



# The PACAP Paradox: Dynamic and Surprisingly Pleiotropic Actions in the Central Regulation of Energy Homeostasis

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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), a pleiotropic neuropeptide, is widely distributed throughout the body. The abundance of PACAP expression in the central and peripheral nervous systems, and years of accompanying experimental evidence, indicates that PACAP plays crucial roles in diverse biological processes ranging from autonomic regulation to neuroprotection. In addition, PACAP is also abundantly expressed in the hypothalamic areas like the ventromedial and arcuate nuclei (VMN and ARC, respectively), as well as other brain regions such as the nucleus accumbens (NAc), bed nucleus of stria terminalis (BNST), and ventral tegmental area (VTA) – suggesting that PACAP is capable of regulating energy homeostasis *via* both the homeostatic and hedonic energy balance circuitries. The evidence gathered over the years has increased our appreciation for its function in controlling energy balance. Therefore, this review aims to further probe how the pleiotropic actions of PACAP in regulating energy homeostasis is influenced by sex and dynamic changes in energy status. We start with a general overview of energy homeostasis, and then introduce the integral components of the homeostatic and hedonic energy balance circuitries. Next, we discuss sex differences inherent to the regulation of energy homeostasis *via* these two circuitries, as well as the activational effects of sex steroid hormones that bring about these intrinsic disparities between males and females. Finally, we explore the multifaceted role of PACAP in regulating homeostatic and hedonic feeding through its actions in regions like the NAc, BNST, and in particular the ARC, VMN and VTA that occur in sex- and energy status-dependent ways.

**Keywords:** proopiomelanocortin (POMC), pituitary adenylate cyclase activating peptide (PACAP), food addiction, sex difference, A10 dopamine neurons, feeding, homeostatic feeding

## INTRODUCTION

The brain receives and processes metabolic signals from multiple sources such as gut hormones, nutrients, and circulating leptin and insulin levels to modulate energy intake and energy expenditure by sending the information to the brainstem and the hypothalamus (1). The hypothalamus and the brainstem coordinately modulate energy homeostasis by controlling food

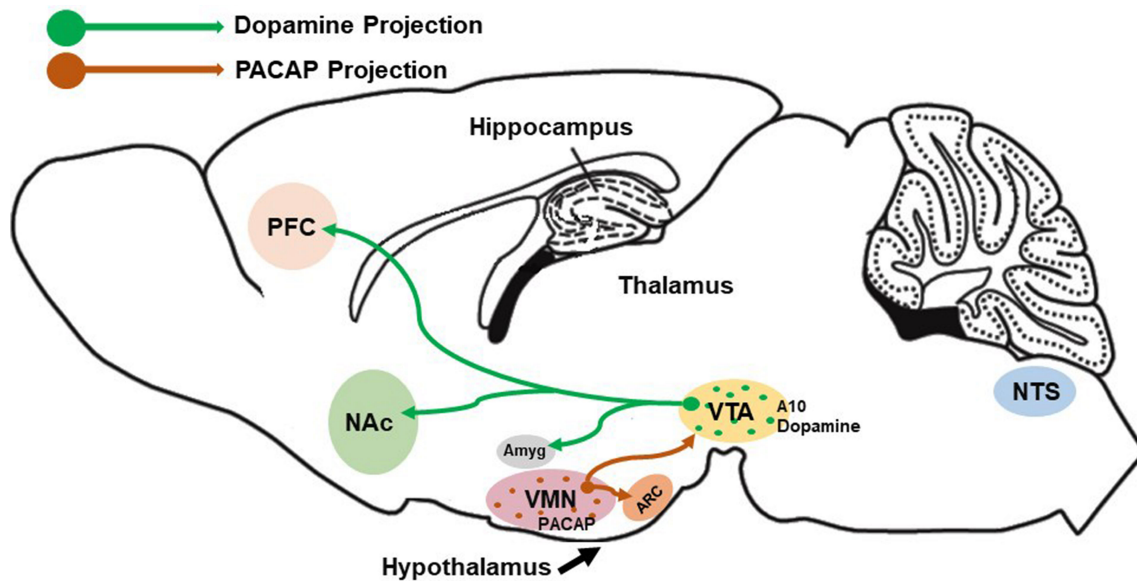
intake and body weight, autonomic regulation of gastrointestinal function (2), as well as energy expenditure through changes in thermogenesis and locomotor activity (3). The dorsal vagal complex is an important center within the brainstem that process signals received through vagal afferents and projects to the hypothalamus and other regions (4). The dorsal vagal complex consists of the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus, and the area postrema, which is easily accessible to peripheral signals due to the incomplete blood brain barrier surrounding it (5). Vagal afferent fibers emanating from the gut have cell bodies in the nodose ganglion and release glutamate from terminals in the NTS to activate group II metabotropic glutamate receptors in these cells. These second order NTS neurons, in turn, employ an array of different neurotransmitters to control gastric functions (2). The NTS also contain other neuropeptides like proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), and glucagon-like peptide-1 (GLP-1) (5–7), which has been touted as anorexigenic (i.e., appetite suppressing) peptides known for their predictive biomarkers of satiety. Indeed, these neurons are stimulated upon food consumption, in part, through the activation of signal transducer and activator of transcription 3 in response to exogenous leptin (8). A recent paper reported that NTS catecholaminergic and neuropeptide Y (NPY) neurons also play a role in the vagal-mediated gut-brain pathway to both stimulate orexigenesis (i.e., hunger or appetite) and suppress feeding in an opposing manner such that the activation of NTS epinephrine neurons co-expressing NPY triggers food consumption while activation of NTS norepinephrine neurons induces satiety, respectively (9). Vagus nerve transmission from the gut to the NTS is extremely important in sensing distension of the gut wall as well as luminal contents, and either acute or chronic stimulation of vagus nerve causes weight loss and food reduction in rats (2, 10). Gut-derived hormones such as GLP-1, cholecystokinin, and peptide YY are released upon sensing food consumption and upon binding to their receptors (11–14), and the vagus nerve conveys this information to the NTS, which, in turn, relays it to the hypothalamus to induce satiety (7, 15). Beside sending signals to the hypothalamus, the NTS receives descending neuronal projections from both the lateral hypothalamus (LH) and the paraventricular nucleus (PVN) (16, 17). As an example, orexin neurons are highly expressed in the LH, extensively projected to many different brain regions, and the administration of orexin A into the caudal brainstem (18) or the hindbrain (19) causes an increase in food intake.

Food consumption is controlled and regulated *via* two circuits: the homeostatic and the hedonic energy balance circuitries. It is well known that the hypothalamus functions in both the modulation of energy homeostasis and feeding regulation. Four hypothalamic regions that contribute to this modulation are: the ventromedial nucleus (VMN), the arcuate nucleus (ARC), the LH, and the PVN. It has been shown that bilateral lesion of the VMN produced severe obesity – indicating that the VMN suppresses appetite and food intake; whereas large lesions of the LH led to death by inanition/starvation (20) – suggesting that the LH promotes food consumption and appetite. Similarly, the ARC

and PVN are also known to regulate feeding as global ablation of the ARC (21) and lesions caudal to the PVN (22) resulted in morbid obesity and overeating. Within these regions, hormones like insulin and leptin released from the pancreas and adipose tissues, respectively, exert anorexigenic effects. In contrast, ghrelin released from the gastric mucosa exerts orexigenic effects. For instance, leptin suppresses appetite *via* the excitatory effect on anorexigenic POMC neurons in the ARC (23–25) that depolarizes these neurons and increases their firing rate through activation of transient receptor potential cation 5 (TRPC5) channels. On the other hand, leptin also exerts inhibitory effects on orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the ARC (26, 27). Similarly, insulin also inhibits and hyperpolarizes NPY/AgRP neurons in the ARC, and it can inhibit SF-1 neurons in the VMN as well *via* the activation of ATP-gated potassium ( $K_{ATP}$ ) channels (28–30). Insulin can regulate POMC neuronal plasticity to control energy balance. For instance, the excitatory effect of insulin depolarizes and stimulates POMC neurons *via* the activation of TRPC5 channels (29, 31); whereas, its inhibitory effect results in hyperpolarization and inhibition of POMC neurons (24, 32–34). Interestingly, it is now known that the balance between excitatory and inhibitory of insulin-induced responses in POMC neurons is dependent on the levels of protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TCPTP) within these cells (34). In contrast to insulin and leptin, ghrelin promotes appetite *via* the excitatory effects on orexigenic NPY/AgRP neurons by depolarizing and increasing the firing rate while also inhibiting and suppressing firing of POMC neurons (35, 36). Satiety and hunger are two physiological responses that can directly reduce food consumption and promote feeding, respectively. If the homeostatic feeding regulates energy intake and expenditure based on survival and physiological needs, the hedonic feeding behavior modulates energy balance pertaining to reward-based or pleasure-driven food intake. The hedonic energy balance circuitry involves the mesolimbic dopamine ( $A_{10}$ ) neurons in its regulation of palatable food. These  $A_{10}$  dopamine neurons emanate from the ventral tegmental area (VTA) and project to several regions such as the nucleus accumbens (NAc), hippocampus, prefrontal cortex (PFC), and amygdala (37–39). Evidence accumulated over the past 20 years indicates that other neuropeptides like PACAP can regulate food intake in both homeostatic and hedonic circuitries. Indeed, the VMN PACAP neurons are ideally suited to do just that (**Figure 1**). In this review, we will focus on the effect of PACAP on the homeostatic and hedonic aspects of feeding. In this context, we aim to describe the *in vivo* and *in vitro* effects of both exogenous and endogenous PACAP in regulating energy intake and energy expenditure.

## THE HOMEOSTATIC ENERGY BALANCE CIRCUITRY WITHIN THE HYPOTHALAMUS

Several seminal lesioning studies conducted in rats and mice during the 50s and 60s denoted the importance of the



**FIGURE 1** | Schematic depiction of VMN PACAP neurons innervating other hypothalamic and mesencephalic structures (e.g., the ARC and VTA, respectively) to regulate homeostatic and hedonic feeding. Postsynaptic targets of these VMN PACAP neurons include POMC neurons and  $A_{10}$  dopamine (DA) neurons, the latter of which project to regions like the amygdala, nucleus accumbens and prefrontal cortex to regulate executive decision-making based on cues associated with anticipated reward.

hypothalamus in feeding regulation (20, 21). This provided a catalyst for future experiments leading to a better understanding of the hypothalamus as the fundamental nexus in controlling the whole-body energy balance within the homeostatic circuitry. The cross-communicating systems are conveyed by various hypothalamic nuclei and their associated neuronal populations, including those in the VMN, ARC, LH, the dorsomedial nucleus (DMN), and the PVN. These hypothalamic nuclei produce both anorexigenic and orexigenic signals to reduce and promote energy intake, respectively.

