Hypothalamic Pituitary Adenylate Cyclase-Activating Polypeptide: Impact On Energy Homeostasis And Glutamate Signaling

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by

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ABSTRACT
HYPOTHALAMIC PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE: IMPACT ON ENERGY HOMEOSTASIS AND GLUTAMATE SIGNALING

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin-glucagon superfamily of peptide hormones, with homology to vasoactive intestinal polypeptide (VIP) and activity at both the VIP receptors and the PACAP specific PAC1 receptor (PAC1R). Abundantly expressed in the hypothalamus, PACAP was recently discovered to regulate energy balance when central injections produced hypophagia and increased metabolic rate. However, the neurocircuitry mediating these effects in the hypothalamus are poorly understood.

To characterize how hypothalamic PACAP signaling affects energy homeostasis we microinjected PACAP site-specifically into the hypothalamic paraventricular (PVN) and ventromedial nuclei (VMN) and examined feeding behavior and metabolism. PACAP injected into both areas significantly decreased food intake, while only VMN injections increased core body temperature and spontaneous locomotor activity. In addition, all responses resulting from hypothalamic PACAP administration were blocked by pretreatment with a PAC1R antagonist. Retrograde-labeling from the PVN or VMN identified PACAP afferents originating from the brainstem, amygdala, and hypothalamus that co-expressed PACAP mRNA. These projections to the PVN and VMN represent the first description of PACAP circuits regulating energy balance.

PACAP signaling is also important for modulating glutamate neurotransmission, however whether glutamatergic signaling is necessary for PACAP-induced hypophagia is unknown. Though PACAP-PAC1R signaling potentiates postsynaptic NMDA receptor activity, PACAP treatment was found to also augment the activity of the astrocytic cystine-glutamate antiporter, system x_c^-, in primary cortical cultures suggesting another possible means of glutamatergic modulation by PACAP. PACAP increased VMN system x_c^- mRNA expression *in vivo*, however, inhibition of system x_c^- activity did not attenuate PACAP-induced hypophagia. Conversely, NMDA receptor antagonism prior to PACAP administration in the VMN did block PACAP-mediated decreases in feeding, suggesting that PACAP neurotransmission in the VMN augments glutamate signaling by potentiating postsynaptic NMDA receptors.

The current findings suggest that PACAP signaling, from both hypothalamic and extrahypothalamic sites, potently regulates energy balance by decreasing food intake and increasing metabolism. Furthermore, the results of our studies involving PACAP-mediated modulation of glutamate neurotransmission indicate that PACAP affects glutamatergic signaling in multiple ways, however, modulation of NMDA receptor activity in the hypothalamus may be the primary mechanism for the regulation of food intake.

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CHAPTER I

INTRODUCTION

General Introduction

Body weight homeostasis is achieved through the balance of energy intake and energy expenditure. The brain maintains this balance by integrating a myriad of hormonal signals and environmental stimuli followed by appropriate behavioral, endocrine, and autonomic nervous system responses (Shin et al. 2009). Thus, aspects of energy regulation such as feeding behavior are the culmination of several neural systems that converge to produce a behavioral output that reflects the body's current energy status (e.g. hunger or satiety; Figure 1.1). However, this delicate balance can be overridden by a number of internal and external factors resulting in imbalances, which over extended periods of time result in serious pathologies such as eating disorders and obesity. In recent decades obesity has grown as a public health concern, as the prevalence has reached epidemic proportions around the globe (Caballero 2007).

Unfortunately, increased body size and negative body image are not the lone drawbacks of becoming overweight or obese, as both are major risk factors for co-morbid diseases including diabetes, cardiovascular disease, cancer, and stroke (Khaodhiar et al. 1999), which, not surprisingly, lead to higher healthcare costs and greater economic and societal burden (Lehnert et al. 2013). Therefore, continued research in the area of central mechanisms of body weight regulation is needed in order to counteract the startling trend of rising obesity rates by learning how imbalances in energy regulation can develop through interactions between pharmacological, environmental, and genetic mechanisms.

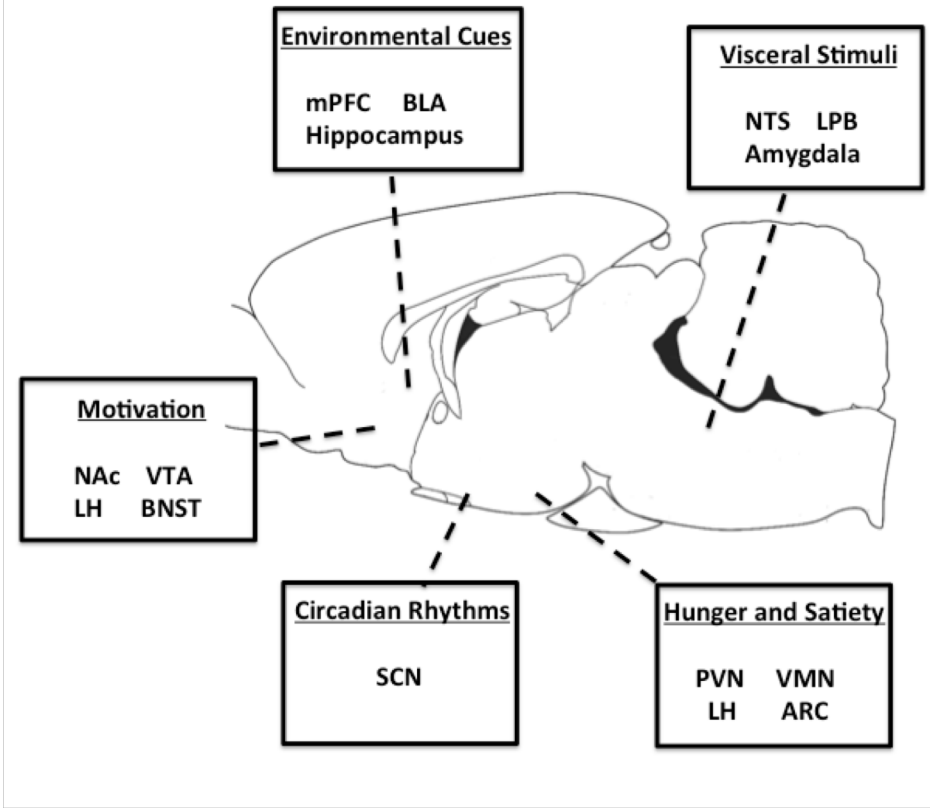

Figure 1.1. Central control of feeding behavior. Food intake is affected by multiple systems regulated by several brain structures throughout the brain. ARC, arcuate nuclei; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; LH, lateral hypothalamus; LPB, lateral parabrachial nuclei; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; NTS, nucleus tractus solitarius; PVN, paraventricular nuclei; SCN, suprachiasmatic nuclei; VMN, ventromedial nuclei; VTA, ventral tegmental area.

Hypothalamic regulation of feeding behavior

As the primary homeostatic regulator in the brain, the hypothalamus controls food intake through bidirectional neural connections between intra- and extrahypothalamic brain regions and detection of circulating hormones that signal either energy deficit or excess (Schwartz et al. 2000). Monitoring energy resources is a critical aspect of energy balance regulation as the hypothalamus sits proximal to the median eminence, a circumventricular organ that allows peripheral hormones to reach hypothalamic cell

groups. By monitoring the peripheral milieu hypothalamic neurons are stimulated or inhibited to affect feeding behavior in a manner that restores energy balance back to a set point (Williams and Elmquist 2012). Though there are countless peripheral signals that affect food intake, leptin, a potent anorectic hormone released from adipocytes (Ingalls et al. 1950; Zhang et al. 1994), and ghrelin, a hormone secreted from the gut to stimulate appetite (Kojima et al. 1999), appear to have the broadest impact on hypothalamic regulation of feeding behavior (Cummings 2006; Elmquist et al. 2005; Nogueiras et al. 2008; Vong et al. 2011; Wren et al. 2001).

Anatomically, the hypothalamus is comprised of several small nuclei, in very close proximity, to which each can be ascribed uniquely distinct functions. Of these nuclei, feeding behavior is generally coordinated by the mediobasal cell groups containing the dorsomedial nuclei (DMN), ventromedial nuclei (VMN) and arcuate nuclei (ARC), in addition to the lateral hypothalamus (LH) and paraventricular nuclei (PVN) that reside outside the mediobasal area (Figure 1.2). Within this collection of hypothalamic nuclei connected with feeding behavior, the VMN may be the most studied but also the most enigmatic. Coined the "satiety center" several decades ago (Kennedy 1950), the VMN were once the primary focus of hypothalamic feeding research (King 2006b). However, as experimental stimulation and lesioning techniques improved and allowed for more precise manipulations, the function of these nuclei became less clear (Gold 1973). Nevertheless, it was established that electrical stimulation of the VMN reduces food intake (Beltt and Keesey 1975; Ruffin and Nicolaidis 1999; Takaki et al. 1992) and both temporary inactivation or permanent lesions generate obesity (Avrith and Mogenson 1978; Brobeck et al. 1943; Choi and Dallman 1999). Despite the decades of

accumulating evidence, whether obesity from lesions to the VMN is the result of hyperphagia is continued to be debated (Cox and Powley 1981).

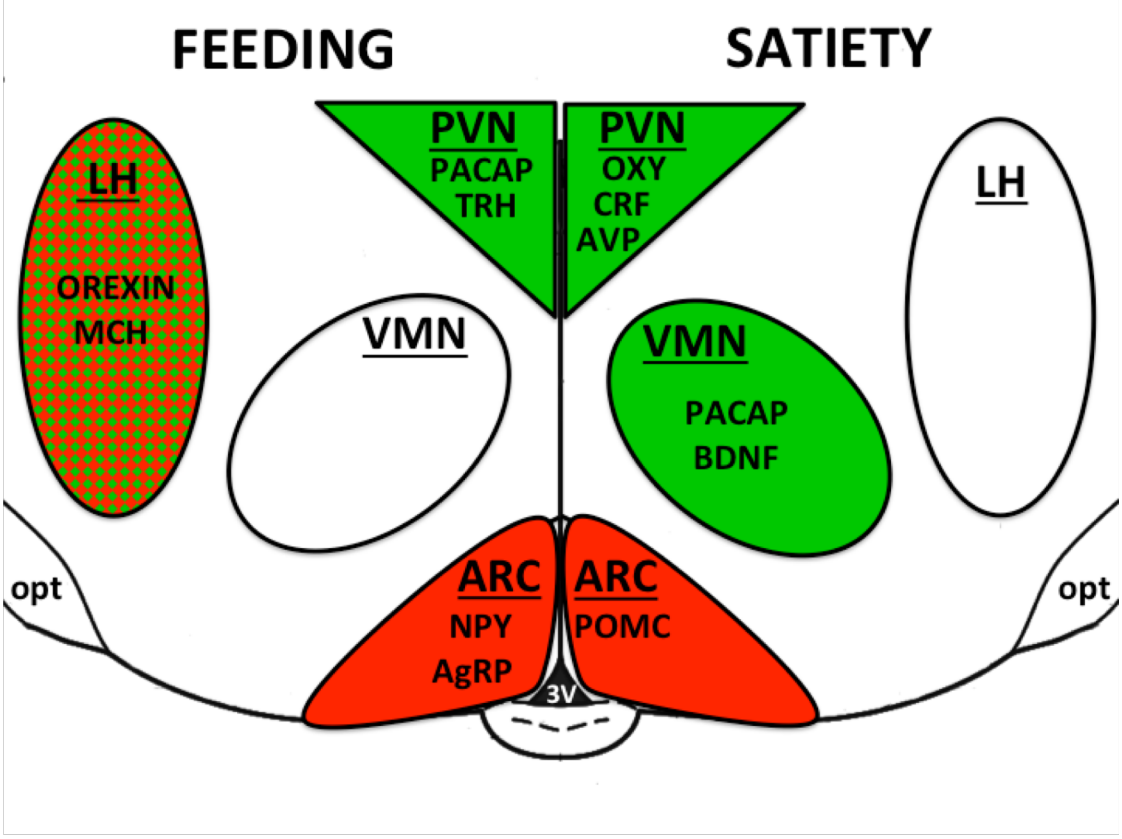

Figure 1.2. Hypothalamic nuclei controlling feeding behavior. The left depicts hypothalamic cell groups that promote feeding and the right highlights areas that promote satiety with known feeding neuropeptides expressed within each nuclei. Green = glutamatergic nuclei; Red = GABAergic nuclei; Green/Red pattern = heterogeneous cell population. ARC, arcuate; LH, lateral hypothalamus; PVN, paraventricular nuclei; VMN, ventromedial nuclei; opt, optic tract; 3V, third ventricle; AgRP, agout-related peptide; AVP, vasopression; BDNF, brain-derived neurotrophic factor; CRF, corticotropin-releasing factor; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OXY, oxytocin; PACAP, pituitary adenylate cyclase-activating polypeptide; POMC, pro-opiomelanocortin; TRH, thryrotropin-releasing hormone.

Although it is known that VMN neurons are almost completely glutamatergic in nature (Fu and van den Pol 2008; Tong et al. 2007; Ziegler et al. 2002), the hypothalamus as a whole is typically thought to regulate feeding behavior exclusively through neuropeptide signaling (van den Pol 2012). Despite the rapid discoveries of

neuropeptides involved in homeostatic regulation, few were ever found to originate in the VMN until recently. These peptides were pituitary adenylate cyclase-activating polypeptide (PACAP) (Segal et al. 2005) and brain-derived neurotrophic factor (BDNF) (Xu et al. 2003), neither of which was previously linked to feeding behavior. Since their detection in the VMN, however, both BDNF and PACAP, which is the focus of this thesis and discussed at length in later sections, have been shown to be critical for VMN control of feeding and metabolism. For instance, VMN/DMN-specific knockout of BDNF produces hyperphagia and obesity (Unger et al. 2007), whereas site-specific injections of BDNF into the VMN produce long-lasting hypophagia (Wang et al. 2007). The VMN also participate in sensing peripheral hormones important for the regulation of feeding and metabolism. Direct injections of leptin into the VMN produce profound hypophagia (Jacob et al. 1997), and VMN-specific deletion of leptin receptors results in obesity (Dhillon et al. 2006).

Just ventral to the VMN are the hypothalamic arcuate nuclei (ARC), containing an immensely heterogeneous population of neurons that both stimulate and inhibit food intake. Neuropeptide Y (NPY)/agouti-related polypeptide (AgRP)-expressing neurons of the ARC drive feeding behavior generally under conditions when energy stores are depleted such as during fasting (Levine and Morley 1984; Li et al. 1998; Liu et al. 2012a). However, acute and specific stimulation of these NPY/AgRP neurons in physiologically replete animals also produces robust feeding behavior suggesting these neurons may be part of a hard-wired motivational feeding circuit (Aponte et al. 2011; Atasoy et al. 2012; Betley et al. 2013; Krashes et al. 2011). By contrast, ARC neurons that express pro-opiomelanocortin (POMC), the precursor to the hypophagia-inducing α-

melanocyte-stimulating hormone (α-MSH), inhibit feeding and are significantly regulated by circulating levels of leptin (Balthasar et al. 2004; Zhan et al. 2013).

The LH and PVN also have considerable roles in regulating feeding behavior especially given their strong afferents from the VMN and ARC. The LH functions to stimulate feeding behavior through extensive stimulatory outputs to the motive circuit, which includes interactions between LH neurons, the ventral tegmental area, and the nucleus accumbens (Leinninger et al. 2009; Stratford and Kelley 1999). Once considered a center of feeding control, in contrast to the VMN, the LH was frequently referred to as the "hunger center" (Anand and Brobeck 1951). While the "dual center" hypothesis (LH = hunger vs. VMN = satiety) has long since fallen out of favor (Elmquist et al. 1999), it still holds that LH lesions produce physiological wasting (Bernardis et al. 1992). Later it was discovered that two unique neuropeptide populations exist within the LH designated melanin-concentrating hormone (MCH) and orexin/hypocretin. Central administration of either of these neuropeptides stimulate food intake consistent with the role of the LH in feeding behavior. Finally, the PVN represents a hub for most intrahypothalamic feeding circuits with extensive innervation coming from both ARC NPY/AgRP and POMC neurons (Atasoy et al. 2012; Betley et al. 2013; Kim et al. 2000). Composed of a very heterogeneous population of neurons itself, lesions to the PVN produce a decidedly hyperphagic obesity (Choi and Dallman 1999; Leibowitz et al. 1981; Tokunaga et al. 1986), perhaps due to the extensive connections with other hypothalamic nuclei, the brainstem, and autonomic ganglia, as well as significant control over pituitary secretions (Swanson and Kuypers 1980; Swanson and Sawchenko 1980; Swanson et al. 1980).

However, the downstream mediators of PVN regulated feeding are still being determined (Atasoy et al. 2012; Krashes et al. 2014).

Hypothalamic regulation of the autonomic nervous system

Control over autonomic nervous system activity, via manipulation of both the sympathetic and parasympathetic arms, is a key means of how the hypothalamus regulates energy expenditure and other aspects of energy balance such as glucose homeostasis (Kalsbeek et al. 2010). Of particular interest in rodent models of energy balance is non-shivering brown adipose tissue (BAT) thermogenesis. Brown fat is composed of adipocytes containing an extraordinary amount of mitochondria giving it a brown appearance in comparison to white adipose tissue (WAT). During BAT thermogenesis, brown adipocytes work to expend energy as heat by reducing ATP production during oxidative phosphorylation in their mitochondria and instead allow for protons leak across mitochondrial membranes due to their expression of uncoupling protein 1 (UCP1) (Cannon and Nedergaard 2004). Thus, heat production through activation of BAT represents a principal means of regulating core body temperature and a significant source of total energy expenditure in the rodent that is commonly altered following hypothalamic manipulations (Morrison et al. 2012). Increased sympathetic activity to BAT drives thermogenesis and creates an upregulation in UCP1 activity and expression that can be a useful marker of increased BAT thermogenesis and sympathetic nervous system activation (Bamshad et al. 1999; Cannon and Nedergaard 2004; Lean et al. 1983). Though once thought to exist only to a small degree, recent evidence suggests that there is actually a considerable amount of BAT in adult humans (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009), making the study of BAT

physiology that much more relevant to understanding human metabolism and not merely a useful index of rodent physiology.

Several hypothalamic nuclei influence BAT function, including those cell groups involved in feeding behavior. Furthermore, the adipostatic hormone leptin has considerable stimulatory effects on BAT thermogenesis, likely mediated by hypothalamic leptin receptors in the DMN and ARC (Kong et al. 2012; Zhang et al. 2011). Another key stimulatory pathway involves orexin-expressing neurons of the LH sending direct excitatory projections to the rostral raphe pallidus (RPa) leading to sympathetic activation of BAT thermogenesis (Tupone et al. 2011). Interestingly, a favored mechanism for unloading excess energy following food intake appears to be activation of BAT (diet-induced thermogenesis) (Lowell and Flier 1997).

The hypothalamus also contains inhibitory circuits to BAT. Excitation of PVN neurons can result in decreased BAT thermogenesis (Madden and Morrison 2009), a circuit that is inhibited by ARC rat insulin promoter (RIP) neurons that do not co-express NPY, AgRP, or POMC (Kong et al. 2012). However, the PVN may also have other mechanisms influencing BAT activity depending on which cell subpopulation is stimulated, as glutamate injections into the PVN are also reported to increase BAT thermogenesis (Amir 1990b). ARC NPY/AgRP neurons may also decrease BAT thermogenesis, as specific stimulation of these neurons significantly reduces energy expenditure (Krashes et al. 2011), likely due, in part, to reduced BAT activity (personal communication).

There are many questions surrounding the role of the VMN in regulation of BAT thermogenesis, primarily stemming from polysynaptic tract tracing studies using

pseudorabies virus resulting in the labeling of all the hypothalamic nuclei discussed above except the VMN, even after long incubation periods (Bamshad et al. 1999; Oldfield et al. 2002). This is a surprising result considering the initial studies involving hypothalamic regulation of BAT demonstrated that the VMN have a stimulatory effect (Amir 1990a; Perkins et al. 1981; Yoshimatsu et al. 1993). Unfortunately, due to the close proximity of the VMN to the DMN and ARC and lack of retrograde tracing from BAT, these pioneering studies, and others like them (Wang et al. 2010), have been dismissed due to lack of confidence in injection site accuracy (Morrison et al. 2012). Recent transgenic approaches have been able to provide a better indication of whether the VMN are involved in BAT regulation. Postnatal elimination of the VMN-specific transcription factor steroidogenic factor 1 (SF-1), allowing for normal development of the VMN, causes a deficit in diet-induced thermogenesis when these animals are challenged with a high fat diet that is also coupled with decreased UCP1 expression (Kim et al. 2011). Still, evidence from anatomical tracing studies may be needed for a universal acceptance of VMN-induced BAT thermogenesis.

In addition to temperature regulation, there are many other aspects of energy metabolism that the hypothalamus governs through regulation of hypothalamic-pituitary axes such as glucocorticoid and thyroid hormone production and through other targets of the autonomic nervous system including the adrenal gland, liver, pancreas, and white adipose tissue to name a few. Many of these hypothalamic processes revolve closely around glucose homeostasis, especially those involving adrenal, hepatic, and pancreatic outputs (Hamelink et al. 2002; Kreier et al. 2006). The capability of this tight hypothalamic control over glycemic levels stems from various nuclei equipped with

glucose-sensing neurons that can either be excited or inhibited by changes in circulating levels of glucose (Karnani and Burdakov 2011). While alterations in glucose levels do not appear to have a significant impact on food intake (Dunn-Meynell et al. 2009), they do lead to changes in autonomic activity to maintain euglycemia (Buijs et al. 2001). As such, the VMN are thought to be necessary for the counterregulatory response to hypoglycemia by stimulating peripheral catecholamine and glucagon release (Borg et al. 1997; Borg et al. 1994; Borg et al. 1995; Fioramonti et al. 2010; Levin et al. 2008; Tong et al. 2007), and have been proposed to excite preautonomic neurons of the PVN to stimulate hepatic glucose production (Yi et al. 2010).

Pituitary adenylate cyclase-activating polypeptide (PACAP)

Isolated from ovine hypothalamus, PACAP was first described to stimulate adenylate cyclase activity in pituitary cells (Miyata et al. 1989). Upon characterization it was discovered that PACAP belonged to the evolutionarily well-conserved secretin-glucagon superfamily of peptide hormones, which also includes gastric inhibitory peptide, growth hormone-releasing factor, and vasoactive intestinal polypeptide (VIP), suggesting this peptide is essential to normal physiology (Sherwood et al. 2000). The PACAP gene encodes a precursor peptide that after posttranslational processing yields the biologically inactive (in mammals) PACAP-related peptide (PRP) and PACAP of either 27 or 38 amino acids (aa) in length (Ogi et al. 1990; Okazaki et al. 1992). Although PACAP27 and PACAP38 undergo different posttranslational processes both peptides are functionally very similar, with PACAP38 the decidedly predominant form in the CNS (Ghatei et al. 1993; Hannibal et al. 1995a). Abundant CNS expression is found in the hypothalamus, limbic system, and brainstem (Ghatei et al. 1993; Hannibal 2002;

Hannibal et al. 1995a; Piggins et al. 1996), where PACAP signaling is functionally linked to a wide array of systems including learning, memory, circadian rhythms, stress, mating, cell survival, autonomic nervous system activity, and energy homeostasis (Agarwal et al. 2005; Ago et al. 2013; Apostolakis et al. 2004; Arimura et al. 1994; Hammack et al. 2010; Hannibal et al. 2000; Harrington et al. 1999; Hawke et al. 2009; Otto et al. 2001; Seaborn et al. 2011; Tanida et al. 2010).

As its name implies, PACAP also has significant effects on endocrine function through stimulation of several pituitary hormones (Rawlings and Hezareh 1996), in addition to expression in the periphery found in the adrenal gland, gonads, pancreas, and peripheral nervous system (Vaudry et al. 2009). It is important to note that the extent to which bidirectional PACAP signaling occurs between central and peripheral compartments is very low, as peripheral PACAP is similar to other peptide hormones that exhibit limited distribution and rapid blood clearance, even though both saturable and nonsaturable mechanisms exist for PACAP transport across the blood-brain barrier (Banks et al. 1993; Bourgault et al. 2011). Furthermore, an extensive efflux component, peptide transport system-6 (PTS-6), in the blood-brain barrier represents a considerable obstacle to PACAP influx from the periphery (Dogrukol-Ak et al. 2009).

The PACAP receptors are all members of the B1 subclass of G protein-coupled receptors (GPCR), and include the PACAP type I receptor (PAC1R) and the VIP receptors, VPAC1R and VPAC2R, due to approximately 68% homology between PACAP and VIP (Dickson and Finlayson 2009). Among the various receptor types, PAC1R has a much greater affinity for both PACAP27 and PACAP38 than for VIP, while VPAC1R and VPAC2R bind PACAP and VIP equally (Table 1.1) (Gottschall et al.

1990; Lam et al. 1990). Because of this overlap in receptor activity between PACAP and VIP several pharmacological agents have been discovered or derived in order to isolate one of the three receptors specifically and determine its function, however, all of the selective ligands thus far are peptidergic in nature and usually display some activity at one of the other PACAP receptors (Table 1.1) (Dickson et al. 2006; Dickson and Finlayson 2009; Fishbein et al. 1994; Gourlet et al. 1995; Lerner et al. 2007; Robberecht et al. 1992; Summers et al. 2003; Vaudry et al. 2009).

Table 1.1. PACAP receptor binding affinities and selective ligands

Receptor	Dissociation Constant (K_d)	Agonists	Antagonists
PAC1R	PACAP ≈ 0.5 nM VIP > 500 nM	Maxadilan	PACAP6-38 (VPAC2R activity) M65
VPAC1R	PACAP ≈ VIP ≈ 1 nM	$[K^{15},R^{16},L^{27}]$VIP(1-7)/ GRF(8-27)	PG 97-269
VPAC2R	PACAP ≈ VIP ≈ 1 nM	BAY 55-9857 (VPAC1R activity)	PG 99-465 (VPAC1R/PAC1R activity)

(Dickson and Finlayson 2009; Vaudry et al. 2009)

As with the rest of the B1 subclass of GPCRs, PACAP receptor signaling is coupled to Gαs and stimulation of adenylate cyclase activity, however, many other signal transduction pathways are activated by either VPAC or PAC1 receptors (Dickson and Finlayson 2009). Consequently, PACAP receptors are also capable of coupling to other G proteins such as Gαq, and can result in a diverse array of intracellular signals outside of cAMP production including activation of phospholipase C, protein kinase C,

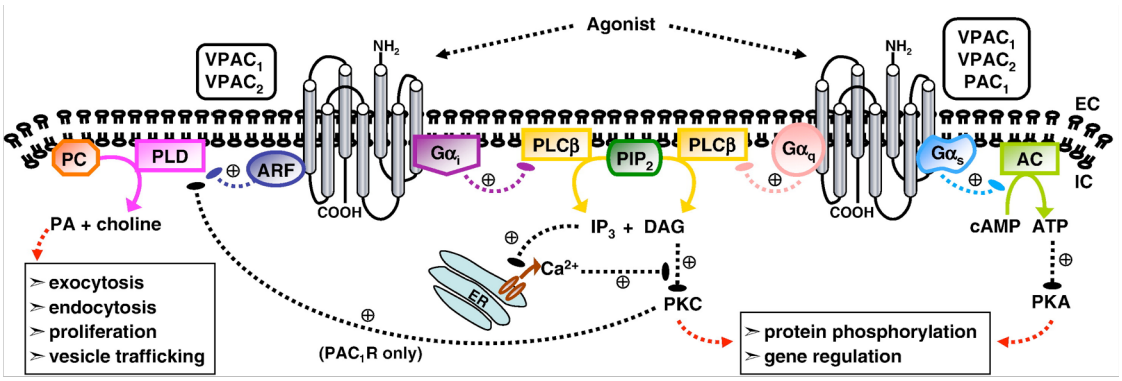

Figure 1.3. PACAP/VIP receptor signaling pathways. G protein-coupling to Gαs, Gαq, or Gαi/o allow for diverse intracellular signal transduction in the cell by PACAP receptors. Figure adapted from Dickson and Finlayson, 2009.

phospholipase D, and/or intracellular calcium release (Figure 1.3) (Dickson and Finlayson 2009). This broad impact mediated by PACAP receptors is attributed to the existence of multiple variants, especially in the PAC1R, as it is described as one of the most alternatively spliced GPCRs known (Dautzenberg et al. 1999; Kilpatrick et al. 1999). Splice variants occur throughout the PAC1 receptor at the N-terminus (Daniel et al. 2001; Dautzenberg et al. 1999; Lutz et al. 2006; Pantaloni et al. 1996), transmembrane domains (Chatterjee et al. 1996), and third intracellular loop (Braas and May 1999; Journot et al. 1995; Spengler et al. 1993), however, the majority of PAC1R variants arise from inclusion or removal of the third intracellular loop cassettes, designated hip (28 aa), hop1 (28 aa), and hop2 (27 aa). No matter the location, all of the approximately 15 PAC1R isoforms have functional consequences resulting in changes in potency of PACAP/VIP and/or second messenger activation (Dickson and Finlayson 2009). The heavy alternative splicing that results in variations of pharmacological profile and

receptor signaling demonstrate the complexity of the PACAP/VIP neuropeptide system, and illustrates the need for development of selective ligands for PAC1 and VPAC receptors (Bourgault et al. 2009), as they are often expressed in the same tissue (Nguyen et al. 1993; Robberecht et al. 1991; Tatsuno et al. 1990).

Regulation of feeding behavior by central PACAP signaling

The first observations of PACAP-mediated regulation of food intake were observed soon after its discovery (Miyata et al. 1989), when intracerebroventricular (icv) injections of PACAP were found to significantly reduce food intake in food-deprived mice (Morley et al. 1992). Later, icv PACAP administration was also observed to decrease feeding behavior in goldfish, chicks, and rats (Matsuda et al. 2005; Mizuno et al. 1998; Tachibana et al. 2003). Pretreatment with PACAP6-38, a truncated form of PACAP that antagonizes the PAC1 receptor (Robberecht et al. 1992), abolishes the hypophagic response to icv PACAP injections indicating that control of food intake can be mediated by PAC1 receptors (Hawke et al. 2009; Mounien et al. 2009). Considering these results, it is surprising that PACAP, PAC1R, and VPAC2R knockout (KO) mice are lean (Adams et al. 2008; Asnicar et al. 2002; Jamen et al. 2000; Nakata et al. 2004; Tomimoto et al. 2008). Furthermore, PACAP KO mice eat significantly less food than their wild-type littermates when normalized for body weight, and exhibit reduced NPY mRNA expression (Nakata et al. 2004). The lean phenotype of PACAP related KO mice conflicts with behavioral data collected from central pharmacological experiments suggesting that peripheral PACAP signaling also contributes to feeding behavior, and possibly that multiple PACAP-expressing circuits in the brain drive either hunger or satiety. Moreover, constitutive deletion of either PACAP or PAC1R results in early

lethality suggesting an essential role for this neuropeptide (Gray et al. 2001; Otto et al. 2004). To date, limited technology allowing for cell-type specific manipulation has hindered the isolation of specific PACAP circuits and their contributions to feeding behavior (until recently (Krashes et al. 2014)), leaving pharmacology and expression analyses as the primary avenues of investigation.

Although central PACAP neurocircuitry is largely unknown, the decreased feeding produced by exogenous PACAP injections has been attributed mainly to signaling within the hypothalamus. This is due, in part, to reports of PACAP-mediated attenuation of NPY-induced feeding (Chance et al. 1995; Morley et al. 1992) and extensive expression of PAC1 receptors in multiple hypothalamic nuclei (Hashimoto et al. 1996; Resch et al. 2011). In further support, there is a distinct abundance of PACAP protein expression in the hypothalamus with greater concentrations than in both the amygdala and extended amygdala and with protein concentrations of approximately 25 times that found in the cerebral cortex (Hannibal et al. 1995a). *In situ* hybridization and immunohistochemistry techniques have specifically identified major hypothalamic cell groups expressing PACAP to include the PVN (Hannibal et al. 1995b; Nomura et al. 2000), VMN, and some subnuclei of the mammillary bodies (Hannibal 2002). In addition, immunohistochemical analysis of PACAP immunoreactivity demonstrates virtually every hypothalamic area receives intense labeling of PACAP nerve fibers (Hannibal 2002; Piggins et al. 1996).

Importantly, hypothalamic PACAP mRNA expression is responsive to energy signaling. Mice maintained on a high energy diet for 8 weeks show increased levels of circulating leptin associated with increased PACAP mRNA expression in the VMN, and

conversely 48 hours of fasting causes significantly decreased levels (Hawke et al. 2009). Of note, leptin treatment in fasted animals normalizes VMN PACAP mRNA expression, and furthermore, leptin deficiency or deletion of leptin receptors from the VMN results in reduced PACAP mRNA expression. The relationship between leptin and PACAP is also demonstrated by pretreatment with the PAC1R antagonist PACAP6-38 eliminating leptin-mediated hypophagia (Hawke et al. 2009) and the attenuated hypophagia observed following icv leptin administration in PACAP KO mice (Tanida et al. 2013). These data indicate that PACAP neurotransmission, likely involving the VMN, may be a mediator of leptin-mediated inhibition of food intake.

Like PACAP, hypothalamic pro-opiomelanocortin (POMC) mRNA expression is also reduced following a 48 hour fast in mice. (Mounien et al. 2009). POMC is the precursor to α-melanocyte-stimulating hormone (α-MSH), a potent neuropeptide signal that has a significant role in body weight regulation through stimulating central melanocortin receptors (MC3/4R) (Zhan et al. 2013). Notably, PACAP also dose-dependently increases POMC mRNA in ARC hypothalamic slices (Mounien et al. 2006b), indicating a possible relationship between the two neuropeptide signals. Remarkably, both PACAP6-38 and the melanocortin receptor antagonist SHU9119 block PACAP-induced decreases in feeding after icv injection, and *in situ* hybridization decisively shows increased c-fos mRNA expression in ARC POMC neurons one hour following PACAP icv injections, perhaps localizing PACAP-induced hypophagia to the ARC (Mounien et al. 2009). Given the relationships between PACAP and both leptin and POMC in the hypothalamus, it is conceivable that leptin stimulates excitatory VMN

PACAP neurons projecting to ARC POMC neurons, leading to decreased food intake (Balthasar et al. 2004; Dhillon et al. 2006; Hawke et al. 2009; Mounien et al. 2009).

Although the hypothalamus appears to be the primary target for regulation of food intake by PACAP signaling, very few have attempted to ascertain the intrahypothalamic PACAP circuitry or site-specific contributions that regulate feeding behavior. The hypothalamus possesses an abundance of PAC1R mRNA in many of its nuclei compared to a more restricted expression pattern of VPAC2R mRNA (Hashimoto et al. 1996; Mounien et al. 2006a; Resch et al. 2011; Usdin et al. 1994; Yi et al. 2010), suggesting that hypothalamic control of feeding utilizes PACAP-PAC1R signaling. Hypothalamic injections of PACAP into the perifornical area of the lateral hypothalamus (PFH) produce potent hypophagia, exemplified by PACAP-mediated inhibition of NPY-induced feeding in the PFH (Chance et al. 1995). Furthermore, animals with restricted access to food continue to respond with decreased food intake following PACAP injections into the PFH demonstrating that PACAP-induced decreases in feeding may not be limited strictly to inhibition of NPY-induced feeding (Chance et al. 1995). The alterations in PFH neuron activity leading to changes in feeding behavior driven by NPY and PACAP are likely a product of cAMP second messenger signaling cascades, as PACAP reduces cAMP response element (CRE) binding in nuclear extracts of PFH tissue, while food deprivation and NPY significantly increase CRE binding (Sheriff et al. 1997). Of note, PACAP signaling in the PFH may also regulate fluid intake, although PFH PACAP administration has been reported to produce both adipsogenic (Chance et al. 1995) and dipsogenic (Puig de Parada et al. 1995) effects. These discrepancies may result from differences in the concentration of PACAP injections delivered into the PFH or an apparent differential

response of fluid intake to rostral and caudal injection sites of the PFH to PACAP. While the circuitry responsible for regulation of ingestive behavior in the PFH is still undefined, it is clear that PACAP has a diverse role in regulating PFH function.

