown adipose tissue (BAT) and thereby increasing the thermogenesis [3, 15, 17]. Notably, this process is independent of food intake [18]. In addition, the DMH also contains NPY-expressing neurons that play a role in the upregulation of energy expenditure of the whole body via the sympathetic nervous system; however, this is largely independent of the mediation of leptin signaling [19].

3. Lateral hypothalamic area (LHA): The LHA contains catecholaminergic and serotonergic circuitries, which play critical roles in the mediation of circadian feeding and sex differences in feeding, and involve in the regulation of spontaneous activity [20]. LHA mainly contains orexin-expressing and melanin-concentrating hormone (MCH)-releasing neurons. Orexin synthesis and secretion can be reduced by leptin directly via LepRb in the LHA neurons or indirectly through the inhibition of the dopaminergic projection from the VTA neurons. Both reactions further reduce the food intake. Moreover, leptin also induces the release of melanocortin in the ARC POMC neurons, which subsequently suppresses the MCH expressing in the LHA neurons. Then, the reduced expression of MCH inhibits neurons activities in the nucleus accumbens (NAc), finally leading to a reduced food intake [13, 21]. In addition, the neurons producing hypocretin (Hcrt)/orexin in the LHA also regulate the corticosterone release and centrally mediate the stress response. Both Hcrt neuronal activity and Hcrt-mediated stress responses can be inhibited by leptin [22].

4. Paraventricular hypothalamic nucleus (PVH): The PVH is a pivotal nucleus in the hypothalamus, located adjacent to the third ventricle. Various leptin signals controlling energy metabolism from ARC, VMH, and DMH are relayed to the PVH [23]. In addition, many non-hypothalamic regions also project to the PVH, including sites in the forebrain and the brain stem, which are able to modulate the ARC-PVH circuit to control energy balance [24, 25].

Further, PVH integrates the leptin signals and mainly acts in the following ways. First, PVH projects to autonomic centers for regulating body temperature, cardiovascular activity, sports activity, and stress response. Second, PVH projects to the pituitary (PIT) portal system to achieve neuroendocrine effects. Third, PVH projects to the nucleus tractus solitaries (NTS) in the brain stem to induce satiety and reduce food intake [13]. Besides, melanocortin-4 receptor (Mc4R) neurons in the PVH also modulate satiety through a projection to the parabrachial nucleus (PBN) in the hindbrain [23, 26]. PVH neurons containing neuronal nitric oxide synthase 1 (Nos1) control both feeding and energy expenditure, at least in part via the PBN and the intermediolateral column of the spinal cord [27].

5. Medial nucleus tractus solitaries (mNTS) and area postrema (AP): The NTS is a series of purely sensory nuclei in the mammalian brain stem, considered as a structure in strategic position to regulate energy balance [28]. The AP is a brain medullary structure that controls vomiting and functions of autonomic nervous

system [29]. During feeding, leptin regulates gastrointestinal satiation signals (e.g., gastric distension, cholecystokinin [CCK], or glucagon-like peptide-1 [GLP-1]) by acting on LepR of the mNTS/AP neurons [30–33]. On the other hand, impairment of LepR in these neurons reduces the signals and thereby attenuates the satiety feeling, ultimately resulting in a significant hyperphagia, markedly increased body weight, and adiposity [30].

6. Ventral tegmental area (VTA): The VTA is located close to the midline on the floor of the midbrain, serving several physiological functions in the reward, motivation, and learning [34, 35]. Further, VTA is also pathologically associated with drug addiction [36]. Generally, leptin can directly inhibit the dopaminergic neurons located in the VTA [37] or also indirectly inhibit these neurons by reducing the orexin expression levels in the LHA [38]. Both processes further prevent sucrose-induced dopamine release into the NAc, downregulating feeding motivation and food reward, which suppresses appetite and reduces food intake [39]. However, LepRb-specific anterograde tracing demonstrates that most VTA LepRb neurons project to the extended CeA (extCeA), but not the NAc. Indeed, LepRb VTA neurons specifically innervate and control the cocaine- and amphetamine-regulated transcript (CART) neurons in the extCeA, primarily contributing to the reward functions [40].

7.2.2 Newly Discovered Brain Nuclei Related to Leptin Function

Recently, several clusters of neurons expressing LepR in certain nuclei have been identified as new structures involved in the regulation of metabolism, which include:

1. Parabrachial nucleus (PBN): The PBN is an area of the dorsolateral pons, which plays an important role as a hub that integrates signals from several brain nuclei, contributing to the regulation of feeding and body weight.

As mentioned above, leptin suppresses ARC AgRP neurons signaling to induce a reduction of food intake and an enhancement of energy expenditure. Notably, this process is closely associated with the activation of PBN neurons, at least in part. As reported recently, there is a critical GABAergic inhibitory projection from AgRP neurons to the PBN neurons, which relays the anorexigenic signals to achieve the leptin functions [41, 42].

Besides, the PBN also receives signals from the NTS to inhibit appetite. Generally, the NTS responds to visceral signals through the vagus nerve and responds to the anorectic serotonin from the raphe obscure (Rob) and raphe magnus (RMg). Subsequently, a subpopulation of neurons in the PBN integrates glutamatergic information from the NTS, ultimately reducing food intake via a projection to the central nucleus of the amygdala (CeA) [42–44]. In addition, Richard D. Palmiter also reports that CCK-expressing NTS neurons can directly activate calcitonin

gene-related protein (CGRP)-expressing PBN ($CGRP^{PBN}$) neurons to suppress feeding [43]. However, a recent research discovers that gut peptides activating the NTS → PBN → CeA anorexia circuit do not activate PBN LepRb neurons [45]. Thus, the concrete mechanism of this pathway still needs further study.

Moreover, hypoglycemia can directly promote the CCK release in the PBN neurons, when humans are in a state of negative energy balance. The activated PBN neurons further activate the VMH neurons, causing a counter-regulatory response (CRR) [45, 46]. Subsequently, through the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic nervous system (SNS), the CRR stimulates glucose production and inhibits glucose uptake, finally restoring a normal blood glucose level [47]. Leptin can inactivate the PBN neurons and suppress the CRR to down-regulate the blood glucose level, thus maintaining the balance of the glucose metabolism.

2. Central nucleus of the amygdala (CeA): The CeA is a nucleus within the amygdala, serving as the major output nucleus of the amygdala and participating in receiving and processing pain information. As mentioned above, leptin signals can project to the CeA via the calcitonin gene-related peptide (CGRP)-expressing neurons in the outer external lateral subdivision of the PBN, suppressing appetite and reducing food intake [44]. Besides, leptin also can stimulate the extCeA neurons through activating the VTA neurons to exert the same effects [40].

3. Hippocampus: The hippocampus is located under the cerebral cortex and in the medial temporal lobe, which is a major component of the brain for learning, memory, and space exploring. The hippocampal-dependent mnemonic functions, which are regulated by both external and internal factors, also mediate feeding behavior [48]. A recent research demonstrate that leptin signals can achieve its functions via the LepR of hippocampal neurons, inhibiting food-related memory processing and reducing food intake [49].

4. Periaqueductal gray (PAG): The PAG is an area of the gray matter in the midbrain that plays a crucial role in the analgesia, defensive behavior, reproductive behavior, and so on. Notably, the PAG LepR-expressing neuron is the largest population of LepR neurons in the brain stem. However, its functions remained unclear for a long time. A recent report finds that PAG LepRb neurons can be activated by the noxious stimuli, which finally mobilize glucose to support an appropriate response. In the process, the activated PAG neurons project to the VMH via the PBN neurons, thus inducing the SNS activation and glucose mobilization [45, 46, 50].

5. Other structures in the brain: A few brain structures expressing leptin receptors have been discovered, including the substantia nigra (SN), certain regions of the cerebral cortices, and so on. The potential roles of leptin in these structures remain to be investigated.

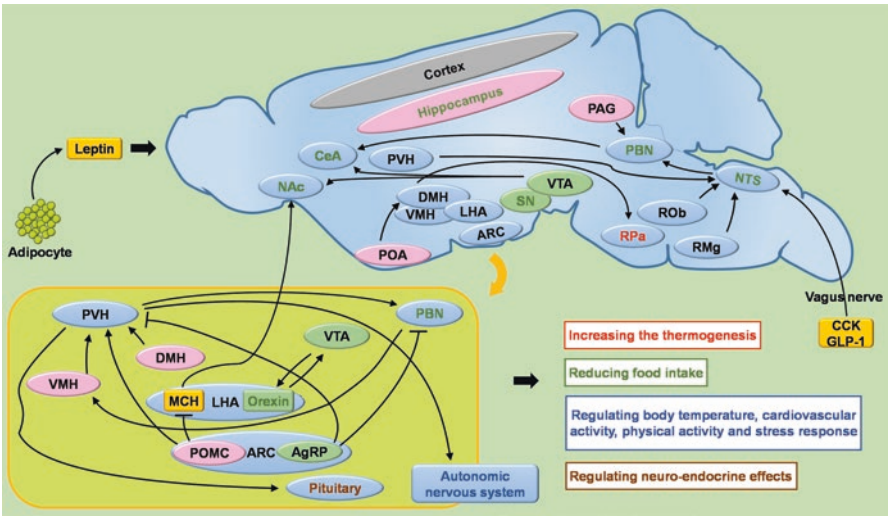

Fig. 7.1 Brain nucleus and neural pathways related to leptin function
——►: Activating. ——┤: Inhibiting. The gray background: function of the LepR-expressing neurons is still unknown in the nuclei with gray background. The green background: leptin inhibits neurons in the nuclei with green background. The pink background: leptin activates neurons in the nuclei with pink background. *ARC* arcuate nucleus, *AgRP* agouti-related peptide, *CCK* cholecystokinin, *CeA* central nucleus of the amygdala, *DMH* dorsomedial hypothalamus, *GLP-1* glucagon-like peptide-1, *LHA* lateral hypothalamic area, *MCH* melanin-concentrating hormone, *NAc* nucleus accumbens, *NTS* nucleus tractus solitaries, *POMC* proopiomelanocortin, *PBN* parabrachial nucleus, *POA* preoptic area, *PVH* paraventricular nucleus, *PAG* periaqueductal gray matter, *RPa* raphe pallidus, *ROb* raphe obscure, *RMg* raphe magnus *SN* substantia nigra, *VMH* ventromedial hypothalamic nucleus, *VTA* ventral tegmental area

7.2.3 Neural Pathways Related to Leptin Function

The classic neural pathways underlying leptin functions mainly include ARC/VMH/DMH → PVH, ARC→LHA, and ARC→PBN. In addition, a few novel pathways have also been discovered in recent years. The classic and novel neural pathways related to leptin functions are summarized and illustrated in the following figure (Fig. 7.1).

7.3 The Cellular Mechanism Underlying Leptin Function

Leptin acts in the brain via the neural structures to regulate energy balance and neuroendocrine function. Furthermore, cellular signaling pathways of leptin are also required for the achievement of these effects. Once leptin binds to the LepR, a series of intracellular reactions will be activated, thereby inducing a reduction in food intake and an increase in energy expenditure. Recently, with the technology

developing, several novel cellular structures and pathways are also reported, helping us to understand the mechanism relative to leptin effects better.

7.3.1 Classic Leptin Signal Transduction Pathways

So far, six classic signaling pathways related to leptin functions have been discovered, which include:

1. LepRb-JAK2-STAT3/5: JAK-STAT signaling is the major signaling pathway through which leptin regulates food intake and energy homeostasis in the hypothalamus. Like other cytokine receptors, LepRb does not contain intrinsic enzymatic activity. Instead, it signals via a noncovalently associated tyrosine kinase of the Jak kinase family (JAK2) [51, 52]. Physiologically, leptin binds to its receptors, which further initiates the downstream signaling through the sequential phosphorylation of the JAK2 and the signal transducer and activators of transcription (STAT), ultimately leading to a reduction of food intake and an enhancement of energy expenditure [53]. STAT molecules are cytoplasmic proteins activated by various factors including cytokines, growth factors, and hormones including leptin. The mammalian STAT family is currently composed of seven members, including STAT1–4, STAT5a, STAT5b, and STAT6, among which STAT3 and STAT5 mainly mediate leptin-induced anorectic effects [54, 55].

STAT3 is widely expressed in the CNS, while its neuron-specific deletion results in hyperphagia, obesity, diabetes, and hyperleptinemia [56]. Tyr1138 phosphorylation of LepRb mediates STAT3 activation during leptin action, whereas its substitution for serine (LeprS1138) specifically disrupts the LepRb-STAT3 signaling [54]. Generally, the activation of STAT3 induces its dimerization and translocation to the nucleus, where STAT3 modulates the transcription of genes involved in food intake and energy homeostasis, facilitating the leptin-mediated transcriptional regulation of key appetite-regulating neuropeptides such as POMC, AgRP, and NPY. In the abovementioned process, the expression of orexigenic POMC is upregulated, while the expressions of the anorexigenic AgRP and NPY are inhibited, which finally reduces food intake [54, 57, 58]. Notably, a single STAT3 activation is not sufficient to promote POMC expression. Simultaneous PI3K (phosphatidylinositol 3-OH kinase)-dependent inhibition of FOXO1 (forkhead box protein O1) expression is also required [59]. Besides, the phosphorylation of STAT3 also increases the transcription of the suppressor of cytokine signaling 3 (SOCS3), which in turn creates a feedback loop, blocking the activation of leptin-STAT signaling [60, 61]. Therefore, the JAK-STAT3 pathway is vital for the maintenance of energy homeostasis through the molecular network of leptin action. STAT5 phosphorylation also contributes to the leptin signaling, which is promoted by the Tyr1077 phosphorylation of LepRb. However, Tyr1138 and STAT3 activations attenuate STAT5-dependent transcription over the long term [55].

2. LepRb-JAK2-IRS-PI3K-Akt-mTOR-S6K: Under physiological conditions, leptin also activates the insulin receptor substrate (IRS)-phosphatidylinositol 3-OH kinase (PI3K) signaling in the hypothalamus to achieve its functions, reducing food intake and increasing energy expenditure [62]. Generally, the leptin-induced PI3K activation can be regulated by the Sar homology family member SH2B1, which recruits IRS proteins to JAK2, thereby promoting IRS phosphorylation and subsequent PI3K activation [63]. In leptin-sensitive neurons, PI3K catalyzes the phosphorylation of phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). The process further enhances sympathetic outflow to the white adipose tissue (WAT), resulting in brown adipose tissue-like transdifferentiation of WAT, ultimately leading to a reduced body fat mass [64].

Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a critical role in multiple metabolic processes, including glucose metabolism, apoptosis, cell proliferation, as well as cell migration. Akt is a PI3K-dependent kinase, and the PH domain of AKT is a direct target of PIP2 and PIP3. With the phosphorylation of PIP2 to PIP3, the rate of Akt activation significantly increases, as do the downstream pathways that depend on Akt for activation [65].

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase, serving as a cellular fuel sensor, which senses nutrient (especially amino acids) availability; stimulates protein synthesis, cell growth, and proliferation; and inhibits autophagy. Physiologically, mTOR is a downstream target of Akt in NPY/AgRP neurons and POMC neurons; it can further activate S6K in these neurons to achieve the leptin functions [66, 67]. In the process, leptin induces a reduction of food intake, stimulates the renal sympathetic nerve outflow, increases energy expenditure, and finally decreases body weight. Intriguingly, activated S6K1 also phosphorylates a2-AMPK at serine 491 in turn, leading to a reduced a2-AMPK activity in the MBH. Thus, mTOR-S6K signaling also serves as an upstream pathway of AMPK in the hypothalamic leptin signaling cascades [68].

3. LepRb-IRS-PI3K-Akt-FoxO1: Forkhead box protein O1 (FoxO1), also known as forkhead in rhabdomyosarcoma, is a transcription factor that plays critical roles in regulation of gluconeogenesis, glycogenolysis, as well as adipogenesis. FoxO1 is a crucial phosphorylation target of the PI3K downstream kinase Akt, mediating the anorectic effects of leptin through regulating the transcription of POMC and AgRP [53]. Generally, FoxO1 is expressed in the neurons of the ARC, VMH, and DMH regions that also express leptin receptors. In these neurons, activated FoxO1 translocates from the cytoplasm to the nucleus, where it increases the expression of the orexigenic NPY/AgRP genes and suppresses the expression of the anorexigenic POMC, thereby increasing food intake [69–71].

Leptin can suppress the FoxO1-mediated transcriptional regulation of POMC, NPY, and AgRP, to achieve its functions. Conversely, activated FoxO1 dampens the ability of leptin to stimulate POMC transcription, reducing the leptin sensitivity [69, 70]. Besides, FoxO1 ablation in POMC neurons enhances the obesity suscep-

tibility gene carboxypeptidase E (Cpe) expression, which induces a decreased food intake without altering energy expenditure [72]. In addition, FoxO1 also antagonizes the activity of STAT3 and thereby downregulates the leptin signaling in hypothalamic neurons, which ultimately causes an increase in food intake and leads to obesity [73].

4. LepRb-IRS-PI3K-PDE3B-cAMP: Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that regulate the cellular levels of the second messengers, cAMP and cGMP, by controlling their rates of degradation. Physiologically, PDE3B is also a downstream regulator of PI3K in neurons of various brain regions, including the hypothalamus. In these neurons, the IRS-PI3K signaling activates PDE3B, which further causes a reduction of cAMP, initiating a chain of events that ultimately decreases food intake and body weight. In this process, the reduced cAMP inhibits the activity of the cAMP response element-binding protein (CREB) and thereby suppresses the expression of NPY via the cAMP-CREB pathway to achieve the anorexic effect of leptin. Notably, the cAMP not only modifies the gene expression of NPY, but it also modifies the action of NPY after it is secreted. Moreover, the PI3K-PDE3B-cAMP pathway also interacts with the JAK2-STAT3 pathway, which reduces food intake together, finally preventing overweight and obesity [74, 75].

5. LepRb-AMPK-ACC: Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase consisting of a catalytic α subunit and regulatory β and γ subunits. It contributes to the regulation of a series of cellular processes including cell growth and autophagy, serving as a crucial intracellular energy sensor activated under conditions of metabolic stress. Activation of the AMPK occurs when the threonine 172 on the catalytic α subunit is phosphorylated, which leads to ATP depletion as well as increased levels of AMP and ADP [76–78]. Generally, AMPK plays a critical role in the regulation of energy homeostasis by integrating hormonal and nutritional signals in both the periphery and the CNS [79].

In the hypothalamus, AMPK is activated by fasting but suppressed by refeeding. While leptin can inhibit AMPK activity in these regions, such as the ARC and the PVN [79], notably, phosphorylated AMPK inactivates the AMP downstream target acetyl-CoA carboxylase (ACC) by phosphorylation and thereby results in a decreased malonyl CoA level, hence enhancing the mitochondrial fatty acid oxidation and increasing the cellular long-chain fatty acyl-CoA (LCFA-CoA) level [80]. Via a chain reaction of these processes, leptin induces anorexia and reduces hepatic glucose production; promotes the sympathetic nerve outflows to the kidney, brown, and white adipose tissues, and ultimately causes a reduction of body weight [81, 82].

On the other hand, AMPK also mediates leptin signaling in the periphery. Circulating leptin stimulates AMPK activity in the skeletal muscle and enhances fatty acid oxidation through both direct and CNS-mediated indirect mechanisms, finally leading to a reduction of body weight [83]. In addition, leptin also modulates the activity of AMPK by acting on AMPK-a2 subunit at Ser491 via a mediation of S6K to achieve its effects in the hypothalamus neurons [68].

6. LepRb-SHP2-MAPKs (ERK1/2): The mitogen-activated protein kinases (MAPKs) are a series of protein kinases specific to the amino acids serine, threonine, and tyrosine. They function to mediate numerous intracellular programs, including cell proliferation, differentiation, survival, and apoptosis, by relaying extracellular signals to the cell. As reported, MAPKs, particularly ERK1/2 (extracellular-regulated protein kinase 1/2), also play crucial roles in the leptin regulation of energy homeostasis [60, 84, 85]. Under physiological conditions, ERK1 and ERK2 are typically activated by leptin in the hypothalamic ARC, which promote the anorectic and weight-reducing effects of leptin and participate in the leptin-induced thermogenesis by controlling sympathetic activity on BAT [85].

Once leptin binds to its receptor in these neurons, the tyrosine residue Tyr985 in the LepR intracellular domain is phosphorylated, which facilitates its interaction with the Src homology 2 (SH2) domain. SHP2 is a SH2-containing tyrosine-specific protein phosphatase, serving as a key modulator of leptin-mediated ERK activation. In this process, leptin activates ERK1 or ERK2, causing a reduction of food intake and an enhancement of energy expenditure, finally decreasing body weight [85, 86]. Notably, SHP2-ERK1/2 pathway also plays a neurotrophic role during the development of hypothalamic neuronal feeding circuits, while disrupted ERK signaling impairs the development of the neuronal circuits [87].

Above all, classic upregulating modulators of leptin signaling mainly contain STAT3, STAT5, Akt, mTOR, S6K, PDE3B, MAPK, and ACC, while downregulating modulators include SOCS3, FoxO1, cAMP, AMPK, PTP1B, etc.

7.3.2 Novel Molecules Related to Leptin Signaling

A few new molecules that regulate leptin signaling have been discovered recently, containing:

1. Rho-kinase1 (ROCK1): Leptin plays a crucial role in the regulation of energy balance, while JAK2 is known as an initial trigger of leptin receptor signaling. However, knowledge of the critical intracellular transducers of leptin signaling remains incomplete. ROCK1 is a protein serine/threonine kinase, serving as a key regulator of actin-myosin contraction and cell polarity. As reported recently, ROCK1 is also a regulator of leptin action, which is required for the leptin-induced activation of JAK2. Under physiological conditions, leptin promotes the physical interaction of JAK2 and ROCK1, thereby enhancing the phosphorylation of JAK2 and the activation of the downstream STAT3 and FOXO1, and ultimately reduces food intake and body weight. Besides, deletion of ROCK1 in the ARC neurons induces an impaired leptin sensitivity, an increased food intake, and severe obesity, identifying ROCK1 as a key modulator of leptin action on energy homeostasis [88].

2. Transient receptor potential C (TRPC): TRPC is a family of transient receptor potential cation channels in the mammalian cells, which underlies the store-operated channels (SOC) to achieve its functions. Recently, it is shown that the TRPC 1, 4, and 5 appear to be key channels in the mediation of the leptin-induced depolarization of hypothalamic neurons, which is thought to be closely associated with the leptin effects.

In the POMC neurons, once leptin binds to the LepRb, the downstream JAK2-IRS-PI3K signaling is activated, which subsequently activates phospholipase C γ1 (PLCγ1) to augment the TRPC channel activity. In addition, PI3K also stimulates a rapid incorporation of functional TRPC channels into the plasma membrane and further increases the depolarization of POMC neurons. Through all of these signaling events, the excitability of the POMC neurons is enhanced, thereby causing a reduction of food intake and body weight [89]. The TRPC channels also mediate the activation of the steroidogenic factor 1 (SF1) neurons within the VMH, contributing to the regulation of energy balance and glucose homeostasis [90]. Besides, leptin also plays crucial roles in female reproductive physiology and spine formation, and TRPC channels are demonstrated to be required in these processes [91, 92].

3. NAD^+-dependent deacetylase sirtuin 1 (SIRT1): SIRT1 is a member of the sirtuin family of proteins, which functions as an intracellular regulatory protein with mono-ADP-ribosyltransferase activity, playing a crucial role in the regulation of epigenetic gene silencing and rDNA recombination. Recently, SIRT1 in certain neurons of the hypothalamus is demonstrated to be a negative regulator of energy balance, while decline in the SIRT1 function contributes to the aging and diet-induced obesity [93–95].

In the arcuate nucleus, overexpression of SIRT1 in POMC neurons stimulates energy expenditure via an increased sympathetic activity in adipose tissue, whereas overexpression of Sirt1 in AgRP neurons suppresses food intake, ultimately preventing weight gain [94]. In the SF1 neurons of the VMH, overexpression of the SIRT1 also protects the mice against the diet-induced obesity, due to an enhanced leptin sensitivity and an increased energy expenditure [95]. Besides, SIRT1 can also improve the hypothalamic leptin sensitivity through inhibiting the activities of negative leptin signaling modulators, including the protein tyrosine phosphatase 1B (PTP1B), the T-cell protein tyrosine phosphatase (TCPTP), and the SOCS3 [94].

4. Heat shock protein 60 (HSP60): HSP60 belongs to the chaperonin family, which is responsible for the mitochondrial protein import and the macromolecular assembly. A recent study shows that leptin regulation of the HSP60 also impacts the hypothalamic mitochondrial function and insulin sensitivity, underlying type 2 diabetes and other obesity-associated metabolic disorders [96, 97].

In the neurons of hypothalamus, the leptin-activated STAT3 further interacts with the HSP60 gene promoter to increase the level of HSP60, finally improving insulin sensitivity in these neurons. On the contrary, knockdown of HSP60 in the hypothalamic neurons impairs the signaling pathway, leading to enhanced ROS production and impaired mitochondrial functions, ultimately inducing diabetes and

obesity [96]. In adipocytes, Hsp60 can activate ERK1/2, Jun NH(2)-terminal kinase (JNK), p38, and nuclear factor (NF)-κB whereas impair Akt phosphorylation, thereby stimulating the secretion of tumor necrosis factor-α, interleukin (IL)-6, IL-8, etc. Thus, Hsp60 may also be a factor closely associated with the adipose tissue inflammation [97].

5. Melanoma antigen-like gene 2 (*MAGEL2*): Prader-Willi syndrome (PWS) is a type of genetic disease, which makes patients obese and retarded. As is reported, the PWS is closely associated with gene mutation of the *MAGEL2*, a gene within the PWS domain that is paternally expressed and maternally silenced [98]. Notably, knockout of the *MAGEL2* reduces leptin sensitivity and inhibits the neural activity of POMC neurons in the ARC and thereby causes a disruption of hypothalamic feeding circuits, ultimately leading to hyperphagia and obesity [99]. These findings suggest that leptin is relevant to the development of obese phenotype of PWS, and the *MAGEL2* may be a novel gene playing a critical role in the mediation of this process. However, a recent study demonstrates that the disruption of hypothalamic feeding circuits appears to be independent of the leptin neurotrophic effects, which remain to be further studied [100].

