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PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR: MULTIPLE SIGNALING PATHWAYS INVOLVED IN ENERGY HOMEOSTASIS

by

Brian Maunze, B.S.

A Dissertation submitted to the Faculty of the Graduate School,
Marquette University,
In Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy

Milwaukee, Wisconsin
May 2022

ABSTRACT

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR: MULTIPLE SIGNALING PATHWAYS INVOLVED IN ENERGY HOMEOSTASIS

Brian Maunze, B.S

Marquette University, 2022

Pituitary adenylate cyclase activating polypeptide (PACAP) exerts pleiotropic effects on ventromedial nuclei (VMN) of the hypothalamus and its control of feeding and energy expenditure through the Type I PAC1 receptor (PAC1R). However, the endogenous role of PAC1R's in the VMN and the downstream signaling responsible for PACAP's effects are unknown.

To determine the endogenous role of PAC1Rs and signaling that may explain PACAP's pleiotropic effects, we knocked down VMN PAC1Rs and pharmacologically inhibited PKA, PKC and PAC1R trafficking. Knocking down PAC1Rs increased meal sizes, reduced total number of meals, and induced body weight gain. Inhibition of either PKA or PKC alone in awake male Sprague Dawley rats, attenuated PACAP's anorectic effects during the dark phase. However, PKA or PKC inhibition resulted in a potentiation of PACAP's hypophagic effects during the light phase. Additionally, PKA or PKC inhibition augmented PACAP's thermogenic effects during the light phase, whereas only PKA inhibition augmented PACAP's locomotor effects given that PKC inhibition had no effect. PACAP administration in the VMN induces PAC1R surface trafficking into the cytosol which was blocked by endocytosis inhibitors. Subsequently, inhibition of PAC1R trafficking into the cytosol attenuated PACAP-induced hypophagia.

PACAP signaling replicates the effects of leptin administration in the VMN and appears to enable leptin regulation of energy homeostasis. However, the manner in which PACAP influences leptin signaling is unknown. We used co-immunoprecipitation to show that VMN PAC1 and leptin receptors are found in the same cell, and they form an immunocomplex. Inhibiting downstream effectors of PACAP signaling, such as PKA and PKC, enhanced or prevented leptin signaling respectively.

The current findings revealed that endogenous PACAP signaling in the VMN has a potent regulatory influence over both energy intake in the form of feeding, and energy output via thermogenesis and locomotor activity. Moreover, PACAP actions in the VMN share a nearly identical sequelae to leptin administration in the same brain region suggesting that these two neuropeptides could functionally intersect. These experiments explored VMN PAC1Rs dependence on PKA, PKC, and receptor trafficking to mediate PACAP's pleiotropic effects on feeding and metabolism as well as potential intersecting points with leptin receptor signaling.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………….…......i

LIST OF TABLES……………………………………..……………..………iv

LIST OF FIGURES ……………………………………………………………v

CHAPTERS

I. INTRODUCTION …………………………………………………..…1

i. General Introduction…………………………………...…………1

ii. Hypothalamic Ventromedial Nuclei Regulation of Feeding.…….3

iii. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)....6

iv. Pituitary Adenylate Cyclase-Activating Polypeptide Receptors..9

v. G-protein coupled receptor signaling and termination…………12

vi. β-arrestins Facilitate Dynamic and Sustained GPCR Signaling Beyond G-protein Signaling...……………………………..……14

vii. GPCR Transactivation Facilitates Interconnected Signaling….16

viii. Leptin and VMN Control of Feeding…………………..………17

II. VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS & CORTICAL CELLS EXPRESS PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR ISOFORMS IN THE RAT BRAIN: CONSEQUENCES FOR SIGANLING & CONTROL OF FEEDING…………………………………………....21

III. DEVELOPMENT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR TARGETING-shRNA AND VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS ADENO-ASSOCIATED VIRUS AND LENTIVIRUS TRANDUCTION……………………………………………...….50

IV. HYPOTHALAMIC VENTROMEDIAL NUCLEI (VMN) PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTORS (PAC1Rs) ARE NECESSARY FOR FEEDING AND BODY WEIGHT REGULATION……………………………..……63

V. PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR (PAC1R) ACTIVATION IN THE HYPOTHALAMIC VENTROMEDIAL NUCLEI DIFFERENTIALLY REGULATES DIURNAL HYPOPHAGIA AND METABOLISM BY RECRUITING PROTEIN KINASE A……………………………………………….84

VI VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS UNIQUELY ENGAGE PROTEIN KINASE C TO REGULATE FEEDING AND ENERGY EXPENDITURE ……………………………………….100

VII PACAP'S HYPOPHAGIC EFFECTS IN THE VMN ARE MEDIATED THROUGH PAC1 RECEPTOR TRAFFICKING………………..…113

VIII VMN CELLS INTEGRATE MULTIPLE COMPLEX SIGNALS TO REGULATE FEEDING AND ENERGY HOMEOSTASIS: PACAP AND LEPTIN SIGNALING……………………………………..133

IX GENERAL DISCUSSION…………………………………………156

BIBLIOGRAPHY………………………………………………………...…175

LIST OF TABLES

CHAPTER IV

Table 1. Meal pattern analysis following PAC1R knockdown……………...…75

LIST OF FIGURES

CHAPTER I

Figure 1.1 Central control of energy balance…………………………………..…2

Figure 1.2 Hypothalamic nuclei control energy balance………………………..3

Figure 1.3 In situ hybridization of PAC1R distribution in the VMN…………….8

Figure 1.4 Structure of PACAP PAC1 receptor………………………...……..9

Figure 1.5 GPCR signaling cycle……………………………..…...…..……..12

Figure 1.6 Leptin signaling pathways in the brain…………………….……....18

CHAPTER II

Figure 2.1 In situ hybridization photomicrograph of PACAP mRNA distribution in rat brain structures…………………………………….……..……...33

Figure 2.2 PAC1 receptor mRNA distribution in rat brain structures……..…34

Figure 2.3 Immunohistochemical staining of PAC1R protein in hippocampus and hypothalamus…………………………………………………….………..35

Figure 2.4 C- PAC1R Extracellular (ECD) and C-terminal intracellular loop 3 ………………..36

Figure 2.5 C- PAC1R isoforms PAC1null and PAC1hop are expressed in rat brain…………………………………………………………………...…37

Figure 2.6 Astrocyte and neuron labeling in the amygdala……………………38

Figure 2.7 Hypothalamic ventromedial nuclei…………………………………..38

Figure 2.8 FACS segregation of GFP negative and positive cells……………39

Figure 2.9 Striatum immune-detection of GFAP…………………………….....40

Figure 2.10 RT-PCR of mRNA products from dissociated rat embryonic day 15 cortical cells…………………………………………………………….41

Figure 2.11 RT-qPCR showing relative gene expression profiles of PAC1R, PACAP, BDNF, xCT and SOCS3……………………………………….42

Figure 2.12 Differential gene expression in rats allowed to eat after fasting……………………………………………………………………43

Figure 2.13 Saporin conjugated PACAP destroys PAC1R expressing cells...44

CHAPTER III

Figure 3.1 PAC1R-shRNA transfection in Neuro2A cells……………………..58

Figure 3.2 AAV8, AAV9 and lentivirus transduce VMN cells…………………60.

Figure 3.3 Green fluorescent protein expression in hypothalamic cells……..60

CHAPTER IV

Figure 4.1 AAV8-mediated transduction of VMN cells with PAC1R shRNA decreases PAC1R protein expression…………………………………..…73

Figure 4.2 Knocking down PAC1Rs in the VMN increases daily food intake and cumulative body weight………………………………………….…74

Figure 4.3 Knocking down VMN PAC1Rs blocks PACAP-dependent hypophagia…………………………………………………………...………76

Figure 4.4 PAC1R-shRNA expression in the VMN prevents PACAP-dependent action potential firing frequency……………………………….77

Figure 4.5 Knocking down PAC1Rs in the VMN downregulates BDNF and SOCS3 mRNA………………………………………………………….78

CHAPTER V

Figure 5.1 KT5720 injection in the VMN dose dependently block PACAP-dependent decreases in food intake ……………………………………91

Figure 5.2 Blocking PKA signaling with KT5750 blocks PACAP-dependent decreases in food intake and attenuates PACAP-induced decrease in body weight ……………………………………………………………….92

Figure 5.3 PKA inhibition with KT5750 facilitates PACAP-dependent decreases in food intake and body weight……………………………..….93

Figure 5.4 Effects of PKA inhibition on PACAP-dependent energy expenditure…………………………………………………………..……..94

CHAPTER VI

Figure 6.1 Blocking PKC with GF109203X transiently reduces PACAP’s hypophagic effects during the dark phase……………………………….107

Figure 6.2 PKC inhibition with GF109203X supersensitized PACAP-dependent decreases in food intake and body weight…………………………………………………………………..…109

Figure 6.3 GF109203X attenuates PACAP’s effects on energy expenditure ……………………………………………………………………………..110

CHAPTER VII

Figure 7.1 PACAP induces surface PAC1R endocytosis …………………...122

Figure 7.2 Dyngo-4a/Pitstop 2 inhibit PAC1R endocytosis ……………..…..123

Figure 7.3 Hypothalamic ventromedial nuclei express β-arrestin 1 and β-arrestin 2……………………………………………………………….124

Figure 7.4 PACAP promotes PAC1R association with β-arrestin 2.……….125

Figure 7.5 β-arrestin-Adaptin association is not required for PACAP-dependent decrease in body weight………………………...………...126

Figure 7.6 Inhibiting surface PAC1R trafficking attenuates PACAP-induced decrease in food intake and exaggerates PACAP-induced decrease in body weight.…………………………………………………………..…….128

Figure 7.7 Inhibiting surface PAC1R trafficking promotes PACAP-induced STAT3 phosphorylation ……………………………………………….……..129

Figure 7.8 Blocking PAC1R trafficking increases STAT3 phosphorylation..130

CHAPTER VIII

Figure 8.1 VMN PAC1 and leptin receptors form an immunocomplex …….143

Figure 8.2. In situ hybridization and immunofluorescence labeling of PAC1 and leptin receptors in VMN and rHypoE-19 neurons ………………...145

Figure 8.3 rHypoE-19 neurons PAC1 receptors are biologically functional ………………………………………………………………………….………146

Figure 8.4 rHypoE-19 neurons PAC1 receptors are biologically functional. ……………………………………………………………..……………147

Figure 8.5 Leptin dose-dependently induces pSTAT3 in rHypoE19 cells …148

Figure 8.6 PACAP6-38 blocks leptin induced pSTAT3 in rHypoE19 cells...149

Figure 8.7 PACAP application in rHypoE-19 neurons induces pSTAT3…..150

Figure 8.8 PACAP induces JAK2 phosphorylation in rHypoE-19 cells ……151

Figure 8.9 Blocking PKA and PKC kinase activity in rHypoE-19 neurons influences leptin induced pSTAT3 ………………………………………153

CHAPTER IX

Figure 9.1 Schematic of PAC1 receptor signaling at the plasma membrane and endocytosis ……………………………………………………………163

Figure 9.2. Schematic of proposed hypothalamic ventromedial (VMN) PAC1 and leptin receptor signaling milieu………………………………………170

CHAPTER I

INTRODUCTION

General introduction

Obesity is a major world public health issue resulting from homeostatic imbalances between energy intake and energy expenditure that can lead to serious and chronic health complications such as heart disease and diabetes (King, 2006; Wilding, 2002; Williams et al., 2001). According to the World Health Organization an estimated 1.5 billion individuals are considered overweight and approximately half a million adults are obese worldwide. The lack of effective treatments or thorough understanding of the mechanisms that lead to obesity contributes to the negative health impact. Efforts made to alleviate complications associated with dysregulated energy homeostasis and obesity will greatly improve quality of life in affected individuals and those caring for them. Various attempts to target neuropeptide systems have shown promise to mitigate obesity, however, none have succeeded, as reviewed in (Boughton & Murphy, 2012). Energy balance is largely regulated by the central nervous system which coordinates information to and from the entire body (J K Elmquist, Elias, & Saper, 1999). Although there is a wide variation in daily food consumption and energy expenditure, most individuals maintain a stable body weight (Leibel, 2008). Evolutionarily, a complex system has evolved to maintain energy homeostasis, primarily centered within the hypothalamus (Figure 1.1) (Andermann & Lowell,

2017; Hetherington & Ranson, 1942; King, 2006), which is a small region at the base of the brain comprised of a collection of highly organized small nuclei that regulate distinct homeostatic functions such as thermoregulation, thirst, hunger, stress, and reproductive needs (King, 2006; Waterson & Horvath, 2015; Wilding, 2002). What is lacking, however, is a comprehensive understanding of how these various neuropeptides regulate the complex network between key hypothalamic nuclei. One of the most enigmatic and often-overlooked cell groups in hypothalamic regulation of energy balance are the hypothalamic ventromedial nuclei (Choi, Horsley, Aguila, & Dallman, 1996; Hetherington & Ranson, 1942; King, 2006) (Figure 1.2).

