Mol Neurobiol (2012) 45:258–278
DOI 10.1007/s12035-012-8241-5

Electrophysiological Analysis of Circuits Controlling Energy Homeostasis

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Received: 1 November 2011 / Accepted: 26 January 2012 / Published online: 14 February 2012

Abstract Since the discovery of leptin and the central melanocortin circuit, electrophysiological studies have played a major role in elucidating mechanisms underlying energy homeostasis. This review highlights the contribution of findings made by electrophysiological measurements to the current understanding of hypothalamic neuronal networks involved in energy homeostasis with a specific focus on the arcuate–paraventricular nucleus circuit.

Keywords Neuronal network · Feeding behavior · Hypothalamus · Peripheral metabolic signals · Membrane current · Intracellular signaling molecules · Adiposity signals · Electrophysiological tools · Leptin · Insulin · Ghrelin · PYY(3–36) · Orexin · Neuropeptides

Introduction

Animals maintain energy homeostasis through a highly coordinated balance between feeding behaviors and energy expenditure that is largely controlled by the central nervous system (CNS). Neuronal circuits within the CNS are responsible for the regulation of animal behaviors in relation to the environment. Multiple techniques have aided efforts directed toward defining neural networks underlying behavioral and physiological responses that control energy homeostasis. Initial studies used crude mechanical and chemical ablation techniques to identify critical brain regions such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamus (LH), and brainstem [1–4]. Through the occurrence of spontaneous genetic obese mutant mice, the identification of leptin and melanocortin circuits, two major regulators of energy homeostasis, provided a better understanding of mechanisms involved in hormonal regulation of energy homeostasis. Hypothalamic brain regions were then categorized into neuronal centers characterized by the receptors and neurotransmitters expressed, and neuronal populations were identified with the potential to respond to stimuli in a predictable manner within a neuronal network.

It is the response of an ensemble of neuronal populations that forms a functional network responsible for the production of a behavior. In order for a network to produce a behavior, a neuron serving as a functional unit receives information from hormones and neurotransmitters released from endocrine organs and upstream neurons. These multiple input signals are integrated and the summation of all hormonal and neuronal inputs arriving onto a neuron at a given time determines the output of that neuron, producing an action potential to continue propagating a message through the circuit or remaining electrically quiet to stop the flow of information. The frequency, pattern, and timing of action potential occurrences determine neurotransmitter release to downstream neurons and specify its contribution to the behavior produced by the collective activity of a network.

Development and use of green fluorescent protein (GFP)-tagged transgenic mice in conjunction with using of various electrophysiological techniques (summarized in Table 1) have furthered our understanding of the neuronal populations affecting both food intake and energy expenditure through assessing the frequency and pattern of action potential firing. Considering the tightly coupled relationship between action potential firing and neurotransmitter release, measurements of firing frequency provide an accurate estimate of signaling to downstream targets. These measurements can be taken from neurons obtained from animals in various metabolic states or, as practiced more commonly, from a neuron in response to exogenous neurotransmitter, hormone, or drug application to

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Table 1 Comparison of major modes of recording of electrophysiological activity

Advantages and disadvantages	Modes of recording		
	In vivo extracellular single unit recordings	Ex vivo (slice preparation) extracellular "cell-attached" recording	Ex vivo (slice preparation) intracellular, "whole-cell" recording
Intact neuronal network	Yes	No	No
Physiology routes for drug administration	Yes	No	No
Mechanical stability during recording	Low	High	High
Visual identification of GFP labeled neurons	No ("blind approach")	Yes	Yes
Obtained information and applications	Action potential firing frequency and pattern	Action potential firing frequency and pattern	Action potential and synaptic activity frequency and pattern Potential across the membrane Voltage clamp
Retains physiological intracellular environment	Yes	Yes	No
Useful for intracellular application of drugs	No	No	Yes
Perturbs chemical composition of cytoplasm "dialysis"	No	No	Yes

assay potential regulators. By assaying electrical activity of neurons from multiple centers within a neural network, one can build a working "circuit diagram" and provide not only of identification of the circuit but also insight into how the network produces a behavior.

This article provides a brief introduction to the advantages and limitations of using electrophysiological approaches compared to other commonly used techniques (Table 2). We will then discuss the contributions that these electrophysiological methods have made toward elucidating the role of ARC neurons as detectors of peripheral energy states by examining effects and molecular mechanism of actions of various peripheral hormones involved in energy homeostasis. We will also highlight the role of hypothalamic neuronal projections, as well as interactions between proopiomelanocortin (POMC) and NPY/AgRP neurons that govern the functional output of the melanocortin circuit responsible for regulating energy homeostasis.

Advantages and Limitations of Electrophysiological Tools Compared to Other Techniques

A thorough understanding of the neuronal circuitry that participates in energy homeostasis will be supported by multiple techniques and approaches that complement each

Table 2 Comparison of various tools of measuring neuronal activity

Characteristics	Techniques of measuring neuronal activity				
	mRNA or protein of immediate early gene products	mRNA or protein of neuropeptides	Rise in intracellular calcium	Electrophysiological tools; action potential and synaptic activity	Electron microscopic study of synaptic connectivity
Sampling distribution: signal is detected across many anatomical regions at the same time	Yes	Yes	Yes	Often sample activity of only one or few neuronal elements	Yes
Detects both increase vs decrease in signal levels	Only increases	Both increase and decrease decreases	Only increases	Both increase and decrease decreases	Both increase and decrease decreases
Identify the pattern of neuronal activity with high temporal and spatial resolution	No	No	No	Yes	No
Direct functional readout	No	No	Yes	Yes	No

other (summarized in Table 2). The nature of interactions between neurons involved in energy homeostasis can be determined by measuring changes in the activity of specific neurons in response to an experimental treatment. The mRNA or protein expression of neuropeptides in a specific brain region has been used to as a marker of neuronal activity. Although changes in synthesis of neuropeptides are usually in parallel to their release, a direct examination of neuronal firing is necessary to conclude synaptic release of the neuropeptides.

Furthermore, measurements of mRNA and protein as a readout of alterations in circuit activity are particularly limited due to the fact that hypothalamic neurons are on average smaller in size and have higher input resistance than neurons in other brain regions such as hippocampus, cerebral cortex, or cerebellum [5]; ligand-mediated changes in mRNA or second messenger levels will be proportionally smaller. This obstacle can be overcome by the use of electrophysiological tools, as they allow for the measurement of subtle changes in electrical activity that cannot be detected with other techniques. For example, a previous study from our lab, using in situ hybridization, reported expression of melanocortin 3 receptor (MC3R) mRNA in approximately one third of ARC NPY-expressing neurons [6]. However, we have recently found that bath application of D-trp-8-gamma-MSH (10–20 nM) on NPY neurons evokes responses in at least 90% of ARC NPY neurons tested (unpublished data). Thus, mRNA measurements underestimate population of neurons expressing functional MC3R by 3-fold lower than by electrophysiological techniques.

Additionally, the expression levels of immediate early gene products such as c-fos have been used by previous studies. This provides an impression of how a signal is intensified across many anatomical regions at the same time, providing an advantage over the use of electrophysiological tools which often sample activity of only one neuronal element within the network at a time and provide no information of the state of other neurons within the network. In contrast, changes in levels of immediate early gene products indicate only increases, but not decreases, in firing activity output. As an instance, peripheral injection of peptide YY (PYY)3–36 generates mild elevation of c-fos in ARC POMC neurons failing to provide support of the peptide action on these neurons. Indeed, slice recording from ARC POMC neurons indicated that this peptide in fact inhibits their neuronal activity [7].

Furthermore, assaying neuronal activity by measuring c-fos fails to identify the pattern of neuronal firing which has been shown to play a crucial role in neuropeptide release from neurons [8]. To demonstrate the functional importance of firing pattern, previous studies have shown that with the same number of action potentials fired by a neuron, a phasic pattern of firing was significantly more efficacious than a continuous repetitive firing in releasing neuropeptides from the posterior pituitary [9]. Distinction between different patterns of firings is only possible by electrophysiological recordings.

Calcium is involved in activation of several intracellular signaling pathways, and a rise in its intracellular concentrations has been used as an indicator of neuronal activation. Although very sensitive, electrophysiological techniques can measure influx of Ca^{2+} across the cytoplasmic membrane, but not the release of Ca^{2+} from intracellular stores. Thus, rises in its intracellular concentration have been measured by examining changes in emission frequency of fluorescent dyes occurring upon binding. Although a reflection of neuronal activity, this technique, as in detection of c-fos expression, harbors limitations in temporal and spatial resolution.

Another approach for studying altered synaptic connectivity within hypothalamic circuitry associated with different metabolic states utilizes electron microscopy (EM) to examine morphological properties of synapses in hypothalamic sections. Although this approach offers high resolution and samples a large number of neuronal contacts, it does not provide a functional readout of the circuit. Increases and decreases in synaptic contacts in EM images are only suggestive of alterations in the strength of signaling through a circuit, however, and cannot provide information whether these synaptic contacts are functional. For example, silent synapses that are morphologically present but functionally inactive limits interpretations from EM studies [10].