6. Steroidogenic factor 1 (SF-1): Critical metabolic roles of FoxO1 in the hypothalamus have been reported recently, which are focused on the melanocortin neurons in the ARC. However, FoxO1 is also highly expressed in many other hypothalamic nuclei, whose functions have yet to be delineated. SF1 is a member of the nuclear receptor family, which plays a crucial role in the regulation of sexual development in the embryo and at puberty. In a recent report, SF1 is also identified to be a direct transcriptional target of FoxO1 in the VMH, participating in the mediation of leptin signaling as well as the regulation of energy balance and glucose homeostasis [101]. Physiologically, in the SF-1 neurons of the VMH, leptin upregulates SF-1 expression through the inhibition of FoxO1 and thereby activates the SF-1 neurons [101, 102]. Subsequently, the activated SF-1 neurons stimulate the whole-body glucose utilization and enhance insulin sensitivity in some peripheral tissues, contributing to the regulation of body-weight homeostasis [101, 103].

7. G protein-coupled receptor 17 (Gpr17): Gpr17 is a G protein-coupled receptor that acts primarily via G proteins by linking to the Gi alpha subunit or the Gq alpha subunit. Recently, Gpr17 is also regarded as an inhibitory factor of leptin signaling, achieving its functions as a FoxO1 target in the AgRP neurons of the ARC. In these neurons, leptin inhibits FoxO1 via a LepRb/IRS/PI3K/Akt/FoxO1 pathway, which further suppresses the Gpr17 activation. Subsequently, this decreases the firing rate of AgRP neurons and activates the STAT3, AKT, as well as PS6, ultimately resulting in a reduced food intake, a lean phenotype, and an improved glucose homeostasis [104, 105].

8. Protein tyrosine phosphatases (PTPs): Protein tyrosine phosphatases are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Biochemically, PTPs serve as key cellular regulatory components to control biological processes, such as cell growth, proliferation, and

differentiation. To date, a few PTPs have also been implicated in leptin signaling, including the SH2-containing PTP 2 (SHP2), the protein tyrosine phosphatase 1B (PTP1B), the protein tyrosine phosphatase epsilon (PTPε), the T-cell protein tyrosine phosphatase (TCPTP), and the phosphatase and tensin homolog (PTEN).

SHP2 is regarded as an important component of the POMC neuron regulation of energy balance, which links LepRb to the MAPK pathway [86, 106]. PTP1B and PTPε both attenuate the leptin-induced JAK2/STAT3 signaling by dephosphorylating the JAK2 [107–109]. Notably, PTP1B also suppresses the IRS1/2 signaling, thereby inhibiting the IRS-PI3K pathway [110]. TCPTP is also an inhibitor of the JAK2/STAT3 pathway, achieving its effects by blocking the Y705 phosphorylation site of STAT3, which is required for STAT3 dimerization and translocation to the nucleus [111]. PTEN is an inhibitory modulator of PI3K signaling, acting by suppressing the PIP3 and further upregulating the downstream factors including Akt and FoxO1 [112].

Overall, PTPs are activated for the attenuation of the signal transductions of leptin signaling pathways, which finally decrease the suppressive efficiency of leptin in the appetite regulation and thereby cause a heightened food intake as well as an increased body weight. In addition, genetic knockout of PTPs in the brain remarkably potentiates leptin signaling and prevents against diet-induced obesity, type 2 diabetes mellitus, and nonalcoholic fatty liver disease (NAFLD), which provides us a new idea for the treatment of the metabolic disorders [113].

9. Angiotensin AT_{1A} receptors: AT_{1A} receptors colocalize with leptin receptors in the ARC, and the cellular co-expression of AT_{1A} receptors and LepR is almost exclusive to the AgRP neurons. Under physiological conditions, leptin contributes to the control of resting metabolic rate (RMR) and blood pressure (BP), which is thought to be through the actions of AT_{1A} receptors in a specific subset of neurons in ARC. Recently, it is reported that angiotensin AT_1 receptors within the brain certainly mediate the leptin regulation of RMR and BP. In the AgRP neurons, AT_{1A} receptor acts as a critical modulator to interact with the signal molecules activated by LepR. Activation of AT1A receptor inhibits the GABA synthesis via suppression of GAD1/2 and vesicular GABA transport (VGAT) and ultimately promotes the RMR via inhibitory GABAergic projections into PVN, LHA, DMH, and VMH. However, the underlying mechanism by which AT_{1A} receptor involves in the regulation of the BP is still illusive, which needs to be further explored [114].

7.3.3 Non-neuronal Cells and Subcellular Structures Related to Leptin Signaling

Recent studies have shown that hypothalamic astrocytes and cilia also participate in the mediation of leptin signaling and its physiological functions.

As Horvath reported, LepR is expressed in hypothalamic astrocytes. Notably, the conditional deletion of LepR in the astrocytes leads to altered glial morphology,

reduced astrocytic coverage of melanocortin cells, and augmented synaptic inputs onto hypothalamic neurons. These further diminish the inhibitory effect of leptin on feeding whereas enhance the fasting or ghrelin-induced hyperphagia, suggesting an active role of glial cells in hypothalamic synaptic remodeling and leptin control of food intake [115].

A recent report also demonstrates that leptin can increase the length of neural cilia by activation of hypothalamic LepRb/JAK2/PI3K signaling pathway or by suppression of the inhibitory leptin signaling mediator including PTEN and glycogen synthase kinase 3β (GSK3β). The induction of long cilia further improves neuronal sensitivity of leptin, which finally reduces food intake and enhances energy expenditure, leading to a catabolic status. Moreover, mice with short hypothalamic cilia exhibits attenuated anorectic responses to leptin, insulin, and glucose, indicating that leptin-induced cilia assembly plays a crucial role in the satiety signal sensing of the hypothalamic neurons [116].

We summarize the classic and novel cellular pathways of leptin signaling as the following figure (Fig. 7.2).

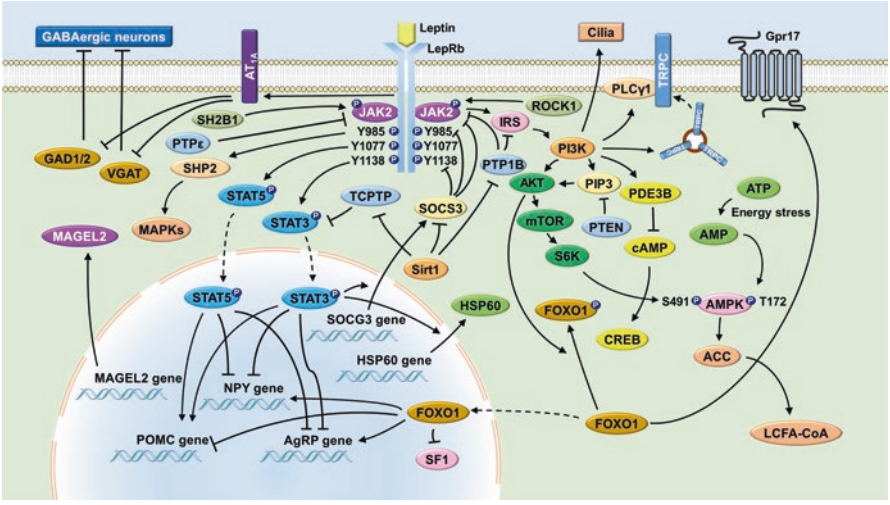

Fig. 7.2 Cellular pathways of leptin signaling
——▶: Activating. ——⊣: Inhibiting. - - - -▶: Translocation. *AMPK* adenosine monophosphate-activated protein kinase, *ACC* acetyl-CoA carboxylase, *AKT* protein kinase B, *AT* angiotensin, *CREB* cAMP response element-binding protein, *ERK* extracellular signal-regulated kinase, *FOXO1* forkhead box protein O1, *GAD* glutamic acid decarboxylase, *Gpr17* G protein-coupled receptor 17, *HSP60* heat shock protein 60, *IRS* insulin receptor substrate, *JAK2* Janus kinase 2, *mTOR* mammalian target of rapamycin, *MAGEL2* melanoma antigen-like gene 2, *PDE3B* phosphodiesterase 3B, *PI3K* phosphatidylinositol 3-OH kinase, *PLCγ1* phospholipase C γ1, *PTEN* phosphatase and tensin homolog, *PTP* protein tyrosine phosphatase, *ROCK1* Rho-kinase1, *S6K* S6 kinase, *SF-1* steroidogenic factor 1, *Sirt1* NAD^+-dependent deacetylase sirtuin 1, *SOCS3* suppressor of cytokine signaling 3, *STAT* signal transducer and activators of transcription, *TCPTP* T-cell protein tyrosine phosphatase, *TRPC* transient receptor potential C, *VGAT* vesicular GABA transporter

7.4 Conclusions

Over the last 20 years, numerous studies have demonstrated the physiological functions of leptin as well as the neural and cellular pathways that mediate the leptin functions. However, the actions of leptin in several brain nuclei are still unclear, and their signaling circuits remain to be established. In addition, the development of leptin-associated drugs to treat obesity has also been disappointing, which is due to safety issues or leptin resistance. Therefore, further studies are needed to solve the problems. More comprehensive studies will be helpful to understanding of the leptin actions, which will ultimately lead to the new strategies for the effective treatment of obesity.

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Chapter 8
The Leptin Resistance

Jiarui Liu, Xiaoning Yang, Siwang Yu, and Ruimao Zheng

Abstract Leptin is an adipocyte-derived hormone, which contributes to the homeostatic regulation of energy balance and metabolism through humoral and neural pathways. Leptin acts on the neurons in certain brain areas such as the hypothalamus, hippocampus, and brain stem to regulate food intake, thermogenesis, energy expenditure, and homeostasis of glucose/lipid metabolism. The pathologically increased circulating leptin is a biomarker of leptin resistance, which is common in obese individuals. Leptin resistance is defined by a reduced sensitivity or a failure in response of the brain to leptin, showing a decrease in the ability of leptin to suppress appetite or enhance energy expenditure, which causes an increased food intake and finally leads to overweight, obesity, cardiovascular diseases, and other metabolic disorders. Leptin resistance is a challenge for clinical treatment or drug discovery of obesity. Until recently, emerging evidence has been showing novel mechanisms of the leptin resistance. Here, we summarized the advances and controversy of leptin resistance and associated diseases, for better understanding the physiology and pathophysiology of leptin as well as the new strategies for treating obesity and metabolic disorders.

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8.1 Leptin Resistance

Leptin resistance is defined by a reduced sensitivity or a failure in response to leptin, showing a decrease in the ability of leptin to suppress appetite or enhance energy expenditure, which causes a heightened food intake and ultimately leads to overweight and obesity [1].

8.1.1 Main Mechanisms Underlying the Leptin Resistance

Leptin resistance can be classified into HFD-induced leptin resistance, leptin-induced leptin resistance, inflammation-induced leptin resistance, site-specific leptin resistance, seasonal leptin resistance, pregnancy/lactation-induced leptin resistance, endoplasmic reticulum stress-induced leptin resistance, etc. These forms of leptin resistance are thought to be underlain by various factors and mechanisms. Until recently, the essential mechanisms related to the leptin resistance are as follows:

1. Disorders of the blood-brain barrier (BBB) transport: Leptin needs to be transported across the BBB to activate the first-order LepRb-expressing neurons, which are connected with the downstream neurons to finally control food intake and energy expenditure. In this process, LepRa in the BBB is required when the circulating leptin is transferred into the CNS. Thus, if the leptin level in the blood plasma is excessively high, it may cause a saturation of the LepRa, which further may reduce the ratio of leptin transport across the BBB, ultimately leading to leptin resistance [2, 3].
2. Competitive inhibition of leptin: Circulating leptin-binding proteins, including the plasma-soluble LepRb and C-reactive protein, can bind to leptin, which are closely associated with development of leptin resistance. Once they bind to leptin, the transport of leptin to the CNS, as well as the binding between leptin and LepRb-expressing neurons in the CNS, will be competitively inhibited, thereby inducing a leptin resistance-related phenotype [4].
3. Mutations of LepR: Certain mutations of the LepRb gene shorten the length of the intracellular signaling domain of LepRb, which impairs the ability of LepRb to mediate leptin signaling. Therefore, with these mutations, even if leptin binds to the receptor, its function cannot be achieved, leading to a severe leptin resistance [5].
4. Impairment of the leptin cellular signaling: Cellular and circulating molecules, such as the IL-6, a proinflammatory cytokine, may lead to an overexpression of leptin signaling inhibitory regulators, including SOCS3, PTP1B, etc. The overexpression of these factors further suppresses leptin signaling, which may be

implicated in the pathogenesis of leptin resistance [6, 7]. In addition, the endoplasmic reticulum (ER) stress and the feedback inhibition of leptin signaling may also have the same effect [8, 9].

Above all, mechanisms of the leptin resistance mainly include two points. First, neurons expressing LepR are not sensitive enough to precisely measure the circulating leptin level, which may cause a decline of the efficacy of leptin binding to their receptors. Second, the brain neurons regulating energy balance and metabolic homeostasis have an impaired signaling ability; thereby leptin cannot achieve its functions in these neurons. In fact, it is difficult to delineate the definition of leptin resistance in a universal and quantifiable manner, but actually, it is necessary to understand the mechanisms that attenuate leptin action, which can provide new insight to decipher the myth of leptin resistance and obesity.

8.1.2 Novel Mechanisms Related to Leptin Resistance

In recent years, with the development of more advanced biotechnology, a number of novel molecules and mechanisms underlying the mediation of leptin signaling have been unveiled, which greatly extends our understanding of the leptin resistance.

1. Brain-derived neurotrophic factor (BDNF): BDNF is a member of the neurotrophin family that is encoded by the BDNF gene. In the brain, BDNF is active in the hippocampus, cortex, and basal forebrain, helping to support the survival of existing neurons and contributing to the growth and differentiation of new neurons and synapses. Recently, it is reported that central BDNF is also closely associated with the neuro-regulation of energy balance [10]. Physiologically, long 3′ UTR (3′ untranslated regions) BDNF mRNA is enriched in the dendrites of hypothalamic neurons, while leptin can stimulate its translation in dendrites. Once the long 3′ UTR of the BDNF mRNA is truncated, the ability of leptin to inhibit food intake is impaired, which causes leptin resistance and ultimately leads to severe hyperphagic obesity. Notably, the obesity can be completely reversed by viral expression of long 3′ UTR BDNF mRNA in the hypothalamus, suggesting a novel mechanism linking leptin action to BDNF expression during hypothalamic-mediated regulation of body weight [11].
2. Myeloid differentiation factor 88 (MyD88): MyD88 is a TLR adaptor molecule that is encoded by the MYD88 gene. Recently, neuronal MyD88-dependent signaling is also reported to be a key regulator of diet-induced leptin resistance in vivo. Generally, saturated fatty acid especially palmitate can act in the central nervous system to inhibit leptin-induced anorexia, and MyD88 play a crucial part in this process. Saturated fatty acid binds to the Toll-like receptors (TLRs) in the hypothalamus neurons, activating the IKK (inhibitor of nuclear factor kappa-B kinase) via the MyD88, thereby upregulating the NF-κB whereas downregulating the STAT3, and ultimately reduces leptin sensitivity. Besides, specific knockout of MyD88 in the CNS leads to an increase of leptin sensitivity

and a reduction of food intake, which contributes to the resistance of obesity induced by high-fat diet. Moreover, CNS-restricted MyD88 deletion also protects from HFD- and palmitate-induced impairment of peripheral glucose metabolism [12].

3. cAMP-Epac (exchange factor directly activated by cAMP) pathway: Epac, also known as Rap guanine nucleotide exchange factor 3, is a cAMP-regulated guanine nucleotide exchange factor for the small G protein Rap1. As is reported recently, elevation of cAMP levels impairs multiple leptin signaling cascades within the hypothalamic neurons, and this process is independent of the PKA activation. In these neurons, overexpressed cAMP activates the downstream Epac, which further upregulates expression of the negative leptin-signaling modulators SOCS3 and PTP1B, whereas inhibits phosphorylation of the positive STAT3 and S6K. Moreover, Epac activation also blunts the leptin-induced depolarization of POMC neurons to suppress leptin functions, leading to a heightened food intake and a decreased energy expenditure, ultimately causing overweight and obesity [13]. In addition, lack of Epac1 leads to enhanced leptin sensitivity, reduced adiposity, and improved glucose homeostasis, further suggesting that cAMP-Epac pathway is closely associated with the development of multiple indices of leptin resistance [14]. However, Epac2a-null mice exhibit obesity-prone nature, which are more susceptible to leptin resistance, indicating that Epac2a plays an opposite role in regulating adiposity and energy balance [15].
4. Methyl-CpG-binding protein 2 (MeCP2): POMC neurons in the ARC regulate energy homeostasis by secreting α-MSH, in response to leptin signaling. Generally, expression of *Pomc* is subject to multiple modes of regulation, including epigenetic regulation. MeCP2 is a kind of nucleoprotein that is essential for neuronal function, interacting with promoters to regulate gene expression, which is reported to be associated with the mediation of *Pomc* expression. In the POMC neurons, deletion of MeCP2 increases the DNA methylation of the hypothalamic *Pomc* promoter and suppresses *Pomc* expression, thereby causing an enhanced food intake and respiratory exchange ratio. Subsequently, it further induces an increase in fat mass and body weight, with an accentuating degree of leptin resistance [16]. These findings suggest that MeCP2 is an important mediator of leptin signaling, which may be a novel target for the treatment of obesity and obesity-associated metabolic disorders.
5. I-kappa-B kinase epsilon (IKKε): IKKε, also known as inhibitor of nuclear factor kappa-B kinase subunit epsilon, is a serine/threonine kinase that plays an essential role in regulating inflammatory responses to viral infection, through the activation of the type I IFN, NF-kappa-B, and STAT signalings. A recent study demonstrates that whole-body knockout of IKKε can also prevent leptin resistance induced by high-fat diet, while IKKε is upregulated in the hypothalamic neurons in obese mice, contributing to leptin and insulin resistance. In these neurons, once IKKε activity is inhibited, IRS-1Ser307 phosphorylation, leptin resistance, and insulin resistance will be reduced, via the improvement of the IR-IRS-1-Akt and JAK2-STAT3 pathways. Furthermore, the increased

insulin and leptin actions in the hypothalamus lead to a decrease in adiposity, an enhancement of energy expenditure, as well as a reduction in hepatic glucose production. Notably, these improvements were independent of body weight and food intake [17].

6. Extracellular signal-regulated kinases (ERKs): ERKs are widely expressed protein kinase intracellular signaling molecules, which are involved in functions including the regulation of meiosis and mitosis in differentiated cells. Recently, ERKs are also reported to be key modulators of leptin signaling, contributing to the transport of leptin from the periphery to the CNS. Under physiological conditions, leptin can act on the brain to reduce food intake by regulating neuronal activity in the mediobasal hypothalamus (MBH). Besides, peripheral administration of leptin also activates LepR in median eminence tanycytes followed by the activation of MBH neurons, which is a process requiring mediation of tanycytic ERK signaling. Once the signal-transducing capability of ERK is impaired, the leptin taken up into the tanycytes will be dampened in median eminence and thus fail to reach the MBH, ultimately leading to an increase in food intake and body weight. Notably, activation of ERK signaling in tanycytes with epidermal growth factor (EGF) reestablishes the leptin transport, which further elicits MBH neuron activation and restores leptin sensitivity, reducing food intake and protecting against obesity. These findings suggest that ERK signaling plays a crucial role in the leptin transport by tanycytes and may be also involved in the pathophysiological process of leptin resistance [18, 19].
7. Mitofusin 2 (MFN2): MFN2 is a GTPase embedded in the outer membrane of the mitochondria, playing critical roles in both mitochondrial fusion and the establishment of mitochondria-endoplasmic reticulum (ER) interactions. Recently, ER stress in the hypothalamus is reported to be a crucial causative factor of leptin resistance, and MFN2 is closely associated with this function. Generally, the mitochondria-ER contacts in the anorexigenic POMC neurons of the hypothalamus are decreased in the obese mice. Both POMC-specific deletion of MFN2 and biallelic MFN2 mutations result in a reduction of mitochondria-ER contacts, which further causes ER stress, thereby leading to leptin resistance and hyperphagia. In the process, the expression of UCP1 in the brown adipose tissue is also suppressed, which inhibits energy expenditure and finally induces obesity. Notably, pharmacological relief of hypothalamic ER stress can reverse these metabolic disorders, suggesting that MFN2 in POMC neurons is essential for the development of ER stress-induced leptin resistance [20, 21].
8. Protein tyrosine phosphatases (PTPs): As mentioned above, PTPs including PTPε and TCPTP play crucial roles in the mediation of intracellular leptin signaling, controlling food intake and energy expenditure. Generally, PTPε-deficient mice are protected from the weight gain induced by high-fat food or aging, exhibiting enhanced leptin sensitivity, improved glucose homeostasis, and decreased adiposity. PTPε is therefore an inhibitor of hypothalamic leptin signaling in vivo, which helps establish obesity-associated leptin resistance. In this process, leptin stimulation induces hypothalamic PTPε phosphorylation at

C terminal Y695, which drives PTPε to downregulate the activity of JAK2 to achieve the effects. Additionally, in obese mice the hypothalamic levels of the TCPTP are also elevated to attenuate the leptin response, through inhibition of the JAK-STAT signaling. Mice lacking TCPTP show leptin hypersensitivity, identifying TCPTP as a critical negative regulator of hypothalamic leptin signaling. These findings suggest that PTPε and TCPTP are essential for the development of leptin resistance [22, 23].

9. Histone deacetylase 5 (HDAC5): HDAC5 is an enzyme encoded by the HDAC5 gene, which plays a critical role in the histone deacetylation to alter chromosome structure and affect transcription factors, thereby participating in the regulation of gene transcription as well as cell cycle progression. Recently, HDAC5 is also demonstrated to be a critical mediator of hypothalamic leptin signaling and organismal energy balance. Generally, pharmacological and genetic inhibition of HDAC5 activity in the mediobasal hypothalamus and global HDAC5 KO all lead to impaired leptin sensitivity, increased food intake, and greater diet-induced obesity. On the contrary, overexpression of hypothalamic HDAC5 improves leptin action, partially protecting against HFD-induced leptin resistance and obesity. In the process, HDAC5 induces STAT3 deacetylation at Lys685 and also initiates STAT3 phosphorylation at Tyr705, which further contribute to the regulation of STAT3 localization and transcriptional activity and finally potentiate leptin signaling. Above all, hypothalamic HDAC5 activity is a factor in the regulation of leptin signaling that controls food intake and body weight, while HDAC5 loss-of-function mutation is closely associated with the development of leptin resistance [24].
10. Oligodendrocyte progenitor cells (OPCs): OPCs, also known as NG2-Glia or polydendrocytes, are a subtype of glial cells in the central nervous system. The loss of OPCs and consequent lack of differentiated oligodendrocytes are identified to be associated with the impairment of myelination and the subsequent inhibition of relative neurological functions. As reported recently, OPCs also play a crucial role in contacting the dendritic processes of the ARC LepR neurons in the median eminence (ME), which is required for leptin sensing and the achievement of leptin effects. Therefore, once the OPCs are eliminated, the dendritic processes will degenerate, ultimately leading to leptin resistance and obesity. These findings indicate that LepR neuron dendrites in the ME are critical for leptin's anorexigenic action and that OPCs are essential for the maintenance of the dendrites [25]. Besides, the OPC proliferation can also be enhanced by leptin via phosphorylation of the ERK, further suggesting that OPCs play an important role in the regulation of leptin signaling and the development of leptin resistance [26].
11. Withaferin A: Withaferin A is a steroidal lactone, which has a wide range of pharmacological activities and cardioprotective, immunomodulatory, anti-inflammatory, anti-angiogenic, anti-metastatic, and anticarcinogenic properties. Celastrol is a well-known naturally occurring compound that acts as a leptin sensitizer, and withaferin A has an effect similar to that of celastrol. Recently, withaferin A is also reported to be a mediator of leptin signaling that

can reverse leptin resistance and thus promote weight loss. In the diet-induced obesity (DIO) mice, withaferin A treatment induces an increase of leptin sensitivity in the hypothalamus, a 20–25% reduction of body weight, and a decrease of metabolic disorders including hepatic steatosis, etc. Notably, the weight-reducing effect is marginal in the ob/ob and db/db mice, both of which are deficient in leptin signaling. In addition, withaferin A, unlike celastrol, also regulates glucose metabolism, which is independent of its leptin-sensitizing effect. Overall, withaferin A is a potential leptin sensitizer with antidiabetic actions, holding therapeutic potential for treating obesity and its associated disorders [27].