Another pair of hypothalamic nuclei that utilize PACAP to regulate feeding behavior are the paraventricular nuclei (PVN). The PVN express a large amount of PAC1R mRNA, as well as an abundance of PACAP-like immunoreactivity in perikarya and terminals (Das et al. 2007; Hannibal et al. 1995b; Hashimoto et al. 1996; Legradi et al. 1998). Furthermore, PACAP synapses terminate specifically onto PVN corticotropin-releasing factor (CRF) neurons, and icv injection of PACAP increases PVN CRF mRNA (Grinevich et al. 1997; Legradi et al. 1998). Importantly, PACAP is hypothesized to excite CRF neurons in the PVN, resulting in stimulation of the hypothalamic-pituitary-adrenal (HPA) axis, which supports the results that icv PACAP injections increase blood corticosterone levels (Dore et al. 2013; Yi et al. 2010). Stress can have a significant impact on feeding and body weight and given the apparent association between PACAP signaling and CRF neurons of the PVN, site-specific PACAP injections into PVN may produce significant alterations in a number of physiological systems including feeding behavior. Behavioral analysis following microinfusion of PACAP into the PVN shows increased face washing and body grooming and decreased rearing and locomotor activity irrespective of whether animals received mild restraint stress (Norrholm et al. 2005). Unfortunately, food intake following PACAP injections into the PVN was not reported leaving a significant gap in knowledge regarding feeding, stress, and PACAP signaling. However, icv pretreatment with a CRF antagonist prior to PACAP administration does

not block hypophagia and reductions in body weight induced by PACAP (Dore et al. 2013), perhaps separating the hypophagic and stress responses to central PACAP.

Autonomic regulation by central PACAP signaling

Multiple sites within the hypothalamus not only control energy intake but also energy expenditure. This is also true of PACAP signaling as central injections dose-dependently reduce feeding under both normal and food-deprived conditions and increase metabolism through regulation of the autonomic nervous system, causing increases in core body temperature (Hawke et al. 2009; Masuo et al. 1995; Pataki et al. 2000), locomotor activity (Masuo et al. 1995), and oxygen consumption (Hawke et al. 2009). The impact of increased energy expenditure driven by these effects on body weight are clearly demonstrated in pair-feeding experiments where animals receiving icv PACAP lose significantly more body weight in a 24 hour period than both controls and animals restricted to the amount of food eaten by PACAP treated animals (Hawke et al. 2009). Furthermore, both PACAP and PAC1R KO mice have notable metabolic deficiencies. The first reports of PACAP/PAC1R KO mice described lean undernourished pups with a high incidence of lethality accompanied by elevated circulating levels of ketones, triglycerides, cholesterol, and corticosterone, in addition to very low liver glycogen (Gray et al. 2001; Otto et al. 2004). Moreover, PACAP/PAC1R null mice show signs of pulmonary hypertension, enlarged hearts, intracellular lipid accumulation in cardiac cells, and increased levels of serum free fatty acids consistent with the possibility of cardiovascular problems contributing to early lethality (Gray et al. 2001; Otto et al. 2004).

Interestingly, survival of PACAP null mice is greatly improved if the animals are housed at an elevated ambient temperature (24° C vs. 21° C) suggesting a crucial role for PACAP in thermoregulation (Gray et al. 2002). Additionally, PACAP knockout mice housed at 24° C challenged with a 21° C climate lost their body temperature significantly faster than wild-type littermates, yet brown adipose tissue (BAT) in these animals appeared to be fully capable of non-shivering thermogenesis (Gray et al. 2002). Therefore, lack of temperature regulation in these mice was attributed to decreased stimulation of brown fat by norepinephrine from sympathetic nerves.

Given pharmacological and genetic data signifying a crucial role for PACAP signaling in thermoregulation, the catabolic response to icv injections of PACAP is likely due to modulation of autonomic nerve activity, as energy expenditure driven by increased core body temperature is often mediated by sympathetic activation of BAT thermogenesis in rodents. Indeed, third ventricle injection of PACAP increases mean arterial pressure and heart rate, as well as altering a host of autonomic nerve pathways. Renal, adrenal, celiac, lumbar, liver, and interscapular brown adipose sympathetic nerve activities all significantly increase following PACAP injections, while parasympathetic gastric vagal and celiac vagal nerve activities decrease (Tanida et al. 2011a; Tanida et al. 2010). As observed with the regulation of feeding behavior, PACAP signaling may work synergistically with leptin and melanocortin signaling to regulate metabolism. Central pretreatment with the PAC1R antagonist PACAP6-38 icv blocks stimulation of white adipose tissue sympathetic nerve activity and hyperthermia induced by leptin injections, suggesting that central actions of leptin may include stimulating PACAP release, perhaps from the VMN (Hawke et al. 2009; Tanida et al. 2013). In addition to the interaction with

leptin, the melanocortin receptor antagonist SHU9119 inhibits some of the effects of PACAP on autonomic nerve activity, including effectively blocking PACAP-induced modulation of the parasympathetic gastric vagal nerve and sympathetic nerves innervating the liver and brown adipose tissue (Tanida et al. 2011a). Meanwhile, SHU9119 does not inhibit PACAP's effect on mean arterial pressure or heart rate, suggesting that the melanocortin system may only participate in the digestive and thermogenic processes regulated by PACAP, but not other physiological systems stimulated by central PACAP injection (Tanida et al. 2011a).

PACAP regulation of glucose homeostasis

PACAP plays a diverse role in glucose homeostasis. Impaired glucose tolerance or insulin hypersensitivity following glucose and insulin tolerance tests are observed in PACAP, PAC1R, and VPAC2R knockout mice (Adams et al. 2008; Asnicar et al. 2002; Jamen et al. 2000; Tomimoto et al. 2008). PACAP knockouts also display low basal levels of insulin (Adams et al. 2008; Tomimoto et al. 2008) and a hypersensitivity to insulin-induced lethality (Hamelink et al. 2002), while PAC1R KO mice exhibit compromised insulin secretion and glucagon response to insulin-induced hypoglycemia (Jamen et al. 2000; Persson and Ahren 2002). These abnormalities of insulin secretion and signaling suggest pancreatic dysfunction, however PACAP knockout mice also show insufficient catecholamine production indicated by low adrenal epinephrine levels and tyrosine hydroxylase activity following insulin challenge (Hamelink et al. 2002), and a blunted glucose response to stressful stimuli (Tanida et al. 2010), all of which point to orchestration of glucose regulation through PACAP signaling in other organs such as the adrenal gland. Given the rich expression of PACAP both centrally and peripherally it is

difficult to pinpoint a single target for the abnormal glucose homeostasis in PACAP knockout animals, especially since PACAP expression exists in a number of peripheral organs such as the pancreas (Filipsson et al. 1998; Portela-Gomes et al. 2003) and adrenal (Ghatei et al. 1993; Kantor et al. 2002; Moller and Sundler 1996).

Central PACAP regulation of glucose does impact peripheral blood glucose independent of peripheral actions via modulation of the autonomic nervous system. Plasma glucose concentrations rise rapidly following central PACAP injections, in addition to plasma glucagon and corticosterone levels. Interestingly, glucose production stimulated by central PACAP occurs through endogenous glucose production largely due to liver glycogenolysis, and to a smaller extent gluconeogenesis (Mounien et al. 2009; Yi et al. 2010). In support of liver involvement, central PACAP administration increases phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA, and drastically decreases glycogen content in the liver. Furthermore, hepatic sympathetic, but not hepatic parasympathetic denervation abolishes the effects of central PACAP injection on plasma glucose (Yi et al. 2010).

Using retrograde tracing from the intermediolateral (IML) column of the thoracic spinal cord, where liver projecting sympathetic neurons reside, in combination with Fos immunohistochemistry, neurons in the paraventricular nuclei (PVN) of the hypothalamus were co-labeled with tracer and Fos immunoreactivity following central PACAP administration, suggesting that PACAP activates preautonomic PVN neurons that stimulate hepatic sympathetic nerve activity and in turn glucose production (Yi et al. 2010). These results are further supported by the increased glucose production that occurs following direct microinjection of PACAP into the PVN (Resch et al. 2013; Yi et al.

2010). Interestingly, the effects of centrally administered PACAP on glucose would appear to be distinct from that of feeding and metabolism because it is not blocked by either PAC1R or melanocortin receptor antagonists. Instead the glycemic response appears to be mediated by the vasoactive intestinal polypeptide 2 receptor (VPAC2R), as VPAC2 receptor antagonism inhibits plasma glucose production induced by central PACAP (Yi et al. 2010).

Glutamate neurotransmission and its modulation by PACAP

Glutamate plays a significant role in several pathways of cellular metabolism including energy production and protein synthesis and degradation (Kelly and Stanley 2001), however, it is also the primary excitatory neurotransmitter in the brain. Elimination of synaptic glutamate release, through targeted gene knockout strategies, is incompatible with life demonstrating that glutamate neurotransmission is absolutely essential to CNS function and survival (Moechars et al. 2006; Wojcik et al. 2004). Glutamate signaling occurs through several ionotropic (Lodge 2009) or metabotropic (Niswender and Conn 2010) receptor subtypes that are expressed in various locations in and around the synapse, leading to complex regulation of excitatory communication (Figure 1.4). Adding to this complicated system are neuromodulatory transmitters that can affect glutamate signaling by altering presynaptic release (Pancani et al. 2014), postsynaptic receptor activity (Kempadoo et al. 2013), or even reuptake (Brown 2000). The neuropeptide PACAP, which is co-expressed with some populations of glutamate neurons (Fig. 5.1) (Engelund et al. 2010; Fahrenkrug and Hannibal 2004; Hannibal et al. 2000), is positioned to be an essential modulator as it appears to have multifaceted effects

on glutamate neurotransmission through signaling both on neurons (Macdonald et al. 2005) and astrocytes (Figiel and Engele 2000).

Activation of ionotropic glutamate receptors provide fast-acting depolarizing current at the postsynaptic neuron following synaptic glutamate release, with each receptor type capable of different subunit compositions that can significantly modify receptor kinetics (Dingledine et al. 1999). The low affinity ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolproprianic acid (AMPA) and kainate are trafficked near to synaptic release sites where concentrations of glutamate are highest upon vesicular release (Franks et al. 2002; Raghavachari and Lisman 2004; Zheng et al. 2008). N-methyl-D-aspartate (NMDA) receptors are high affinity ionotropic glutamate receptors, however, they also require the presence of co-agonists glycine or D-serine to become activated (Johnson and Ascher 1987; Schell et al. 1995). Furthermore, within the ion channel of NMDA receptors exists a voltage-dependent magnesium block that is removed by depolarization, usually done so by AMPA/kainate activation, which exhibit rapid gating (Nowak et al. 1984). Contributing to glutamate receptor activation, PACAP signaling through both VPAC and PAC1 receptors appear to potentiate activity of ionotropic glutamate receptors, specifically AMPA (Cho et al. 2012; Costa et al. 2009) and NMDA receptors (Harrington et al. 1999; Macdonald et al. 2005; Wu and Dun 1997; Yaka et al. 2003), however presynaptic modulation of glutamate release may also be involved (Otto et al. 1999). Likewise, depression of AMPA and NMDA receptor currents have been reported under conditions of excessive PACAP concentrations in the hippocampus (Costa et al. 2009; Macdonald et al. 2005).

In addition to ionotropic forms, multiple subtypes of high affinity metabotropic glutamate receptors (mGluR) exist. The mGluRs are divided into three categories based on sequence homology, G protein-coupling, and ligand selectivity (Niswender and Conn 2010). Group I mGluRs ($mGluR_{1,5}$) are generally postsynaptic and excitatory through coupling to $G\alpha q_{/11}$ and activation of phospholipase C (Hermans and Challiss 2001). By contrast, group II ($mGluR_{2\text{-}3}$) and group III ($mGluR_{4,6\text{-}8}$) receptors are often located presynaptically (especially group III) and inhibit neurotransmitter release (Niswender and Conn 2010). Considering both mGluRs and PACAP signaling have modulatory roles it is not surprising that little evidence exists for interactions between them, however, PACAP signaling in the suprachiasmatic nuclei may reduce the calcium induction caused by group I mGluR activation (Kopp et al. 2001).

Glial glutamate regulation and the potential role for PACAP

Glutamate homeostasis may be more dependent on the function of glia than any other cell type in the CNS. This is supported by the importance of sodium-dependent glial glutamate transporters (GLT-1 and GLAST) to remove vesicular glutamate release from the synapse in order to prevent spillover into other synaptic compartments (Figure 1.4) (Asztely et al. 1997; Diamond and Jahr 2000) and to signal for glycolysis activation to replenish energy resources following increased neuronal activity (Pellerin and Magistretti 1994). However, a poorly studied aspect of glutamate neurotransmission is astrocytic glutamate release (Baker et al. 2002; Parpura and Haydon 2000; Ye et al. 2003), which may be capable of coordinating neuronal activity on a network level (Bushong et al. 2002; Ogata and Kosaka 2002). In particular, the astrocytic cystine-glutamate antiporter, system x_c^-, which exchanges one extracellular molecule of cystine

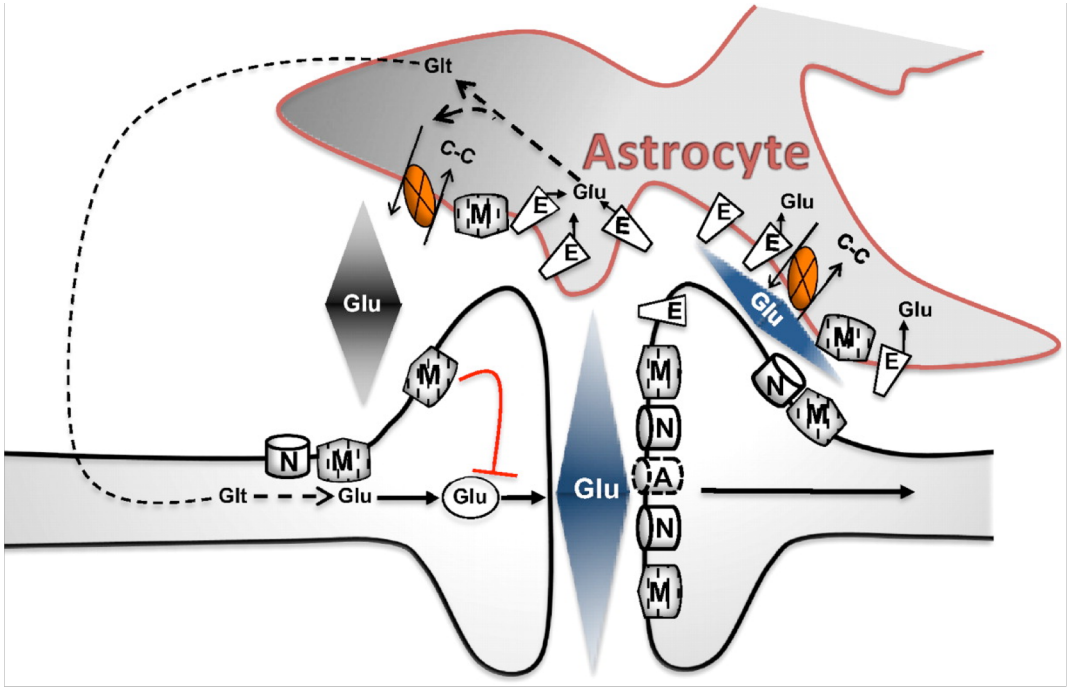

Figure 1.4. Schematic representation of a typical glutamatergic synapse. Glutamate (Glu) signaling occurs both within the synapse and extrasynaptically to regulate neuronal excitability, much of which is regulated by astrocytes through synaptic glutamate uptake (E; EAATs), glutamate release (via cystine (c-c)-glutamate antiporter), and the glutamate-glutamine (Glt) cycle responsible for replenishing glutamate availability in neurons that have only few glutamate reuptake transporters. M, metabotropic glutamate receptor; A, AMPA receptor; N, NMDA receptor. Figure adapted from Bridges et al. 2012.

for one intracellular molecule of glutamate (Bannai and Kitamura 1980) has been implicated in maintaining the extrasynaptic pool of glutamate (Baker et al. 2002). Dysregulation of system x_c^- activity results in altered concentrations of extrasynaptic glutamate, altered neurotransmitter release, and upregulation of postsynaptic glutamate receptor clustering, which can lead to pathology (Augustin et al. 2007; Baker et al. 2008; Baker et al. 2003; De Bundel et al. 2011; Featherstone et al. 2002; Knackstedt et al. 2009; Kupchik et al. 2012; Moran et al. 2005). Moreover, excessive extrasynaptic glutamate concentrations, perhaps through system x_c^-, may also contribute to excitotoxic neuronal injury or death suggesting the regulation of this antiporter may be tightly regulated by the

synaptic environment (Buckingham et al. 2011; de Groot and Sontheimer 2011; Fogal et al. 2007; Jackman et al. 2010; Liu et al. 2014; Sontheimer 2011).

Importantly, PACAP also displays significant interactions with glia, especially astrocytes, with all three PACAP/VIP receptors showing astrocytic expression (Ashur-Fabian et al. 1997; Grimaldi and Cavallaro 1999; Grimaldi and Cavallaro 2000; Jaworski 2000; Suzuki et al. 2003; Tatsuno et al. 1991; Tatsuno et al. 1990; Tatsuno et al. 1996a). Remarkably, PACAP signaling on astrocytes involves many of the glutamatergic systems in place to regulate cell excitability and energy metabolism. For example, PACAP treatment of astrocytes appears to upregulate expression and function of the glial glutamate transporters GLT-1 and GLAST through activation of both PKA and PKC-dependent pathways (Figiel and Engele 2000). PACAP has also been shown to play a role in glial glycogen metabolism (Cardinaux and Magistretti 1996; Magistretti et al. 1998), and may mediate its neurotrophic role through protecting against oxidative stress and excitotoxicity by signaling on astrocytes (Brown 2000; Masmoudi-Kouki et al. 2011; Morio et al. 1996; Stumm et al. 2007; Zusev and Gozes 2004).

Current Studies

This thesis continues to build upon the foundation of knowledge involving hypothalamic regulation of energy balance by investigating the role of PACAP signaling on feeding behavior and metabolism. Characterizing specific components of PACAP neurocircuitry and receptor signaling as they pertain to hypothalamic energy regulation could make great strides toward eventual therapeutic tools to treat obesity and diabetes. While the research field is aware of the potent effects of PACAP on feeding and metabolism, much of the data is marred by behavioral pharmacology based on non-

specific central injections of the peptide or global gene knockout strategies. The current work begins to answer questions about the hypophagic and hypermetabolic effects observed following icv injections of PACAP by carefully restricting our injections to distinct hypothalamic nuclei.

Remarkably at the onset of this dissertation project, the PACAP neurocircuitry mediating its regulation of body weight was only speculative. Consequently, we set to 1) identify the site(s) of action responsible for the hypophagic response to PACAP in the hypothalamus, 2) determine if these "satiety centers" were also involved in other aspects of energy homeostasis such as controlling energy expenditure and glucose homeostasis, 3) delineate the sources of endogenous PACAP signaling projecting into these nuclei, and 4) investigate how PACAP modulates glutamatergic neurotransmission to produce behavioral and physiological effects. Through site-specific behavioral pharmacological analysis, retrograde tract tracing, and examination of the interaction between glutamate and PACAP both *in vitro* and *in vivo*, the resulting data from these experiments not only strongly suggests a critical role for PACAP in the regulation of feeding behavior and metabolism but also towards the modulation of glutamate neurotransmission.

CHAPTER II

REGULATION OF FOOD INTAKE BY PACAP SIGNALING IN THE VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS

Introduction

The ventromedial nuclei of the hypothalamus (VMN) are important regulators of feeding behavior. Temporary inhibition, with procaine (Berthoud and Jeanrenaud 1979; Yadav et al. 2009) and colchicine (Avrith and Mogenson 1978; Choi and Dallman 1999), or permanent lesion of the VMN result in hyperphagia (Brobeck et al. 1943), while electrical stimulation produces reduced feeding in rats (Beltt and Keesey 1975; Ruffin and Nicolaidis 1999; Stenger et al. 1991). Furthermore, many signaling molecules including norepinephrine (Shimazu et al. 1986), brain-derived neurotrophic factor (BDNF) (Wang et al. 2007), and leptin (Jacob et al. 1997) have been discovered to affect feeding behavior when administered into the VMN, however the effect of a particular transmitter on feeding behavior is dependent upon receptor expression within the VMN and whether the signal results in excitation or inhibition of VMN neurons. Understanding specific neurotransmitters and how they modulate VMN activity provides insight into the neurocircuitry controlling VMN-dependent behaviors and potential therapeutic targets for obesity.

The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is positioned to have a significant role in hypothalamic regulation of feeding behavior, but its signaling specifically within the VMN has yet to be clearly characterized. Intracerebroventricular (icv) administration of PACAP robustly decreases food intake

(Dore et al. 2013; Hawke et al. 2009; Mizuno et al. 1998; Morley et al. 1992; Mounien et al. 2009). In addition, PACAP has been linked to both leptin and BDNF signaling (Fukuchi et al. 2005; Hammack et al. 2009; Hawke et al. 2009; Pellegri et al. 1998; Yaka et al. 2003), both of which decrease food intake when administered into the VMN (Jacob et al. 1997; Wang et al. 2007). Interestingly, PACAP mRNA expression decreases following fasting (Hawke et al. 2009; Mounien et al. 2009) while chronic high fat diet increases PACAP mRNA in the VMN (Hawke et al. 2009), suggesting that PACAP-expressing neurons of the VMN are important energy sensors that monitor and regulate body weight.

PACAP is a 38 amino acid neuropeptide that belongs to the secretin super family of peptides that also includes vasoactive intestinal polypeptide (VIP) (Hannibal et al. 1995a; Vaudry et al. 2009). Because of their high sequence homology PACAP not only binds and activates the PACAP receptor (PAC1R), but also the VIP receptors VPAC1 and VPAC2. All three receptors are G protein-coupled with PACAP demonstrating an approximate 2-fold higher affinity for the PACAP receptor (PAC1R) compared to the VIP receptors. Interestingly, PACAP and VIP display equal affinity for the VIP receptors, whereas VIP has a very low affinity for the PAC1R (Vaudry et al. 2009). All PACAP receptors stimulate adenylate cyclase activity, however the receptors, especially PAC1R, are heavily alternatively spliced and several variants have been shown to activate other secondary messenger pathways (Dickson and Finlayson 2009; Ushiyama et al. 2007).

Much of the work thus far involving PACAP and feeding behavior has focused on icv injections or global knockouts, neither of which can speak to specific sites of action

by which PACAP induces its effects (Adams et al. 2008; Hawke et al. 2009; Morley et al. 1992; Mounien et al. 2009; Nakata et al. 2004). PAC1R mRNA expression is reported to be widespread throughout the central nervous system, with abundant expression in the hypothalamus, specifically in areas known to regulate feeding behavior such as the paraventricular nuclei (PVN), VMN, and arcuate nuclei (ARC) (Hashimoto et al. 1996).

Given its discrete and abundant expression within the VMN, PACAP and its receptor may yield novel mechanisms for the control of feeding behavior. To further investigate the importance of highly localized PAC1R signaling we microinjected PACAP site-specifically into the VMN and subsequently measured feeding behavior. We further confirmed that changes in feeding behavior were first, mediated through the PAC1R subtype, and second, not a product of malaise. Our studies demonstrate that PACAP signaling within the hypothalamic VMN promotes a negative energy balance by decreasing food intake.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 225-250 g were individually housed in a climate controlled room with a 12 hr light/dark cycle (lights on at 0500h). Animals had free access to Harlan standard diet (8604 formulation) and water unless stated otherwise in specific experiments. Food consumption was measured with a BioDAQ Food Intake Monitor (Research Diets; New Brunswick, NJ) or calculated by pre-weighing food in each bin and subtracting the weight of non-ingested and spilled

food at the end of each measurement period. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; ip) cocktail and placed in a stereotaxic apparatus. 26 gauge bilateral guide cannulae (Plastics One; Roanoke VA) were placed 3 mm dorsal to the hypothalamic ventromedial nuclei (VMN) in all animals, and secured to the surface of the skull with an acrylic resin. The stereotaxic coordinates for the VMN injection site were: anterior/posterior, -2.5 mm from bregma; medial/lateral, ±0.6 mm from midline; dorsal/ventral, -9.2 mm from surface of the skull based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos and Watson 2007). Injectors extended 3 mm past the ventral tip of the cannulae reaching an injection site of -9.2 mm ventral from the surface of the skull. The upper incisor bar was positioned -3.3mm below horizontal zero. A bilateral dummy stylet placed in the guide cannulae was used to maintain patency. The animals were given at least five days to recover after cannulae installation before receiving drug or vehicle injections. During this time the animals were handled and guide cannulae were removed and replaced daily to acclimate the animals to the physical handling necessary during the experiment. Correct cannulae placements were confirmed at the conclusion of each experiment by microscopic examination of Nissl stained sections and only those with correct placement were included in the studies.

Microinjections and Injection Spread

In all experiments, 0.25 µl/side of PACAP (PACAP38; California Peptide Research; Napa, CA) or saline was microinjected through bilateral guide cannulae over approximately two minutes in awake animals. Following each injection an additional minute elapsed before removing injectors to minimize backflow of injected material. At the completion of each experiment, brains were collected following rapid decapitation for *in situ* hybridization or confirmation of cannulae placement. The optimal spread of injection within the VMN was determined using different volumes (0.1, 0.25 µl) of biotinylated PACAP (PACAP38-Biotin; Anaspec; Fremont, CA). One hour following biotinylated PACAP injections animals were anesthetized with a ketamine/xylazine/acepromazine cocktail, and transcardially perfused with 0.9% NaCl and 4% paraformaldehyde in phosphate-buffered saline. Brains were removed, sucrose embedded, and sectioned on a freezing microtome at 30 µm and stored in cryoprotectant solution at -20° C. Brain sections containing the injection sites and surrounding areas were then immunohistochemically stained using a primary antibody against biotin. Immunohistochemical signal was then compared to the cresyl violet staining of adjacent sections to determine surgical accuracy and the spread of the biotinylated PACAP injection within the VMN.

Experiments

Feeding behavior

Animals (n=4 per group) were acclimated to the BioDAQ Food Intake Monitor for at least 7 days before the onset of the experiment. Approximately 2 hours prior to

lights off rats were weighed and injected bilaterally with vehicle, 25, or 50 pmol PACAP. Feeding measurements were collected for the next 18 hours at which time body weights were measured again. Afterwards, brains were collected to confirm cannula placement as well as retroperitoneal (including perirenal; rWAT) and epididymal white adipose tissue (eWAT), and interscapular brown adipose tissue (iBAT). iBAT was cleaned of white adipose tissue for measurement of uncoupling protein 1 (UCP1) mRNA content.

To examine effects of PACAP on feeding behavior following an overnight fast, a separate cohort of animals (n=10 per group) was used. Food was removed from all animals just prior to lights off (1700h) the day before the experiment. The following morning (approximately 16 hours later) animals received bilateral injections of vehicle or 50 pmol PACAP. 10 minutes after the injection, food was returned and feeding was measured for the next hour.

Conditioned flavor avoidance

To determine whether PACAP-induced hypophagia in the VMN was a consequence of malaise, a conditioned flavor avoidance (CFA) behavioral paradigm was used (Deutsch and Hardy 1977; Rinaman and Dzmura 2007). A baseline experiment was performed by water depriving naïve rats for 22 hours, and giving the animals a two-bottle choice test with one bottle containing novel vanilla flavored water (0.5% McCormick vanilla extract) and the other bottle containing novel almond flavored water (0.5% McCormick almond extract). For the CFA test, rats (n=5 per group) were water deprived for 22 hours then allowed 30 minutes of access to novel almond flavored water (0.5% McCormick almond extract) or novel vanilla flavored water (0.5% McCormick vanilla extract). Flavors were divided among each group. Following the ingestion of flavored

water animals received a bilateral injection of 50 pmol PACAP into the VMN. Normal water was returned 30 minutes later and rats were allowed *ad libitum* access to water for the next 24 hours. Afterwards, rats were again water deprived for 22 hours then allowed 30-minute access to the opposite flavor as on day 1 (see Fig. 3A). Subsequent to access to flavored water, animals received a bilateral vehicle injection into the VMN and water was again returned for the next 24 hours. After both pairing days the rats were water deprived a final time for 22 hours followed by the test day in which rats were given a two-bottle choice with both flavors for 30 minutes. A preference ratio was generated from the test day to determine if rats avoided the flavor paired with the VMN PACAP injection. As a CFA positive control, another group of animals (n=5 per group) was included using lithium chloride (LiCl; 0.15M; 2% body weight, ip), as LiCl is well known to induce malaise. This group of rats was treated similar to the PACAP treated animals, but receiving only vehicle injections into the VMN and an ip injection of either saline or LiCl.

PACAP receptor antagonism

Approximately 1 hour before dark, rats (n=6 per group) were weighed and given a bilateral VMN pretreatment of either vehicle or 2.2 nmol of the specific PACAP receptor (PAC1R) antagonist, PACAP6-38 (Anaspec; Fremont, CA). After five minutes, rats received a second bilateral VMN injection of either vehicle or 220 pmol of PACAP. Food intake was measured manually for the next three hours, followed by a final 24-hour post-injection measurement of food intake and body weight.

In addition to food intake and body weight, changes in hypothalamic arcuate nuclei (ARC) neuropeptide mRNA expression were measured. In a similar experimental

design, rats (n=4 per group) received either bilateral VMN injections of vehicle or 220 pmol PACAP and after one hour the animals were euthanized via rapid decapitation. Brains were removed quickly and frozen for analysis using semi-quantitative *in situ* hybridization for pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and agouti-related polypeptide (AgRP) mRNA levels.

In Situ Hybridization

Brains were cryostat sectioned coronally at 12 μm, thaw-mounted onto electrostatically clean slides, and stored at -80°C until post-fixed. Prior to hybridization, sections were post-fixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25% acetic anhydride. Sections were dehydrated through graded ethanol concentrations, de-lipidated in chloroform, re-hydrated to 95% ethanol, and air-dried. Standard *in vitro* transcription methods were used to generate riboprobes against PAC1R (Choi; Milwaukee, WI), NPY (Sabol, Bethesda, MD), AgRP (Schwartz, Seattle, WA), and POMC (Wilkinson, Seattle, WA), which were subsequently diluted in hybridization cocktail (Amresco; Solon, OH) with tRNA. Sections were hybridized overnight at 55°C with each ^{33}P-labeled riboprobe. After hybridization, slides were rinsed in 2x SSC buffer (pH 7.0). They were treated with RNase A in a 0.5 M sodium chloride, 10 mM Tris, 1 mM EDTA buffer for 30 min at 37°C and then washed in the same buffer without RNase A for 30 min at 37°C. After a subsequent 1x SSC wash for 15 min at room temperature, slides were stringently washed in 0.5x SSC for 30 min at 65°C (AgRP, NPY, PAC1R) or 68°C (POMC). Slides were then coated with Kodak autoradiographic emulsion NTB (Rochester, NY) and exposed for 11-22 days depending on the specific riboprobe to

produce silver grains. Following standard autoradiography development, NTB emulsion-dipped sections were counterstained with 0.5% cresyl violet.

Image Analysis

Semi-quantitative analysis of silver grains was conducted using dark field microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY). Optical transmission (OT) was determined from the integration of scattered light captured with a 5x objective and analyzed for intensity of region of interest and total area of reflected light. Background values measured in adjacent areas of tissue not containing hybridized probe were subtracted from all measurements. As such, the reported units are arbitrary and will vary based on the proportion of the area of transmitted light compared to the background. Since the area of distribution for each population of mRNA varies relative to the size of the background, the arbitrary units do not represent relative differences between various brain regions but can be used to measure changes within the same brain region. Analyses of hypothalamic cell groups include the following distances posterior to Bregma: the arcuate nuclei for NPY and AgRP analysis: -3.1mm to -3.6mm, and POMC analysis: -2.6mm to -3.8mm.

Immunohistochemistry

Floating coronal sections were incubated in primary antibody against biotin (goat anti-biotin; 1:5,000,000; Vector labs; Burlingame, CA) or PACAP (rabbit anti-PACAP38; 1:10,000; Bachem; Torrance, CA) for 48 hours at 4°C. After washes in PBS sections were incubated in biotinylated secondary antibody (1:600; Vector labs) for 1 hour at room temperature. Next, sections were washed again and incubated in a

peroxidase-based avidin/biotin solution using the Vectastain Elite ABC kit (Vector labs) for 1 hour at room temperature. Finally, immunohistochemical staining was visualized using a 3,3'-diaminobenzidine (DAB) chromogen solution containing nickel.

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Tukey HSD analysis was used for all post-hoc group comparisons. Statistical analyses were performed using JMP9 (SAS Institute; Cary, NC). P values less than 0.05 were considered statistically significant.

Results

Hypothalamic PAC1R mRNA expression and microinjection spread

Figure 2.1A shows *in situ* hybridization for PAC1R in the rat brain, confirming high levels of mRNA expression in the hypothalamus (Hashimoto et al. 1996). Note the most abundant mRNA expression is found in the medial cell groups of the hypothalamus, which include the dorsomedial nuclei (DMN), VMN, and arcuate nuclei (ARC; Fig 2.1B and C). In Figure 2.2, immunohistochemical staining of biotinylated PACAP confirmed that the 0.25 µl microinjection spread was an optimal volume (Fig 2.2A), as it did not exceed the boundaries of the VMN (right panel) delineated in the adjacent nissl stained section (left panel). Figure 2.2B shows PACAP immunoreactivity within the VMN of both neuronal cell bodies and terminals suggesting endogenous PACAP neurotransmission in this region.

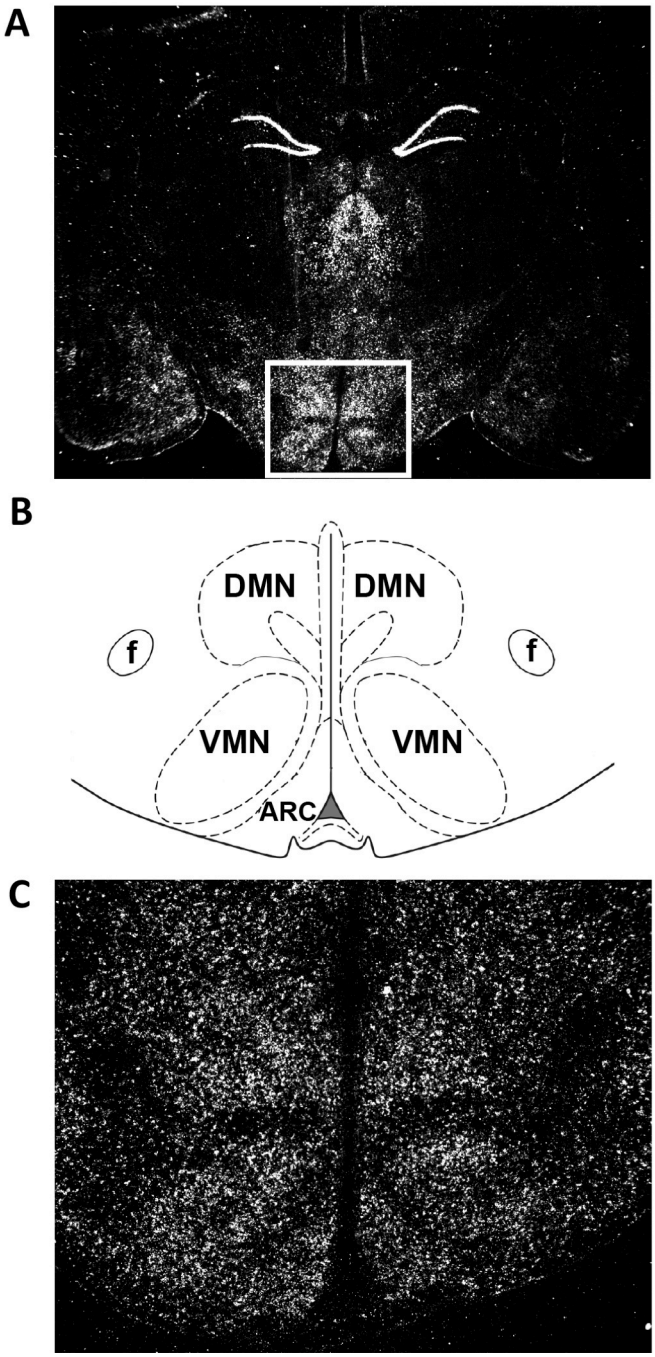

Figure 2.1. *In situ* hybridization of hypothalamic PAC1R mRNA distribution. (a) Darkfield photomicrograph (1.25X) of PAC1R mRNA expression in the rat. (b and c) Schematic and darkfield photomicrograph (5X) of region highlighted in (a) illustrating the dense expression of PAC1R mRNA in the medial nuclei of the hypothalamus. DMN = dorsomedial nuclei, VMN = ventromedial nuclei, ARC = arcuate nuclei, f = fornix. Anterior/Posterior coordinate of section depicted is -3.0 mm relative to Bregma.