Hormonal Mechanisms Regulating Activity of the ARC–Paraventricular Nucleus Circuitry

The ARC is located in the most ventral part of the diencephalon where the capillary blood brain barrier is thought to be more permeable compared to other regions of the brain [11]. Thus, these ARC neurons are in an ideal location for sensing peripheral signals [12]. Consistent with this role, the ARC neurons express receptors for many peripherally produced hormones that regulate energy homeostasis, including leptin [13], insulin [14], ghrelin [15], and PYY [16]. Categorized by their neuropeptide production, POMC and NPY/AgRP are two major ARC cell populations involved in energy homeostasis. Each of these populations has also been further subdivided based on receptor expression and functional responses to stimuli [17]. The ARC cell population that produces POMC peptide products including α-MSH and γ-MSH, agonist for the melanocortin 3 and 4 receptors (MC3/4R), and β-endorphin, an agonist for the μ opioid receptor (MOR) project to over a hundred brain nuclei known to participate in energy homeostasis including PVN, LH, dorsomedial (DMH), and ventromedial nucleus of hypothalamus (VMH). In addition, a great majority of POMC neurons have been found to express another less

characterized peptide, CART; approximately 60% have been estimated to produce the fast excitatory neurotransmitter, glutamate; and approximately 40% have been estimated to express the inhibitory neurotransmitter GABA [18, 19]. A recent study suggested that expression of glutamate and GABA is mutually exclusive, implying the existence of glutamatergic and GABAergic subtypes of POMC neurons [20]. The second major ARC cell population produces NPY, a broadly expressed orexigenic peptide, and AgRP, a MC3/4R inverse agonist. These neurons express exclusively GABA, which is essential for the orexigenic function of these neurons [21].

As was originally shown by lesioning of ARC, the genetic or pharmacological deletion of hypothalamic POMC signaling generates an obesity phenotype comparable to MC4R deletion-induced obesity [3, 22], indicating the critical role of these neurons as sensors of peripheral energy stores. Although much data support a strong orexigenic role for both NPY and AgRP, disruption of each of these genes has failed to produce a significant lean phenotype in rodents [23, 24], possibly due to compensatory increase in GABA release. In fact specific ablation of NPY/AgRP neurons that also eliminates GABAergic neurotransmission has been shown to produce severe hypophagia and leanness associated with death during adulthood [25]. Furthermore, a brief selective electrical or chemical stimulation NPY/AgRP neurons in mice causes immediate voracious drive for food intake, underscoring the important orexigenic role of these neurons [26, 27]. In fact, Aponte et al. [26] have recently provided evidence suggesting that the drive for food intake induced by selective stimulation AgRP neurons may not require MC3/4 receptors, as previously eluded to [28].

The generation of transgenic mice that express GFP in specific neurons coupled with electrophysiological techniques has provided a powerful tool for investigating responses of these two major ARC cell populations to peripheral hormones [29]. In the most of transgenic mouse models used in original studies, levels of GFP expression are directly regulated by the promoter of the gene of interest, such as POMC, NPY, and orexin gene (e.g., [30]). However, in some mouse models, levels of GFP production are constitutively driven after the upstream floxed stop codon is removed by Cre recombinase protein that was once expressed by a promoter of gene of interest. This approach can potentially cause an error if the gene driving Cre is no longer expressed in the cell. This "switch" in phenotype during development has been shown to occur conspicuously in ARC neurons [31]. Therefore, if this developmental "switch" does occur, the use of the Cre X flox system to express GFP reporter or delete a gene of interest may significantly mislead our interpretation of electrophysiological as well as in vivo findings.

In using hypothalamic brain slices, bath application onto ARC neurons of peripherally produced peptides at plasma concentrations is predicted to mimic the in vivo milieu, allowing for a controlled system to probe the effects of individual peptide on specific subpopulations of neurons.

Leptin Signaling in ARC

Leptin is a hormone produced by the white adipocytes in direct proportion to fat stores. In vivo administration suppresses food intake, while leptin deficiency, as first observed in naturally occurring mutations, produces a severe obesity phenotype [32]. Leptin receptor is widely expressed throughout the CNS [33]. Binding of leptin to its receptor activates signaling pathways that mediate acute effects through membrane potential regulation and long-term effects through transcriptional regulation, thereby regulating diverse systems including reproduction, neuronal growth, and energy homeostasis.

The ARC was shown to have one of the densest expression patterns of leptin receptor in the brain. In fact, both POMC- and NPY/AgRP-expressing neurons express leptin receptor [34–36], as do yet-unidentified GABAergic ARC neurons [37]. Thus, ARC neurons likely represent a prominent site for leptin-mediated regulation of energy homeostasis. Leptin concomitantly increases ARC POMC and decreases ARC NPY/AgRP neuronal activity, as measured by changes in both mRNA and protein [38]. Furthermore, leptin increases c-fos gene expression, an early activity marker, in ARC POMC but not NPY/AgRP neurons [39]. In addition to a large body of evidence elucidating a role of ARC in mediating energy homeostatic effect leptin, studies have also indicated a role of leptin receptor signaling in the brainstem in regulating short as well as long-term energy homeostasis [40].

Leptin Electrophysiology

Cowley et al. [30] used electrophysiological methods to examine effects of leptin on the firing activity of POMC cells in ARC by recording from POMC-GFP neurons in acute hypothalamic slices from transgenic mice (Table 3). Bath application of leptin induced rapid increases in action potential firing activity associated with membrane potential depolarization of ARC POMC neurons mediated by activation of non-selective cationic currents [30]. These findings confirm direct activation of ARC POMC-expressing neurons by leptin and strongly support the hypothesis that leptin increases α-MSH release (and other POMC derivatives) at downstream neuronal targets. In addition, recordings from POMC neurons also showed that leptin reduced the frequency of $GABA_A$-mediated inhibitory synaptic currents, causing an additional excitatory effect through dis-inhibition. This inhibitory synaptic input was suggested to originate from local NPY/AgRP neurons, which were

Table 3 Central effects and sites of action of some hormones/peptides involved in regulation of energy homeostasis

Hormones/peptides	Site of action	Effects	Intracellular signaling pathway	Ion channels affected by signaling
Leptin (anorexigenic)	POMC cells	Depolarization (dis-inhibition)	PI3K-mediated depolarization [41]	Activation of non-selective cationic currents [30, 41]
			Mediated through a Jak2–PI3k–PLCγ pathway [42]	Activation of transient receptor potential cation channels [42]
			Through JAK2–phosphatidylinositol 3-kinase [43]	Increasing amplitude of high voltage-activated transient Ca^{+2} currents ($I_{(HVA)}$) [43]
	NPY/AgRP cells	Hyperpolarization (inhibition)		Activation of a $K_{(ATP)}^{+}$ current [44]
			Mediated by JAK2–MAPK pathway [43]	Decreasing the peak amplitude of Ca^{+2} current ($I_{(HVA)}$) [43]
Insulin (anorexigenic)	POMC cells	Hyperpolarization	ATP, PI3K mediated [17, 41, 45, 46]	
	NPY/AgRP cells	Hyperpolarization	Sulfonylurea receptor (SUR1) [47, 48, 49]	Activation of $K_{(ATP)}^{+}$ current [47]
		Depolarization	PI3K mediated [49]	
	VMH cells	Hyperpolarization	Sulfonylurea receptor (SUR1) [50]	Activation of $K_{(ATP)}^{+}$ current [50]
Ghrelin (orexigenic)	POMC cells	Inhibition	Increase in GABA mediated synaptic activity	An increase in inhibitory synaptic activity [51]
			EM study	Reduction in asymmetric (excitatory) synaptic contacts reported by electron microscopy [52]
	NPY/AgRP neurons	Depolarization [51]	Increase internal Ca^{2+} through adenylate cyclase–PKA [53]	Activation of N-type Ca^{2+} channels [53]
			AMP-activated protein kinase [54]	
			Increase levels of phosphorylated AMPK, PI3K, acetyl-CoA carboxylase [54]	
			c-fos [55], mRNA and peptide for NPY/AgRP [56, 57, 58]	
PYY3–36 (anorexigenic)	POMC cells	Increase in firing activity [59]	GABA mediated [59]	Decrease in the activity of inhibitory postsynaptic potentials [59]
		Hyperpolarization and decrease in firing activity [7, 60]	Direct Y2R mediated effects [7, 60]	Activating K^{+} currents [7, 60]
	NPY/AgRP cells	Hyperpolarization and inhibition		Activating K^{+} currents [60, 59]
Orexin (orexigenic)	POMC cells	Hyperpolarization and Inhibition [61].	Gi/Go-coupled GPCR [62]	Attenuated Ca^{2+} current oscillations and decreased Ca^{2+} currents [62]
				Hyperpolarization of membrane potentials of postsynaptic cells, in addition to presynaptic increases in frequency of GABAergic inputs [61]

Table 3 (continued)

Hormones/peptides	Site of action	Effects	Intracellular signaling pathway	Ion channels affected by signaling
				Presynaptic enhancement of excitatory and inhibitory input onto these cells [63]
				The sodium–calcium exchanger [63]
			EM study	Increase in symmetrical (inhibitory) synaptic contacts [64]
	NPY/AgRP cells	Depolarization	Through phospholipase C and protein kinase C [62]	Increase in intracellular Ca^{2+} and Ca^{2+} influx through N-type Ca^{2+} channel [53, 62]
			cAMP, PKA mediated activation of N-type Ca^{2+} channel [53]	Oscillations of membrane potential: depolarizing low-threshold Ca^{2+} conductance followed by hyperpolarization [44]
				Ca^{2+} mobilization from intracellular stores by activation of a sodium–calcium exchange current (Na^{+}/Ca^{2+}) [65]

later shown to be directly inhibited by leptin through hyperpolarization of membrane potential by activation of a ATP-sensitive K^+ channel (K_{ATP}) mediated current (Fig. 1) [44]. These findings are in agreement with the observed effects of leptin on mRNA and protein expression of POMC and NPY/AgRP neurons and suggest an important role for these ARC neurons in sensing and relaying signals of peripheral energy stores to downstream neuronal targets.