As mentioned above, the VMN has long been considered a central satiety center. SF-1 is a transcription factor encoded by the NR5A1 gene, and the SF-1 expressing neurons play crucial roles in the control of energy balance demonstrated by the studies of conditional knock-out mice (40–42). SF-1 can directly impact the VMN development such that the deletion of SF-1 or leptin receptor in SF-1 neurons leads to obesity in mice (42–44). In addition, the brain-derived neurotrophic factor (BDNF) as well as its neurotrophic receptor kinase 2 (NTRK2) receptor are also strongly expressed in the VMN, and the deletion of BDNF and its receptor leads to hyperphagia and obesity in humans and mice (45, 46). In contrast, the administration of BDNF either by central venous or peripheral venous results in body weight loss and food reduction through melanocortin 4 receptors (MC4R) signaling (47).

Excitatory input from VMN SF-1 neurons activates the anorexigenic POMC neurons, which leads to a reduction in energy intake and an increase in energy expenditure (48–50). POMC neurons in the ARC make up the melanocortin system of the hypothalamus. Alpha-melanocyte-stimulating hormone

( $\alpha$ -MSH) consists of 13-amino acid residues and is derived from posttranslational modification of the POMC precursor peptide along with other bioactive peptides like the endogenous opioid  $\beta$ -endorphin (51–53). The ARC also houses another population of neurons that co-express orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) (54). POMC/CART and NPY/AgRP cells together with downstream target neurons expressing the MC4R (or MC3R) constitute the melanocortin system (55, 56). Studies have shown that  $\alpha$ -MSH plays a critical role in the central nervous system (CNS) in suppressing food intake (55, 57) such that the MC4R knock-out mice exhibit obesity, hyperphagia, hyperleptinemia, and hyperglycemia (58). Similar results were observed with AgRP, a high-affinity competitive antagonist of MC4R, which directly inhibits POMC neurons and ultimately impedes the anorexigenic signaling of  $\alpha$ -MSH to promote feeding and decrease energy expenditure (55, 56).

Previous studies have reported that  $\mu$ -opioid agonists, inhibit POMC cells by inducing outward current and hyperpolarization (59–62) and promote appetite in rodents (63, 64) by physiologically antagonizing the effects of  $\alpha$ -MSH on downstream melanocortin receptors (65). In contrast,  $\beta$ -endorphin knock-out male, but not wild-type male mice exhibit hyperphagia, hyperleptinemia, hyperinsulinemia, and greater adiposity (66). Additionally, the anorexigenic neuropeptide CART is co-expressed in an appreciable number of POMC neurons (67).

Of the orexigenic neuropeptides in the ARC, both NPY and AgRP can directly modulate energy balance. NPY/AgRP neurons inhibit POMC neurons *via* synaptic release of the inhibitory amino acid neurotransmitter gamma-aminobutyric acid

(GABA) from these cells (68). NPY acts at five different receptors known as Y1, Y2, Y4, Y5, and Y6; however, concrete evidence suggests that postsynaptic Y1 and Y5 receptors mediate the effects of NPY under *ad libitum*-fed conditions (69, 70). For instance, direct administration of NPY into specific hypothalamic sites like the VMN, PVN and LH (71–73), as well as injection into the lateral ventricle (72, 74–76) swiftly induces feeding responses in rats. Similarly, chronic central administration of AgRP reduces energy expenditure, induces food intake, and causes obesity (77–79). In congruence with these studies, Krashes and colleagues employed designer receptors exclusively activated by designer drugs (DREADDs) and chemogenetic approaches to acutely stimulate AgRP neurons through action on MC4R to induce feeding over a prolonged period as compared to the rapid stimulation of feeding by GABA and NPY – indicating that AgRP plays a role in a different phase of appetite regulation (80, 81). Despite these reports demonstrating the effects of NPY and AgRP as positive modulators of energy balance, some genetic studies have shown contradicting results as NPY- and AgRP- knock-out mice failed to show changes in feeding behavior and body weight (82–84). In contrast, several papers have indicated that AgRP ablation in adult mice eventually leads to death due to starvation but completely ineffective in neonatal mice (85). There is also a population of nociceptin/orphanin FQ (N/OFQ)-expressing neurons in the ARC. These cells are glucose- and leptin-sensitive, and their excitability is enhanced by acute exposure to a high-fat diet (HFD) (86). ARC N/OFQ neurons co-release GABA, and optogenetic stimulation of these cells inhibits neighboring POMC neurons *via* a combination of fast GABA<sub>A</sub> receptor-mediated neurotransmission and activation G protein-gated, inwardly rectifying K<sup>+</sup> (GIRK) channels (86, 87).

Besides the VMN and ARC, the LH also plays a crucial role in the regulation of energy balance (88). Two neurons that are abundantly expressed in the LH are melanin-concentrating hormone (MCH) and orexin. MCH has been recognized as a feeding stimulator in mammalian brain because MCH mRNA levels are not only upregulated by threefold in *ob/ob* (i.e., leptin deficient) mice under *ad libitum* fed, but MCH mRNA expression also increase under fasting condition in both *ob/ob* and normal mice (89). Several studies have demonstrated that intracerebroventricular (ICV) injections of MCH produce an increase in food intake in both rats (90, 91) and mice (92); whereas, mice with reduced MCH mRNA levels or disruption of the MCH1 receptor remain lean and hypophagic (93, 94). Orexin neurons, on the other hand, are found to have increased in mRNA expression under fasting conditions (95) and stimulation of these neurons increases both food intake and energy expenditure (96). Central administration of orexin not only promotes food consumption (95, 97), but also increases behavioral responses to food reward (98). Taken together, it could be said that neurons found within the LH, such as those containing orexin or MCH, can relay orexigenic signals. For example, orexin enhances GABAergic and diminishes glutamatergic inputs onto POMC neurons, which electrically silences these cells (99). On the other hand, endocannabinoids elicit its orexigenic effect by retrogradely inhibit GABAergic

input onto MCH neurons in a leptin-dependent manner, which may account in part for the hyperphagic effect of these cells (100).

The PVN and DMN are also very crucial in the control of energy balance and regulation of food intake (74, 101, 102) and also play important roles in physiological processes including thermoregulation, stress, and appetite (103, 104). The PVN expresses high levels of MC4R and MC3R, and it receives innervation not only from the POMC and AgRP neurons from the ARC, but also from the NTS (105, 106). Direct injection of NPY or anti-NPY  $\gamma$  IgG into the PVN causes stimulatory or inhibitory effects on food consumption, respectively (107, 108). On the other hand, the DMN receives projections from the ARC and sends projections to the PVN and LH. Several studies reported that NPY expression in the DMN is increased in rodents with obesity (103, 109), and the increased NPY levels play a significant role in the development of diet induced obesity (DIO) as well as the regulation of thermogenesis (104).

Besides playing a significant role in the regulation of energy balance, these neurons are susceptible to the influence of peripheral hormones like insulin, leptin, ghrelin, and sex hormones. Leptin was first discovered by Zhang and his colleagues in 1994 which opened up new avenues of research in the regulation of body weight homeostasis and obesity (110). Leptin is synthesized primarily in mature adipocytes within white adipose tissue and acts as an anorexigenic hormone after being released into the bloodstream (110, 111). Leptin acts not only as an appetite suppressor, but also plays a role in stimulating metabolism and reducing excessive stored energy (112). There are several isoforms of leptin receptor (LepR) that have been cloned like Ob-Ra, Ob-Rb, Ob-Rc (113–116), but the long form (LepRb) is the most crucial to bring out the effects of leptin and is abundantly expressed in the hypothalamus (113). For instance, activation of LepRb *via* the Janus kinase (JAK)/signal transducer and activator of transcription (JAK/STAT) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathways depolarizes both the ARC POMC and VMN SF-1 neurons leading to ions entering and increase in firing *via* the activation of TRPC5 channels (23–25, 44). By contrast, the inhibitory effect of leptin on NPY/AgRP neurons is mediated by the activation of K<sub>ATP</sub> channel; leading to hyperpolarization and decrease in firing (26). Studies of NPY/AgRP and POMC neurons from *ob/ob* mice as well as DIO guinea pigs and NR5A1-cre mice reveal that they undergo extensive synaptic plasticity under conditions of obesity; with the former getting more excitatory glutamatergic inputs and fewer inhibitory GABAergic inputs, and the latter receiving large number of GABAergic inputs and less glutamatergic input (49) (117); however, fasting can diminish the strength of the excitatory inputs onto POMC neurons (48). Previous reports have shown that a deficiency in leptin (i.e., *ob/ob*) or LepRb (i.e., *db/db*) not only causes hyperphagia and reduces energy expenditure in both humans and mice (118–120), but also results in morbid obesity in mice (114, 115).

Like leptin, insulin is known as an anorectic hormone in the hypothalamus and is synthesized in pancreatic  $\beta$ -cells. Insulin receptor (IR) is highly expressed in the hypothalamic areas that



play a role in the regulation of energy balance (121, 122) and also colocalizes with POMC and AgRP neurons (123, 124). Though the absence of IR in these POMC and AgRP neurons did not significantly alter energy homeostasis, deficiencies in the ability of insulin to decrease hepatic glucose production and suppress adipocyte lipolysis were observed (124–126) – indicating that insulin not only acts as an anorexigenic hormone in the CNS but also plays an important role in glucose metabolism. While LepRb activity is mediated *via* JAK/STAT and PI3K signaling as well as TRPC5 and  $K_{ATP}$  channels, the inhibitory effects of insulin are mediated *via* similar yet distinct pathways. For instance, insulin inhibits NPY/AgRP (29, 127) and VMN SF-1 (28) neurons *via* the activation of  $K_{ATP}$  channels. On the other hand, insulin can exert both excitatory (*via* activation of TRPC5 channels) and inhibitory effects (*via* PI3K) on POMC neurons (24, 29, 32, 33, 128). The directionality of these effects depends on the levels of PTP1B and TCPTP expressed by POMC and NPY/AgRP neurons, which fluctuate with changes in energy status (34, 129, 130). The phosphatases PTP1B and TCPTP are highly expressed in the ARC and can directly regulate leptin and insulin signaling in POMC neurons such that PTP1B attenuates leptin activity while TCPTP decreases insulin signaling *via* dephosphorylation of JAK2 tyrosine kinase and IR. However, in NPY/AgRP neurons, TCPTP decreases only insulin signaling (129–131). Furthermore, deletion of both phosphatases in POMC neurons, or of TCPTP in NPY/AgRP neurons, from obese mice prevents DIO in animals fed a high-fat diet, increases energy expenditure, and enhances leptin and insulin signaling to promote weight loss (129, 130).