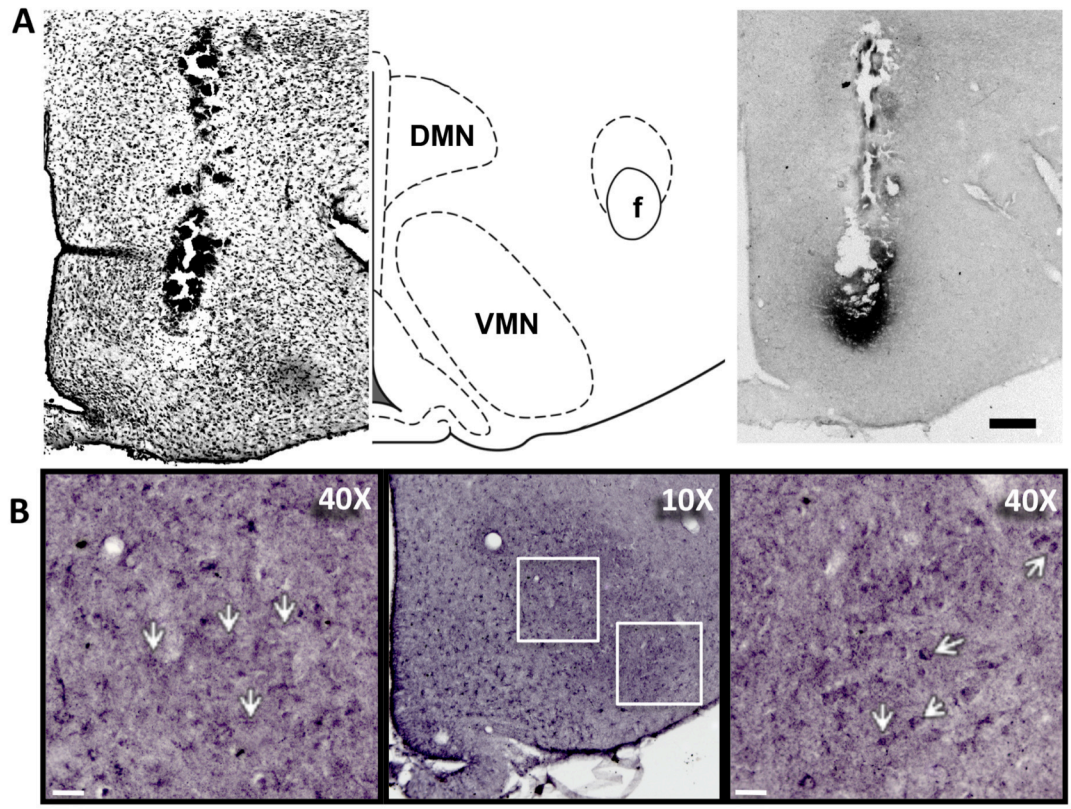

Figure 2.2. Ventromedial nuclei injection spread of biotinylated PACAP and endogenous PACAP protein immunohistochemistry. Staining for biotin-labeled PACAP (a; far right panel; scale bar = 200 µm) accompanied by adjacent cresyl violet staining (a; far left panel) shows that spread of injectate is contained within the boundaries of the VMN. Schematic image corresponding to the level of injection site is shown in the middle panel (a). PACAP immunoreactivity in the VMN is shown at low magnification (b; middle panel), with a left inset (b; far left panel) showing terminal labeling in the VMN (white arrows), and the right inset (b; far right panel) showing neuronal cell body immunoreactivity (white arrows). Anterior/posterior coordinate of section depicted is -2.76 mm relative to Bregma. Scale bar = 30 µm.

Feeding Behavior

PACAP injected into the VMN decreased normal feeding as demonstrated by significant main effects by repeated measures ANOVA ($P < 0.05$; Fig. 2.3A). At three hours post-injection both 25 and 50 pmol doses of PACAP significantly reduced cumulative food intake compared to saline controls, with 25 pmol reducing feeding by

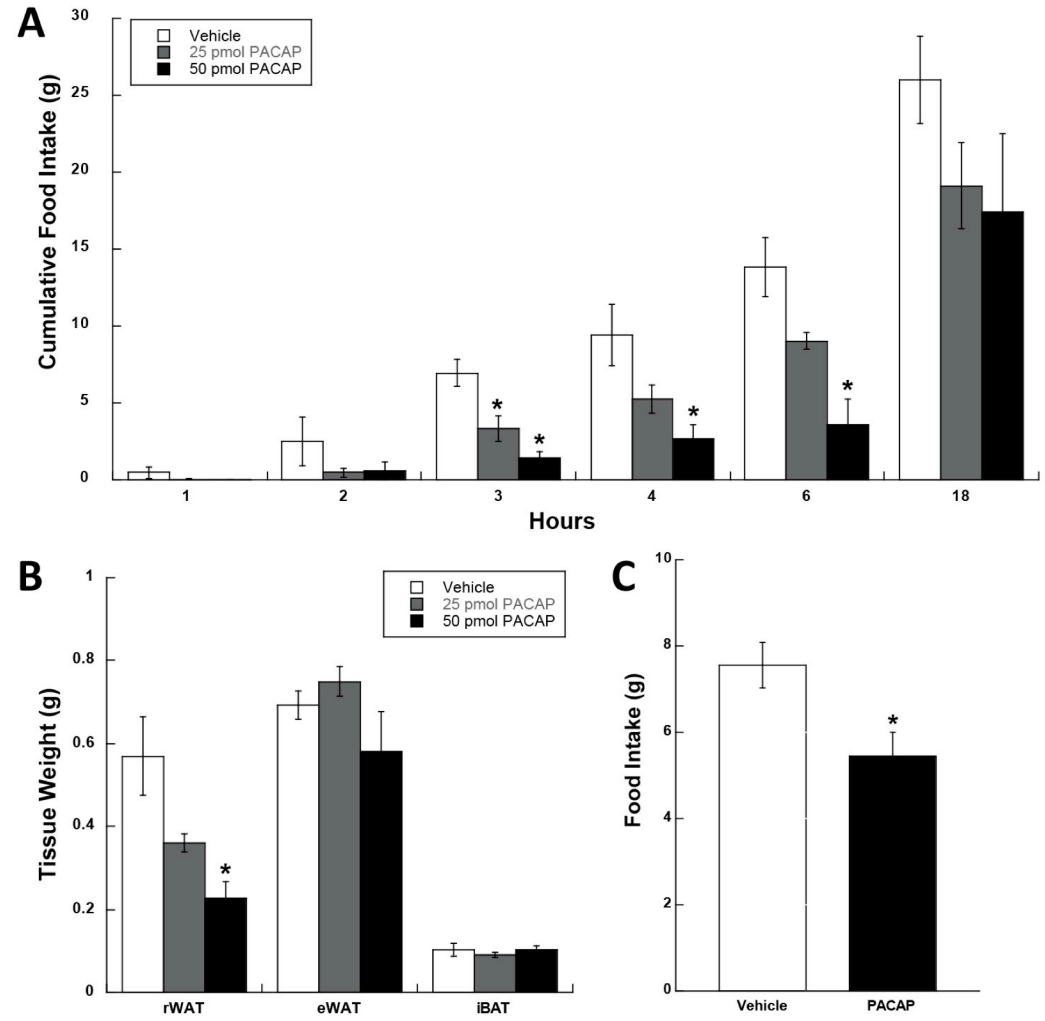

Figure 2.3. PACAP injections into the VMN dose dependently decrease feeding behavior and retroperitoneal white adipose tissue (rWAT). (a) Both 25 pmol and 50 pmol bilateral PACAP injections (0.25 µl/side) result in significantly reduced cumulative food intake after three hours, while the effect of 50 pmol PACAP lasts for six hours post-injection. (b) PACAP injections specifically reduce rWAT mass after 18 hours. (c) 50 pmol PACAP reduces refeeding (1 hour after PACAP administration) in animals following an overnight fast. Data are expressed as mean ± SEM, * = $P < 0.05$ compared to vehicle.

52% ($P < 0.05$) and 50 pmol by 80% ($P < 0.01$). Furthermore, the 50 pmol dose of PACAP continued to significantly decrease cumulative food intake at four hours ($P < 0.05$) and six hours post-injection ($P < 0.01$). 18 hours after injections no significant

differences were observed across any groups for food intake, but fat pad analysis revealed significant reduction in rWAT weight in animals receiving bilateral 50 pmol PACAP infusion ($P < 0.05$; Fig. 2.3B). No significant changes in eWAT or iBAT weights were found.

PACAP also significantly reduced food intake following an overnight fast. Bilateral VMN-injected PACAP at a dose of 50 pmol decreased food intake compared to saline controls after one hour of refeeding during the light cycle ($P < 0.05$; Fig. 2.3C).

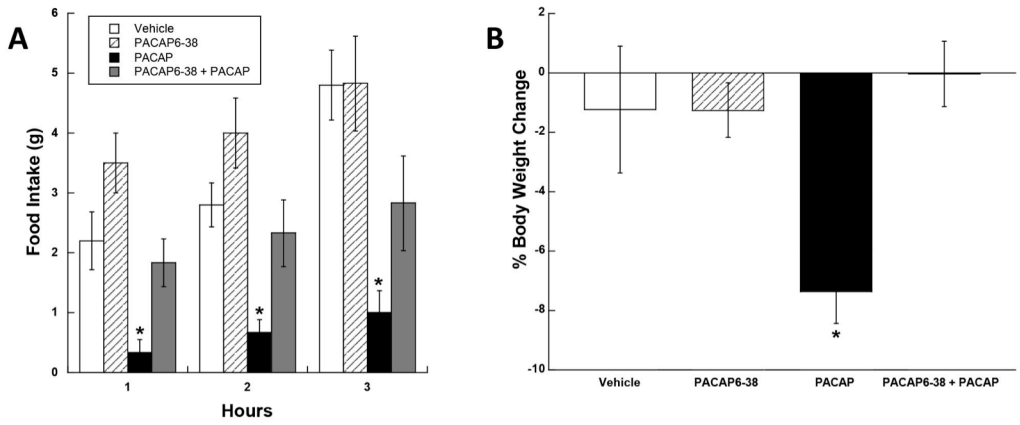

Figure 2.4. PACAP effects on feeding are mediated by the PAC1 receptor. (a) PACAP-induced hypophagia (220 pmol), and (b) body weight loss (24 hours) are prevented by the PAC1R antagonist, PACAP6-38 (2.2 nmol). Data are expressed as mean ± SEM, * = $P < 0.05$ compared to vehicle.

PACAP receptor antagonism

Specific antagonism of PAC1R prior to injection of PACAP completely blocked PACAP-induced decreases in food intake and body weight. Rats received a bilateral pretreatment of saline or PACAP6-38 approximately five minutes prior to bilateral saline or PACAP injections into the VMN. Significant main effects were observed by repeated

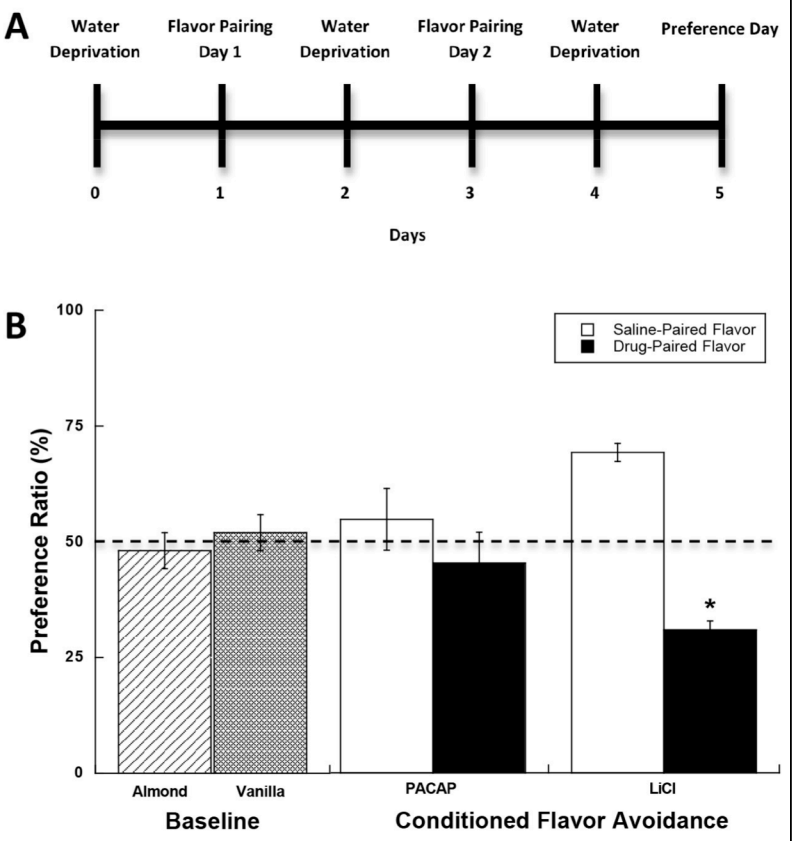

Figure 2.5. Hypothalamic PACAP injections do not produce conditioned flavor avoidance. (a) Schematic of the conditioned flavor avoidance behavioral paradigm shows the procedural timeline of the experiment. (b) Preference ratios expressed as mean ± SEM for PACAP (50 pmol) and LiCl (0.15 M; 2% body weight, ip) treated animals show a clear avoidance of the LiCl-paired flavor, but not the PACAP-paired flavor. There was no difference in baseline intake of either flavor. * = P<0.01 compared to saline-paired flavor.

measures ANOVA for food intake ($P < 0.05$; Fig. 2.4A). Animals receiving vehicle as a pretreatment prior to PACAP showed significantly reduced food intake compared to vehicle controls with a reduction in food intake by 85% ($P < 0.05$) over the first hour, 76% ($P < 0.05$) over two hours, and 79% ($P < 0.01$) over three hours. Food intake levels in animals receiving pretreatment with PACAP6-38 prior to PACAP did not differ from vehicle controls. Body weight measurements 24 hours after injections showed a

significant decrease in the vehicle/PACAP group compared to all other groups ($P < 0.05$; Fig. 2.4B).

Conditioned flavor avoidance

Baseline consumption of the two novel flavors were tested by giving water deprived naïve rats a two-bottle choice test for 30 minutes, and measuring intake of both almond and vanilla flavored water. There was no difference in intake volumes of the two flavors in the baseline experiment (52% almond, 48% vanilla; Fig. 2.5A). In the conditioned flavor avoidance experiment (CFA) bilateral infusion of 50 pmol PACAP on pairing day one was evenly distributed between the two flavors. Furthermore, intake volumes on both pairing days were measured, and rats drank at least 13 ml of flavored water on each pairing day demonstrating that every animal experienced both flavors prior to the test day. PACAP had no effect on the two-flavor choice test, with animals drinking similar volumes of both the PACAP-paired and vehicle-paired flavor (Fig. 2.5B). As a positive control, a separate group of animals receiving ip injections of LiCl showed significant avoidance of the LiCl-paired flavor compared to saline treated controls ($P<0.01$; Fig 2.5B).

Neuropeptide mRNA expression changes

Semi-quantitative *in situ* hybridization analyses of AgRP, NPY, and POMC mRNA in the hypothalamic arcuate nuclei following bilateral PACAP injections into the VMN were performed at one hour post-injection (Fig 2.6A). Quantification of the *in situ* hybridization revealed a significant increase in arcuate POMC mRNA expression by 52%

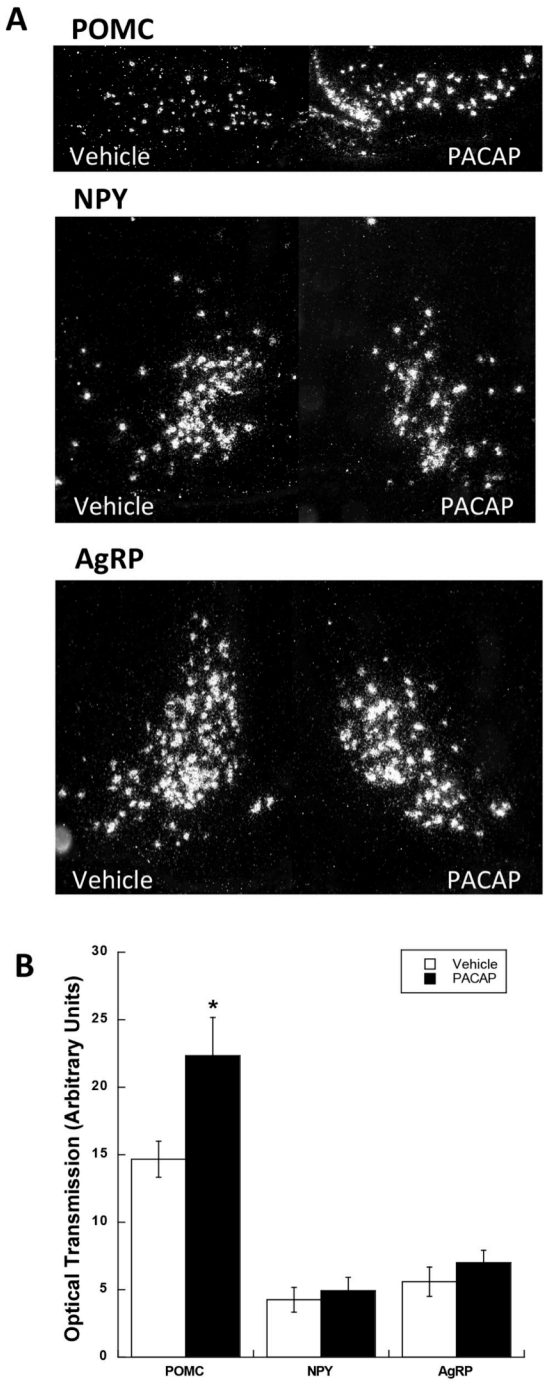

Figure 2.6. *In situ* hybridization of hypothalamic arcuate nuclei neuropeptide mRNA expression levels following PACAP injection into the VMN. (a) Representative darkfield photomicrographs showing mRNA expression levels of POMC, NPY, and AgRP in the ARC from vehicle treated (left side of photo) and PACAP treated (220 pmol; right side of photo) animals. (b) Graphical representation of optical transmission from *in situ* hybridization signal for each neuropeptide. Data are expressed as mean ± SEM, * = $P < 0.05$ compared to vehicle.

($P < 0.05$; Fig. 2.6B), while no significant differences were observed in either arcuate AgRP or NPY mRNA expression.

Discussion

Bilateral injections of PACAP into the VMN produce a marked reduction in feeding under both normal and food restricted conditions. Importantly, this reduction in feeding is not the result of malaise induced by PACAP administration, as there was no evidence of conditioned flavor avoidance. To diminish potential confounding contributions of PAC1R stimulation in surrounding hypothalamic cell groups, we made efforts to confine our PACAP injections within the boundaries of the VMN, which was clearly demonstrated by the biotinylated PACAP.

Under normal feeding conditions, injections of either 25 pmol or 50 pmol PACAP per side into the VMN significantly decreased in cumulative feeding by three hours post-injection, and lasting for up to six hours post-injection in animals receiving the higher dose of PACAP. The delayed effect on feeding behavior may be a result of injections administered approximately two hours prior to lights off, a time when normal food intake is still considerably low suggesting that PACAP-induced suppression of feeding behavior occurs when endogenous feeding behavior is actively stimulated. This is further supported by results following an overnight fast, where PACAP injections effectively reduced feeding in food-deprived animals one hour post-injection, as well as in the PACAP receptor antagonist study where animals injected with PACAP more proximal to the onset of dark did significantly reduce feeding after one hour. In addition to influencing feeding behavior, we also observed a significant reduction in rWAT weight at 18 hours post-injection with no differences in any of the other fat depots measured. While

the reduction in only rWAT is curious and the underlying mechanism still unknown, others have also found rWAT to be more labile during conditions of mild/moderate weight loss (Giordano et al. 2005; Li et al. 1998).

Results obtained following PACAP injections into the VMN closely resemble reports of ventricular PACAP injections (Hawke et al. 2009; Morley et al. 1992; Mounien et al. 2009), which suggests that the medial hypothalamus may be an important site for PACAP signaling pertaining to energy homeostasis. This is further supported by the dense expression of PAC1R mRNA found in multiple subdivisions of the medial hypothalamus such as the dorsomedial (DMN), arcuate (ARC), and VMN, all of which are major contributors to the control of energy balance (Fig. 2.1). Although resulting in different patterns in feeding behavior, lesions of all three hypothalamic subgroups produce obese rats (Choi and Dallman 1999). To begin identifying the circuitry involving PACAP signaling and energy regulation, we demonstrate that changes in feeding behavior following PACAP administration into the VMN are specifically mediated through the PAC1R. In addition, while our chosen dose of PAC1R antagonist did not produce significant differences in feeding from vehicle treated controls, food intake was elevated in these animals suggesting that a higher dose of PACAP6-38 may have produced a significant increase. Changing the timing of PAC1R antagonist injections to a period of minimal feeding behavior would aid in making conclusions about the role of endogenous PACAP signaling in the VMN and feeding behavior. As an initial point of observation, the VMN may function as an important, but not exclusive, site of action for mediating the behavioral effects of PACAP signaling. For example, PACAP's actions in the hypothalamus may also include direct or indirect activation of feeding and/or

metabolic circuitries involving other hypothalamic cell groups such as the PVN and ARC. As mentioned earlier, both the PVN and ARC have prominent roles in regulating energy balance and are both known to contain both PACAP receptors and PACAP protein, making these areas attractive targets for future studies.

Previous reports have suggested PACAP-induced hypophagia following icv injection in mice may stimulate melanocortin signaling in the ARC, a well characterized system known to decrease feeding behavior (Mounien et al. 2009). Indeed, PACAP receptors have been found on both POMC and NPY containing neurons in the ARC through which activation leads to stimulation of POMC mRNA, c-fos expression and cytosolic Ca^{2+} concentrations (Mounien et al. 2006a; Mounien et al. 2006b; Mounien et al. 2009; Nakata et al. 2004). However, the current results demonstrate that bilateral injections of PACAP directly into the VMN also increase mRNA expression of POMC in the ARC one hour after injection without affecting NPY or AgRP expression. Since the vast majority of neurons in the VMN are glutamatergic (Fu and van den Pol 2008), activation of PACAP receptors in the VMN could have an excitatory effect on VMN neurons through modulation of AMPA and NMDA currents, as seen in the hippocampus (Costa et al. 2009; Macdonald et al. 2005; Yaka et al. 2003), leading to VMN-mediated excitation of POMC neurons in the ARC (Sternson et al. 2005). Additionally, strong efferent connections from the VMN to the nucleus of the solitary tract (NTS) have been established, and it is possible that stimulation of the VMN by PACAP may cause increased POMC signaling in the NTS as well (Canteras et al. 1994). In support of the current data, increased POMC signaling in the NTS has also been associated with inhibition of food intake (Li et al. 2007; Zhang et al. 2010).

While there has been evidence for PACAP stimulation of NPY neurons in the ARC (Mounien et al. 2006a; Nakata et al. 2004), our data did not demonstrate any effect at one hour post-injection, possibly arguing for POMC-specific ARC activation by PAC1R expressing neurons from the VMN (Sternson et al. 2005). Given the initial constellation of effects produced by icv or *ex vivo* application of PACAP such as hypophagia, thermogenesis, increased POMC and NPY signaling, further studies are needed to delineate PACAP's integrated actions on multiple hypothalamic cell groups (Masuo et al. 1995; Mounien et al. 2009; Nakata et al. 2004; Resch et al. 2011).

Although the VMN is known to express large amounts of PACAP, which appear to be responsive to varying nutritional conditions (Hawke et al. 2009; Mounien et al. 2009), the origin of PACAP neurons synapsing onto VMN neurons is not known. In light of the current results, brain regions releasing PACAP into the medial hypothalamus that are sensitive to signals of nutritional status are likely sources of PACAP input into the VMN. One such candidate is the medial amygdala because of its dense PACAP mRNA expression, identified connections to the VMN, and its glucose sensing capability (Anand et al. 1964; King 2006a; Sakaguchi and Bray 1987; Sudo et al. 1991; Zhou et al. 2010). In addition to extra-hypothalamic regions of PACAP input, paracrine/autocrine signaling from VMN neurons themselves may also provide a notable source of PACAP signaling, especially since the VMN are well known to have extensive intrinsic and contralateral VMN-VMN synaptic circuitry (Kiss et al. 2011; Nishizuka and Pfaff 1989). Further anatomical studies are needed to identify hypothalamic circuitry involving PACAP signaling.

CHAPTER III

MULTIPLE SITES OF ACTION FOR PACAP-INDUCED HYPOPHAGIA IN THE HYPOTHALAMUS: FOCUS ON GLUTAMATERGIC CELL GROUPS

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a key regulator of several hypothalamic systems, including stress (Agarwal et al. 2005), osmoregulation (Gillard et al. 2006), thermoregulation (Gray et al. 2002), and body weight (Hawke et al. 2009). PACAP was first discovered to influence energy homeostasis through inhibition of feeding in mice following a single intracerebroventricular (icv) injection of the peptide (Morley et al. 1992). These results combined with reports of diet-specific alterations of PACAP mRNA expression in the hypothalamic ventromedial nuclei (VMN) suggest that PACAP neurons are responsive to nutritional status and directly tied to the regulation of feeding (Hawke et al. 2009; Mounien et al. 2009).

As a ligand, PACAP binds to three different G protein-coupled receptors (GPCR), the PAC1 receptor (PAC1R), and the receptors originally discovered as targets of vasoactive intestinal polypeptide (VIP), VPAC1R and VPAC2R (Dickson and Finlayson 2009). Although PACAP stimulates all three receptor subtypes it has the highest affinity for the PAC1R, whereas VIP is less likely to utilize the PAC1R due to its low binding affinity compared to the VPACRs (Gottschall et al. 1990; Lam et al. 1990).

Due to the limited availability of conditional PACAP transgenic models, site-specific injections of PACAP and related pharmacological agents directly into discrete hypothalamic nuclei are necessary to selectively investigate the role of PACAP signaling

within the hypothalamus. Recently, we reported that site-specific injections of PACAP isolated to the VMN reduce food intake via PAC1R signaling (Resch et al. 2011). Despite abundant PACAP receptor expression in several different hypothalamic nuclei, little attention has been given to other hypothalamic cell groups with regard to PACAP's effects on food intake and body weight. In the few studies that have examined PACAP signaling in the hypothalamus, the paraventricular nuclei (PVN) are reported to show PACAP terminal immunoreactivity (Das et al. 2007; Legradi et al. 1998), and following direct administration of PACAP into the PVN changes in grooming behavior (Norrholm et al. 2005) and hepatic glucose production (Yi et al. 2010). However, these studies did not report on PACAP-induced changes in feeding or body weight. Given that PVN lesion studies demonstrate an imperative role for this area of the hypothalamus in the regulation of energy balance (Leibowitz et al. 1981), and both the PVN and PACAP have been linked to melanocortin signaling involved in control of body weight (Balthasar et al. 2005; Mounien et al. 2009), further examination of the relationship between PACAP signaling and PVN-mediated energy homeostasis is warranted. In order to examine the effects of PACAP signaling in the hypothalamus on food intake we have executed site-specific PACAP injections in two separate hypothalamic nuclei, the PVN and VMN.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 225-250 g were individually housed in a climate controlled room with a 12 hr light/dark cycle. Animals had free access to Harlan standard diet (8604 formulation) and water. Food consumption

was measured with a BioDAQ Food Intake Monitor (Research Diets; New Brunswick, NJ) or calculated by pre-weighing food in each bin and subtracting the weight of non-ingested and spilled food at the end of each measurement period. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; ip) cocktail and placed in a stereotaxic apparatus. 26 gauge bilateral guide cannulae (Plastics One; Roanoke VA) were placed 3 mm dorsal to the target site in all animals, and secured to the surface of the skull with an acrylic resin. The stereotaxic coordinates for the PVN injection site were anterior/posterior, -1.7 mm from bregma; medial/lateral, ±0.5 mm from midline; dorsal/ventral, -4.9 mm, and for the VMN anterior/posterior, -2.5 mm from bregma; medial/lateral, ±0.6 mm from midline; dorsal/ventral, -6.2 mm from surface of the skull based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos and Watson 2007). Injectors extended 3 mm past the ventral tip of the cannulae reaching an injection site of -7.9 mm for PVN and -9.2 mm for VMN ventral from the surface of the skull. The upper incisor bar was positioned -3.3 mm below horizontal zero. A bilateral dummy stylet placed in the guide cannulae was used to maintain patency. The animals were given at least five days to recover after cannula installation before receiving drug or vehicle injections during which time the animals were handled and dummy stylets were removed and replaced daily in order to acclimate the animals to the physical handling necessary during experiments. Correct cannulae placements were confirmed at the conclusion of each experiment by microscopic

examination of Nissl stained sections and only those with correct placement were included in the studies.

Microinjections and Injection Spread

In all experiments, PACAP (50 pmol/0.25 µl/side; PACAP38; California Peptide Research; Napa, CA), PACAP6-38 (500 pmol/0.25 µl/side; Anaspec; Fremont, CA), VIP6-28 (500 pmol/0.25 µl/side; Bachem; Torrance, CA), or saline vehicle was microinjected through bilateral guide cannulae over approximately two minutes in awake animals while gently restrained. Following each injection an additional minute elapsed before removing injectors to minimize backflow of injected material. The optimal injection volume and subsequent spread within the VMN was determined previously (Resch et al. 2011), and the same procedure was used for PVN injection spread analysis. Briefly, biotinylated PACAP (50 pmol/0.25 µl/side; PACAP38-Biotin; Anaspec; Fremont, CA) was injected into the PVN or VMN. One hour following biotinylated PACAP injections animals were perfused with 0.9% NaCl and 4% paraformaldehyde in phosphate-buffered saline, and brains were removed. Brain sections containing the injection sites and surrounding areas were then immunohistochemically stained using a primary antibody against biotin. Immunohistochemical signal was then compared to the cresyl violet staining of adjacent sections to determine surgical accuracy and the spread of the biotinylated PACAP injection within the target injection site.

Feeding Behavior

Animals were weighed daily and acclimated to the BioDAQ Food Intake Monitor for at least 7 days before the onset of the experiment. On the experiment day,

approximately 1 hour prior to lights off rats were injected bilaterally with vehicle or 50 pmol PACAP. Feeding measurements were collected for the next 24 hours, as well as a final measurement of body weight at 24 hours post-injection. For BioDAQ meal pattern analysis the data were analyzed over the first six hours post-injection to determine latency to meal onset, meal amount, duration, and eating rate. Meals were defined as food intake of 0.2 g or more with less than 15 minutes elapsing between feeding bouts (Dunn-Meynell et al. 2009; Farley et al. 2003). For studies involving antagonism of PACAP receptors, rats were bilaterally pretreated with saline, PACAP6-38 (500 pmol/0.25 µl/side; Anaspec; Fremont, CA), or VIP6-28 (500 pmol/0.25 µl/side; Bachem; Torrance, CA). PACAP6-38 is a widely used PAC1R antagonist however it also has antagonistic properties at the VPAC2R (Gourlet et al. 1995; Hawke et al. 2009; Mounien et al. 2009; Robberecht et al. 1992), while VIP6-28 is reported to be a potent nonselective VPAC receptor antagonist (Fishbein et al. 1994; Mohney and Zigmond 1998; Shoge et al. 1998) shown to be effective in the hypothalamus (Hermes et al. 2009). Five minutes after administering the antagonist rats received a second bilateral injection of either saline or 50 pmol of PACAP followed by subsequent physiological and behavioral measurements.

In Situ Hybridization

Brains were sectioned coronally at 12 µm using a cryostat, thaw-mounted onto electrostatically clean slides, and stored at -80°C until post-fixed. Prior to hybridization, sections were post-fixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25% acetic anhydride. Standard *in vitro* transcription methods were used to generate both sense and antisense riboprobes recognizing VPAC2R and PAC1R

transcripts (Choi; Milwaukee, WI), which were subsequently diluted in hybridization cocktail (Amresco; Solon, OH) with tRNA. Sections were hybridized overnight at 55°C with either digoxigenin (DIG) or fluorescein isothiocyanate (FITC) labeled riboprobes. After hybridization, slides were treated with RNase A, and stringently washed in 0.5x SSC at 65°C (VPAC2R and PAC1R) for 30 minutes. Slides were then incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche; Indianapolis, IN) overnight at 4° C. Riboprobe signal was further enhanced using the TSA-plus fluorophore system with either fluorescein or Cy3 (Perkin Elmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

Biotinylated PACAP injection spread was visualized following standard free-floating immunohistochemical techniques. Floating coronal sections were incubated in primary antibody against biotin for 24 hours at 4°C. After washing in PBS, sections were incubated in biotinylated secondary antibody (Vector labs; Burlingame, CA) for 1 hour at room temperature. Following a second wash, sections were incubated in a peroxidase-based avidin/biotin solution using the Vectastain Elite ABC kit (Vector labs; Burlingame, CA) for 1 hour at room temperature. Immunohistochemical staining was visualized using a nickel enhanced 3,3'-diaminobenzidine (DAB) chromogen solution.

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fischer LSD analysis was used for all post-hoc group comparisons. Statistical analyses were

performed using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $P < 0.05$ were considered statistically significant.