Recent studies have re-examined the ionic nature of the current(s) generated by leptin. Using combined whole-cell recording from rat-dissociated neurons and post hoc immunohistochemistry, leptin was observed to increase the amplitude of a nifedipine-sensitive, high voltage-activated transient Ca^{2+} currents (I_{HVA}) evoked by voltage steps through a Janus kinase 2–phosphatidylinositol 3-kinase (JAK2–PI3K) pathway in POMC neurons. Furthermore, leptin was shown to decrease the peak amplitude of I_{HVA} mediated by JAK2–mitogen-activated protein kinase (MAPK) pathway in NPY/AgRP neurons [43]. Using hypothalamic slices from POMC-GFP mice, another group showed that leptin depolarizes POMC neurons through activation of a non-selective cationic current that is mediated through a JAK2–PI3K–phopholipase C γ (PLCγ) pathway to open the transient receptor potential cation (TRPC) channels [42]. A possible link between these two currents and how they can modulate neuronal excitability remains to be determined. However, it is plausible to propose that the activation of the non-selective cationic current through TRPC channels activates the PI3K-mediated Ca^{2+} influx (I_{HVA}) resulting in neuronal excitability (Table 3).

These data support the observation that deletion of PI3K in POMC neurons prevented leptin-mediated depolarization of POMC neurons and muted the decrease in food intake associated with acute leptin administration [41]. Thus, leptin-mediated suppression of food intake appears to involve POMC neuronal activation. Surprisingly, specific deletion of PI3K in POMC neurons did not disrupt body weight regulation [41]. Perhaps this is due to the multiple signaling pathways activated by leptin receptor and different subtypes of POMC cells that contribute to leptin effects on energy homeostasis [17, 41, 66].

Leptin is known to regulate energy homeostasis in part through decreasing the activity of orexigenic AgRP-expressing neurons and thus dis-inhibiting ARC POMC neurons [37]. Studies showing that restricted deletion of the leptin receptor or its signaling on POMC or AgRP neurons results in modest alterations in body weight compared to that of complete leptin receptor deficient *db/db* mice, indirectly suggests a role of non-ARC centers in the regulation of long-term energy homeostasis [67, 68]. VMH deletion of the leptin receptor provides direct evidence for non-ARC effects of leptin. Mice lacking the leptin receptor in the VMH display about 20% of the body weight phenotype observed in the global leptin receptor null, *db/db*,

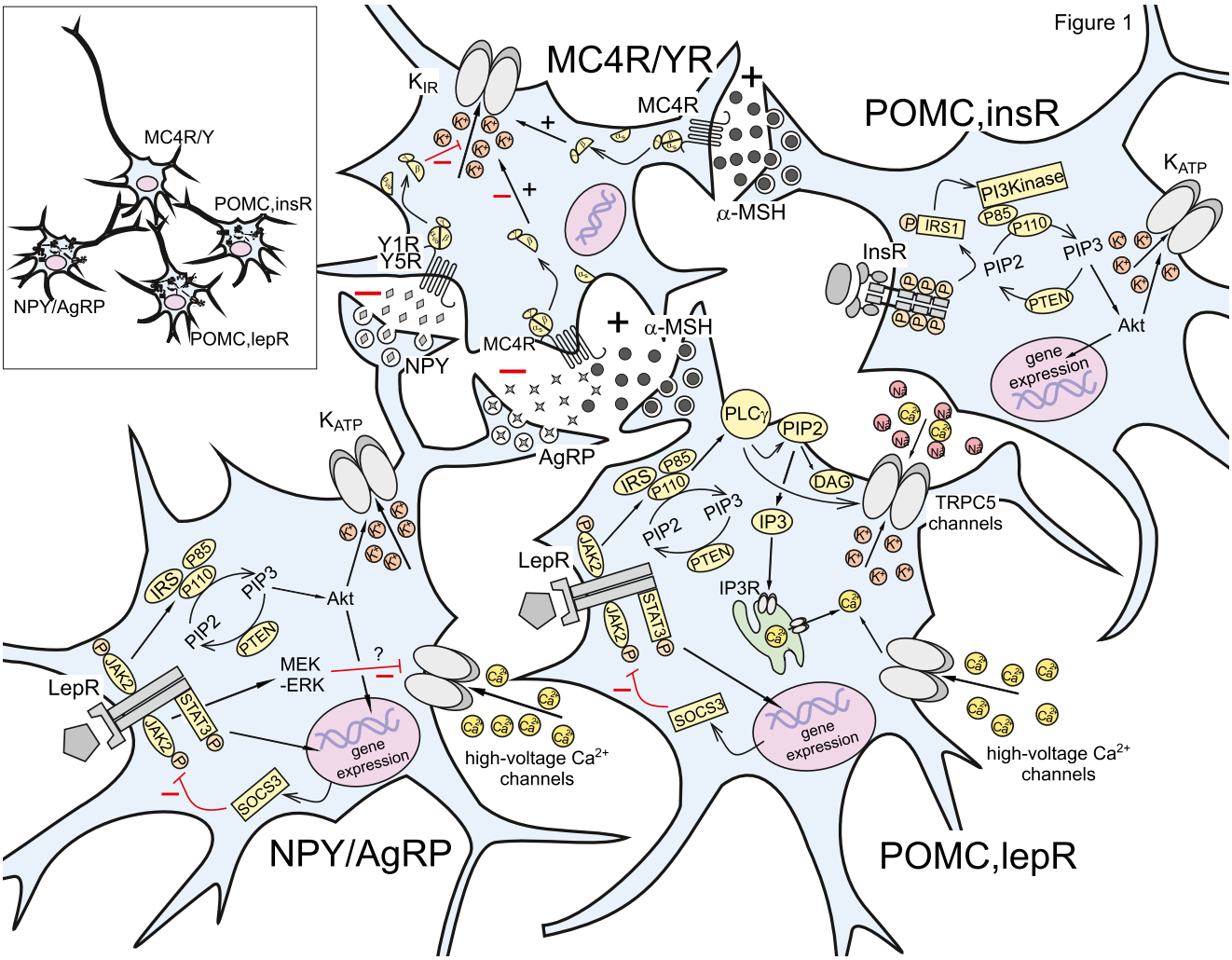


Fig. 1 Intracellular signaling pathways initiated by activation of leptin and insulin receptor in ARC NPY/AgRP and ARC POMC neurons. Schematic model of arcuate NPY/AgRP and POMC neurons projecting to effector PVN neurons depicting intracellular signaling pathways initiated by activation of leptin and insulin receptor. Note that a network of mutually exclusive leptin- and insulin-responding ARC POMC neurons in connection with ARC NPY/AgRP and MC4R/YR PVN neurons may mediate the PI3K-mediated cross-desensitization between leptin or insulin receptor signaling [17]. In ARC POMC neurons, activation of leptin receptor signaling results in PI3K-dependent activation PLCγ which causes activation TRPC, directly, and high voltage-activated (*HVA*) transient Ca^{2+} channels, indirectly. Insulin signaling inhibits these cells through PI3 kinase-dependent activation of K_{ATP} channels. Inhibition of NPY/AgRP neurons by leptin is also mediated by activation of K_{ATP} and inactivation of HVA Ca^{2+} channels

mouse [69]. Furthermore, we have recently shown that neurons in PVN are directly regulated by leptin, through melanocortin-independent mechanisms, to regulate the thyroid hormone release and reduction of food intake [70, 71]. Continued investigations into leptin-mediated effects on additional targets will expand our understanding of the complete neuronal circuitry regulating energy balance.

Insulin Signaling in ARC

Insulin is synthesized and secreted from pancreatic β cells in response to glucose, and serum insulin level generally increases as insulin resistance develops in obesity. Chronic central administration of insulin causes a reduction of food intake and of body weight, regardless of peripheral effects on glucose homeostasis [72, 73]. Insulin can act at neurons within the hypothalamus as it is transported through the blood–brain barrier by saturable transporters [74]. Insulin receptor (IR) is also widely expressed in the brain [75]. A clear role of hypothalamic IR signaling in regulation of glucose homeostasis has been emerging in recent years. Whole-body IR signaling knockout mice display greater hyperglycemia than the peripheral IR knock out ones [76]. Insulin signaling in hypothalamus is required for regulation of hepatic glucose production (HGP) [77]. Moreover, hypothalamic disruption of IR signaling causes hyperphagia and insulin resistance by blunting effects of peripheral insulin in

reduction of HGP in rodents [78], and this condition can be mimicked by central, but not peripheral, inhibitor of PI3K [79]. Furthermore, specific deletion of IR signaling from AgRP, but not from POMC, neurons significantly abolishes insulin-mediated suppression of HGP examined during euglycemic–hyperinsulinemic clamp [47].

The crucial role of AgRP neurons in regulating HGP was later confirmed by Lin et al. [80] who showed that the selective expression of IR signaling in AgRP, but not POMC, neurons in mice lacking IR signaling (L1 mice) restored the insulin-induced suppression of HGP, hence proposing divergent central pathways regulating the peripheral energy and glucose homeostasis [80]. Additionally, hepatic vagotomy or sympathectomy independently can abolish the hypothalamic IR-mediated regulation of HGP, further elucidating the neuronal pathways underlying central regulation of HGP [48, 81]. Electrophysiological characterization of insulin responsive neurons has increased the understanding of mechanisms underlying centrally mediated insulin effects on energy homeostasis.

Insulin Electrophysiology

By recording from ARC and VMH neurons obtained from lean Zucker rats, Spanswick et al. [50] showed that physiological concentrations of insulin can inhibit the spontaneous firing frequency associated with hyperpolarization of glucose-responsive neurons. This hyperpolarization involved postsynaptic mechanisms mediated by opening K_{ATP} channels [50].