12. Protein tyrosine phosphatase receptor type J (PTPRJ): PTPRJ, the protein tyrosine phosphatase receptor type J, plays a crucial role in the regulation of key cellular processes including cell proliferation and transformation. Accelerated PTPRJ is associated with a reduced cell proliferation and migration in various human cancers [28]. Recently, the PTPRJ expressed in hypothalamic neurons together with LepR is also regarded as a negative regulator of leptin signaling. In these neurons, PTPRJ inhibits the activation of JAK2 through the dephosphorylation of Y813 and Y868 in JAK2 autophosphorylation sites, thereby incurring an increase of food intake and body weight. Notably, diet-induced obesity or leptin treatment upregulates PTPRJ expression in the hypothalamus, while the overexpression of PTPRJ further induces leptin resistance. Thus, PTPRJ is an important factor contributing to the development of leptin resistance. Moreover, the inhibition of PTPRJ may be a potential strategy for the treatment of obesity [29].
13. c-Jun N-terminal kinases (JNKs): JNKs are a series of kinases belonging to the mitogen-activated protein kinase family, which are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. As reported recently, JNK activation in the AgRP neurons of the hypothalamus also increases spontaneous action potential firing of the AgRP cells, which is sufficient to induce an enhanced food intake, thereby increasing body weight and fat mass with a development of leptin resistance [30]. Besides, pharmacological inhibition of the JNK reduces food intake and sensitizes the anorectic signaling actions of leptin [31]. Notably, JNK3 deficiency in the AgRP neurons does not exhibit the effects mentioned above. On the contrary, it leads to hyperphagia selectively in high-fat diet-fed mice, which provides a mechanism that contributes to the regulation of energy balance in response to metabolic stress [32].
14. Activating transcription factor 4 (ATF4): ATF4, also known as CREBP2, belongs to the CREBP families, which is ubiquitously expressed in many tissues and brain nuclei, including the hypothalamus. It is involved in the regulation of various processes, including memory formation, osteoblast differentiation, amino acid deprivation, and redox homoeostasis [33]. Recent studies demonstrate that ATF4 also plays a crucial role in the regulation of energy balance. In the AgRP neurons of the hypothalamus, specific deletion of ATF4 induces a decreased food intake, an enhanced energy expenditure, and a

reduced body weight, with improved leptin and insulin sensitivity. Notably, in these neurons, the expression of FOXO1 is directly inhibited via the cAMP-responsive element site on its promoter [34]. Besides, ATF4-specific knockout in the POMC neurons also exhibits the same effects, and the ATF4/ATG5 (autophagy protein 5) axis may be closely associated with this process [35]. Taken together, these findings identify a critical role of hypothalamic ATF4 in the regulation of food intake and energy expenditure, which may be a new potential therapeutic target for treating obesity and obesity-related metabolic disorders.

Besides, several novel factors mediating leptin functions also have been discovered recently. As is reported by Feuillan and his partners, obesity is a feature relative to some syndromes of cilia dysfunction, such as Bardet-Biedl syndrome (BBS); and the obesity of the BBS mutant mice is closely associated with the development of leptin resistance. In the leptin-responsive POMC neurons, induction of the cilia loss leads to increased food intake and body weight. However, in the preobese state, these mice still respond to the anorectic effects of leptin, with no phenotypes associated with defective leptin signaling. Furthermore, thermoregulation and activity measurements in the cilia mutant mice are inconsistent with the phenotypes observed in the leptin-deficient ob/ob mice, suggesting that leptin resistance is present only when mutants are obese [36].

In addition, RPGRIP1L (retinitis pigmentosa GTPase regulator-interacting protein-1-like) protein, encoded by the *RPGRIP1L* gene vicinal to the FTO locus, is a cilia-centrosomal multidomain protein, whose mutations also lead to leptin resistance and obesity. In the process, the number of cilia in hypothalamic neurons is diminished, the convening of the leptin receptor to the vicinity of the cilium is impaired, and the expression of STAT3 in these neurons is downregulated [37, 38]. These findings indicate that RPGRIP1L may be partly responsible for the obesity susceptibility signal at the FTO locus.

Moreover, environmental factors also have a strong impact on leptin sensitivity. Mice under environmental enrichment (EE) conditions are stimulated by various information, which show an increased STAT3 expression level in the ARC neurons, a heightened leptin sensitivity, and a reduced adipose mass compared with mice under drab environments [39]. Hypothalamic BDNF is selectively upregulated by EE, whereas BDNF knockdown blocks the effect of EE, suggesting the BDNF/leptin axis may mediate the process [40].

We summarized the novel molecules and mechanisms underlying leptin resistance in the following figure (Fig. 8.1).

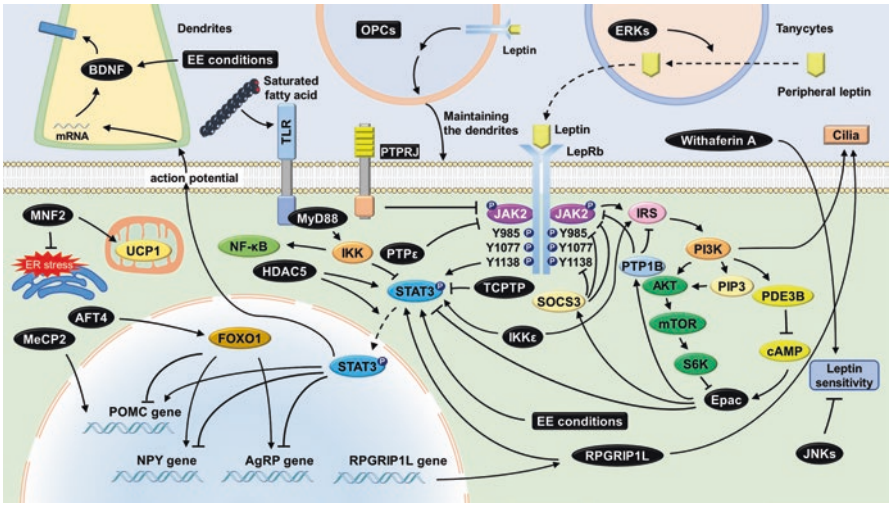

Fig. 8.1 Newfound molecules and mechanisms relative to leptin resistance
——►: Activating. ——┤: Inhibiting. - - - - ►: Translocation. The black background: factors related to leptin resistance. *AKT* protein kinase B, *ATF4* activating transcription factor 4, *BDNF* brain-derived neurotrophic factor, *EE* environmental enrichment, *Epac* exchange factor directly activated by cAMP, *ER* endoplasmic reticulum, *ERKs* extracellular signal-regulated kinases, *FOXO1* forkhead box protein O1, *HDAC5* histone deacetylase 5, *IKK* inhibitor of nuclear factor kappa-B kinase, *IRS* insulin receptor substrate, *JAK2* Janus kinase 2, *JNKs* c-Jun N-terminal kinases, *MeCP2* methyl-CpG-binding protein 2, *MFN2* mitofusin 2, *mTOR* mammalian target of rapamycin, *MyD88* myeloid differentiation factor 88, *OPCs* oligodendrocyte progenitor cells, *PI3K* phosphatidylinositol 3-OH kinase, *PTP* protein tyrosine phosphatase, *PTPRJ* protein tyrosine phosphatase receptor type J, *RPGRIP1L* retinitis pigmentosa GTPase regulator-interacting protein-1 like protein, *S6K* S6 kinase, *SOCS3* suppressor of cytokine signaling 3, *STAT3* signal transducer and activators of transcription 3, *TCPTP* T-cell protein tyrosine phosphatase, *TLRs* Toll-like receptors, *UCP1* uncoupling protein 1

8.2 Leptin-Associated Diseases

Leptin is an adipocyte-derived hormone and cytokine, which contributes to the homeostatic regulation of energy balance through a wide range of functions. Physiologically, leptin acts on the neurons of hypothalamus and other regions in the brain, to regulate food intake, thermogenesis, and the blood glucose level. Furthermore, increased circulating leptin, a marker of leptin resistance, is common in obesity and independently associated with various metabolic diseases. The diseases related to leptin effects mainly include:

1. Cardiovascular disease (CVD): CVD is a class of diseases that involve the heart or blood vessels, including coronary artery diseases (CAD), stroke, heart failure, hypertensive heart disease, etc. Generally, almost all of the above diseases involve atherosclerosis. On the one hand, ob/ob mice are resistant to atherosclerosis, and LepR is detected in the atherosclerotic lesions, suggesting that leptin

may be a directly atherogenic modulator [41, 42]. On the other hand, atherosclerosis is regarded as an inflammatory disease; thus the reciprocal modulation of leptin and inflammatory pathways is also closely associated with the cardiovascular risk.

Leptin contributes to the regulation of human immune functions, while activation of the innate immunity upregulates leptin expression in humans [43, 44]. Under physiological conditions, leptin stimulates central T-cell production and a peripheral shift of T cells, which activates the proinflammatory T helper (Th) 1 adaptive immune responses, whereas inhibits the anti-inflammatory Th2 responses. Further, the inflammatory milieu that fosters atherosclerosis is augmented, increasing the risk of CVD [45]. In this setting, leptin plays a crucial role as a proinflammatory cytokine in the induction of atherosclerosis and CVD.

Notably, multiple downstream inflammatory biomarkers, including C-reactive protein (CRP), are independent predictors of CVD. In the process, leptin induces the expression of CRP in human coronary artery endothelial cells, where CRP further promotes atherosclerosis [46, 47]. In addition, levels of several macrophage and T-cell cytokines, such as IL-18 and IL-6, also participate in the development of coronary heart disease [48]. Overall, a series of inflammatory pathways, which can be modulated by leptin signaling and leptin resistance, is relative to atherosclerosis and multiple CVDs in human.

As reported recently, leptin also acts on human coronary artery endothelial cells by promoting the cellular adhesion molecules (CAMs) and tissue factor (TF) expression, thereby inducing the development of atherosclerosis [49]. Besides, leptin is a newly found regulator of aldosterone synthesis that enhances aldosterone production via calcium-dependent mechanisms. Subsequently, the leptin-mediated aldosterone secretion causes the endothelial dysfunction and the pro-fibrotic marker expression in the heart, finally leading to CVD [50]. Moreover, leptin-associated diabetes, hypertension, thrombosis, and myocardial injury also contribute to the development of CVDs [51].

2. Diabetes mellitus (DM): DM, commonly referred to as diabetes, is a series of metabolic disorders in which there are high blood glucose levels over a prolonged period. Interacting with other hormonal regulators of energy metabolism, such as insulin, leptin is closely associated with the development of DM. Notably, with leptin replacement, the diabetic features of leptin-deficient mice and humans can be improved, even before the reduction of body weight [52, 53]. On the contrary, leptin resistance might induce hyperinsulinemia and insulin resistance independently of BMI, which ultimately causes obesity and DM [54].

   Consistent with the bidirectional adipoinsular axis, insulin and glucose can stimulate the secretion of leptin in adipocytes. On the other hand, leptin can further suppress insulin secretion and hepatic glucose production in response [55–57]. The regulation of this process is closely associated with the mediation of IRS-PI3K-PDE3B-cAMP pathway, at least in part [58]. In addition, leptin also mediates the glucose metabolism in the CNS. In POMC neurons of the ARC, leptin acts via the PI3K signal pathway, while knockout of PI3K in these neurons reduces insulin sensitivity, leading to a lower glucose tolerance [59, 60].

As reported recently, AgRP neurons in the ARC also mediate the antidiabetic effect of leptin [61]. Besides, leptin plays a crucial role in the downregulation of the activity of the hypothalamus-pituitary-adrenal (HPA) axis, which further suppresses glucocorticoid release and attenuates ketogenesis, thereby reversing diabetes [62]. Moreover, leptin can decrease the blood glucose levels via the activation of the hypothalamic neurons without increasing insulin sensitivity, and in insulin-deficient mice, leptin can also relieve the symptoms of type 1 diabetes [63, 64].

3. Hypertension (HTN): Hypertension, also known as high blood pressure (HBP), is a long-term medical condition in which the blood pressure in the arteries is persistently elevated. As is shown in various studies, hypertension is closely associated with obesity. However, the mechanism of this process remains unclear. Recently, several studies reveal that leptin appears to mediate the increase in blood pressure (BP) relative to obesity.

   Generally, increased leptin levels in mice of diet-induced obesity (DIO) drive the development of HTN, which is not seen in the leptin- or LepR-deficient animals. In addition, loss-of-function mutations of leptin and LepR also lead to low BP despite severe obesity in humans, suggesting that leptin signaling may play a crucial role in the stimulation of HTN [65]. In the POMC neurons of the ARC, leptin activates the α-MSH to agonize the MC4R and MC3R in the brain, which further leads to a decreased food intake and an enhanced energy expenditure, thereby preventing overweight and obesity. However, activation of the MC4R also increases sympathetic nervous outflow to the kidney and the heart, ultimately exacerbating hypertension [66, 67]. Besides, AgRP/NPY neurons also regulate the melanocortin system to mediate this process [66, 68]. Neuronal circuits in the DMH and VMH are also involved in the leptin effects on BP, independently of changes in weight [69]. In addition, leptin administration directly into the NTS dose-dependently increases the renal SNA, which suggests that extrahypothalamic regions, such as NTS, may also participate in the leptin-mediated control of HTN developing in obesity [70]. Moreover, the role that leptin plays in the development of HDT might also be closely associated with the renin-angiotensin-aldosterone system (RAAS) [71, 72].

   However, a recent report challenges the above results. Phillip Gorden and his partners find that more than one third of the patients with lipodystrophy, who have low levels of leptin, show hypertension in the leptin-deficient state. Notably, after 12 months of leptin treatment, there is no increase in either systolic or diastolic blood pressure in these patients. These findings highlight that mouse models may not be able to predict human adverse events, and it appears to be premature to regard leptin as a mediator of HTN associated with human obesity [73].

4. Cancer: Cancer is a group of diseases, which are induced by abnormal cell growth, with the potential to invade or spread to other parts of the body. Epidemiological data have demonstrated a link between obesity and multiple types of cancer [74–76]. However, molecular mechanisms underlying this

process are still unclear. As reported recently, the alteration of leptin signaling in obesity appears to participate in the mediation of cancer development.

Leptin levels are closely correlated with adiposity in humans. In the obese state, there is an increase in the size and number of adipocytes, resulting in an enhanced secretion of leptin and inflammatory cytokines. Further, leptin may function as a growth factor via the JAK2-STAT3, PI3K-AKT, and ERK signaling pathways, stimulating the growth of cancer cells and thereby contributing to the cancer formation and progression [77–82]. In addition, leptin also can promote the epithelial-mesenchymal transition (EMT) in the process, thus playing an important role in the tumor invasion and metastasis [83, 84].

Several epidemiological researches have examined the association of leptin levels with cancer. For example, in a study on Scandinavian men, leptin is correlated with an increased prostate cancer risk; nevertheless, it is only true for intermediate leptin levels [85]. Besides, an analysis on Japanese women with colorectal cancer also shows an association between leptin levels and the cancer risk, which is independent of BMI [86]. Whereas, there are also some studies finding different results, in which leptin is not associated with cancers [87, 88]. Thus, the relation between leptin and cancer remains to be unveiled, which may provide preventive and therapeutic strategies to reduce the cancer risk and mortality in obesity.

5. Immunological diseases: Leptin is a well-known adipocyte-derived hormone, playing an important role in the central control of energy metabolism. In the peripheral tissue, leptin is also a crucial regulator of the immune system, which functions as a link between metabolism and the immunological diseases.

   Generally, leptin can activate immune cells, including monocytes, granulocytes, natural killer (NK) cells, and T cells, and promote the release of proinflammatory cytokines such as TNF-α, IL-2, and IFN-γ, thus stimulating the innate and adaptive immunity and participating in the mediation of inflammation [89–93]. Notably, serum leptin levels are shown to be closely associated with the development of the autoimmune diseases, including the rheumatoid arthritis (RA), the systemic lupus erythematosus (SLE), the multiple sclerosis (MS), etc. [94–96]. On the other hand, leptin-deficient mice show resistance to the progression of these diseases, further suggesting that leptin may be a key mediator of the autoimmune diseases [97]. In addition, epidemiological studies have also demonstrated the association between obesity and allergic diseases and the important part leptin plays in the process [98].
6. Other diseases related to leptin: As reported recently, several diseases are also closely associated with the leptin levels, including the Alzheimer disease (AD), the frontotemporal dementia (FTD), etc. However, the role leptin plays in these diseases, and the underlying mechanism, remains to be unveiled [99–101].

8.3 Conclusions

In the last 20 years, since Friedman named the hormone as leptin, there have been various studies relative to leptin signaling and leptin resistance. These studies reveal that leptin plays a critical role in the regulation of energy balance and metabolic homeostasis and leptin resistance is a crucial pathogenic factor causing obesity as well as some other metabolic disorders. In addition, novel anti-obesity and antidiabetic poly-pharmacotherapies have also been invented based on these findings. Thus, we can believe that, with the development of science, obesity and its complications will be treated on the base of leptin, paving the way for a new era in obesity research.

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Chapter 9
Ghrelin Signaling in Immunometabolism and Inflamm-Aging

Chuo Fang, Hang Xu, Shaodong Guo, Susanne U. Mertens-Talcott, and Yuxiang Sun

Abstract Intracellular changes in immune cells lead to metabolic dysfunction, which is termed immunometabolism. Chronic inflammation is a hallmark of aging; this phenomenon is described as inflamm-aging. Immunometabolism and inflamm-aging are closely linked to obesity, insulin resistance, type 2 diabetes (T2D), cardiovascular diseases, and cancers, which consequently reduce life span and health span of the elderly. Ghrelin is an orexigenic hormone that regulates appetite and food intake. Ghrelin's functions are mediated through its receptor, growth hormone secretagogue receptor (GHS-R). Ghrelin and GHS-R have important roles in age-associated obesity, insulin resistance, and T2D. In this chapter, we have discussed the roles of ghrelin signaling in diet-induced obesity and normal aging as it relates to energy metabolism and inflammation in key metabolic tissues and organs. The new findings reveal that ghrelin signaling is an important regulatory mechanism for immunometabolism and inflamm-aging. Ghrelin signaling offers an exciting novel therapeutic strategy for treatment of obesity and insulin resistance of the elderly.

Keywords Aging · Ghrelin · Inflammation · Insulin resistance · Adipose tissue macrophages

9.1 Introduction

Obesity is a global epidemic that affects all age groups. The prevalence of obesity-related chronic diseases increases with age, rising from an average 25% in young adults to 75% in Americans aged 65 years and older [1, 2]. Obese elderly people are more prone to metabolic syndrome. In large-scale observational studies involving obese elderly people, a direct relationship has been observed between abdominal

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adiposity and the increased probability of developing type 2 diabetes (T2D), hypertension, and cardiovascular diseases [3–5]. Additionally, obesity is linked to increased risk of other age-associated chronic diseases such as arthritis, pneumonia, T2D, and cardiovascular diseases [6]. Consequently, these chronic conditions reduce both life span and health span.

Aging is a process accompanied by progressive impairment of many physiological functions [7]. Over the past several decades, aging research has provided exciting innovations in preventive medicine, particularly the discoveries in genetics and neuroendocrine and mitochondrial functions, which may delay aging [7, 8]. Aging is commonly accompanied by obesity and inflammation [7]. An in-depth understanding of the pathogenesis of aging and age-associated obesity and inflammation will have profound impacts on aging process and attenuation of chronic diseases in the elderly. The orexigenic hormone ghrelin signaling promotes obesity and insulin resistance and is activated in aging [9]. In this chapter, we review the current findings relating to ghrelin and its receptor, growth hormone secretagogue receptor (GHS-R), and the impacts of ghrelin signaling in the regulation of age-associated inflammation, energy metabolism, and insulin resistance in animals and humans. We hope that further research in this area will lead to interventions targeting ghrelin signaling to improve longevity and treat age-associated chronic diseases.

9.2 Obesity-Associated Inflammation and Metabolic and Glycemic Dysregulations

Chronic disease clusters are the leading causes of death in the USA, based on the data released in 2010 by the Centers for Disease Control and Prevention (CDC). The top four contributors are heart disease, cancer, stroke, and T2D, representing approximately 60% of all deaths [10]. About half of U.S. adults have at least one chronic disease [11]. Obesity, as measured by excessively high body mass index (BMI >30 kg/m^2), is one of the major causes of chronic metabolic diseases, affecting over one-third of U.S. adults [12]. Obesity is a major pathogenic factor of many chronic diseases. Substantial evidence has shown that obesity-associated low-grade inflammation leads to many other diseases such as T2D, cardiovascular diseases, hypertension, and cancers [13–15]. Chronic disease-associated morbidity, mortality, and health-care costs pose major challenges to our society (Fig. 9.1).

9.2.1 Obesity-Induced Inflammation and Insulin Resistance in Adipose Tissue, Liver, and Skeletal Muscle

Ample studies have shown the direct cause and effect relationship between obesity and insulin resistance in animals and humans [16]. It is known that obesity-induced inflammation leads to the development of insulin resistance through a cascade of pro-inflammatory cytokine-induced multi-tissue dysfunctions [17].

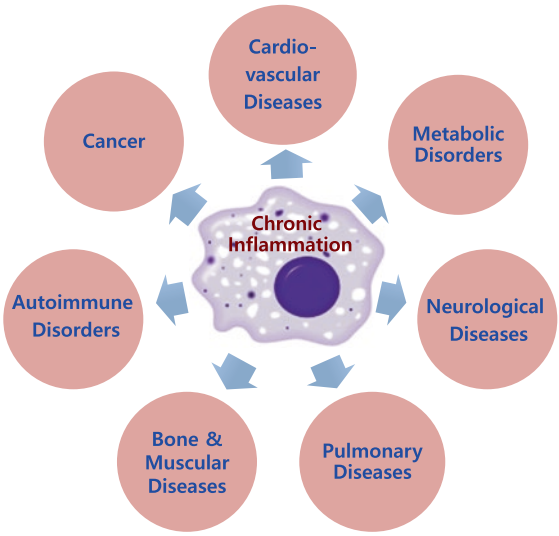

Fig. 9.1 Chronic inflammation-associated chronic diseases Obesity-associated low-grade inflammation leads to many chronic diseases such as type 2 diabetes, cardiovascular diseases, hypertension, cancers, neurological diseases, pulmonary diseases, autoimmune disorders, and bone and muscular diseases

Adipose tissue is classified as either white adipose tissue (WAT) or brown adipose tissue (BAT). WAT is known for its primary role in storing energy in the form of triacylglycerides (TAG) during energy surplus, and TAG is mobilized following energy depletion. WAT also acts as an endocrine organ secreting adipokines, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α), as well as hormones such as leptin and adiponectin; these peptides modulate inflammatory states and metabolic functions of tissues in the entire body [18, 19]. In contrast, BAT contains large amounts of mitochondria that use lipids to generate heat (non-shivering thermogenesis) to maintain normal body temperature and protect against cold stress under cold exposure. Upon cold stimulation, the sympathetic nervous system (SNS) is activated. Nerve terminals release norepinephrine (NE) into BAT; NE binds to β3-adrenergic receptor (β3-AR). The activation of β3-AR leads to lipolysis, which converts TAG into free fatty acid (FFA). As a result, uncoupling protein 1 (UCP1) recruits FFA into mitochondria to enhance respiration, subsequently promoting substrate oxidation and heat production [20, 21].

Under an obese and insulin-resistant state, WAT undergoes lipid dysfunction. This results in increased lipolysis, leading to increased TAG degradation and elevated FFA. FFA causes inflammation and insulin resistance in insulin-targeting tissues such as the adipose tissue, liver, and skeletal muscle. This provides an important link between obesity, inflammation, and the development of insulin resistance. Increased FFA concentration results in the accumulation of lipid derivatives (fatty acid CoA and diacylglycerol [DAG]), which subsequently increases production of reactive oxygen species (ROS), and promotes the activation of pro-inflammatory pathways of c-Jun NH_2-terminal kinase (JNK) and IKK/IκB/NF-κb. Consequently, FFA-induced activation of protein kinase C (PKC) interrupts insulin signaling in the liver and skeletal muscle by impairing insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS1) and IRS2 [22].

In adipose tissue, diet-induced obesity (DIO) is associated with increased adiposity and pro-inflammatory cytokine secretion. Adipose tissue macrophages (ATMs) are responsible for most TNF-α and IL-6 production. TNF-α promotes perilipin-mediated lipolysis and the release of FFA from adipose tissue into the bloodstream [23, 24]. Also, TNF-α mRNA expression in obese subjects is correlated to the plasma concentrations of fasting glucose, insulin, and TAG [25]. In addition, elevated IL-6 concentration is directly correlated with insulin resistance in the liver [26]. Insulin resistance is considered a predictor for the development of T2D and myocardial infarction [27, 28]. Therefore, increased secretion of pro-inflammatory cytokines by adipose tissue can lead to secondary inflammation and insulin resistance in other metabolic organs such as the liver [29] and skeletal muscle [17].

Under normal physiological conditions, insulin affects the liver by stimulating glycogen synthesis. However, defective glycogen synthesis in metabolic disorders has a causative role in insulin resistance and T2D. Nonalcoholic fatty liver disease (NAFLD) is a complex metabolic condition in which lipids are deposited in the liver due to non-alcohol-related causes. Hepatic insulin resistance is mainly attributed to impaired insulin-stimulated tyrosine phosphorylation of IRS1 and IRS2, which are both associated with the activation of PKC and JNK [30, 31]. Elevated levels of hepatic FFA in obesity induce hepatic insulin resistance by promoting PKC translocation from the cytosol to the membrane [32, 33], as well as impairing insulin suppression of endogenous glucose production (EGP) [32]; this in turn leads to impairment of IRS-associated phosphoinositide 3-kinase (PI3K) activity and downstream insulin signaling. In addition, dysregulated secretion of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines by adipose tissue is implicated in hepatic insulin resistance [34]. Chronic liver inflammation can stimulate hepatic fibrosis and cirrhosis as a consequence [35].

Skeletal muscle is the main target organ for glucose uptake during exercise, accounting for 80% of glucose uptake in humans [36, 37]. Elevated levels of FFA lead to insulin resistance in skeletal muscle, partly due to the inhibition of insulin-stimulated glucose transport [38]. Moreover, FFAs promote the release of pro-inflammatory cytokines in skeletal muscle, as demonstrated by increased levels of CD154, CD163, and TNF-α [39]. Muscle-specific overexpression of IL-10 increases tyrosine phosphorylation of IRS1, which is considered an indicator of improved insulin sensitivity [40].

Increased FFA and pro-inflammatory cytokine secretions in obesity have detrimental impacts on systemic insulin resistance, further exacerbating insulin resistance in metabolic tissues, particularly in insulin-targeting organs of the liver, muscle, and adipose tissues (Fig. 9.2). Given the pivotal role of inflammation in insulin resistance and T2D, control of inflammatory response would have profound effects on insulin resistance and is a therapeutic strategy for chronic diseases.