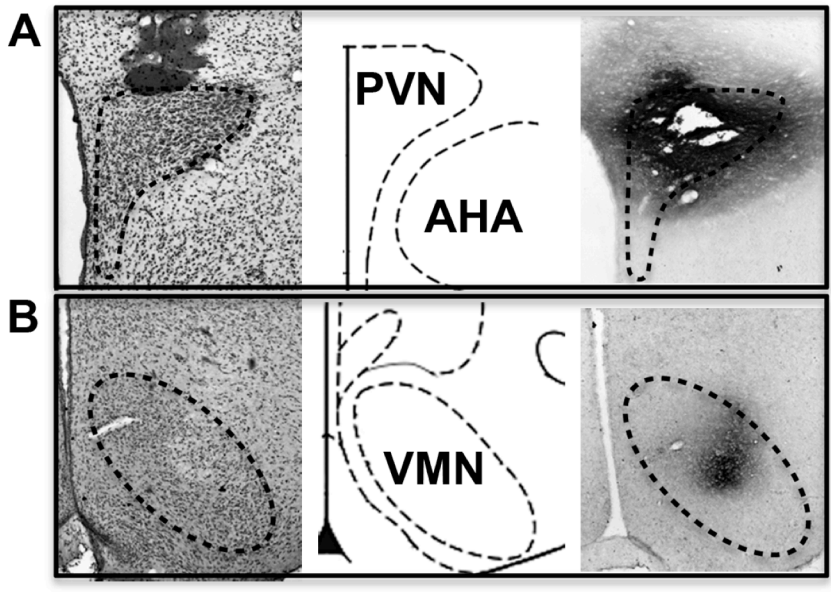

Figure 3.1. Injection spread of biotin-labeled PACAP within the PVN and VMN. Staining for biotin-labeled PACAP (far right panel) accompanied by adjacent cresyl violet staining (far left panel) shows that spread of injectate is contained within the boundaries of the PVN (A; Bregma -1.8 mm) or VMN (B; Bregma -3.0 mm). Schematic image corresponding to the level of injection site is shown in the middle panel adapted from *The Rat Brain in Stereotaxic Coordinates 6th Edition* (Paxinos and Watson 2007). PVN, paraventricular nuclei; AHA, anterior hypothalamic area; VMN, ventromedial nuclei

Results

To assess diffusion of PACAP microinjections into our target nuclei we injected a biotinylated PACAP peptide to simulate behavioral experiments followed by histological analysis of injection sites. Following immunohistochemical detection of the biotinylated PACAP (Fig. 3.1) we confirmed that peptide injection volumes were appropriate and did not diffuse outside of the borders of the PVN or VMN. Our prior studies have indicated that bilateral PACAP injections of 50 pmol into the VMN significantly decreased food

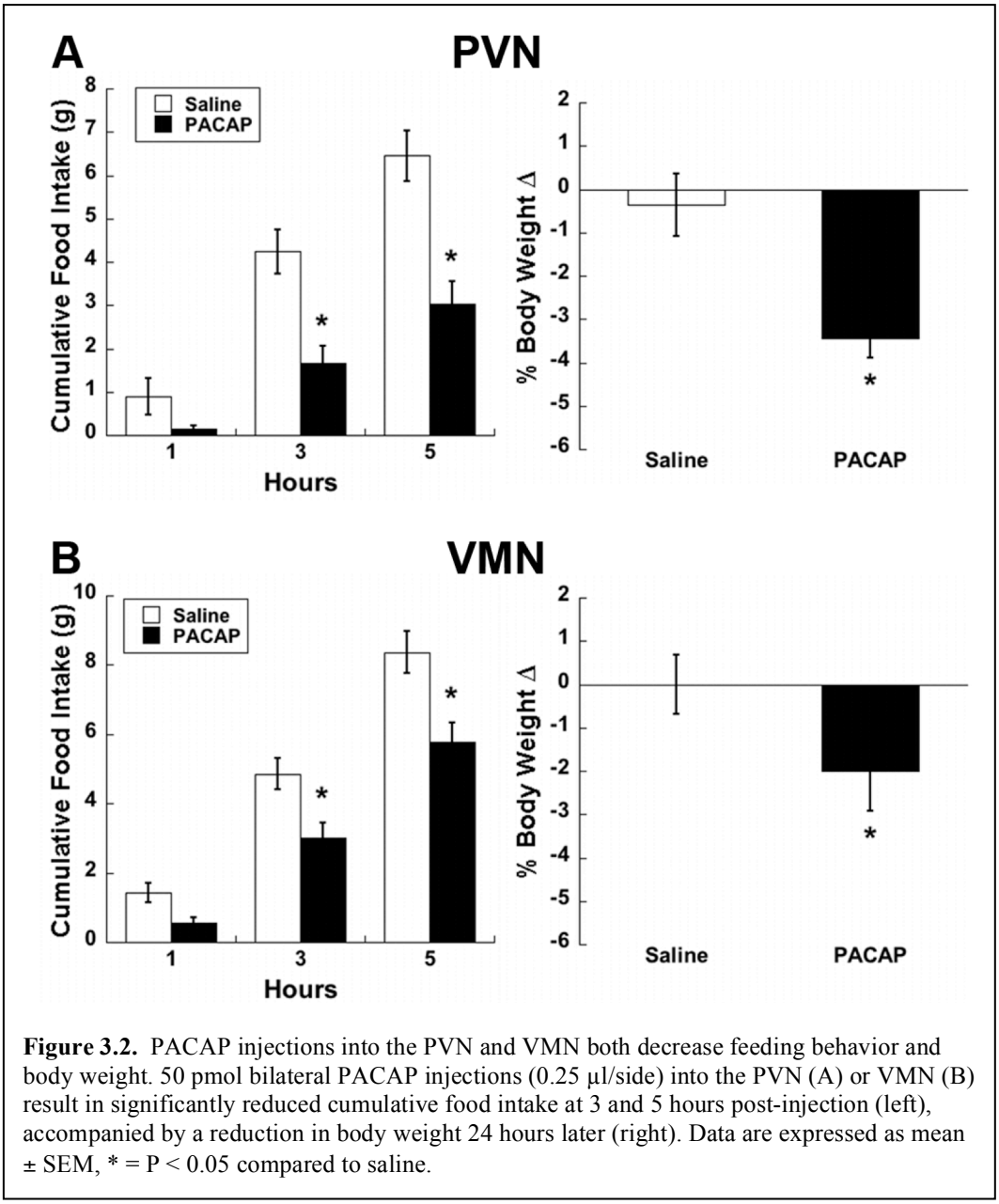

Figure 3.2. PACAP injections into the PVN and VMN both decrease feeding behavior and body weight. 50 pmol bilateral PACAP injections (0.25 µl/side) into the PVN (A) or VMN (B) result in significantly reduced cumulative food intake at 3 and 5 hours post-injection (left), accompanied by a reduction in body weight 24 hours later (right). Data are expressed as mean ± SEM, * = $P < 0.05$ compared to saline.

intake (Resch et al. 2011), but the effects of PACAP injections into the PVN on food intake had yet to be reported. We performed feeding behavior experiments under free-feeding conditions starting at approximately 1 hour prior to lights off following saline or PACAP injections into the PVN and compared subsequent responses to PACAP-induced

hypophagia in the VMN. Feeding behavior in both PVN and VMN animals treated with PACAP showed significant main effects by two-factor repeated measures ANOVA (treatment $P < 0.01$; time $P < 0.001$; interaction $P < 0.05$). Multiple comparison analysis performed at 1, 3, and 5 hours post-injection resulted in significant differences at 3 (PVN, $P < 0.001$; VMN, $P < 0.01$) and 5 hours (PVN, $P < 0.001$; VMN, $P < 0.001$) compared to controls (Fig 3.2). Body weight following the injection day was also significantly decreased (Fig. 3.2) with PVN-PACAP treated animals losing 3% ($P < 0.001$) and VMN-PACAP treated animals losing 2 % ($P < 0.05$) of their pre-injection body weight compared to saline injections which left body weight unchanged.

Table 3.1. Meal pattern analysis following PACAP treatment in the PVN and VMN

	PVN		VMN	
	Saline	PACAP	Saline	PACAP
Latency to 1st meal (min)	47.9 ± 12.8	105.9 ± 25.1*	29.6 ± 6.8	89.3 ± 27.5*
Latency to 2nd meal (min)	93.9 ± 13.1	75.6 ± 15.6	62.8 ± 10.1	60.2 ± 11.6
Duration of 1st meal (min)	42.6 ± 7.6	14.5 ± 4.0*	37.4 ± 5.8	48.8 ± 7.1
Duration of 2nd meal (min)	31.9 ± 4.8	15.4 ± 1.8*	28.8 ± 4.5	22.9 ± 3.6
Amount of 1st meal (g)	2.8 ± 0.7	1.0 ± 0.2*	2.8 ± 0.4	2.1 ± 0.3
Amount of 2nd meal (g)	3.0 ± 0.4	1.4 ± 0.3*	3.1 ± 0.5	2.5 ± 0.5
Average meal size (g)	3.1 ± 0.3	1.5 ± 0.2*	2.8 ± 0.2	2.3 ± 0.3
Meal Frequency	2.6 ± 0.2	2.5 ± 0.4	3.7 ± 0.3	3.3 ± 0.3
Total time spent eating (min)	91.2 ± 8.4	56.3 ± 14.5*	107.2 ± 6.9	104.6 ± 10.6
% of total time spent eating	25.3 ± 2.3	15.6 ± 4.0*	30.5 ± 1.7	29.1 ± 3.0
Rate of eating (mg/min)	22.0 ± 1.8	13.1 ± 2.7*	27.6 ± 1.1	21.0 ± 2.2*
24 hr intake (g)	19.4 ± 1.2	16.9 ± 1.0	21.7 ± 0.8	19.3 ± 1.1

Meal patterns were analyzed for six hour post-injection. Data are mean ± SEM. * = $P < 0.05$ compared to appropriate anatomical control

In order to better characterize the hypophagia produced by hypothalamic PACAP signaling we evaluated meal patterns from the experiment shown in Figure 3.2 (Table

3.1). PVN injections of PACAP significantly decreased meal size (1st meal, $P < 0.05$; 2nd meal, $P < 0.01$), meal duration (1st meal, $P < 0.01$; 2nd meal, $P < 0.01$), average meal size ($P < 0.01$), time spent eating (total time, $P < 0.05$; % time, $P < 0.05$), and rate of eating ($P < 0.01$), while also increasing the time it took to initiate the first meal (1st meal, $P < 0.05$; 2nd meal, $P = 0.375$). In contrast, PACAP administration into the VMN only produced a decrease in the rate of eating ($P < 0.05$), and increased the latency to initiate the first meal (1st meal, $P < 0.05$; 2nd meal, $P = 0.863$). While PACAP treated animals ate less, neither PVN nor VMN injected animals showed significantly altered 24-hour intake.

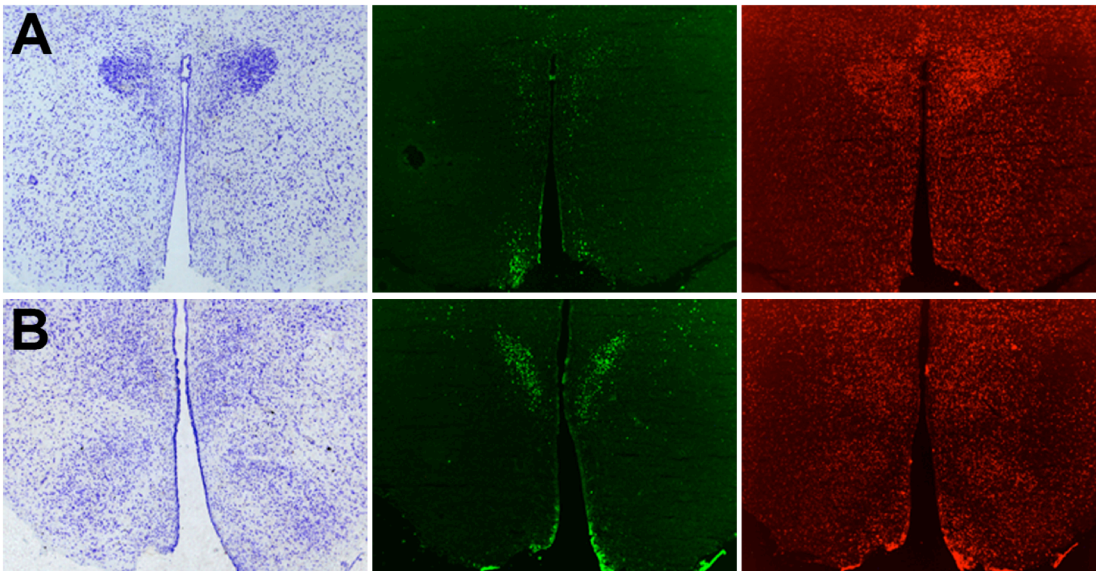

Figure 3.3. *In situ* hybridization of hypothalamic PACAP receptor mRNA. mRNA expression of the VPAC2R (middle panel) and PAC1R (far right panel) in the hypothalamic paraventricular (A; Bregma -1.8 mm), and ventro/dorsomedial nuclei (B; Bregma -3.0 mm) accompanied by adjacent sections stained with cresyl violet depicting the corresponding hypothalamic anatomy in the far left panel.

The diversity of effects that PACAP produces physiologically and behaviorally may be due to its multiple receptor subtypes, and the anatomical distribution of specific receptor expression. Fluorescent *in situ* hybridization revealed that the VPAC2R and

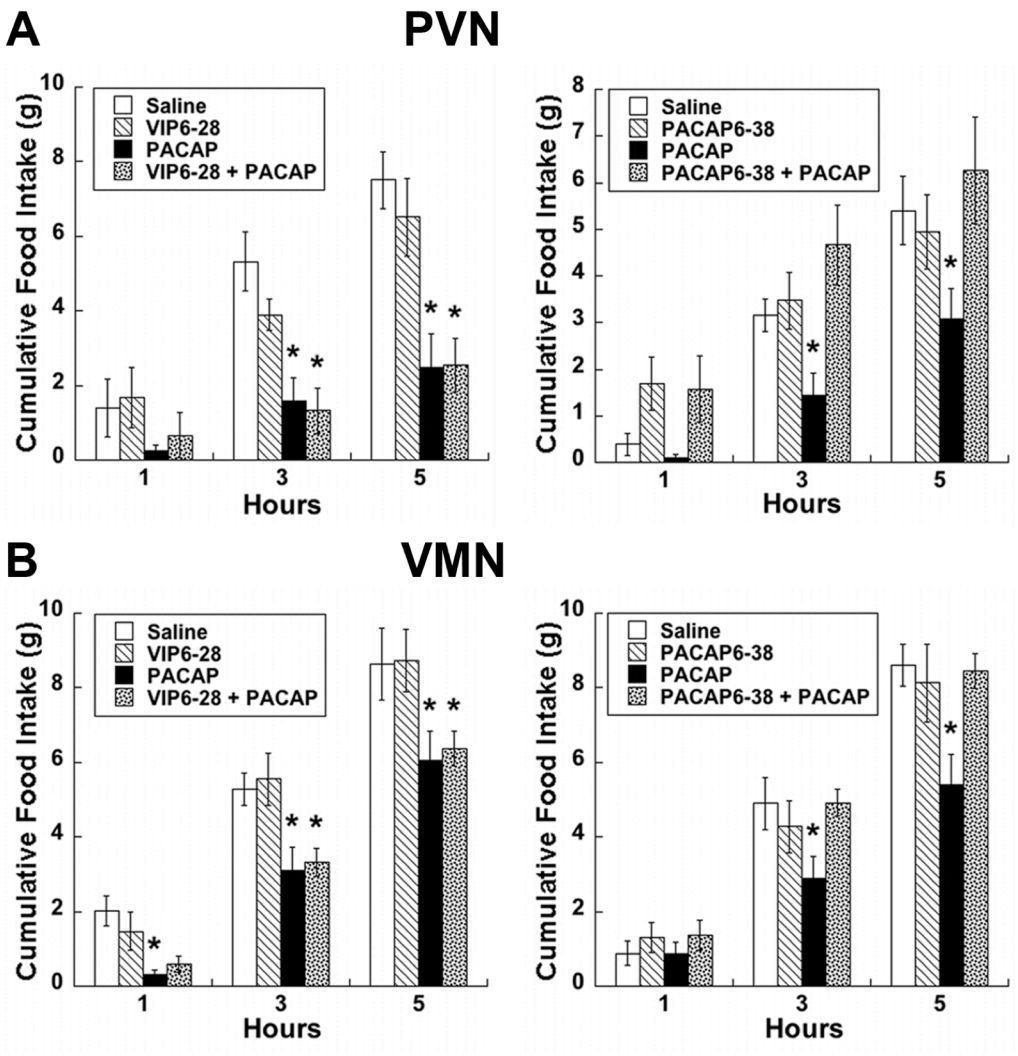

Figure 3.4. PACAP effects on feeding are mediated by PAC1R in both the PVN and VMN. In both the PVN (A) and VMN (B), PACAP-induced hypophagia (50 pmol/side) is prevented by the PAC1R antagonist, PACAP6-38 (500 pmol/side; right), but not the VPACR antagonist VIP6-28 (500 pmol/side, left). Data are expressed as mean ± SEM, * = $P < 0.05$ compared to saline.

PAC1R in the hypothalamus each have distinctive expression patterns (Fig. 3.3). VPAC2R mRNA expression was abundant in the central dorsomedial nuclei (DMN) and the medial PVN, while VPAC2R expression in the VMN was not detected. On the other hand, PAC1R mRNA expression was much more widely distributed, with abundant expression found throughout the hypothalamic nuclei, including PVN, DMN, VMN, and

ARC nuclei. Furthermore, the wide distribution of PAC1R mRNA expression may represent expression in both glial and neuronal cell types (Figiel and Engele 2000). Hybridization with sense probes to control for nonspecific binding did not show any signal for either receptor probe (data not shown).

Due to the expression of multiple PACAP receptors in the hypothalamus, we assessed the involvement of PAC1 and VPAC receptors in the regulation of feeding behavior by administering receptor antagonists prior to PACAP injections. Pretreatment with VIP6-28 did not alter the suppression of feeding behavior produced by PACAP in either the PVN or VMN compared to controls (Fig. 3.4). However, administration of PACAP6-38 completely reversed the effects of PACAP administration into the PVN at both 3 ($P < 0.05$) and 5 hr ($P < 0.01$) time points (Fig 3.4A). Similarly, pre-treatment with PACAP6-38 blocked the effects of PACAP administration into the VMN at both 3 ($P < 0.05$) and 5 hrs ($P < 0.001$) post-injection (fig 3.4B).

Discussion

The current study demonstrates that site-specific PACAP injections into either the ventromedial (VMN) or paraventricular nuclei (PVN) of the hypothalamus both produce long-lasting reductions in food intake, as well as changes in meal patterns, altered glucose homeostasis, and significant body weight loss 24 hours post-injection. Importantly, site-specific hypothalamic injections do not cause malaise (Resch et al. 2011) that can sometimes arise following exogenous peptide administration. Although there are multiple PACAP receptor subtypes expressed in the hypothalamus, the reduction in feeding behavior appears to be mediated primarily by the PAC1R subtype which is in agreement with prior feeding studies (Hawke et al. 2009; Mounien et al.

2009; Resch et al. 2011). Specifically, in both the PVN and VMN, the effects of PACAP could be blocked by the VPAC2R/PAC1R antagonist PACAP6-38, but not by the VPAC1R/VPAC2R antagonist VIP6-28.

To specifically address the impact of PACAP signaling on feeding behavior we placed site-specific microinjections of PACAP into both the PVN and VMN, which produced significant reductions in feeding. However, there were differences in PACAP-mediated meal pattern changes in the PVN versus the VMN. PACAP injected into the PVN produced several alterations in meal patterns, including increased latency to meal initiation, decreased meal size, decreased meal duration, decreased time spent eating, and decreased rate of eating. In contrast, PACAP injected into the VMN only produced an increase in the latency to meal initiation, and a decrease in the rate of eating. Taken together with the cumulative food intake data, PACAP's functional role in the PVN may be more specific to the regulation of feeding behavior compared to its actions in the VMN. These results are consistent with loss of function studies involving the PVN and VMN where there is a pronounced hyperphagia in PVN-lesioned animals, while loss of function following VMN-lesions produces a more modest hyperphagia often restricted to the light period (Choi and Dallman 1999). In light of the current data, combined with anatomical and biochemical reports involving PACAP, stress, and the hypothalamus (Agarwal et al. 2005; Choi et al. 1996; Dore et al. 2013; Hammack et al. 2010) it is plausible that central stress pathways may play a substantial role in the anorectic response caused by PACAP within these hypothalamic nuclei. Further investigation of stress and satiety mechanisms utilizing PACAP signaling will be important to the understanding of the role of this pleiotropic neuropeptide.

Due to the heterogeneous PACAP receptor population in the hypothalamus, and in particular the PVN, it was necessary to determine which PACAP receptor subtype mediated the effects on food intake. The most abundant and widely distributed PACAP receptor in the hypothalamus is the PAC1R, with high mRNA expression in all hypothalamic mediobasal cell groups. There is little evidence for any appreciable expression of VPAC1R mRNA in the hypothalamus, however the VPAC2R is expressed in several hypothalamic areas including the suprachiasmatic nuclei (SCN), PVN, and dorsomedial nuclei (DMN) (Usdin et al. 1994). Expression of multiple PACAP receptor subtypes in the hypothalamus suggests PACAP is a multifunctional neuropeptide signal that contributes to several behavioral and physiological systems. Feasibility of this concept is supported by reports linking PACAP to stress (Ressler et al. 2011), anxiety (Hammack et al. 2009), feeding (Hawke et al. 2009; Mounien et al. 2009; Resch et al. 2011), and glucose mobilization (Yi et al. 2010), all systems that may work synergistically, and converge on PACAP-enriched anatomical areas, such as the hypothalamus and amygdala (Hannibal et al. 1995a).

We examined both the VPAC2R/PAC1R antagonist PACAP6-38 and the VPAC1R/VPAC2R antagonist VIP6-28 (Fishbein et al. 1994) on their ability to inhibit the affects of PACAP on feeding. Even though there is no data to support VPAC1R expression in the hypothalamus, we used VIP6-28 to antagonize both the VPAC1R and VPAC2R subtypes to fully eliminate the participation of the VPAC receptors (Usdin et al. 1994). Furthermore, because PACAP6-38 has antagonistic activity at the VPAC2 receptor, VIP6-28 was utilized to rule out possible contributions of VPAC2R antagonism by PACAP6-38. Our findings were clear that, in both the PVN and VMN, pretreatment

with PACAP6-38 prior to PACAP injection completely abolished the effects of PACAP on feeding. While we had reported previously that the PAC1R was important for the feeding phenotype observed following PACAP injection into the VMN (Resch et al. 2011), it is worthy to note that VIP6-28 did not alter the effects of PACAP in the PVN on feeding behavior despite recent reports demonstrating the importance of PVN VPAC2Rs for the regulation of PACAP-induced hepatic glucose production (Yi et al. 2010). Additionally, PAC1R antagonist treatment alone caused increased feeding in the PVN, however the results were not significant, which suggests a dose-response of PACAP6-38 at higher concentrations may result in significantly increased food intake and provide stronger evidence for endogenous PACAP signaling. Taken together there may be anatomical and receptor specific roles for PACAP in the PVN with PAC1R governing the feeding behavior response, while VPAC2R may govern the sympathetic-driven glycemic response to PACAP.

CHAPTER IV

SITE-SPECIFIC ACTIVATION OF ENERGY EXPENDITURE WITHIN THE HYPOTHALAMUS BY PACAP

Introduction

PACAP signaling is an important regulator of feeding behavior (Chance et al. 1995; Hawke et al. 2009; Morley et al. 1992; Mounien et al. 2009; Resch et al. 2011; Resch et al. 2013). Yet, pair-feeding experiments demonstrate that body weight loss from PACAP injections is not solely due to decreased food intake, suggesting that PACAP may also increase energy expenditure (Hawke et al. 2009). In fact, icv injections of PACAP increase core body temperature, oxygen consumption, and locomotor activity (Hawke et al. 2009; Masuo et al. 1995; Mizuno et al. 1998), as well as increases in sympathetic nerve activity and heart rate (Tanida et al. 2010).

In contrast to pharmacological data, PACAP knockout mice are not metabolically insufficient nor are they overweight. Instead, PACAP and PAC1R knockout mice are lean, temperature sensitive, and have impaired glucose homeostasis (Adams et al. 2008; Gray et al. 2002; Hamelink et al. 2002; Jamen et al. 2000; Tomimoto et al. 2008). Given the disparity between phenotypes of PACAP knockout mice and pharmacological data from central injections, and reports of PACAP and PAC1R knockout mice exhibiting complications that result in early lethality (Gray et al. 2001; Otto et al. 2004), a more specific approach to investigate the regulation of metabolism by PACAP is needed.

PACAP and PAC1R expression is abundant in the hypothalamus (Hannibal 2002; Hannibal et al. 1995a; Hashimoto et al. 1996; Resch et al. 2011; Resch et al. 2013), and

PACAP signaling exerts control over the autonomic nervous system in order to regulate glycemia and metabolism. Recently we reported that intrahypothalamic injection of PACAP into the PVN or VMN reduces food intake and body weight (Resch et al. 2011; Resch et al. 2013), however each of these nuclei are also involved in regulation of energy expenditure and glucose homeostasis. The VMN are particularly important for the counterregulatory response to hypoglycemia, stimulating hormonal release that drives glucose production in times of low blood glucose (Borg et al. 1997; Borg et al. 1994; Borg et al. 1995; Fioramonti et al. 2010; Levin et al. 2008). Similarly, the PVN play a role in sympathetic activation of hepatic glucose production, which is reported to involve PACAP signaling (Kalsbeek et al. 2004; Yi et al. 2010). Furthermore, both the PVN and VMN may regulate brown adipose tissue (BAT) thermogenesis, a critical means of heat generation and energy expenditure for rodents, however findings arguing for and against this notion have been described (Amir 1990a; Amir 1990b; Bamshad et al. 1999; Kim et al. 2011; Madden and Morrison 2009; Oldfield et al. 2002; Perkins et al. 1981; Sakaguchi and Bray 1987; Yoshimatsu et al. 1993). To assess the affects of PACAP on indices of energy expenditure and glucose homeostasis within the hypothalamus we did site-specific PACAP injections into the PVN and VMN similar to the feeding experiments (see Chapter 2 and 3). In these studies, we assessed the responses of temperature, locomotor activity, blood glucose, and pancreatic hormones following central PACAP injections.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 225-250 g were individually housed in a climate controlled room with a 12 hr light/dark cycle. Animals had free access to Harlan standard diet (8604 formulation) and water. Food consumption was calculated by pre-weighing food in each bin and subtracting the weight of non-ingested and spilled food at the end of each measurement period. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; ip) cocktail and placed in a stereotaxic apparatus. 26 gauge bilateral guide cannulae (Plastics One; Roanoke VA) were placed 3 mm dorsal to the target site in all animals, and secured to the surface of the skull with an acrylic resin. The stereotaxic coordinates for the PVN injection site were anterior/posterior, -1.7 mm from bregma; medial/lateral, ±0.5 mm from midline; dorsal/ventral, -4.9 mm, and for the VMN anterior/posterior, -2.5 mm from bregma; medial/lateral, ±0.6 mm from midline; dorsal/ventral, -6.2 mm from surface of the skull based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos and Watson 2007). Injectors extended 3 mm past the ventral tip of the cannulae reaching an injection site of -7.9 mm for PVN and -9.2 mm for VMN ventral from the surface of the skull. The upper incisor bar was positioned -3.3 mm below horizontal zero. A bilateral dummy stylet placed in the guide cannulae was used to

maintain patency. The animals were given at least five days to recover after cannula installation before receiving drug or vehicle injections during which time the animals were handled and dummy stylets were removed and replaced daily in order to acclimate the animals to the physical handling necessary during experiments. Correct cannulae placements were confirmed at the conclusion of each experiment by microscopic examination of Nissl stained sections and only those with correct placement were included in the studies.

Microinjections and Injection Spread

In all experiments, PACAP (50 pmol/0.25 µl/side; PACAP38; California Peptide Research; Napa, CA), VIP (50 pmol/0.25 µl/side; California Peptide Research; Napa, CA), maxadilan (50 pmol/0.25 µl/side; a generous gift from Dr. Ethan Lerner), PACAP6-38 (500 pmol/0.25 µl/side; Anaspec; Fremont, CA), or saline vehicle was microinjected through bilateral guide cannulae over approximately two minutes in awake animals while gently restrained. Following each injection an additional minute elapsed before removing injectors to minimize backflow of injected material. The optimal injection volume and subsequent spread within the PVN and VMN was determined previously (Resch et al. 2011; Resch et al. 2013).

Thermogenesis and activity

At the time of cannulation surgery animals were also implanted intraperitoneally with telemetry probes (Mini-Mitter Inc.; Sunriver, OR). Animals were allowed to recover for five days before initiation of experiments. For telemetry experiments, rats received bilateral VMN injections of vehicle or 50 pmol PACAP during the light cycle, when

temperature and activity are at their circadian trough, and placed back into their home cages positioned over a telemetry receiver platform. Telemetric data for core body temperature and spontaneous locomotor activity were collected every 5 minutes. Core body temperature data were averaged by the hour, and spontaneous locomotor activity data was either summed into one-hour bins or summed to give cumulative activity over a specified amount of time.

Isolation of Total RNA and Quantitative PCR

Total RNA was isolated from interscapular brown adipose tissue (iBAT) by TRIzol extraction (Invitrogen; Carlsbad, CA). Subsequently, cDNA was constructed with 1 μg of total RNA using the Reverse Transcription System (Promega; Madison, WI). Quantitative PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems; Carlsbad, CA), and PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersberg, MD) according to the manufacturer's protocol. Quantification of UCP1 expression was done using a relative standard curve and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for each gene were designed to span an exon-exon junction and had efficiencies of approximately 95%. Product sizes for each primer set were 126 bp for UCP1 and 126 bp for GAPDH. Melt curve analysis of experiments confirmed a single product for each reaction. Primers for UCP1 were 5'-GGTCAGAATGCAAGCACAAA-3' and 5'-TTAGGAGTCGTCCCTTTCCA-3', and primers for GAPDH were 5'-CTCCCATTCTTCCACCTTTGA-3' and 5'-ATGTAGGCCATGAGGTCCAC-3'.

Glucose, pancreatic hormone, and BAT triglyceride measurements

Blood was collected via tail vein before and after injections of saline or PACAP into the PVN or VMN in tubes containing EDTA, and then immediately chilled on ice until centrifugation. Plasma was then collected and stored at -20°C until analyzed. Glucose measurements were performed via a glucose oxidase colorimetric assay (Sigma-Aldrich; St. Louis, MO). Insulin and glucagon levels were measured by radioimmunoassay (Millipore; Billerica, MA). For triglyceride measurements, interscapular brown adipose tissue (BAT) was harvested three hours after PACAP injections into the VMN and snap frozen in liquid nitrogen. BAT was homogenized in radioimmunoassay buffer (Boston BioProducts Inc; Ashland, MA) containing protease inhibitor cocktail (Roche; Indianapolis, IN). Triglyceride content of BAT was measured using a colorimetric assay (Sigma-Aldrich; St. Louis, MO) and normalized to protein concentration using the DC protein assay (Bio-Rad; Hercules, CA).

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fischer LSD analysis was used for all post-hoc group comparisons. Statistical analyses were performed using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $P < 0.05$ were considered statistically significant.

Results

Glucose and pancreatic hormones

Both the PVN and VMN are hypothalamic regions known to regulate peripheral glucose as well as feeding behavior (Choi and Dallman 1999; Kalsbeek et al. 2004; Tokunaga et al. 1986; Tong et al. 2007). Similarly, central PACAP has been implicated in glucose homeostasis (Yi et al. 2010). The effect of PACAP infusion into the PVN or VMN on plasma glucose was assessed in animals fasted overnight (Table 4.1). PACAP administration in both the PVN and VMN produced significant increases in plasma glucose concentrations one hour post-injections (PVN, $P < 0.05$; VMN, $P < 0.05$). Next, in non-fasted animals, we analyzed insulin and glucagon levels to assess the effects of PACAP on pancreatic hormone secretion. Neither PVN nor VMN injections of PACAP significantly altered pancreatic hormone levels, although insulin did show a trend to decrease with PACAP injections into the VMN ($P = 0.11$).

Table 4.1. Change in levels of glucose and pancreatic hormones 60 minutes following PACAP treatment

	PVN		VMN	
	Saline	PACAP	Saline	PACAP
Glucose Δ (fasted; mg/dl)	19.1 ± 2.2	29.8 ± 4.5*	9.2 ± 2.5	17.5 ± 2.0*
Insulin Δ (ng/ml)	0.03 ± 0.18	-0.06 ± 0.13	-0.01 ± 0.21	-0.55 ± 0.18
Glucagon Δ (pg/ml)	-6.6 ± 6.2	5.5 ± 6.5	-2.9 ± 4.2	-13.2 ± 13.1

Data are mean ± SEM. * = $P < 0.05$ as compared to appropriate anatomical control

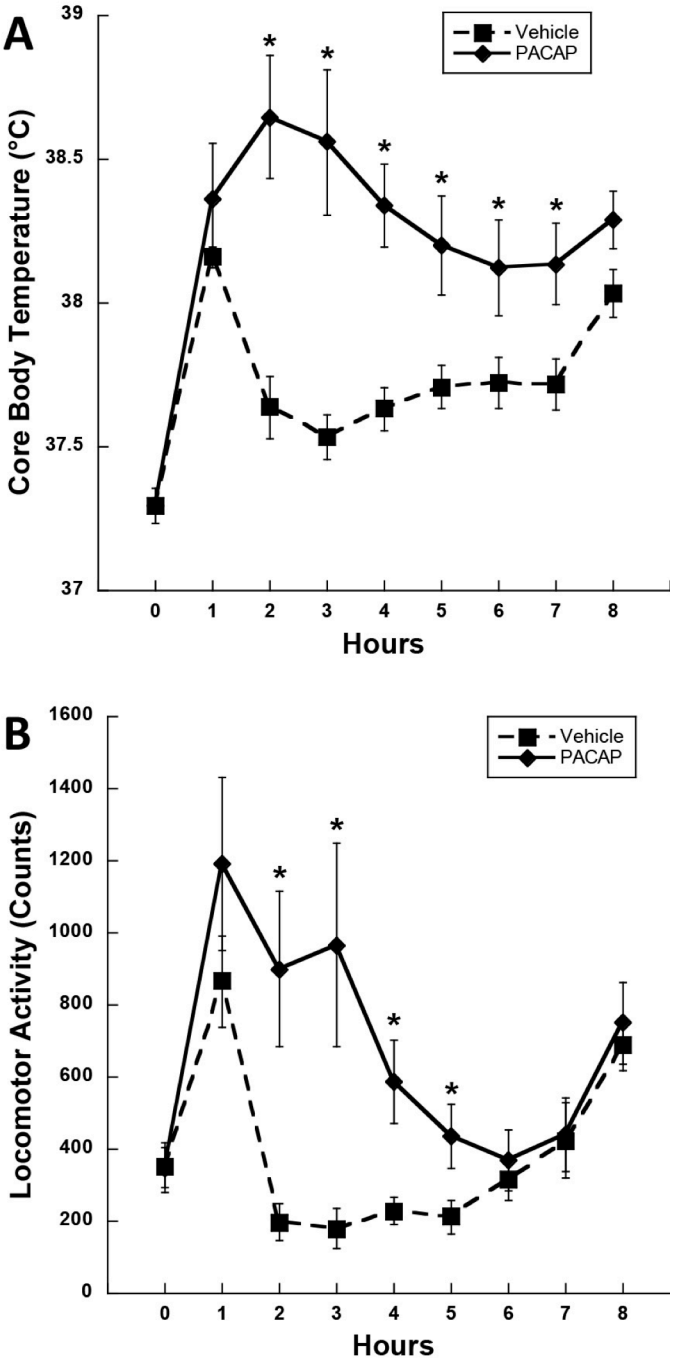

Figure 4.1. VMN injections of PACAP increase thermogenesis and activity. (a) Core body temperature and (b) spontaneous locomotor activity (averaged into 1 hour bins) increase significantly following PACAP injections (50 pmol) into the VMN. Data are expressed as mean ± SEM, * = $P < 0.05$ compared to vehicle.

Thermogenesis and activity

We began telemetry experiments first with bilateral infusions of PACAP into the VMN. These telemetric experiments were similar to our previous feeding studies except they were performed at the onset of the light phase, when core body temperature is at its circadian trough. PACAP injections induced increases in core body temperature with significant main effects of time and treatment by repeated measures ANOVA ($P < 0.01$; Fig. 4.1A), and the increase in thermogenesis was sustained for up to 7 hours after a single injection ($P < 0.05$). In addition to core body temperature, spontaneous locomotor activity was also increased in PACAP treated animals, again showing significant main effects of time and treatment by repeated measures ANOVA ($P < 0.05$; Fig. 4.1B). Specifically, activity was significantly increased in PACAP-treated animals from 2-5 hours following injections ($P < 0.05$).

To gain further insight as to how hypothalamic PACAP signaling impacts energy expenditure we expanded our injection sites in order to compare temperature and activity responses from animals administered PACAP into the PVN vs. VMN. Surprisingly, PACAP infusion into the PVN had no effect on either core body temperature (Fig. 4.2A & B) or activity (Fig. 4.2C & D) compared to saline treated controls. By contrast, animals that were injected with PACAP into the VMN again displayed marked and long-lasting increases in both temperature (Fig. 4.1A & B, $P < 0.05$) and activity (Fig. 4.2C & D, $P < 0.01$) compared to saline-treated controls.

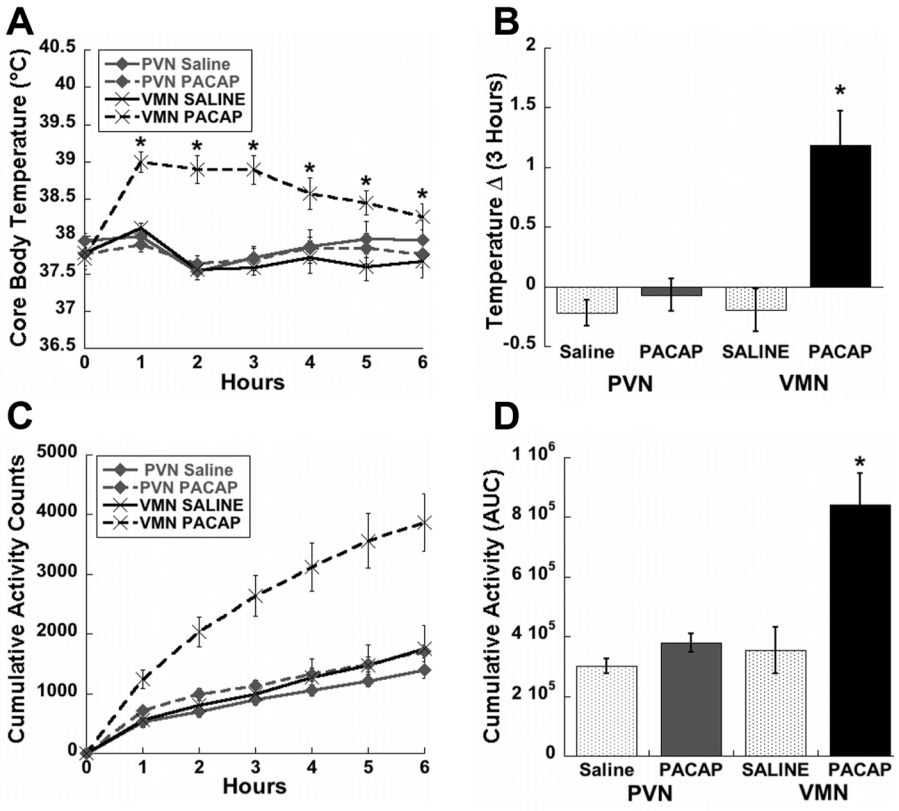

Figure 4.2. VMN but not PVN injections of PACAP increase thermogenesis and activity. (A) Timecourse of core body temperature and (B) 3 hour temperature change (Δ) show a significant increase following PACAP injections (50 pmol) into the VMN, however injections into the PVN do not affect temperature. (C) Timecourse for cumulative activity and (D) area under the curve (AUC) analysis depict a similar significant increase only in animals receiving injections of PACAP into the VMN. Data are expressed as mean ± SEM, * = $P < 0.05$ compared to saline.