More recently, Könner et al. [47] showed that insulin causes hyperpolarization of membrane potential of AgRP neurons by activating K_{ATP} channels [47]. Interestingly, Pocai et al. showed that the activation K_{ATP} channels in hypothalamus, by diazoxide, a SUR1 agonist, decreases plasma glucose levels by inhibiting expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, enzymes regulating glycogenolysis and gluconeogenesis. The authors showed that hepatic vagus nerve and activation of hypothalamic K_{ATP} channels are required for a central role of insulin in regulating HGP and serum glucose [48]. The authors proposed a model in which IR signaling inhibits AgRP neurons, through activation of K_{ATP} channels, resulting in hyperpolarization of membrane potential and down-regulation of enzymes participating in glycogenolysis and gluconeogenesis thereby depressing HGP.

Insulin and Leptin Cross-Desensitization

The first association of leptin and insulin receptor cross-talk was suggested by Spanswick et al. [50] who noticed that insulin failed to activate K_{ATP} channels when tested on hypothalamic ARC and VMH neurons obtained from obese leptin-insensitive Zucker rats. Recent studies of intracellular signaling indicate that PI3K mediates signaling through both leptin and insulin receptors and that PI3K is in fact involved in cross-desensitization of these two signaling cascades. This explains how insulin resistance at the intracellular signaling level may cause a relative resistance to leptin, resulting in an increase in body weight [82–85]. However, electrophysiological studies indicate opposing effects of insulin and leptin on ARC POMC neuronal activity. Leptin causes membrane depolarization associated with an activation action potential firing in ARC POMC cells, while insulin causes hyperpolarization associated with inhibition of the firing activity in these neurons [41]. Interestingly, the opposite responses of neuronal firing activity in ARC POMC neurons to leptin and insulin both require PI3K [41]. Williams et al. [17] have recently clarified this puzzle by showing that although both hormonal responses were mediated through PI3K, these responses were compartmentalized into two differing subpopulations of ARC POMC neurons, insulin-inhibited, and leptin-excited neurons (Fig. 1).

Ghrelin Signaling in ARC

Ghrelin is synthesized in stomach and intestine as a preprohormone, which undergoes a posttranslational acylation by the enzyme ghrelin *O*-acyltransferase [86]. Approximately 20% of total circulating ghrelin is in the biologically active acylated form [87] that can reliably trigger both growth hormone secretion and food intake in vivo [88]. Exogenous administration studies show that ghrelin promotes meal initiation and causes a long-term increase in adiposity [89, 90].

Mechanisms underlying the orexigenic effects of ghrelin have been controversial. Ghrelin receptor (GHSR-1) is widely expressed in the various brain regions including the hypothalamus and the brainstem as well as vagal afferent neurons in nodose ganglion [91, 92]. ICV administration of ghrelin induces c-fos in the dorsal motor nucleus of the vagus [93] and direct injections of ghrelin into the dorsal vagal complex stimulate feeding [94] suggesting a role of vagus system for ghrelin-induced stimulation of food intake. Studies examining effects of vagotomy on ghrelin-initiated food intake have produced conflicting results, with some showing that vagotomy can inhibit ghrelin's effects [95, 96] and other showing that effects of ghrelin on food intake remained intact after bilateral vagotomy [97].

There is also a large body of evidence suggesting that ghrelin stimulates food intake by acting on ARC neurons possibly mediated through the central melanocortin system. In ARC, ghrelin receptor (GHSR-1) expressed exclusively in NPY neurons [15]. Ghrelin has been shown to increase

the expression of c-fos in NPY/AgRP neurons [55], NPY/AgRP mRNA expression [56], and the NPY/AgRP neuronal firing activity [51]. Furthermore, the orexigenic effect of ghrelin is greatly reduced by NPY receptor antagonists [98] or by disrupting melanocortin signaling [99]. Thus, it is widely believed that at least in part ghrelin action on energy balance is mediated by the hypothalamic central melanocortin system.

Ghrelin activates multiple intracellular signal transduction pathways, some of which mediate changes in intracellular Ca^{2+}. For example, in hypothalamic neurons, ghrelin has been demonstrated to increase Ca^{2+} through adenylate cyclase–protein kinase A (AC–PKA) [53] and AMP-activated protein kinase (AMPK) [54]. Ghrelin also increases intracellular Ca^{2+} concentrations in a majority of NPY immunoreactive positive neurons. The ghrelin-induced rise in internal Ca^{2+} is mediated by activation of PKA and Ca^{2+} influx through N-type Ca^{2+} channels [53]. Ghrelin can also increase acetyl-CoA carboxylase (ACC) and levels of phosphorylated AMPK, an intracellular energy sensor in hypothalamic NPY neurons [54]. Ghrelin-mediated activation of AMPK was also reported in PVN neurons [100]. Interestingly, since Kohno et al. [101] have shown that leptin can suppress the ghrelin-induced rise in intracellular Ca^{2+} concentrations in ARC NPY neurons [101], AMPK and PI3K have been introduced as avenues to link ghrelin and leptin signaling pathways.

Ghrelin Electrophysiology

The effect of ghrelin on neuronal firing activity was assessed in a hypothalamic slice preparation from NPY or POMC-GFP transgenic mice. Consistent with the increased expression of NPY and AgRP in ARC neurons, Cowley et al. [51] showed that bath application of ghrelin caused a significant increase in spontaneous action potential firing activity of NPY/AgRP neurons and caused a less dramatic decrease in the firing activity of ARC POMC neurons [51] (Fig. 2 and Table 3). However, it remains to be elucidated whether the ghrelin-induced Ca^{2+} influx mediated by either cAMP-PKA or AMPK pathways is responsible for its excitatory effects on NPY/AgRP neurons.

The inhibitory effects of ghrelin on firing activity of POMC neurons were associated with an increase in inhibitory synaptic activity that was blocked by NPY receptor subtype Y1 (Y1R) or $GABA_A$ receptor antagonists, suggesting a presynaptic site of action. These findings were also consistent with a reduction in asymmetric (excitatory) synaptic contacts in ARC POMC neurons reported by EM studies [102]. In contrast, effects of this peptide on NPY/AgRP neurons were shown to be a direct action on postsynaptic cells [51], consistent with rises in intracellular Ca^{2+} concentration through activation of N-type Ca^{2+} channels observed by Kohno et al. [53] (Fig. 2). All together, these observations suggest that the stimulation of food intake by ghrelin may at least in part be due to its direct excitatory effects on NPY/AgRP and inhibitory effects on POMC neurons of ARC.

Consistent with in vivo findings, electrophysiological studies also have provided evidence depicting an action of ghrelin in the hindbrain. Cui et al. [103] using brainstem slice preparation showed that ghrelin inhibits frequency of spontaneous and mini-excitatory postsynaptic currents (sEPSCs and mEPSCs) onto catecholamine-expressing, cholecystokinin-sensitive afferent neurons in nucleus of tractus solitarius (NTS) resulting in a decrease in their basal firing rate. The investigators also showed that an 18-h fast accentuated the ghrelin-induced inhibition of EPSCs recorded from NTS neurons [103]. In another study, Fry and Ferguson [104] showed that ghrelin can dose-dependently evoke responses in frequency of spontaneous action potential firing activity in area postrema (AP) neurons with around 20% stimulated and 20% inhibited. The investigators proposed an activation of a non-selective cation current and a voltage-gated K^+ currents responsible for generation of, respectively, hyperpolarization and depolarization of these neurons by ghrelin [104]. These findings were consistent with in vivo findings of that lesioning the AP can blunt ghrelin-induced food intake [105].

PYY

PYY is an enteric hormone that is produced and released into the circulation upon arrival of a meal into the gut lumen [106]. Serum concentration profiles suggest that it initiates an appetite-suppressing drive during a meal [107]. Although its central administration results in an increase in food intake [108], slow peripheral administration of this peptide reduces food intake in rodents and human [59]. As described below, mechanisms underlying the anorexigenic action of this peptide remain controversial.

Electrophysiology of PYY3–36

In a preliminary study, PYY3–36 was reported to increase action potential firing in ARC POMC neurons, associated with a decrease in the activity of inhibitory postsynaptic potentials [59]. This study also reported only 11% of POMC cells expressed c-fos after PYY3–36 treatment. Since Y2R was expressed in NPY/AgRP neurons [109], the authors hypothesized that PYY3–36 directly inhibit NPY/AgRP neurons, and the consequent decrease in release of GABA was proposed to cause dis-inhibition of POMC neurons. During a more exhaustive study, we observed that application of PYY3–36 actually inhibited firing activity of POMC neurons directly by acting on postsynaptic Y2R. In fact, across a wide range of concentrations (from 10 pM to 1 mM), this peptide was never observed to cause an increase