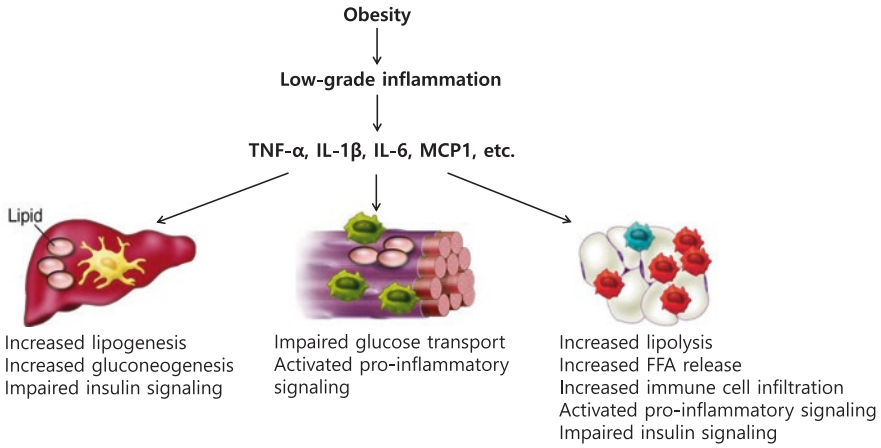

Fig. 9.2 Mechanisms of obesity-induced inflammation and insulin resistance in the liver, muscle, and adipose tissue
In obesity, white adipose tissue (WAT) exhibits increased lipolysis and free fatty acid (FFA) secretion, subsequently leading to inflammation and insulin resistance in insulin-targeting tissues. Increased adiposity is associated with increased immune cell infiltration, as well as the activation of pro-inflammatory signaling pathways. Hepatic insulin resistance is mainly attributed to enhanced de novo lipogenesis and impaired suppression of endogenous glucose production. Increased FFA leads to insulin resistance in skeletal muscle, partly due to impaired insulin-stimulated glucose transport and increased release of pro-inflammatory cytokines. Thus, increased secretions of FFA and pro-inflammatory cytokine secretions such as TNF-α, IL-1β, IL-6, and MCP1 elicit tissue inflammation in insulin-targeting organs of the liver, skeletal muscle, and adipose tissue, further exacerbating systemic insulin resistance

9.2.2 Role of ATMs in Inflammation and Insulin Resistance

9.2.2.1 Macrophage Classification and Activation

Inflammatory response is a highly regulated process in which adipose tissue immune cells are involved in initiation, maintenance, and resolution [41]. Adipose tissue contains many types of cells including preadipocytes, mature adipocytes, and vascular cells, as well as immune cells such as macrophages, T cells, and natural killer cells. ATMs are the major cell population in adipose tissues, constituting up to 40% in obese mice [42]. ATMs infiltrate into adipose tissue and secrete monocyte chemotactic protein-1 (MCP-1), IL-6, and inducible nitric oxide synthase (iNOS), which exacerbates the progression of obesity-induced insulin resistance [43, 44]. Based on distinct functions, macrophages are classified into two major populations: the classically activated M1 macrophages and the alternatively activated M2 macrophages. M1 macrophages (pro-inflammatory, $F4/80^{+}CD11c^{-}$) are driven by T-helper 1 (Th-1) cytokines such as interferon-γ (IFN-γ) and TNF-α or by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) [45]. M1

macrophages secrete high levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, possessing strong microbicidal and tumoricidal characteristics [46]. Chronic low-grade inflammation can be counteracted by protective effect of anti-inflammatory M2 macrophages ($F4/80^{+}CD11c^{-}$ $CD206^{+}$). There are several types of M2 macrophages, and they predominate in lean adipose tissues. M2a macrophages are typically driven by Th2 cytokines, which can be induced by IL-4 and IL-13; M2b macrophages, on the other hand, are driven by IL-1β and LPS. Both M2a and M2b macrophages possess immune-regulatory properties through the downregulation of IL-1β, IL-6, and TNF-α. In contrast, M2c macrophages are induced by IL-10 and transforming growth factor-β (TGF-β) [47].

9.2.2.2 Macrophage Polarization

Macrophage polarization is a process in which macrophages fully acquire the pro-inflammatory M1 phenotype or anti-inflammatory M2 phenotype in response to microenvironmental changes. Obesity-induced insulin resistance is associated with increased secretion of pro-inflammatory factors such as FFA, resistin, leptin, TNF-α, IL-6, and IL-1β. These factors activate the inflammatory signaling pathways in macrophages [22, 48, 49]. Increased FFA concentration induces macrophage inflammation through Toll-like receptor 2 (TLR2) and TLR4, leading to elevated expressions of MCP-1, IL-6, and IL-1β [50]. TLRs induce M1 polarization via transcription factors such as NF-κb, STAT1, caspase1, and interferon regulatory factor 5 (IRF5) [51, 52]. A deficiency of TLR4 reduces pro-inflammatory cytokine expression in the liver and adipose tissue and promotes the alternative activation of M2 macrophages, protecting mice from diet-induced insulin resistance [53, 54]. In addition, the macrophage shift from pro-inflammatory phenotype M1 to anti-inflammatory M2 is associated with IL-4 and IL-13 secreted by eosinophils, IL-10 secreted by regulatory T cells, or adiponectin secreted by adipocytes [45]. Taken together, these findings indicate that M2 macrophages have an important role in protecting surrounding tissues from inflammation and obesity-induced insulin resistance.

Thus, therapeutic strategies promoting macrophage polarization from M1 to M2 may hold great potential for the treatment of obesity-induced insulin resistance and T2D.

9.2.2.3 Macrophage Dysfunction in Aging

It is recognized that aging is a significant factor in deterioration of the immune system, as indicated by macrophage dysfunction in TLRs, phagocytosis, antibacterial defenses, wound healing, and cytokine and chemokine secretion. TLRs function through the recognition of a wide variety of PAMPs, resulting in the secretion of antibacterial peptides that can destroy bacteria, fungi, and viruses [55]. However, TLRs' expression and function in macrophages decline with aging, leading to

increased susceptibility to infections and diminished immune responses in the elderly [56]. In addition, TLRs work synergistically with adenosine A receptors to greatly increase expression of vascular endothelial growth factor (VEGF) and promote wound healing [57]. Thus, age-associated declines in TLRs' expression and function contribute to delayed wound healing. Phagocytosis is an essential process in immune defense and wound healing, acting through the ingestion and elimination of pathogens. Aging impairs phagocytosis of peritoneal and alveolar macrophages [58, 59]. Macrophages are the main sources of cytokines, chemokines, reactive oxygen, and some proteases and enzymes [60]. The impacts of age on cytokine and chemokine production and secretion by macrophages are not well defined. Age-associated changes in the pro-inflammatory cytokine and chemokine levels have been reported in both in vitro and in vivo studies. The majority of rodent studies have reported that old mice have substantially lowered production of TNF-α, IL-6, IL-1β, and IL-12 in response to LPS; this is accompanied by increased production of IL-10 [61, 62]. Conversely, human monocytes from aged individuals have been shown to secrete higher levels of IL-6 and IL-8 than monocytes from young individuals [63]. Recently, age-related reduction of TNF-α and IL-6 production in human monocytes has been reported in a flow cytometry study [64].

The effects of aging on macrophage phenotype polarization toward either pro-inflammatory M1 or anti-inflammatory M2 have been examined. The expressions of a panel of M1 phenotype markers (iNOS and TNF-α) and M2 phenotype markers (CD 206 and arginase-1) were compared in bone marrow macrophages isolated from young and aged mice. Aged mice showed higher ratios of M1/M2 phenotype markers [65]. These results suggest a decline of macrophage immune response functions and a tendency to pro-inflammatory macrophage polarization with aging. A better understanding of changes in macrophage activation and polarization during aging will provide novel therapeutic strategies for age-associated chronic diseases.

9.2.3 Effects of Inflammation and Immunosenescence on Aging Metabolism

The effects of aging on the immune system are manifest in many aspects: increased inflammation and oxidative stress, impaired mitochondrial function, and decreased growth hormones and IGF1, all of which can result in weakened immune systems [66]. Inflamm-aging refers to the chronic low-grade inflammation commonly seen in aging. Chronic low-grade inflammation, characterized by elevated levels of pro-inflammatory cytokine, affects body composition, energy metabolism, immune responses, and neuronal functions in the elderly [67]. However, the mechanism of how inflamm-aging contributes to adverse health outcomes in the elderly remains largely unknown.

Inflamm-aging is largely attributed to the age-related decrease in immunity, also referred to as "immunosenescence" [68]. Age-related alterations in immunity are

well-established in both innate and adaptive immune systems. The production of cytokines and chemokines by innate immune cells is reported to substantially change with age, contributing to increased susceptibility to infection. Increased pro-inflammatory cytokine levels such as IL-6, IL-1β, TNF-α, and TGF-β are observed in the elderly [69–71]. Also, macrophages not only function as phagocytic cells for the elimination of pathogens but also as immune mediators for the initiation and propagation of inflammatory responses. Age-associated macrophage dysfunctions are characterized by altered macrophage population, TLRs' expression, and cytokine secretion, as well as reduced phagocytosis, antibacterial defense, and wound repair. All of these physiological changes contribute to compromised inflammatory responses and higher susceptibility to infectious diseases.

9.3 Ghrelin Signaling in Inflammation, Energy Metabolism, and Insulin Resistance during Aging

9.3.1 Ghrelin and GHS-R in Aging Tissues and Organs

Ghrelin, a 28-amino acid peptide, is a circulating orexigenic hormone. Ghrelin stimulates food intake, adiposity, and insulin resistance [9]. The concentration of ghrelin in plasma increases during fasting and declines after food intake [72]. The biological functions of ghrelin are mediated by its receptor, GHS-R. The activation of GHS-R elicits a wide variety of signaling pathways involved in energy homeostasis, glucose and lipid metabolism, and immune responses [9].

Aging is associated with reduced appetite and food intake, which contributes to malnutrition, metabolic impairment, and adverse health outcomes in the elderly [73]. We have investigated the impact of age on ghrelin and GHS-R levels in plasma and the brain of aging mice. In plasma, levels of ghrelin increase with age [74]. In the brain, ghrelin mRNA expression in mice at 18 and 24 months of age is significantly higher compared to that of 12- and 28-month-old mice, respectively [74]. Ghrelin is ubiquitously expressed, while expression of its receptor GHS-R1a is more limited. To fully understand ghrelin's effect on target tissues, the distribution of the mRNA of GHS-R1a (ghrelin's functional receptor) was examined in multiple tissues and organs; the pituitary glands and brain showed the highest expressions. Furthermore, GHS-R1a mRNA expression in pituitary glands varies in different age groups: mice aged 1–2 months had the highest mRNA expression, while its expression in brains was not significantly changed with age [74]. It is also worth noting that in young mice, GHS-R1a is not detected in the liver, epididymal fat, or brown fat [74].

9.3.2 GHS-R in Age-Associated and Diet-Induced Obesity and Insulin Resistance

Aging is associated with increased adiposity in WAT. GHS-R1a mRNA is not expressed in young mice, but its expression increases in epididymal WAT and interscapular BAT of aged mice [74]. We have reported that 24-month-old GHS-R knockout (*Ghsr*$^{-/-}$) mice exhibit reduced body weight and adiposity and increased lean mass when compared to wild-type (WT) mice. Deletion of GHS-R reduced adipogenic gene expression (C/EBPα and aP2) in WAT and also reduced levels of TG, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and FFA in the circulation of *Ghsr*$^{-/-}$ mice. Old *Ghsr*$^{-/-}$ mice showed greater glucose clearance capacity and reduced insulin secretion than WT mice, indicating improved insulin sensitivity during aging [75]. These data suggest that GHS-R deletion protects against age-associated obesity and insulin resistance.

9.3.2.1 GHS-R in ATM-Associated Adipose Inflammation

Age-associated increases of adipose tissue inflammation are closely associated with the development of insulin resistance. GHS-R expression in mature peritoneal macrophages is increased during aging [76]. GHS-R deletion reduced pro-inflammatory cytokine expression (MCP-1, TNF-α, IL-1β, and CD206) in both WAT and BAT. Moreover, the ratio of pro-inflammatory M1 (F4/80^{+}CD11c^{-}) vs. anti-inflammatory M2 (F4/80^{+}CD11c^{+}) (M1/M2 ratio) was significantly reduced in WAT of old *Ghsr*$^{-/-}$ mice compared to WT mice. In agreement, we detected reduced M1 peritoneal macrophages and increased M2 peritoneal macrophages in the old *Ghsr*$^{-/-}$ mice, which indicates that GHS-R deletion promotes macrophage phenotypic shift from M1 to M2 [76]. Consistently, the expression of M1 markers (MCP1, TNF-α, IL-1β, IL-6, and CD11c) was decreased, but the expression of M2 markers (CD206 and CD301) was increased [76]. As a result, GHS-R mediates inflammatory responses through modulation of both macrophage polarization and ATM-associated adipose tissue inflammation.

We also observed that in cultured RAW264.7 macrophages, GHS-R antagonist [D-Lys3]-GHRP-6 suppressed the LPS-induced expression of TNF-α and IL-1β. Moreover, GHS-R knockdown reduced the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1) and pro-inflammatory macrophage marker (CD11c) in macrophages [76]. These findings suggest that GHS-R antagonists may serve as a novel therapy for age-associated inflammation and insulin resistance.

9.3.2.2 GHS-R in Age-Associated Thermogenic Impairment

Aging is also associated with impaired thermogenesis in BAT. Mainly through non-shivering thermogenesis (heat production), BAT regulates energy homeostasis and adiposity in rodents and humans. It is known that BAT mass and activity decline with age in both men and women. In one study, BAT of old male adults (aged 56–82 years) showed a dramatic decrease of 95% in mass and 75% in activity when compared to young adults (aged 11–43 years) [77]. The impaired thermogenesis in BAT contributes to obesity and insulin resistance during aging. Ghrelin and obestatin are two peptide hormones derived from the same preproghrelin gene, but they have opposite effects on thermogenic markers: ghrelin decreases UCP1 expression, whereas obestatin enhances UCP1 expression in a dose-dependent manner [78]. Old mice exhibited higher plasma concentrations of ghrelin compared to young mice but unchanged obestatin concentrations. Deletion of GHS-R led to increased energy expenditure and enhanced thermogenic gene expressions (UCP1) in BAT of old mice, despite GHS-R deletion had no effect on food intake [75]. We found that GHS-R deletion activated insulin signaling cascade IR-IRS1, thermogenic cascade PKA-Creb-UCP1, and lipolytic cascade PKA-HSL-UCP1, ameliorating age-associated impairment of thermogenesis and insulin resistance in BAT [20].

Mitochondria are dynamic organelles that continuously join (fusion) and divide (fission). Imbalance between the processes of fusion and fission is implicated in disease states such as insulin resistance and cardiovascular diseases [79]. Fusion is mediated by mitofusins (Mfns) and optic atrophy gene 1 (OPA1); fission is mediated by dynamin-related protein 1 (Drp1) and fission 1 (Fis1). We found that *Ghsr*$^{-/-}$ mice exhibited increased expressions of both fusion- and fission-related genes (Drp1, Fis1, OPA1, Mfn1, Mfn2), which indicates improved mitochondrial dynamics [20]. *Ghsr*$^{-/-}$ mice also exhibit increased Cox2 and Cox10 expressions, indicative of increased mitochondrial biogenesis [20]. Collectively, our findings suggest that GHS-R deletion improves both mitochondrial dynamics and biogenesis in aging.

9.3.2.3 Ghrelin and GHS-R in the Liver and Skeletal Muscle

GHS-R deletion also attenuates steatohepatitis in old mice. H&E and Oil Red O staining indicates that lipid content in the livers of *Ghsr*$^{-/-}$ mice is much lower than in the livers of WT mice [80]. It was further revealed that expressions of F4/80 and TNF-α were dramatically lower in the livers of *Ghsr*$^{-/-}$ mice [80], indicating decreased lipid accumulation and inflammation in the liver. These results support that GHS-R deletion reduces aged-associated steatohepatitis, thus improving insulin sensitivity. Because GHS-R1a is not expressed in the liver, the effect on the liver is most likely through the paracrine action of macrophages on hepatocytes.

Insulin resistance in skeletal muscle is mainly attributed to excessive ROS production and inflammation. Ghrelin is also called acylated ghrelin, because of the unacylated ghrelin in the circulation. The unique acylation of acylated ghrelin allows it to activate GHS-R, while unacylated ghrelin cannot activate GHS-R. Weight gain and hyperglycemia are primarily caused by acylated ghrelin, but not unacylated ghrelin [81]. It has been reported that treatment of pharmacologic doses of ghrelin increases muscle strength of old mice [82]. Administration of unacylated ghrelin has been reported to result in not only reduced mitochondrial ROS generation and pro-inflammatory cytokine expressions but also in enhanced AKT signaling and glucose uptake in rodent skeletal muscle [83]. Because unacylated ghrelin cannot activate GHS-R, the effect of unacylated ghrelin on skeletal muscle is not likely to be mediated by GHS-R. The gene expression of GHS-R in skeletal muscle is reported to be low [84]. The function of GHS-R in skeletal muscle remains to be determined.

9.3.2.4 Neuronal GHS-R in Diet-Induced Obesity

Studies involving global GHS-R knockout have illustrated the roles of GHS-R in obesity and insulin resistance. It should however be noted that GHS-R is primarily expressed in the brain, with the highest expression in mice found in agouti-related peptide (AgRP) neurons of the hypothalamus [84]. The hypothalamus controls appetite and food intake via complex neural networks and mechanisms. To further determine the sites of action of GHS-R, we generated GHS-R knockout mice to target all neurons (*Syn1-Cre;Ghsr*$^{f/f}$) and AgRP neurons (*AgRP-Cre;Ghsr*$^{f/f}$). GHS-R deletion in all neurons completely prevents DIO [85]. GHS-R deletion in AgRP neuron neurons attenuates DIO [84]. Both models show increased energy expenditure and thermogenesis and improved insulin resistance [84, 85]. AgRP neurons are essential for regulation of obesity and energy homeostasis; expression of AgRP is regulated by hormones such as ghrelin and leptin, as well as nutritional conditions such as fasting and feeding [86, 87]. *AgRP-Cre;Ghsr*$^{f/f}$ mice showed decreased ghrelin-induced growth hormone secretion, acute food intake, and increased energy expenditure with no change in total energy intake [84]. Moreover, deletion of GHS-R in AgRP neurons activates thermogenic signaling in both WAT and BAT [84]. Together, our findings have shown that suppressing GHS-R has many beneficial effects in aging mice, leading to lean and insulin sensitive metabolic healthy phenotype (Fig. 9.3).

Collectively, these new findings highlight the regulatory roles of ghrelin signaling not only in appetite and adiposity but also in age-associated inflammation, thermogenic impairment, and insulin resistance. These findings strongly suggest that controlling ghrelin signaling may present a novel strategy in the prevention/treatment of obesity and insulin resistance.

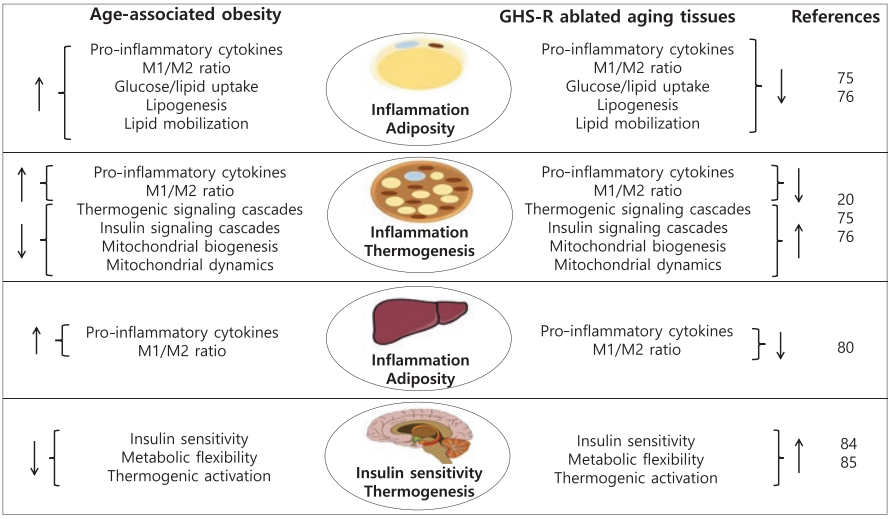

Fig. 9.3 Ghrelin/GHS-R in metabolic tissues during aging
Compared to age-associated obesity, deletion of GHS-R reduces inflammation (e.g., decreased pro-inflammatory cytokine expressions and M1/M2 ratio) and adiposity in both WAT and the liver. Moreover, deletion of GHS-R attenuates age-associated thermogenic impairment in BAT by mediating thermogenic and insulin signaling cascades. GHS-R has a regulatory role in neurons, particularly in agouti-related peptide (AgRP) neurons of the hypothalamus. The neuron-specific deletion of GHS-R improves insulin sensitivity, metabolic flexibility, and thermogenic signaling cascades

9.4 Conclusion and Perspective

Ghrelin is a multifunction hormone. The multifunctional roles of ghrelin and GHS-R in energy homeostasis, inflammation, and thermogenesis make them key regulators of obesity and insulin resistance in the elderly. Ghrelin and GHS-R expression increase with age, which makes them very relevant to aging biology. Ghrelin signaling is therefore an important regulatory mechanism for immunometabolism and inflamm-aging. Targeting the ghrelin receptor and ghrelin signaling may provide powerful means for combating age-associated obesity and insulin resistance, and GHS-R antagonists hold great potential as anti-obesity and anti-diabetic drugs.

Nomenclature

AgRP-Cre;Ghsr$^{f/f}$	AgRP neuron-specific GHS-R knockout
AMPK	AMP-activated protein kinase
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue

BMI	Body mass index
CDC	Centers for Disease Control and Prevention
Cox1	Cyclooxygenase 1
Cox2	Cyclooxygenase 2
DAG	Diacylglycerol
DIO	Diet-induced obesity
Drp1	Dynamin-related protein 1
EGP	Endogenous glucose production
FFA	Free fatty acid
Fis1	Fission 1
GHS-R	Growth hormone secretagogue receptor
$Ghsr^{-/-}$	GHS-R knockout
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
iNOS	Inducible nitric oxide synthase
IRF5	Interferon regulatory factor 5
IRS	Insulin receptor substrate 1
JNK	Jun NH_2-terminal kinase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MCP-1	Monocyte chemotactic protein-1
Mfns1	Mitofusins 1
Mfns2	Mitofusins 2
NAFLD	Nonalcoholic fatty liver disease
NE	Norepinephrine
OPA1	Optic atrophy gene 1
PAMPs	Pathogen-associated molecular patterns
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
SNS	Sympathetic nervous system
$Syn1\text{-}Cre;Ghsr^{f/f}$	Neuron-specific GHS-R knockout
T2D	Type 2 diabetes
TAG	Triacylglycerides
TGF-β	Transforming growth factor-β
Th1	T-helper 1
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor

VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
WT	Wild-type
β3-AR	β3-Adrenergic receptor

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Chapter 10
Electrophysiological Mechanism of Peripheral Hormones and Nutrients Regulating Energy Homeostasis

Zhuo Huang and Kuo Xiao

Abstract In organism, energy homeostasis is a biological process that involves the coordinated homeostatic regulation of energy intake (food intake) and energy expenditure. The human brain, particularly the hypothalamic proopiomelanocortin (POMC)- and agouti-related protein/neuropeptide Y (AgRP/NPY)-expressing neurons in the arcuate nucleus, plays an essential role in regulating energy homeostasis. The regulation process is mainly dependent upon peripheral hormones such as leptin and insulin, as well as nutrients such as glucose, amino acids, and fatty acids. Although many studies have attempted to illustrate the exact mechanisms of glucose and hormones action on these neurons, we still cannot clearly see the full picture of this regulation action. Therefore, in this review we will mainly discuss those established theories and recent progresses in this area, demonstrating the possible physiological mechanism by which glucose, leptin, and insulin affect neuronal excitability of POMC and AgRP neurons. In addition, we will also focus on some important ion channels which are expressed by POMC and AgRP neurons, such as K_{ATP} channels and TRPC channels, and explain how these channels are regulated by peripheral hormones and nutrients and thus regulate energy homeostasis.

Keywords Glucose · Leptin · Insulin · Energy homeostasis · Proopiomelanocortin · Agouti-related protein · Neuropeptide Y · POMC neuron · AgRP\NPY neuron · K_{ATP} channel · TRPC channel

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10.1 Energy Homeostasis in the Control of POMC and AGRP Neurons' Electrophysiological Properties

In organism, energy homeostasis is a biological process that involves the coordinated homeostatic regulation of energy intake (food intake) and energy expenditure. Impairment of energy homeostasis is involved in a range of metabolic disorders like obesity. Obesity can lead to a bunch of other medical conditions such as diabetes, hypertension, and coronary heart disease. It is not only considered a health risk but also an economic burden [1]. From energy regulation's perspective, obesity can be considered as a state of excessive energy intake and insufficient energy expenditure [2, 3]. Therefore, the most important step to treat obesity is to disclose how the energy homeostasis is maintained under physiological condition.

It has been shown that arcuate (ARC) nucleus within the hypothalamus plays an important role in regulating energy homeostasis [4, 5]. The arcuate nucleus is located near the third ventricle and lies above the median eminence and contains populations of neurons including POMC- and AgRP/NPY-expressing neurons [6]. The arcuate nucleus has a unique structure in which blood-brain barrier is incomplete in ARC-median eminence area. Thus, proteins and other signaling molecules such as peripheral hormones and nutrients can cross the barrier and regulate the excitability of POMC and AgRP/NPY neurons in this area.