In the VMN, PACAP6-38 significantly attenuated the increased thermogenesis produced by PACAP administration alone from 2-5 hours post-injection (Fig 4.3A and B; $P < 0.05$). A separate cohort of animals was used to assess UCP1 mRNA expression via qPCR in iBAT one and three hours following PACAP injection into the VMN. The results of the qPCR analysis showed a trend to increase iBAT UCP1 mRNA at one hour and a significant increase at three hours post-injection ($P < 0.05$; Fig 4.3C). Additionally,

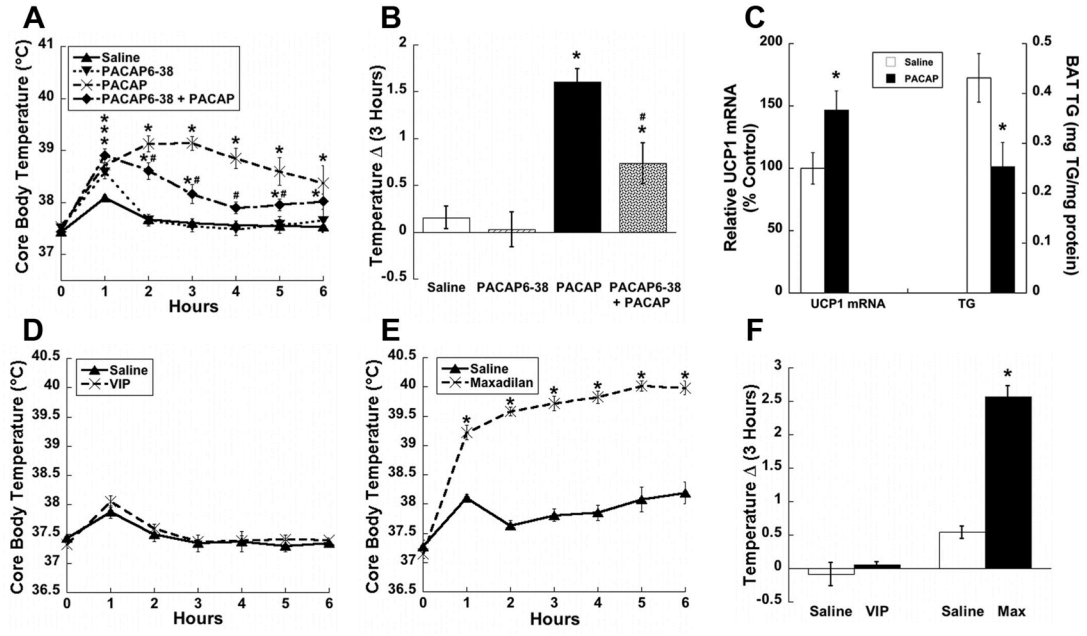

Figure 4.3. PACAP-mediated increases in temperature by the VMN are PAC1R-dependent. (A & B) The PAC1R antagonist PACAP6-38 (500 pmol/side) attenuates the timecourse and 3 hour temperature change (Δ) of PACAP-induced thermogenesis (50 pmol/side). (C) Interscapular brown adipose tissue (iBAT) UCP1 mRNA and triglyceride (TG) content was analyzed. Quantitative PCR of iBAT UCP1 mRNA showed a significant increase at three hours following injections of PACAP into the VMN. At the same timepoint iBAT TG content was significantly reduced. (D) VMN injections of the VPACR agonist VIP (50 pmol/side) did not alter the timecourse of core body temperature, while the PAC1R specific agonist maxadilan (50 pmol/side) caused significant increases in the (E) timecourse and (F) 3 hour temperature change (Δ) in core body temperature similar to PACAP injections. Data are expressed as mean ± SEM. * = $P < 0.05$ compared saline group, and # = $P < 0.05$ compared to PACAP group.

interscapular brown adipose tissue (iBAT) triglyceride content per mg protein at 3 hours post-injection into the VMN was significantly decreased (Fig. 4.3C, $P < 0.05$). Although PACAP6-38 only attenuated the effects of PACAP-induced thermogenesis, we used agonists for the VPAC and PAC1 receptors, VIP and maxadilan respectively, to confirm the specific involvement of PAC1R receptors. As predicted, VIP injections into the VMN did not affect body temperature (Fig. 4.3D & 4.3F), whereas the PAC1R-specific agonist, maxadilan, produced a large and long-lasting increase in core body temperature (Fig. 4.3E & 4.3F, $P < 0.001$) similar to PACAP-induced thermogenesis.

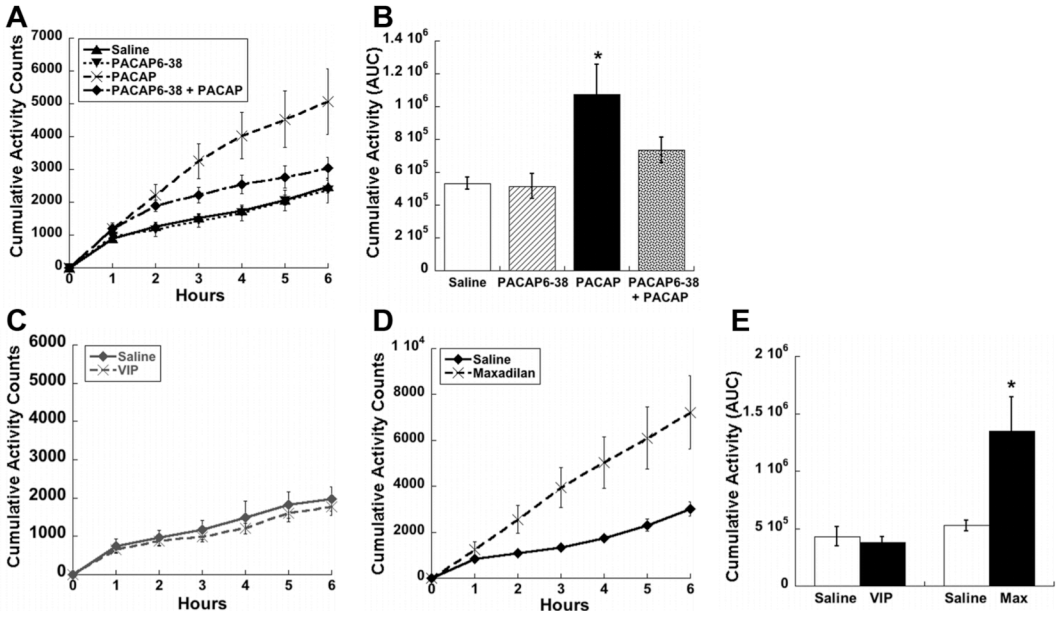

Figure 4.4. PACAP-mediated increases in activity by the VMN are PAC1R-dependent. (A) The timecourse of PACAP-induced spontaneous locomotor activity (50 pmol/side) is blocked by the PAC1R antagonist, PACAP6-38 (500 pmol/side), shown by (B) area under the curve (AUC) analysis. (C) VMN injections of the VPACR agonist VIP (50 pmol/side) did not alter the timecourse of activity, while the (D & E) PAC1R specific agonist maxadilan (50 pmol/side) caused significant increases in the timecourse of locomotor activity shown by AUC analysis similar to PACAP injections. Data are expressed as mean ± SEM. * = $P < 0.05$ compared saline group.

The PAC1 receptor also mediates the increased activity following PACAP infusion into the VMN. PACAP6-38 blocked the PACAP-induced increase in spontaneous locomotor activity (Fig. 4.4A & B, $P < 0.05$) unlike the effect on temperature, which was only partially inhibited. Further investigation with VIP and maxadilan confirmed the increase in locomotor activity induced by PACAP is dependent on the PAC1R. VIP did not alter activity compared to controls (Fig. 4.4C & E), while maxadilan produced large increases in cumulative activity compared to saline treated controls (Fig. 4.4D & E, $P < 0.05$).

Discussion

The current study establishes the PVN and VMN as important targets for PACAP signaling likely involved in autonomic nervous system regulation of glucose homeostasis and metabolism. Following intrahypothalamic PACAP injections, both the PVN and VMN produced increases in blood glucose availability without altering insulin and glucagon levels one-hour post-injections. Although the physiological relevance is still unknown, PACAP has previously been shown to stimulate hepatic glucose production through activation of VPAC2 receptor-expressing neurons of the PVN, which regulate hepatic sympathetic nerve activity by sending efferent projections to the intermediolateral column of the thoracic spinal cord (Yi et al. 2010). Less is known about glucose responses emanating from VMN activation by PACAP leaving the mechanism involved in driving glucose production unclear, however the VMN have been associated with catecholaminergic and glucagon responses to hypoglycemia (Borg et al. 1997; Borg et al. 1994; Borg et al. 1995).

Numerous other studies have linked PACAP with autonomic activation including those showing PACAP synapsing onto PVN neurons, which are known to control autonomic nervous system activity (Das et al. 2007; Grinevich et al. 1997; Legradi et al. 1998; Yi et al. 2010). Also, icv injection of PACAP in anesthetized rats alters autonomic nerve activity (Tanida et al. 2010), some of which can be blocked by the melanocortin receptor antagonist SHU9119 (Tanida et al. 2011b), further supporting that PACAP-mediated effects on energy are at least partially dependent on melanocortin signaling (Mounien et al. 2009; Resch et al. 2011). Interestingly, PACAP knockout mice are highly thermosensitive and require higher ambient temperatures to survive into adulthood,

adding to the evidence that PACAP plays a necessary role in thermoregulation (Adams et al. 2008; Gray et al. 2001; Gray et al. 2002).

Reduction in body weight is consistently found following central PACAP injections (Dore et al. 2013; Hawke et al. 2009; Resch et al. 2011), however pair-feeding studies suggest that the body weight loss is not entirely due to decreased feeding, but rather an increased metabolic rate (Hawke et al. 2009). Here we find that PACAP signaling in the VMN augments core body temperature and spontaneous locomotor activity, both of which requiring PAC1R signaling. While pretreatment of the VMN with the PAC1R antagonist only attenuated the increased thermogenesis and activity produced by PACAP it is likely that a higher dose of PACAP6-38 would fully inhibit these effects, as injections of the specific PAC1R agonist maxadilan in the VMN produced the same pattern of behaviors demonstrated by PACAP administration, while VIP injections had no effect on either temperature or activity. By contrast, stimulation of the PVN by PACAP did not significantly alter these measures of energy expenditure, despite evidence of PVN regulation of sympathetic outflow to BAT (Amir 1990b; Oldfield et al. 2002), and its control over the sympathetically driven PACAP-induced glycemic response (Yi et al. 2010). However, these results are in agreement with reports that populations of PVN neurons inhibit sympathetic activity to BAT (Kong et al. 2012; Madden and Morrison 2009).

Sympathetic thermogenesis independent of physical activity or shivering behavior can be positively correlated with UCP1 content in BAT (Himms-Hagen 1990). At one hour following PACAP administration into the VMN, increases in iBAT UCP1 mRNA expression did not reach statistical significance (data not shown), but by 3 hours the

increase in UCP1 mRNA was significant. Furthermore, VMN injected animals exhibiting increased thermogenesis and activity also displayed decreased BAT triglyceride stores, which is consistent with increased sympathetic nerve activation and lipolysis but not pyrogenic action (Cannon and Nedergaard 2004; Okamatsu-Ogura et al. 2007). While transynaptic retrograde tracing studies from the interscapular brown adipose tissue often fail to detect VMN neurons (Bamshad et al. 1999; Oldfield et al. 2002), there is ample evidence supporting VMN activation of BAT thermogenesis, including both VMN-specific microinjection and genetic studies (Amir 1990a; Kim et al. 2011; Perkins et al. 1981). It is feasible that the VMN exerts its influences over hypothalamic structures that are labeled by transynaptic tracers from BAT such as the medial preoptic area, dorsomedial nuclei, or lateral hypothalamus (Oldfield et al. 2002; Zhang et al. 2011) allowing for indirect and downstream effects on BAT thermogenesis that are not observed in more proximal polysynaptic tracing experiments. Future studies are needed to reconcile the discrepancy between the anatomical and functional studies regarding VMN-mediated BAT thermogenesis.

The elevation of spontaneous locomotor activity observed after PACAP injections into the VMN is in contrast to previous reports of ventricular injections in mice where no increases in activity were noted (Hawke et al. 2009), however increased activity has been observed in rats (Masuo et al. 1995). This may reflect that direct or specific stimulation of the VMN is necessary to alter locomotor circuits, similar to reports showing that direct injection of $GABA_A$ receptor antagonists or kainate into the VMN produces running activity (Narita et al. 1993; Yokawa et al. 1989). Moreover, VMN lesion studies have consistently demonstrated diminished locomotor activity and delayed onset of normal

diurnal running activity. Such evidence is congruous with the current study and contributes to the notion that locomotor activity pathways involve the VMN (Challet et al. 1995; Challet et al. 1996; Choi et al. 1998).

PACAP neurocircuitry mediating energy homeostasis is not well defined, however the present results suggest at least partially distinct circuits of PACAP innervation between the PVN and VMN. Analysis of PACAP expression suggests several strong candidate brain regions that may provide afferent input of PACAP into the hypothalamic regions under study including the medial amygdala and perhaps the VMN itself (Hannibal 2002; Nishizuka and Pfaff 1989). Future directions should address this gap in knowledge through functional mapping studies of PACAP-expressing PVN and VMN circuits, and by overlaying this information in terms of the role of PACAP in the regulation of feeding and metabolism.

CHAPTER V

HYPOTHALAMIC AFFERENTS EXPRESSING PACAP: POTENTIAL CIRCUITS MODULATING FEEDING BEHAVIOR AND METABOLISM

Introduction

The hypothalamus contains the highest concentration of PACAP protein expression within the brain (Hannibal et al. 1995a). Given the high expression of PACAP and its receptors in the hypothalamus, it's signaling is likely vital to normal hypothalamic function. This is supported by the large deficits in temperature regulation and glycemic control exhibited by PACAP knockout mice (Adams et al. 2008; Gray et al. 2001; Gray et al. 2002; Hamelink et al. 2002; Tomimoto et al. 2008), and the robust changes in food intake and energy expenditure observed following central injections of PACAP (Chance et al. 1995; Hawke et al. 2009; Morley et al. 1992; Mounien et al. 2009; Resch et al. 2011; Resch et al. 2013). Remarkably, the hypothalamic neurocircuitry regulating feeding and metabolism through PACAP signaling has not been characterized.

Many hypothalamic nuclei contain some expression of PACAP mRNA, but the majority of PACAP-expressing neurons reside within the VMN and mammillary bodies (Hannibal 2002). Importantly, fasting causes a decrease in hypothalamic PACAP mRNA expression indicating a relationship between feeding behavior and PACAP signaling (Mounien et al. 2009). Moreover, decreased PACAP mRNA expression following fasting is specifically found in the VMN, and by contrast, mice chronically fed a high energy diet display increased expression (Hawke et al. 2009). Yet, the efferent targets of

hypothalamic PACAP neurons, specifically emanating from the VMN, that are potentially responsible for body weight regulation, are not known.

In addition to intrahypothalamic sites of PACAP mRNA expression, there are several extrahypothalamic regions that express PACAP and have a role in maintaining energy homeostasis, including the amygdala and areas of the brainstem (Hannibal 2002). Both the amygdala and brainstem possess heavy bidirectional connectivity with the hypothalamus, and are critical for regulation of many homeostatic systems including feeding, temperature, glucose, and stress regulation (Hammack et al. 2010; King 2006a; Kong et al. 2012; Ulrich-Lai et al. 2011; Wu et al. 2009; Zhou et al. 2010; Ziegler et al. 2012).

The PVN and VMN were previously shown to decrease food intake, while increasing peripheral glucose levels in response to PACAP injections. Furthermore, PACAP signaling specifically in the VMN causes augmented thermogenesis and activity (Resch et al. 2013). In order to initiate the functional mapping of PACAP circuits in the hypothalamus that drive these behaviors we performed retrograde neuronal tract tracing from the PVN and VMN to examine PACAP-expressing circuits that project to these regions of the hypothalamus. Retrograde signal from the PVN or VMN was then co-labeled with PACAP mRNA signal using fluorescent *in situ* hybridization to define afferent circuits expressing PACAP that may control feeding behavior and metabolism.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 225-250 g were individually housed in a climate controlled room with a 12 hr light/dark cycle. Animals had free access to Harlan standard diet (8604 formulation) and water. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; ip) cocktail and placed in a stereotaxic apparatus. The stereotaxic coordinates for the PVN injection site were anterior/posterior, -1.7 mm from bregma; medial/lateral, ±0.5 mm from midline; dorsal/ventral, -7.9 mm, and for the VMN anterior/posterior, -2.5 mm from bregma; medial/lateral, ±0.6 mm from midline; dorsal/ventral, -9.2 mm from surface of the skull based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos and Watson 2007). The upper incisor bar was positioned -3.3 mm below horizontal zero. Correct injection sites were confirmed by microscopic examination of Nissl stained sections and immunohistochemical signal for the retrograde tracer at the injection site. Only those with correct placement were included in the analysis.

In Situ Hybridization

Brains were sectioned coronally at 12 µm using a cryostat, thaw-mounted onto electrostatically clean slides, and stored at -80°C until post-fixed. Prior to hybridization,

sections were post-fixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25% acetic anhydride. Standard *in vitro* transcription methods were used to generate both sense and antisense riboprobes recognizing VGLUT2 (Cullinan; Milwaukee, WI) and PACAP transcripts (Choi; Milwaukee, WI), which were subsequently diluted in hybridization cocktail (Amresco; Solon, OH) with tRNA. Sections were hybridized overnight at 55°C with either digoxigenin (DIG) or fluorescein isothiocyanate (FITC) labeled riboprobes. After hybridization, slides were treated with RNase A, and stringently washed in 0.1x SSC at 65°C for 30 minutes. Slides were then incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche; Indianapolis, IN) overnight at 4° C. Riboprobe signal was further enhanced using the TSA-plus fluorophore system with either fluorescein or Cy3 (Perkin Elmer; Waltham, MA). For dual fluorescent *in situ* hybridization, following the first probe visualization the remaining HRP activity was quenched with 30% H_2O_2. Following the peroxide quench, slides were treated for visualization of the second probe. Image capture was performed using fluorescent microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

Tract tracing and Immunohistochemistry

For PVN and VMN tract tracing experiments, 0.1 µl (PVN) or 0.2 µl (VMN) of biotinylated cholera toxin subunit B was stereotaxically microinjected over 10 minutes, and injectors were left in place for an additional 5 minutes before removal. 7 days later animals were euthanized and brains were collected. Brains were sectioned by cryostat at 12 µm and prepared for PACAP or VGLUT2 fluorescent *in situ* hybridization as

described above. Following *in situ* hybridization, slides were then probed with a primary antibody against CTB (goat anti-CTB; List Biological Laboratories; Campbell, CA) overnight at 4° C, and the antibody signal was visualized using a donkey anti-goat Alexafluor 594 conjugated secondary antibody (Life Technologies; Grand Island, NY). Upon completion of fluorescent staining, representative sections from regions containing both PACAP signal from *in situ* hybridization and CTB immunolabeling were counted for total number of CTB positive cells and the number of CTB positive cells also expressing PACAP mRNA.

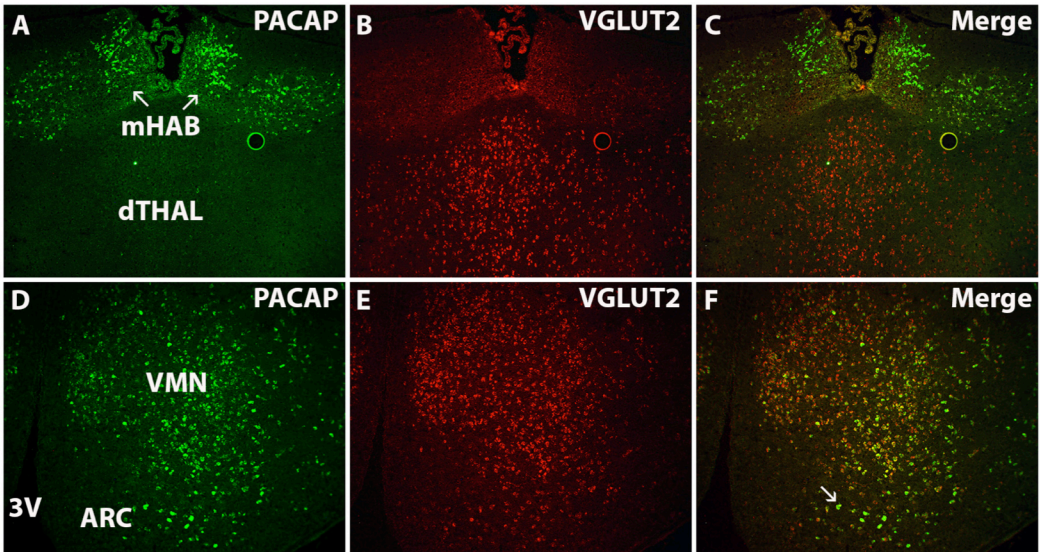

Figure 5.1. PACAP and Vglut2 mRNA are co-expressed in the VMN. *In situ* hybridization for PACAP (FITC) and Vglut2 (Cy3) mRNA in the dorsal thalamus, habenula (A-C), and ventromedial nuclei of the hypothalamus (D-F) shows minimal co-expression in the dorsal thalamic region (C), but extensive co-labeling in the ventromedial hypothalamus (F). 3V, third ventricle; ARC, arcuate nuclei; dTHAL, dorsal thalamus; mHAB, medial habenula; VMN, ventromedial nuclei.

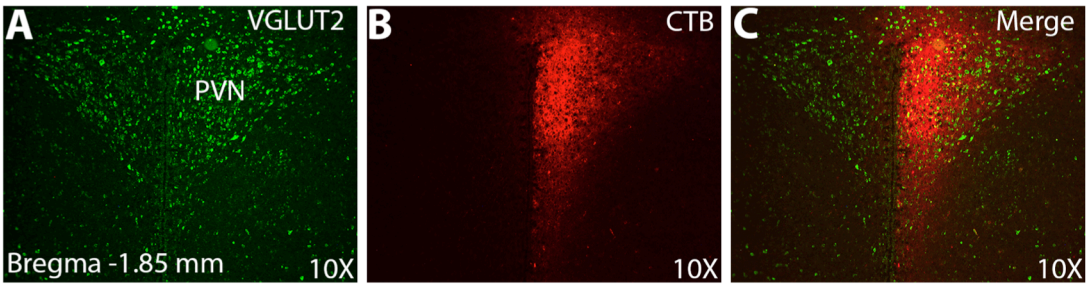

Figure 5.2. Cholera toxin subunit B injection site for the PVN. For retrograde labeling of PVN afferent circuits the neuronal tracer cholera toxin subunit B (CTB) was injected site-specifically into the PVN. Boundaries of PVN are denoted by Vglut2 mRNA signal (green; A), with CTB injection signal (red; B) found to be confined within the PVN (C).

Results

To characterize VMN neuron mRNA expression of PACAP and the vesicular glutamate transporter 2 (Vglut2) dual fluorescent *in situ* hybridization was used. Analysis of PACAP and Vglut2 expression was done in the dorsal thalamus and VMN. The dorsal thalamus was almost completely devoid of PACAP mRNA expression. However, within the same habenular area expression of PACAP mRNA was very robust, especially in the medial portion (PACAP = green fluorescence; Fig. 5.1A). In contrast, Vglut2 mRNA expression was abundant in the dorsal thalamus with little expression in the medial habenula, and no cells were observed to express both PACAP and Vglut2 in this region (Vglut2 = red fluorescence; Fig. 5.1B & C). Conversely, the VMN contain rich expression of both PACAP and Vglut2 mRNA, with almost all PACAP positive ($PACAP^{+}$) neurons co-expressing Vglut2 (~95%; Fig. 5.1D-F). However, there were a few PACAP neurons residing in the lateral portions of the arcuate (ARC) that were not co-labeled with Vglut2 (Arrow; Fig. 5.1F).

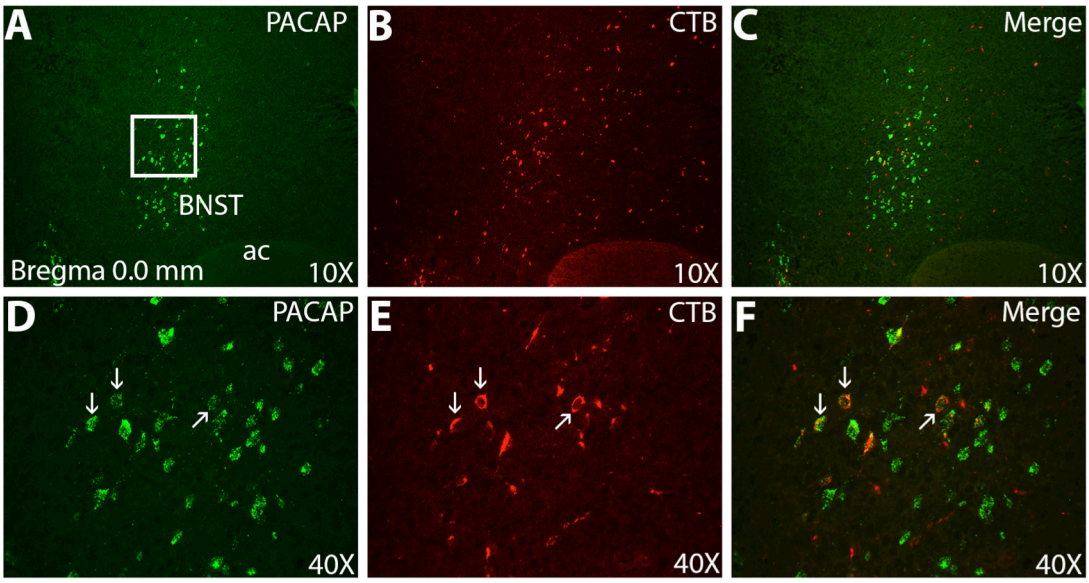

Figure 5.3. PACAP-expressing cells of the bed nucleus of the stria terminalis project to the PVN. Left column: fluorescent *in situ* hybridization for PACAP mRNA, middle column: immunofluorescence for CTB, and right column: merged images. Approximately 50% of cells identified projecting from the bed nucleus of the stria terminalis (BNST) to the PVN were positive for PACAP mRNA. Anterior medial BNST at 10X (A, B, C) and 40X (D, E, F). Box within the left panels represent the area of subsequent high magnification pictures. Arrows indicate representative dual-labeled cells. ac, anterior commissure; BNST, bed nucleus of the stria terminalis; CTB, cholera toxin subunit B.

PACAP signaling in the hypothalamus drives several behaviors leading to reduced body weight, however PACAP-expressing afferent circuits of the PVN and VMN are not well defined. We performed tract tracing with the neuronal tracer cholera toxin subunit B (CTB), coupled with fluorescent *in situ* hybridization for PACAP and VGLUT2 mRNA (Fig. 5.2 and 5.6). VGLUT2 fluorescent *in situ* hybridization was used as an anatomical marker for both the PVN and VMN. One week following unilateral stereotaxic injections of CTB into the PVN and VMN, we examined the distribution of both the neuronal tracer and PACAP mRNA. CTB retrograde signal from the PVN (Fig. 5.3-5.5) co-localized with PACAP mRNA most notably in the anterior medial bed nucleus of the stria terminalis (BNST), VMN, and lateral parabrachial nucleus (LPB).

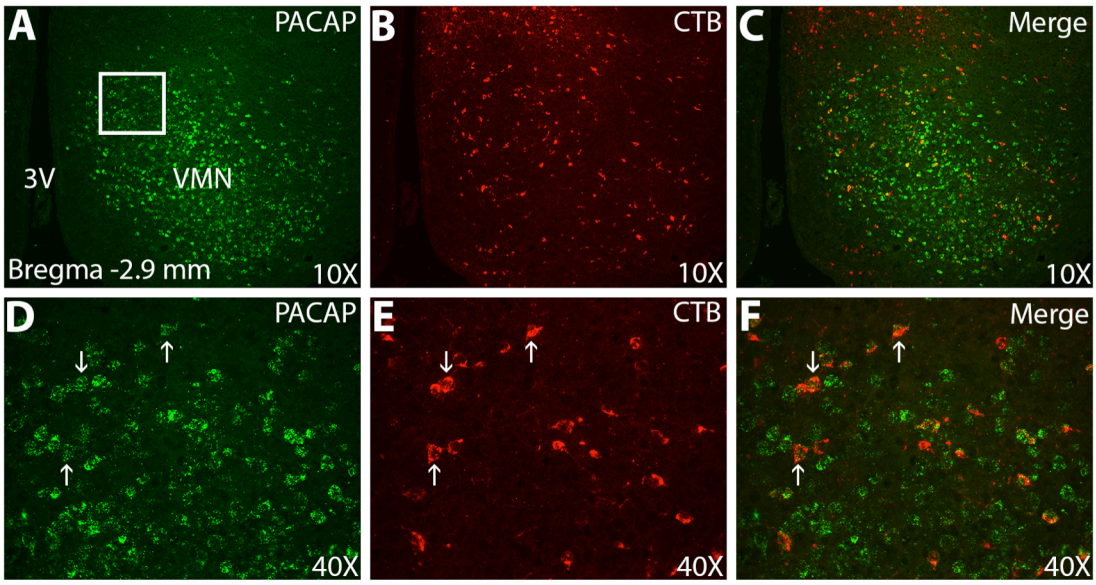

Figure 5.4. PACAP-expressing cells of the VMN project to the PVN. Left column: fluorescent *in situ* hybridization for PACAP mRNA, middle column: immunofluorescence for CTB, and right column: merged images. The majority of cells (~90%) in the VMN that project to the PVN were positive for PACAP. VMN at 10X (A, B, C) and 40X (D, E, F). Box within the left panels represent the area of subsequent high magnification pictures. Arrows indicate representative dual-labeled cells. 3V, third ventricle; CTB, cholera toxin subunit B; VMN, ventromedial nucleus.

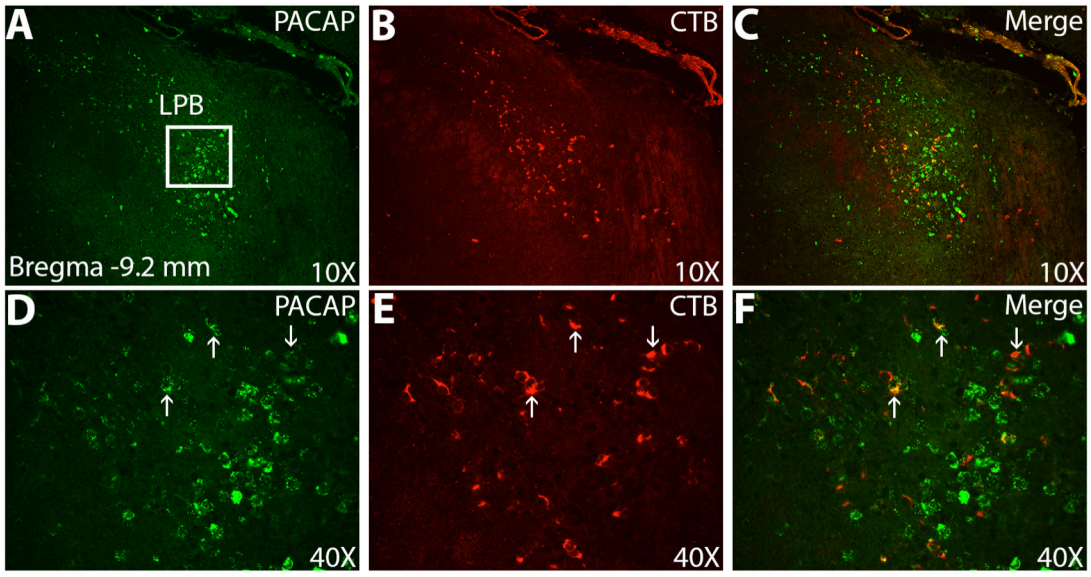

Figure 5.5. PACAP-expressing cells of the lateral parabrachial nuclei project to the PVN. Left column: fluorescent *in situ* hybridization for PACAP mRNA, middle column: immunofluorescence for CTB, and right column: merged images. The majority of cells (~90%) in the lateral parabrachial nuclei (LPB) projecting to the PVN were positive for PACAP. LPB at 10X (A, B, C) and 40X (D, E, F). Box within the left panels represent the area of subsequent high magnification pictures. Arrows indicate representative dual-labeled cells. CTB, cholera toxin subunit B; LPB, lateral parabrachial nucleus.

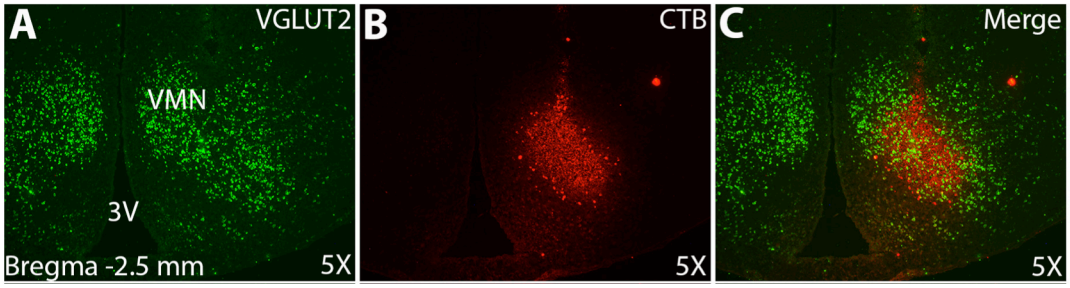

Figure 5.6. Cholera toxin subunit B injection site for the VMN. For retrograde labeling of VMN afferent circuits the neuronal tracer cholera toxin subunit B (CTB) was injected site-specifically into the VMN. Boundaries of VMN are denoted by Vglut2 mRNA signal (green; A), with CTB injection signal (red; B) found to be confined within the VMN (C).

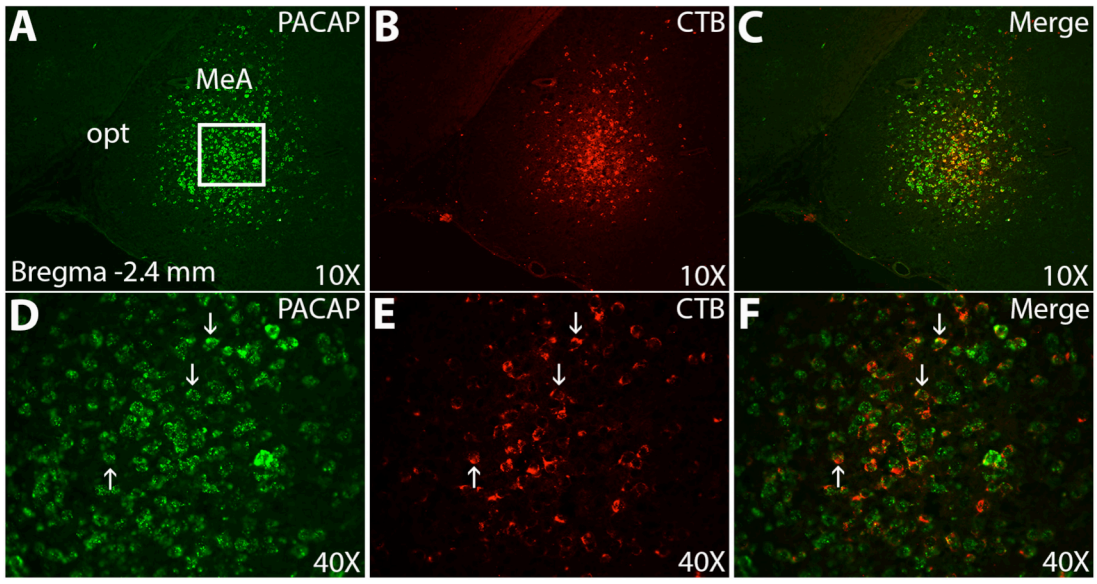

Figure 5.7. PACAP-expressing cells of the medial amygdala project to the VMN. Left column: fluorescent in situ hybridization for PACAP mRNA, middle column: immunofluorescence for CTB, and right column: merged images. The majority of cells projecting to the VMN from the medial amygdala (MeA) were positive for PACAP (~85-90%). MeA at 10X (A, B, C) and 40x (D, E, F). Box within the left panels represent the area of subsequent high magnification pictures. Arrows indicate representative dual-labeled cells. CTB, cholera toxin subunit B; opt, optic tract.

Few dual labeled cells were also found in the medial amygdala (MeA; data not shown). With the exception of the BNST, which had 56% of CTB positive cells co-expressing PACAP mRNA, the vast majority of CTB cells in the VMN (95%) and LPB (92%) co-

expressed PACAP mRNA. Retrograde CTB tracing from the VMN (Fig. 5.7-5.8) was abundantly co-localized with PACAP mRNA in the MeA (85%) ipsilateral to the injection site, as well as in the LPB (90%).