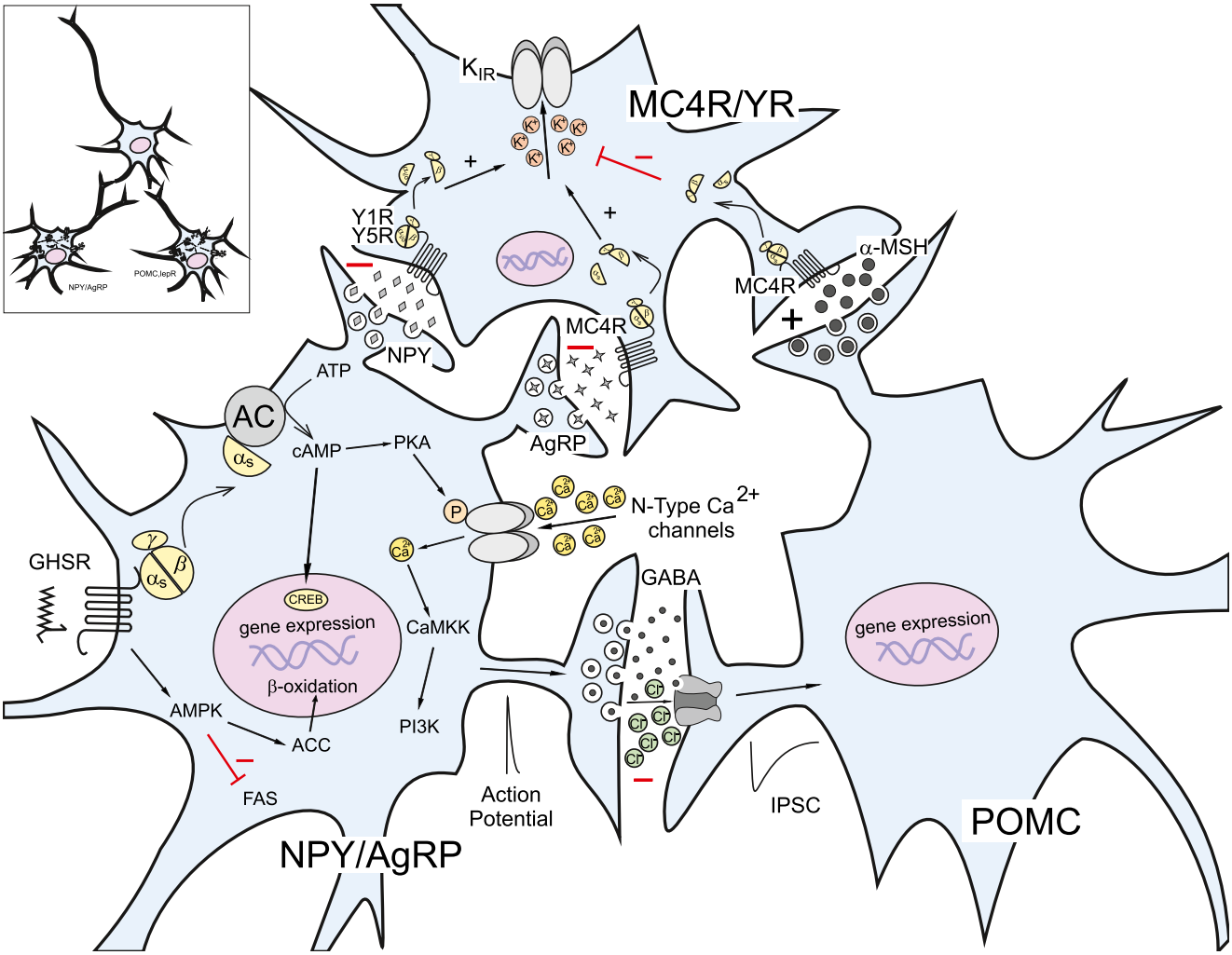


Fig. 2 Intracellular signaling pathways initiated by activation of ghrelin receptor in ARC NPY/AgRP neurons. Schematic model of arcuate NPY/AgRP and POMC neurons projecting to effector PVN neurons depicting the NPY/AgRP neurons as the primary site of ghrelin action in ARC. Activation of GHSR by ghrelin activates adenylate cyclase/PKA-dependent activation of N-type Ca^{2+} channels and CREB signaling in ARC NPY/AgRP neurons. GHSR-mediated phosphorylation of AMPK, ACC, and β-oxidation in ARC NPY/AgRP neurons has also been proposed. Subsequent activation of ARC NPY/AgRP neurons inhibits ARC POMC neuronal activity through $GABA_A$ and NPY neurotransmission. This reduces concentrations of α-MSH released from ARC POMC neurons on to effector MC4R PVN neurons

in firing activity of ARC POMC neurons [7] Another independent study confirmed that PYY3–36 consistently inhibited action potential firing of ARC POMC cells and hyperpolarized the membrane potential associated with activation of an inwardly rectifying K^+ channel currents [60]. They also reported that PYY3–36 had similar inhibitory effects on identified orexigenic NPY/AgRP neurons. Given these effects, Acuna-Goycolea et al. speculated that by inhibiting both NPY and POMC cells, PYY3–36 reduces the contribution of ARC neuronal output to downstream PVN neurons, allowing for an enhanced regulation by other CNS loci (Table 3). These findings further argue that the original finding of stimulation of ARC POMC neurons was an artifact, consistent with the low level of c-fos activation of these cells by PYY3–36, and indicates that the appetite-suppressing effect of PYY3–36 is mediated through mechanisms independent of the central melanocortin pathway. This model was further supported by findings obtained from various mouse models. In fact, the inhibitory effect PYY3–36 on food intake was not abolished in MC4R knockout mouse [110] the POMC knockout mouse [111] or the obese agouti mouse [112], a naturally occurring mutant that expresses a MC4R antagonist. Thus, the activity of melanocortin circuitry does not appear to play an essential role in inhibition of feeding by PYY3–36, and the sites and mechanisms of action remain to be fully described.

Orexin

There are two orexin peptides, orexin A and B, both exclusively produced by neurons in the LH and posterior

hypothalamus. They activate two known receptors with overlapping affinity. These peptides, as their name suggests, promote feeding when injected intracerebroventricularly while endogenous mRNA expression decreases with feeding [113]. A portion of the orexin effect on food intake is mediated through regulation of the central melanocortin pathway via direct input to ARC neurons. Several groups have demonstrated orexin-immunoreactive (IR) nerve terminals originating from LH making direct synaptic contact with both NPY/AgRP and POMC neurons of ARC [64, 114]. In particular, orexin-IR nerve terminals projecting to POMC ARC neurons at axo-somatic or axo-dendritic synapses were detected and categorized as symmetrical synapses, suggesting a GABAergic inhibitory regulation of anorexigenic POMC neurons [64]. In addition, both NPY/AgRP and POMC-expressing cells in the ARC are orexin receptor–IR [115, 116].

Orexin Electrophysiology

Bath application of orexin to an in vitro slice preparation increased firing activity in the majority of ARC neurons, mediated through direct action on the postsynaptic cells. Approximately, half of these ARC neurons were leptin-inhibited, and thus presumably NPY neurons and around 10% were leptin-excited thus presumably POMC neurons. [117]. In AgRP/NPY neurons, orexin induces bursts of action potential firing generated by a depolarizing low-threshold Ca^{2+} conductance followed by a hyperpolarizing transient outwardly rectifying potassium conductance, appearing as peptide-induced oscillations of membrane potential in the postsynaptic neurons [44].

In glutamate decarboxylase-67-positive GABAergic neurons, orexin activates action potential firing by mobilizing Ca^{2+} from intracellular stores in the postsynaptic cell (Fig. 3). The authors suggested that the orexin-induced Ca^{2+} mobilization was caused by the activation of a sodium–Ca^{2+} exchange current (Na^{+}/Ca^{2+}) resulting in membrane depolarization [65].

Corroborating these findings, orexin was also shown to increase intracellular Ca^{2+} concentrations and Ca^{2+} currents in isolated ARC NPY-IR neurons [53] with evidence of downstream signaling through PLC and protein kinase C (PKC) [62]; however, Kohno et al. [53] suggested a role of cAMP, PKA, and N-type Ca^{2+} channel-dependent signaling downstream to orexin receptor.

The evidence for orexin-mediated regulation of ARC POMC-expressing ARC neurons is more controversial, likely due to subtypes of POMC-expressing neurons. Muroya et al. [62] examined orexin-mediated effects on isolated POMC neurons and observed attenuated Ca^{2+} current oscillations and decreased Ca^{2+} currents [62]. These effects were pertussis toxin-sensitive, suggesting involvement of a G-protein-coupled receptor (GPCR) subunit type, Gi/Go. These findings were in agreement with previous findings demonstrating symmetrical (inhibitory) synaptic contacts between orexin-IR axon terminals and ARC POMC neurons [64].

Consistent with these findings, a subsequent study of POMC-GFP neurons reported that orexin-mediated suppression of spontaneous action potential firing was associated with hyperpolarization of membrane potentials of postsynaptic cells in addition to the previously reported presynaptic increases in frequency of GABAergic inputs [61]. The findings presented so far (Fig. 3 and Table 3) strengthen the model in which the appetite-promoting peptide orexin directly activates NPY/AgRP and inhibits POMC neurons in order to exert its effects.

However, this view has been challenged by the findings published by Acuna-Goycolea and van den Pol [63], in which they showed that orexin excited median eminence-projecting POMC neurons in ARC by postsynaptic activation of the sodium–calcium (Na^{+}/Ca^{2+}) exchanger and by presynaptic enhancement of excitatory and inhibitory input onto these cells [63]. The underlying causes of this discrepancy with findings of Ma et al. [61] could be that Ma et al. [61] did not isolate subtypes of ARC POMC neurons and, in fact, examined mostly PVN and VMH projecting ARC POMC neurons, as opposed to Acuna-Goycolea and van den Pol [63] who examined median eminence-projecting ARC POMC neurons that regulate pituitary function.

Neuronal Mechanisms Regulating Activity of the ARC Neuronal Output to the Melanocortin Network

Intrinsic Regulatory Mechanisms

As an animal goes through natural cycles of feeding and hunger, levels of metabolic signals in circulation, such as leptin and insulin, undergo significant fluctuations and have the potential to affect firing activity of ARC neurons. ARC neurons are likely equipped with an auto-inhibitory mechanism that prevents excessive firing, as has been previously described in magnocellular neurosecretory cells in supraoptic vasopressin neurons regulating serum osmolality [118]. Evidence supports potential auto-inhibitory regulation of ARC neurons, as well a NPY/AgRP–POMC reciprocal neuronal inhibition (Fig. 4).