POMC gene is expressed in a number of tissues including skin, the immune system, pituitary, and hypothalamus neurons. In hypothalamus POMC neurons, prohormone convertase 1 (PC1) cleaves POMC to ACTH. After that prohormone convertase 2 (PC2) cleaves ACTH to α-MSH, which can activate melanocortin-4 receptor (MC4R) through secretion. MC4R is located in the paraventricular nucleus (PVN) neurons in the hypothalamus, and MC4R is an important molecule underlying appetite control and energy homeostasis. In conclusion, when POMC neuron is depolarized, it will lead to α-MSH release, activate MC4R neurons in PVN, and eventually inhibit feeding and reduce body weight. Functional mutation of MC4R can cause obesity and leptin deficiency, but the specific pathway of MC4R regulation still needs to be further illustrated [7].

AgRP and NPY are also secreted by ARC neurons. However, the functions of AgRP and NPY are completely opposite with POMC [8]. The system of AgRP/NPY is considered to have an orexigenic effect. The synthesis and secretion of AgRP and NPY are regulated by situations of energy deficiency or increased metabolic demand. Furthermore, both of them have various projection sites in the hypothalamus. AgRP and NPY regulate energy homeostasis mainly though MC4R. AgRP is a selective antagonist of MC4R; it can stop α-MSH's binding with MC4R and thus inhibit the food intake decrease effect of MC4R. In contrast to AgRP, NPY's effect in increase food intake is rather short-lived. Central administration of AgRP in rodents leads to food intake increase for days, but for NPY, it will only last for an hour [9].

The arcuate nucleus has a unique structure in which blood-brain barrier is incomplete in ARC-median eminence area. Therefore, numerous peripheral neuropeptides

such as insulin and leptin and nutrients such as glucose and fatty acids from the gastrointestinal tract and other organs cross the barrier and regulate the excitability of POMC and AgRP/NPY neurons in this area. The functional regulation relies on the plastic alteration of ion channels expressed in these neurons. Specially, K_{ATP} channels and TRPC channels on POMC and AgRP neurons play an important role in the regulation of neuronal excitability. The detailed mechanism of these two ion channels by which POMC and AgRP neuron are regulated will be introduced in later part of the review.

10.2 Signaling Pathway of Glucose Regulating POMC and AgRP Neurons' Excitability

It is undoubtedly true that there are multiple regulation factors of POMC and AgRP neurons. These factors including glucose, amino acids, fatty acids, and numerous peripheral hormones like leptin and insulin transfer the information about energy metabolism from the peripheral to central nervous system. Here, we will discuss the well-studied messenger, glucose, in order to gain an insight into the mechanism by which glucose regulates the excitability of POMC and AgRP neurons.

The regulation of energy homeostasis by glucose is primarily induced by glucose-sensing neurons. Glucose-sensing neuron is a population of neurons that respond to changes in extracellular glucose concentration [10]. In the hypothalamus, there are two major types of glucose-sensing neurons: those excited and those inhibited by glucose. Glucose-sensing neuron plays a vital role in the brain, because unlike other organs, the brain needs to use glucose constantly and depriving glucose supply for a short time could lead to permanent brain damage. Therefore, brain evolved a sophisticated regulatory system to guarantee the adequate concentration of glucose around CNS.

ATP-dependent potassium (K_{ATP}) channel has a key role in regulating glucose homeostasis. In peripheral pancreatic beta cells, glucose enters the cells via the GLUT2 transporters and is phosphorylated by glucose kinase. Then it is metabolized to produce ATP [11]. The increase in the cell's ATP level consequently closes K_{ATP} channels, which leads to reduced potassium current, depolarizing the membrane, eventually increasing the cell's excitability [12, 13]. Several lines of evidence suggested that the glucose-sensing mechanism of POMC and AgRP neurons could be the same as that in pancreatic beta cells. K_{ATP} channels are widely expressed in POMC and AgRP neurons. Intriguingly, glucose-sensing ability of POMC neurons is affected by blocking K_{ATP} channels [14]. Further study has also showed that the glucose tolerance is impaired in K_{ATP} channel mutation mice [15]. Taken together, K_{ATP} channel may provide a possible mechanism for regulating glucose homeostasis (Fig. 10.1).

Recent studies indicate that there may be other mechanisms for POMC and AgRP neurons to sense glucose concentration [16, 17]. This hypothesis is that

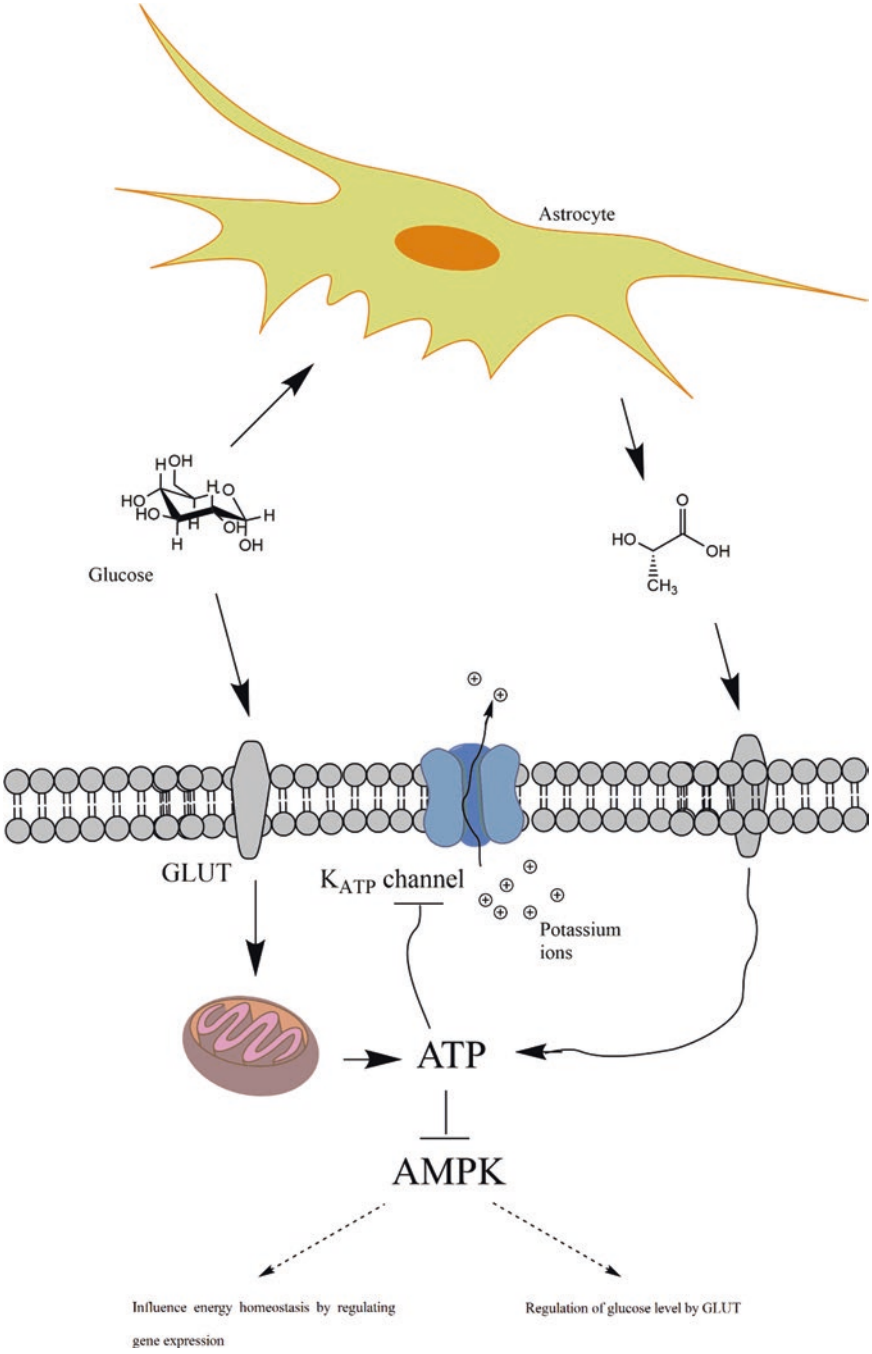

Fig. 10.1 Glucose-sensing mechanism of AgRP and POMC neurons. Glucose influences the excitability of POMC and AgRP neurons in two different ways. Firstly, glucose enters neurons through GLUT, and it is metabolized to produce ATP. Then, ATP level regulates K_{ATP} channels to influence neural excitability. The other way is via astrocyte. Astrocytes take in glucose, transfer it into lactate. Then lactate enters neuron and is metabolized into ATP, thus regulates K_{ATP} channels to change neural excitability. In addition, glucose also affects AMPK level, which has strong influence on many aspects of energy homeostasis

glucose change is detected with the help of astrocytes in the hypothalamus. The astrocyte's glucose-sensing mechanism is also related to K_{ATP} channels. Astrocytes take in glucose, change it into lactate, and release lactate to neurons, and then neurons use lactate to produce ATP; eventually the increased concentration of ATP causes K_{ATP} channels to close. This intriguing hypothesis is supported by a lot of evidence. A study shows when astrocytes in the hypothalamus are exposed to a high concentration of glucose, it will increase its ATP produce, which will lead to lactate release [18]. The same study also shows that high concentration of lactate elevates cytosolic ATP level and closes K_{ATP} channel. After several years, a new finding also supports this hypothesis when people have access to GLTU2 knockout mice [16, 19]. GLUT2 knockout mice suffer from glucagon deregulation, but when re-expressing GLUT2 in astrocytes, these mice restore normal control of glucagon levels. These evidences clearly demonstrated astrocytes also have an important role in glucose-sensing mechanism (Fig. 10.1).

Furthermore, AMPK signaling pathway may also be involved in the glucose-sensing. Glucose-insensitive mutant mice show low levels of AMP-activated protein kinase (AMPK) expression in POMC neurons [20]. AMPK is a sensor of cellular AMP/ATP ratio. Mice lacking AMPK show decreased energy expenditure, which leads to fat. However, these data remains controversial. Other investigations have demonstrated that there was no direct link between AMPK and K_{ATP} channels. These facts indicate there could be other regulation pathways for sensing glucose apart from K_{ATP} channels. Recent study show AMPK is regulated by leptin and adiponectin, and AMPK is a downstream target of PI3K, which is leptin's signal pathway. Therefore, in glucose-sensing neurons, the interplay between glucose and leptin signal pathway needs to be further investigated [21].

Glucose-sensing function requires a complex system, and there are plenty of theories to explain the specific mechanism of this function. But like many other biological phenomenon, it is unlikely to work for only one signaling pathway. From known evidence now, we know that glucose sensing is related to K_{ATP} channel and AMPK, both of which plays a very important role in the process. But there may still be other factors to be found, and the link between these two known pathways is also to be further studied.

10.3 Signaling Pathway of Leptin and Insulin Regulating POMC and AgRP Neurons' Excitability

Aside from glucose, many hormones such as insulin and leptin are important in energy homeostasis regulation. High levels of leptin are frequently observed in obese patients. This phenomenon may be the consequence of leptin resistance, that is, patients have high leptin concentration, but leptins fail to decrease energy intake [22]. Moreover, leptin knockout mice are associated with massively obesity and hyperglycemia. These mice also show hyperinsulinemia and insulin resistance.

Treating these mice with ICV leptin shows rapid weight loss and normoglycemia. Based on these facts, there would be no doubt that leptin is closely correlated with obesity and plays a key role in energy homeostasis regulation [23–25].

Leptin is secreted by fat tissue, and its action is mediated by leptin receptor. Interestingly, leptin receptors (LepR) are widely expressed in the hypothalamus, while its expression is especially high in POMC and AgRP neurons. To investigate leptin's exact mechanism of energy homeostasis regulation, CRE-LOXp technology have been used. POMC or AgRP selectively LepR knockout mice show changes in body weight and glucose homeostasis [26]. Many studies have shown that knockout LepR in POMC neurons promotes obesity. This kind of knockout-induced obesity could be caused by either diminished food intake or increased energy expenditure. Re-expressing LepR in these knockout mice improves glucose regulation and insulin sensitivity [27, 28]. Based on these results, it is clear that leptin is involved in POMC and AgRP neurons' energy balance regulation function.

Like leptin, insulin's function in energy homeostasis regulation has been well recognized for a long time. Insulin is secreted by pancreatic beta cells. It is transferred by blood to plenty of organs, and it influences any metabolic progresses. Interestingly, insulin receptors are also expressed in the hypothalamus like leptin [29]. The functional studies of insulin start from tests on hypothalamus insulin receptor knockout mice. Tests show these knockout mice are associated with increased food intake and obesity. Furthermore, insulin receptor in the hypothalamus has also been shown to have essential functions in glucose regulation [30, 31]. But strangely, POMC or AgRP neuron-specific insulin receptor knockout mice show no effect on body weight and food intake [12]. However, these mice do show changes in glucose regulation, indicating insulin has influences on POMC and AgRP neurons in the regulation of glucose homeostasis but it does not influence body weight. Further experiments show re-expressing insulin receptor in POMC neuron increases glucose production and food intake [32]. Taken together, these facts clearly show insulin plays an important role in POMC and AgRP neurons' energy homeostasis regulation.

To illustrate the exact molecular mechanisms of leptin and insulin actions in POMC and AgRP neurons, leptin's and insulin's signaling pathways should be investigated. As state above, leptin and insulin both have important anorexigenic effects in POMC and AgRP neurons. Plenty of tests on genetic modification animals have been done to demonstrate why these hormones can have their effects on POMC and AgRP neurons. The next part of review will focus on the exact pathways of insulin and leptin, to demonstrate their effects on neuron excitability and energy regulation function.

Recent researches have showed the importance of STAT3 in energy regulation. Deletion of STAT3 has been found to decrease the POMC mRNA level. STAT3 is a transcription factor. It binds to POMC promoter and stimulates POMC expression. Leptin can activate STAT3 by the Janus kinase (JAK). JAK can phosphorylate leptin receptor and activate STAT3 [33]. Many tests have been done to STAT3 POMC- or AgRP-specific deletion of mice. In POMC neurons, deletion of STAT3 can cause a mild decrease in POMC expression and increase in body weight. Experiment results

indicate this body weight increase is associated with increased food intake [34]. Interestingly, POMC-specific STAT3 deletion mice also show hyperleptinemia, demonstrating them suffering from leptin resistance. On the other hand, injecting leptin in deletion mice cannot repeat the same result that shows in wildtype mice. Body weight or food intake changes have not been observed in these knockout mice. This result clearly shows that STAT3 is important in POMC neuron-mediated leptin effects [35]. Furthermore, this study also shows STAT3's activation leads to insulin resistance, indicating STAT3 is a mediator of both leptin and insulin. In the meantime, STAT3's function in AgRP neuron has also been investigated. Overexpression of STAT3 in AgRP neurons leads to increased locomotor activity with no effect on neuropeptide expression [36]. And deletion of STAT3 in AgRP neurons shows a mild gain in mouse body weight [37]. Constitutive activation of STAT3 in AgRP neurons changes energy balance regulation. This change is likely caused by decreased AgRP gene expression, which is a target of STAT3 [38]. Considering all evidence, STAT3 have an important role in leptin and insulin's regulation of energy homeostasis through POMC and AgRP neurons. Different results can be elicited to POMC and AgRP neurons indicating the complexity of hypothalamus energy homeostasis regulation.

Another well-studied signaling pathway of leptin and insulin regulation is the PI3K-PKD1-FoxO1 signaling pathway. Insulin and leptin have been shown to regulate the primary factor of this pathway, the phosphatidylinositol3-kinase (PI3K). It has been shown that PI3K plays a fundamental part in regulation of insulin and leptin. Without PI3K, these hormones cannot activate or inhibit POMC and AgRP neurons anymore [12, 39, 40]. When PI3K is activated, it will phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Accumulation of PIP3 can activate other kinases, like 3-phosphoinositide-dependent protein kinase (PDK1), to continue the signaling cascade. PDK1 is involved in leptin and insulin's action on POMC and AgRP neurons [41–43]. Deletion of PDK1 in POMC neurons leads to increased food intake and decreased POMC expression [41]. Furthermore, in these knockout mice, glucose regulation is impaired, and leptin cannot show its effects anymore. These results show PDK1 is essential for leptin's action on POMC and AgRP neurons [41]. Other experiments show PDK1 is also involved insulin's regulation. Deletion of PDK1 in POMC neurons causes insulin sensitivity change. Moreover, this deletion also affects glucose homeostasis [43]. In contrast, deletion of PDK1 in AgRP neurons causes body weight loss because of decreased food intake and increased locomotor activity [42].

PDK1 regulates many kinase and transcriptional factors; between them, FoxO1 is one of the most well-studied transcriptional factors that is related to energy homeostasis. FoxO1's activation can increase POMC's expression and, in the meantime, decreases AgRP's expression. POMC-specific deletion of PDK1 model mice can prove FoxO1's function. PKD1 deletion decreases FoxO1 phosphorylation. Because the phosphorylation of FoxO1 is reduced, FOX1 will remain in the nucleus and inhibit POMC expression, rather than come out of the nucleus and not have this inhibit effect [42]. This experiment shows FoxO1 could be the final effector of

leptin signaling. Further studies have been focused on the FoxO1's effects on leptin and insulin's energy regulation function. After specific deletion of FoxO1 in POMC neurons, body weight loss and decreased food intake have been observed in these gene modified mice [44, 45]. However, this change is not caused by regulating POMC expression but by directly influencing α-MSH, which, as stated above, is the target of POMC. These knockout mice also show increased leptin sensitivity, indicating FoxO1's important role in leptin action. In the meantime, deletion of FoxO1 in AgRP neuron leads to decreased food intake and increased locomotor activity, but body weight is not changed in this model [46]. Furthermore, glucose homeostasis is affected by FoxO1 knockout. Though deletion of FoxO1, glucose production is increased, mostly because of insulin sensitivity changes in AgRP neurons.

Electrophysiology research about leptin and insulin's function returns a very complicated result. As stated above, leptin and insulin shared almost the same signaling pathway in POMC and AgRP neurons, but their influences on neuron's excitability are completely opposite. While leptin can lead to cell membrane depolarization and increase in firing rate, insulin makes cell membrane hyperpolarization and decreases its firing rate. Researchers believe both of these changes are related to the K_{ATP} channels [13, 39]. Through the same channel, this peculiar phenomenon could be caused by a different concentration of PIP3. The hypothesis researchers proposed is that when certain amount of PIP3 accumulate in the membrane, some kinds of cation channels (likely to be TRPC channels) will open, leading to depolarization. Nevertheless, more PIP3 leads to opening of KATP channels and then hyperpolarizing the membrane (Fig. 10.2).

However, recent studies have proposed a new theory [47]. Researchers have identified two subtypes of SF1 neurons. Although it is not POMC or AgRP neurons, SF1 neuron is also a population of hypothalamic neurons that control energy homeostasis, and it is regulated by leptin and insulin as well. This study might provide some insight for the paradoxical phenomenon in the research of POMC and AgRP neurons' electrophysiological properties. The theory is that there are two subtypes of neurons, one is activated by leptin, and the other is inhibited by leptin. In the meantime, they are both insulin-inhibited. These two types of neurons perform different functions. Leptin-depolarizing neurons are mediated by the putative transient receptor potential C (TRPC) channel, which is activated by the PI3K p110β catalytic subunit. Meanwhile, leptin and insulin's hyperpolarizing effect requires the PI3K p110α or p110β catalytic subunits and was mediated by K_{ATP} channel. This theory explains how PI3K can induce two different effects. For there are two types of SF1 cells and PI3K effects are different between them. Same situation could happen to POMC and AgRP neurons too, further investigation need to focus on the subtype of POMC and AgRP neurons.

In conclusion, STAT3 and PI3K-PDK1-FoxO1 signaling pathways are crucial in leptin and insulin's action. These factors influence POMC and AgRP neurons from a variety of aspects. Many experiments have proved functions of these factors on food intake, energy expenditure, glucose metabolism, and, above all, energy homeostasis regulation. More importantly, these factors cannot work by their own. There must be a synergistic interaction between pathways to achieve the final function.

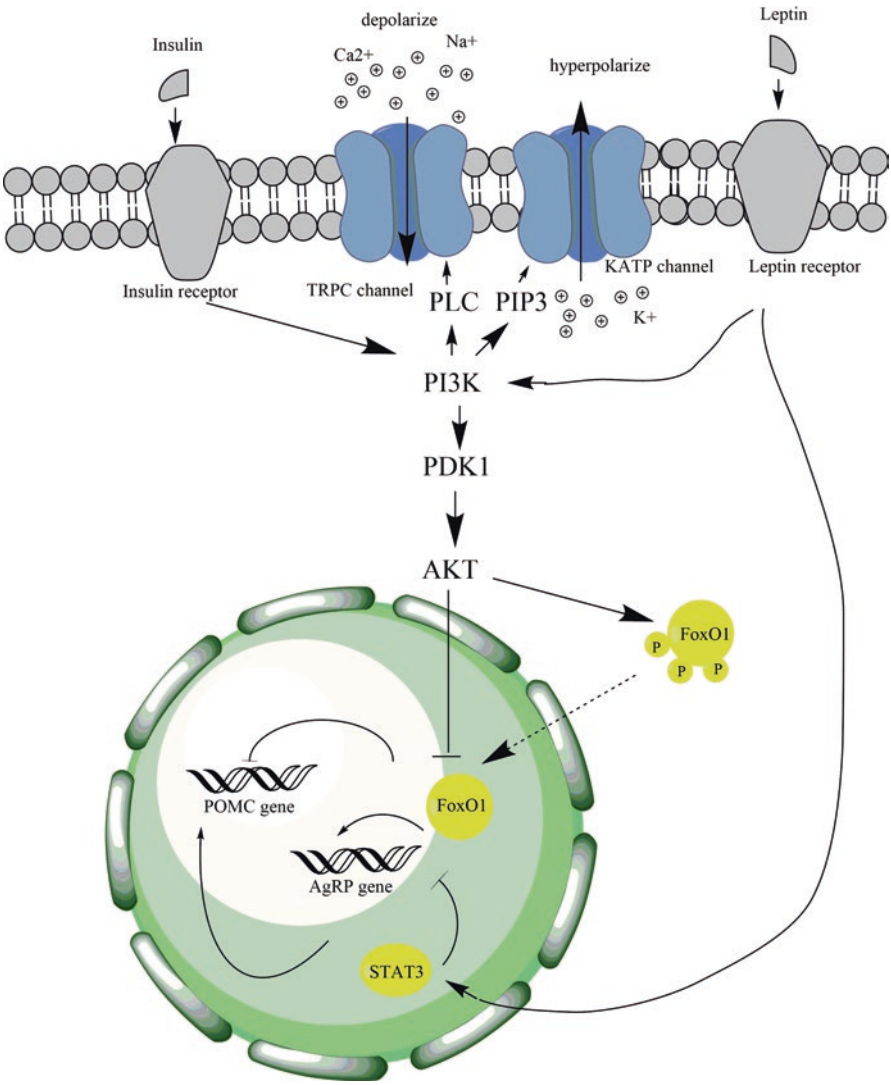

Fig. 10.2 Insulin and leptin action on POMC and AgRP neurons

Insulin and leptin share the same signaling pathway on POMC and AgRP neurons. The signaling pathway is primarily mediated by PI3K. PI3K can lead to FoxO1 change to influence POMC or AgRP gene expression. However, leptin and insulin have different effect. This may be caused by different ion channels they influence. Through K_{ATP} channel, neuron can be hyperpolarized, whereas TRPC channel can cause neuron depolarization. These two types of channel may be expressed in different subtypes of POMC and AgRP channels, indicating to further investigate the subgroups of these neurons.

10.4 K_{ATP} Channels in Energy Regulation of POMC and AgRP Neurons

Two types of ion channels have important regulation effects on POMC and AgRP neurons, the K_{ATP} channel and the TRPC channel. Today, we think these two types of channels work together to achieve energy regulation function of POMC and AgRP neurons. Like the theory stated last part, leptin and insulin have different regulation functions through the same PI3K pathway, and this phenomenon is caused by primarily K_{ATP} and TRPC channels. Recent studies support this theory by some new experimental findings. Using whole-cell recording technique and selective pharmacological drugs, researchers find that insulin depolarizes POMC neuron via the activation of TRPC channel and, in the meantime, hyperpolarizes AgRP neuron by activation of K_{ATP} channel [48]. Therefore, in this part of review, we will focus on K_{ATP} and TRPC channels to get a better understanding of POMC and AgRP neurons' action on energy regulation.

About K_{ATP} channel, we have discussed it in the last part; thus here we will reiterate some important facts again. K_{ATP} channel have been studied for a long time; the history of K_{ATP} channel's study is remarkable. K_{ATP} channel's most important characteristic is that it is regulated by ATP. When ATP level increases, ATP will bind to one of the K_{ATP} channel's subunits – Kir6 – to cause channel closure [49]. The intracellular ATP concentration is closely connected to metabolism, so K_{ATP} channel is considered probably to have a role in energy regulation. Further experiments in pancreatic beta cell proved this postulation; K_{ATP} channels do have a pivotal role in the energy regulation process of pancreatic beta cells. K_{ATP} channel has been proved to help pancreatic beta cells to maintain its resting potential. When K_{ATP} channel is closed, pancreatic beta cell will be depolarized, and calcium channel will open, leading to insulin release. After the mechanism of K_{ATP} channel regulation has been found, when people are investigating the hypothalamus's energy regulation function, there is no surprise to speculate K_{ATP} channel may also play a role in it, since this channel have been proved to have similar effects peripheral. It turned out to be true, K_{ATP} channel does have the effect researchers postulated, and here is some evidence to prove it. Firstly, K_{ATP} channel is highly expressed in POMC and AgRP neurons. Secondly, knockout of K_{ATP} channel in POMC or AgRP neurons impairs energy regulation functions on these cells. Nowadays, there is no doubt that K_{ATP} channels have important regulation functions in POMC and AgRP neurons anymore [50].