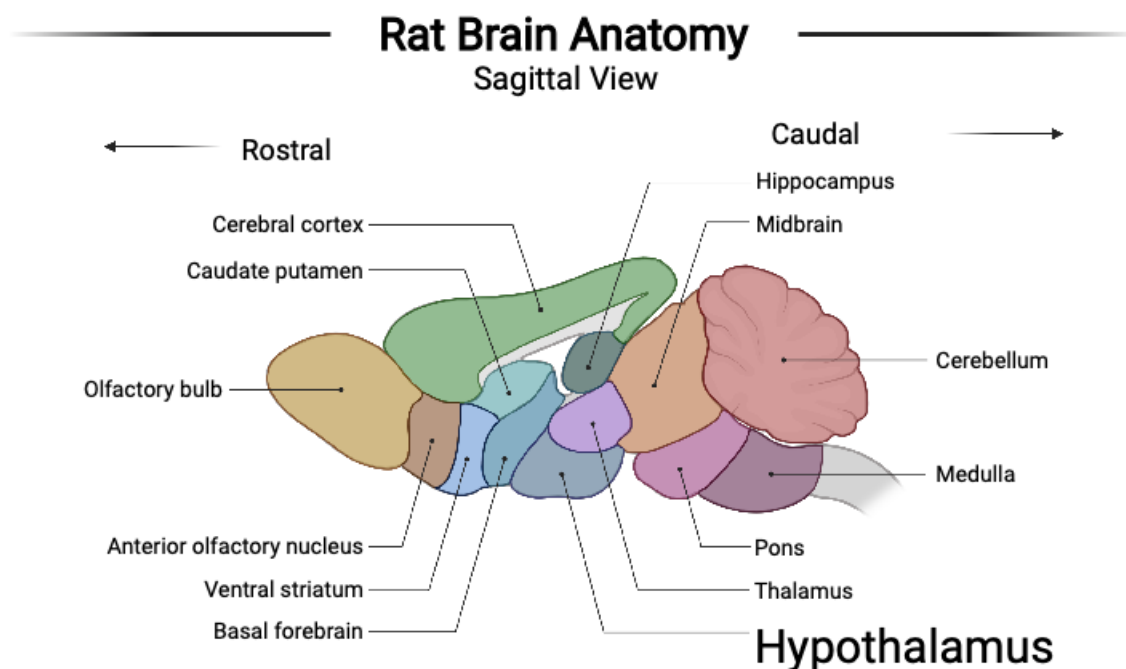


Figure 1.1. Central control of energy balance. The hypothalamus integrates central and peripheral signals to maintain energy balance. Created with BioRender.com

Hypothalamic ventromedial nuclei regulation of feeding

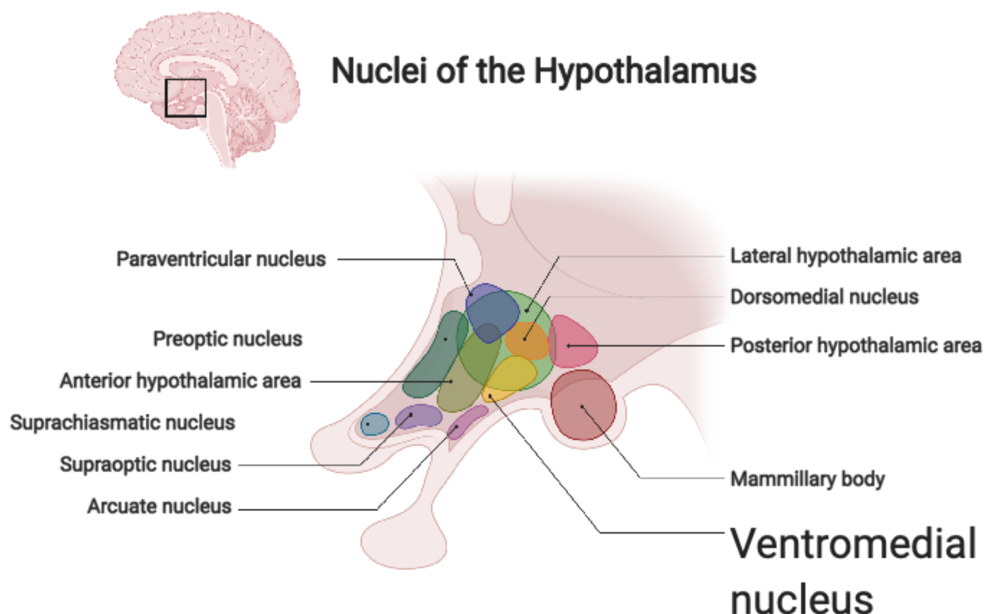


Figure 1.2. Hypothalamic nuclei control energy balance. Energy balance is maintained by several hypothalamic nuclei including the hypothalamic ventromedial nuclei. Created with BioRender.com

The ventromedial hypothalamic nuclei (VMN) constitute a group of cells found at the base of the hypothalamus and that early on were shown to demonstrate that disruption by bilateral lesions produced hyperphagia and obesity in several different species, including humans (Choi et al., 1996; Hetherington & Ranson, 1942; King, 2006). By contrast, electrical stimulation of the VMN reduces food intake in hungry rats (Beltt & Keesey, 1975). Lesions of the VMN produce an obesity that is distinct from other medial hypothalamic obesities, in that it is characterized not only by increased food intake and body weight but also increased circulating corticosterone and insulin concentrations

during the light phase and decreased metabolism (Choi, Wong, Yamat, & Dallman, 1998). In nocturnal animals such as the rat, food intake and activity levels peak during the dark phase of their circadian rhythm. This led to VMN being designated as the “satiety center” (Choi et al., 1996) also reviewed in (King, 2006; Williams et al., 2001). Rodents with VMN lesions eat a significant amount of food and gain twice as much weight as any other lesion in the hypothalamus (Hetherington & Ranson, 1942). These early studies identifying the VMN as an important center for energy homeostasis were confirmed by more targeted (Choi et al., 1998) and restricted lesions involving steroidogenic factor 1 (SF1), a well-established and exclusive marker of VMN cells (Davis et al., 2004). However, it is important to note that the VMN is not the only hypothalamic cell group to be important for energy regulation. It should be recognized that the VMN are a part of a highly connected network of hypothalamic and extra-hypothalamic cell groups that participate in the regulation energy balance (Hawke et al., 2009; Kalkhoff, Gossain, Matute, & Wilson, 1976; Resch et al., 2013). Several hypothalamic nuclei receive both direct and indirect projections from the VMN, including the arcuate, dorsomedial, paraventricular and lateral hypothalamic nuclei (Bai et al., 1985; Elias et al., 1998; Horst & Luiten, 1986)

The sympathetic nervous system also plays a role in the development of hypothalamic VMN obesity. Previous studies that produce VMN lesions report a reduction in sympathetic nervous system activity and subsequently reducing the activation of interscapular brown adipose tissue (BAT) (Sakaguchi, Bray, & Eddlestone, 1988). Both white adipose tissue (WAT) and BAT function as a

major means of storing energy but unlike WAT, BAT is a major source of non-shivering and diet-induced thermogenesis (Foster & Frydman, 1979; Himms-Hagen, 1990). Thus, sustained decreases in sympathetic input to BAT decreases non-shivering and diet-induced thermogenesis, and therefore, decreases overall energy expenditure (Himms-Hagen, 1990). The VMN have been demonstrated to have direct regulatory connections and function of sympathetic output to targets such as BAT (Bozadjieva-Kramer et al., 2021; Foster & Frydman, 1979).

Although the VMN have been established as a key center for feeding behavior and metabolism (Choi et al., 1998), the broader regulation of energy homeostasis requires a more intricate neuroendocrine system involving numerous neuropeptides that have either orexigenic or anorexigenic effects and/or governance over energy expenditure. Within the VMN can be found many neuropeptide and neurohormone-responsive receptors involved in feeding and metabolism including cholecystokinin (CCK), brain derived neurotrophic factor (BDNF) receptor Trkb, neuropeptide Y receptors, orexin receptors, melanocortin-4 receptors, and the long-form leptin receptor (lepRb) just to list a few. However, the known number of neuropeptides originating from within the VMN are still few (Flak et al., 2020; Koizumi et al., 2020). Brain derived neurotrophic factor (BDNF) and pituitary adenylate cyclase-activating polypeptide (PACAP) have been shown to be abundantly expressed in the VMN, and both are necessary for energy balance regulation (King, 2006; Resch et al., 2011; Zink et al., 2004). Elucidating the endogenous role of PACAP and the mechanisms mediating the actions of PACAP in the VMN will be the focus of this dissertation.

Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP is a 38-amino acid peptide from the vasoactive intestinal peptide (VIP) glucagon secretin superfamily (Dickson & Finlayson, 2009; Gottschall, Tatsuno, Miyata, Arimura, & Gottschall, 1990; LAM et al., 1990; Lu & DiCicco-Bloom, 1997; Miyata et al., 1989, 1990; Ohtakis et al., 1993; Vaudry et al., 2009) and was first isolated from ovine hypothalamic tissue. PACAP is the most evolutionarily conserved member of the glucagon family according to sequence length that determine the identity of amino acids (Sherwood, Krueckl, & McRory, 2000). PACAP is identified in more than 15 vertebrate species from humans to tunicates, suggesting that PACAP is biologically important for survival (Hosoya et al., 1992; Sherwood et al., 2000). This point is well illustrated by the fact that congenital PACAP knockout is lethal (Mounien et al., 2009). PACAP has a myriad of functions in several regions of the body including the nervous, endocrine, cardiovascular and immune systems (Sherwood et al., 2000). PACAP is encoded by the gene (ADCYAP1), and following transcription and post-translational proteolytic processing of a larger PACAP precursor protein generates polypeptides in varying sizes including PACAP38 (38 amino acids long) and PACAP27 (27 amino acids long) (Miyata et al., 1989; Ohtakis et al., 1993; Vaudry et al., 2009). PACAP38 is the predominant form of PACAP that is expressed in the central nervous system (H. Hashimoto et al., 2000; Miyata et al., 1990), although both peptides, PACAP27 and PACAP38, are functionally similar (Fahrenkrug & Hannibal, 2004). On the other side of neuronal signaling, PACAP has two receptor subtypes that it binds to with equal affinity, Type I and

Type II, that belong to the class B family of G-protein coupled receptors (GPCRs) (Apostolakis, Riherd, & O'Malley, 2005; Arimura, 1998; Jozwiak-Bebenista, Dejda, & Nowak, 2007; Vaudry et al., 2009; Warfvinge & Edvinsson, 2020). The two receptors under the Type II receptor category, VPAC1 and VPAC2, also bind vasoactive intestinal polypeptide whereas the Type I or PAC1 receptor (PAC1R) selectively binds PACAP. PACAP binds PAC1R with a 1000-fold higher affinity than VIP, compared to VPAC1 and VPAC2 receptors, which bind PACAP and VIP with similar affinity (Vaudry et al., 2009). Looking at the distribution of PAC1R within the brain (Figure 1.3), the PAC1 receptor is abundantly expressed in the hypothalamus and shown to be a potent pleiotropic regulator of energy homeostasis within several nuclei (Chang et al., 2021; Hawke et al., 2009; Resch et al., 2011, 2013; Resch, Maunze, Phillips, & Choi, 2014). It is therefore not surprising why PACAP is tightly conserved in evolution as organisms inherently need metabolism for survival. Acute PACAP administration in hypothalamic ventromedial nuclei (VMN) decreases food intake with concomitant increases in core body temperature and spontaneous locomotor activity, which results in rapid reductions in body weight (Resch et al., 2011, 2013, 2014). In the VMN, these hypophagic and positive metabolic affects were confirmed to be mediated specifically by PAC1 receptors with no apparent involvement of the VPAC receptors (Resch et al., 2011, 2013). Moreover, there are early indicators that PACAP integrates within a broader context of energy homeostatic systems with evidence showing PACAP administration in the VMN is accompanied by

increases in STAT3 phosphorylation, and BDNF and SOCS3 transcription (Hurley et al., 2020, 2016; Resch et al., 2013, 2014) all of which have been independently shown to regulate feeding behavior and metabolism.

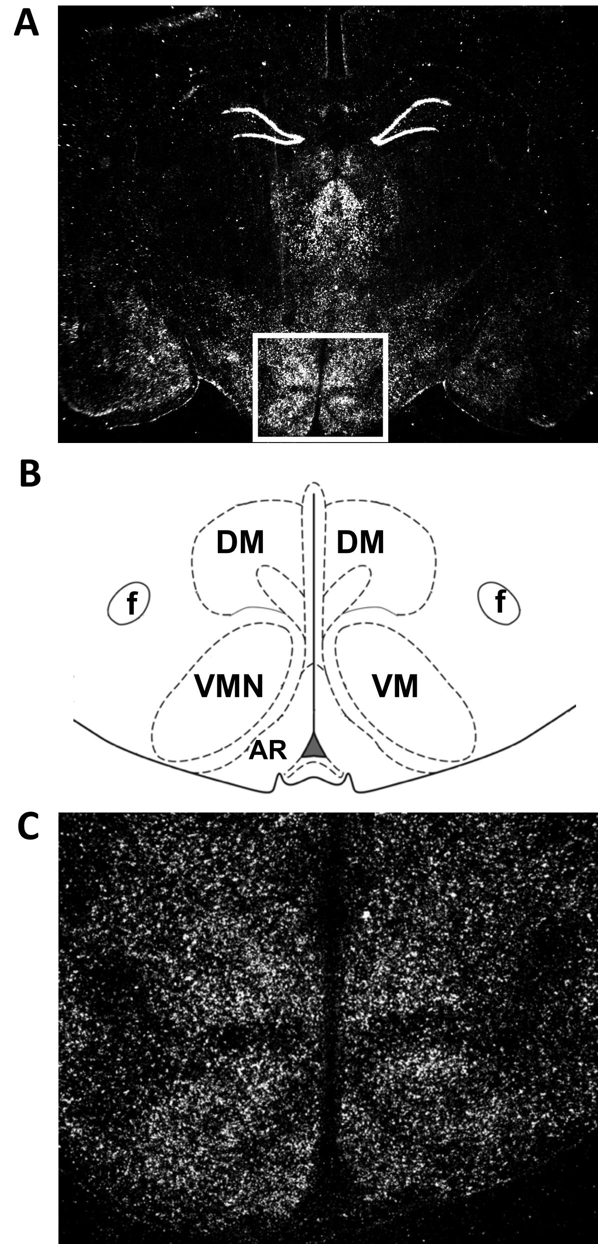


Figure 1.3. In situ hybridization of PAC1R distribution in the ventromedial hypothalamic region. 1) 1.25X magnification of a coronal rat brain section, B) schematic, C) 5X magnification. Resch et al., AJP Integr Comp Physiol 301; 2011.

Pituitary adenylate cyclase-activating polypeptide receptors

As a class B GPCR, PAC1R signals through coupling to G-alpha subunits Gs and Gq that typically activate adenylyl cyclase (AC) and phospholipase Cβ enzymes, respectively (Marinissen & Gutkind, 2001; W. Wang, Qiao, & Li, 2018). A recent analysis of the PAC1R C-terminus (Lyu, Germano, Joon Ki Choi, Le, & Pisegna, 2000) revealed essential amino acid motifs that are necessary for Gs, Gq-protein coupling, and receptor trafficking. Numerous studies have now confirmed that PAC1Rs are coupled to both Gαs/adenylyl cyclase/protein kinase A (Gαs/AC/PKA) and Gαq/phospholipase C/protein kinase C (Gαq/PLC/PKC), while also undergoing trafficking following stimulation (Liao, Remington, May, &

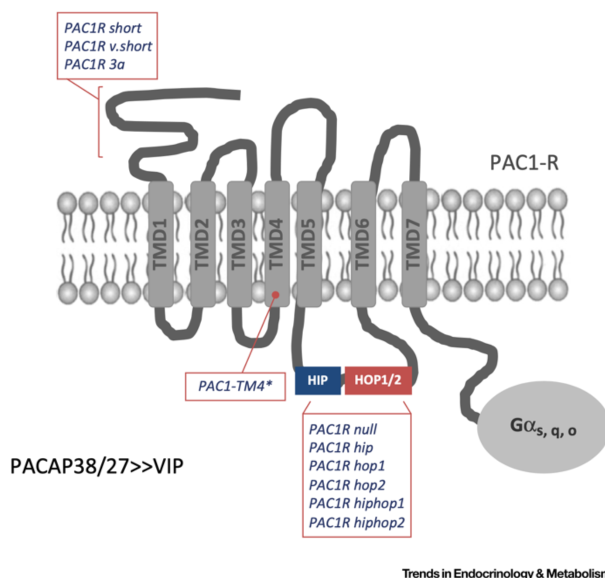


Figure 1.4. Structure of the PACAP-specific PAC1 receptor showing the location of the known splice variants within the structure. TMD=transmembrane domain. Figure reproduced from Rudecki and Gray (2016) Trends in Endocrinology and Metabolism 27(9): 620-632).