POMC Auto-inhibition

In ARC neurons, the expression of MC3R mRNA in POMC and NPY neurons has been shown by double immunohistochemistry/in situ hybridization [119]. γ-MSH, a high affinity ligand for MC3R [120] and a POMC-derived peptide released from POMC dendrites, likely functions as an

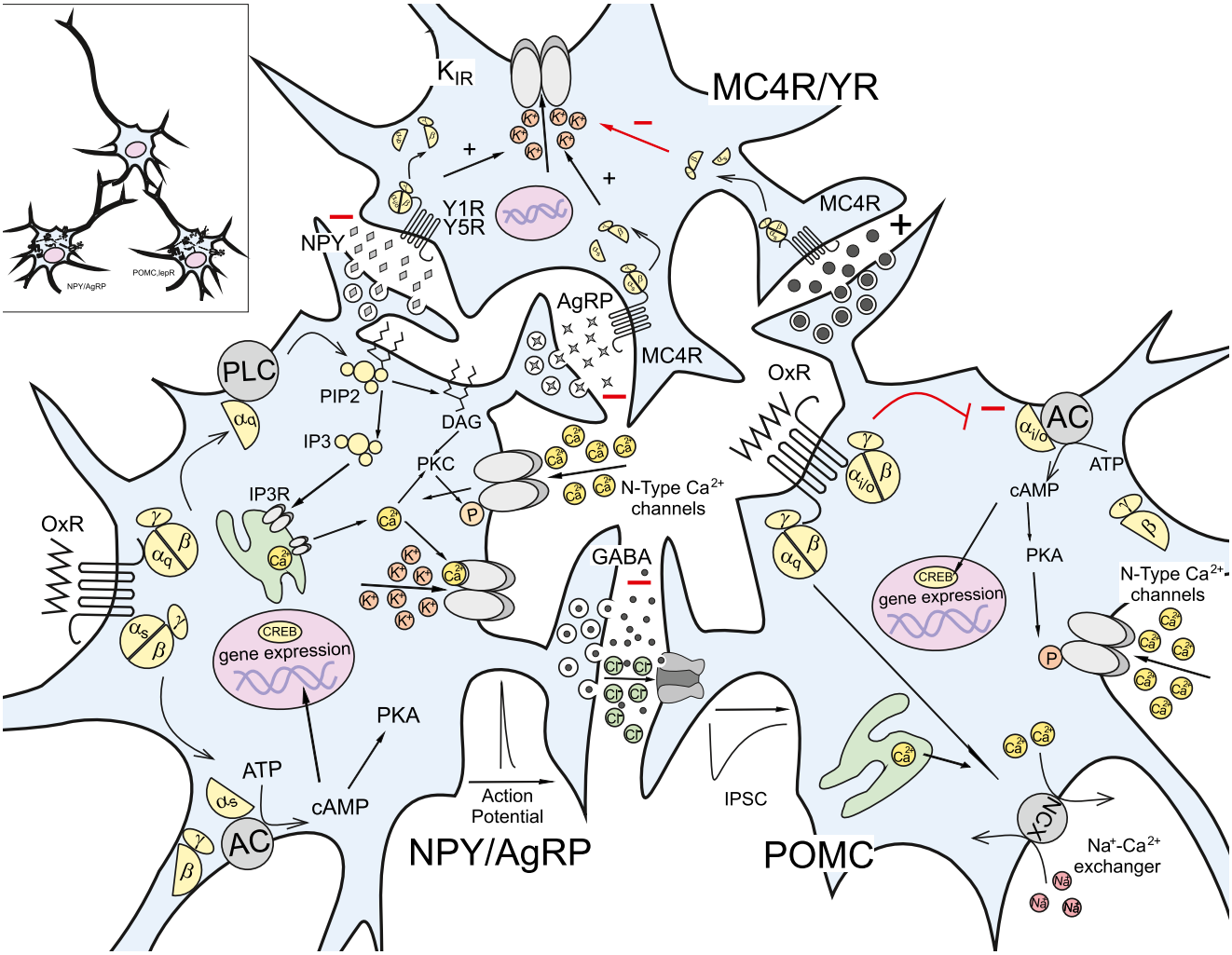


Fig. 3 Effects of orexin in ARC and intracellular signaling pathways initiated by activation of orexin receptor (*OxR*) in ARC NPY/AgRP and ARC POMC neurons. Schematic model of arcuate NPY/AgRP and POMC neurons projecting to effector PVN neurons depicting sites of action of orexin in ARC and intracellular signaling pathways initiated by activation of OxR in ARC NPY/AgRP and ARC POMC neurons. Orexin receptor signaling in ARC NPY/AgRP neurons causes PLC–IP3–DAG–PKC-dependent activation of N-type Ca^{2+} channels. OxR signaling may also cause activation of AC–PKA pathway that activates N-type Ca^{2+} channels. Intracellular Ca^{2+} accumulation caused by influx of Ca^{2+} from extracellular space and the internal stores activates Ca^{2+}-dependent K^+ channels to repolarize the membrane potential, a process that appears as oscillations of the membrane potential. In addition to receiving indirect inputs from ARC NPY/AgRP neurons, the activity of ARC POMC cells is directly regulated OxR signaling. Mediated by the G protein, Giα, this receptor signaling inhibits AC–PKA-dependent activation of N-type Ca^{2+} channels, while by Gqα, this receptor can activate ARC POMC cells through the Na^+/Ca^{2+} exchangers

endogenous ligand for MC3R expressed on POMC neurons and is postulated to mediate auto-inhibitory regulation of these neurons. Peripheral injection of the MC3R-specific agonist, d-Trp8-γ-MSH, stimulates feeding [121], suggesting that MC3R in circumventricular regions plays a role in regulation of food intake, perhaps by inhibiting POMC neurons. In addition, application of *d*-Trp8-γ-MSH, with an IC_{50} around 10 nM, inhibited action potential firing associated with hyperpolarization of POMC neurons recorded from hypothalamic slices [30]. These findings support that MC3R signaling inhibits POMC neuronal activity and may represent an auto-inhibitory mechanism promoting food intake; however, in apparent paradox, a more recent study showed that bath applications of 50 nM *d*-Trp8-γ-MSH increased action potential firing associated with depolarizing membrane potentials of POMC neurons recorded from mouse hypothalamic slices [122]. These discrepancies may originate from a relatively similar affinity of MC3R and MC4R for γ-MSH, as indicated by only 15-fold lower IC50 for MC3R compared to MC4R [123]. In addition to MC3R, there have been indications of other auto-regulatory mechanisms in ARC POMC neurons, with particular attention focused on β-endorphin auto-inhibition through the MOR. Opioid antagonists stimulate POMC