By contrast, ghrelin, acts as an orexigenic peptide and its circulating levels increase in response to negative energy balance. Within the ARC, ghrelin, the orexigenic peptide hormone, plays a crucial role in regulating energy intake and energy expenditure. To promote food intake, ghrelin acts on its growth hormone secretagogue receptor (GHSR), which is highly expressed in the ARC and is located near the median eminence, a site known to allow the swift access of circulating ghrelin (132). A growing body of evidence shows that peripheral administration of ghrelin selectively increases *c-Fos* expression in the ARC, and ghrelin fails to stimulate food consumption in ARC-ablated rats (133–135). Ghrelin regulates food intake and energy balance by stimulating the NPY/AgRP neurons to elicit an inward current coupled with depolarization and an increase in firing rate, and mice lacking NPY- and AgRP-expression are insensitive to the exogenous administration of ghrelin (35, 136). Similarly, ghrelin also increases NPY and AgRP mRNA expression (137, 138). The ghrelin-induced depolarization of NPY/AgRP neurons can occur directly through activation of postsynaptic sulfonylurea receptor 1/transient receptor potential melastatin 4 receptors as well as indirectly given that tetrodotoxin did not completely block the depolarization and decrease in input resistance caused by the peptide (139). The ghrelin-induced activation of NPY neurons causes an increase in inhibitory postsynaptic currents in POMC neurons, and the GABA<sub>A</sub> receptor-mediated inhibition of POMC neurons is secondary to the excitatory effect on NPY/AgRP neurons – indicating that ghrelin is most likely stimulating

the release of GABA from NPY neurons to induce this hyperpolarization (140). Indeed, the inhibition of both NPY Y1 and GABA<sub>A</sub> receptors reverses the hyperpolarization of POMC neurons – suggesting that ghrelin stimulating the release of both NPY and GABA from NPY/AgRP/GABA nerve terminals (140). This increase in the firing of NPY/AgRP neurons enhances GABAergic input onto POMC neurons such that deletion of the vesicular GABA transporter in AgRP-cre mice blunts the ghrelin's hyperphagic effect (68). This is an indicative that presynaptic GABA release from NPY/AgRP neurons is an essential mediator of ghrelin's effect on food intake. Moreover, ghrelin enhances NPY's feeding stimulant action and NPY's effects on respiratory quotient (141). The fasting-induced increase in circulating ghrelin concentrations (142) is consistent with the increase in spontaneous firing rate in NPY/AgRP neurons from food-deprived mice compared to those from *ad libitum*-fed animals (143). Thus, either peripherally originated ghrelin or ghrelin neurons in the periventricular hypothalamus or ARC that send synaptic inputs onto various ARC neurons can regulate the activity of these cells by controlling the release of neuropeptides and inhibitory amino acid neurotransmitter (140, 144). Besides the ARC, the orexigenic effect of ghrelin may also be attributed to its actions in the PVN. Intranuclear administration of ghrelin into the PVN, where ghrelin GHSR-expressing cells are found (145), promotes appetite (146). Interestingly, it has been reported that blockade of cannabinoid CB1 receptors in the PVN not only stimulates fasting-induced hyperphagia but also increases ghrelin's effect in *ad libitum*-fed rats (147). On the other hand, the orexigenic effects of both cannabinoids and ghrelin appear to involve the activation of AMP-activated protein kinase (AMPK) (148, 149). Lastly, it has been recently discovered that liver-expressed antimicrobial peptide-2 functions as an endogenous antagonist of the GHSR (150).

AMPK is known as a central regulator of lipid metabolism and of energy homeostasis. AMPK is a serine/threonine kinase comprising of two alternative  $\alpha$  catalytic subunits ( $\alpha 1$  and  $\alpha 2$ ) as well as two regulatory  $\beta$  and  $\gamma$  subunits (151). Activation of AMPK is dependent on the cellular AMP : ATP ratio such that increasing in AMP level while decreasing in ATP level stimulates its activity (151, 152). According to Yan and colleagues, AMPK activity is regulated by direct allosteric activation and by activation loop phosphorylation, in which, ATP inhibits, and AMP triggers, AMPK in these mechanisms (152). As indicated, the orexigenic effects of ghrelin and cannabinoids are mediated by the activation of AMPK. There exists a ghrelin-cannabinoid interaction responsible for this, such that ghrelin requires an intact endocannabinoid system to promote food intake and fails to induce the same effect in CB1 knock-out mice (153). Furthermore, the AMPK-stimulating effect of cannabinoids was shown to be CB-1 dependent, which was blocked by rimonabant but had no effect in CB-1 knock-out animals (153). Taken together, the GHSR-mediated decrease in excitatory input onto parvocellular neurons in the PVN is mediated by the activation of AMPK as well as the increased production of endocannabinoids *via* subsequent activation of

CB1 receptor (153, 154). Leptin and insulin also modulate the activity of energy-sensing AMPK, such that they inhibit both AMPK and its downstream targets in the hypothalamus (155, 156). Leptin does this by phosphorylation on serine491 of the  $\alpha 2$ AMPK subunit, which was found to be crucial for leptin's effects on food intake and body weight (157). On the other hand, insulin does this by inducing direct phosphorylation *via* Akt (protein kinase B) on serine485 of the  $\alpha 1$ AMPK subunit, which was shown to inhibit threonine172 phosphorylation and thus promote cell survival and proliferation (158).

As mentioned above,  $K_{ATP}$  channels play a crucial role in the regulation of energy and glucose homeostasis (159, 160). They are present in most cell types, and are critical in regulating the membrane potential of neurons (161). The electrochemical gradient for  $K^+$  is such that its equilibrium potential is considerably more negative than the resting membrane potential of most neurons; therefore, the opening of  $K^+$  channels induces outward currents that result in  $K^+$  efflux and the hyperpolarization of the cell (160). Because  $K^+$  channels have a major role in setting the resting membrane potential, reducing action potential duration and decreasing firing rate, this indicates that these channels play a profound role in suppressing cell excitability (161).  $K_{ATP}$  (aka. Kir6) channels belong to a subfamily of the weak inward rectifier  $K^+$  channels that are classified into Kir6.1 and Kir6.2 subtypes (162). While Kir6.1 genes are mainly expressed in the mitochondria (163, 164), Kir6.2 subunits are involved in most functional  $K_{ATP}$  channels that are present in pancreatic  $\beta$  cells, cardiac muscle cells, smooth muscle cells, and all brain regions (162, 165, 166). These channels are negatively gated by ATP. For instance, a decrease in the ratio of ATP/ADP opens cardiac  $K_{ATP}$  channels and results in hyperpolarization, which stabilizes cardiac myocytes (162, 165). In contrast, an increase in the ratio of ATP/ADP in hyperglycemic conditions closes  $K_{ATP}$  channels of pancreatic  $\beta$  cells and leads to depolarization and insulin secretion (162). The antagonism of  $K_{ATP}$  channels can also be induced by tolbutamide, a  $K_{ATP}$  channel blocker that binds preferentially to the regulatory sulfonylurea receptor subunits SUR1 (165).

## THE HEDONIC ENERGY BALANCE CIRCUITRY AND THE MESOLIMBIC DA PATHWAY

While the homeostatic-hypothalamic circuitry regulates energy balance based on physiological needs, the hedonic circuit modulates energy intake and expenditure based on food reward. A growing body of literature suggests that both the consumption of palatable foods and substance abuse converge on a shared pathway within the limbic system in humans and rodents alike to mediate motivated behaviors (167–169). This indicates that the consumption of palatable food involves the  $A_{10}$  neurons that originate from the VTA and project to the NAc, hippocampus, prefrontal cortex, and amygdala (37–39). The dopamine (DA) neurons were first discovered ~50 years ago and were classified anatomically based on the clustering of

somata into three areas: the retrorubral area ( $A_8$ ), the substantia nigra pars compacta (SNc,  $A_9$ ), and the ventral tegmental area (VTA,  $A_{10}$ ) (170–172). These neurons share the rate limiting enzyme tyrosine hydroxylase and dopa decarboxylase, which catalyzes an irreversible decarboxylation reaction to produce dopamine (173). The VTA is made up predominantly of three types of neurons including GABAergic, glutamatergic and  $A_{10}$  DA cells. Beier and colleagues employed viral trans-synaptic tracing to demonstrate that the GABAergic and  $A_{10}$  DA neurons receive direct inputs from similar brain regions; the VTA GABAergic neurons, however, receive inputs mostly from the central amygdala (a region regulates emotions such as fear and aggression) and the anterior cortex (174). Consistent with this finding, stimulation of VTA GABAergic neurons induces place aversion and disruption in food consumption *via* the inhibition of DA activity; whereas, their inactivation promotes reward by enhancing the  $A_{10}$  DA signaling (175–177). In contrast, activation of DA neurons within the NAc promotes reward seeking behavior (178–181). In addition,  $A_{10}$  DA neurons have been shown to express vesicular glutamate transporters 2 (VGlut2), which reportedly enhances DA storage, neuronal growth and survival, the density of DA innervation of the NAc, as well as promotes the corelease of DA and glutamate, the latter of which induces a fast excitatory postsynaptic current (182–185). The knockout of VGlut2 gene, in contrast, reverses all the effects by abolishing glutamate release from  $A_{10}$  DA neurons and reducing the excitatory signal to the NAc (184, 185). In addition, local superfusion of glutamate at the VTA not only selectively activates  $A_{10}$  DA neurons but also enhances the excitatory synaptic signaling, whereas the superfusion of quinpirole, a D2 DA receptor agonist, increases the activation threshold of DA neurons and inhibits the excitatory synaptic event (182). This may be indicative that glutamatergic co-transmission plays a crucial role in conveying signals related to incentive salience. It is indeed the case as the activation of glutamatergic fibers in the anterior NAc shell causes a temporary cessation of feeding and promotes reward-seeking behavior (186).