Li, 2021; May, Lutz, MacKenzie, et al., 2010; Rudecki & Gray, 2016). As an added layer of signaling complexity, PAC1Rs exist in multiple isoforms that can

generate diverse and complex signaling by coupling to multiple G-proteins that can modulate channel function and cell responses in different tissues (Blechman, Levkowitz, & Vaudry, 2013; Fukuchi et al., 2015; Holighaus, Mustafa, & Eiden, 2011; Lyu et al., 2000; Zhou et al., 2001). PAC1 receptor isoforms are generated from alternative splicing of the transcript from a single gene and inclusion or exclusion of one or two cassettes, the hip and hop cassettes (Blechman et al., 2013; Holighaus et al., 2011; Sherwood et al., 2000). Initially, seven PAC1R splice variants were isolated from rat brain. Figure 1.4. illustrates the various isoforms and the location of splice variants found in the rat brain which include PAC1null, PAC1hop1, PAC1hop2, PAC1hip, PAC1hip-hop, PAC1-short, and PAC1-very short (Amir-Zilberstein et al., 2012; Apostolakis et al., 2005; Blechman et al., 2013). Interestingly, except for the PAC1hip variant, all PAC1R variants stimulate adenylyl cyclase (AC) equally through Gs (Blechman et al., 2013; Holighaus et al., 2011; Sherwood et al., 2000). However, PLC/PKC accumulation is stimulated to varying degrees through Gq/PLC/PKC (Blechman et al., 2013; Hardwick et al., 2017; Sherwood et al., 2000). Recent studies show that PAC1hop contains a consensus PKC phosphorylation (Blechman et al., 2013; Holighaus et al., 2011; May, Buttolph, Girard, Clason, & Parsons, 2014; Sherwood et al., 2000). These unique variants may serve a purpose such as regulation of mRNA stability, G-protein coupling and receptor desensitization and trafficking (Holighaus et al., 2011; May et al., 2014). For example, in HEK293, cardiac, and dentate cells, the PAC1hop isoform is internalized following PACAP stimulation and is necessary for induction of kinase signaling and action potential

generation (Johnson, Parsons, May, & Hammack, 2020; May et al., 2014; May, Lutz, MacKenzie, et al., 2010). Although G-protein coupling has been the primary focus of PAC1R signaling, recent evidence in cardiac and dentate granule neurons have demonstrated that PAC1R endocytosis facilitates neuronal excitation, which further adds to the complex PACAP signaling repertoire (Johnson et al., 2020; May, Lutz, MacKenzie, et al., 2010; May & Parsons, 2017; Parsons & May, 2018).

Although VMN and PACAP studies have made tremendous progress in characterizing energy homeostasis regulation, the endogenous role of PACAP, and the intracellular signaling mechanisms that determine the comprehensive responsivity of VMN cells to PACAP is poorly understood. Considering its clear and marked involvement in regulating energy homeostasis, it becomes essential to characterize down-stream consequences of PACAP ligand binding in order to identify potential etiologies of obesity as well as exploring possible future therapies. Examination of the activated receptor cascade may help to differentiate the mechanisms by which PACAP can exert a diverse array of actions on specific cellular and physiological processes that may ultimately impact disease states such as obesity. For example different PAC1R isoforms have been shown to influence sleep (Hannibal, Georg, & Fahrenkrug, 2016), stress (Amir-Zilberstein et al., 2012), locomotion (Vaudry et al., 2009), and post-traumatic stress disorder (PTSD) (Ressler et al., 2011). This raises the question, how does PAC1R signaling regulate disparate aspects of feeding and metabolism in the VMN? Unique characteristics of class B family of G-protein

coupled receptors may explain PACAP's pleiotropic effects in the VMN by enabling PAC1R-dependent regulation of feeding and metabolism through G-protein coupling and PAC1R trafficking.

G-protein coupled receptor signaling and termination

GPCRs are seven transmembrane spanning proteins that form the largest family of cell-surface receptors (Marinissen & Gutkind, 2001; Nogueras-Ortiz & Yudowski, 2016). Classically, GPCRs are activated by extracellular ligands that induce intracellular signaling by coupling to transducer proteins from four families of heterotrimeric G-proteins (Eichel & Von Zastrow, 2018; Jalink & Moolenaar, 2010; W. Wang et al., 2018). It has now been demonstrated that individual GPCRs produce complex signaling that allow for multidimensional regulation of

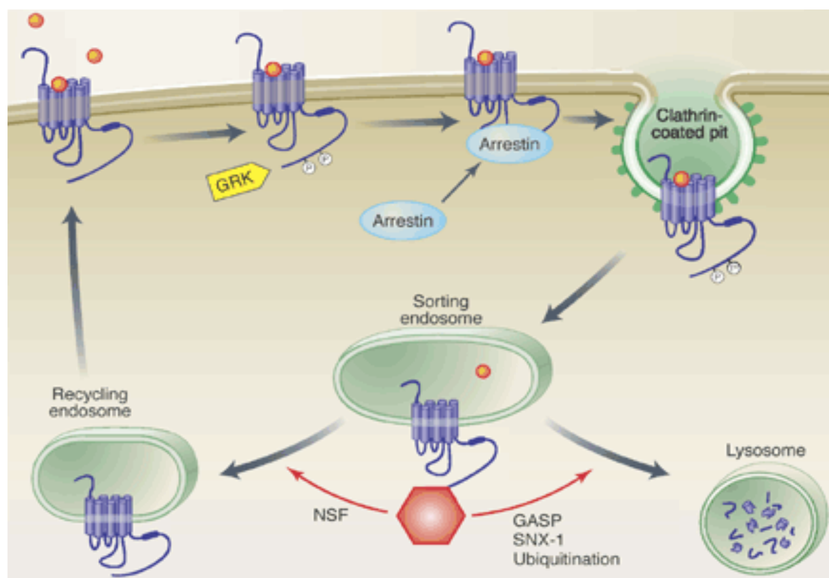


Figure 1.5. GPCR signaling cycle. Figure reproduced from , A Last GASP for GPCRs?, Volume: 297, Issue: 5581, Pages: 529-531, DOI: (10.1126/science.1075453)

cellular function (Eichel & Von Zastrow, 2018). PAC1R stimulation has been shown to engage Gαs, Gαq, and Gαi signaling (Blechman et al., 2013).

PAC1 receptor signaling is regulated in a similar manner to other GPCRs, through desensitization and endocytosis of the receptors (May, Lutz, Mackenzie, et al., 2010; Tompkins et al., 2018a). Canonically, GPCR desensitization and endocytosis are regulated by serine/threonine kinases such as PKA and PKC, and G-protein receptor kinases (GRKs) (Di Fiore & von Zastrow, 2014; Eichel & Von Zastrow, 2018). Once phosphorylated, GPCRs are recognized by β-arrestins and other nucleating protein complexes such as adaptor proteins that facilitate clathrin aggregation (Delom & Fessart, 2011; DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Lobingier & von Zastrow, 2019). This results in plasma membrane invagination that is cleaved by dynamin to form endosomes, that is either recycled to the membrane or tagged for lysosomal degradation (Cosker & Segal, 2014; Scita & Di Fiore, 2010; Sposini et al., 2017). Classically, GRKs (Dautzenberg & Hauger, 2001; Ferguson, 2007; Hupfeld & Olefsky, 2007) phosphorylate the C-terminal tail of GPCRs to attenuate G protein signaling, allowing for the binding and activation of β-arrestins which mediate receptor desensitization and internalization (Cahill et al., 2017; Di Fiore & von Zastrow, 2014). However, recent works have shown that some GPCRs engage in sustained G-protein signaling from within internalized cellular compartments rather than desensitizing including the PAC1 receptor (May & Parsons, 2017; Sposini et al., 2017; Yarwood et al., 2017).

β-arrestins facilitate dynamic and sustained GPCR signaling beyond G-protein signaling.

Generation of second messengers by GPCRs and its termination was thought to occur exclusively at the plasma membrane. However, this paradigm is not consistent with recent observations that PAC1R stimulation continues to suppress food intake and increase metabolism long after the peptide has likely been cleared from the synapse, and membrane signaling has been terminated. β-arrestins were classically considered as signaling terminating proteins (Cahill et al., 2017; DeWire et al., 2007), however, these scaffolding proteins are also critical signal transducers at the GPCRs in the membrane and downstream of numerous GPCRs via their ability to nucleate signaling complexes containing members of the MAPK and Src kinases (Reinecke & Caplan, n.d.; Shukla, Xiao, & Lefkowitz, 2011; Thomsen, Jensen, Hicks, & Bunnett, 2018). Recent data demonstrate that PAC1R forms a complex with β-arrestins (Broca et al., 2009; Merriam et al., 2013; Shintani et al., 2018), and that the complex formed between β-arrestins and PAC1R is critical for neuronal excitability and sustained cell signaling (May et al., 2021; May & Parsons, 2017; Parsons & May, 2018) Endocytosis is considered a master organizer of signal circuits with a primary role to maintain the fidelity and resolution of signals in space and time (Scita & Di Fiore, 2010; Sigismund et al., 2012). The integration of endocytosis is thought to be critical for determining the net signaling output by sustaining signals that originate from the plasma membrane and for generating unique signals that are prohibited at the plasma membrane thus contributing to the signal diversity (Di

Fiore & von Zastrow, 2014; Lobingier & von Zastrow, 2019; McMahon & Boucrot, 2011).

The unique small volume of endosomes is a key feature for signaling because it supports receptor-ligand association and sustains receptor activity, and this might help to explain peptide neurotransmission where production, packaging and release is energy consuming (Di Fiore & von Zastrow, 2014; Scita & Di Fiore, 2010). Endosomes are enriched with lipids and proteins such as phosphatidylinositol-3-phosphate and lipid-raft adaptor protein, affording specific scaffolding surfaces on which signaling complexes can nucleate (Scita & Di Fiore, 2010; Sigismund et al., 2012). Endosomes are also unique because they contain rapid microtubule-mediated transport of molecules which allow transmission of signals over long distances, such as from the plasma membrane to the nucleus, while also providing an acidic pH which is necessary for a variety of specific signaling pathways (Di Fiore & von Zastrow, 2014; Lobingier & von Zastrow, 2019; Sigismund et al., 2012; Sposini et al., 2017). For example, retinal ganglion cells respond to blue light and induce PAC1R nuclear translocation (Yu, Lin, Ouyang, Tao, & Fan, 2021), creating the possibility for direct gene regulation.

GPCR transactivation facilitates interconnected signaling.

GPCRs can transactivate and can be reciprocally transactivated by other receptor types such as receptor tyrosine kinases and cytokine receptors (Moody, Lee, & Jensen, 2020; W. Wang et al., 2018). This relationship is an important aspect of the GPCR repertoire as it would allow GPCRs access to a broader array of highly interconnected signaling mechanisms that influence numerous physiological and pathological conditions (Campbell & Smrcka, 2018; Gavi, Shumay, Wang, & Malbon, 2006). Recent studies using *in vitro* models have demonstrated that PAC1Rs cause transactivation of receptor tyrosine kinases EGFR and HER 3 to exacerbate cell proliferation in non-small cell lung cancer (Moody et al., 2020; Moody, Ramos-Alvarez, & Jensen, 2021). Another study demonstrated that PAC1Rs transactivate EGFR to prevent damage to corneal endothelial cells (Moody et al., 2020). These studies demonstrate that PAC1R transactivation-related signaling mechanisms are important for physiological and pathological processes. More recently, in the VMN, PAC1R activation has been shown to stimulate a number of downstream markers of the JAK2-STAT3 signaling pathway, a downstream leptin receptor signaling cascade (Hurley et al., 2020). The crosstalk between PACAP and leptin signaling will be the subject of chapter VIII.

Leptin and VMN control of feeding

As mentioned earlier, the VMN contains an abundance of leptin receptors that respond to the peripheral hormone, leptin (Dhillon et al., 2006; Joel K. Elmquist, Bjørbæk, Ahima, Flier, & Saper, 1998; Koizumi et al., 2020). Leptin or leptin receptor mutations lead to overeating and excessive weight gain that can ultimately lead to morbid obesity, suggesting that intact leptin and its long-form receptor is necessary for maintaining body weight and energy balance (J K Elmquist et al., 1999; Wilding, 2002). The high abundance of leptin receptors in VMN neurons suggest these cells are responsive to circulating leptin (Hawke et al., 2009; Hurley et al., 2020). Leptin, the ob gene product, is a cytokine secreted by adipocytes into the blood and its circulating concentrations are approximately proportional to fat stores (J K Elmquist et al., 1999; King, 2006). Thus, leptin has colloquially been referred to as a "lipo-stat". Leptin delivery into the VMN likely occurs through the blood brain barrier of the median eminence and/or transported into the cerebrospinal fluid via the choroid, from where it then can enter the hypothalamus (Ronghua Yang & Barouch, 2007a).

Unlike G-protein coupled receptors, the leptin receptor is a single membrane-spanning receptor belonging to the class I cytokine receptor family and expresses six different isoforms (a-f) (Bjørbæk et al., 2000; Mancour et al., 2012) (Figure 1.5.). Of these isoforms, the long-form (b) is the active form and is typically denoted as lepRb or ObRb (Banks, Davis, Bates, & Myers, 2000; Bjørbæk et al., 2000). Like many cytokine receptors, lepRb is associated with tyrosine kinases placing it within the category of receptor tyrosine kinase

(Bjørbæk et al., 2000; Ghilardi & Skoda, 1997; Procaccini, Lourenco, Matarese, & La Cava, 2009; Vaisse et al., 1996). Upon binding to the lepRb, leptin engages the JAK-STAT pathway, which involves the activation of JAK2 (Ghilardi & Skoda, 1997; Vaisse et al., 1996) (via phosphorylation) leading to the phosphorylation of STAT3 (Lundin, Rondahl, Walum, & Wilcke, 2000; Procaccini et al., 2009; Wilding, 2002). The phosphorylation of STAT3 causes its

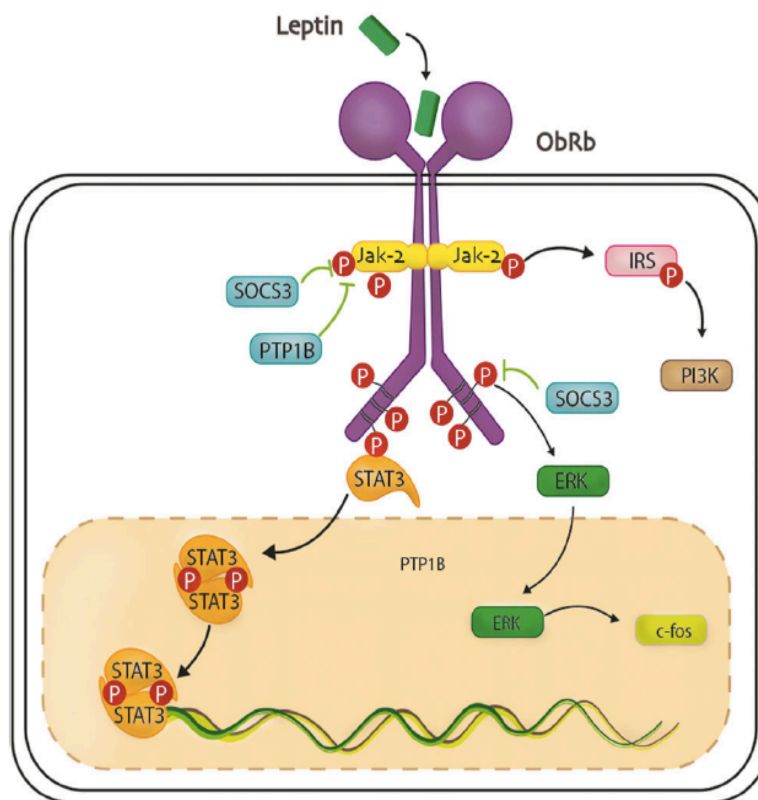


Figure 1.6. Leptin signaling pathways In the brain, the binding of leptin to the ObRb receptor activates janus-tyrosine kinase 2 (JAK2), which leads to the phosphorylation of two tyrosine residues in the cytoplasmic tail of the Ob-Rb receptor, producing the activation the signal transducer and activator of transcription 3 (STAT3) signaling pathways. SOCS-3 negatively regulates leptin receptor signal transduction. Figure reproduced from King et al (2018) Metab Brain Dis. 2018 Aug;33(4):1097-1110.

homodimerization, which allows it to enter the nucleus and bind to specific DNA targets (Devos et al., 1997). The JAK-STAT pathway is tightly regulated by

phosphatases such as suppressors of cytokine signaling 1-7 (SOCS1-7) and protein inhibitors of activated STAT (Lundin et al., 2000; Procaccini et al., 2009; Ronghua Yang & Barouch, 2007b).