New technologies are helping people to find more crucial details about K_{ATP} channel. Crystal structure studies show K_{ATP} channel is consists of four Kir6 subunits and four sulfonylurea receptor (SUR) subunits. Between them, Kir6 subunits form the pore of K_{ATP} channel, and the function of SUR subunits is mainly regulatory [51]. On the other hand, K_{ATP} channel's unique characteristics are unveiled through more electrophysiology experiments. K_{ATP} channel is only regulated by ATP; this property has some profound meanings. It means K_{ATP} channel is not voltage-dependent or time-dependent, and that is very unique in potassium channels. It is opened when ATP level is low and closed when ATP level is high [52]. Because

of its time-independent nature, if ATP level is appropriate, K_{ATP} channel will stay open, and a K_{ATP} current can always be observed. These findings suggest a ATP-dependent regulatory system existed in POMC and AgRP neurons. When ATP level is low, K_{ATP} channel will open, an outward potassium current will occur, and consequently the neuron will be hyperpolarized. And with more intracellular ATP, K_{ATP} channel will be closed, causing outward potassium current to decrease and neuron to depolarization. Finally, regarding energy regulation function, change of POMC and AgRP neurons' membrane potential can cause POMC or AgRP release, leading to its orexigenic or anorexigenic effects.

Noticeably, electrophysiological experiments have reported contradict results about the research on POMC and AgRP neurons. This controversy might be caused by record method [7]. Tradition electrophysiology data is collected by patch clamp technique. But there are problems with currently used whole-cell recording method. Firstly, researches use different concentrations of glucose in their bath solutions. This might cause inaccuracy in research, for real glucose concentration in hypothalamus especially in arcuate nucleus is fluctuating and hard to measure. Next potential problem is when recording pipette puncture the neuron, it will change the cell's ATP/GTP ratio to pipette's preset concentration. This process can directly influence the ATP/GTP ratio in neuron, disrupt its normal physiological metabolism, causing changes in membrane potential. Therefore, existing recording method need to be improved to meet the need of studying POMC and AgRP neurons.

10.5 Regulation Function of TRPC5 Channel on POMC and AgRP Neurons' Excitability

Early theories to answer the question why insulin and leptin have different effect on POMC and AgRP neurons mentioned a specific kind of cation channel. Further experiments have shown this cation channel is TRPC5, because leptin can activate TRPC5 channel [48]. To gain a better understanding of leptin and insulin action on energy homeostasis, this part of review will focus on TRPC5 channel and its electrophysiology property, demonstrating its role in neuron excitability regulation.

The TRPC family has seven members, the names of which are TRPC1 to TRPC7. Between them, TRPC5 is very similar to TRPC4 and TRPC1, the sequence identity of which are 65% and 45% [53]. The structure similarity is shown in membrane topology and high selectivity to cations over anions. TRPC5 channel subunit consists six transmembrane segments, which are linked by extracellular and intracellular segments and flanked with cytoplasmic N and C termini. The regain between transmembrane 5 segment and transmembrane 6 segment lies the channel pore. And a total of four subunits form a complete channel. The N-terminus of TRPC5 channel contains ankyrin repeats, which is a binding site to many binding partners. Beside that, the C-terminus contains calmodulin-binding sites, indicating its regulation by calmodulin. Furthermore, there are other sites found to bind to plenty of binding partners like cAMP or caveolin. Near transmembrane 6 segments, there is the TRP

box, which is expressed in all TRPM family and is used to mediate dynamic protein interaction. In TRPM5's downstream of the TRP domain, there is a site to interreact with PIP2, which is the substrate for the phospholipase C enzyme (PLC), and related to the PI3K pathway [54, 55].

TRPC5 is a nonselective cation channel, but its permeability of calcium is bigger than sodium. Unlike the K_{ATP} channel, TRPC5 channel is voltage-dependent, and its voltage-dependent characteristic is unique and remarkable. TRPC4 channel shares the same voltage-dependent characteristic like TRPC5. The conductance-voltage curve can help understand the voltage dependence of TRPC5. When membrane is depolarized, TRPC5 channel will firstly open. But at around −50 mV, TRPC5 channel begins to close, leading conductance to decrease. And this decline stops at 20 mV, after that TRPC5 channel begin to open again, so as the conductance begins to increase. This peculiar behavior is called N-shaped voltage dependence. The voltage dependence of TRPC4 is very similar to TRPC5, but TRPC4's activation range is about 65 mV positive than TRPC5. Another intriguing phenomenon of TRPC5 is that when it is activated by G-protein, its I-V curve will move toward positive direction like TRPC4. This phenomenon is also observed in many other TRP channels and is considered as a hallmark of TRP channel. The appearance of this N-shaped voltage dependence of TRPC channel can be explained by magnesium ion. Researchers believe TRPC channel is blocked by intracellular Mg^{2+} ions, and this blockage is voltage-dependent. The hypothesis of Mg^{2+} ions is supported by the fact that without Mg^{2+}, the cannel conductance is influenced and the N-shaped voltage dependence is impaired [56, 57].

TRPC5 channel is well recognized to be regulated by multiple factors like G-protein or Ca2+ ions. Recent findings suggest lipids can also be a key regulator of TRPC5 channel. These findings indicate various phospholipases (like PLC) can also regulate TRPC5 function via lipids. Between them, PIP2 have shown great influence on TRPC5. PIP2 can directly activate TRPC5, while PIP2 depletion reduces TRPC5 current. The major signaling pathway of leptin and insulin is PI3K pathway; PIP2 is regulated by PI3K as well. Experiments have proved insulin and leptin have influence on TRPC5. From the aspect of PI3K signaling pathway, this influence is possibly induced by changed PLC and subsequently changed PIP2. Through PIP2 pathway, insulin and leptin can regulate TRPC5 channel function, lead to an inward cation current, cause cell depolarization, eventually generate a different effect from K_{ATP} channel. In conclusion, through TRPC5 channel, the contradictory effect of PI3K pathway can be well explained [54, 56].

10.6 Outlooks

Although people have learned a lot about POMC and AgRP neurons action on energy homeostasis regulation [7], there are still plenty of jobs to do in this particular area. First of all, we now know that there is an interaction in signaling pathway of leptin and insulin. But the detailed molecular mechanism of this interaction is

still unknown. Now we only know a vague structure of this interaction, and the exact factors that would change in this progress are not well identified. Still, there might be some other factors that take part in this interaction, and the full picture of it remains to be illustrated. Secondly, despite signaling pathways of insulin and leptin involve almost the same factors, their effects on neurons are different. And we know it is due to K_{ATP} and TRPC5 channels. But there are still some points of this theory which need to be investigated, such as the determination of the exact PIP3 concentration that leads TRPC5 channel to open. Otherwise, this theory will remain a hypothesis.

Furthermore, during the electrophysiological experiments, there are still some problems. Protocols of experiment need to be changed to fit the true environment of POMC and AgRP neurons; otherwise results of experiments cannot be trusted. Thus, it is necessary to repeat at least some of the electrophysiology experiments, so that we can learn the mechanism of energy regulation more accurately in POMC and AgRP neurons. Another important issue is to clearly separate all subpopulations of POMC and AgRP neurons. There is evidence showing that different subpopulations of POMC neurons have different sensitivity to insulin and leptin and the regulation effects between these subpopulations are different too. As more techniques have been used to identify different subpopulation in the brain, for example, people have already successfully identified 13 distinct populations of oligodendrocytes. The same techniques can be used in POMC and AgRP neurons to help identify all of its subgroups. After that, the study of POMC and AgRP neurons can be more precise and accurate.

The regulation effect of nutrients and hormones on POMC and AgRP neurons has been well tested and proven. It is clear that nutrients and hormones act as messengers to regulate energy homeostasis. Remarkably, more results indicate that these regulation effects tend to void in obese patients. Thus, studies in this area may provide a new aspect to treat obesity and restore normal energy homeostasis in these patients. As obesity is becoming increasingly common disease today, finding a way to stop this pandemic seems to be of great importance. Researches in this area provide a brilliant potential treatment to obesity. Traditional way to fight obesity usually need patients to exercise or control food intake. But if we can illustrate the exact mechanism in the central nervous system regulating energy homeostasis, we can use it to change the state of obesity and restore normal energy balance. It does not require hard work; patient must suffer. Instead, we will only need to design a drug aiming to important regulation factors and change the abnormal status, and obesity can be cured effectively. At today's point of view, this is merely a beautiful dream, but with better knowledge of energy homeostasis, this vision can become true someday.

In conclusion, studies in POMC and AgRP neurons are vital to understand central regulation of energy homeostasis. This review has described glucose and leptin and insulin actions on POMC and AgRP neurons. Further study should be focused on the global mechanism of these factors and eventually gain a systematic understanding of central energy regulation. From therapeutic point of view, this would help people fight obesity and its associated metabolic syndrome.

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Chapter 11
Regulation of Lipolysis in Adipose Tissue and Clinical Significance

Xin Li and Kai Sun

Abstract Lipolysis is a critical process to hydrolyze triglyceride in adipose tissue, thereby breaking down the stored lipid and maintaining energy homeostasis. Recent studies have made significant progress in understanding the steps of lipolysis. This chapter discusses the major pathways that regulate lipolysis in adipose tissue. Specifically we focus on the mechanisms by which the activities of critical lipolytic enzymes are regulated. We further discuss how the lipolysis is regulated by other factors, including insulin and neurotransmitters, in particular catecholamines and the role of sympathetic nervous system in the whole process. Finally we provide clinical perspectives about the novel therapeutic strategies to target or promote adipose tissue lipolysis for treatment/prevention of obesity and type 2 diabetes.

Keywords Adipose tissue · Triglyceride · Lipolysis · Lipase · Sympathetic nervous system (SNS) · Catecholamine

11.1 Introduction

Lipolysis is the catabolic process of triglyceride (TG) stored in cellular lipid droplets. In mammals TG stored in adipose tissue (AT) serves as the major energy reservation which is under a dynamic balance of lipolysis and reesterification [1]. In periods of energy shortage such as fasting, exercise, and cold exposure, TG in white adipose tissue (WAT) is hydrolyzed to liberate fatty acids (FAs) which are released into the circulation system to serve as the substrates for β-oxidation and ATP production in other organs and tissues [2]. Basically, three sequential steps are needed to ultimately break down TG into FAs and glycerol: adipose triglyceride lipase (ATGL) initiates lipolysis and hydrolyzes TG to diacylglycerol (DG); hormone-sensitive lipase (HSL) enzymes DG to monoacylglycerol (MG) and MG lipase

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(MGL) is responsible for the ultimately hydrolysis of MG into fatty acid and glycerol. WAT lipolysis is orchestrated by a wide range of factors involving both endocrine system and autonomic nervous system. Catecholamines are the classical regulators of lipolysis, with specific impacts depending on the subtypes of adrenergic receptors they bind with on the surface of adipocytes. Upon activation of adrenergic receptors, coupled G-protein subunit is released to activate or inhibit adenylyl cyclase (AC) activity depending on the subtype of G-proteins [3]. Activated AC increases cytoplasmic level of cAMP and subsequently results in activation of protein kinase A (PKA, also known as cAMP-dependent protein kinase), which then phosphorylates HSL and strongly activates lipolysis [3]. It used to be accepted that adrenal medullary catecholamines were the primary stimulators of lipolysis. However, abundant data, especially some direct evidence of neuro-adipose junction in WAT visualized in vivo recently, indicate that sympathetic nervous system (SNS) innervation of WAT is necessary and even sufficient to stimulate fat lipolytic process [4]. This chapter will review the current knowledge about lipolysis and its regulation, especially the SNS innervation of WAT on lipolysis.

11.2 The Basic Process of Lipolysis by Lipases

Lipolysis requires three consecutive steps of hydrolysis to liberate three molecules of fatty acids with different enzymes acting on each step. Adipose triglyceride lipase (ATGL) hydrolyzes TG into diacylglycerol (DG), initiating the process of lipolysis; HSL catalyzes DG into monoacylglycerol (MG); and MGL ultimately hydrolyzes MG into fatty acid and glycerol.

ATGL was first described as TG hydrolase in 2004 by three different groups [5–7]. It belongs to the family of patatin-like phospholipase domain-containing protein A (PNPLA) with officially name as PNPLA2. On structure, the NH_2-terminal half of ATGL contains the patatin domain, and the COOH-terminal contains a hydrophobic region for lipid droplets (LDs) binding mediating the interaction between ATGL and LDs, thus regulating enzyme activity [8, 9]. ATGL specifically hydrolyzes TG with very limited activity toward other lipid substrates [7]. ATGL is highly expressed in adipose tissue; meanwhile it's also expressed in the heart, skeletal muscle, liver, and other tissues [6, 10]. Global ATGL-deficient mice exhibit improved insulin sensitivity and glucose tolerance under both normal diet and high-fat diet situations despite severe systemic TG accumulation in both adipose tissue and non-adipose tissues including heart, liver, and skeletal muscle [11–13], suggesting that ectopic TG storage per se does not lead lipotoxicity [14, 15]. On the other hand, increased WAT lipolysis leading fatty acid overload in non-adipose tissue is associated with systemic insulin resistance, inflammation, and impaired metabolism. Given the crucial role in lipolysis, ATGL has been considered as interesting pharmacological target for hyperlipidemia and associated metabolic disorders. Atglistatin, the first small-molecule inhibitor of ATGL, has been reported to effectively reduce adipose tissue lipolysis, insulin resistance, weight gain, and

nonalcoholic fatty liver in mice fed with high-fat diet [16, 17]. Notably, even long-term treatment doesn't cause ectopic lipid accumulation, which means pharmacological inhibition of ATGL is a potential and powerful therapeutic strategy for metabolism disorders [1].

ATGL hydrolase activity is regulated by phosphorylation modification. AMP-activated kinase (AMPK) phosphorylates ATGL at Ser406 and activates the lipolysis process [18]. AT-specific knockout of both α1 and α2 catalytic subunits of AMPK causes blunted phosphorylation of ATGL at Ser406 and TAG hydrolase activity [19]. Despite of these findings, the role of AMPK in the regulation of ATGL phosphorylation is controversial with other data as reviewed [20, 21]. Ser430 in murine ATGL can also be phosphorylated. But the function and regulation of this site is unclear currently [22].

Some other proteins can regulate the catalytic activity of ATGL. In adipocytes, comparative gene identification-58 (CGI-58) activates the enzyme activity of ATGL by anchoring on LDs with N-terminal region [23] and interacting with the patatin domain of ATGL [24]. Both the localization and the protein-protein interaction of CGI-58 to ATGL are essential for the activation of ATGL. In basal state, CGI-58 forms complex with perilipin 1 (PLIN1) on LD, preventing the accessibility of ATGL [25]. Upon activation by protein kinase A (PKA), phosphorylated PLIN1 releases CGI-58 [26], which increases CGI-58 availability for ATGL co-activation [27]. On the other hand, G0/G1 switch gene 2 (G0S2) is identified as an inhibitor of ATGL by binding with the catalytic patatin domain and suppressing activation [28]. The mRNA expression of G0S2 is reduced during fasting condition and increased after feeding [28, 29], thus modulating lipolysis activity basing on nutrition situation.

Hormone-sensitive lipase (HSL) was the first found lipolytic enzyme. Its activity is sensitive to hormones including catecholamines, adrenocorticotropin, and glucagon [30]. Predominantly function of HSL is to liberate the fatty acid residues in the sn-1 or sn-3 position of diacylglycerols [31, 32]. HSL also hydrolyzes ester bonds of triacylglycerols, monoacylglycerols, cholesteryl esters, and short-chain carbonic acid esters [1, 33, 34].

Similar to ATGL, HSL is highly expressed in adipose tissue and has detectable expression levels in other tissues including muscle, testis, steroidogenic tissues, and pancreatic islets [35]. HSL-deficient mice have intracellular DG accumulated in AT, skeletal muscle, cardiac muscle, and testis but do not suffer from severe ectopic TG accumulation [36] indicating HSL has predominant activity on DG catabolism during the whole lipolysis process. Genomic mutation casing HSL deficiency in humans was recently reported [37]. Mutation carriers, in the absence of HSL protein, show impaired lipolysis, insulin resistance, and inflammation [37].

HSL activity is strongly activated by β-adrenergic stimulation and inhibited by insulin [21]. Upon the activation β-adrenergic stimulation, protein kinase A (PKA) or protein kinase B (PKB) phosphorylates HSL at specific serine residues such as Ser552, Ser469, and Ser650 in humans, in which phosphorylation at different residues causes distinct functions including translocation of HSL to LDs and catalyti-

cally activation [20]. Insulin signaling attenuates PKA-dependent stimulation of lipolysis process [38]. More details will be discussed in the following section of this chapter.

Last but not least, MGL is required for the final step of fat hydrolysis, which was identified as a MG-specific lipase with no affinity to TG, DG, or cholesteryl esters [39, 40]. MGL is constitutively expressed in adipose tissue with no published data

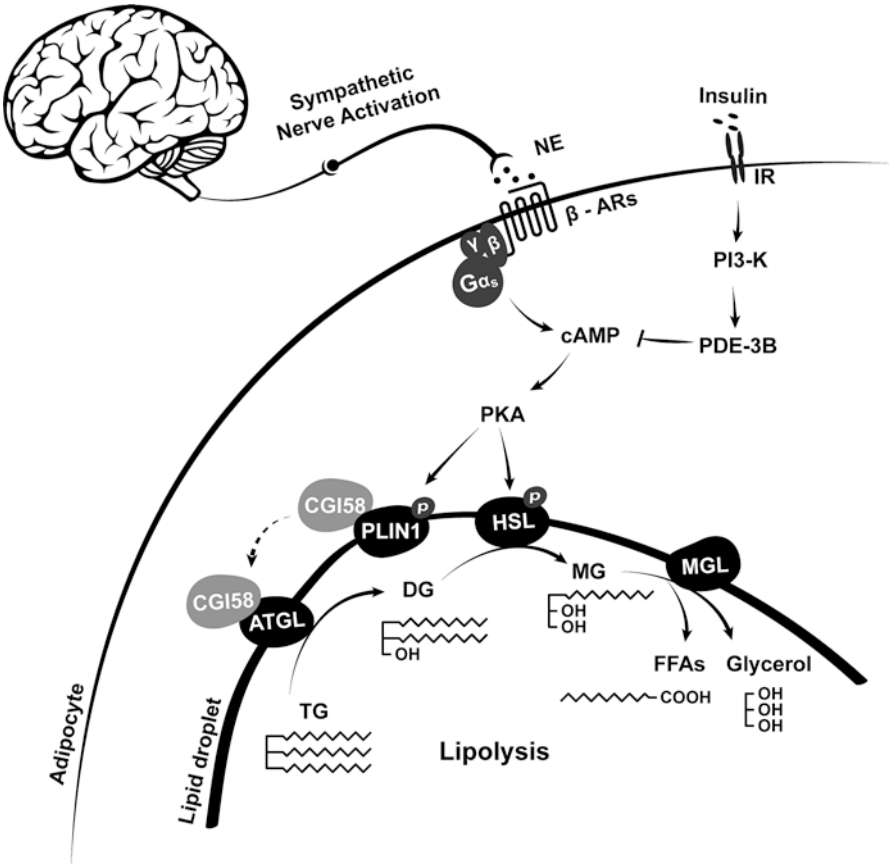

Fig. 11.1 *Regulation of lipolysis by activation of sympathetic nerve system in adipose tissue*. Stimuli, such as cold exposure and exercise stimulate activation of sympathetic nerve system (CNS). Increased NE upon CNS activation binds to β-ARs which further trigger the downstream G-protein-PKA signaling pathway. Critical lipases, such as HSL are phosphorylated and activated by PKA, therefore accelerating lipolysis. Other lipolysis-regulatory proteins, such as PLIN1 are also phosphorylated by PKA, resulting the releasing of CGI58 for the initiation of lipolysis. Intriguingly, insulin signal inhibits lipolysis process by decreasing the levels of cAMP which hence blunts the PKA pathway. Of note, lipolysis can also be regulated by other sympathetic nervous system-independent pathways

NE Norepinephrine, *β-AR* β-adrenergic receptors, *IR* Insulin receptor, *ATGL* triglyceride lipase, *HSL* Hormone-sensitive lipase, *MGL* Monoacylglyceride lipase, *TG* Triacylglycerol, *DG* Diacylglycerol, *MG* Monoacylglycerol, *FFAs* Free fatty acids, *PLIN1* Perilipin 1, *PKA* Protein kinase A, *CGI58* Comparative gene identification-58

indicating any of its regulation, either expression or activity, by nutritional status or any hormones [40]. MGL-deficient mice exhibit MG accumulation in WAT, brain, and liver [41]. Amino acid residues of Ser122, Asp239, and His269 are involved in the lipase activities of MGL [42], while the detailed roles are still elusive. MGL plays a crucial role in endocannabinoid signaling by hydrolyzing 2-arachidonoylglycerol, and selective inactivation of MAGL has obtained desirable effects in several diseases including cancer, cannabinoid addiction, and neurodegenerative disorders (Fig. 11.1) [43, 44].

11.3 Regulation of Lipolysis by Physiological and Pathological Signaling Pathways

Lipolysis is highly regulated by a wide range of upstream signaling inputs. Among them, numerous factors such as catecholamines, hormones, cytokines, and peptides can stimulate fat mobilization, while insulin is the major stimulator of antilipolytic process [20].

Catecholamines are the primary positive regulators of lipolysis which exert diverse functions by binding with different types of adrenergic receptors (AR). AR belongs to G-protein-coupled proteins receptor (GPCR) family. Upon binding with ligand, the receptor is activated and releases associated G-protein submitted to interact with adenylyl cyclase (AC), either activating (G_s subunit) or deactivating (G_i subunit) AC function [45]. Upon activation, AC catalyzes the conversion of ATP to cAMP, increasing intracellular cAMP level, thus resulting in activation of protein kinase A (PKA, also known as cAMP-dependent protein kinase) [46]. Activated PKA phosphorylates both LD-associated protein PLIN1 [47] and ATGL coactivator CGI-58 [27], causing the release of CGI-58 from PLIN1 and further promoting the interaction between CGI-58 and ATGL which finally facilitates ATGL activation and initiates lipolysis cascade. At the same time, activated PKA also phosphorylates HSL, which leads to the rapid translocation from cytosol to the surface of the lipid droplet and activation of HSL, and enhances the lipolysis process [48]. Among the different subtypes of AR, only α2, β1, and β2 (in human) or β3 (in mice) isoforms are involved in lipolysis. β-AR is associated with G_s subunit, conducting simulative signals for lipolysis, while α2-AR is associated with G_i subunit, mediating antilipolytic process [45].

In addition to catecholamines, cardiac natriuretic peptide (NP), released from the atrial and ventricular walls of the heart, is another important positive regulator of lipolysis which could stimulate fat mobilization to the same degree as nonselective β-AR agonist [49, 50]. Upon the binding of NP with NP receptor, the linked guanylyl cyclase (GC) is activated to convert GTP to cGMP resulting in activation of protein kinase G (PKG). Similar to PKA, PKG also phosphorylates PLIN1 and HSL and activates lipolysis [51]. At present, the detail of the mechanism by which NPs regulate lipolytic activity in vivo is poorly understood. Some data indicate that natriuretic peptide system works together with SNS in an integrated manner [52, 53].

Besides what are discussed above, several other factors can also regulate lipolytic process in adipose tissue, either through receptor-mediated signal pathways or by indirectly remodeling lipolysis cascade. GPCR-AC-cAMP signaling pathway particularly plays a central role in modulating TG hydrolysis [20]. Thyroid-stimulating hormone (TSH) activates AC by stimulated G_s-coupled TSH receptor [54]. TSH is found to be more important than catecholamines in lipolysis process in neonates and newborns because of their high physiological levels of TSH [55]. Growth hormone (GH) activates AC by selectively stimulating $G_i\alpha 2$ subunit and removing inhibition of cAMP production [56], resulting in fat mobilization and thus influencing body composition and muscle mass accretion [3]. GH is essential for the increased fat mobilization rate responding to prolonged fasting, while in daily fed-fast cycle, the GH effect on lipolysis is very limited [57].

Insulin signaling pathway plays the major role in antilipolytic process [58]. Insulin receptor (IR) is a tyrosine kinase receptor whose activation upon ligand binding sequentially promotes the activation of phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase (PDK), and Akt [59, 60]. Akt further activates phosphodiesterase 3B (PDE3B), which mediates degradation of cAMP to 5′-AMP and attenuates PKA-dependent stimulation of lipolysis process [38]. In addition, the phosphorylation of PLIN1, but not HSL, is also alleviated by a noncanonical insulin pathway independent of Akt [61]. Considering the important role of PLIN1 in the process of lipolysis, this noncanonical regulation by insulin pathway may provide new insight to the regulation of lipolysis. Besides the inhibitory effect on lipolysis, insulin also promotes lipid storage by facilitating the process of uptake, synthesis, and storage of triglyceride in adipocytes (Fig. 11.1) [62].

Recently, chaperone-mediated autophagy (CMA) is reported to be required for the initiation of lipolysis [1]. CMA is activated when the organism undergoes nutrient deprivation, in which condition intracellular TG stores is hydrolyzed to generated free fatty acid for energetic purpose. CMA-mediated degradation of lipid droplet proteins PLIN2 and PLIN3 facilitates the recruitment of cytosolic lipases to LD and macroautophagy ATGs [63]. AMPK-dependent phosphorylation of PLIN2 is regarded as the trigger of CMA degradation [64]. In addition, the intrinsic activity of lipases is regulated by autophagy. Both ATGL and HSL exhibit multiple LC3-interacting region motif, and mutating a single LIR motif on ATGL blocks ATGL-mediated lipolysis [65]. Hence, the details of the regulatory effect of autophagy in lipolysis are still unclear.