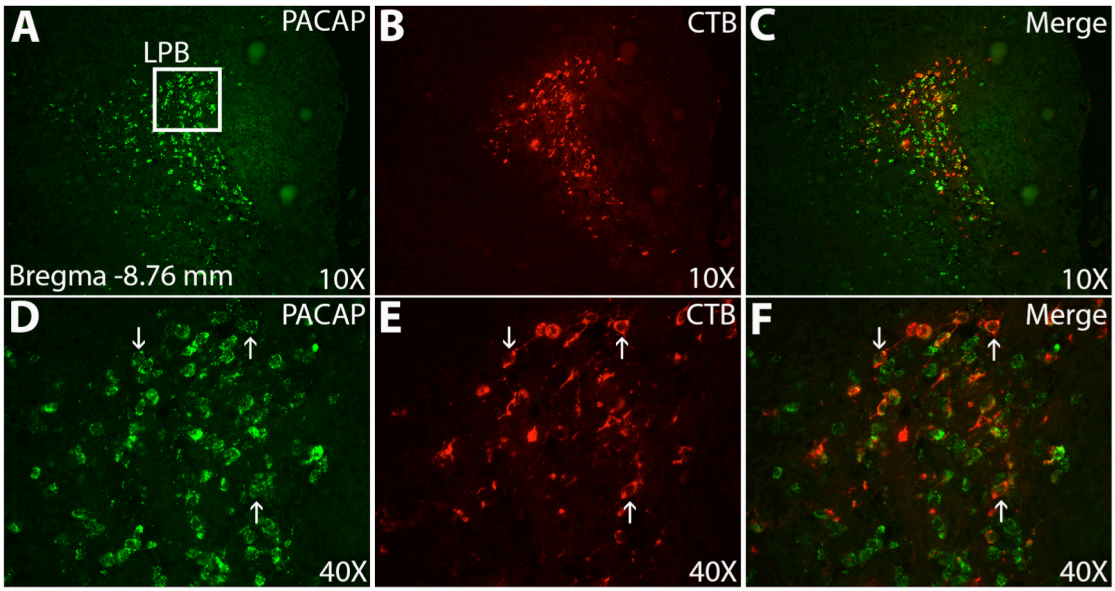

Figure 5.8. PACAP-expressing cells of the lateral parabrachial nuclei project to the VMN. Left column: fluorescent in situ hybridization for PACAP mRNA, middle column: immunofluorescence for CTB, and right column: merged images. The majority of cells projecting to the VMN from the lateral parabrachial nuclei (LPB) were positive for PACAP (~85-90%). LPB at 10x (A, B, C) and 40X (D, E, F). Box within the left panels represent the area of subsequent high magnification pictures. Arrows indicate representative dual-labeled cells. CTB, cholera toxin subunit B; LPB, lateral parabrachial nucleus.

Discussion

PACAP signaling within the hypothalamus and at downstream extrahypothalamic sites is critical for the control of feeding behavior and metabolism. The VMN possess a high degree of PACAP mRNA expression (Hannibal 2002), and are comprised of predominantly glutamatergic neurons that express Vglut2 (Fu and van den Pol 2008; Tong et al. 2007; Ziegler et al. 2002). While PACAP and glutamate have been reported to

be co-stored within the same cell previously (Engelund et al. 2010; Fahrenkrug and Hannibal 2004; Hannibal et al. 2000), we found evidence of co-storage of PACAP and glutamate in VMN neurons using Vglut2 as a marker of glutamate releasing neurons (Fig. 5.1D-F). Importantly, not all PACAP-expressing neurons co-express Vglut2, as there are $PACAP^{+}$ neurons in the lateral portions of the ARC that do not express Vglut2. Interestingly, these ARC PACAP neurons may also express POMC and acetylcholine (Durr et al. 2007), yet whether these ARC PACAP neurons are still glutamatergic is unknown. Moreover, analysis of habenular neurons revealed no co-labeling of PACAP and Vglut2 signal. It should be noted, however, that $PACAP^{+}$ neurons of the habenula are also likely glutamatergic but express Vglut1 instead of Vglut2 (Barroso-Chinea et al. 2007).

Hypothalamic PACAP mRNA is predominantly expressed in the VMN (Hannibal 2002; Hawke et al. 2009), suggesting that a prominent source of PACAP release in the PVN may originate from the VMN. In fact, much work has been done to characterize PACAP-containing afferents of the PVN. PACAP immunolabeling of synaptic terminals (Legradi et al. 1998) and expression of PACAP receptor subtypes have been identified within the PVN (Hashimoto et al. 1996; Resch et al. 2013; Sheward et al. 1995; Usdin et al. 1994). Furthermore, retrograde tracing from the PVN demonstrates co-labeling of neurons with PACAP immunoreactivity (Das et al. 2007), however results using PACAP immunohistochemistry are often difficult to interpret since available PACAP antibodies typically have low sensitivity and require the use of colchicine and antigen retrieval methods to obtain even low numbers of immunoreactive labeling of neuronal cell bodies

(Das et al. 2007). The current study demonstrates that a number of known PVN afferent pathways utilize PACAP signaling including an input from the VMN. Taking into consideration the similar feeding behavior responses following stimulation of the VMN and PVN by PACAP injections (Resch et al. 2013), the established circuitry of glutamatergic VMN neurons projecting to the PVN (Ulrich-Lai et al. 2011), and the extremely high PACAP mRNA expression found in the VMN (Hannibal 2002), it is not surprising that the VMN may contribute functionally important PACAP-expressing afferent inputs to the PVN. However, other significant sources of PACAP innervation to the PVN were also found in the anterior medial bed nucleus of the stria terminalis (BNST), the lateral parabrachial nucleus (LPB), and sparse dual labeling in the medial amygdala (MeA) (Figure 5.9). Although PACAP containing cells from the LPB and MeA appear to project more extensively to the VMN, the BNST is well characterized to be a critical regulator of stress and anxiety with known inputs to the PVN, which could suggest a potential role of PACAP-mediated hypophagia during times of stress (Dong and Swanson 2006; Hammack et al. 2009; Hammack et al. 2010).

In contrast to the PVN, the circuitry of PACAP-containing afferents to the VMN are far less studied, with no prior published reports to date. While the possibility of contralateral and intrinsic PACAP signaling within the VMN still exists (Kiss et al. 2011; Nishizuka and Pfaff 1989), cholera toxin subunit B retrograde tracing following injections into the VMN robustly co-labeled with fluorescent *in situ* hybridization signal for PACAP mRNA in two distinct regions, the MeA and LPB (Figure 5.9). Both brain regions have been strongly implicated in energy balance, suggesting that the MeA and LPB may be two distinct central pathways that converge on the PVN and VMN to

manage both feeding behavior and metabolic systems. Lesions to the MeA produce hyperphagia and obesity, especially in female rats (King 2006a). Moreover, the MeA is also a glucose-sensing area of the brain that may respond to states of hypoglycemia further strengthening a connection with the well-characterized glucostatic role of the VMN (Zhou et al. 2010). The LPB also contributes to body weight regulation as demonstrated by the loss of GABAergic signaling to the parabrachial nuclei from agouti-related polypeptide (AGRP) neurons of the arcuate leading to starvation (Wu et al. 2009). Given their effect on feeding, the PVN and VMN may be significant efferent targets of the LPB whereby, LPB disinhibition could lead to increased excitation of these areas and anorexia. Furthermore, the LPB are critical structures in the thermoregulation pathway (Nakamura and Morrison 2008; Nakamura and Morrison 2010), thereby positioning the VMN as potential downstream targets of LPB signaling that, in turn, could modulate thermogenic responses.

The differential response to PACAP signaling within the PVN and VMN regarding feeding, temperature, and locomotor activity may reflect an anatomical divergence segregating the PVN as the predominant sight of action for PACAP-mediated hypophagia, while PACAP stimulation of the VMN may primarily serve to stimulate energy expenditure. The distinct and widespread population of PACAP receptors in the hypothalamus enables functionally diverse signaling by PACAP. Thus, it would be intriguing to determine if multiple subtypes of PACAP receptors are expressed on the same cell, or more importantly at the same synapse.

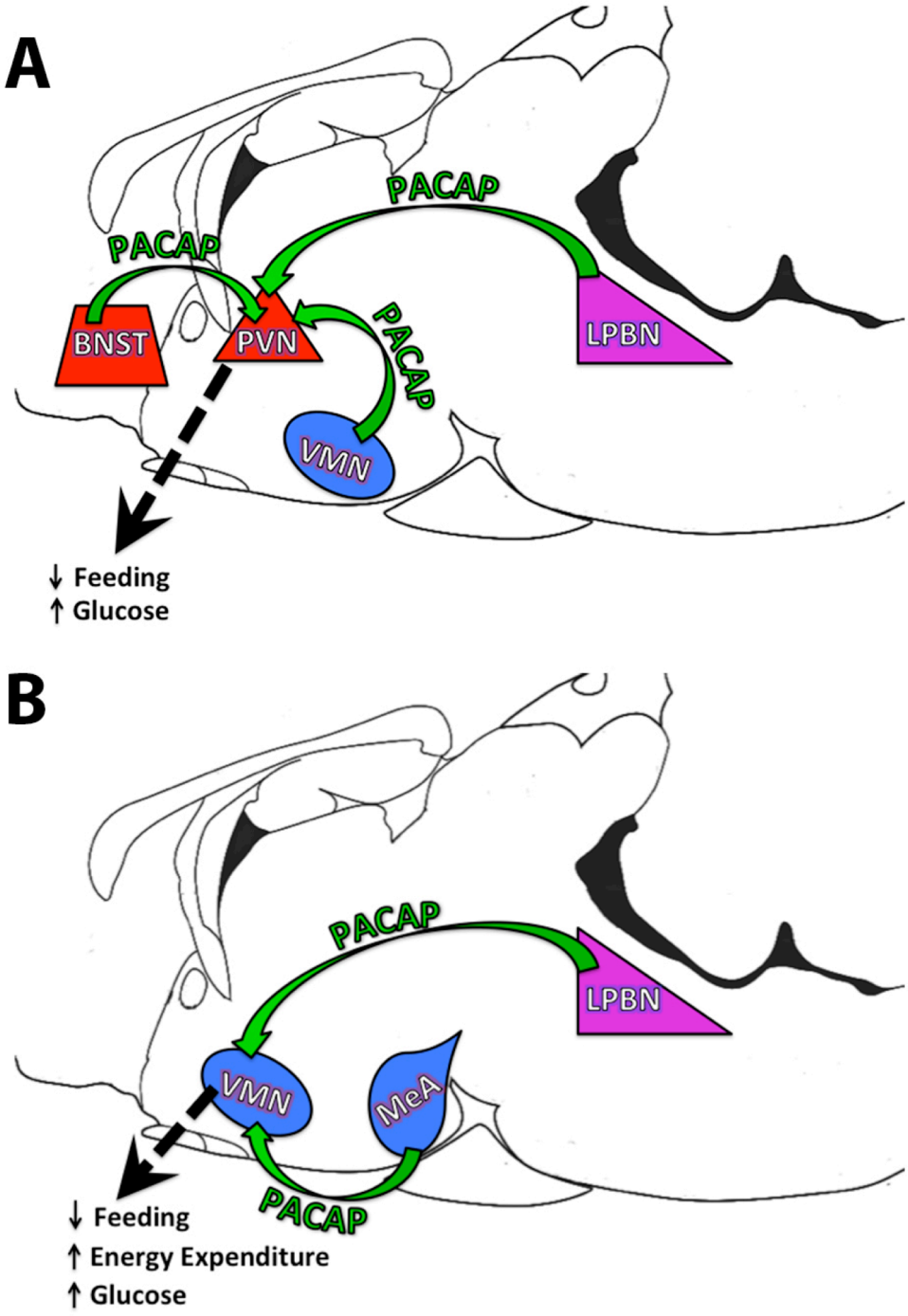

Figure 5.9. Sagittal schematic of PACAP-expressing afferent circuits of the paraventricular (PVN) and ventromedial (VMN) nuclei. (A) Afferents of the PVN that may regulate energy homeostasis through PACAP signaling originate from the bed nucleus of the stria terminalis (BNST), lateral parabrachial nuclei (LPBN), and VMN. (B) Afferents of the VMN that potentially regulate energy balance through PACAP signaling originate from the LPBN and medial amygdala (MeA).

CHAPTER VI

PACAP MODULATION OF GLUTAMATE: REGULATION OF ASTROCYTIC CYSTINE-GLUTAMATE EXCHANGE

Introduction

The inhibition of feeding behavior in response to PACAP signaling in the hypothalamus is mediated by the PAC1 receptor (Hawke et al. 2009; Mounien et al. 2009; Resch et al. 2013). Because the PAC1 receptor is G protein-coupled, the exact mechanism driving excitation of cells in the hypothalamus by PACAP is not clear. Given evidence that PACAP receptor activation potentiates glutamate signaling in several brain regions (Chen et al. 1999; Cho et al. 2012; Costa et al. 2009; Kopp et al. 2001; Macdonald et al. 2005; Martin et al. 1995; Pellegri et al. 1998; Yaka et al. 2003), it is probable that PACAP modulates glutamate signaling to increase the excitation of hypothalamic neurons. However, glutamate signaling can be enhanced not only by conventional means, such as increased presynaptic release and potentiation of postsynaptic receptor currents, but also through nonconventional mechanisms including decreased synaptic clearance via glutamate transporters or glutamate release from astrocytes. Often overlooked, nonconventional modulation of glutamate signaling by astrocytes is critical to CNS function, which is especially relevant since astrocytes are heavily targeted by PACAP signaling (Figiel and Engele 2000; Magistretti et al. 1998; Tatsuno et al. 1991; Tatsuno et al. 1990; Tatsuno et al. 1996a; Tatsuno et al. 1996b). Identifying novel aspects of glutamate modulation by PACAP will be valuable towards

understanding more complex aspects of PACAP signaling that affect physiology and behavior.

In the central nervous system (CNS) astrocytic cystine-glutamate exchange is critical for both protection against reactive oxygen species (ROS) (Kranich et al. 1998; Sagara et al. 1993b), as well as the maintenance of extracellular glutamate concentrations (Baker et al. 2002; Moran et al. 2005; Murphy and Baraban 1990). The cystine-glutamate antiporter, system x_c^-, composed of the light chain xCT and heavy chain 4F2hc subunits, is a sodium independent amino acid transporter that exchanges one molecule of extracellular cystine for one molecule of intracellular glutamate (Bannai 1986; Bannai and Kitamura 1980; Sato et al. 1999). System x_c^- is the principal mode of cystine entry into the cell (Sagara et al. 1993a), which is immediately reduced to cysteine, the rate-limiting substrate for glutathione (GSH) production (Sagara et al. 1993b). Maintaining sufficient levels of GSH is paramount to the cellular defense against oxidative stress, which is thought to significantly impact the development of several disease states including cancer, diabetes, and numerous neurodegenerative diseases (Bridges et al. 2012; Lewerenz et al. 2013).

Nonvesicular extrasynaptic glutamate release by system x_c^- may be important for activation of extrasynaptic NMDA and group II/III metabotropic glutamate receptors that, in turn, affect neuronal excitability (Kupchik et al. 2012; Moran et al. 2005; Pow 2001). In support, nonvesicular glutamate released by the cystine-glutamate antiporter is reported to be an essential regulator of extrasynaptic glutamate concentrations in the CNS (Baker et al. 2002). Moreover, dysregulation of extrasynaptic glutamate concentrations resulting from altered system x_c^- activity has been implicated in glutamate excitotoxicity

(Piani and Fontana 1994) and psychiatric disorders (Baker et al. 2008; Baker et al. 2003). With the large array of pathological states in the CNS stemming from both insufficient glutathione synthesis and dysfunctional glutamate homeostasis, system x_c^- is positioned to heavily influence a variety of pathologies ranging from compulsive disorders to neurodegenerative disease (Berman et al. 2011; de Groot and Sontheimer 2011; Grant et al. 2007; Grant et al. 2009; Knackstedt et al. 2009; Park et al. 2004; Sontheimer 2011; Zhou and Kalivas 2008). Unfortunately, the regulation of this heterodimeric amino acid transporter is poorly understood.

PACAP is a pleiotropic neuropeptide with widespread expression throughout the central nervous system (CNS) and periphery (Vaudry et al. 2009). PACAP regulates multiple aspects of astrocyte signaling (Magistretti et al. 1998; Masmoudi-Kouki et al. 2007; Tatsuno et al. 1991; Tatsuno et al. 1996a; Watanabe et al. 2006) including transcriptional activation of the glial glutamate transporters GLT-1 and GLAST (Figiel and Engele 2000). Three receptors have been identified for this peptide including the VIP receptors VPAC1 and VPAC2, and PAC1R, which PACAP binds to with the highest affinity (Dickson and Finlayson 2009). All of the PACAP receptors are expressed on astrocytes to some degree (Ashur-Fabian et al. 1997; Grimaldi and Cavallaro 1999; Grimaldi and Cavallaro 2000; Masmoudi-Kouki et al. 2007; Suzuki et al. 2003; Tatsuno et al. 1991) suggesting a significant role for PACAP signaling in the regulation of astrocyte function. Moreover, PACAP synthesis and transmission may be exclusively released from glutamate neurons in the CNS (Engelund et al. 2010; Fahrenkrug and Hannibal 2004; Hannibal et al. 2000) allowing PACAP to have a potential modulatory role on glutamate signaling at both the neuron as well as on the surrounding glia.

While PACAP appears to be a critical signal for astrocytes (Masmoudi-Kouki et al. 2007), as well as a key modulator of glutamate signaling (Magistretti et al. 1998), the affects of PACAP signaling on system x_c^- function are unknown. However, evidence of the neuroprotective actions of PACAP under conditions of both oxidative stress and excitotoxicity (Masmoudi-Kouki et al. 2011; Morio et al. 1996; Shintani et al. 2005) suggests these effects could be mediated through activation of system x_c^-, a mechanism that alters both GSH production and glutamate signaling. To test this hypothesis, we treated primary cortical cultures with PACAP and found a potentiation of system x_c^- activity. Subsequent experiments were performed to determine the specific mechanism mediating the increased cystine-glutamate exchange produced by PACAP application in these cultures.

Materials and Methods

Materials

Timed pregnant female Swiss Webster mice (Charles River Laboratories; Wilmington, DE) were housed in a climate controlled room with a 12 hr light/dark cycle. Animals had free access to standard diet (Harlan 8604 formulation) and water until preparation of cell cultures. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee. PACAP was obtained from California Peptide Research (Napa, CA), PACAP6-38 from Anaspec (Fremont, CA), and VIP, VIP6-28, PG 97-269, PG 99-465 from Bachem (Torrance, CA). (S)-4-carboxyphenyglycine (CPG) was purchased from Tocris Bioscience (R&D

Systems; Minneapolis, MN), and H89 from Sigma (St. Louis, MO)**.** All other common chemicals were purchased from Sigma (St. Louis, MO).

Cortical cell cultures

Primary mixed cortical cultures containing both neurons and glia were prepared from embryonic day 15-16 mouse pups as previously described (Lobner 2000). Dissociated cortical cells suspended in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum (Atlanta Biologicals; Lawrenceville, GA), 5% fetal bovine serum (Atlanta Biologicals), 2 mM glutamine and glucose (total 21 mM) were plated on 24-well plates coated with poly-D-lysine and laminin. Neuronal-enriched cultures were prepared in an identical manner with the addition of 10 μM cytosine arabinoside 48 hours after plating cultures in order to inhibit glial replication. Neuronal-enriched cultures using this method have astrocyte levels of <1% of the total cell population (Dugan et al. 1995; Rush et al. 2010). Astrocyte-enriched cultures were prepared using a similar protocol to that of the mixed cortical culture, however cortices were obtained from postnatal day 1-3 mouse pups (Choi et al. 1987; Rush et al. 2010; Schwartz and Wilson 1992). To obtain microglial cultures, microglia were collected after shaking astrocyte-enriched cultures and then plated in media containing 10 ng/ml colony stimulating factor (CSF) (Barger and Basile 2001). All cultures were maintained in humidified 5% CO_2 incubators at 37°C.

Radiolabeled cystine uptake

Radiolabeled cystine uptake was measured as previously described (Liu et al. 2012b). Briefly, prior to treatment on *in vitro* day 15, cultures were washed in serum-free media and incubated for a specified period with or without drug. Following incubation, cultures were then washed with HEPES buffered saline solution and immediately exposed to 0.3 μM ^{14}C-cystine (Perkin Elmer; Waltham, MA) for 20 minutes. Following ^{14}C-cystine exposure the cultures were washed with HEPES buffered saline solution and dissolved in 250 μl of 0.1% sodium dodecyl sulfate. Of the 250 μl sample, 200 μl were used for scintillation counting, with the remaining sample used for protein quantification using the Bio-Rad DC protein assay (Hercules, CA). Counts were normalized to ^{14}C-cystine uptake in controls on the same experimental plate. To inhibit system x_c^- activity, 200 μM CPG was added to the ^{14}C-cystine solution during the uptake experiment.

Quantitative reverse transcription PCR

Cultures used for qRT-PCR were treated identically to those used for radiolabeled cystine uptake assays. However, following drug incubation, cells were harvested for total RNA extraction using TRiZOL (Life Technologies; Grand Island, NY). Single-stranded cDNA synthesis was performed with 1 μg total RNA using the Promega Reverse Transcription System (Madison, WI). Real-time quantitative PCR was performed with the StepOne real-time PCR system (Applied Biosystems; Carlsbad, CA) using PerfeCTa SYBR Green FastMix containing ROX (Quanta Biosciences; Gaithersberg, MD). Relative quantification of xCT transcripts was analyzed via the $\Delta\Delta C_t$ method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for each gene were designed to span an exon-exon junction and had efficiencies of approximately 95%. Product sizes

for each primer set were 88 bp for xCT and 116 bp for GAPDH. Melt curve analysis of experiments confirmed a single product for each reaction. Primer sequences were as follows: xCT forward – AGG GCA TAC TCC AGA ACA CG; xCT reverse – GAC AGG GCT CCA AAA AGT GA; GAPDH forward – AAG GGC TCA TGA CCA CAG TC; and GAPDH reverse – GGA TGC AGG GAT GAT GTT CT.

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance. Fischer LSD analysis was used for all post-hoc group comparisons. Statistical analyses were performed using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $P < 0.05$ were considered statistically significant.

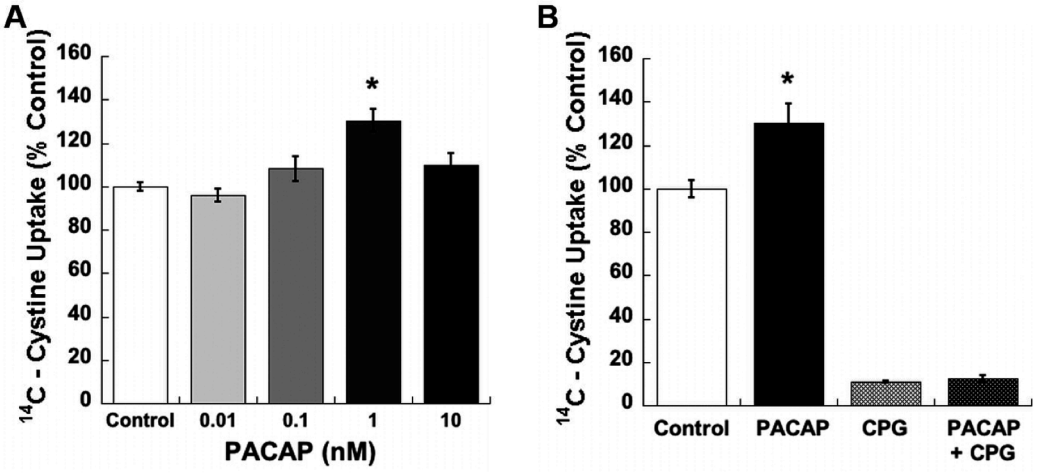

Figure 6.1. PACAP increases cystine uptake through system x_c^- in a dose-dependent manner. (A) Dose response of 24-hour PACAP treatment on primary mixed cortical cultures on radio-labeled cystine uptake. (B) Inhibition of system x_c^- with (S)-4-carboxyphenylglycine (CPG; 200 μM) blocks cystine uptake in both control and PACAP (1 nM) treated primary mixed cortical cultures. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

Results

The effect of PACAP on radiolabeled cystine uptake was initially tested in primary mixed cortical cultures using doses ranging from 0.01 and 10 nM PACAP. By 24 hours post-treatment, 1 nM PACAP showed a significant increase in radiolabeled cystine uptake (Fig. 6.1A; $P < 0.01$). Consequently, we used the 1 nM dose of PACAP for all future experiments examining cystine uptake following PACAP treatment. To determine whether the increased cystine uptake induced by PACAP was attributed to system x_c^- activity, we applied the system x_c^- inhibitor CPG (200 μM) during uptake experiments. CPG inhibited approximately 90% of radiolabeled cystine uptake in control cells as well as the increase in system x_c^- activity following 24-hour PACAP treatment (Fig. 6.1B).

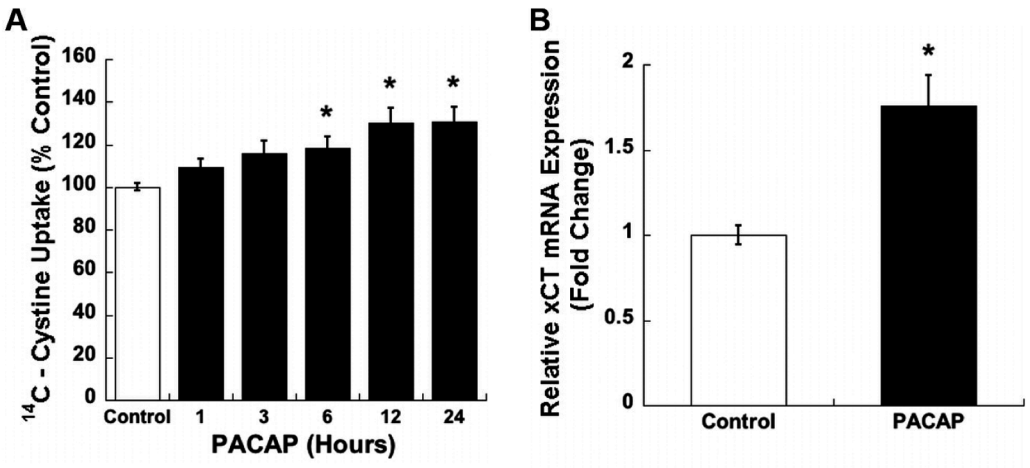

Figure 6.2. Time course of cystine uptake and quantitative PCR for xCT mRNA expression following PACAP treatment. (A) A time course revealed a delayed effect of PACAP (1nM) on cystine uptake in primary mixed cortical cultures. PACAP treatment for 6, 12, and 24 hours yielded significant increases in radiolabeled cystine uptake. (B) Quantitative PCR from RNA isolated from control and 24-hour PACAP (1 nM) treated primary mixed cortical cultures. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

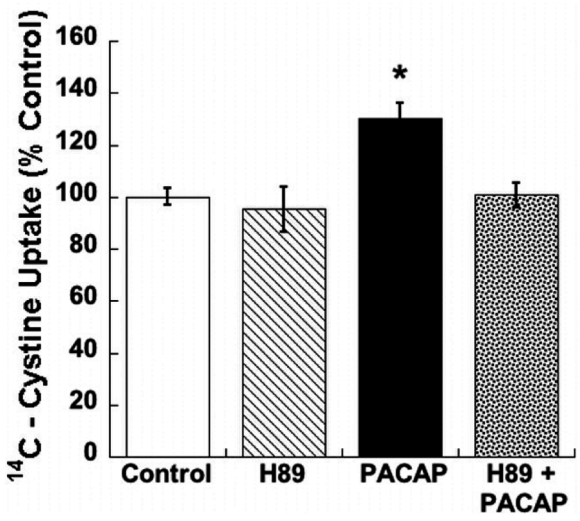

Figure 6.3. PACAP increases cystine uptake via a PKA-dependent pathway. 10 μM of the PKA inhibitor H89 completely blocked increases in cystine uptake induced by 24-hour PACAP (1 nM) treatment in primary mixed cortical cultures. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

Subsequently, mixed cortical cultures were incubated with 1 nM PACAP for 1, 3, 6, 12, and 24 hours prior to the radiolabeled cystine uptake assay to determine the optimal incubation period for PACAP treatment needed to augment system x_c^- activity. PACAP treatment produced significant increases in cystine uptake following incubation times ranging from 6-24 hours (Fig 6.2A; $P < 0.05$). The necessity for long-term treatment with PACAP to significantly increase cystine uptake in primary mixed cortical cultures suggests that PACAP may be involved with the transcriptional regulation of the cystine-glutamate antiporter. Using quantitative real-time PCR (qPCR) we measured mRNA expression of the unique functional subunit of system x_c^-, xCT. Relative quantification for xCT mRNA was performed on total RNA extracted from control or 24-hour PACAP treated primary mixed cortical cultures normalized to the housekeeping

gene GAPDH. In primary mixed cortical cultures, 24-hour treatment with PACAP produced significantly increased xCT mRNA levels (Fig. 6.2B; $P < 0.01$).

PACAP signaling through its G protein-coupled receptors often utilizes Gαs to increase adenylyl cyclase, a process leading to cyclic AMP formation and increased activation of protein kinase A (PKA) (Dickson and Finlayson 2009). To determine whether the PKA pathway contributed to the augmentation of system x_c^- activity, mixed cortical cultures were treated with 10 μM H89 (Figiel and Engele 2000), a PKA inhibitor, during 24-hour PACAP treatment. Inhibition of PKA activity by H89 completely blocked the increase in radiolabeled cystine uptake induced by 24-hour PACAP treatment (Fig. 6.3).

To identify the PACAP receptor type mediating the effects on cystine-glutamate exchange, cortical cultures were co-incubated with antagonists for the PAC1 receptor and VPAC receptors. Inhibition of the PAC1R with 100 nM PACAP6-38 did not block PACAP facilitated radiolabeled cystine uptake, but instead potentiated the effects of PACAP treatment alone (Fig 6.4A; $P < 0.05$ compared to PACAP treatment). The nonspecific VPAC receptor antagonist VIP6-28 at a dose of 100 nM was effective in attenuating the increased cystine uptake following 24 hour 1 nM PACAP treatment (Fig. 6.4B). To further investigate which VPAC receptors were involved in the augmentation of system x_c^- activity, primary mixed cortical cultures were pre-treated with specific antagonists for VPAC1R and VPAC2R. A 100 nM concentration of the VPAC1R antagonist PG 97-269 blocked the increased cystine uptake produced by 24 hour PACAP (1 nM) treatment in mixed cortical cells (Fig. 6.4C), while similar treatment with the VPAC2R antagonist PG 99-465 had no effect (Fig. 6.4D). Finally, a dose response using

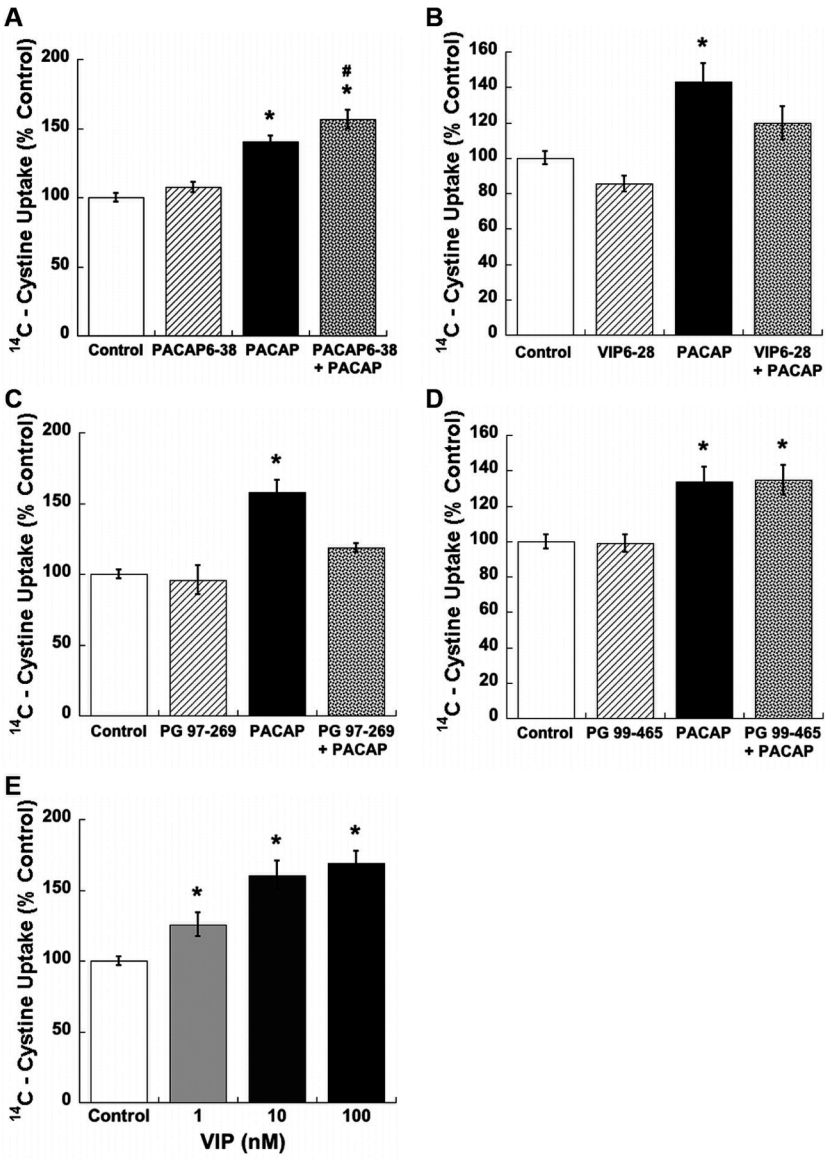

Figure 6.4. Increases in cystine uptake induced by PACAP are mediated by VPAC1 receptors. (A) The PAC1R antagonist PACAP6-38 (100 nM) caused a potentiation of 1 nM PACAP on cystine uptake. (B) The VIP receptor antagonist VIP6-28 (100 nM) significantly attenuated the increased cystine uptake caused by PACAP treatment. (C) Treatment with 100 nM PG 97-269 (specific VPAC1R antagonist) abolished the 1 nM PACAP effect on cystine uptake, while (D) 100 nM PG 99-465 (VPAC2R antagonist) had no effect. (E) A dose response of 24-hour VIP treatment showed that VIP increases cystine uptake at a similar dose to PACAP, as well as at the higher dose ranges where PACAP was ineffective. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group. # = $P < 0.05$ compared to PACAP group.

VIP concentrations ranging from 1, 10, and 100 nM also produced significant increases in radiolabeled cystine uptake (Fig. 6.4E; $P < 0.05$).

To isolate which cell type in a mixed cortical preparation primarily contributed to the increased radiolabeled cystine uptake following incubation with PACAP or VIP, we applied similar measures to neuronal, astrocyte, and microglia-enriched cultures. Optimal concentrations determined from previous dose response experiments in mixed cortical cultures resulted in the use of 1 nM PACAP (Fig. 6.1A) and 10 nM VIP (Fig. 6.4E) doses. Incubation with either PACAP or VIP produced significant increases in radiolabeled cystine uptake in astrocyte-enriched cultures only (Fig. 6.5B; $P < 0.01$), with no changes detected in neuronal or microglia-enriched cultures.

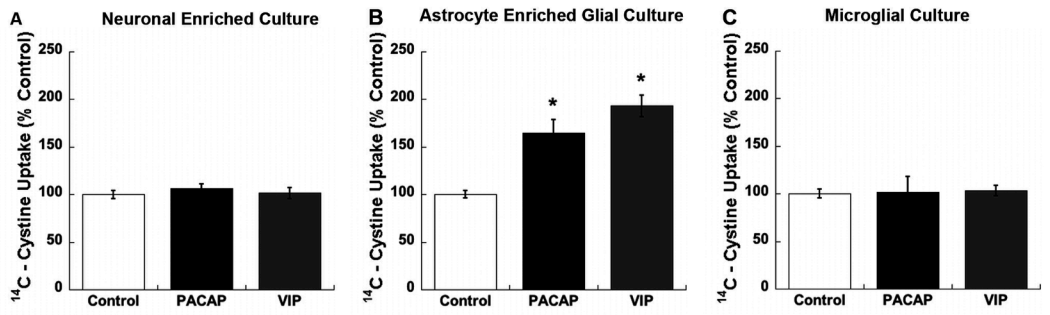

Figure 6.5. Cell type specific analysis of PACAP and VIP treatment on cystine uptake. (A) Cystine uptake was unaffected following PACAP (1 nM) and VIP (10 nM) treatment in neuronally-enriched cultures. (B) Both PACAP and VIP significantly increased cystine uptake in astrocyte-enriched cultures. (C) There was no affect of PACAP or VIP on microglial cystine uptake. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

Discussion

Previous reports have indicated that both neuropeptides and growth factors may be potential regulators of the cystine-glutamate antiporter, system x_c^- (Johnson and Johnson 1993; Liu et al. 2012b). The present study demonstrates that the pleiotropic

neuropeptide PACAP is a novel regulator of system x_c^- activity. 24-hour PACAP treatment of primary cortical cultures significantly increased radiolabeled cystine uptake. Inhibition of cystine uptake following CPG treatment indicated that PACAP facilitated cystine uptake was mediated specifically through system x_c^-. Furthermore, the increases in cystine-glutamate exchange appear to be mediated, in part, by increased transcription of the specific light chain subunit of system x_c^-, xCT. This induction of cystine-glutamate exchange activity appears to be driven primarily via VPAC1R signaling through a PKA-dependent pathway that appears to be specific to astrocytes in our primary cortical culture models.