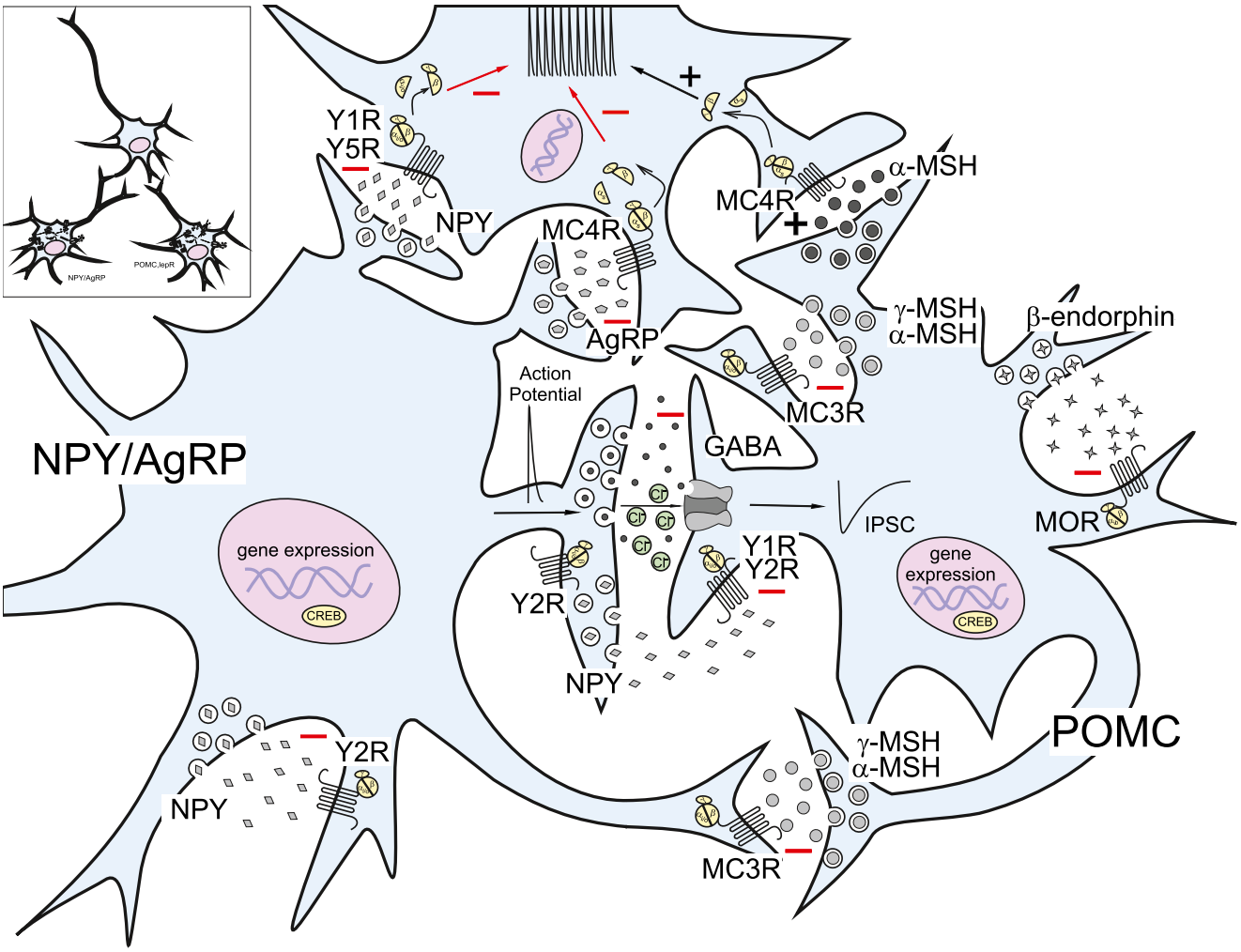


Fig. 4 Auto- and reciprocal-inhibitory mechanisms regulating the activity of ARC NPY/AgRP, ARC POMC. The diagram depicts auto- and reciprocal-inhibitory mechanisms believed to regulate the activity of ARC NPY/AgRP, ARC POMC, and their effector MC4R/YR PVN neurons. The ARC NPY/AgRP neurons inhibit activity of neighboring POMC neurons through $GABA_A$ and Y1/2R signaling. The dendritic release of NPY also causes auto-inhibition of these neurons acting through Y2R. ARC POMC neurons by releasing melanocortins thought to inhibit activity of ARC NPY/AgRP neurons through MC3R signaling. Somatodendritic release of melanocortins as well as β-endorphin from ARC POMC neurons also generates auto-inhibitory effects by acting respectively through MC3R and MOR signalings. Acting on MC4R, the axonal release of α-MSH from ARC POMC neurons increases firing activity of the effector PVN neurons. This effect of melanocortins on PVN neurons is antagonized by axonal release of NPY and AgRP acting, respectively, on Y1/Y5 and MC4 receptors

gene expression and regulate the level of POMC-derived neuropeptides in the rat hypothalamus [124]. Similarly, opioid antagonists also enhance β-endorphin and γ-MSH release from hypothalamic explants [125]. Opioid agonists have been shown to hyperpolarize ARC POMC neurons in rat hypothalamus [126], and the occurrence of MOR mRNA has been demonstrated in ARC POMC neurons [127]. These observations, collectively, suggest that β-endorphin released from somatodendritic vesicles exerts direct auto-inhibition on ARC POMC neurons (Fig. 4). However, a direct examination of the role of MOR and MC3R on ARC POMC neurons in regulating neuronal activity through auto-inhibitory mechanisms is overdue.

Reciprocal Inhibition of ARC POMC and NPY/AgRP Neurons

Although effects of neuropeptides on each group of ARC neurons have been extensively studied, direct neuronal interactions between POMC and NPY/AgRP neurons and how the functional output of their ensemble activity is determined are not completely understood. Electrophysiological recordings from hypothalamic slices of POMC-GFP mice have shown that ARC POMC neurons receive a dense population of $GABA_A$-mediated synaptic inputs [30], and using immunohistochemistry, it has also been shown that nerve endings in apposition to POMC somata co-express

both NPY and GABA [128]. Furthermore, it was shown that activation of ARC NPY neurons in hypothalamic slices increases the frequency and amplitude of $GABA_A$-mediated inhibitory synaptic events recorded from POMC neurons [30]. Additionally, ARC POMC neurons have been shown to express Y2R [7], and bath application of NPY inhibits action potential firing of ARC POMC neurons in hypothalamic slice preparations [129]. All of these findings suggest that ARC NPY/AgRP neurons inhibit firing activity of their neighboring POMC neurons through both $GABA_AR$ and Y2R signaling (Fig. 4). Whether ARC POMC neurons exert similar inhibitory inputs onto NPY/AgRP neurons has been controversial. Previous studies have provided evidence of MC3R expression in ARC NPY/AgRP neurons [130]. Low concentrations of γ-MSH consistently inhibit activity NPY/AgRP neurons, suggesting that MC3R signaling in NPY/AgRP neurons mediates inhibitory feedback from POMC to NPY/AgRP neurons (M G-L, unpublished data). These findings suggest existence of reciprocal feedback inhibitory mechanisms between POMC and NPY/AgRP neurons in ARC. However, one study failed to observe α-MSH-immunoreactivity in nerve endings in apposition to NPY/AgRP neurons [131]. It is quite possible that splicing of POMC into final peptide products could be differential along nerve endings. Moreover, in human basomedial hypothalamus, just as NPY-IR varicosities were observed in juxtaposition to all α-MSH-IR neurons, the majority of NPY-IR neurons were also contacted by α-MSH-IR varicosities [132]. Collectively, these findings imply existence of reciprocal-inhibitory feedback between ARC POMC and NPY/AgRP neurons.

Extrinsic Regulatory Mechanisms

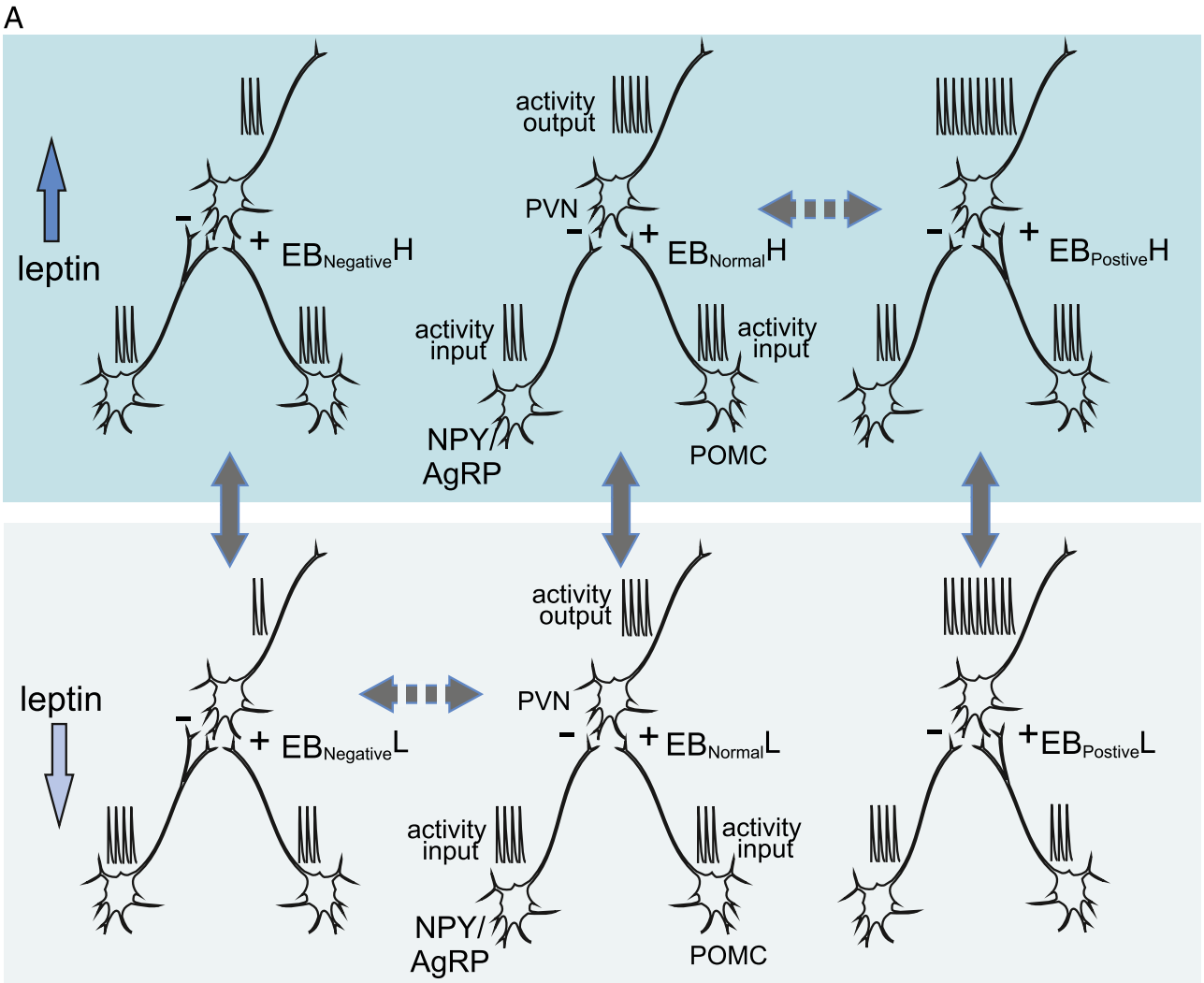
Neuronal Projections Regulating Activity of ARC POMC Neurons

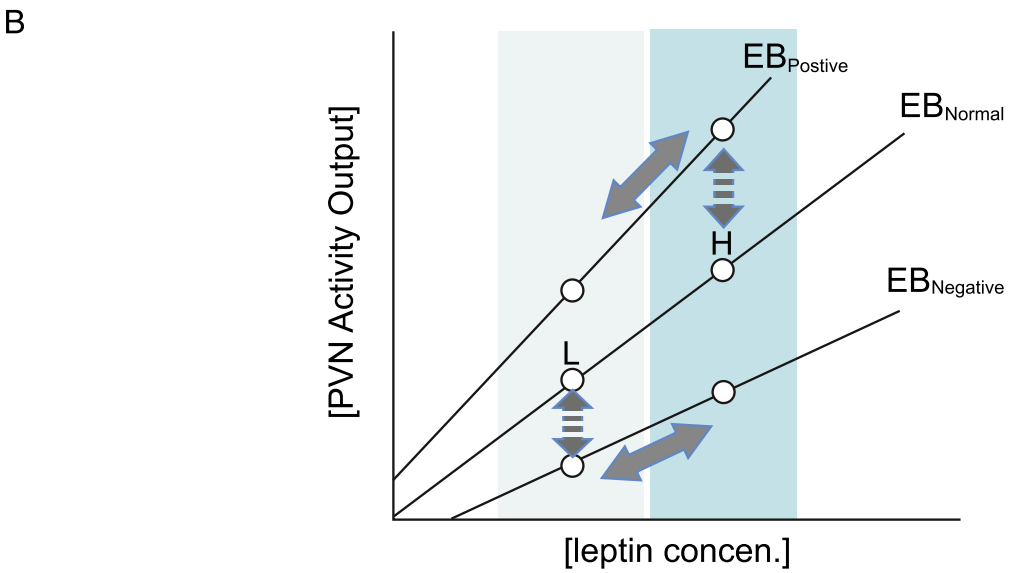
The activity of ARC neurons is regulated by synaptic input from other neuronal centers. EM studies have suggested that the density of excitatory and inhibitory synaptic input to ARC POMC neurons changes with metabolic state or with exposure to leptin or ghrelin [102], and recent studies suggest that a major mechanism of leptin action may involve a diverse set of GABAergic to ARC POMC neurons. In fact, based on anatomical studies, the leptin receptive neuronal projections to ARC originating from both hypothalamic and extrahypothalamic sites are numerous [133]. In a functional study, the significance of VMH to ARC projections was tested by focally stimulating presynaptic VMH neurons using laser scanning photostimulation to induce glutamate release onto postsynaptic NPY/AgRP or POMC neurons. Using this method, VMH neurons were shown to provide excitatory input to ARC POMC, but not NPY/AgRP neurons [134]. This is consistent with the observed increases in body weight in mice lacking leptin receptor signaling in VMH neurons [69].