11.4 Role of Sympathetic Nervous System in Lipolysis in Adipose Tissue

In time of energy shortage (e.g., fasting) or increased energy expenditure (e.g., exercise and cold exposure), sympathetic nervous system (SNS) will be activated to stimulate the lipolysis which breaks down the fat storage in WAT into free fatty

acids to fuel other organs [66, 67]. Epinephrine and norepinephrine are the dominating positive regulators of fat mobilization. They bind with β-adrenergic receptors and subsequently activate PKA pathway, which results in phosphorylation of HSL and PLIN1 and triggers fat mobilization. For a long time, adrenal medullary catecholamines, especially epinephrine, were thought to be the primary stimulator of lipolysis in WAT. However, blocking circulating epinephrine by bilateral adrenal demedullation (ADMEDx) does not completely abolish fat mobilization induced by fasting, glucoprivation, exercise, or electrical stimulation of hypothalamus [4], indicating that adrenal medullar is not the only source of lipolysis-inducing catecholamines. The commanding function of SNS on lipolysis has been suggested as long as 100 years [68]. Abundant data provided evidence that SNS innervation to WAT is necessary for the lipolysis induced by a wide range of stimuli including fasting, glucoprivation, cold exposure, photoperiod, and estradiol (as reviewed in [69]). Innervation of WAT by sympathetic nerve tone was firstly proved by Youngstrom in 1995 [70], and several hypothalamic nuclei and many sites of the neuro-axis are revealed as part of central nervous system sympathetic outflow to WAT [4]. Surgical and selectively chemical sympathetic WAT denervation, but not ADMEDx, blocks the long-day associated obesity in Siberian hamsters [71, 72]. Together, all these data could support the necessity and sufficiency of SNS for the initiation and activation of lipolysis in WAT (Fig. 11.1).

Although previous studies could support the idea that SNS innervation is necessary for WAT lipolysis, the direct evidence of neuronal projections into adipocytes has always been expected. In the previous studies, the neural projection in WAT was traced by pseudorabies virus which could be retrogradely transported across synaptic contact resulting in infection along the nerve fibers from periphery to central [73]. Besides, immunohistochemistry (IHC) and immunofluorescence (IF) were also used to visualize the SNS penetration in WAT [4]. However, these methods were not able to show functional innervation of SNS to WAT. Excitingly, the direct function of sympathetic tone on lipolysis has been recently demonstrated by the optogenetic approach [74]. By using this advanced optogenetic technique, Zeng et al. clearly showed the functional neural projections onto adipocytes [74, 75]. They further demonstrated that local stimulation of sympathetic inputs leads to the release of catecholamines, increase of local lipolysis, and depletion of white adipose mass [74]. Interestingly, our group reported that angiogenesis mediated by VEGF-A may lead to smaller adipocytes and adipose tissue mass, suggesting enhanced lipolysis in the tissue [76]. We are now carrying out experimental studies to demonstrate the critical role of sympathetic activation in the process.

11.5 Clinical Significance

Lipolysis in adipose tissue has been found to have dual roles in metabolic diseases. On the one hand, due to rapid expansion of adipose tissue during obesity, even basal level of lipolysis in adipocytes may dramatically increase the circulating free fatty

acid (FFA) levels which may further cause lipotoxicity in other metabolically active tissues, ultimately leading to insulin resistance, the characteristic feature of type 2 diabetes [77]; on the other hand, obese patients have been reported to have impaired lipolysis in adipose tissue [78, 79]. The lipolytic defects could be caused by down-regulation of critical lipases, perilipins, beta-adrenergic receptors, as well as many other anti-lipolytic responsiveness [80]. Therefore, improvement of lipolysis in these patients might help to break down the abnormally accumulated lipids in adipocytes. Given that lipolysis plays dual roles in obesity and type 2 diabetic patients, one might consider to provide different therapeutic strategies to treat/prevent the diseases. For the patients with type 2 diabetes, targeting the lipolysis to reduce the circulating FFA levels might be an effective way. Indeed, blockade of the activities of critical lipases, such as HSL and ATGL with the specific inhibitors as well as inhibition of other lipolytic factors in adipose tissue, has been under consideration in the past years [80]. Conversely, for prevention of obesity development, stimulation of enhanced lipolysis might be a conceivable method. In this regard, exercise and cold exposure both have been reported to enhance lipolysis via sympathetic activation. While there is a remaining concern about the increased circulating FFA levels upon lipolysis, increased lipolysis in adipose tissue does not necessarily cause abnormal FFA levels in circulation at least in animal models [81, 82]. Intriguingly, our laboratory found that VEGF-A is upregulated upon exercise and cold exposure and it triggers enhanced lipolysis via activation of sympathetic nervous system (unpublished observation), highlighting VEGF-A as a critical factor with clinical significance.

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Chapter 12
Current Genetic Techniques in Neural Circuit Control of Feeding and Energy Metabolism

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Abstract The current epidemic of obesity and its associated metabolic syndromes imposes unprecedented challenges to our society. Despite intensive research focus on obesity pathogenesis, an effective therapeutic strategy to treat and cure obesity is still lacking. The obesity development is due to a disturbed homeostatic control of feeding and energy expenditure, both of which are controlled by an intricate neural network in the brain. Given the inherent complexity of brain networks in controlling feeding and energy expenditure, the understanding of brain-based pathophysiology for obesity development is limited. One key limiting factor in dissecting neural pathways for feeding and energy expenditure is unavailability of techniques that can be used to effectively reduce the complexity of the brain network to a tractable paradigm, based on which a strong hypothesis can be tested. Excitingly, emerging techniques have been involved to be able to link specific groups of neurons and neural pathways to behaviors (i.e., feeding and energy expenditure). In this chapter, novel techniques especially those based on animal models and viral vector approaches will be discussed. We hope that this chapter will provide readers with a basis that can help to understand the literatures using these techniques and with a guide to apply these exciting techniques to investigate brain mechanisms underlying feeding and energy expenditure.

Keywords Inducible and conditional gene targeting · Genome editing · Optogenetics · Chemogenetics · Neural circuit mapping · Energy balance · Body weight

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12.1 Introduction

The current epidemic of obesity and its associated metabolic syndromes imposes unprecedented challenges to our society. Despite intensive research focus on obesity pathogenesis, an effective therapeutic strategy to treat and cure obesity is still lacking. The obesity development is due to a disturbed homeostatic control of feeding and energy expenditure, both of which are controlled by an intricate neural network in the brain. Given the inherent complexity of brain networks in controlling feeding and energy expenditure, the understanding of brain-based pathophysiology for obesity development is limited. One key limiting factor in dissecting neural pathways for feeding and energy expenditure is unavailability of techniques that can be used to effectively reduce the complexity of the brain network to a tractable paradigm, based on which a strong hypothesis can be tested. Excitingly, emerging techniques have been involved to be able to link specific groups of neurons and neural pathways to behaviors (i.e., feeding and energy expenditure). In this chapter, novel techniques especially those based on animal models and viral vector approaches will be discussed. We hope that this chapter will provide readers with a basis that can help to understand the literatures using these techniques and with a guide to apply these exciting techniques to investigate brain mechanisms underlying feeding and energy expenditure.

12.2 Conditional and Inducible Gene Targeting Through Cre/loxP Recombination

The complete gene knockout (KO) in mice through homologous recombination permits investigation of the biological roles of specific genes in vivo [1, 2]. However, this technology becomes obsolete as a result of some obvious caveats, including embryonic lethality, no desirable phenotype, and lack of specificity for genes simultaneously expressed in multiple cell or tissue types [3, 4]. To overcome these obstacles, Cre recombinase isolated from bacteriophage P1 has emerged as a popular tool to achieve site-specific gene targeting in mouse models [5–8]. The Cre recombinase catalyzes excision of a "floxed" DNA fragment, a region which is flanked by two 34-bp *loxP* elements, placed in direct orientation [9]. The 34-bp long sequence of *loxP,* which is absent in the endogenous mouse genome, is made of two 13-bp inverted repeats as the Cre-binding sites with an 8-bp spacer region in the middle [10–13]. To achieve tissue-specific knockout of gene of interest (GOI) in a mouse model, the following steps are required for successful implementation of this *Cre/loxP* binary system [5] (Fig. 12.1):

1. The floxed mouse can be created by homologous recombination (HR) in ES cells, in which one or multiple essential exons of the GOI are flanked by two loxP sites located in intronic regions. The *loxP* sites should not disturb gene

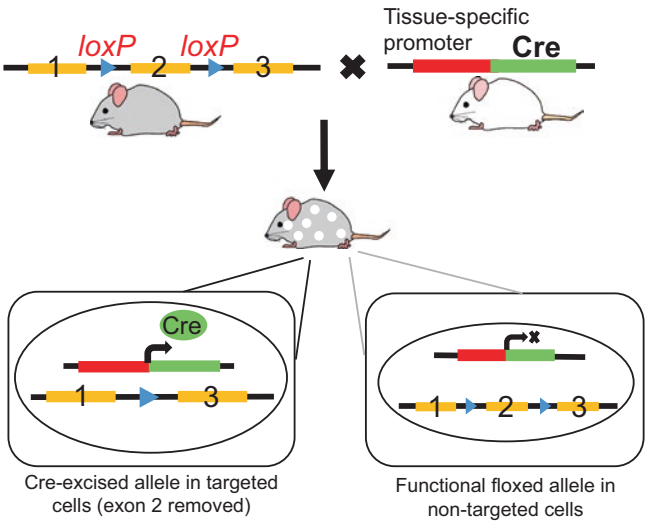

Fig. 12.1 Schematic representation of the conventional Cre/loxP-mediated conditional gene knockout

transcription so that the floxed mouse carrying two floxed alleles displays a wild-type phenotype.

2. To generate a *Cre*-driver mouse, the coding sequence of Cre recombinase is introduced by either knock-in or BAC transgenic approach to ensure ectopic expression of Cre recombinase under the control of a carefully chosen promoter. The promoter practically determines the cell lineage or tissue specificity of the Cre expression profile. The pattern of Cre-mediated gene targeting can be validated by breeding the Cre mouse with a "reporter" mouse line in which a reporter allele (such as beta-galactosidase and green fluorescent protein or GFP) is driven by a strong and ubiquitous promoter but is interrupted by a transcription *STOP* cassette flanked by *loxP* sites [14–16]. A reporter expression pattern would indicate whether the *Cre* driver is expressed in the desired cell types.
3. Breeding the floxed mouse with the *Cre*-driver mouse leads to irreversible excision of the floxed exons in cells expressing Cre recombinase, thereby rendering gene inactivation specifically in Cre-expressing tissues or cells [17–19]. To perform gain-of-function genetic analysis, a *loxP-STOP-loxP* cassette is introduced upstream of the first exon of the targeted gene, leading to disrupted gene expression, i.e., KO. As the next step, breeding a *Cre* mouse with this floxed KO mouse removes the *STOP* cassette, and normal gene expression can be restored exclusively in the Cre-expressing cell types [20–23].

Over the past decades, the *Cre/loxP* strategy has been evolved as the most popular binary recombination system, allowing versatile and efficient control of gene expression. One successful case in application of *Cre/loxP* binary expression system in energy metabolism research is the use of a *Pro-opiomelanocortin (Pomc)-Cre*

line for targeting metabolism-relevant genes in a defined subpopulation of hypothalamus neurons [24, 25]. This strategy unraveled the mechanistic insights of an array of hormonal signaling systems, such as leptin and ghrelin, in mediation of energy homeostasis through differential action upon POMC neurons [26]. Furthermore, characterization of mice carrying *Pomc-Cre* and floxed genes that governs critical cellular processes including endoplasmic reticulum stress, mitochondrial uncoupling proteins, and autophagy system demonstrated their unique yet critical roles in control of body weight and metabolism [27–31].

The classical *Cre/loxP* binary expression, though achieved great success in genetic manipulation, lacks temporal control of gene expression. In the strategy by breeding *Cre* mice with floxed mice, genetic manipulation occurs as soon as the promoter-driven Cre expression is turned on during the early developmental stage. The early Cre expression, either transiently or persistently, may trigger developmental compensation as commonly displayed by global KO approach, thus masking the desired phenotypes. Furthermore, because the expression pattern of some promoters in early stage is markedly different from that of adult stage, early Cre expression may lead to widespread recombination in many undesired places. This caveat potentially makes the functional data difficult to interpret. To achieve a better understanding of physiological roles of genes, the onset of Cre-mediated recombination is desirable to be controlled independently from the endogenous regulatory elements [8, 9].

As an alternative yet efficient approach, Cre recombinase can be introduced to floxed mouse through viral transduction. Cre-expressing adenovirus (Ad) or adeno-associated virus (AAV) can be injected through various routes to target genes within individual peripheral organs or a restricted brain area [32–34]. Used in adult animals, this strategy circumvents the developmental compensation and often results in rapid and efficient recombination within a restricted surrounding area at the injection site [21–23, 35–37]. Despite the lack of inheritance, some studies indicated that phenotypes from AAV-mediated genetic knockout were maintained for up to 6 months [38].

The tamoxifen-based Cre^{ER} system was developed in the late 1990s and has been emerged as a popular tool to achieve inducible control of gene expression [39] (Fig. 12.2). This technique takes advantage of the nuclear localization capacity of a ligand-binding domain of human estrogen receptors (ER) fused with the coding sequence of Cre recombinase. This strategy ensures the restriction of HSP90-bound Cre^{ER} within the cytoplasm unless exposed to an estrogen receptor antagonist, tamoxifen or 4-hydroxytamoxifen [6]. The Cre^{ER} fusion protein translocates into the nucleus to cause cell-specific recombination once transgenic mice are treated with tamoxifen at a desired development or post-development stage [40]. To date, a large repertoire of conditional mutant mice carrying floxed alleles, floxed reporters, or *Cre/*Cre^{ER} drivers have been generated by numerous mouse genetic labs and several large-scale collaborative initiatives, including the GENSAT Project (Rockefeller University), AIBS Transgenic Mouse Project (Allen Institute for Brain Science), RIKEN (Japan), MMRRC (NIH), and JAX [5, 14, 41–43]. Majority of these mouse strains are maintained as either live or cryo-stocks and readily to be distributed to researchers. The ever-increasing amount of *Cre/loxP* mouse inventory will undoubt-

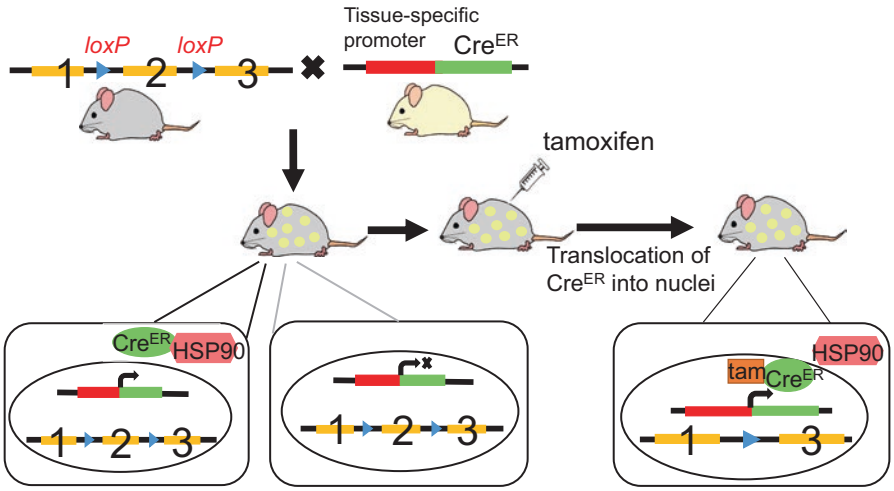

Fig. 12.2 Schematic representation of the inducible Cre^{ER}/loxP-mediated gene knockout

edly facilitate a more complete understanding of the biological processes underlying energy homeostasis.

Emerging evidences suggest that the Cre^{ER} system possesses some non-negligible caveats in addressing fundamental biological issues, such as weak recombination, silencing effects, as well as Cre^{ER}- and tamoxifen-mediated metabolic side effects and cytotoxicity in particular organs [44–50]. To overcome these limitations, a new nsCre system by introducing a premature termination codon (PTC) into the coding sequence of *Cre* gene (*nsCre*) was designed (Fig. 12.3) [51]. It is conceivable that the mRNA transcribed from *nsCre* transgene cannot be fully translated into functional Cre recombinase unless suppressing the PTC by aminoglycoside (AG)-based compounds [52, 53]. Results from in vitro and in vivo proof-of-concept paradigms demonstrated that administering AG compounds into a pre-defined brain region of adult transgenic animals within any desired time window achieved rapid and specific control of gene targeting [51]. This new inducible nsCre system has been successfully applied in the genetic disruption of GABA biosynthesis in AgRP neurons 4 days after administration of AG [51]. Rapid and total deletion of GABA signaling from AgRP neurons in young adult animals abolished feeding response, increased energy expenditure, exacerbated glucose tolerance, and ultimately resulted in severe starvation manifesting the AgRP neuron ablation model [51]. Unlike lipid-soluble tamoxifen, applicable AG derivatives cannot penetrate the blood-brain barrier or diffuse far from the site of injection [54]. Thus, the new nsCre system is particularly useful to distinguish between central and peripheral contributions when endogenous expression profile of the gene of interest exists in both the brain and periphery. Targeting specific genes with a widely distributed pattern within the brain may also be easily achieved. Together, this nonsense suppression-based genetic system provides an ideal strategy in achieving conclusive results for many complicated, compensatory mechanism-protected, physiological, and neurological processes.

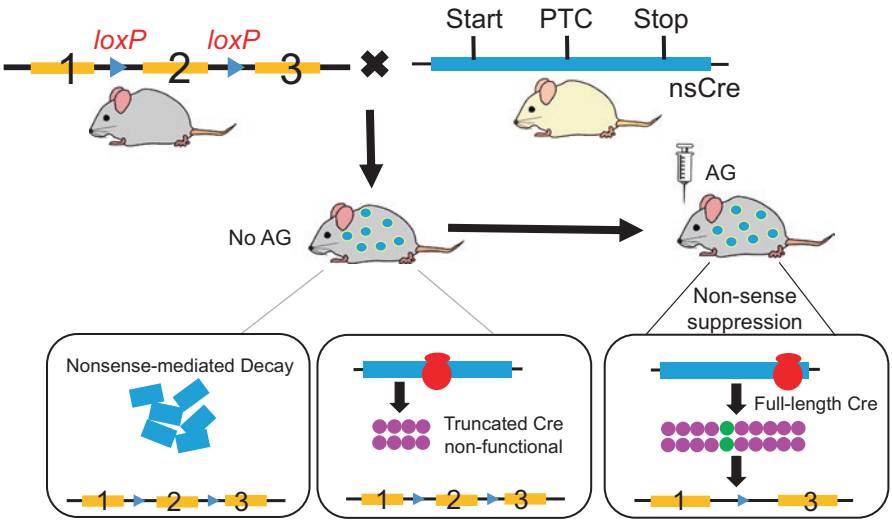

Fig. 12.3 Schematic representation of the inducible nsCre/loxP-mediated gene knockout. The nonsense mutation that generates a premature termination codon (PTC) is inserted into the coding sequence of Cre recombinase (*nsCre*). Transgenic mice carrying the nsCre and homozygous floxed gene of interests are made. When in the absence of aminoglycoside (AG), PTC-containing mRNA is predominantly degraded through the process of nonsense-mediated decay (NMD), while the remainder is translated to truncated nonfunctional Cre. When AG (e.g., Geneticin) is administered into adult animals, AG suppresses the decay of the PTC-containing pre-mRNA at the site of transcription, represses the proofreading mechanism of the ribosomal complex inserting a random amino acid at the PTC, and finally generates full-length, functional, Cre proteins. This whole process is termed nonsense suppression. The functional Cre then catalyzes the genetic recombination of the floxed gene. Once AG is metabolized by the Cre-expressing cells or tissues, the nonsense suppression is rapidly reinstated to stop further production of functional Cre

12.3 Dissection of Neural Circuit by Recombinant Rabies Virus

Deciphering the neural mechanisms in control of feeding behavior and body weight requires knowledge of the synaptic connectivity of relevant neural circuits as well as the relationship between each defined neural circuit and physiological function. Rabies virus, a negative-sense single-stranded RNA [(-)ssRNA], has outstanding capacity as a retrograde tracer of neuronal populations that are synaptically connected [55–58]. There are several advantages of rabies virus over another family of retrograde viral tracers, i.e., α-herpes viruses which include herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV):

1. Rabies virus displays a significantly reduced cytotoxicity.
2. Rabies virus has the capacity to infect the brain of primates.
3. Herpes viruses typically have a lysis phase that often leads to the release of viral particles from host cells and infect nearby cells through non-synaptic manner.

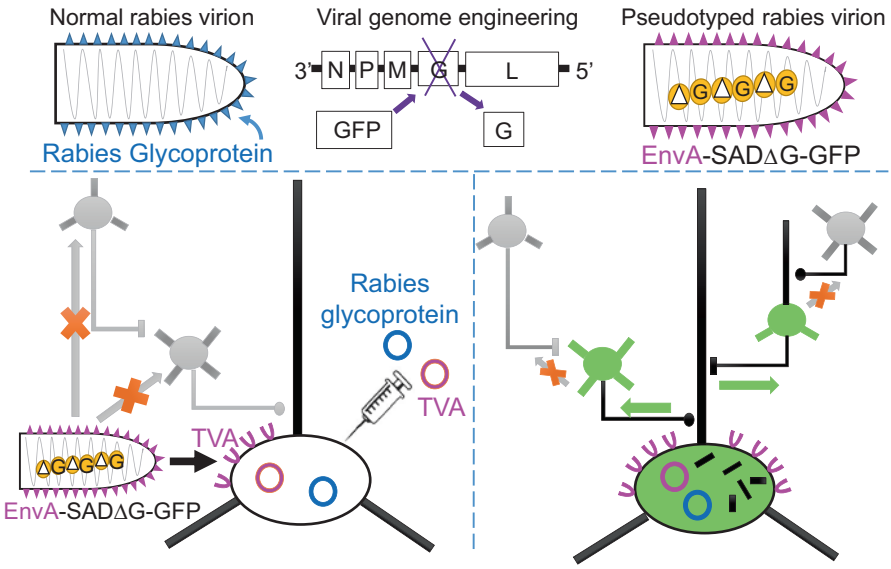

Fig. 12.4 Schematic representation of pseudotyped rabies virus for retrograde monosynaptic tracing

4. Rabies virus can be easily genetically engineered due to the fact that it has a genome of only ~12 kb that harbors 5 coding genes, while herpes viruses have genomes of ~150 kb that code for more than 70 genes [55].

Altogether, these features make rabies virus a useful retrograde tracer for studying the functional connectivity of neural circuit [56, 59]. However, like other trans-synaptic viruses, wild-type rabies virus is a polysynaptic tracer that leads to potential ambiguity in determination of how many synaptic steps have been crossed at any given time [55]. This caveat poses a challenge to dissection of a precise pattern of synaptic connectivity, considering the vast cohort of neuronal types within the brain, the large number of synapses existing on each neuron, and the high degree of connectivity in intact neural circuits. Furthermore, it is not possible to apply this method for combined circuit tracing and gene manipulation of the first-order neurons, since high-order neurons will also be affected (see more details below).

In recent years, a glycoprotein-deleted (ΔG) rabies virus has been developed and recognized as a robust toolbox for monosynaptic circuit analysis [60–62] (Fig. 12.4). The envelope glycoprotein (RG) is essential not only for the assembly of infectious viral particles during the natural cycle of rabies virus but also for the trans-synaptic crossing of the virus [63, 64]. Genetic deletion of RG gene or swap-out with a GFP reporter from the SAD-B19 rabies genome leads to the restriction of viral infection to the first-order neurons at the injection site [61]. To restore the trans-synaptic capacity, RG-deleted rabies virus is pseudotyped with an avian virus envelope protein named EnvA; such virus is therefore called EnvA-SADΔG-GFP [61]. Once the virus is injected into the brain of wild-type mice, it cannot infect any neurons

because the mammalian neurons lack the cognate receptor for EnvA, called TVA. However, pretreatment of AAV vector carrying the coding gene for TVA to the same brain region warrants efficient infection of TVA-expressing neurons by EnvA-SADΔG-GFP rabies virus [55].

Since RG is required for packaging new viral particles and trans-synaptic propagation, and SADΔG-GFP does not carry the coding sequence for RG, RG should be introduced to the same group of neurons typically through AAV-mediated viral transduction [65]. If TVA but not RG is expressed, infection with EnvA-SADΔG-GFP rabies virus (with GFP marker) would be restricted to TVA-positive neurons and cannot spread over to the presynaptic neurons. If neurons express both TVA and RG, TVA would allow initial infection of rabies virus, while RG in those cells allows trans-synaptic propagation and GFP labeling of the second-order neurons with direct synaptic projection to these cells. Importantly, continued propagation beyond the second-order neurons cannot occur, because these presynaptic neurons do not express RG and there is no RG-coding sequence in the rabies genome [56]. Therefore, this system warrants monosynaptic spread of rabies virus, which eliminates the ambiguity about the number of synapses that have been crossed. More interestingly, the pseudotyped rabies tracing strategy can be further adapted to *Cre*-driver mouse lines to achieve cell-/tissue-specific control of TVA and RG expression [66].

One of the great advantages of ΔG rabies virus-mediated monosynaptic tracing over other existing approaches (such as electron microscopy [EM] and paired electrophysiological recording) is the capacity to identify long-range, direction-specific, synaptic connectivity [60, 67, 68]. A few recent studies successfully applied this new technology to interrogate hypothalamic circuits in control of appetitive and cognitive behaviors [67, 68]. In order to decipher the excitatory inputs to AgRP neurons that control caloric-deficiency-induced activation, the Cre-dependent AAV-FLEX-RG and AAV-FLEX-TVA-mCherry viruses (Fig. 12.4) were co-injected into the arcuate nucleus (ARC) where AgRP neurons are located [67]. As the next step, EnvA-SADΔG-GFP rabies virus was injected to the same region in the ARC to allow rabies infection of AgRP neurons that express TVA-mCherry and RG. The rabies virus was thus supposed to retrograde from AgRP neurons to presynaptic cells which can be visualized by the GFP reporter carried from trans-synaptically propagated rabies virus. Using this technique, these authors made a surprising discovery that major excitatory inputs to the AgRP emanate from two discrete neuronal populations in the hypothalamic paraventricular nucleus (PVN). Application of a similar tracing strategy leads to identification of a novel connection between oxytocin-expressing neurons within the PVN and neurons in the central amygdala, a pathway which is critical for the control of fear response [68]. In this study, synaptic connections were further supported by several lines of results derived from optogenetic manipulation and EM-based histological data [68]. Nevertheless, rabies virus tracing results, for the first time, established the monosynaptic property of this critical neural circuit.