Cell culture models have shown that overexpression of xCT yields increased glutathione content and neuroprotection against toxicity induced by glutamate and reactive oxygen species (Shih et al. 2006), thus suggesting that signals such as PACAP or VIP may achieve their neuroprotective effects via increased expression and activity of the cystine-glutamate antiporter. In addition, spontaneous deletion of the xCT gene characterized in sut/sut mutant mice results in significant brain atrophy (Shih et al. 2006). However, it should be noted that genetically engineered xCT knockout mice do not appear to have significant behavioral or morphological deficits (Lewerenz et al. 2013), and increased system x_c^- activity may produce excitotoxicity under certain circumstances such as in primary brain tumors (de Groot and Sontheimer 2011) and interleukin-1β mediated hypoxic neuronal injury (Fogal et al. 2007; Jackman et al. 2010).

Alterations in system x_c^- activity and expression have been implicated in various disease states such as gliomas, schizophrenia, and drug abuse (Baker et al. 2008; Baker et al. 2003; Ye et al. 1999). Oxidative stress and glutamate dysfunction lead to the

development of numerous pathologies, demonstrating that system x_c^- may be an attractive target for drug development. Similarly, the potential therapeutic value of PACAP as a neuroprotective drug is high, and others have begun the task of designing drugs targeting PACAP and its receptors for the treatment of neurodegenerative disorders (Bourgault et al. 2011). Thus, PACAP/VIP signaling on astrocytes may be a critical regulator of cystine-glutamate exchange mediating neurotrophic effects in the central nervous system.

PACAP and VIP are neurotrophic factors that have numerous effects on both astrocyte function and glutamate homeostasis (Arimura et al. 1994; Brenneman et al. 1998; Figiel and Engele 2000; Hannibal et al. 2000; Magistretti et al. 1998; Martin et al. 1995; Masmoudi-Kouki et al. 2011; Morio et al. 1996; Seaborn et al. 2011; Yuhara et al. 2001). As an excitatory neuropeptide expressed in glutamatergic neurons, PACAP enhances both NMDA and AMPA currents in the hippocampus through PAC1R signaling (Costa et al. 2009; Hannibal et al. 2000; Macdonald et al. 2005; Yaka et al. 2003). Moreover, PACAP ameliorates glutamate toxicity and oxidative stress suggesting that its signaling mechanism contributes to multiple aspects of glutamate modulation and cell survival. One such mechanism of protection may be the result of enhanced sodium-dependent glial excitatory amino acid transporter (EAAT) expression following PACAP treatment, leading to an increase in synaptic glutamate clearance following high intensity neurotransmitter release (Figiel and Engele 2000). In addition, VIP and PACAP also regulate astrocytic glycogen metabolism through cAMP-dependent activation of the transcription factor CCAAT/enhancer binding protein (Cardinaux and Magistretti 1996; Magistretti et al. 1998; Sorg and Magistretti 1991). Thus, increases in cystine-glutamate exchange activity is yet another mechanism enabling PACAP/VIP to exert its

neuroprotective potential by increasing cystine/cysteine availability for glutathione production to decrease free reactive oxygen species, as well as increasing extrasynaptic glutamatergic tone on the presynaptic group II/III metabotropic glutamate autoreceptors, dampening the potentially harmful excessive synaptic release of neurotransmitter.

Twenty-four hour treatment of primary mixed cortical cultures with PACAP produced a dose-dependent increase in cystine uptake, at an optimal dose of 1 nM, while an elevated dose of 10 nM PACAP did not produce any changes in cystine uptake (Fig. 6.1A). The physiological relevance of prolonged neuropeptide treatment *in vitro* is still somewhat unclear as there is no rapid clearance mechanism for neuropeptides, and diffusion is not an applicable process when neuropeptide concentrations are uniform in the cell culture media. Furthermore, the slow degradation of neuropeptides by aminopeptidases likely result in peptide fragments that are commonly used as neuropeptide receptor antagonists in pharmacology experiments. In the case of PACAP, PACAP6-38 is likely generated a few hours after application, and the consequences of this process may be reflected in some of our data, such as the inverted U-shape curve of cystine uptake observed at high doses of PACAP. Additionally, this increase in cystine uptake in primary mixed cortical cultures at 1 nM PACAP was relatively modest compared to the increases observed in astrocyte-enriched cultures (Fig. 6.5B) suggesting that PACAP-mediated regulation of system x_c^- is primarily driven by signaling on astrocytes, and PACAP stimulation of neurons may evoke other signaling mechanisms that inhibit increases in cystine uptake via system x_c^-. Interestingly, the PAC1R antagonist, PACAP6-38, actually potentiated the effect of 1 nM PACAP treatment in mixed cultures on cystine uptake (Fig. 6.4A), perhaps demonstrating a role for neuronal

PAC1R activation in reducing cystine-glutamate exchange. Furthermore, a dose-response of VIP treatment did not produce the same U-shaped profile for cystine uptake in primary mixed cortical cultures as PACAP (Fig. 6.4E), indicating that VIP-neuron interactions do not suppress system x_c^- activity.

Surprisingly, the nonspecific VIP receptor antagonist VIP6-28 and the VPAC1R antagonist PG 97-269 both blocked PACAP-induced increases in cystine uptake suggesting that regulation of system x_c^- in mixed cortical cells is achieved through VPAC1R signaling and not VPAC2R or the PACAP-specific PAC1R. Importantly, primary cortical neurons express PAC1 and VPAC2 receptor subtypes, while primary cortical astrocytes express all three receptor subtypes including VPAC1 (Grimaldi and Cavallaro 1999). The expression of the VPAC1R is distributed throughout the central nervous system, with very high levels of immunoreactivity reported in cortical layers (Joo et al. 2004). Since, regulation of feeding behavior by hypothalamic PACAP signaling is dependent upon PAC1R and central VIP injections have little effect on food intake (Mounien et al. 2009), it appears unlikely that control of feeding behavior by PACAP is mediated by system x_c^- activity. However, the current studies have not addressed the possible rapid regulation of cystine-glutamate exchange by PACAP which could significantly affect behavior, and similar in vitro studies using cultured hypothalamic cells may be necessary to examine whether the mechanisms of glutamate modulation by PACAP receptors is anatomically specific.

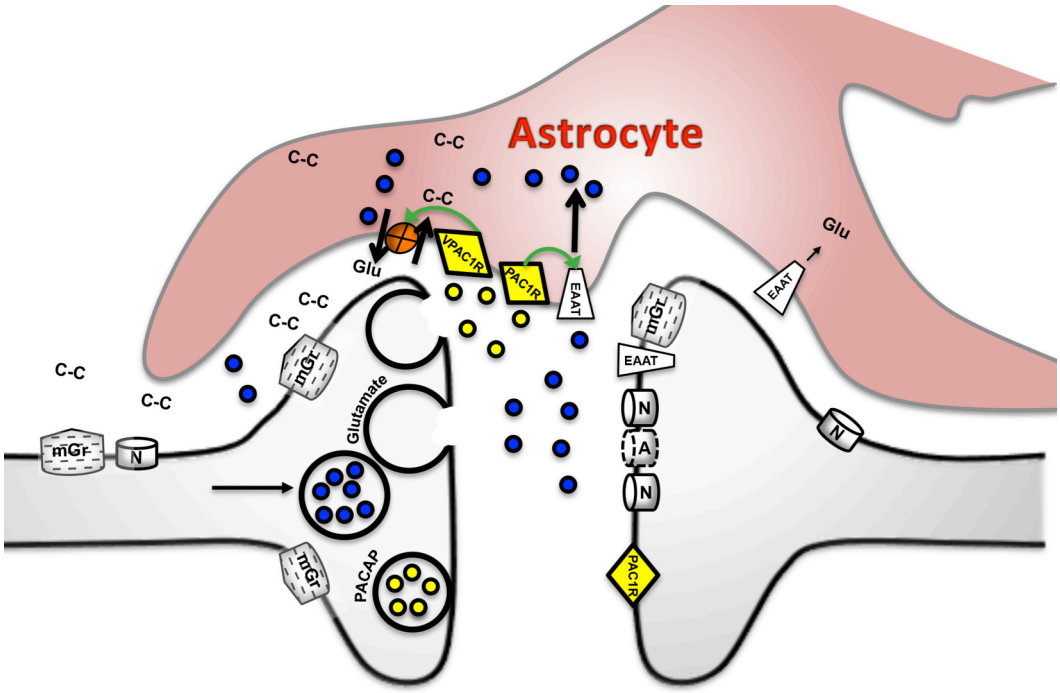

Figure 6.6. Modulation of astrocytic system x_c^- activity by PACAP. PACAP released from Glutamate neurons targets astrocytic receptors altering glutamate signaling by regulating EAATs and system x_c^-. C-C, cystine; mGr, metabotropic glutamate receptor; A, AMPA receptor; N, NMDA receptor.

The current study identifies a novel neuropeptide signal that regulates the function of the cystine-glutamate antiporter (Figure 6.6). PACAP/VIP signaling may critically modulate system x_c^- activity throughout the central nervous system conveying signals to astrocytes of increased activity and oxidative stress at the synapse. Future studies are needed to examine the potentially harmful affects of abnormal PACAP/VIP signaling on system x_c^- function, as well as the therapeutic potential of targeting these neuropeptide signals in order to drive system x_c^- activity for the treatment of disease.

CHAPTER VII

PACAP MODULATION OF GLUTAMATE: HYPOPHAGIA INDUCED BY PACAP IN THE VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS IS MEDIATED BY NMDA RECEPTORS

Introduction

Hypothalamic glutamate neurotransmission is crucial to energy regulation (Fuente-Martin et al. 2012; Liu et al. 2012a; Sternson et al. 2005; Tong et al. 2007; Xu et al. 2013a; Xu et al. 2013b), in part, through the regulation of feeding behavior (Guyenet et al. 2013; Stanley et al. 1993a; Takaki et al. 1992). This is in contrast to classical views of hypothalamic signaling that have focused on the importance of neuropeptides for homeostatic regulation. However, recent investigation into mechanisms of neuropeptide function has indicated that modulation of the fast-acting amino acid neurotransmitters, glutamate and GABA, in addition to effects on cell morphology and gene expression, may be the primary role for neuropeptide signaling (van den Pol 2012). This is demonstrated by neuropeptides, such as orexin or neuropeptide Y, that potently increase feeding behavior through glutamate receptor-dependent pathways (Doane et al. 2007; Lee and Stanley 2005).

The hypothalamic ventromedial nuclei (VMN) are critical regulators of body weight and possess both high levels of glutamate and all glutamate receptor subtypes (Fu and van den Pol 2008; Meeker et al. 1994; Tong et al. 2007; Ziegler et al. 2002). Stimulation of these nuclei produces reductions in food intake and increased metabolic rate (Amir 1990a; Beltt and Keesey 1975; Ruffin and Nicolaidis 1999; Takaki et al. 1992; Yoshimatsu et al. 1993). Likewise, microinjection of PACAP into the VMN also inhibits

feeding behavior through activation of PAC1 receptors even after food deprivation (Resch et al. 2011), however, the signaling downstream of the PAC1R-expressing neurons of the VMN that results in decreased food intake is not clear. Still, previous demonstration of synergy between PACAP and glutamate (Hannibal et al. 2000; Harrington et al. 1999; Macdonald et al. 2005) suggests that PACAP-PAC1R signaling in the VMN may result in augmented glutamate neurotransmission.

Co-localization of PACAP and glutamate immunoreactivity in retinal ganglion cells, as well as in nerve terminals located in the suprachiasmatic nuclei (SCN) support a mechanism of co-release at synapses of the retinohypothalamic tract (Engelund et al. 2010; Fahrenkrug and Hannibal 2004; Hannibal et al. 2000). Functionally, PACAP application to SCN slices produces dose-dependent phase shifts in circadian rhythms through modulation of NMDA receptor activity (Harrington et al. 1999). Moreover, PACAP enhances NMDA receptor activity in the hippocampus, reportedly by two separate mechanisms involving Src tyrosine kinase signaling. The first of which involves cAMP/PKA-dependent activation of Fyn, a member of the Src tyrosine kinase family, leading to phosphorylation of multiple tyrosine residues on the GluN2B subunit of the NMDA receptor (Yaka et al. 2003). The second was shown to occur via PAC1R activation of a phospholipase C pathway leading to Src tyrosine kinase activation and augmented hippocampal NMDA receptor function (Macdonald et al. 2005). Both PACAP-mediated signaling pathways suggest that modulation of NMDA receptors can occur through Src kinase activity, which has been implicated in the regulation of feeding behavior by lateral hypothalamic neurons (Khan et al. 2004).

Glutamate is also substantially regulated by astrocytes, which are major targets of PACAP signaling (Masmoudi-Kouki et al. 2007; Tatsuno et al. 1996a; Tatsuno et al. 1996b), yielding another potentially important mechanism for glutamate modulation by PACAP. Astrocytes impact glutamate neurotransmission most notably through removal of synaptic glutamate by sodium-dependent excitatory amino acid transporters (EAATs) (Asztely et al. 1997; Diamond and Jahr 2000; Zheng et al. 2008) and glutamate release from astrocytes themselves (Baker et al. 2002; Kupchik et al. 2012; Moran et al. 2005; Parpura and Haydon 2000; Ye et al. 2003). Given that a single astrocyte can interact with numerous synapses (Bushong et al. 2002; Ogata and Kosaka 2002), their influence over glutamate signaling and overall network activity is immense. Therefore, investigation into how glutamate neurotransmission is influenced by astrocytes may reveal new insights into signaling mechanisms that are unique to glutamatergic synapses and the manner by which they influence behavior. In terms of PACAP signaling, this neuropeptide stimulates expression of both GLAST and GLT-1 (EAAT1 and EAAT2 respectively) in primary cortical astrocyte cultures (Figiel and Engele 2000) and augments cystine-glutamate exchange through increasing system x_c^- activity (Resch et al. 2014 under review), illustrating that separate mechanisms driven by the same neuropeptide signal can increases both uptake and release of glutamate by astrocytes. Despite the extensive study of how astrocytic control of glutamate affects hypothalamic function (Fuente-Martin et al. 2012; Gordon et al. 2009; Oliet et al. 2001; Potapenko et al. 2012) and knowledge that PACAP potently regulates feeding behavior (Hawke et al. 2009; Morley et al. 1992; Mounien et al. 2009; Resch et al. 2013), whether or not

modulation of astrocytic glutamate signaling by PACAP influences food intake has yet to be investigated.

In order to determine whether modulation of glutamate signaling underlies the regulation of feeding behavior by PACAP in the hypothalamus we measured food intake following administration of pharmacological inhibitors of system x_c^- or NMDA receptor function within the VMN prior to PACAP injection. Our results suggest that PACAP interacts with and possibly potentiates both system x_c^- and NMDA receptor activity, however only modulation of NMDA receptors significantly altered PACAP-induced hypophagia in the VMN.

Methods

Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 225-250 g were individually housed in a climate controlled room with a 12 hr light/dark cycle. Animals had free access to Harlan standard diet (8604 formulation) and water. Food consumption was measured with a BioDAQ Food Intake Monitor (Research Diets; New Brunswick, NJ) or calculated by pre-weighing food in each bin and subtracting the weight of non-ingested and spilled food at the end of each measurement period. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; ip) cocktail and placed in a stereotaxic apparatus. Bilateral guide cannulae (26 gauge; Plastics One; Roanoke VA) were placed 3 mm dorsal to the target site in all animals, and secured to the surface of the skull with an acrylic resin. The stereotaxic coordinates for the VMN were anterior/posterior, -2.5 mm from bregma; medial/lateral, ±0.6 mm from midline; dorsal/ventral, -6.2 mm from surface of the skull based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos and Watson 2007). Injectors extended 3 mm past the ventral tip of the cannulae reaching a VMN injection site of -9.2 mm ventral from the surface of the skull. The upper incisor bar was positioned -3.3 mm below horizontal zero. A bilateral dummy stylet placed in the guide cannulae was used to maintain patency. All animals were given at least five days to recover after cannula installation before receiving drug or vehicle injections, during which time the animals were handled and dummy stylets were removed and replaced daily in order to acclimate the animals to the physical handling necessary during experiments. Correct cannulae placements were confirmed at the conclusion of each experiment by microscopic examination of Nissl stained sections and only those with correct placement were included in the studies.

Feeding Behavior Experiments

Animals were weighed daily and acclimated to the BioDAQ Food Intake Monitor for at least 7 days before the onset of the experiment. In all experiments, approximately 1 hour prior to lights off rats received bilateral microinjections of an antagonist, which

included sulfasalazine (SSZ; 10 - 10,000 pmol in 10 % dimethylsulfoxide (DMSO)/0.25 µl/side; Anaspec; Fremont, CA), (S)-4-carboxyphenylglycine (CPG; 10 - 10,000 pmol in saline/0.25 µl/side; Tocris Bioscience; Minneapolis, MN), D-(-)-2-amino-5-phosphonopentanoic acid (AP5; 10 - 10,000 pmol in saline/0.25 µl/side; Tocris Bioscience), 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP1; Src kinase inhibitor; 1 - 100 pmol in 10 % DMSO/0.25 µl/side; 1 nmol in 25% DMSO/0.25 µl/side; Tocris Bioscience), or vehicle alone over approximately two minutes in awake animals while gently restrained. When necessary, DMSO was used as a vehicle for water insoluble substances such as SSZ and PP1, as it has been previously verified to be suitable for such feeding experiments, causing no significant alterations in food intake when administered alone, even with solutions at concentrations as high as 75% (Blevins et al. 2002; Khan et al. 2004). Upon completion of antagonist injections an additional minute elapsed before removing injectors to minimize backflow of injected material. Five minutes later (15 minutes for PP1 studies) rats received a second bilateral injection of either saline or PACAP (50 pmol/0.25 µl/side; PACAP38; California Peptide Research; Napa, CA) followed by subsequent feeding measurements. The optimal injection volume of 0.25 µl and subsequent spread within the VMN was determined previously (Resch et al. 2011). Feeding measurements were collected for the next 24 hours, with the greatest emphasis placed on the first 3-5 hours after microinjections were delivered.

Quantitative reverse transcription PCR

Three hours post-injection of saline or PACAP (50 pmol/0.25 µl/side) into the VMN, bilateral dissections of the VMH were collected and snap frozen in liquid nitrogen.

Total RNA was isolated from VMH punches by TRIzol extraction (Invitrogen; Carlsbad, CA). Subsequently, cDNA was constructed with 1 μg of total RNA using the Reverse Transcription System (Promega; Madison, WI). Quantitative PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems; Carlsbad, CA), and PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences; Gaithersberg, MD) according to the manufacturer's protocol. Quantification of xCT expression was done using the ΔΔCT method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primers for each gene were designed to span an exon-exon junction and had efficiencies of approximately 95%. Product sizes for each primer set were 112 bp for xCT and 126 bp for GAPDH. Melt curve analysis of experiments confirmed a single product for each reaction. Primers were as follows: xCT - AGGGCATACTCCAGAACACG and ATGCTCGTACC CAATTCAGC; GAPDH - CTCCCATTCTTCCACCTTTGA and ATGTAGGCCATGAGGTCCAC.

Western blot analysis of GluN2B tyrosine phosphorylation

Thirty minutes following saline or PACAP (50 pmol/0.25 μl/side) injections into the VMN, bilateral dissections of the ventromedial hypothalamus (VMH) were collected. VMH tissue was homogenized in ice-cold homogenization buffer (320 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10mM EDTA, 10mM EGTA) containing Halt protease and phosphatase inhibitor cocktail (Pierce; Rockfork, IL) with a Teflon homogenizer (10 strokes), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4° C to remove nuclei and large debris. The resulting supernatant was further centrifuged at 10,000 X g for 30 minutes at 4° C to obtain a crude membranal pellet that was resuspended in solubilization buffer (1% Triton x-100, 150 mM NaCl,

10nM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGT, protease & phosphatase inhibitor cocktail). Protein quantification of samples was determined using a bicinchoninic (BCA) assay (Pierce). Membrane protein (20 µg) was run on an 8% gel by SDS-PAGE and transferred to a polyvinylidine fluoride (PVDF) membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.1 % Tween-20 (TBS-T). Blots were then probed with rabbit anti-pY1336 GluN2B antibody (Rockland Immunochemicals; Gilbertsville, PA) overnight at 4° C, followed by washes with TBS-T and incubation with an HRP-conjugated mouse anti-rabbit secondary antibody (Jackson Immunoresearch; West Grove, PA) at room temperature for 2 hours. Band intensities for pY1336 were developed using SuperSignal West Femto chemiluminescent substrate (Pierce) and visualized using the Kodak Image Station 4000MM. After visualization of pY1336 signal, blots were stripped and reprobed in an identical fashion for total GluN2B expression using mouse anti-GluN2B (Rockland Immunochemicals) and HRP-conjugated goat anti-mouse (Jackson Immunoresearch) antibodies. Band densities were measured using Kodak Molecular Imaging Software v4.0.

Radiolabeled cystine uptake from VMN punches

Brain punches containing the ventromedial hypothalamus (VMH) which included the VMN and other nearby hypothalamic regions were collected using a blunt 16 gauge needle and incubated in 95% O_2: 5% CO_2 slice buffer (118 mM NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 2.7 mM $MgSO_4$, 26 mM $NaHCO_3$, 3.2 mM $CaCl_2$, and 7.8 mM glucose) for 20 minutes for acclimation. Punches were then treated with 1 µM ^{14}C-Cystine and 10 µM TBOA for 20 minutes ±500 µM sulfasalazine (SSZ) or 1 mM S-(4)-carboxyphenylglycine (CPG). Following radiolabeled cystine incubation punches were

washed three times with ice-cold PBS, and dissolved with 1N NaOH. Radioactivity was measured using a scintillation counter and normalized for protein content using the Bio-Rad DC protein assay (Hercules, CA). Data are presented as % radiolabeled cystine uptake of control VMH punches.

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fischer LSD analysis was used for all post-hoc group comparisons. Statistical analyses were performed using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $P < 0.05$ were considered statistically significant.

Results

Prior to determining the affects of cystine-glutamate exchange on feeding behavior, we first tested for functional system x_c^- activity in the hypothalamus by assessing radiolabeled cystine uptake in ventromedial hypothalamus (VMH) punches *ex vivo*. VMH punches were incubated with or without a system x_c^- inhibitor, 500 μM sulfasalazine (SSZ) or 1 mM 4-(S)-carboxyphenylglycine (CPG), in the presence of radiolabeled cystine for 20 minutes. Both SSZ and CPG dramatically reduced radiolabeled cystine uptake in VMH punches to approximately 30% of controls (Fig. 7.1A; $P < 0.05$). Although PACAP modulates both system x_c^- activity and mRNA expression in primary cortical cultures (Resch et al. 2014 under review), there are no reports of similar mechanisms in the hypothalamus. In order to assess whether PACAP signaling affects *in vivo* system x_c^- expression in the hypothalamus we measured

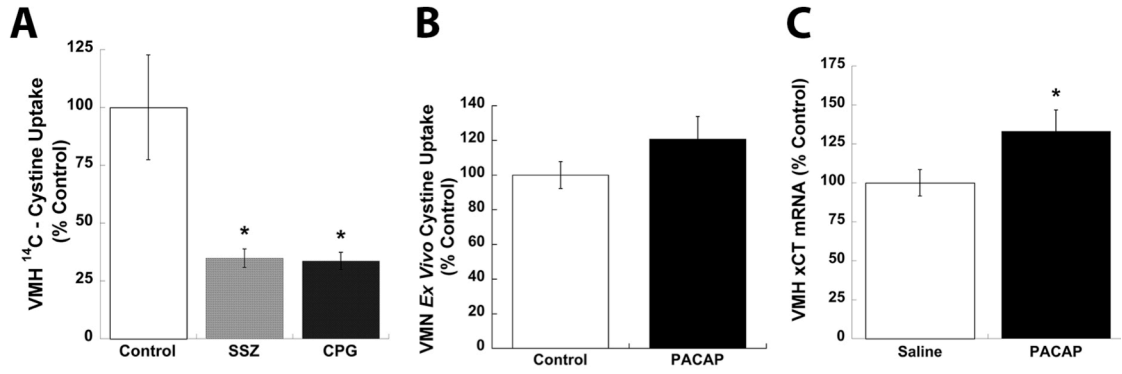

Figure 7.1. Demonstration of system x_c^- activity in the ventromedial hypothalamus and its regulation by PACAP. (A) Radiolabeled cystine uptake in ex vivo VMH punches was significantly reduced with the system x_c^- inhibitors sulfasalazine (SSZ) and 4-(S)-carboxyphenylglycine (CPG). (B) Radiolabeled cystine uptake from *ex vivo* VMH punches treated with 1 nM PACAP was increased compared to controls.(C) Quantitative PCR demonstrates that PACAP injections into the VMN result in increased xCT mRNA expression 3 hours post-injection. Levels of xCT expression were normalized to GAPDH using the ΔΔCt method. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

radiolabeled cystine uptake in VMH punches treated with PACAP and xCT mRNA three hours following microinjections of saline or PACAP into the VMN. PACAP increased cystine uptake by approximately 20% above controls, although, it was not a statistically significant increase. By contrast, PACAP-treated VMH samples demonstrated a significant increase in xCT mRNA compared to saline treatment (Fig. 7.1B; $P < 0.05$).

After confirming system x_c^- activity and PACAP-induced stimulation of xCT mRNA expression in the VMN, we examined whether impairment of cystine-glutamate exchange in the VMN altered feeding behavior using the system x_c^- inhibitor sulfasalazine (SSZ). No concentration of SSZ (50 pmol, 500 pmol, or 5 nmol/side) injected into the VMN injections altered food intake (Fig. 7.2A) nor did SSZ (500

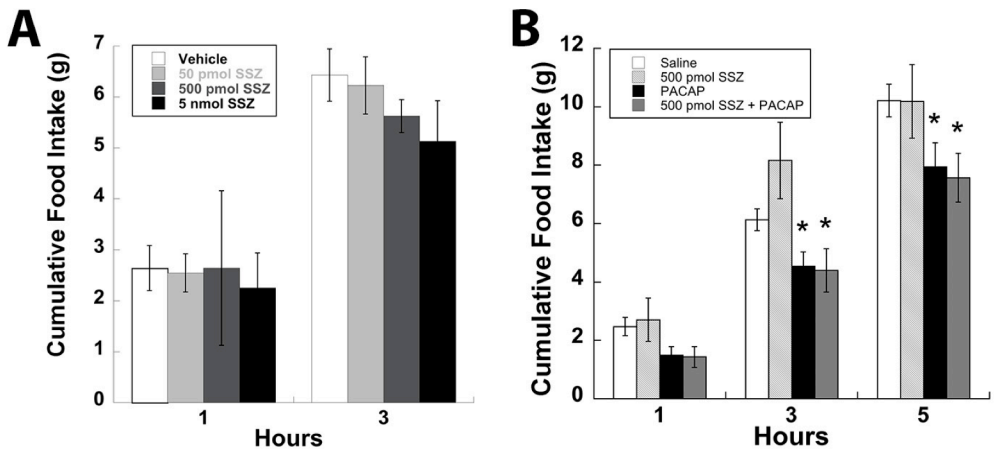

Figure 7.2. Inhibition of the cystine-glutamate antiporter in the VMN with sulfasalazine has no effect on food intake. (A) Dose response of SSZ injections into the VMN shows no differences in food intake. (B) Bilateral pretreatment with 500 pmol of SSZ into the VMN does not attenuate decreased food intake caused by PACAP. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

pmol/side) attenuate PACAP-mediated decreases in food intake (Fig. 7.2B). However, to be certain that the lack of effects on feeding were not due to the choice of system x_c^- inhibitor used, we also tested a second cystine-glutamate antiporter inhibitor, CPG, to determine whether it would attenuate the effects of PACAP on feeding behavior. Multiple doses of CPG (10 pmol - 1 nmol/side) were injected into the VMN prior to PACAP administration, however, like SSZ none of the CPG treatments were effective in blocking the hypophagic response to PACAP in the VMN (Fig. 7.3).

Considering that inhibition of cystine-glutamate antiporter function did not affect feeding behavior or attenuate hypophagia induced by PACAP, we proceeded to test whether glutamate signaling via the NMDA receptor was necessary for PACAP-mediated reductions in food intake. Initially, the NMDA receptor antagonist AP5 was administered into the VMN at multiple doses ranging from 10 pmol to 10 nmol to investigate its

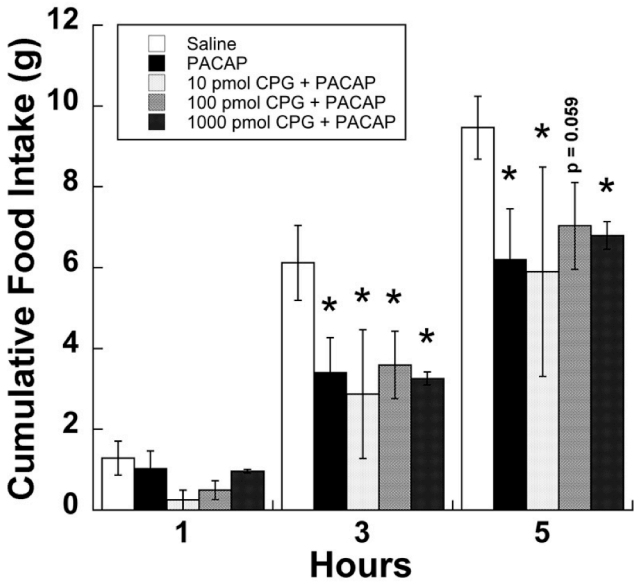

Figure 7.3. Inhibition of system x_c^- activity with 4-(S)-carboxyphenylglycine (CPG) does not block PACAP-mediated hypophagia. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

contribution to normal feeding behavior. Surprisingly, only bilateral injections of 10 nmol AP5 significantly altered feeding behavior by decreasing food intake (Fig. 7.4A; $P < 0.05$). An unexpected result considering inhibition of the VMN produces increased feeding behavior (Berthoud and Jeanrenaud 1979), however, administration of AP5 at concentrations of 10 nmol/side into the VMN also dramatically reduced locomotor activity, which may explain the reduction in food intake (data not shown). To determine whether NMDA receptor function is necessary for PACAP-mediated decreased food intake animals received AP5 (10 pmol/side) injections into the VMN prior to PACAP (50 pmol/side). Although AP5 treatment alone again had no effect on feeding, it did successfully block the effects of PACAP injections into the VMN on food intake (Fig. 7.4B; $P < 0.05$).

With the evidence that NMDA receptor function appeared to be necessary for decreased food intake induced by PACAP, we examined whether similar mechanisms of NMDA receptor potentiation by PACAP were at play in the hypothalamus as have been

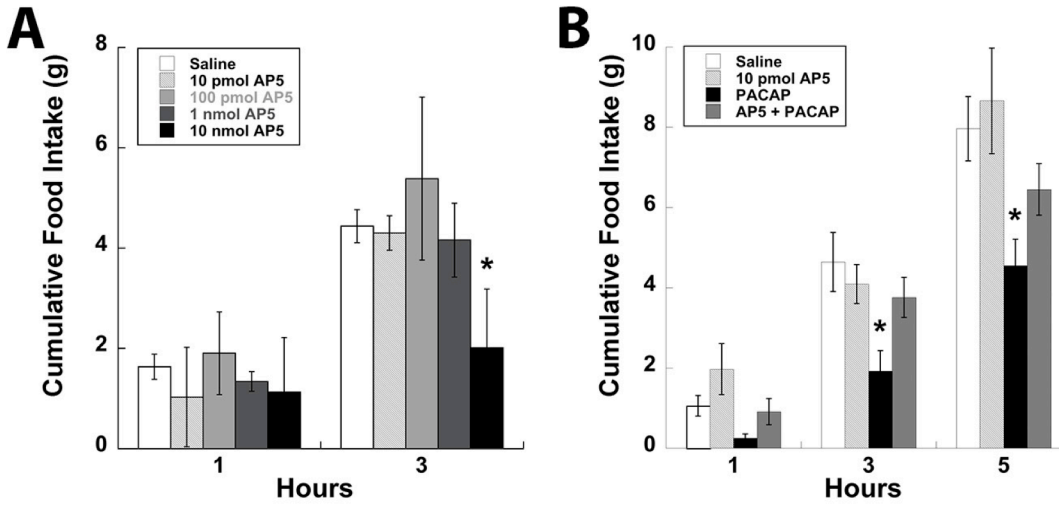

Figure 7.4. NMDA receptor antagonism attenuates the hypophagic effects of PACAP administration into the VMN. (A) Feeding responses to a dose-response of bilateral AP5 microinjections into the VMN. (B) Bilateral injection of 10 pmol AP5 into the VMN blocks the inhibitory effect of PACAP on feeding behavior. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

reported in the hippocampus. PACAP treatment of hippocampal slices was found to produce increased phosphorylation of tyrosines 1252, 1336, and 1472 specifically on the GluN2B subunit of NMDA receptors (Yaka et al. 2003). To test for tyrosine phosphorylation in our feeding behavior model we performed microinjections of saline or PACAP in a similar manner to prior feeding behavior experiments but then collected VMH tissue 30 minutes post-injection for GluN2B tyrosine phosphorylation analysis. In western blot experiments we examined phosphorylation levels of tyrosine 1336 (pY1336) on the GluN2B subunit of the NMDA receptor and normalized band intensities to total

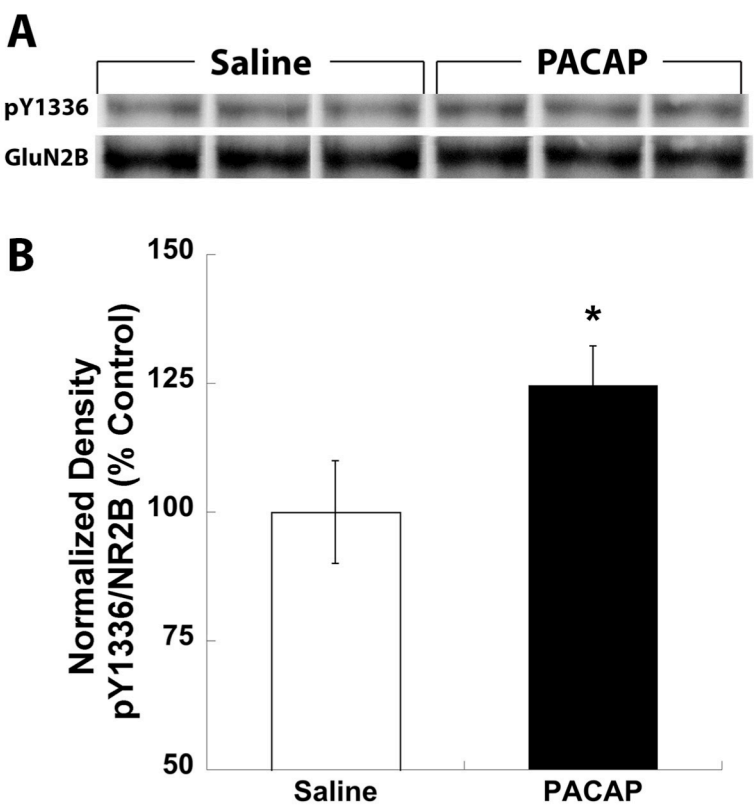

Figure 7.5. PACAP administration into the VMN results in increased tyrosine phosphorylation of the GluN2B subunit of the NMDA receptor. (A) Representative VMH protein samples from saline or PACAP (50 pmol/side) microinjections into the VMN probed for phosphorylation of tyrosine 1336 of the GluN2b subunit (pY1336) and total GluN2B protein expression. (B) Semi-quantitative analysis of band densities for pY1336 normalized to total GluN2b expression expressed as a percent of saline treatment. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

GluN2B expression. Indeed, semi-quantitative analysis demonstrated that VMN PACAP treatment increased pY1336 expression by approximately 25% (Fig. 7.5A & B; $P < 0.05$).