As stated above, the  $A_{10}$  DA neurons receives direct input from other hypothalamic areas. For instance, it has been shown that a subpopulation of ARC POMC neurons project to the VTA to inhibit  $A_{10}$  DA neurons and induce hypophagia as a result (187). The local stimulation of VTA GABAergic neurons disrupts food intake and induces place aversion (175, 176), whereas photoactivation of LH GABAergic neurons projecting to the VTA instigates the opposite effect. This is because photostimulation of LH GABAergic neurons-VTA projection inhibits local VTA GABAergic neurons, increases DA release, and promotes social interaction and approaching, whereas photoactivation of VTA-projecting LH glutamatergic neurons reduces DA release and promotes avoidance (188). In addition, they noticed that the inhibition of LH GABAergic-VTA projection in food-restricted suppresses behavioral responses illustrated by significantly decreasing the time spent feeding and investigating the objects (188); however, the activation of LH GABAergic-VTA projection promotes feeding in sated mice (189). Within the LH, the two neuropeptides, orexin and MCH,

have also been studied extensively to better understand how they modulate feeding and reward seeking behavior in the VTA. While both peptides project widely throughout the brain, orexin-expressing LH neurons also project to the A<sub>10</sub> DA neurons to promote intake, but MCH-expressing LH neurons do not. For instance, orexin signals emanating from the LH to the VTA to promote neuronal activity by enhancing excitation *via* orexin receptors in the VTA and triggering DA neurons and thus promoting food intake (190, 191). On the other hand, MCH-expressing LH neurons project to the NAc to elevate food intake and reward reinforcement (168, 192, 193). Since neuropeptides from the LH project to the VTA and NAc to regulate energy balance, it is indicative that there must be a neural circuit conveying information from the homeostatic to the hedonic energy balance circuitry.

Interestingly, leptin and its receptors have been shown to modulate these two energy balance circuitries. Activation of VTA LepRb neurons decreases motivation for food reward and food intake in food-restricted mice (194), whereas the activation of VTA-projecting LH LepRb neurons, which form inhibitory synapses with non-DA VTA neurons and promote motivation for food intake (195). In addition, optogenetic activation of LH LepRb neuron-VTA projections drives appetitive learning while not affecting consummatory behaviors *in vivo* (196). Taken together, the neuropeptides and LepRb neurons in the LH can modulate energy balance within the hypothalamic-VTA-NAc circuits.

Hedonic eating is linked to the A<sub>10</sub> system, which is known for its regulation of reward-related behaviors (197, 198) and its role in binge eating episodes (199, 200). Berridge and colleagues were one of the first teams to distinguish the role of A<sub>10</sub> DA neurons in hedonic feeding by terming them as reward 'liking' and reward 'wanting' (201, 202). While 'liking' is associated with the hedonic pleasure triggered by a rewarding stimulus, such as highly palatable food, 'wanting' is related to incentive salience and is a mesolimbic-induced event that enhances reward-seeking (201, 202). In addition, 'liking' has been measured by observing changes in facial expressions provoked by taste stimulus in which sweet tastes are associated with lip smacking while bitter tastes showed a combination of face expressions like moving the brows, nose and face (203). In contrast, 'wanting' is established *via* neural interactions between the NAc, VTA, and amygdala, which is driven by both physiological state and a reward cue (201, 202). Though 'wanting' and 'liking' occur simultaneously, they can be psychologically and neurally distinguishable from one another and both are needed to achieve a full reward (202).

Pharmacological and genetic studies demonstrate that peripheral hormones such as leptin, insulin and ghrelin can act on the A<sub>10</sub> DA systems to control food intake. For instance, leptin is involved in negative affective encoding within the DA reward system to regulate hedonic feeding behavior and energy balance. Several reports indicate that administration of leptin into the VTA decreases feeding, while adeno-associated virus (AAV)-mediated LepRb knockdown in the VTA leads to an increase in both food intake and the sensitivity to palatable foods (204, 205). Leptin does this by reducing the firing of DA neurons through the activation of an intracellular JAK/STAT pathway

(204, 205). Like leptin, acute microinjection of insulin in the VTA induces long-term depression to suppress excitatory synaptic transmission onto A<sub>10</sub> DA neurons and reduces food consumption (206–208). Insulin does this by suppressing alpha-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA)-mediated excitatory post synaptic currents onto A<sub>10</sub> DA neurons as well as inducing the activation of cannabinoid CB1 receptors, that in turn, inhibiting glutamate release (207). On the other hand, ghrelin binds to its GHSR to modulate DA release in the NAc and regulate hedonic feeding in a dose-dependent manner (209, 210). It has been reported that direct administration of ghrelin into the VTA or NAc triggers hedonic feeding. Ghrelin does this by triggering the cannabinoid CB1 signaling in VTA neurons to regulate release of GABA and glutamate, as well as increasing DA neuronal activity and turnover in a GHSR-dependent manner *via* the activation of Gq/phospholipase C (PLC)/diacylglycerol (DAG) protein signaling pathway and Gs/cyclic adenosine monophosphate (cAMP)/cAMP-response element binding protein (CREB) pathway (149, 209–211). Moreover, while the neuropeptide nociceptin/orphanin FQ (N/OFQ) is largely considered orexigenic through its actions within the homeostatic energy balance circuitry (212), it inhibits A<sub>10</sub> DA neurons and attenuates binge feeding behavior when administered into the VTA (87). These findings reinforce the notion that A<sub>10</sub> DA neurons are an important neural substrate in the regulation of incentive salience, motivated behaviors and hedonic feeding.

## SEX DIFFERENCES IN, AND ACTIVATIONAL EFFECTS OF GONADAL HORMONES ON, THE REGULATION OF ENERGY BALANCE

Sex differences in the context of energy homeostasis have been studied extensively. Some reports have shown that men have a higher prevalence of obesity in early adulthood than their age-matched female counterparts, but this gender difference tapers off as reproductive senescence is approached (213, 214). Other studies have reported that women exhibit a higher chance of developing eating disorders and extreme obesity (215, 216). Sex differences exist in the regulation of energy balance as men and women not only crave different kinds of food but also show diverse changes under the condition of negative energy balance. For instance, previous studies reported that women experience more frequent episodes of state craving (217–219) while men report 15.6% fewer cravings for sugar-sweetened drinks (219, 220). Compared to men, women were reported to exhibit greater neural reactivity in the orbitofrontal cortex and insula in response to highly palatable food under food deprivation (221). This is congruent with other lines of evidence depicting that women have a reduced ability to control food desire, higher cortical and limbic activation when presented with visual, olfactory, gustatory cues, and increased susceptibility to episodes of food-craving compared to men (222–225). Sex is also thought to be one of the main determinants for susceptibility



to food-related disorders, including binge eating disorder (226, 227). Indeed, binge-prone female rats endured high levels of foot shock for access to Oreo cookies in comparison to males, confirming the ‘continued use despite negative consequences’ criteria for substance dependence (228). Similarly, the N/OFQ-induced suppression of binge feeding is greater in females than in males, and more robust in obese subjects than in lean ones (87). Consistent with these findings, Yu and co-workers assessed sex differences using self-reported of the Yale Food Addiction Scale and Eating Attitude Test, and they reported that female college students were 3 times more likely to exhibit disordered eating behaviors that was positively associated with food addiction regardless of weight status (229).

The role of sex hormones, especially the circulating estrogen levels, play in an important role in feeding regulation as feeding decreases during the estrous stage when serum estrogen levels are at the highest (230). Consistent with this result, the administration of estrogen, either centrally or peripherally, greatly reduces food intake and decreases body weight (230–235).  $17\beta$ -estradiol ( $E_2$ ) is the predominant form of estrogen in the non-pregnant state.  $E_2$  diffuses through biological membranes and interact with two well-known estrogen receptors (ER):  $ER\alpha$  and  $ER\beta$ .  $ER\alpha$  is highly expressed in the ARC and the VMN, whereas  $ER\beta$  is abundantly expressed in other regions of the brain but scarcely expressed in the ARC (236). It has been shown that  $ER\alpha$  is involved in  $E_2$ -mediated effects on body weight and food intake, as mice with  $ER\alpha$  deficiency have increased body weight and heightened food consumption in both male and female mice (237). Estrogenic actions are mediated by two different cellular responses; one is characterized by changes in gene transcription when estrogen binds to nuclear ERs (aka.  $ER\alpha$  and  $ER\beta$ ), and the other involves rapid signaling initiated by  $E_2$  binding to membrane-bound  $ER\alpha$ ,  $ER\beta$  or the Gq-coupled membrane ER (Gq-mER), which activates second messengers signaling molecules such as PLC and protein kinase A (PKA) (238–240). Gq-mER has been shown to induce opposite effects on POMC and NPY/AgRP neurons. For instance, the activation of Gq-mER by  $E_2$  or the nonsteroidal compound STX uncouples  $\mu$ -opioid, CB1, nociceptin opioid and GABA<sub>B</sub> receptors from GIRK channels in POMC neurons; resulting in an increase in membrane excitability illustrated by diminished outward currents and hyperpolarizations in these cells (240–243). In contrast, the activation of Gq-mER by  $E_2$  or STX enhances the ability of GABA<sub>B</sub> receptors to activate GIRK channels in NPY/AgRP neurons thus eliciting robust but reversible inhibitory outward currents (244). Interestingly, there is evidence to suggest that kappa opioids are involved in the weight gain associated with the hypoestrogenic state (245). Together, these findings confirm that the estrogenic actions mediated *via* ERs and Gq-mER play a crucial role in regulating energy homeostasis