Beyond the tremendous success in neural tracing analysis, a variety of ΔG rabies virus variants have recently been developed by amalgamating with some newly

developed genetic toolboxes. These variants include ΔG rabies vectors carrying *trans*-acting factors such as Cre and FLP recombinase, genetically encoded Ca^{2+} sensors, and molecules for activation or silencing of neural circuits such as channelrhodopsin-2 and allatostatin receptors [69, 70]. These emerging techniques, when combined with *Cre/FLP*-driver mice or conditional mouse models, allow precise genetic and functional analysis in defined neuronal circuits.

12.4 Optogenetics

Optogenetics, as reflected by its name, uses a combination of light and genetic methods to enable precise perturbation of neurocircuits in living animals. This technique utilizes light-activated microbial opsins, which can be genetically expressed in neurons to achieve rapid control of neuron activity by light [71, 72]. The principle underlying light-mediated activation is photoisomerization of the chromophore retinal. Similar to light-sensing in retina of vision sensing, photo isomerizes the all-trans retinal, which leads to a series of conformational changes of opsins [73]. These conformational changes result in ion transports across the membrane, which, in stark contrast to light sensing in the retina, results in changes in signal transduction instead of ion transport [73]. This striking difference makes it possible to effectively apply microbial opsins in mammalian systems without interfering endogenous signaling pathways by light. Interestingly, the endogenous retinal in mammalian neurons can tolerate well with foreign microbial opsins, and no additional retinal is required for microbial opsin to mediate light-induced ion transport in mammalian neurons. In turn, microbial opsins can also tolerate well with fusion with other proteins such as green and red fluorescent proteins (GFP and RFP) without affecting their ion transport ability or light sensitivity, which makes it easy to track transgenic and functional expression of microbial opsins in mammalian neurons. In addition, low intensity of light illumination (below 10 mW) is required to activate these opsins, which greatly limits potential damage to tissues directly by light illumination. Due to its capability of rapid control of neuron activity with a high level of fidelity and precision, noninvasive control by light, and reversibility, the optogenetics has gained rapid popularity. Since the first application of microbial opsins in mammalian neurons, a variety of microbial opsins as well as various mutations and hybrids have been generated with an aim to achieve better performance. The distinct features of these opsins have been intensively reviewed elsewhere [73–77]. Discussions on application of optogenetics in the investigation of a variety of brain disorders, including mood disorders, anxiety, addiction, Parkinson's diseases, etc., have also been reviewed elsewhere [78–82]. This section will briefly introduce the most utilized opsins including channelrhodopsin-2 (ChR2), halorhodopsin (Halo), and archaerhodopsin (Arch), which have been used for neuronal excitation (ChR2) or inhibition (Halo and Arch) as well as their application in understanding brain control of feeding and energy expenditure (Fig. 12.5).

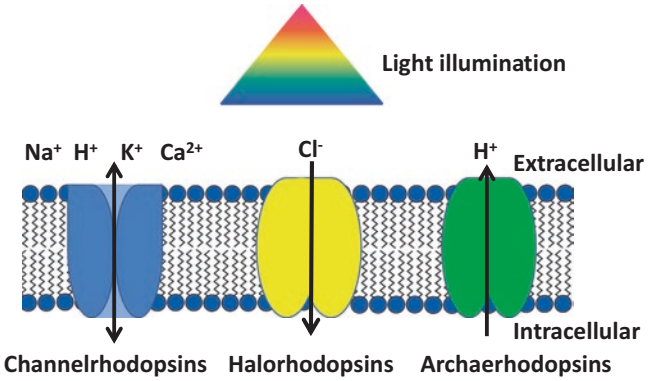

Fig. 12.5 Schematic representation of channelrhodopsins, halorhodopsins, and archaerhodopsins

ChR2 was first identified to be a light-gated cation channel in 2002 [83–85] and was demonstrated to conduct nonselective cation influx in neurons and therefore reliably control neuron activity in 2005 [72, 86, 87]. ChR2 can be expressed stably and safely in mammalian neurons, in which blue light illumination can reliably induce sustained activation with a rapid kinetics (milliseconds). The firing of neurons can be induced in a scalable way by delivery of a large range of frequency and duration of light stimulation [86]. ChR2, upon receiving light illumination, will undergo conformational changes and open its channel for passive conductance of cations, rendering it as an efficient cation channel [73, 88]. Importantly, ChR2 expression, without light illumination, causes no discernable effects on neurons [86]. Thus, ChR2 is an ideal candidate as a tool to activate neurons and investigate their physiological effects. To overcome some limitation with native ChR2, mutations have been made on ChR2 with an aim to be able to use light to better control neuron activity with precision. Toward this, many mutations have been generated. ChR2 with H134R mutation (ChR2/H134R) exhibits reduced desensitization and increased light sensitivity [74, 87], which increases photocurrents and is so far the most popular tool in the field of brain control of metabolism.

To complement the application of ChR2 that activates neurons, Halo was explored to silence neurons based on its light-driven chloride pumping activity (Fig. 12.5). When Halo from *Natronomonas pharaonis* (NpHR) was expressed in neurons, long-duration light pulses induce long-term steady inhibition, and short-duration light pulses reliably inhibit single action potentials induced by current injection across a broad range of frequencies [71, 89]. Similar to ChR2, light activation of NpHR induces Cl^--mediated currents with fast kinetics within milliseconds [71, 89]. So NpHR can effectively serve as an optical inhibitor of neuronal activity. A major difference between NpHR and GABAA receptors, a chloride channel, is that the former is a pump and will only cause membrane potential changes, but not input resistance, while the latter will cause a reduction in input resistance. Excitingly, due to differential action spectrums of ChR2 and NpHR by light, it is possible to use distinct wavelengths of lights to activate and silence the same neurons [71, 89],

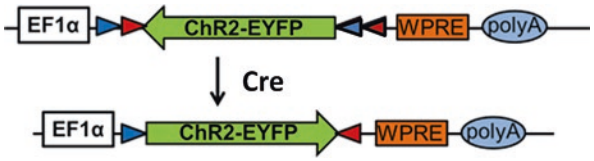

Fig. 12.6 Schematic diagram showing DIO/FLEX strategy in which the expression of ChR2-EYFP in the viral vector is dependent on Cre-mediated flip of the inverted ChR2-EYFP coding sequence

allowing a simultaneous bidirectional control of neuron activity with light. Thus, use of combination of ChR2 and NpHR will be able to test the sufficiency and requirement for brain neurons in physiology simultaneously. To overcome poor membrane targeting, an advanced version of NpHR has been generated (eNpHR3.0), which, when expressed in mammalian neurons, exhibits improved membrane targeting and photocurrents [90]. However, a brief episode of eNpHR3.0 activation tends to increase the probability of spiking in response to a volley of presynaptic action potentials [91], presumably due to its depletion of normal Cl^- gradient of neurons. This alteration of baseline neuron activity might confound and therefore limit the use of this opsin in mammalian neurons.

Archaerhodopsin (Arch) is a green-yellow light-driven outward proton pump and therefore can hyperpolarize neurons (Fig. 12.5). When expressed in mammalian neurons, Arch can also produce hyperpolarizing current with millisecond temporal precision [92]. Compared to eNpHR3.0, the advanced version of Arch, eArchT3.0, showed better membrane targeting, higher sensitivity to light, and higher photocurrents [90, 93]. Importantly, it appears that the outward proton pump activity is not associated with any alterations in intracellular pH due to the intrinsic compensatory mechanism [91]. This feature is in contrast to eNpHR3.0, which, as discussed above, is able to cause changes in Cl^- gradient. Thus, eArchT3.0 appears to be a better choice to silence mammalian neurons.

A combination of optogenetics and Cre-loxP technology provides an unprecedented specificity in manipulating neuron activity. The Cre-loxP technology provides a specificity in the expression of opsins, and the optogenetic approach provides a specific regulation by light in a rapid inducible and reversible fashion. Currently, a variety of viral vectors are available with Cre-dependent expression of ChR2, eNpHR3.0, eArchT3.0, or other opsins. The Cre-dependent expression is achieved by an elegant design of double-floxed inverted open reading frame (DIO) or flip-excision (FLEX) strategy, in which the inverted opsin reading frame is flanked by two sets of floxed sequences (Fig. 12.6). Cre-mediated inversion of DIO/FLEX will allow functional expression of opsins. A typical example of using a combination of optogenetics and Cre-loxP technology is ChR2-assisted circuit mapping (CRACM), by which monosynaptic connectivity can be identified if light-stimulated presynaptic neurons induce a time-locked postsynaptic current within a few milliseconds (Fig. 12.7). Due to irregular distribution and diffusive projections of hypothalamic neurons, monosynaptic connectivity between these neurons is particularly difficult

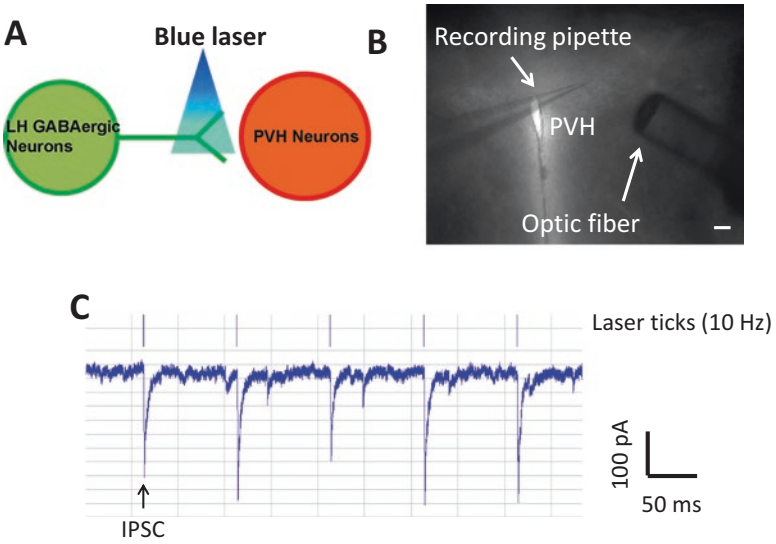

Fig. 12.7 A representative example showing CRACM. (**a**) Diagram showing GABAergic projections from lateral hypothalamic (LH) to PVH neurons. (**b**) Picture showing an actual recording setup with a brain section containing PVH neurons, a recording pipette, and an optic fiber. (**c**) Actual current traces showing time-locked (less than 5 ms) inhibitory postsynaptic currents (IPSCs) in response to each blue light stimulation

to ascertain. As discussed below, CRACM has been frequently used to determine monosynaptic connectivity between hypothalamic neurons.

The optogenetics approach has greatly enhanced our understanding on brain neural pathways controlling feeding and metabolism. It is well established that hypothalamic neurons expressing agouti-related protein (AgRP neurons) are important for feeding [94, 95]. However, it was unknown how and whether these neurons act alone or in concert with other neurons to promote feeding. With blue light activation of AgRP neurons expressing ChR2 in live animals, the animals exhibit robust feeding, and the feeding behavior correlates with light activation [96], demonstrating that AgRP neurons are sufficient to orchestrate feeding without training or co-activation of any other neurons. Furthermore, light activation of AgRP neurons induces monosynaptic inhibitory currents in nearby POMC neurons as well as those in the paraventricular hypothalamus (PVH), proving a direct GABAergic projection from AgRP neurons to POMC and PVH neurons [97]. In contrast, light activation of POMC neurons fails to induce any monosynaptic responses in AgRP neurons [97], demonstrating a unidirectional interaction between AgRP and POMC neurons. It is perceivable that clear delineation of neurocircuits and their roles in feeding wouldn't have been established without the application of optogenetics. Similarly, light stimulation of ChR2-expressing GABAergic neurons in the bed nucleus of the stria terminalis (BNST) stimulates feeding, while that of glutamatergic neurons in the LHA inhibits feeding [98], identifying a previously unknown role for these neurons

in potently regulating feeding behavior. Notably, light inhibition of eArchT3.0-expressing GABAergic neurons in the BNST inhibits feeding in food-deprived animals [98], demonstrating an importance of these neurons in feeding inhibition. This is one of first examples of using eArchT3.0-mediated neuron inhibition for feeding regulation.

Efficient expression of ChR2 in remote fibers allows specific activation of these fibers by light in vivo, which can be used to examine specific physiological roles of distinct remote sites that mediate the function of ChR2-expressing neurons. For example, specific activation of ChR2-expressing AgRP fibers in the PVH, BNST, lateral hypothalamic area (LHA), paraventricular thalamus (PVT), or central amygdalar nucleus (CEA) all produces robust feeding behavior [99], suggesting parallel and redundant projections from AgRP neurons to these discrete sites in feeding promotion. Similarly, light stimulation of ChR2-expressing local fibers in the CEA of calcitonin gene-related peptide (CGRP) neurons in the parabrachial nucleus (PBN) suppresses feeding [100], demonstrating a role for PBN CGRP projections in the CEA in feeding suppression. In addition, local stimulation of GABAergic local fibers in the PVH coming from the LHA increases feeding [101], suggesting a role for PVH in mediating the feeding behavior by stimulating LHA GABAergic neurons. Furthermore, local stimulation of GABAergic fibers in the VTA coming from LHA GABAergic neurons also stimulates feeding [102]. Thus, LHA GABAergic neurons promote feeding also through parallel and redundant pathways, and PVH and VTA are at least part of downstream brain sites mediating the feeding behavior.

As discussed above, single use of excitatory ChR2 and inhibitory eArchT3.0 has been used to study neuron function in feeding regulation. However, to delineate neural pathways, it is imperative to determine whether the putative downstream neurons are responsible for mediating the feeding effect. To achieve this goal, dual stimulations with light to control the activities of both upstream and downstream neurons are required. Notably, due to a difference in action spectrum, different rhodopsins can be concurrently activated by respective lights at a distinct wavelength without affecting each other [71, 89, 103]. This multimodal independent control of neuron activity allows interrogation of downstream neurons that mediate behaviors. As illustrated as an example of occlusion experiments in Fig. 12.8, excitation of neuron A releases glutamate onto neuron B and inhibits feeding. To determine whether excitation of neuron B is required for the feeding inhibition, one could express ChR2 in neuron A and eArchT3.0 in neuron B, allowing simultaneous activation of local GABAergic fibers originated near neuron A and inhibition of neuron B with illumination of both blue and yellow lights. If inhibition of neuron B by yellow light reduces the feeding inhibitory effect elicited by blue light activation of neuron A, then neuron B is at least one of downstream mediators of neuron A in feeding inhibition. This method has nicely been employed in establishing that inhibition of PVH neurons is required to mediate AgRP feeding promotion [97] as well as that excitation of parabrachial neurons is required to mediate PVH MC4R-expressing neurons in feeding inhibition [104].

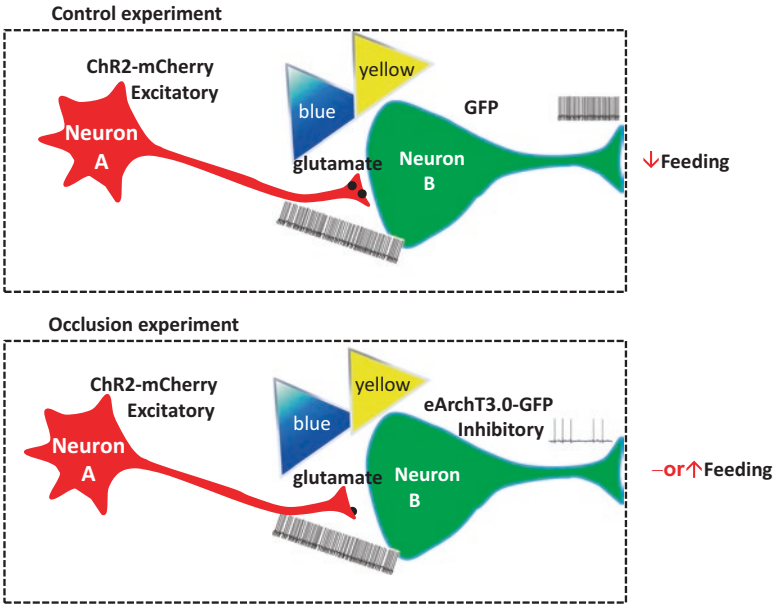

Fig. 12.8 Schematic diagram showing an occlusion experimental model

With the development of more advanced versions of opsin and new lines of Cre animals with more specific and restricted expression in the brain, the application of optogenetics approach to understand brain mechanism in feeding/energy balance regulation will become more versatile and powerful. The noninvasive light-mediated control of neuron activity with millisecond kinetics and reversibility offers an unprecedented precision to correlate neuron activity with feeding behavior. However, despite the current popularity of using optogenetics, cautions must be exercised in applying this technique, and a few drawbacks have been noticed with this technique [105]. Most studies using optogenetics on freely living animals require implantation of optic fibers for light delivery, which will inevitably cause damage to brain tissues and will potentially confound the ongoing studies. Associated with light illumination is light-induced heat production, which itself may alter neuron activity. In addition, ChR2-mediated excitation can sometimes induce depolarization block [106], and inhibitory opsins can also induce hyperpolarization-induced activation of cation channels and therefore rebound action potential firings [107, 108], both of which may confound interpretation of experimental results. Another major drawback is that optogenetic control of neuron activity is purely artificial and the magnitude of excitation (ChR2) or inhibition (eArchT3.0 or eNpHR3.0) may never be experienced in any physiological circumstances and, therefore, behavioral phenotypes (i.e., feeding) may be artificial. Thus, additional physiological experiments are required to extrapolate the significance of behavior phenotypes from optogenetic studies. Finally, artificial manipulations of neuron activity with optogenetics are conducted without real-time monitoring of neuron

activity. Real-time monitoring of neuron activity is key to establish a causal relationship between neuron activity and behavior [109]. For example, light stimulation of ChR2-expressing AgRP neurons causes voracious feeding, and the duration of feeding depends on duration of light stimulation [96], which might indicate that AgRP neuron activation drives feeding behavior. However, recent studies with real-time monitoring AgRP neuron activity in live animals demonstrate that initial feeding requires AgRP neuron activation but continuous feeding is not and, instead, is associated with reduced AgRP neuron activity [110, 111]. These contrasting results suggest a need to exercise caution when using optogenetics to blindly manipulate neuron activity to study behavior.

To address potential issues associated with optogenetics, more efforts have been investigated to improve its performance. Wireless delivery of light has been actively pursued to achieve remote delivery of light for optogenetic control of deep brain neurons [112–114]. New opsins with faster kinetics and better sensitivity to light have been actively sought, for example, a recent study reported a new, natural anion channelrhodopsin, which requires less than one thousandth of the light intensity than required by the most efficient currently available optogenetic rhodopsins [115]. Importantly, a close-loop strategy has been proposed to use with optogenetics to achieve optic control of neuron activity based on real-time monitoring neuron activity and behavior output [109]. These technical and conceptual advances will lead to a more efficient application of optogenetics in the identification of key neurons and neural pathways in the brain that control feeding and energy expenditure.

12.5 Chemogenetics

Chemogenetics, also termed designer receptors exclusively activated by designer drugs (DREADD), involves expression of an artificial receptor, normally a G-protein-coupled receptor, which lacks endogenous ligands but can effectively engage intracellular signaling pathways, normally through G-protein-mediated signaling, by an artificial ligand, which is usually a chemical but with no endogenous activity [116, 117]. Thus, pharmacological application of ligands can achieve remote control of G-protein-coupled signaling pathways. Since ligands activate G-protein-coupled signaling, this approach can virtually be used for all kinds of cells with G-proteins. This approach was initially developed by Dr. Bryan Roth of the University of North Carolina at Chapel Hill.

Through mutation of human muscarinic acetylcholine receptors, an excitatory DREADD receptor was generated (hM3Dq), which completely loses the binding of acetylcholine, but can be effectively activated by an otherwise pharmacologically inert drug, clozapine-N-oxide (CNO) [118]. Importantly, once activated by CNO, hM3Dq receptors will activate Gq-protein-coupled signaling pathways, leading to an elevation of intracellular Ca^{2+} and thus activation of neurons [97, 119–121]. With a similar approach, the inhibitory DREADD (hM4Di) receptors, which induce Gi-protein-coupled signaling pathways by activation of CNO, were generated and

can be used to achieve remote inhibition of neurons that express this receptor [97, 118, 121, 122]. Recently, Gs-DREADD has also been generated to induce the cAMP pathway once activated by CNO [123, 124]. Given the fact that it utilizes the intracellular pathways and involves in vivo pharmacology, the DREADD approach can be applied to address questions in a more "physiological" sense and has gained rapid popularity in the field of neuron control of feeding and metabolism. Similar to optogenetics, a few animal models [119, 124, 125] and a variety of vectors with Cre-dependent expression of DREADDs have been generated [97, 116, 121]. These vectors can be used in combination with Cre-loxP technology to achieve a high level of controlled specificity in targeted expression and activation. Of note, mice with knock-in of Cre-dependent expression of Gs-, Gi-, or Gq-coupled DREADD receptors have all become available from the Jackson Laboratory, which will greatly expedite the research using DREADD approaches for neuronal control of feeding and other behaviors. The controlled expression, inducibility, and reversibility (degradation of CNO within a few hours) make the DREADD approach powerful to link neuron activity with behavior.

The showcase of the application of both hM3Dq and hM4Di has been demonstrated on AgRP neurons. CNO effectively increases and reduces the excitability of AgRP neurons that express hM3Dq and hM4Di, respectively [121]. Mice with specific expression of hM3Dq in AgRP neurons respond to CNO with voracious feeding and those with hM4Di in the AgRP neurons with reduced feeding [121]. These results illustrate the effectiveness of in vivo pharmacological CNO in controlling AgRP neuron activity. With a combination of DREADD and mouse genetics, differential roles of neurotransmitters, GABA, NPY, and AgRP, have been shown to mediate feeding behavior of AgRP neurons [126]. For POMC neurons, although it is well-established that these neurons suppress feeding, how these neurons located in different brain regions (hindbrain versus hypothalamus) regulate feeding is unknown. With specific hM3Dq expression in different brain regions of POMC neurons, it has been shown that POMC neurons in the hindbrain mediate short-term feeding suppression, whereas those in the hypothalamus mediate long-term feeding suppression [127]. DREADD-mediated activation of leptin receptor neurons in the dorsomedial hypothalamus increases energy expenditure and reduces body weight [128], demonstrating a sufficiency for these neurons in promoting energy expenditure. Notably, DREADD is also able to efficiently activate tyrosine hydroxylase (TH)-expressing neurons in rats to regulate feeding behavior [129], suggesting DREADD as a versatile approach in different species.

Of interest, DREADD has recently been explored to probe functions of subcellular regions in neurons. Using an axon-targeting approach, the inhibitory hM4Di is targeted preferentially to remote axon terminals of PVH single-minded 1 (Sim1) neurons. Stereotaxic delivery of CNO to distinct projection sites of Sim1 neurons reveals that isolated inhibition of glutamate release from Sim1 neurons restricted to the periaqueductal gray (PAG) regions is sufficient to promote feeding, nicely demonstrating a role for PAG neurons in mediating PVH Sim1 neurons in feeding regulation [130]. Importantly, DREADD can be used in conjunction with the optogenetics to identify novel neural pathways for feeding and energy metabolism. For

example, the CRACM technology has helped identification of a novel excitatory drive from PVH neurons that express thyroid-releasing hormone (TRH neurons) to AgRP neurons [67]. With a combination of excitatory hM3Dq expressed in TRH neurons and inhibitory hM4Di expressed in AgRP neurons, it has been demonstrated that feeding promotion by PVH TRH neurons is mediated by AgRP neurons [67]. These sets of experiments convincingly identified a previously unknown glutamatergic pathway from PVH TRH neurons to AgRP neurons in feeding regulation.

In comparison to optogenetics, the DREADD approach has a much slower kinetics. Long diffusion (minutes) and clearance (hours) time associated with CNO administration renders this approach to lack a precise temporal control of neuron activity, which may potentially confound delineation of complex neuronal circuits for behavior. However, this long duration of action may provide an advantage to studies that require long-duration observations of behaviors such as feeding and energy expenditure. For example, measurements of energy expenditure require a relatively long duration and enclosed chambers, which are not compatible with prevalent optogenetic applications with tethered optical cables. Using DREADD, the function of a group of arcuate GABAergic neurons in promoting energy expenditure has been elegantly demonstrated [131]. Thus, for a given study, it may be more effective to combine optogenetics and DREADDs, as illustrated above in delineation of TRH neuron to AgRP neuron projection in feeding regulation [67]. Another potential complication arises from the fact that DREADDs rely on endogenous G-protein signaling pathways to control neuron functions. Thus, the ability of DREADDs to effectively control neuron activity is determined by a combination of DREADD expression levels, coupling of DREADD and G-protein, and inherent action of the G-protein signaling pathways on the function of the studied neurons. This may be the reason that a wide range of CNO doses are reported to elicit behavioral effects.

12.6 Conclusion

It is noteworthy that an approach that combines light control with intracellular signaling pathways is on the horizon. Based on a chimeric opsin, intracellular signaling can be controlled in a temporally precise manner by light [132, 133]. Stemmed from the light-oxygen-voltage (LOV) family domain of proteins, a variety of LOV-based optogenetic tools for control of intracellular signaling have been proposed and tested [134, 135]. It is without doubt that new approaches such as optogenetics and DREADD have revolutionized our understanding of brain neural circuits in controlling feeding and energy metabolism. With the rapid development of novel technology, more advanced approaches will likely become available and will inevitably expedite our research progress toward more complete understanding of brain control of feeding and provide a mechanistic rationale for effective drug targeting against feeding disorders and associated obesity and diabetes.

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