Tyrosine phosphorylation of the NMDA receptor mediated by PACAP signaling has been shown to be a result of increased Src family kinase activity (Macdonald et al. 2005; Yaka et al. 2003). This pathway has also been implicated in the regulation of feeding behavior by the lateral hypothalamus (Khan et al. 2004), and in support of studies conducted in the hippocampus (Yaka et al. 2003), the phosphorylation appears to be

specific to the GluN2B subunit of the NMDA receptor. We confirmed increased GluN2B tyrosine phosphorylation following PACAP administration into the VMN, however, analysis of other NMDA receptor subunits was not performed in our studies. Taken together it is probable that PACAP-PAC1R signaling results in tyrosine phosphorylation of the GluN2B subunit of the NMDA receptor in the VMN through activation of an Src kinase similar to prior reports. To assess this possibility the Src kinase inhibitor PP1 (1 - 1000 pmol/0.25 µl/side) was injected into the VMN followed by measurements of food intake. No concentration of PP1 used in these studies significantly altered food intake when injected into the VMN alone (Fig. 7.6A). However, pretreatment with PP1 (10 pmol/side) in the VMN prior to PACAP injections did significantly attenuate the hypophagic effects of PACAP (Fig. 7.6B; $P < 0.05$).

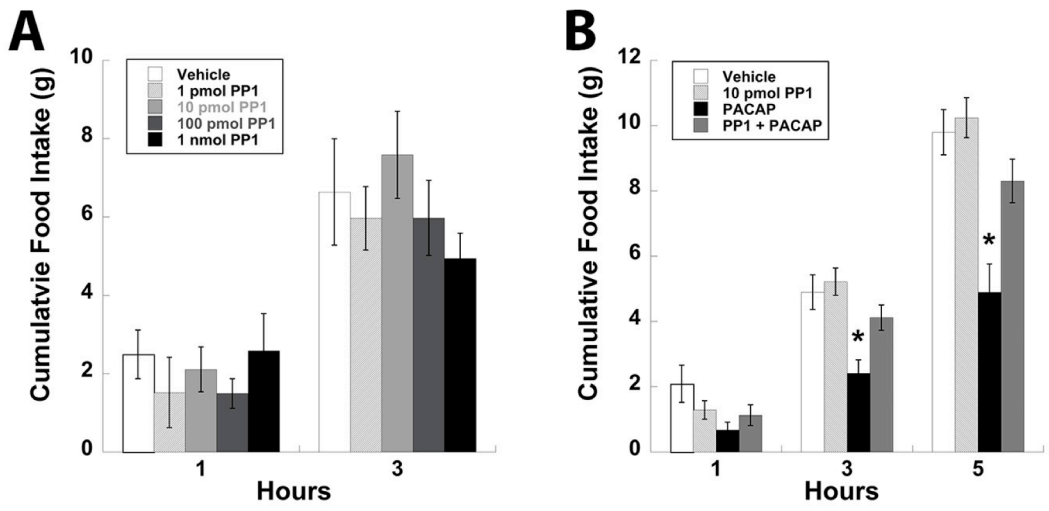

Figure 7.6. Inhibition of Src kinase activity with PP1 attenuates PACAP-induced decreases in food intake in the VMN. (A) Feeding response to PP1 injections into the VMN. (B) Pretreatment of the VMN with PP1 prior to PACAP injections prevents PACAP-mediated hypophagia. Data are expressed as mean ± SEM. * = $P < 0.05$ compared to control group.

Discussion

By inhibiting aspects of glutamatergic signaling we were able to examine the contribution of cystine-glutamate exchange as well as NMDA receptor activity to PACAP-mediated decreases in food intake in the VMN. While functional system x_c^- activity was detected within the VMN, in addition to transcriptional regulation of xCT by PACAP signaling, inhibition of system x_c^- through SSZ or CPG administration failed to mitigate the effects of PACAP on feeding behavior. In contrast, pretreatment with the NMDA receptor antagonist AP5 successfully attenuated the hypophagic response to PACAP administration into the VMN. These data build upon previous findings indicating that PACAP-PAC1R signaling may potentiate postsynaptic NMDA receptor activity (Figure 7.7), perhaps through phosphorylation of the GluN2B subunit (Yaka et al. 2003). Upon examination of GluN2B phosphorylation following *in vivo* VMN PACAP injections, we detected an increase in phosphorylation at tyrosine 1336, a previously identified site of PACAP-induced tyrosine phosphorylation on the GluN2B subunit of the NMDA receptor (Yaka et al. 2003). This Src family of non-receptor tyrosine kinases was previously described to facilitate NMDA receptor phosphorylation mediated by PACAP (Macdonald et al. 2005; Yaka et al. 2003), and hypothalamic Src activity plays a role in controlling feeding behavior (Khan et al. 2004). These findings led us to test whether Src may be a downstream mediator of PACAP-induced hypophagia in the VMN. Inhibition of Src family tyrosine kinases with PP1 effectively blocked the effects of PACAP on feeding behavior, further supporting PACAP-mediated potentiation of NMDA receptors via an Src-dependent pathway.

Although we did not detect an effect feeding behavior following inhibition of system x_c^- activity, altered cystine-glutamate exchange significantly impacts memory, psychosis, and motivated behaviors (Baker et al. 2008; Baker et al. 2003; De Bundel et al. 2011; Knackstedt et al. 2009; Zhou and Kalivas 2008). Furthermore, under certain conditions it is possible that augmented glutamate release from the cystine-glutamate antiporter may lead to increased excitation of VMN neurons and decreased feeding through activation of extrasynaptic NMDA or metabotropic glutamate receptors. Indeed, our dose-response of SSZ injections into the VMN showed alterations in feeding behavior at higher doses, and while not significant this suggests an endogenous glutamatergic signal at extrasynaptic NMDA and metabotropic glutamate receptors in the VMN. In addition, our current studies focused only on natural nocturnal feeding behavior, so while PACAP does appear to regulate cystine-glutamate exchange, under our experimental conditions changes in system x_c^- activity does not contribute significantly to PACAP-mediated decreases in food intake in the VMN, but may under different circumstances.

Given that system x_c^- is predominantly found on astrocytes, and astrocytes are critical to both glutamate and glucose homeostasis in the hypothalamus (Fuente-Martin et al. 2012; Gordon et al. 2009; Potapenko et al. 2012), a more likely role for system x_c^- in energy balance may exist in glycemic regulation. In support of this hypothesis, both the VMN and third ventricle tanycytes are critical to hypothalamic glucose sensing (Borg et al. 1997; Garcia et al. 2003; Sanders et al. 2004; Tong et al. 2007) and express xCT mRNA (Sato et al. 2002). Furthermore, stimulating cystine-glutamate exchange via systemic administration of the cysteine prodrug N-acetylcysteine improves glucose

tolerance and attenuates weight gain from a high sucrose diet (Novelli et al. 2009; Souza et al. 2008). Although the current experiments did not examine glucose regulation, there is evidence that PACAP administration increases both blood glucose (Resch et al. 2013) and system x_c^- activity (Resch et al. 2014 under review) suggesting that activation of system x_c^- by PACAP in the VMN may impact peripheral glucose availability.

Similar to our current data combining NMDA receptor antagonists with PACAP injections into the VMN, inhibition of NMDA receptors also blocks hyperphagia induced by orexin and neuropeptide Y in the lateral hypothalamus (Doane et al. 2007; Lee and Stanley 2005). Furthermore, AMPA/kainate and NMDA glutamate receptor agonists produce robust feeding in sated rats, and AP5 significantly reduces refeeding following a fast when administered into the lateral hypothalamus (Stanley et al. 1993a; Stanley et al. 1996; Stanley et al. 1993b). Although treatment with AP5 in the VMN did not produce increased feeding behavior as expected in our experiments, AP5 was administered at a time when feeding behavior was naturally at its peak, possibly reducing our ability to detect AP5-induced alterations in feeding behavior. Moreover, we unexpectedly observed significant decreases in food intake following high doses of AP5 (10 nmol/0.25 µl/side), however, high doses of AP5 appeared to concomitantly produce immobility in animals post-injection (data not shown) likely due to VMN regulation of physical activity (Challet et al. 1995; Challet et al. 1996; Choi et al. 1998; Narita et al. 2002; Narita et al. 1993; Resch et al. 2013; Yokawa et al. 1989). In light of our current findings that VMN PACAP injections produce NMDAR-dependent hypophagia and GluN2B tyrosine phosphorylation, GluN2B containing NMDA receptors may significantly regulate feeding behavior. Interestingly, GluN2B expression is widespread throughout the

hypothalamus including in the VMN. In the lateral hypothalamus, antagonism of GluN2B containing NMDA receptors with ifenprodil attenuates the feeding response to both fasting and microinjection of NMDA into the lateral hypothalamus, although ifenprodil concentrations used in these studies may have also antagonized GluN2A containing NMDA receptors (Khan et al. 1999; Khan et al. 2000). Furthermore, the phosphorylation state of the GluN2B subunit may be critical for augmenting NMDA receptor activity induced by PACAP signaling, and appears to be regulated by the Src family of tyrosine kinases (Macdonald et al. 2005; Yaka et al. 2003), a family of kinases which have also been reported to reduce GluN3A trafficking to the synaptic membrane (Chowdhury et al. 2013). Importantly, inhibition of Src family tyrosine kinases by PP1 has previously been reported to block NMDAR-dependent feeding behavior in the lateral hypothalamus (Khan et al. 2004), similar to our data regarding PACAP signaling in the VMN.

Although our investigations of PACAP-mediated feeding behavior in the VMN cannot directly demonstrate potentiation of postsynaptic NMDA receptors by PACAP signaling, extensive biochemical and electrophysiological analysis of glutamate signaling in the hippocampus has demonstrated this modulatory pathway. PACAP application yields enhancement of field excitatory postsynaptic potentials, brain derived neurotrophic factor mRNA expression, and tyrosine phosphorylation of the GluN2B subunit of the NMDA receptor by Fyn tyrosine kinase, which is a member of the Src family of tyrosine kinases (Yaka et al. 2003). Further coupling PACAP to glutamate signaling, PACAP augments NMDA currents following Schaffer collateral stimulation through a PAC1R-dependent pathway that activates Src tyrosine kinase (Macdonald et al. 2005). While two different mechanisms of NMDA receptor potentiation by PACAP have been identified,

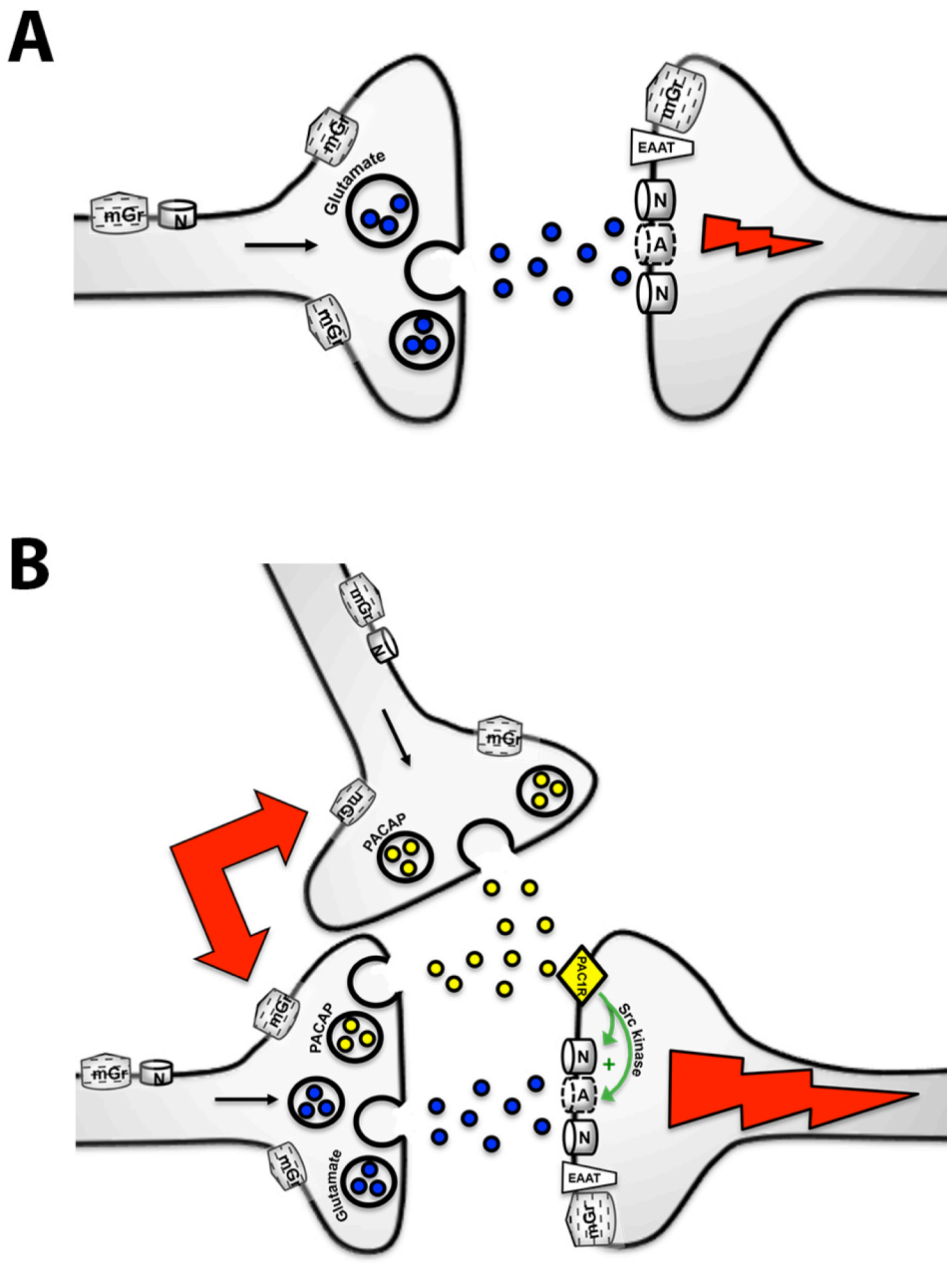

Figure 7.7. Modulation of NMDA receptor signaling by PACAP. (A) Conventional glutamatergic synapse showing no modulation of glutamate neurotransmission leading to lesser excitatory postsynaptic potential (EPSP). (B) Proposed potentiation of EPSP following modulation of glutamate receptors by PACAP released from glutamate and PACAP co-expressing neurons or PACAPergic neurons.

both occur as a result of tyrosine phosphorylation of NMDA receptors by a member of the Src family of tyrosine kinases (Fyn and Src).

PACAP signaling may also modulate other aspects of glutamate signaling including the AMPA receptor, as PAC1R-dependent potentiation of AMPA receptors at low PACAP concentrations and VPAC2R-mediated depression of AMPA receptors at high concentrations of PACAP have been reported in the hippocampus (Costa et al. 2009). Furthermore, excitatory transmission from the basolateral amygdala to the central amygdala is augmented through PACAP-VPAC1R increases in AMPA receptor activity (Cho et al. 2012). However, without further behavioral pharmacology examining glutamate receptor-mediated properties of PACAP-induced hypophagia in combination with the appropriate electrophysiology, the role of other modes of glutamatergic neurotransmission cannot be ascertained. Nevertheless, our experiments using AP5 and PP1 attenuated the effects of PACAP on feeding suggesting a similar mechanism of NMDA receptor modulation by PACAP likely exists in the hypothalamus as it does in the hippocampus.

CHAPTER VIII

General Discussion

Summary

The studies described in this thesis highlight some of the first experiments investigating site-specific effects of PACAP signaling on feeding and metabolism in the hypothalamus. This dissertation demonstrates that PACAP administration either into the PVN or VMN produced hypophagia mediated by the PAC1R, while increases in energy expenditure via elevated activity and core body temperature were only observed following PACAP administration into the VMN. Retrograde tracing from the PVN and VMN combined with fluorescent *in situ* hybridization for PACAP mRNA suggest partially overlapping PACAP circuits innervating these two hypothalamic nuclei, however, distinct efferent circuits could easily account for the dichotomy in energy expenditure (Chapter IV), as well as the perceived increased potency of PACAP-induced decreases in feeding from PVN administration (Chapter III).

While the effects of icv and hypothalamic PACAP administration on body weight regulation appear to be consistent across studies (Chance et al. 1995; Dore et al. 2013; Hawke et al. 2009; Masuo et al. 1995; Mizuno et al. 1998; Morley et al. 1992; Mounien et al. 2009; Pataki et al. 2000; Resch et al. 2011; Resch et al. 2013), the mechanisms by which PACAP modulates neurotransmission to affect feeding and metabolism are still unclear. In a number of brain regions, PACAP is co-expressed with glutamatergic markers (Figure 5.1) (Engelund et al. 2010; Hannibal et al. 2000) and has been implicated to regulate glutamate signaling (Cho et al. 2012; Figiel and Engele 2000; Macdonald et

al. 2005), leading us to investigate potential mechanisms of glutamate modulation by PACAP both *in vitro* and *in vivo*. Expanding on previous reports of astrocytic glutamate transport regulation (Figiel and Engele 2000), we found PACAP to significantly increase astrocytic glutamate signaling via the cystine-glutamate antiporter, system x_c^-, both *in vitro* and *in vivo* (Chapters VI and VII). Despite the stimulatory effects of PACAP on system x_c^- function, augmenting glutamate signaling via PACAP-mediated increases in cystine-glutamate exchange did not appear to contribute to changes in feeding behavior as demonstrated by the ineffectiveness of intra-VMN sulfasalazine (SSZ) or (4)-(S)-carboxyphenylglycine (CPG) to block PACAP-induced hypophagia. On the other hand, potentiation of postsynaptic glutamate receptor signaling by PACAP has been reported in areas of the brain such as the hippocampus and amygdala (Cho et al. 2012; Harrington et al. 1999; Macdonald et al. 2005; Yaka et al. 2003) suggesting that PACAP may augment NMDA or AMPA receptor activity in the hypothalamus. Indeed, NMDA receptor antagonism blocked the effects of PACAP on feeding behavior in the VMN corroborating PAC1R-NMDAR signaling pathways proposed earlier (Chapter VII). Overall these results indicate that PACAP acts as a modulator of glutamate neurotransmission, thereby, augmenting excitatory signaling, which in the case of the VMN leads to decreased food intake and increased metabolism, while maintaining an optimal synaptic environment through astrocytic regulation of glutamate homeostasis. The following sections discuss the importance of the results described within this thesis, as well as remaining questions that should be addressed in the future to further understand hypothalamic PACAP signaling.

What is the endogenous role of PACAP signaling in terms of energy balance?

Central PACAP signaling appears to function as a significant satiety factor, as well as a stimulator of metabolism (Chance et al. 1995; Hawke et al. 2009; Mizuno et al. 1998; Morley et al. 1992; Mounien et al. 2009; Resch et al. 2011; Resch et al. 2013). Unfortunately, the circumstances under which PACAP becomes important for the regulation of energy balance remain unclear. While neuropeptides such as oxytocin and vasopressin are released dendritically and only from brief high-frequency action potentials (Kombian et al. 1997; Leng and Ludwig 2008), data characterizing the release dynamics of PACAP is lacking. Furthermore, in vivo estimates of neuropeptide diffusion and rates of degradation within the hypothalamus are very difficult to predict, but the long duration of PACAP-induced effects may suggest that the neuropeptide is active in some form for quite some time following its release.

Clues to PACAP's physiological importance have emerged in recent years, linking it to several critical hypothalamic signals such as leptin, corticotropin-releasing factor (CRF), and melanocortin signaling (Dore et al. 2013; Hawke et al. 2009; Mounien et al. 2009; Tanida et al. 2013; Tanida et al. 2011a). However, many of these studies administer antagonists along with agonists into the brain's ventricular system, which often results in effective attenuation of agonistic properties (Dore et al. 2013; Hawke et al. 2009; Mounien et al. 2009; Tanida et al. 2013; Tanida et al. 2011a). Experimental designs utilizing ventricular injection routes cannot account for separate but parallel circuits involving PACAP and other neuromodulators. For example, Hawke et al. reported that the PAC1R antagonist PACAP6-38 completely blocked the effects of leptin on feeding and metabolism, which leads to the assumption that PACAP neurons mediate

leptin signaling. Yet, there are several other neuronal populations, not just in the hypothalamus but throughout the brain, that express leptin receptors and affect energy balance independent of PACAP signaling (Balthasar et al. 2004; DiLeone 2009; Elmquist et al. 2005; Kong et al. 2012; Leinninger et al. 2009; Zhang et al. 2011). It is more likely that PACAP6-38 and leptin merely have separate but opposing effects that result in neutralization of the signals leading to false interpretations. Furthermore, PACAP neurons are almost exclusively glutamatergic and leptin regulation of body weight is predominantly mediated through GABAergic neurons (Vong et al. 2011). Therefore, instead of presuming that PACAP has a specific physiological role similar to CRF and stress, it may be more accurate to suggest that PACAP is simply pleiotropic in nature, acting within multiple neural systems as a modulator of glutamate signaling. The pattern of PACAP expression in the brain corroborates this notion, with PACAP mRNA expression found in several brain regions (Hannibal 2002) with a wide range of functions, regulating affect (Dore et al. 2013; Hammack et al. 2009), fear (Ressler et al. 2011; Stevens et al. 2014), and body weight (Hawke et al. 2009; Krashes et al. 2014; Mounien et al. 2009; Resch et al. 2013) among others.

Significance of afferent PACAP circuits to the hypothalamus

The PVN and VMN are both glutamatergic cell populations in the hypothalamus (Ziegler et al. 2002) that produce obesity when lesioned (Choi and Dallman 1999; Tokunaga et al. 1986). Given that icv PACAP administration results in decreased feeding behavior we hypothesized that these two nuclei are likely targets for PACAP-mediated hypophagia. Site-specific PACAP injections into the PVN and VMN did indeed produce decreased feeding similar to icv administration, but alterations to thermogenesis stemmed

from PACAP injections only into the VMN. With this in mind we began to map the PACAP-containing circuits that mediate satiety using retrograde tracing from the PVN and VMN combined with *in situ* hybridization for PACAP mRNA (Figure 8.1). These anatomical studies identified the lateral parabrachial nuclei (LPB) to contain PACAP-expressing neurons that project to both the PVN and VMN indicating that the LPB are prominent sources of PACAP release into these structures. LPB neurons are activated by the satiety signals cholecystokinin (CCK) and amylin (Becskei et al. 2007), as well as by substances that are perceived to be toxic such as LiCl or lipopolysaccharide (LPS) (Carter et al. 2013) producing hypophagia through signaling downstream of the LPB. In addition, activation of ascending fibers carrying cutaneous thermosensory information excite LPB neurons in order to regulate body temperature, thereby identifying this circuit as potentially relevant to VMN induction of BAT thermogenesis by PACAP. More studies investigating the stimuli necessary for activation of PACAP-expressing LPB neurons are needed to understand the specific physiological role of these neurons with respect to feeding and metabolism.

VMN-specific afferents expressing PACAP originated from the medial amygdala (MeA), a brain region less familiar to the study of body weight regulation. While the MeA does possess a small population of glucose-sensing neurons (Zhou et al. 2010) and produces obesity when lesioned, especially in females (King 2006a), its main function appears to be involved in the processing of olfaction, as the MeA receives considerable input from the olfactory bulb (Park et al. 2013) leading to activation of its neurons in the presence of pheromones or predator odors (Bian et al. 2008; Butler et al. 2011; Kondo 1992; Masini et al. 2009; Staples et al. 2008). Therefore, this vomeronasal pathway may

involve the VMN in the activation of the sympathetic fight or flight response and/or dampen parasympathetic activity in times of psychosocial stress, as the VMN have been implicated in mating behaviors and aggression (Yang et al. 2013).

PVN-specific afferents expressing PACAP included the anteromedial bed nucleus of the stria terminalis (BNST), a well-characterized region of the brain regulating stress and anxiety-related behaviors. This PACAP-expressing circuit may be responsible not only for regulation of the hypothalamic-pituitary-adrenal (HPA) axis by the PVN, but also the hypophagia that often corresponds with stressful and anxiogenic stimuli. Interestingly, we found a PACAP-expressing neuronal population in the anteromedial BNST that projects to the PVN that we propose regulates feeding behavior in some fashion, however, a recent study by Kocho-Schellenberg et al. reports that PACAP administration into the posterior BNST produces significant decreases in feeding behavior and body weight, while the the anterior BNST did not (Kocho-Schellenberg et al. 2014). Though these findings appear to be in contradiction, PACAP injections into areas of the BNST stimulate neurons expressing PACAP receptors, and not necessarily the neurons expressing PACAP mRNA that we propose inhibit feeding by projecting to the PVN.

Finally, a subset of PACAP neurons of the VMN project to the PVN, perhaps to the preautonomic subpopulation, providing a connection that allows VMN neurons to produce hypophagia or activation of glucose production in response to hypoglycemia. VMN PACAP neurons also project to pro-opiomelanocortin (POMC) neurons of the hypothalamic arcuate nuclei (ARC) (Krashes et al. 2014) suggesting that stimulation of the VMN by PACAP contributes to additional routes that inhibit feeding behavior.

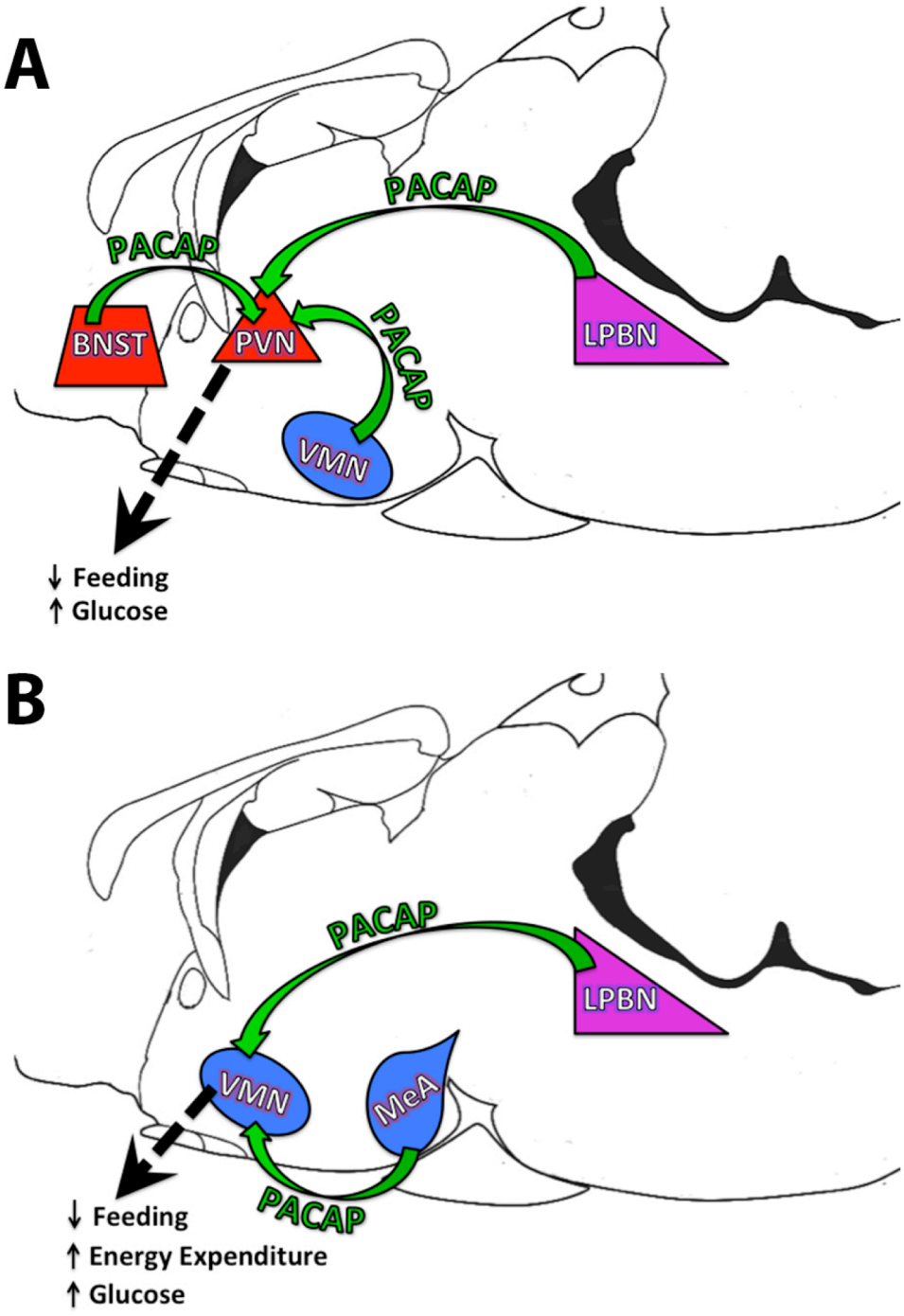

Figure 8.1. Review of PACAP-expressing afferent circuits of the paraventricular (PVN) and ventromedial (VMN) nuclei. (A) Afferents of the PVN that may regulate energy homeostasis through PACAP signaling originate from the bed nucleus of the stria terminalis (BNST), lateral parabrachial nuclei (LPBN), and VMN. (B) Afferents of the VMN that potentially regulate energy balance through PACAP signaling originate from the LPBN and medial amygdala (MeA).

Notably, PACAP administration into the PVN did not affect indices of energy expenditure in our experiments, perhaps suggesting that ARC POMC neuron activation by VMN neurons are responsible for these effects, as they are known to project to and stimulate preganglionic sympathetic neurons of the intermediolateral spinal cord coordinating aspects of sympathetic nervous system activity (Elias et al. 1998).

Significance of efferent PACAP circuits from the hypothalamus

Decades of research focusing on hypothalamic regulation of feeding behavior have referred to both the PVN and VMN as "satiety centers" following demonstrations of stimulation-induced hypophagia and lesion-induced hyperphagia and obesity (Beltt and Keesey 1975; Choi and Dallman 1999; Kennedy 1950; Leibowitz et al. 1981; Ruffin and Nicolaidis 1999; Stenger et al. 1991; Tokunaga et al. 1986). Despite this wealth of data, remarkably little is known about the downstream circuitry that produces satiety, as both of these nuclei have considerable efferent targets making it difficult to isolate the feeding circuits from circuits controlling other aspects of physiology. The utilization of gene targeting approaches allowing for the examination of function within distinct circuits will provide giant steps towards providing a wiring diagram for hypothalamic feeding circuits.

Nonetheless, there are some known efferents that produce satiety involving these nuclei including PACAP projections from the VMN to ARC POMC, but not AgRP/NPY neurons (Krashes et al. 2014; Resch et al. 2011; Sternson et al. 2005), and projections to the PVN (Resch et al. 2013). Efferents from the PVN to areas such as the nucleus tractus solitarius (NTS) have been shown to inhibit brown adipose tissue thermogenesis without effects on satiety (Kong et al. 2012). Recently, however, a pro-feeding circuit was recently discovered involving a subpopulation of PVN neurons that express PACAP

and/or thyrotropin-releasing hormone (TRH). These neurons project specifically to ARC AgRP neurons and provide intense excitatory stimulation under fasting conditions thereby completely transforming our presumptions about PACAP and PVN function (Krashes et al. 2014).

What hypothalamic cell populations express PACAP receptors?

As the organization of the ARC and PVN alone demonstrate, hypothalamic neurocircuitry is extraordinarily complex, often with multiple cell populations within the same nuclei having completely opposing or seemingly unrelated functions. This demonstrates the importance of understanding which specific cell populations are mediating the effects of PACAP signaling on feeding and metabolism. This point is exemplified by PACAP receptor expression in the PVN, which when activated by PACAP injections decrease feeding behavior and increase peripheral glucose levels (Resch et al. 2013). Though we were able to attribute the hypophagic effect of PACAP to activation of the PAC1 receptor we did not identify the receptor involved in modulating glucose availability, although another research group has indicated that VPAC2 receptors are important for stimulation of hepatic glucose production following PACAP injections into the PVN (Yi et al. 2010). It is unclear whether the same PVN cells express both types of PACAP receptors yielding those effects, or if distinct PACAP circuits target different cell populations within the PVN. Currently, expression patterns of PAC1 and VPAC receptors are not well characterized amongst the many cell phenotypes of the PVN, or in any of the other hypothalamic nuclei outside of the ARC where the PAC1 receptor has been linked to feeding and to show expression on both POMC and

NPY/AgRP neurons (Krashes et al. 2014; Mounien et al. 2006a; Mounien et al. 2006b; Mounien et al. 2009).

Glutamate modulation by PACAP

Our results from Chapters VI and VII demonstrate a relationship between PACAP and glutamate signaling that results in increased astrocytic system x_c^- activity, as well as apparent potentiation of NMDA receptor activity in the VMN. Furthermore, our findings demonstrate that tyrosine phosphorylation of the GluN2B subunit of the NMDA receptor in the VMN stimulated by PACAP injections and the attenuation of PACAP-induced hypophagia by inhibition of Src kinases agree with prior reports that an Src-dependent pathway resulting in NMDA receptor potentiation is driven by PACAP (Macdonald et al. 2005; Yaka et al. 2003). However, without evidence of PAC1R signaling acting directly on the NMDA receptor through electrophysiological investigation we cannot completely rule out the possibility of indirect modulation of the NMDA receptor through other PACAP-related mechanisms. While pharmacological inhibition of the NMDA receptor with AP5 and Src with PP1 both attenuated the effects of PACAP on feeding behavior, we acknowledge that eliminating such essential components of cell signaling may render VMN neurons nonfunctional independent of PAC1R signaling pathways. Although we do not believe this is the case, as pharmacological inhibition of NMDA receptors or Src kinase alone did not produce behavioral effects, and others have used similar means to examine NMDA receptor (Stanley et al. 1996) and Src kinase (Khan et al. 2004) activity with regard to feeding behavior.

Modulating other aspects of glutamate signaling to affect feeding behavior is also a viable possibility. As with NMDA receptors, PACAP has been shown to augment

AMPA receptor activity in the hippocampus and amygdala suggesting that multiple second messenger signals with numerous targets could be stimulated by PACAP receptor activation (Cho et al. 2012; Costa et al. 2009). In addition to postsynaptic localization of the PAC1 receptor with ionotropic glutamate receptors (Cho et al. 2012; Costa et al. 2009; Harrington et al. 1999; Macdonald et al. 2005; Shioda et al. 1997; Yaka et al. 2003), PACAP receptors also exist presynaptically on mossy fibers of the hippocampus and cerebellum likely having a significant impact on presynaptic neurotransmitter release (Otto et al. 1999). While postsynaptic mechanisms of PAC1R signaling appear to dominate the literature, we have not specifically addressed the possibility of presynaptic regulation and thus, it cannot be ruled out.

Non-neuronal expression of PACAP receptors, especially on astrocytes, may substantially contribute to PACAP-mediated behaviors by significantly impacting glutamate neurotransmission. Although PACAP regulation of the astrocytic glial glutamate transporters GLT-1 and GLAST could have considerable ramifications on synaptic signaling, we have yet to address such actions by PACAP signaling in the hypothalamus. However, we have demonstrated augmented astrocytic cystine-glutamate exchange by PACAP signaling, with the initial studies conducted in primary cortical cultures, while acknowledging the unique cellular physiology compared to hypothalamic cells *in vivo*. Although we did detect significant increases in xCT mRNA in the VMN following PACAP injections, inhibition of system x_c^- activity did not block PACAP-mediated alterations in feeding behavior. But, without verification of direct action of PACAP signaling both in and out of the synapse it is difficult to unequivocally confirm the exact mechanism by which PACAP modulates glutamate to alter feeding behavior.

Conclusions

The impact of PACAP signaling on body weight is complex. Not only do we report hypophagia and increased energy expenditure from site-specific hypothalamic injections (Resch et al. 2013), recent reports indicate that PACAP-expressing circuits that drive hunger may also exist in the hypothalamus (Krashes et al. 2014). Moreover, PACAP's mechanism of action is still unknown. Even though it appears to have a stimulatory effect via PAC1R that is dependent upon NMDA receptor signaling, PACAP and its receptors have multiple modulatory actions on glutamate signaling in and around the synapse that may be critical to their function. Thus, future directions for investigating the mechanisms by which PACAP controls hypothalamic function should continue to focus on mapping PACAP circuits to and from the hypothalamus, determine which subpopulations of neurons express the feeding responsive PAC1 receptor, and confirm the specific actions of PACAP signaling on glutamate homeostasis, although the latter may prove challenging as differentiating between signals emanating from neurons and astrocytes *in vivo* will necessitate the development of new tools outside of those used here.

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