Similar to PACAP, leptin injections into the VMN potently induces hypophagia and hyperthermia, while also inducing pSTAT3 and SOCS3 mRNA transcription (Hurley et al., 2020). Moreover, disruption to leptin receptor signaling in the VMN leads to obesity (Bingham, Anderson, Reuter, Stallings, & Parker, 2008; Choi, Sparks, Clay, & Dallman, 1999; Dhillon et al., 2006). In situ hybridization analysis shows that leptin and PACAP receptor transcripts co-localize within the same VMN cells (Hawke et al., 2009; Hurley et al., 2020). More importantly, blocking PAC1 receptors prevents leptin’s ability to engage its downstream signaling and influence feeding and energy expenditure (Hawke et al., 2009; Hurley et al., 2020). Given that PACAP and leptin signaling in the VMN appear to have a high degree of commonality on their impact on feeding, metabolism, and gene transcription, the possibility that both peptides alter feeding and energy balance via a shared intracellular pathway could pose some distinct advantages. As a collaborative pair, leptin may supply constant or tonic activation of the system in order to prime it for activation upon the release of PACAP, a more phasic-patterned signaling molecule. Despite the highly speculative nature of this potential model, data collected and presented in this thesis may provide preliminary support for such a model. Collectively, these findings suggest that elucidating the signaling mechanisms underlying the cross talk between PACAP and leptin signaling may be useful to understanding the

etiologies of obesity, while providing potential therapeutic avenues to treat obesity. In both humans and animals, an obese phenotype is often accompanied by leptin insensitivity, which poses a barrier to therapeutic measures necessary to achieve weight loss. The functional relationship between PACAP receptors on leptin receptors may provide insight into an origin of leptin insensitivity or a potential mechanism for overcoming leptin insensitivity.

CHAPTER II

VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS & CORTICAL CELLS EXPRESS PITUITARY ADENYLATE CYCLSE-ACTIVATING POLYPEPTIDE RECEPTOR ISOFORMS IN THE RAT BRAIN: CONSEQUENCES FOR SIGNALING & CONTROL OF FEEDING

Introduction

Neurons and astrocytes constitute the fundamental infrastructure for central nervous system (CNS) signaling and function. Within these unique cell types are gene products and their respective proteins that can define and facilitate how cells function and communicate nuanced information processing throughout the CNS. One gene in particular that has emerged as a critical signaling component in neurons and astrocytes is the peptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its cognate receptors (Ciranna & Costa, 2019; Jozwiak-Bebenista et al., 2007; Kong et al., 2016). PACAP and PACAP receptors are abundantly expressed in numerous CNS regions implicated in feeding, reward seeking, stress responses, and learning and memory (Eiden, Hernández, Jiang, & Zhang, 2021; Gottschall et al., 1990; H. Hashimoto, Ishihara, Shigemoto, Mori, & Nagata, 1993; Resch et al., 2011; Limei Zhang, Hernandez, et al., 2021). Interestingly, examination of PACAP and PACAP receptor distributions throughout the CNS show that the neuropeptide and its receptors are co-expressed with glutamatergic and GABAergic markers suggesting a putative involvement in excitatory and inhibitory neurotransmission in the CNS (Engelund, Fahrenkrug, Harrison, & Hannibal, 2010; Fahrenkrug &

Hannibal, 2004; Resch et al., 2013; Limei Zhang, Hernández, et al., 2021). In addition, PACAP signaling is important for synaptic function through synaptogenesis and receptor/ion channel signaling regulation (Ciranna & Costa, 2019; Girard, Keller, Schutz, May, & Braas, 2004; Macdonald et al., 2005). Disruptions in PACAP or its receptor have been implicated in a dichotomy of nervous system disorders including post-traumatic stress disorder (Ressler et al., 2011), depression (R. Hashimoto et al., 2010; Pinhasov et al., 2011), migraine (Amin et al., 2014; Edvinsson, Tajti, Szalárdy, & Vécsei, 2018; Schytz et al., 2009), and anorexia (Kocho-Schellenberg et al., 2014; Resch et al., 2011, 2013, 2014), suggesting that PACAP and its receptors are important for normal CNS infrastructure and function. Although recent advances in molecular biology have expanded our understanding of PACAP signaling, there is still a paucity in our knowledge and understanding of cell-type-specific expression of PACAP and the PAC1 receptor and how their genes may change or influence CNS transcriptomes, and activity under physiological and pathophysiological states

The PAC1 receptor gene precursor mRNA is alternatively spliced to generate several receptor proteins with pleiotropic properties that are relevant for cell signaling and physiology. Although PACAP and PAC1R isoform signaling cascades have previously been discussed and reviewed (Blechman et al., 2013; Girard et al., 2004; Holighaus et al., 2011), none have examined the cell-type-specific PACAP and PAC1 receptor variant expression and how this may influence signaling associated with stress, motivation and feeding behaviors. The functional roles of these receptor variants have been proposed to influence

ligand selectivity and/or G-proteins (Liao et al., 2019, 2021). Our lab has previously shown PACAP influences cortical and hypothalamic regulation of motivation, feeding, metabolism and gene transcription (Hurley, 2018; Hurley et al., 2020; Resch et al., 2011, 2013). Tract tracing experiments show that the cortical motive circuit and VMN are each highly connected to other brain regions that express PACAP and PAC1R and have been shown to share functionally consistent roles (Resch et al., 2013). It is however unknown whether PAC1Rs and associated genes are responsive to alterations to motivational and energy states. Additionally, the neuronal and astrocyte distributions of PAC1Rs and genes that change in response to PACAP are unknown. Addressing cell-type-specific PAC1R isoform expression and how their unique signaling pathways influence cell physiology and gene expression under physiological stressors may reshape our understanding of peptide control of neurotransmission under physiological and pathophysiological conditions

To address the cell-type-specific and physiological-dependent changes in gene expression of PAC1 receptor isoforms, we corroborated previously identified distributions of PACAP and PAC1R mRNA expression in select brain regions including the hippocampus, thalamus, amygdala and VMN. Subsequently, we matched PAC1R mRNA expression and distribution using a PAC1R antibody and then surveyed PAC1R isoform expression in tissue and or dissociated neurons and astrocytes from cortex, nucleus accumbens, medial amygdala, hypothalamic paraventricular nuclei (PVN) and VMN. Next, we assessed energy-state dependent changes in mRNA expression of PAC1Rs and

known PACAP-responsive genes in the amygdala, VMN and nucleus accumbens, brain regions associated with stress, feeding and motivation. Finally, we showed that PACAP treatments in the VMN target all PAC1 receptor isoforms, and targeted reduction of PAC1 expressing cells leads to loss of all PAC1 receptor isoform mRNA expression.

Materials and Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) and transgenic GFAP-lck-eGFP (Medical College of Wisconsin, Milwaukee, WI) knock-in rats were individually housed in standard cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

GFAP-Lck-eGFP Rats

GFAP-Lck-eGFP transgenic rats were generated in collaboration with Dr. Aron Geurts at the Medical College of Wisconsin. The *Sleeping Beauty* transposon system was used to generate a GFAP-Lck-eGFP to express membrane targeted enhanced green fluorescent protein (eGFP) under the

regulation of the glial fibrillary acidic protein (GFAP) promoter (Katter et al., 2013). eGFP was targeted and incorporated into the cell membrane by fusing green fluorescent protein (GFP) with a peptide containing myristoylation and palmitoylation domains of Lck, a Src kinase family tyrosine kinase. Together, the use of a GFAP promoter and GFP fused to Lck resulted in GFP expression exclusively in astrocytes membranes.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). A 26-gauge stainless steel unilateral injector (Plastics One, Roanoke, VA) was stereotaxically lowered 9.2 mm to reach the hypothalamic ventromedial nuclei (VMN) in all animals. The stereotaxic coordinates for the VMN injection sites 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 & Watson, 2007). The upper incisor bar was positioned -3.3 mm below horizontal zero.

In situ Hybridization

Rat brains were sectioned coronally at 12 μm and then postfixed 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 PAC1R and PACAP (Choi, Milwaukee, WI) transcripts, which were subsequently diluted in hybridization cocktail (Amresco, Solon, OH) with tRNA. Sections were hybridized overnight at 60 °C with either digoxigenin (DIG) or fluorescein (FITC)-labeled riboprobes. After hybridization, slides were treated with RNase A and stringently washed in 0.3X SSC at 65 °C (PAC1R) for 30 min. Slides were incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche) overnight at 4 °C. Riboprobe signal was amplified using the TSA-Plus fluorophore system with either fluorescein or Cy3 (PerkinElmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2; Zeiss, Thornwood, NY) and Axiovision image analysis software (Zeiss, Thornwood, NY).

Immunohistochemistry

Brains were collected via rapid decapitation and flash frozen in OCT with a dry ice/ethanol bath. Brains were kept at -80 °C before being cut on a cryostat into coronal sections (12 μm thick) which were thaw-mounted onto electrostatically clean slides. Brain sections were stored at -80 °C until immunohistochemistry procedures. Prior to immunohistochemical staining, brain sections were post-fixed in 4% PFA for 10 minutes on ice and rinsed in 0.1 M PBS (pH 7.4). Brain sections were incubated in blocking buffer (0.05M KPBS, 3% normal donkey serum, 0.1% Triton X-100) for 1h at room temperature followed

by an overnight incubation at 4 °C in blocking buffer containing either primary antibody against GFAP (rabbit anti-GFAP, #G9269; Signal-Aldrich; St. Louis, MO), primary antibody against neuronal nuclear protein (NeuN) (rabbit anti-NeuN, #ABN78; Millipore, Temecula, CA) or primary antibody against PAC1R (rabbit anti-PAC1R, #; Alomone; Israel). After several rinses in 0.05M KPBS, sections were incubated in blocking buffer containing donkey anti-rabbit Alexafluor 594 or Alexafluor 488 conjugated secondary antibody (1:250; Life Technologies; Grand Island, NY). Upon completion of fluorescent staining, representative sections containing eGFP, GFAP, NeuN, PAC1R were imaged using fluorescent microscopy (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

Cell Dissociation

Brain tissue dissections were collected from male GFAP-lck-eGFP or WT Sprague Dawley rats and placed immediately into ice-cold Hanks´ Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Sigma-Aldrich, St Louis, MO). Tissues were further minced before enzymatic digestion to obtain single cell suspension with Neural Tissue Dissociation Kit - P (Miltenyi Biotec, San Diego, CA), following manufacturer’s protocol. 1% BSA, 1mM EDTA and 12.5 U/ml DNase DNase I (Sigma, Cat. DN25) were added to single cell suspensions to reduce cell clustering. Cells were incubated with 3μM Calcein Violet 450 AM Viability Dye (eBioscience, San Diego, CA) on ice for 10min to stain live cells.

Fluorescence Activated Cell Sorting (FACS)

Following cell dissociation, single cells were filtered through a 70 μm filter and subsequently washed in fluorescence-activated cell sorting (FACS) buffer containing 2% BSA and 1 mM EDTA. FACS analysis was performed using FACSDiva software, 6.1.3 (BD Biosciences). A primary gate based on the physical parameters of fluorescently labeled cells (side light scatter, SSC and forward light scatter, FSC) will be used to select for fluorescent cells to exclude double cells, dead cells and debris. The fluorescence background level was estimated by using cells from non-fluorescent brain tissue processed at the same time.

Isolation of RNA and Quantitative Polymerase Chain Reaction

Total RNA was extracted from brain tissue or sorted cells by Trizol extraction (Invitrogen; Carlsbad, CA) method. Total RNA was treated with DNase (Life Technologies, Carlsbad, CA) to remove contaminating genomic DNA. Subsequently, 1 μg total RNA was reverse transcribed into complementary DNA (cDNA) using a mixture of random primers, oligodT primers and reverse transcriptase (Reverse Transcription System, Promega; Madison, WI).

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

PCR was performed with *GoTaq* Green Master Mix (Promega, Madison, WI) on a thermocycler with parameters as follows. Initial denaturation (3min at

95°C), Amplification (35 cycles of the following: 95°C for 30s, 60°C for 45s, 72°C for 45s), and Extension (72°C for 10min). PCR products were then examined with electrophoresis on an agarose gel (1.8%). Sequences of primers used to amplify PAC1R isoforms and the following housekeeping genes along with a 100bp DNA Ladder (Promega, Madison, WI) used to locate target PCR products.

PAC1 Forward 5' GGC CCC GTG GTT GGC TCT ATA ATG G 3'

PAC1 Reverse 5' GAG AGA AGG CGA ATA CTG TG 3'

PAC1hop Reverse 5' AGA GTA ATG GTG GAT AGT TCT GAC A 3'

PAC1hip Reverse 5' TGG GGA CTC TCA GTC TTA AA 3'

GAPDH Forward 5' GTT ACC AGG GCT GCC TTC TC 3'

GAPDH Reverse 5' GGG TTT CCC GTT GAT GAC C 3'

Experiments

PACAP, PAC1R mRNA and PAC1R Protein Distribution in Rat Brain

Male Sprague-Dawley rats were euthanized by rapid decapitation approximately 7 hours after the onset of lights on. Rat brains were frozen in OCT and coronal sections measuring 12-14 μm in thickness were collected using a cryostat. Rat coronal brain slices containing hippocampus, thalamus, amygdala, and hypothalamic nuclei were selected for qualitative detection of PACAP, PAC1R mRNA and PAC1 receptor protein using fluorescent in situ hybridization and immunohistochemical assays respectively.

PAC1 Receptor Isoform Expression in Rat Brain

Male Sprague-Dawley rats were euthanized at the onset of lights off and PVN, VMN, amygdala, and nucleus accumbens tissue was collected for total RNA extraction. One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) for PCR analyses of PAC1 receptor extracellular domain (ECD) and intracellular loop 3 (IC3) isoform expression. Gel electrophoresis was used to separate and visualize PAC1R isoforms amplified from PVN, VMN, amygdala, and nucleus accumbens cDNA on a 1.5% agarose gel. PAC1 receptor isoforms PCR products were identified using a 100 base pair ladder.