As mentioned,  $ER\alpha$  is abundantly expressed in the ARC, which houses both POMC and NPY/AgRP neurons. A growing body of evidence has reported that the effects of  $E_2$  on feeding largely occur through multifaceted actions on synapses involving these cells (238, 244, 246, 247), while  $E_2$  in the VMN regulates

brown adipose tissue (BAT) thermogenesis (246, 248, 249). In addition, central administration of  $E_2$  reduces food intake in controls but not in AgRP knock-out mice (250). Furthermore,  $E_2$  dampens the refeeding response and suppresses c-Fos activation in NPY/AgRP neurons of fasted female mice (250) – suggesting that estrogen may regulate NPY/AgRP neurons indirectly *via* presynaptic neurons that express  $ER\alpha$ .  $E_2$  might also do this by influencing other peripheral hormones, as  $E_2$  was reported to inhibit ghrelin’s orexigenic effect (251); ghrelin, in turn, regulates food intake and energy balance in large part through its effects on NPY/AgRP neurons.  $E_2$  has been shown to affect the M-current flowing through KCNQ channels, which is a voltage- and time-dependent, non-inactivating outward  $K^+$  current that is often targeted by G-protein-coupled receptors (252). The KCNQ 2, 3 and 5 subunits are abundantly expressed in the NPY/AgRP and POMC neurons. Fasting attenuates M-current and increases the excitability in NPY neurons by reducing the mRNA expression of KCNQ 2 and 3 subunits, whereas  $E_2$  escalates M-current and reduces the excitability of NPY neurons to decrease food intake by increasing the expression of KCNQ5 while not affecting KCNQ2 and KCNQ3 subunits (252). In addition, targeted knockout of KCNQ3 has been shown to reduce M-current in NPY neurons which correlated to an increase in body weight of high-fat diet (HFD)-fed mice but did not affect food consumption (253). In contrast, the inhibition of M-current has been shown to enhance the activity of POMC neurons in *ad libitum* chow-fed mice demonstrated by the reduction of food intake and the control of glucose homeostasis (254, 255). The Kisspeptin/Neurokinin B/Dynorphin (KNDy) neurons in the ARC also plays an important role in controlling of energy balance as optogenetic stimulation of the kisspeptin neurons directly excites POMC neurons and induces a depolarization (256, 257). In congruence with these findings,  $E_2$  has been shown to increase the ARC KNDy neuronal activity *via* ghrelin-induced inhibition of the M-current in female mice (258), and also enhances glutamate release from KNDy neurons that excites POMC neurons and inhibits NPY/AgRP neurons *via* subsequent activation of metabotropic group I and group II/III receptors, respectively (259). This, in turn, likely contributes to the anorexigenic actions of the steroid. Finally, ERs are also found within the VTA, as intra-VTA injection of  $E_2$  significantly reduced sucrose-seeking behaviors within an hour after injection (260).

In contrast to estrogen, testosterone in males rapidly increases food intake caused by the activation of cannabinoid CB1 receptors that leads to the decrease in glutamatergic input onto POMC neurons (261, 262). Testosterone induces the increase in endocannabinoid tone *via* the activation of AMPK, which leads to suppression of glutamate release at VMN SF-1/ARC POMC synapses *via* the upregulation of DAG lipase- $\alpha$  (261, 262). Interestingly, these effects are intensified in obese males due to reduced PI3K signaling in the ARC (49, 128). In addition, the appetite-stimulating effect of N/OFQ, as well as its ability to inhibit POMC neurons *via* activation of GIRK channels and presynaptically inhibit glutamatergic input onto these cells, is greater in males than their female counterparts (263).



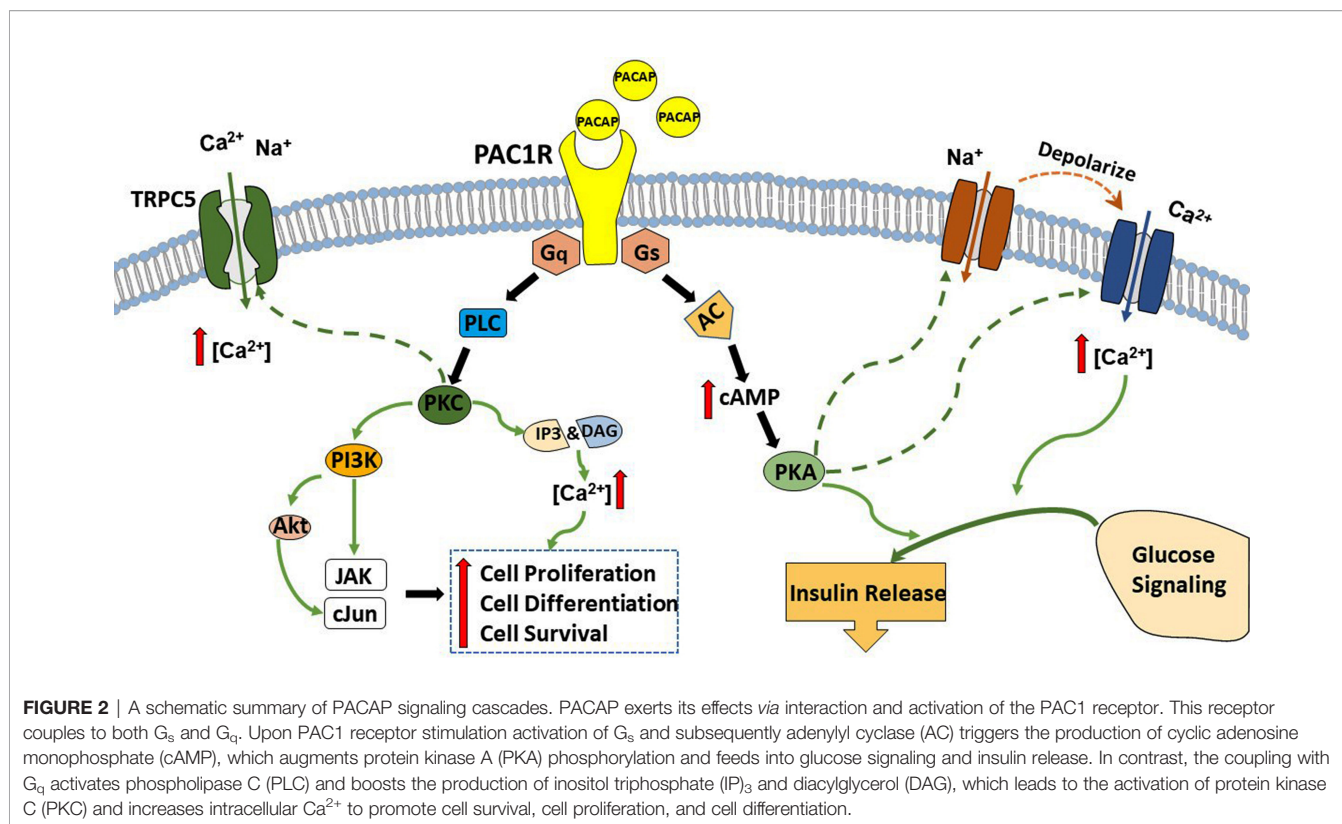
## DYNAMIC AND PLEIOTROPIC INVOLVEMENT OF PACAP IN THE REGULATION OF HOMEOSTATIC AND HEDONIC ENERGY BALANCE CIRCUITS

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated in 1989 from ovine hypothalamus (264). It has since been revealed that PACAP is highly conserved among vertebrates in terms of its amino acid sequence, which remained almost unchanged during an evolutionary period of ~700 million years (265–267); indicating its critical role in a number of different physiological responses (266, 268, 269). PACAP presents in two isoforms: a neuropeptide with 38 amino acid residues named PACAP<sub>1-38</sub> or a C-terminally truncated version with 27 residues called PACAP<sub>1-27</sub> as well as PACAP-related peptides (266, 267). PACAP exhibits high homology to vasoactive intestinal peptide (VIP), and is considered to belong to the VIP/secretin/growth hormone-releasing hormone/glucagon superfamily (268). PACAP is localized throughout the central and peripheral nervous system (CNS - PNS); thus, PACAP is equipped to act as a neurotransmitter, hormone, as well as a trophic factor in various cell types (268). In the rat brain, the highest concentration of PACAP can be found in the hypothalamus (270), which helps regulate many metabolic processes and activities of the autonomic nervous system. PACAP exerts a wide range of biological effects such as the regulation of hormone secretion, the control of neurotransmitter release, as well as neurotrophic and neuroendocrine actions (266, 269). There are two classes of PACAP receptors. The type I receptor is termed the PACAP-specific receptor (PAC1R), which has high binding affinity and selectivity for PACAP. On the other hand, type II receptors were originally called VIP-PACAP 1 and 2 receptors but were later reclassified as VPAC1R and VPAC2R, and they exhibit high binding affinity for both PACAP and VIP (267). The PACAP/PAC1 receptor system is distributed throughout the central nervous system, and is highly expressed in hypothalamic nuclei including the ARC, DMN, VMN, and PVN (266, 268, 271, 272).

There are two separate pathways that can be stimulated upon PAC1 receptor activation; the G protein coupled receptors Gs and Gq pathways. The insulinotropic effect of PACAP is triggered *via* the Gs pathway. PACAP binds to PAC1 receptor and activates Gs, which turns on adenylate cyclase (AC), leads to the production of cAMP, and activates PKA. PKA, in turn, opens Ca<sup>2+</sup> and Na<sup>+</sup> channels and augments glucose-induced insulin secretion (269). In addition, activation of the Gs by PACAP also leads to increase in firing and depolarize the membrane potential of magnocellular neurons in rats brain slices (273–275). PACAP also has a neuroprotective effect which is accomplished through the Gq pathway. Briefly, PACAP binds to the PAC1 receptor and stimulates Gq, which activates PLC. PLC generates inositol triphosphate (IP3) and DAG, which boosts protein kinase C (PKC) activity to stimulate cell proliferation, cell survival, cell differentiation, as well as activate TRPC5 channels (50, 268). Known for its pleiotropic effects, PACAP can act differentially in multiple brain regions to stimulate signaling factors and modulate several ionic currents. The signaling pathways of PACAP will be summarized in the schematic shown in **Figure 2**.

PACAP and VIP are known to exert both neurotrophic and neuroprotective effects (268). These effects are stimulated through the activation of different signaling pathways. Several reports suggest that PACAP can restore not only dopaminergic transmission but also hippocampal cholinergic transmission (276–278). This is indicative that PACAP may be a promising therapeutic agent to fight against cognitive decline in Parkinson's and Alzheimer's disease. Parkinson's disease is a neurodegenerative disorder that is second most common after Alzheimer's disease, and a cure has yet to be found. Along these lines, PACAP exhibits promising neuroprotective properties as it has been shown to rescue DA neurons from neurodegeneration and improve locomotor function in rat parkinsonian models (278).