By taking a more global approach, Vong et al. [37] deleted leptin receptor from *GAT* (inhibitory) and *GLUT2* (excitatory) expressing neurons and showed that the leptin receptor signaling in GABAergic neurons mediates the majority (greater than 80%) of the anti-obesity effects of leptin. Since the VMH, the ventral premammillary nucleus (PMv), raphe nucleus, and NTS/dorsal motor nucleus of the vagus (DMV) are predominantly glutamatergic, the investigators excluded their role in mediating leptin's effects. The GABAergic DMH, LH, and ARC neurons express high levels of the leptin receptor signaling molecule, p-STAT3. Since deletion of GABA neurotransmission in AgRP neurons had only a small effect on body weight, the authors concluded that the majority of the leptin effect is mediated through presynaptic, non-NPY/AgRP expressing, GABAergic neurons located in ARC. They further suggested that leptin's action on presynaptic GABAergic nerve endings contacting with ARC POMC neurons is essential for leptin's effect on regulation body weight.

Despite these findings, many studies have established a significant role for glutamatergic neurons in regulating leptin receptor signaling. Leptin receptor signaling has been detected in PMv neurons that are shown to innervate the ARC [135]. Interestingly, deletion of leptin receptor from these neurons in males causes obesity indistinguishable from that of the *db*/*db* male mice [136]. As mentioned earlier, deletion of leptin receptor in glutamatergic POMC and VMH neurons also generates mild obesity, although the number of leptin expressing neurons depleted of leptin receptor are much fewer than the study by Vong et al.

Fig. 5 A model describing effects of continuous extreme leptin concentrations on the functional gain of the ARC-PVN neuronal output. The diagram depicts a model describing how continuous extreme leptin concentrations observed in perturbed states of energy homeostasis can change the functional gain of the ARC- PVN neuronal output. **a** In the normal states of energy balance (Energy $Balance_{Normal}$, the *middle panels*), physiological increase or decrease in concentrations of leptin (Energy $Balance_{Normal}$ H and Energy $Balance_{Normal}$ L) affects the output of ARC-PVN neuronal activity by stimulating and inhibiting ARC POMC neuronal activity, respectively. Under continuous positive energy balance (Energy Balance, $EB_{positive}$), leptin-induced activation of ARC POMC neurons causes increases in efficacy of POMC-PVN synapse, hence generating a new set point of homeostasis featured by an increase in the ARC-PVN synaptic gain, resulting in higher PVN neuronal firing with the same leptin concentrations. Under continuous negative energy balance (Energy Balance, $EB_{negative}$), a decline in leptin concentration causes activation of ARC NPY/AgRP neurons hence increasing the efficacy of their synapse with the PVN neurons and decreasing the functional gain of ARC-PVN neuronal output, resulting in lower PVN neuronal firing with the same leptin concentrations. **b** The plot of PVN neuronal firing as a function of concentrations of leptin during various states of energy homeostasis as depicted in **a**. Note that changes in efficacy of ARC POMC and ARC NPY/AgRP synapse with their target neurons can, respectively, increase and decrease the gain of the circuitry





[37]. Considering these findings, it is plausible to propose that multiple centers in the hypothalamic neuronal network contribute to conveying leptin receptor signaling to ARC neurons and hence regulate energy homeostasis.

The ARC neurons are also innervated by projections from the leptin receptor-expressing brainstem areas such as lateral parabrachial nucleus, raphae nucleus, nucleus of tractus solitarius, and the dorsal motor nucleus of the vagus, which relay visceral information regarding food intake and metabolism to effector ARC POMC cells [133]. Abundant projections also arrive onto ARC neurons from other hypothalamic sites including the periventricular and PVN, suprachiasmatic nucleus, median preoptic nucleus, VMH, and the PMv. The activity of these projections has strongly been linked to long-term energy homeostasis in animal models, and a role in energy homeostasis is supported by expression of leptin receptor and its regulatory molecules [137].

It should be noted that these studies while increasing our understanding of homeostatic feeding are limited in their scope and largely ignore hedonic feeding. Direct projections from cortical areas, medial amygdala, and bed nucleus of the stria terminalis relay information regarding sensory modalities, e.g., olfactory, gustatory and visual memories, reward expectations, and emotional experience associated with food to the ARC [133].

Synaptic Plasticity in ARC Neurons Induced by Leptin

Serum leptin concentrations fluctuate diurnally, peaking several hours after beginning of food intake. Leptin levels in cerebrospinal fluid also vary reflecting its levels in serum [32]. Furthermore, DIO rodents express increased levels of serum and CSF leptin, suggesting that leptin may regulate neuronal function by acting on neuronal processes, in addition to affecting firing activity. In fact, EM studies have shown augmentation of excitatory synapses on POMC ARC neurons induced by leptin and decline in inhibitory synapse by ghrelin, providing evidence to support the model of synaptic plasticity in energy homeostasis [102]. Additionally, the frequency and/or amplitudes of inhibitory postsynaptic currents and EPSCs recorded from POMC and NPY ARC neurons are modulated by leptin, ghrelin, corticosteroids, estrogen as well as metabolic states [52, 138–140]. These findings support a role of synaptic plasticity in regulating the functional output of ARC POMC and ARC NPY/AgRP neurons synapsing to their effector PVN neurons. Based on these observations (Fig. 5), increases or decreases in concentrations of leptin will affect the output of ARC neurons by stimulating or inhibiting action potential firing of POMC neurons, respectively. Continuous high leptin concentrations, as observed in perturbed states of energy homeostasis, however, can also increase synaptic contacts of POMC neurons with the PVN neurons, resulting in higher PVN neuronal firing with the same leptin concentrations. In contrast, in continuous negative energy balance, a decline in leptin concentrations increases synaptic contacts of NPY/AgRP neurons with their effector neurons, resulting in lower PVN neuronal firing with the same leptin concentrations (Fig. 5).

Future Investigations of Energy Homeostasis Using Electrophysiological Techniques

Our accumulated knowledge indicates that there is redundancy in neuronal connectivity between various hypothalamic nuclei that regulate food intake, presumably as mechanisms against starvation ensuring animal survival. Consistent with this, second-order neurons, such as PVN neurons, receive a synaptic input from many brain regions other than ARC. As shown in the first-order ARC NPY/AgRP and POMC neurons [37, 141], there is now evidence that activity of these neurons are directly regulated by hormones, such as leptin or insulin, that are able to cross the BBB and enter cerebrospinal fluid circulation [70, 71, 142].

Many important questions remain: What is the ARC contribution to regulation of firing activity of the second-order neurons in PVN, VMH, or LH? Despite a large body of evidence depicting ARC neurons an important regulator of energy homeostasis, deletion of leptin receptor from ARC NPY/AgRP or POMC neurons has caused minimal effects on body weight. An alternative explanation could be that other neuronal centers also significantly contribute into regulating the activity of the second-order neurons.

What are the neuronal projections that regulate the activity of ARC POMC neurons? The findings of Vong et al. [37] suggest a crucial role of GABAergic neurons in relaying anti-obesity effects of leptin. In addition to intra ARC neurons, a role of GABAergic neurons from DMH, LH, and extra-hypothalamic leptin receptor-expressing GABAergic neurons ought to be investigated. As an approach to cross-examine the hypothesis, it would be valuable to express leptin receptor into GABAergic neurons of db/db obese mice.

What is a role of brain stem projecting ARC NPY/AgRP and POMC neurons in regulation of energy homeostasis? Deletion of AgRP-expressing neurons causes severe starvation resulting in death, and this effect can be rescued by brain stem injection of GABA, underscoring importance of this subset of ARC neurons. Is the activity of this subset of AgRP neurons primarily driven by hormones such as leptin receptor signaling or projections from other neuronal centers?

As obesity continues to grow, so does the need to understand the circuits that regulates energy homeostasis. Innovative techniques such as optogenetics or the designer receptors exclusively activated by designer drugs [26, 27] will be exploited to define and uncover the complex circuitry involved in energy homeostasis. Such techniques will be used in combination with well diagnosed clinical conditions characterized by perturbations of energy homeostasis to identify novel therapeutic modalities for treatment of obesity and cachexia.

Acknowledgments I am greatly thankful to Drs. Amanda Vanhoose, Roger D Cone, Benjamin Renquist, and David Jacobson for their assistance in preparation of this manuscript.

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