GFAP-Lck-eGFP Rat Characterization

GFAP-Lck-eGFP rat brains were collected via rapid decapitation and flash frozen in OCT with a dry ice/ethanol bath. Brains were cut on a cryostat into coronal sections which were thaw-mounted onto electrostatically clean slides and fixed in 4% paraformaldehyde. Immunohistochemistry analyses for endogenous transgene, enhanced green fluorescent protein (eGFP), GFAP, and NeuN were performed on slides containing striatum, VMN and amygdala sections.

Neuron and Astrocyte-Specific PAC1R Isoform Expression

Adult VMN and embryonic day 15 (E15) striatum tissue was dissociated and sorted by fluorescence activated cell sorting. Neuronal markers NeuN and SCG10, and the astrocytic markers Aldh1L1 and GFAP were used to confirm GFP negative (GFP-, neurons) and GFP positive (GPF+, astrocytes) cells. Total RNA was extracted from neurons and astrocytes for cDNA synthesis. Nested PAC1 receptor isoform primers were used for PCR and quantitative PCR to identify PAC1R isoforms in dissociated neurons and astrocytes.

Energy-State and PAC1R Isoforms Expression

Male Sprague-Dawley rats were food restricted for 22 hours followed by a 2-hour access to feeding. Rats were euthanized 90 minutes following 2 hours of feeding and brain tissue was collected from VMN, amygdala and nucleus accumbens for total RNA extraction. One microgram total RNA was reverse transcribed to generate tissue-specific cDNA libraries. Nested PAC1 receptor isoform primers were used to amplify changes in PAC1R isoform mRNA expression. Primers for PACAP, PAC1R, BDNF, SOCS3, STAT3 and xCT were also used to quantitate changes in mRNA expression.

PACAP-Mediated Saporin annihilation of VMN Cells Expressing PAC1 Receptors

Male Sprague Dawley rats received bilateral injections of saporin (ZAP), a ribosomal inactivating toxin alone or PACAP conjugated to saporin (PACAP-

ZAP) in the VMN to selectively target PAC1R expressing cells. Rats were returned to their home cages where we measured ad libitum access to food intake and body weight for 2 weeks. Rats were euthanized by rapid decapitation and VMN tissue was collected for total RNA extraction. RNA was reverse transcribed into cDNA in order to use PCR to measure changes in PACAP, PAC1R, BDNF, and xCT mRNA expression.

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean. Statistical analyses were performed using SigmaPlot 11 (Systat Software, Inc, San Jose, CA). P values less than 0.05 were considered statistically significant.

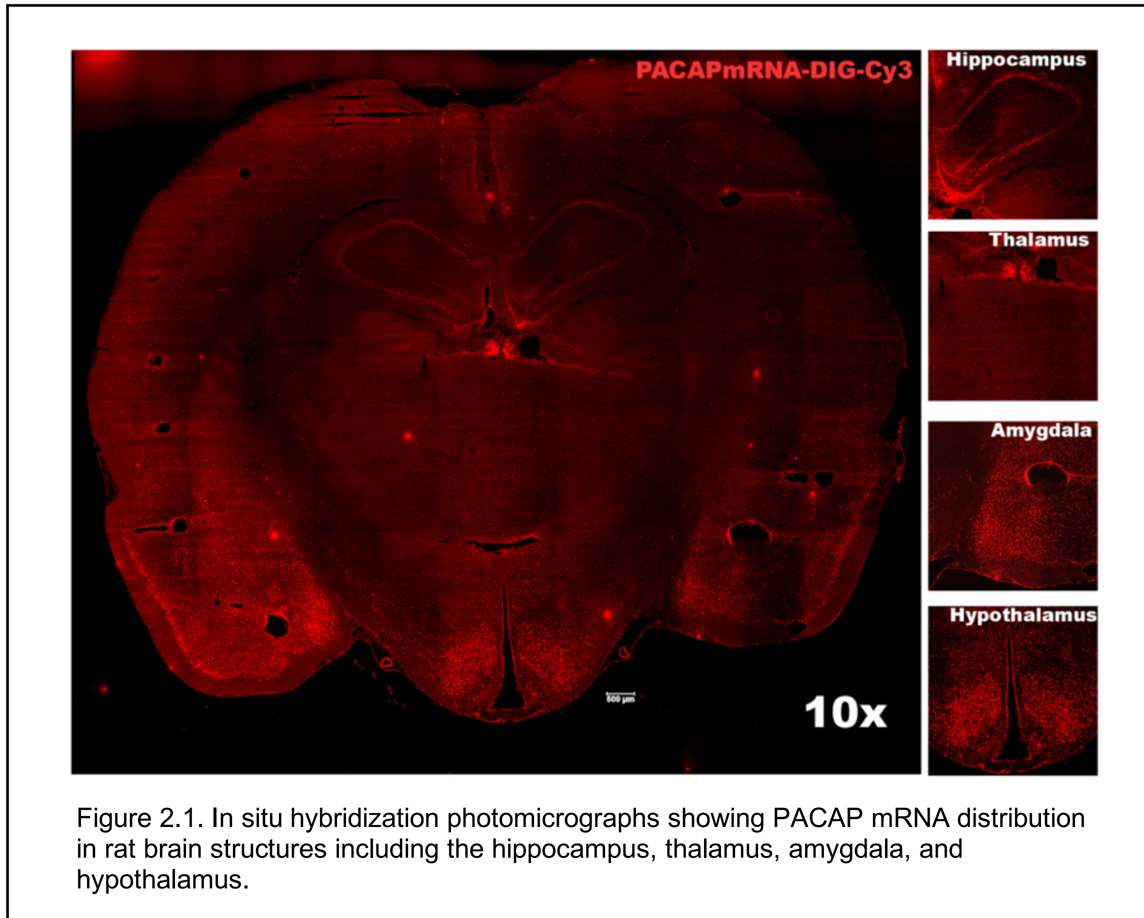


Figure 2.1. In situ hybridization photomicrographs showing PACAP mRNA distribution in rat brain structures including the hippocampus, thalamus, amygdala, and hypothalamus.

Results

Analysis of fluorescent in situ hybridization images show that PACAP (Fig 2.1) and PAC1R mRNA (Fig 2.2) are widely distributed in rat brain. More specifically, we detected PACAP, PAC1R mRNA expression in the hippocampus, thalamus, amygdala, and several hypothalamic nuclei including the VMN. In addition to PACAP and PAC1R mRNA, we found that PAC1R protein is similarly

distributed in the hippocampus, and hypothalamic nuclei (Fig 2.3).

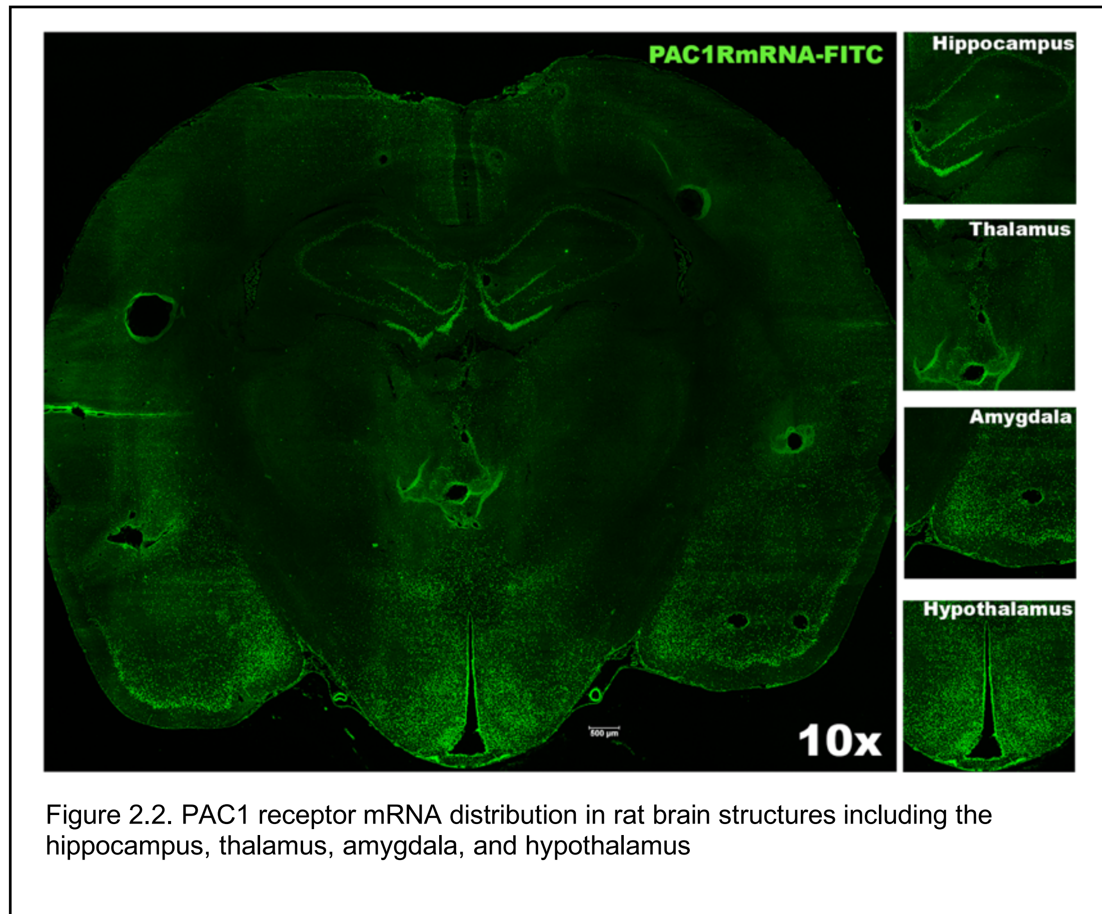


Figure 2.2. PAC1 receptor mRNA distribution in rat brain structures including the hippocampus, thalamus, amygdala, and hypothalamus

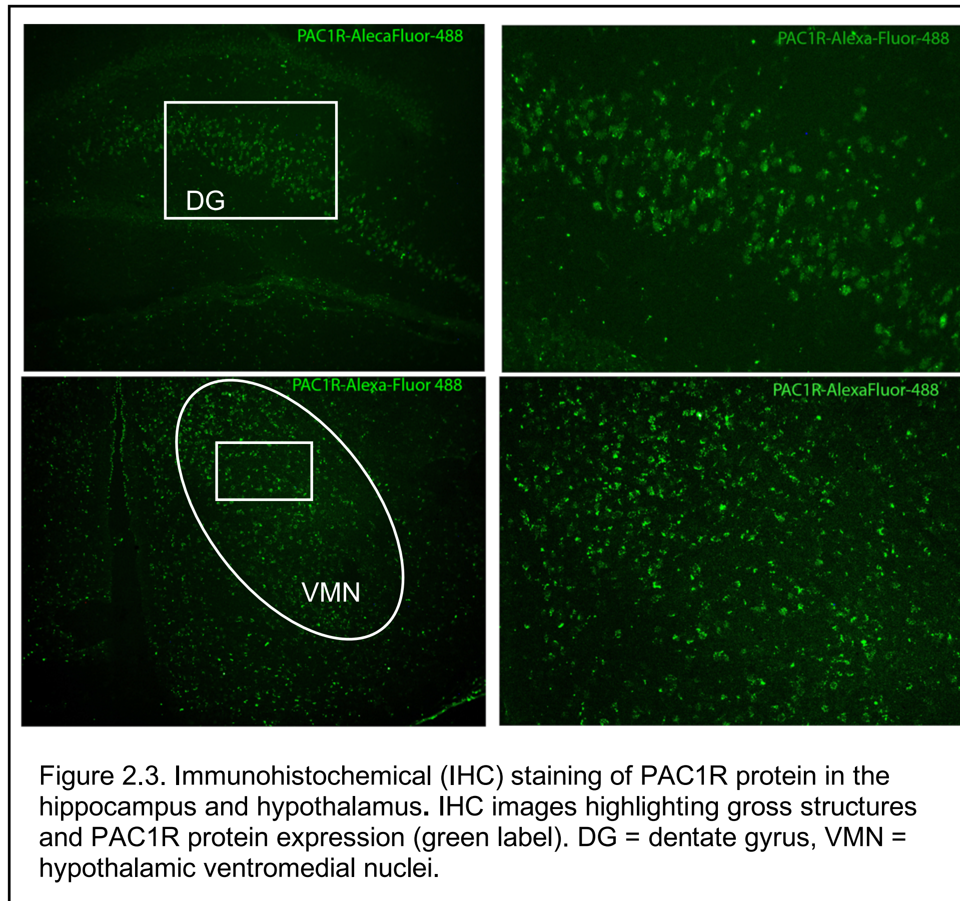


Figure 2.3. Immunohistochemical (IHC) staining of PAC1R protein in the hippocampus and hypothalamus. IHC images highlighting gross structures and PAC1R protein expression (green label). DG = dentate gyrus, VMN = hypothalamic ventromedial nuclei.

Rat cortex and VMN cells express PAC1 receptor isoforms

PCR analysis for PAC1 receptors isoforms demonstrated that PAC1ECD, PAC1null, and PAC1hop isoforms are expressed in the paraventricular nuclei of the hypothalamus (PVN), VMN, medial amygdala (MeAmy), and nucleus accumbens brain tissue. These brain structures are well established to modulate feeding, stress, and motivational behaviors.

PCR digital gel shows that the N-terminal extracellular domain (ECD), and C-terminal intracellular loop 3 (IC3) PAC1R isoforms PAC1ECD, PAC1null and

PAC1hop are expressed in hypothalamic paraventricular nuclei (PVN), VMN, medial amygdala (MeAmy), and nucleus accumbens (NAc) (Fig 2.4). However, the isoforms PAC1hip or PAC1hip-hop isoforms were not detected in any of the adult rat brain structures mentioned above. This is consistent with previous reports showing that the major isoforms found in adult rat brain are PAC1ECD, PAC1null and PAC1hop.

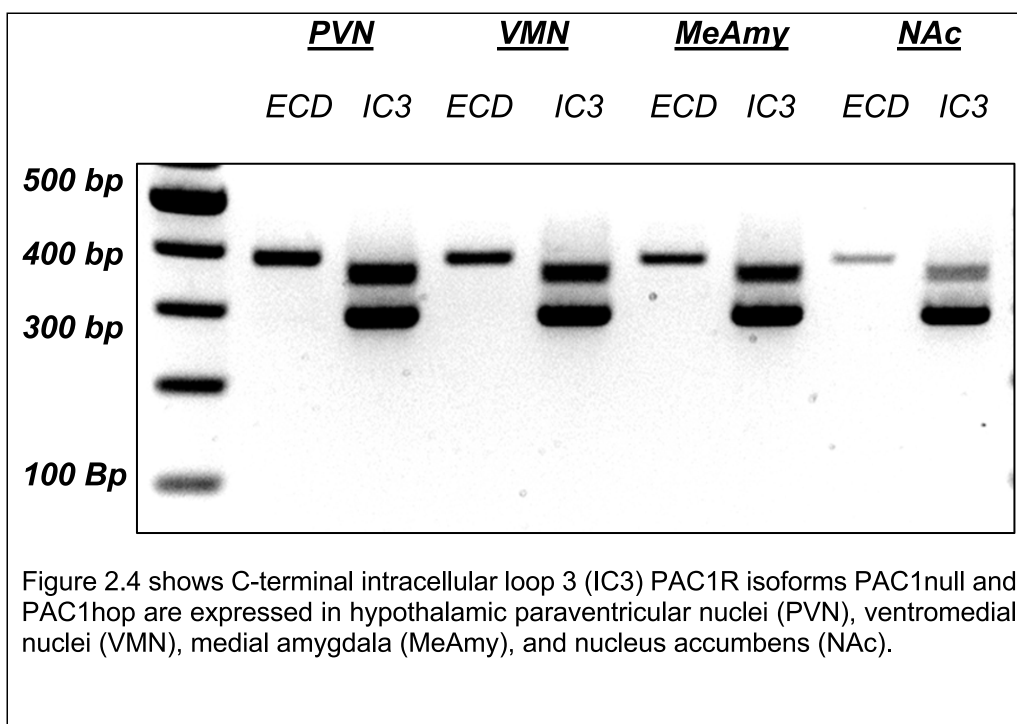


Figure 2.4 shows C-terminal intracellular loop 3 (IC3) PAC1R isoforms PAC1null and PAC1hop are expressed in hypothalamic paraventricular nuclei (PVN), ventromedial nuclei (VMN), medial amygdala (MeAmy), and nucleus accumbens (NAc).