As stated, the hypothalamic areas such as the ARC, VMN and PVN play an important role in the regulation of energy expenditure, energy intake, and thermogenesis. Furthermore, these regions highly express PACAP and its PAC1 receptor, suggesting that PACAP must also play a role in these processes (268, 270, 279). Studies that employed laser microdissection revealed a number of VMN-enriched genes; including that encoding for PACAP, the expression of which is diminished in SF-1 knock-out mice (280). PACAP is also found to colocalize with SF-1 within the VMN (281). Administration of PACAP has been shown to notably diminish food consumption either by intraperitoneal (IP) injection (282, 283), ICV (271, 284, 285), intra-PVN (272), intra-VMN (272, 286, 287), intra-VTA (288), or intra-ARC (50, 212) under *ad libitum*-fed, HFD-fed, and fasting conditions. Several studies found that PACAP given ICV or intra-VMN in CD-1 mice and Sprague-Dawley rats increases locomotor activity, raises in O<sub>2</sub> consumption and core body temperature (272, 281, 286) as well as elevates uncoupling protein 1 mRNA expression while reducing interscapular brown adipose tissue level (272). In contrast, intra-PVN administration of PACAP is without effect on locomotor activity and core body temperature (272). Interestingly, ICV injection of PACAP at higher doses reduced locomotion in C57BL/6J mice (289) – suggesting the difference in doses of this peptide may affect the final results when used in different species. Similarly, intra-ARC administration of PACAP significantly increases O<sub>2</sub> consumption and metabolic heat production while decreasing in respiratory exchange ratio (RER), and these effects are attenuated in DIO male mice (50). In ovariectomized (OVX) females, PACAP also significantly decreases cumulative energy intake, and increases O<sub>2</sub> consumption, CO<sub>2</sub> production, and metabolic heat production. The anorexigenic effect, but not the catabolic effects, of PACAP in OVX females are potentiated by E<sub>2</sub> (50). Consistent with this finding, McMillan and colleagues reported that PACAP is likely involved in the melanocortin system regulation of sympathetic nerve activity that triggers thermogenesis. Although treatment with melanotan II, a melanocortin receptor agonist, did not affect body weight, white adipose tissue and lipid content of brown adipose tissue, melanotan II partially rescued the impaired thermogenic capacity of PACAP deficient mice under cold-acclimated condition compared to the controls (290). The appetite-suppressing and metabolic-enhancing effects of PACAP administered into the mediobasal hypothalamus are most likely due PAC1 receptor-mediated excitation of POMC

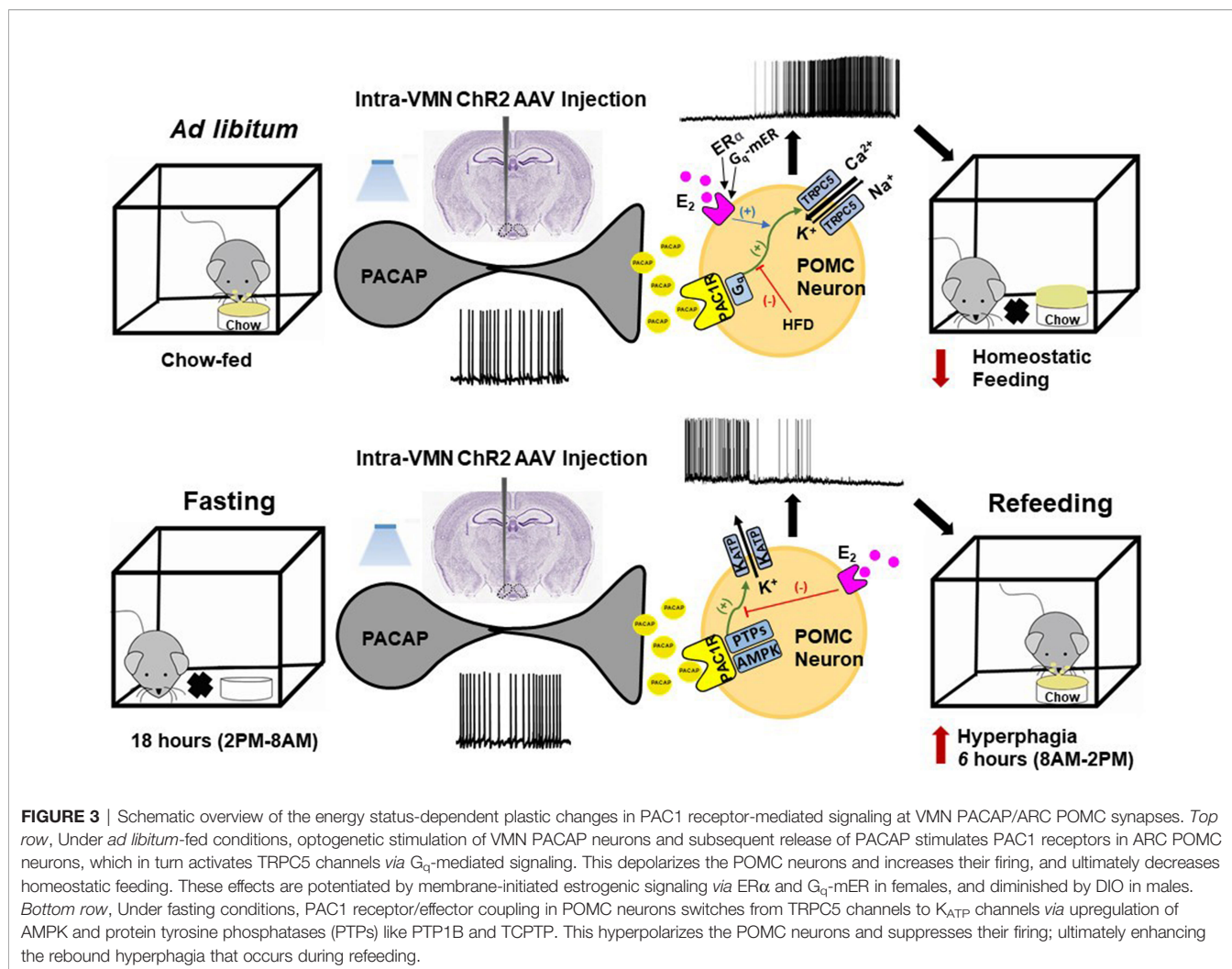


neurons *via* activation of TRPC5 channels. This effect can be attributed to a  $G_q$ -coupled signaling pathway involving PI3K, potentiated by  $E_2$  in females *via* activation of  $ER\alpha$  and  $G_q$ -mER, and diminished by DIO in males (50). Optogenetic stimulation of VMN PACAP neurons elicited the same excitatory effect in POMC neurons, which was again augmented by  $E_2$  in females (50). Similarly, IP injection of PACAP prior to the dark cycle decreases food intake in wildtype but not PAC1 receptor *fl/fl* mice and it does this in a dose-dependent manner (283). Furthermore, only the highest dose of PACAP [10  $\mu$ M] consistently and significantly suppressed bout duration, bout frequency, meal size, time spent in feeding, and rate of consumption during the first 8-hour after injection compared to the vehicle, while the lowest dose [100 nM] only significantly attenuated rate of consumption (283) – illustrating that these effects were achieved *via* the coupling of PACAP/PAC1 receptor system.

How PACAP exerts its anorexigenic or orexigenic effect to regulate the homeostatic energy balance depend not only on its site of injection but also on the anatomical location of particular PACAP neuronal populations as well as the ambient energy status, with *ad-libitum* (chow or HFD) feeding reflecting a more positive energy balance relative to fasting. Food-restriction (i.e., fasting) affects the levels of endogenous PACAP. Some have reported that PACAP expression is elevated in the hypothalamus of food-deprived rats as well as in chickens; suggesting that PACAP acts as a regulator of food intake (291). This was confirmed in mice in a sex differentiated manner such that hypothalamic PACAP levels increased to a greater extent in

male than in female mice with food deprivation, while they decreased to a greater extent in female than in male mice under water deprivation (292). The result enhances the idea that male and female PACAP systems react differently under food deprivation, which may explain their differences in the choice of food and amount of food consumption in response to starvation (221). Still, others report that positive energy states upregulate PACAP levels in the VMN of mice (281), food restriction reduces the PACAP mRNA expression in mice in both the hypothalamus and more specifically the VMN (271, 281). In addition, food-restricted mice show low levels of POMC and PACAP mRNA expression coupled with high NPY mRNA expression, and ICV injection of PACAP decrease energy intake after 30-min of refeeding (271, 281). Interestingly, and yes paradoxically, we found that fasting completely reversed the anorexigenic effect of an intra-ARC injection of PACAP by significantly increasing the cumulative energy intake of wildtype mice, which is greatly reduced by  $E_2$  in OVX female mice (212). This reversal was due to a switch in the polarity of the response in POMC neurons from excitatory to inhibitory caused by a shift in the coupling of PAC1 receptors from TRPC5 channels to  $K_{ATP}$  channels brought on by upregulated AMPK as well as PTP1B and TCPTP in these cells (212). These fasting-induced changes in PAC1 receptor-mediated signaling in ARC POMC neurons is highlighted in **Figure 3**.

In other studies, researchers found that PACAP-deficient mice showed reduction in food intake, carbohydrate intake, and NPY mRNA expression (293), suggesting that endogenous PACAP



affects carbohydrate consumption *via* the orexigenic effect of NPY/AgRP neurons as PACAP and its receptors including PAC1R and VPAC2R are highly expressed on NPY neurons (294). In congruence with these findings, PACAP neurons in the PVN provide excitatory input onto NPY/AgRP neurons that can drive homeostatic feeding (295). Additionally, PACAP knock-out mice show reduced nocturnal and daily food intake as compared to the controls, but diurnal intake was increased; indicating that food intake may be dependent on time of the day for these PACAP knock-out mice (296). In addition, PACAP knock-out mice exhibit increased POMC mRNA expression and decreased AgRP mRNA level (296), and AgRP levels were higher in the wildtype compared to PACAP knock-out mice. Furthermore, ICV administration of PACAP<sub>6-38</sub> lead to a reduction in food intake, body weight, and AgRP expression in wildtype mice, while no changes were observed in POMC expression (296). This is indicative that PACAP can act as either an orexigenic or anorexigenic neuropeptide in the hypothalamus, and PACAP is capable of regulating the release of the peptides  $\alpha$ -MSH and AgRP. As a neuropeptide that is intimately involved in energy homeostasis, PACAP may also be capable of regulating the release of other peptides like leptin, insulin, and

ghrelin. This was indeed the case under the fasting state and postprandially where insulin and leptin expression levels were reduced in PAC1 knock-out mice, while ghrelin levels were greatly elevated by overnight fasting and postprandially in PAC1 knock-out mice compared to the wildtype (283). Additionally, evidence suggests that PACAP may mediate the anorexigenic effect of leptin (297). As with POMC neurons, leptin depolarizes VMN SF-1/PACAP neurons and increases their firing rate (44). Leptin injection into the VMN reduced food intake compared to saline-treated animals and the effect was completely blunted by PACAP<sub>6-38</sub>; however, administration of PACAP<sub>6-38</sub> per se into the VMN showed no effect on either food consumption or body weight (297). This gives the indication that leptin may exert its effect *via* PAC1R activation in the VMN. Furthermore, they found not only that PACAP expression is colocalized with BDNF mRNA, but PAC1R expression was also co-expressed with BDNF and LepRb. With this information, they administered PACAP in the VMN and observed STAT3 phosphorylation as well as increased BDNF and suppressor of cytokine signaling 3 mRNA levels – just like with LepRb activation (297). Again, these effects of leptin and PACAP were blocked by PACAP<sub>6-38</sub>; suggesting that PACAP is downstream



of leptin and mediating leptin's effect on energy balance (296). Indeed, this may be how leptin activates the anorexigenic VMN PACAP/ARC POMC circuit under normophysiological conditions (50, 212). Taken together, PACAP is capable of exerting dynamically pleiotropic actions within the homeostatic energy balance circuitry that include mediating the anorexigenic actions of leptin and regulating the activity of POMC and NPY/AgRP neurons as well as other peptide systems such as  $\alpha$ -MSH, AgRP, and leptin.

The BNST plays a crucial role in regulating anxiety caused by long-term threats, and it is also important in mediating stress-induced anorexia and associated weight loss (298–300). The term “stress” refers to processes involving appraisal, perception, and response to stimuli or noxious events (301). Stress is manifested through two different pathways: the sympathetic adrenal medullary system, which releases catecholamines during periods of acute stress, and the hypothalamic-pituitary-adrenal (HPA) axis (302). The infusion of PACAP in the posterior BNST in both female and male Sprague-Dawley rats mimics weight loss induced by long-term stress exposure (300). Interestingly, the effect was not seen with PACAP administration ICV or in the anterior BNST – suggesting that PACAP exerts its effect only in the posterior BNST.

Given the role of PACAP in the posterior BNST, and the significance of the BNST in regulating food intake and the response to stress, this suggests that the PACAP/PAC1 receptor system may also be involved in stress-induced eating disorders. Prolonged stress increases glucocorticoid secretion, which can work synergistically with insulin to promote abdominal fat deposition, decrease HPA axis activity, and consequently affect energy homeostasis and eating behavior (303). It has been shown that chronically stressed rats tend to eat more under acute stress conditions and show a preference to consume palatable food (304, 305). Similarly, stress produces a greater intake of palatable food in overweight or obese individuals as compared to lean individuals (306, 307), and promotes food-seeking even in the absence of hunger and homeostatic need for calories (308). Stress-induced eating may contribute to the development of obesity (304), and may also adversely impact meal pattern and food preference (304). As mentioned above, stress increases the consumption of palatable food (304, 305), which might be an indicator of elevated DA release from A<sub>10</sub> DA neurons. Indeed, DA release in the dorsal striatum is enhanced in obese, binge eating disorder (BED) participants as compared to non-BED obese participants during exposure to food cues (309, 310). The hallmark of BED is the excess consumption of food (311), which could result in an elevated body mass index (BMI). In addition, BED is significantly and strongly associated with severe obesity (BMI > 40) (312, 313). Taken together, stress increases vulnerability to binge-eating behavior and promotes irregular eating patterns, and these effects may be intensified in overweight and obese individuals.

In addition to its critical functions within the homeostatic energy balance circuitry, PACAP can also regulate the consumption of palatable food *via* the hedonic energy balance circuitry. PACAP effects within the hedonic circuit were demonstrated when Hurley and coworkers microinjected

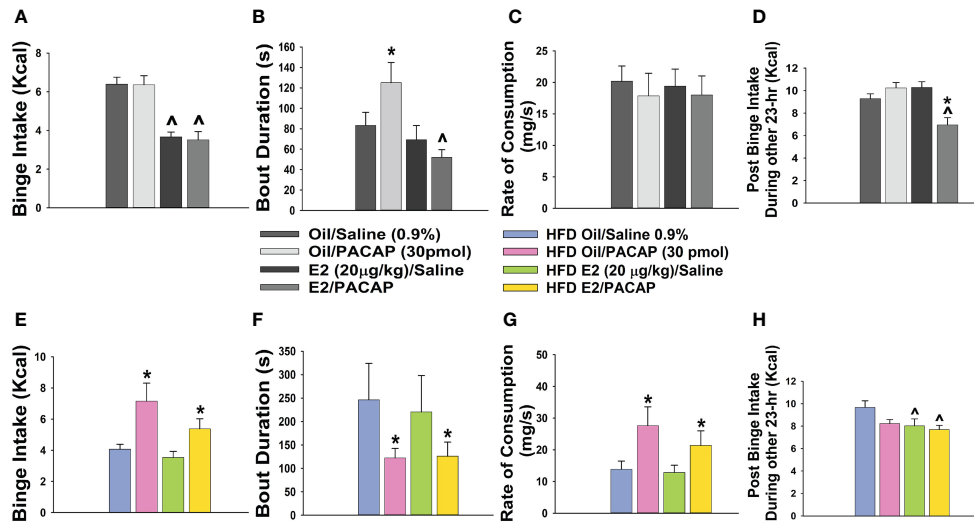
PACAP into the NAc, which then mimicked the actions of GABA agonists to reduce hedonic feeding as well as hedonic drive (287, 314). By contrast, while intra-VMN microinjection of PACAP replicates the AMPA-induced reduction in homeostatic feeding, it was without effect on hedonic feeding (287). Similarly, we have shown that PACAP administered into the VTA attenuates the binge-like consumption of palatable food (limited to one hour/day for five consecutive days) in lean male wildtype mice (288). This can be attributed to the PAC1 receptor-mediated activation of K<sub>ATP</sub> channels in A<sub>10</sub> DA neurons following bath application of PACAP, which results in membrane hyperpolarization and the cessation of firing. This inhibitory effect is mirrored by optogenetic stimulation of VTA-projecting VMN PACAP neurons (288). Surprisingly, intra-VTA PACAP is without effect on binge feeding behavior in either estradiol- or vehicle-treated OVX wildtype females (288) (**Figure 4A**). Estradiol did abrogate the PACAP-induced increase in bout duration (**Figure 4B**), and homeostatic feeding observed during the remaining 23 hours of the day was reduced in estradiol- and PACAP-treated OVX females (**Figure 4D**). However, neither E2 nor PACAP have any effect on rate of consumption (**Figure 4C**). Given the links between obesity and BED, we then investigated whether PACAP could suppress the rampant binge feeding observed in DIO OVX females (87). Unexpectedly, intra-VTA PACAP increased rather than decreased binge feeding in these animals (**Figure 4E**). This is associated with a decrease in bout duration (**Figure 4F**), and a sizable increase in the rate of consumption (**Figure 4G**). We followed this up with *in vitro* recordings in mesencephalic slices from lean, chow-fed and DIO, HFD-fed OVX TH-cre mice. We found that the PACAP-induced change in the excitability of A<sub>10</sub> DA neurons switched from predominantly inhibitory in recordings from lean, chow-fed animals to excitatory seen in the majority of cells from DIO, HFD-fed animals (**Figure 5**), and this is illustrated schematically in **Figure 6**.

## CONCLUDING REMARKS

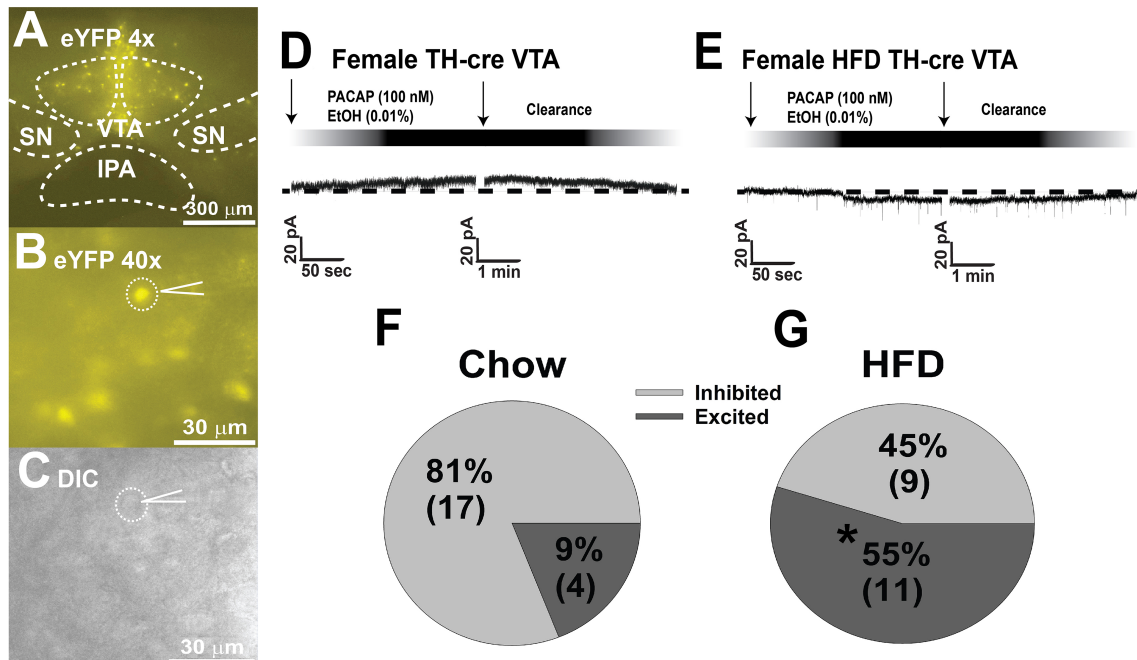
Extensive ongoing research for the past several decades has identified many of the major players comprising the circuits involved in homeostatic and hedonic energy balance regulation. We now understand that the components of the homeostatic energy balance circuits are deeply interconnected and ultimately converge at sites of integration within the PVN. We also know that peripheral hormones like leptin, insulin and ghrelin impart energy status-dependent signals to the neural substrates of these circuitries; changing their excitability in ways that profoundly impact appetitive behavior and satiety. More importantly, there is a growing appreciation for how connections between the hypothalamus and VTA as well as the NAc allow for cross-talk between the homeostatic and hedonic energy balance circuitries that coordinates nutrient- and palatability-based energy intake.