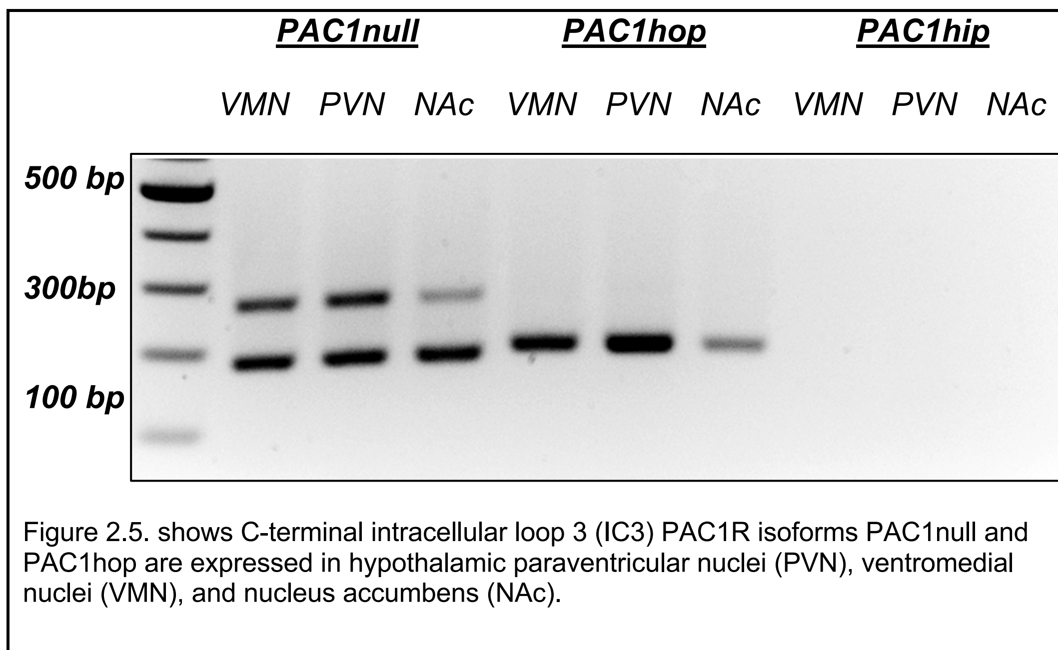


Figure 2.5. shows C-terminal intracellular loop 3 (IC3) PAC1R isoforms PAC1null and PAC1hop are expressed in hypothalamic paraventricular nuclei (PVN), ventromedial nuclei (VMN), and nucleus accumbens (NAc).

To determine the distribution of PAC1 receptors in rat brain cell types, we developed a rat model with green fluorescent protein expressed in astrocytes exclusively. We used immunohistochemistry and fluorescent microscopy to characterize successful expression of GFP and GFAP labeling in astrocytes alone. We found successful GFP expression in GFAP-Lck-eGFP rat astrocytes which was confirmed by co localization with GFAP labeling. In addition, we found that GFP expressing cells do not co-localize with NeuN, a protein exclusively expressed in neurons.

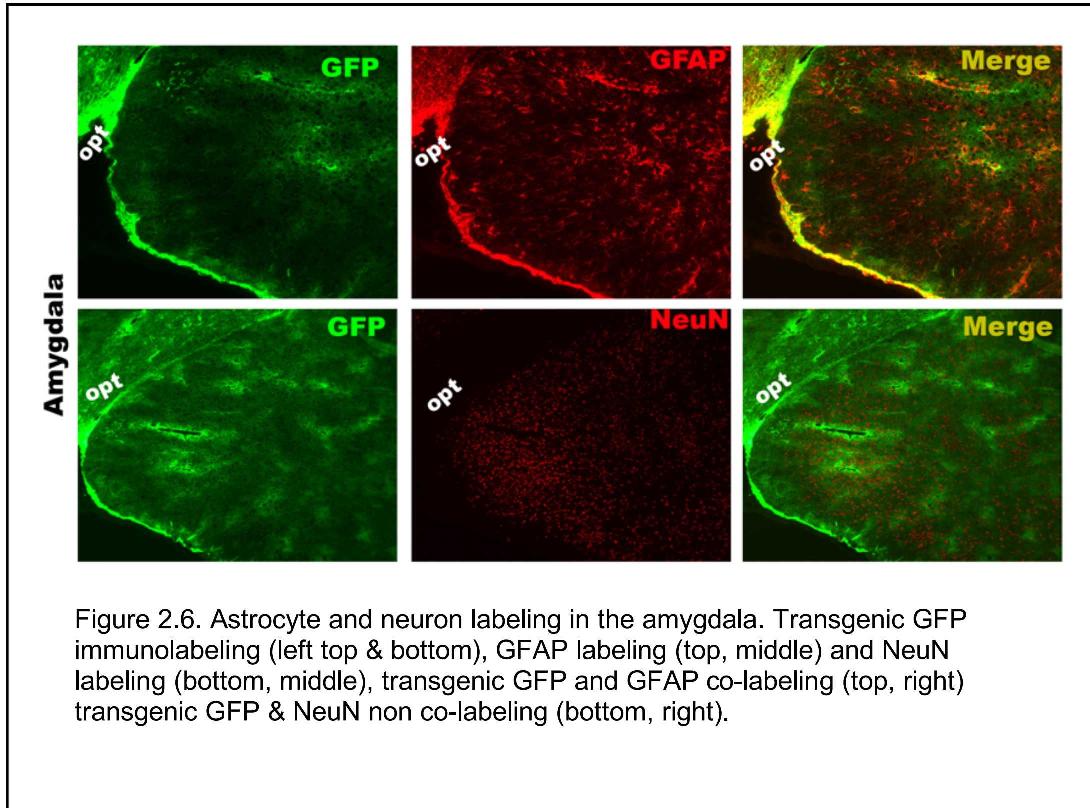


Figure 2.6. Astrocyte and neuron labeling in the amygdala. Transgenic GFP immunolabeling (left top & bottom), GFAP labeling (top, middle) and NeuN labeling (bottom, middle), transgenic GFP and GFAP co-labeling (top, right) transgenic GFP & NeuN non co-labeling (bottom, right).

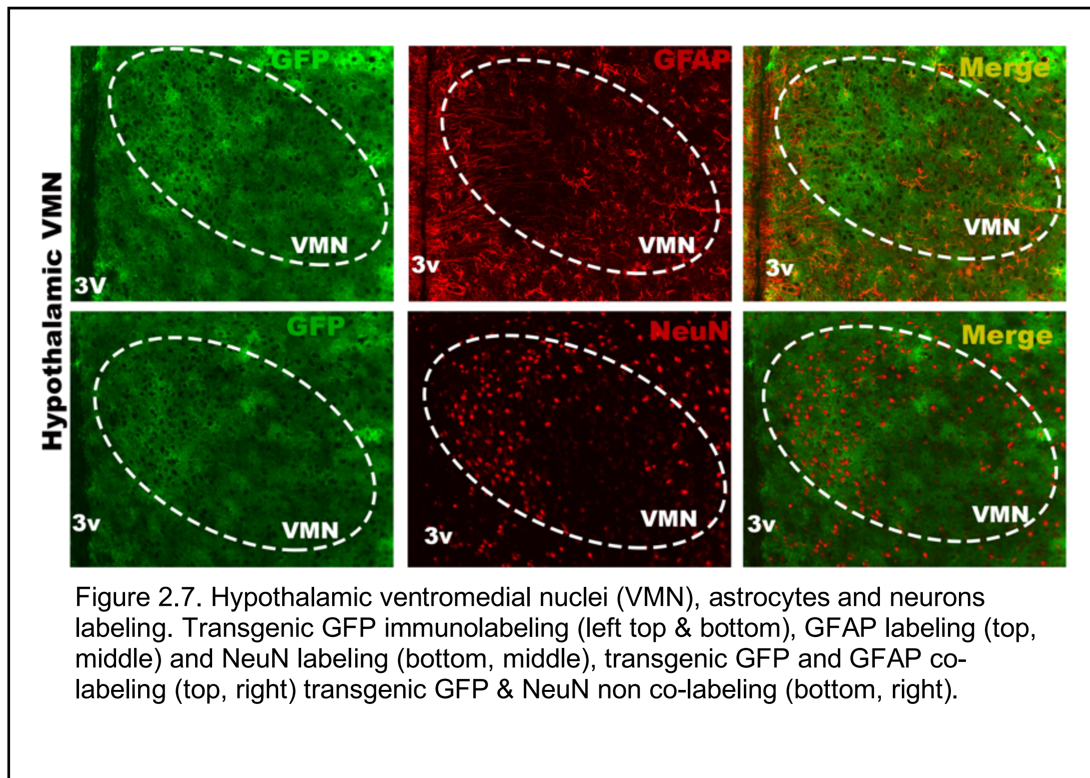


Figure 2.7. Hypothalamic ventromedial nuclei (VMN), astrocytes and neurons labeling. Transgenic GFP immunolabeling (left top & bottom), GFAP labeling (top, middle) and NeuN labeling (bottom, middle), transgenic GFP and GFAP co-labeling (top, right) transgenic GFP & NeuN non co-labeling (bottom, right).

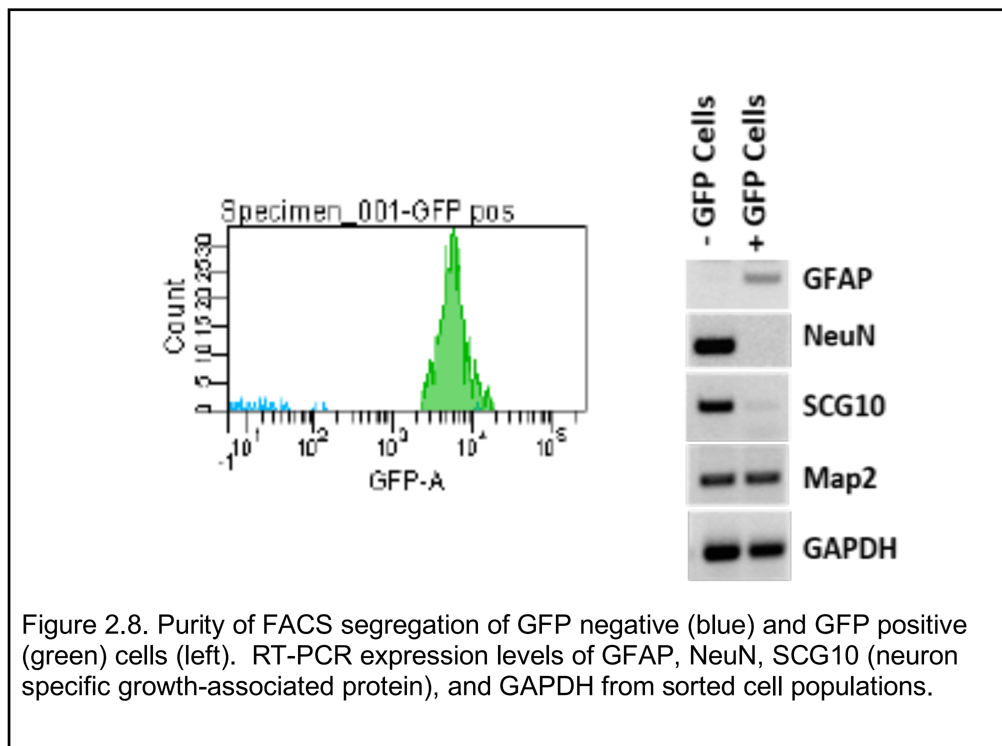


Figure 2.8. Purity of FACS segregation of GFP negative (blue) and GFP positive (green) cells (left). RT-PCR expression levels of GFAP, NeuN, SCG10 (neuron specific growth-associated protein), and GAPDH from sorted cell populations.

To characterize PAC1 receptor isoform expression in sub-populations of rat brain cells, we used immunohistochemistry to verify that our transgenic GFAP-Lck-eGFP rats expressed GFP in astrocytes alone. Analysis of GFP expression and GFAP staining was done in the hypothalamus (Fig 2.7), amygdala (Fig 2.6), and striatum tissue (Fig 2.9). Cells in both the striatum and hypothalamic nuclei show robust GFP expression and GFAP staining that colocalize (Figs 2.6, 2.7, 2.9).

Next, with the help of Kong et al (Kong, 2009) we used fluorescence activated cell sorting (FACS) to successfully isolate striatum neurons and astrocytes from cells dissociated from GFAP-Lck-eGFP rat brain tissue. PCR analysis of segregated astrocyte and neuron cell populations show that astrocytes express GFAP and the neuronal cell population expressed NeuN amplicons respectively. In addition, neurons and astrocytes in rat cortex and

hypothalamic nuclei express both PAC1null and PAC1hop receptor isoforms. Interestingly, only rat embryonic tissue show differential expression of PAC1 receptor isoforms in neurons vs astrocytes. At this stage of development both neurons and astrocytes express PAC1null, PAC1hop, and PAC1hip isoforms whereas, only neurons express the PAC1hip-hop isoform. This suggests there may be a developmental role for PAC1hip-hop-dependent PACAP signaling that is not present in adult rat brain.

Rat brain neurons and astrocytes differentially express PAC1R isoforms

The results show that PAC1R isoforms are developmentally and differentially expressed in neurons vs astrocytes. Embryonic day 15 (E15) cortical neurons express PAC1null, PAC1hop, PAC1hip, and PAC1hip-hop isoforms, whereas astrocytes only express PAC1null, PAC1hip, and PAC1hop and not the PAC1hip-hop isoform.

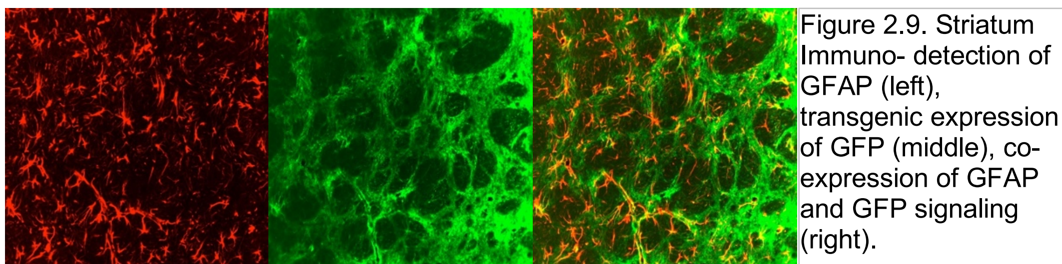


Figure 2.9. Striatum Immuno- detection of GFAP (left), transgenic expression of GFP (middle), co-expression of GFAP and GFP signaling (right).

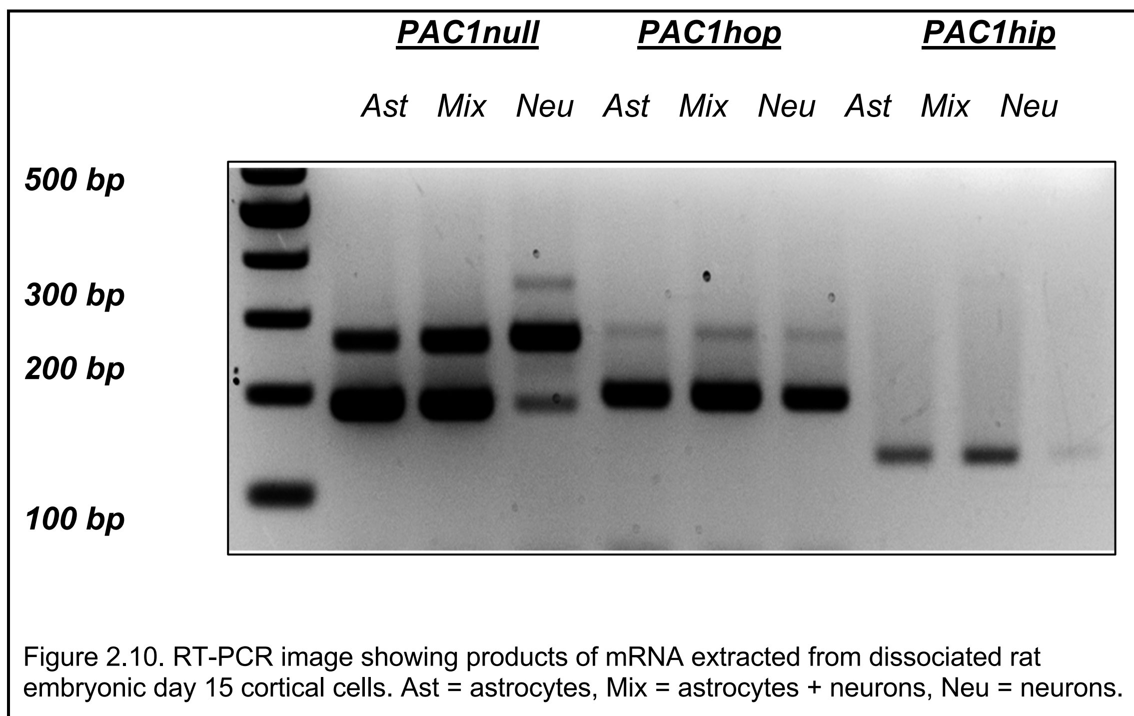


Figure 2.10. RT-PCR image showing products of mRNA extracted from dissociated rat embryonic day 15 cortical cells. Ast = astrocytes, Mix = astrocytes + neurons, Neu = neurons.

RNA extracted from dissociated cortical neurons and astrocytes (E15) show differential gene expression profiles. Quantitative mRNA analysis shows PAC1R, PACAP, BDNF, xCT, and SOCS3 mRNA are enriched in neurons at the onset of lights off, compared to astrocyte expression (Fig 2.11). BDNF is upregulated in both neurons and astrocytes, and leptin receptor mRNA is downregulated (Fig 2.11).

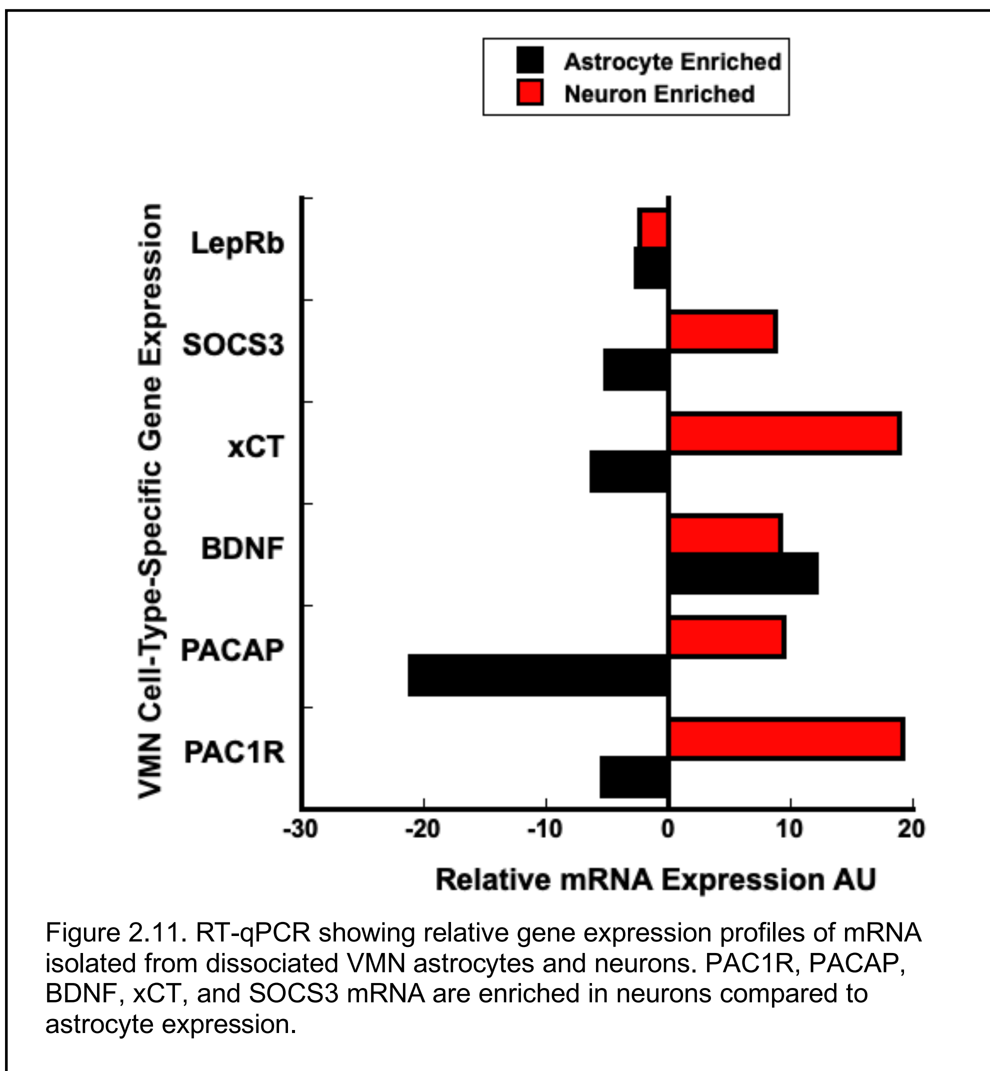


Figure 2.11. RT-qPCR showing relative gene expression profiles of mRNA isolated from dissociated VMN astrocytes and neurons. PAC1R, PACAP, BDNF, xCT, and SOCS3 mRNA are enriched in neurons compared to astrocyte expression.

Analysis of gene expression profiles show that food ingestion following a fast induced an overall decrease in PACAP, PAC1Rs, BDNF, SOCS3, STAT3 and xCT mRNA in tissue collected from the nucleus accumbens (Fig 2.12). By contrast, mRNA analysis from amygdala tissue revealed that feeding following a fast stimulated an overall increase in PAC1Rs, BDNF, SOCS3, and xCT, whereas PACAP and STAT3 decreased (Fig 2.12). In the VMN, food ingestion following a fast resulted in increased PACAP, PAC1Rs, BDNF, and STAT3

mRNA expression, however, SOCS3 and xCT decreased (Fig 2.12). A close analysis of PAC1R isoforms expression revealed that food presentation following a fast does not differentially influence VMN PAC1R isoform expression. Interestingly, PAC1R isoforms appear differentially regulated in the amygdala and nucleus accumbens. In the amygdala, it appears that PAC1hop isoform in higher in expression compared to the PAC1null isoform. In the nucleus accumbens, PAC1hop isoform decreased more that PAC1Rnull isoform. However, whether these magnitude increases are functionally relevant remain to be determined.

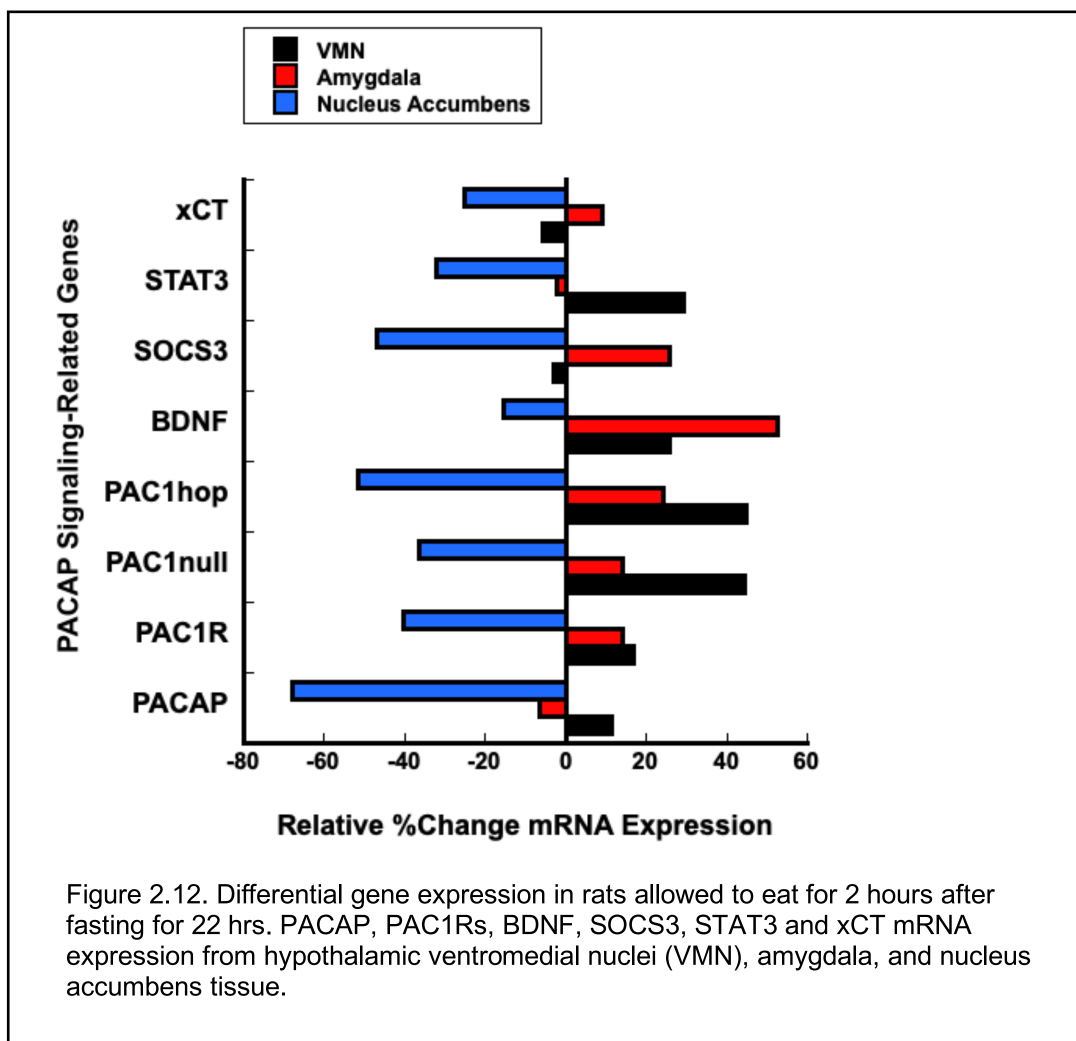


Figure 2.12. Differential gene expression in rats allowed to eat for 2 hours after fasting for 22 hrs. PACAP, PAC1Rs, BDNF, SOCS3, STAT3 and xCT mRNA expression from hypothalamic ventromedial nuclei (VMN), amygdala, and nucleus accumbens tissue.

Effect of PACAP-ZAP on VMN PAC1R Expression

Hypothalamic VMN injections of PACAP conjugated to saporin caused a complete loss of PAC1null, and PAC1hop mRNA expression (Fig 2.13). PAC1 receptor DNA band detection following gel electrophoresis shows missing VMN PAC1null and PAC1hop isoforms compared to PVN, and nucleus accumbens tissue that did not receive conjugated PACAP-Saporin injections (Fig 2.13). This indicates that the PACAP-Saporin complex was sufficient to recognize and ablate PAC1R expressing VMN cells.

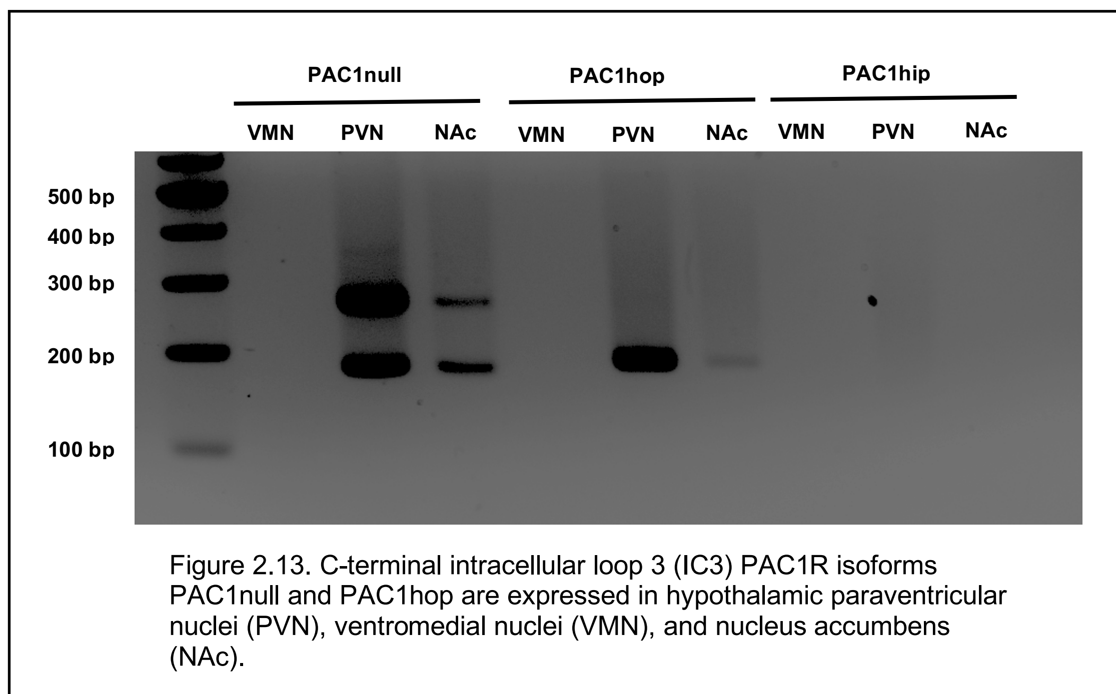


Figure 2.13. C-terminal intracellular loop 3 (IC3) PAC1R isoforms PAC1null and PAC1hop are expressed in hypothalamic paraventricular nuclei (PVN), ventromedial nuclei (VMN), and nucleus accumbens (NAc).