PACAP has emerged as another major player in the regulation of energy homeostasis. A considerable body of evidence accumulated over the past 30 years indicates that the PACAP/PAC1 receptor system exerts perplexingly pleiotropic actions in regulating both the homeostatic and hedonic energy

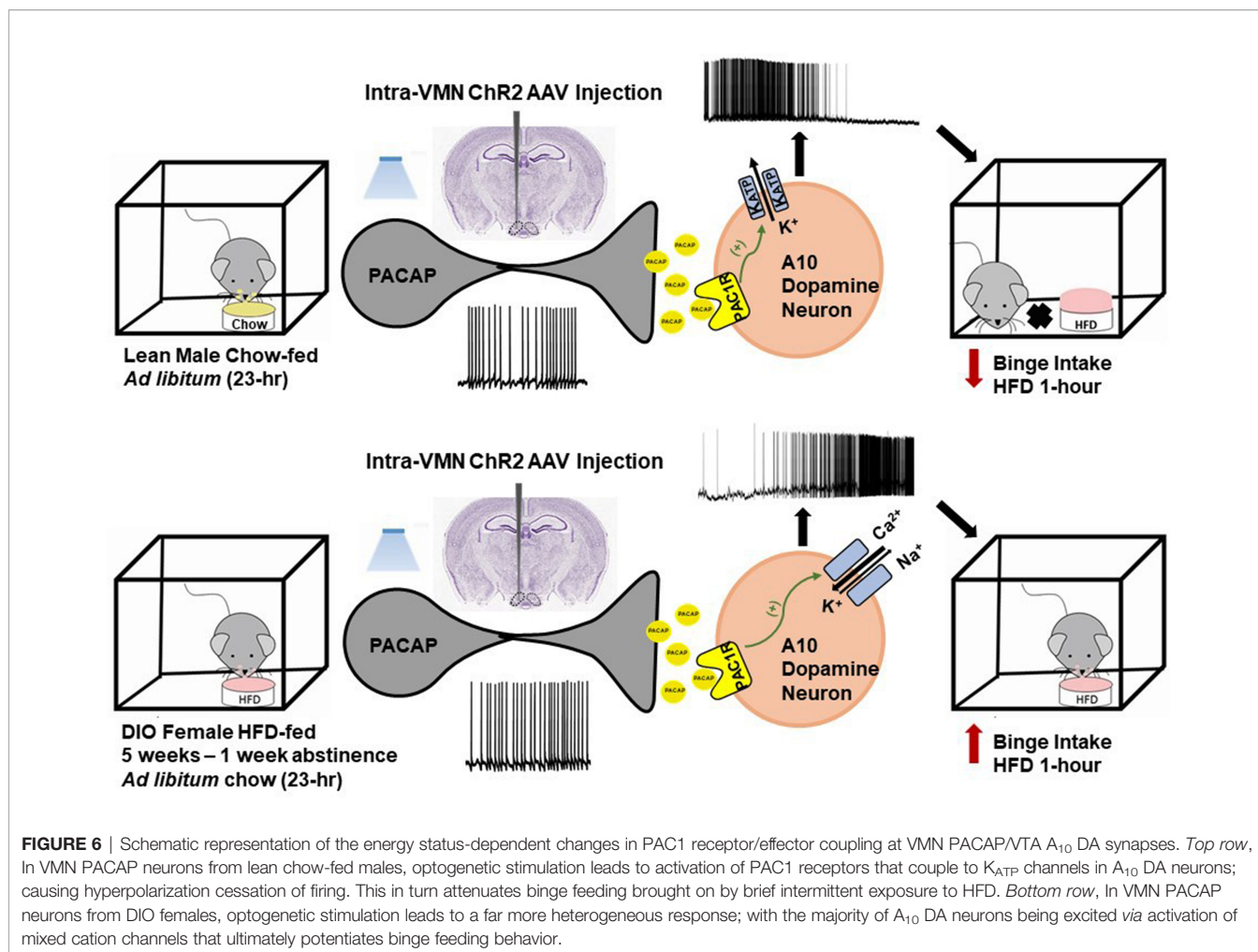




**FIGURE 4** | Dichotomous effects of intra-VTA PACAP on binge feeding in OVX, lean vs obese, sesame oil- vs EB-treated female mice. In lean chow-fed mice, EB (20 µg/kg; s.c.) but not PACAP (30 pmol) decreases binge intake (A), and significantly reduces bout duration in PACAP-treated animals (B). Neither E2 nor PACAP have any effect on rate of consumption (C). EB and PACAP work synergistically to decrease chow intake during the remaining 23 hours (D). In obese HFD-fed animals given a one-week respite prior to implementation of the binge paradigm, PACAP increases binge intake in both oil- and EB-treated females (E), which is associated with decreased bout duration (F) and increased rate of consumption (G). EB reduces homeostatic feeding during the remaining 23 hours (H). Bars represent means and lines 1 SEM. ^, p < 0.05; with respect to sesame oil; \*, p < 0.05; with respect to saline; repeated measures, multi-factorial ANOVA/LSD (A–H); n = 6 for all treatment groups.



**FIGURE 5** | DIO-induced alterations in the response profile of A<sub>10</sub> DA neurons from OVX female TH-cre mice to the postsynaptic effect of PACAP. (A, B) enhanced yellow fluorescent protein signal from A<sub>10</sub> DA neurons in VTA slices captured at 4X and 40X. (C) differential interference contrast image of the recorded A<sub>10</sub> DA soma seen in (B). (D, F) PACAP (100 nM; n = 17) produces a reversible outward current in the considerable majority (17/21; 81% vs males): of A<sub>10</sub> DA neurons from lean, chow-fed females, which is very similar to the percentage of PACAP-inhibited A10 DA neurons seen lean, chow-fed males (21/24; 87%). (E, G) Conversely, PACAP (n = 11) exerts a more heterogenous response in A<sub>10</sub> DA neurons from obese, HFD-fed females, with the majority (11/20; 55%) of them being an excitatory inward current. Arrows indicate where I/Vs were conducted. \*, p < 0.05; with respect to chow-fed controls, Chi-squared test.



balance circuitries. The anorexigenic and orexigenic effects of PACAP are dependent on site of injection, the anatomical location of the PACAP neuronal populations (i.e., VMN vs PVN), and strongly influenced by the ambient energy status not to mention the animal model and strain used in any given study. We have demonstrated that PACAP reduces homeostatic feeding and increases energy expenditure in lean, sated chow-fed animals, due largely to its excitatory actions on ARC POMC neurons. These appetite-suppressant and catabolic effects are diminished by DIO in males, whereas the anorexigenic (but not the metabolic) effect is potentiated by E<sub>2</sub> in OVX females, and completely reversed under negative energy balance upon fasting. On the other hand, we have shown that PACAP dampens hedonic binge feeding through its inhibitory actions on A<sub>10</sub> DA neurons in the VTA. This effect is sexually differentiated in that it occurs in lean, otherwise chow-fed males but not OVX females, and diet-dependent in that PACAP actually increases binge feeding in DIO OVX females.

Nevertheless, future research will be imperative to fully evaluate the paradoxical function of PACAP in regulating energy homeostasis not only under normophysiological conditions and positive energy (i.e., DIO) but also under

negative energy states (i.e., food-restriction). In addition, the underlying mechanism of how PACAP exerts its pleiotropic actions not only in the hypothalamic areas and other brain regions, but also in the context of sex differences will need to be further addressed. For example, how might ER- and PAC1 receptor-mediated signaling converge to modulate the ingestion of palatable food in DIO females? What forms of synaptic plasticity might be occurring that would allow projections of VMN PACAP neurons to potentially target other VTA neural substrates in DIO females? Lastly, what are the activational effects of testosterone in males that may contribute to sexually differentiated PAC1 receptor-mediated regulation of energy homeostasis? Only in this way will we unravel the mysteries and further advance our understanding of how PACAP and its receptor adaptively regulate energy balance in response to dynamic changes in energy status.

## AUTHOR CONTRIBUTIONS

NL, SS, and VM-P performed all metabolic studies. NL performed all electrophysiological recordings. NL and EW

created all figures and performed all statistical analyses. NL and EW generated the manuscript, while all authors edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

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## GLOSSARY

$\alpha$ -MSH	Alpha-melanocyte-stimulating hormone
A <sub>10</sub> DA neurons	Mesolimbic Dopamine neurons
AAV	Adeno-associated virus
AgRP	Agouti-related peptide
AMPA	alpha-amino-3-hydroxyl-5-methyl-4 -isoxazole
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of stria terminalis
CART	cocaine-and amphetamine-regulated transcript
DIO	Diet-induced obesity
DMN	Dorsomedial nucleus
DREADDs	Designer receptors exclusively activated by designer drugs
GABA	neurotransmitter gamma-aminobutyric acid
GHSR	growth hormone secretagogue receptor
GLP-1	Glucagon like peptide-1
Gq-mER	Gq-coupled membrane estrogen receptor
HFD	High-fat diet
ICV	Intracerebroventricular
IP	Intraperitoneal
IR	Insulin receptor
JAK/STAT	Janus kinase/signal-transducer- and activator-of-transcription
K <sub>ATP</sub>	ATP-gated potassium
LepRb	Leptin receptor
LH	Lateral hypothalamus
MCH	Melanin-concentrating hormone
MC4R	Melanocortin 4 receptor
NAc	Nucleus accumbens
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OVX	Ovariectomized
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PAC1R	PACAP-specific 1 receptor
PI3K	phosphatidylinositol-4,5-bisphosphate 3 kinase
POMC	proopiomelanocortin
PVN	Paraventricular nucleus
SF-1	Steroidogenic factor-1
TRPC5	Transient receptor potential cation 5
VMN	Ventromedial nucleus
VTA	Ventral Tegmental Area
VGlut2	Vesicular glutamate transporters 2