Discussion

PACAP is a biologically relevant neuropeptide whose sequence has been highly conserved throughout evolution from tunicates to mammals (Vaudry et al., 2009). It is not surprising that PACAP and its receptor mRNA are broadly distributed in key central nervous system cell groups. We used in situ hybridization and immunohistochemical staining to label rat brain cell containing PACAP, PAC1R mRNA and PAC1R protein as demonstrated in (Figs. 2.1, 2.2, 2.3). We found that PACAP, PAC1R mRNA and PAC1R protein are highly expressed in several brain regions including the hippocampus, thalamus, amygdala, and hypothalamus. Hypothalamic nuclei exhibited the densest PAC1R mRNA staining as previously published by Resch et al (Resch et al., 2011, 2013). We further confirmed that the protein for PAC1R is highly expressed in brain regions where PAC1R mRNA is expressed suggesting that the PAC1R gene is not only transcribed but it is also translated into functional protein that can influence key signaling events across numerous brain regions.

PACAP binding to PAC1 receptors engages multiple second messenger signaling pathways throughout the central nervous system to regulate gene transcription, neurotransmission, and numerous behaviors (Blechman et al., 2013; Holighaus et al., 2011; May, Lutz, Mackenzie, et al., 2010; May & Parsons, 2017; Shintani et al., 2018; Limei Zhang, Hernandez, et al., 2021). Previous studies have demonstrated that the PAC1R gene is highly spliced and generates multiple isoforms that display differing sensitivity to PACAP and bias toward

unique 2nd messenger signaling. To that end, we probed for PAC1R isoform expression in brain regions our lab and others have previously shown to influence feeding, stress, and motivation (He et al., 2021; Hurley et al., 2020, 2016; Loh, Herzog, & Shi, 2015; Resch et al., 2011, 2014). We found that PAC1R isoforms PAC1ECD, PAC1null and PAC1hop were expressed in rat hypothalamic paraventricular and ventromedial nuclei, amygdala, and the nucleus accumbens, suggesting that PACAP signaling has another layer of complexity that could be afforded by PAC1R isoforms. For the numerous studies showing functional consequences of PACAP/PAC1R signaling, consideration could and should be made towards further nuanced roles of endogenous PACAP release parameters and the PAC1 receptor subtypes mediating physiological outcomes.

It is now well established that both neurons and astrocytes uniquely and coordinately regulate neurotransmission by responding to and releasing neurotransmitter and neuropeptide factors. Recent evidence show neurons and astrocytes both express PAC1 receptors and that both cell types can directly respond to PACAP and influence neurotransmission (Jozwiak-Bebenista et al., 2007; Kong et al., 2016). For example, we and others have demonstrated that PACAP signaling influences glutamatergic signaling at the synapse (Kong et al., 2016; Macdonald et al., 2005; Yaka, He, Phamluong, & Ron, 2003) with more recent opportunity to closely examine neuron and astrocyte-specific gene profiles using our newly developed GFAP-Lck-eGFP transgenic rat. This transgenic rat model allowed us to confidently isolate astrocytes from neuronal cells as the

GFAP-Lck-eGFP transgenic rat was designed to express a green fluorescent protein in astrocytes exclusively. We confirmed this isolated expression using immunohistochemistry and PCR. Photomicrographs confirmed that GFP positive cells co-localized with GFAP, an astrocyte-specific marker, but not the neuronal marker, NeuN or SCG10, in several brain regions including the striatum, amygdala, and the VMN (Figs. 2.6, 2.7, 2.9). Thus, the GFAP-Lck-eGFP transgenic rat model is an appropriate tool for dissociating gene profiles of astrocytes from other cell types. Using dissociated astrocyte and neuron cell populations, we found that neurons and astrocytes in rat cortex and hypothalamic nuclei express the PAC1null and PAC1hop receptor isoforms. Although Barres et al (Cahoy et al., 2008) had previously demonstrated PACAP and PAC1R mRNA are present in developing and mature forebrain neurons and astrocytes, this is the first time PAC1R isoform distributions have been shown in rodent astrocytes and neurons. Interestingly, only rat embryonic tissue shows differential expression of PAC1 receptor isoforms in neurons vs astrocytes. While neurons and astrocytes express PAC1null, PAC1hop, and PAC1hip isoforms, only embryonic neurons express the PAC1hip-hop isoform, whereas astrocytes did not. This suggests a differential and developmental role for PAC1 receptor isoforms that may be absent in adults. This could potentially explain specific PACAP and PAC1R contributions to feeding and metabolism during development that may differ from function in adult stages (Bakalar et al., 2022).

In addition to several brain regions expressing PAC1 receptor isoforms, PAC1null and PAC1hop also have been shown to have different sensitivity to

PACAP and shown to engage differing downstream signaling and gene transductions, which together illustrate that PACAP signaling through PAC1Rs cannot be generalized throughout the brain. For example, PACAP stimulation of PAC1Rs influence NMDA receptor function to increase hippocampal and VMN cell firing frequency but decreased firing in the striatum. These region-specific differences may be mediated by PAC1R isoform function in either neurons and/or astrocytes (Hurley et al., 2016; Macdonald et al., 2005; Resch et al., 2014; Yaka et al., 2003). To date, the importance of specific receptor splice variants in engaging combinatorial transduction pathways to regulate neurotransmission has yet to be delineated. Another consideration to make is the PAC1R isoform sensitivity to PACAP and resulting activation of G-protein coupling, which would implicate another complex signaling code for PACAP-regulated neurotransmission that differs across different cell types. Intersection with G-proteins may provide a platform to integrate other signaling systems that could take into consideration changes in physiological state or milieu. Such complex PAC1R-mediated effects might serve to illustrate the various regulatory mechanisms involved in the pleiotropic actions of PACAP during behaviors that engage stress, motivational, and energy homeostasis systems. Our laboratory has previously shown that PACAP stimulation of PAC1 receptors in hypothalamic VMN induced multiple effects on food intake and metabolism (Hurley et al., 2016; Maunze et al., 2022; Resch et al., 2013). In the current study, we examined the consequences of food deprivation and food presentation on changes in PACAP, PAC1R isoforms, BDNF, SOCS3, STAT3 and xCT mRNA in tissue collected

from the nucleus accumbens and VMN. Food presentation following a fast increased in amygdala PAC1Rs, BDNF, SOCS3, and xCT whereas, PACAP and STAT3 decreased. The profile was notably different in the VMN in animals that fed following a fast with increased PACAP, PAC1Rs, BDNF, and STAT3 mRNA expression, and decreases in SOCS3 and xCT. A closer analysis of PAC1R isoforms expression revealed that food presentation following food deprivation does not influence VMN PAC1R isoform expression. However, PAC1R isoforms were differentially regulated in the amygdala and nucleus accumbens. The PAC1hop isoform in the amygdala had higher expression compared to the PAC1null isoform in contrast to the marked decrease in PAC1hom observed in the nucleus accumbens compared to the PAC1null isoform. To further illustrate the need to be more specific regarding PAC1 receptor related signaling, we showed that VMN PACAP-saporin injections resulted in the loss of all PAC1R isoforms. As a result, future therapeutic strategies employed in treating disease-states associated with PACAP and PACAP gene products could be less effectively deployed due to missed opportunities to recognize more selective PACAP and PACAP receptor function in neurons and astrocytes and their responsiveness under physiological and pathophysiological states.

CHAPTER III

DEVELOPMENT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR TARGETING-shRNA AND VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS ADENO-ASSOCIATED VIRUS AND LENTIVIRUS TRANDUCTION

Introduction

Dysregulation of energy homeostasis is associated with environmental and genetic factors that may lead to eating disorders or obesity (Andermann & Lowell, 2017; J K Elmquist et al., 1999; Mou et al., 2015; Timper & Brüning, 2017). In the hypothalamus, several genes including melanocortin-4 receptor (MC4R), pro-opiomelanocortin (POMC), brain derived neurotropic factor (BDNF) and leptin gene products have been tested for their involvement in feeding and metabolic regulation in rodents and humans, and their dysfunction has directly been implicated in overeating and obesity (Andermann & Lowell, 2017). Another emerging candidate gene whose role in feeding and metabolism regulation is pituitary adenylate cyclase-activating polypeptide (PACAP) and its cognate receptor PAC1R (H. Hashimoto et al., 2000; Resch et al., 2014; Rudecki & Gray, 2016). PACAP and PAC1 receptors are highly expressed in the hypothalamus with the highest expression observed in the ventromedial nuclei of the hypothalamus (VMN) (Resch et al., 2011, 2013). Previously, our lab and others have employed global gene knockdown and pharmacological manipulations to elucidate putative roles for PACAP signaling in several brain regions including the VMN control of feeding and metabolism (Bakalar et al., 2022; Bozadjieva-Kramer et al., 2021; H. Hashimoto et al., 2000). These studies have repeatedly

demonstrated that PACAP and PAC1R gene knockouts and pharmacological manipulations lead to alterations in feeding and metabolism. While these methods greatly contributed to our general knowledge about the role PACAP signaling may play in energy homeostasis, there are limitations with the methods that we and the field have had to acknowledge while determining the nuances of PACAP signaling. For example, global PACAP and PAC1R gene knockouts cause developmental lethality, modify PACAP and PAC1R DNA regulatory element recognition by trans-acting factors, and produce cell specific PACAP and PAC1R contributions to feeding and metabolism during development that may differ from function in adult stages (Bakalar et al., 2022). In addition, pharmacological antagonists used to infer necessity have been demonstrated to act as inverse agonists thereby potentially obscuring endogenous roles of PACAP in the VMN control of feeding and other functions (Hurley, 2018).

To address the challenges and limitations associated with global gene knockout and pharmacological antagonism in future PACAP studies, we have designed and tested tools to deliver short hairpin ribonucleic acid nucleotides (shRNA) in vitro to induce RNA interference in adult rat VMN cells to knockdown PAC1R in a confined and delineated manner. Our approach employs lentivirus and adeno-associated viruses (AAVs) which are reliable means for delivering gene editing tools in cell culture and brain tissue (Burger et al., 2004; de Backer, Brans, Luijendijk, Garner, & Adan, 2010; Giering, Grimm, Storm, & Kay, 2008; Hioki et al., 2007). Lentivirus and AAV vectors transduce non-dividing neurons to deliver and express gene cassettes over long periods of time that allow for

assessment of gene functions over broader ranges of time. Additional unique feature of AAVs is that they can selectively transduce specific cell types in vitro and in vivo using promotors or AAV DNA packaging with different serotype capsids (Burger et al., 2004) thereby, allowing for additional controlled gene manipulations and more selective interpretation of outcomes.

In the current studies, we designed and tested shRNA targeting PAC1 receptors in vitro using Neuro2A cells expressing PAC1 receptors. We also tested lentivirus and adeno-associated virus serotype transduction in VMN cells. Finally, we packaged shRNA targeting PAC1R mRNA into a lentivirus vector and transduced VMN cells in male Sprague-Dawley rats and determined through PCR analysis subsequent mRNA expression of PAC1R, BDNF, and suppressor of cytokine signaling-3 (SOCS3) all of which have been shown to be responsive to PACAP signaling. The PAC1R-targeting shRNA nucleotide sequence we selected was sufficient to knockdown PAC1R mRNA in vitro in a dose dependent manner and demonstrated that lentivirus and AAV serotypes AAV8, AAV9 containing this sequence successfully transduce VMN cells. Consequently, transduction of the VMN with lentivirus- or AAV-containing PAC1R-targeting shRNA significantly decreased PAC1R mRNA expression.

Materials & Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow and water. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Cell culture and plasmid transfection

Mouse neuroblastoma Neuro2a cells (N2a; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% (vol/vol) fetal bovine serum (FBS) at 37°C and 5% CO2. Prior to plasmid transfections, cells were seeded in 24-well plates and allowed to reach 80% confluence overnight. Transfection of cells with control or lenti-shRNA plasmid was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

PAC1R shRNA Plasmid Construction & virus production

Hairpin RNA designed to target PAC1R mRNA sequence (CGGAATCCACTACACAGTATT) was cloned into a lentivirus shRNA plasmid (Dharmacon GE (RHS3979-201740301) pLKO.1 lentiviral vector) for transfection

in vitro. Lentivirus and adeno-associated virus (AAV) 8 and 9 particles measuring $1x10^{13}$ titer units/mL and $15.25X10^{12}$ viral genomes (VG)/mL were used to transduce hypothalamic ventral medial nuclei.

Immunohistochemistry

Whole brain tissue was harvested by transcardial perfusion using saline and fixed in 4% paraformaldehyde. Coronal brain sections were cut at 30μm and mounted on Superfrost Plus slides. Sections were washed five times with phosphate buffered saline (PBS) and cover-slipped using Vectashield mounting medium. Image capture was conducted on a fluorescent microscope (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

RNA extraction and quantitative reverse transcription

Total RNA was extracted from brain tissue or sorted cells by Trizol extraction (Invitrogen; Carlsbad, CA) method. Total RNA was treated with DNase (Life Technologies, Carlsbad, CA) to remove contaminating genomic DNA. Subsequently, 1μg total RNA was reverse transcribed into complementary DNA (cDNA) using a mixture of random primers, oligodT primers and reverse transcriptase (Reverse Transcription System, Promega; Madison, WI). Quantitative PCR was performed using a StepOne Real-Time PCR System (AppliedBiosystems; Carlsbad, CA), and PerfeCTa SYBR Green FastMix with ROX (QuantaBiosciences; Gaithersberg, MD) according to the manufacturer's

protocol. Quantification of PAC1R, BDNF, SOCS3 mRNA expression was done using a relative standard curve and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for reach gene were designed to span an exon-exon junction and had efficiencies of approximately 95%. Melt curve analysis of experiments confirmed a single product for each reaction.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). A 26-gauge stainless steel unilateral injector (Plastics One, Roanoke, VA) was stereotaxically lowered to reach the hypothalamic ventromedial nuclei (VMN) in all animals. The stereotaxic coordinates for the VMN injection sites 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 & Watson, 2007). The upper incisor bar was positioned -3.3 mm below horizontal zero.

Experiments

PAC1R Knockdown in Neuro2A Cells

Neuro2A cells were seeded in 24-well plates and allowed to reach ~80% confluence overnight. Transfection experiments were prepared with n=6 wells/treatment per reaction. Neuro2A cells were transfected with either 0.5 μg, 1.0 μg, or 1.5 μg total PAC1R-shRNA plasmid DNA in lipofectamine and incubated for 48 h. Following transfection, cells were harvested for total RNA extraction using Trizol reagent and quantitative PCR.

AAV8, AAV9 and lentivirus GFP injections in rat VMN

0.25 μl AAV8, AAV9 and lentivirus particles containing plasmids expressing green fluorescent protein (GFP) were unilaterally microinjected in the VMN cells over a 30-minute period. Animals were given 2 mg/kg ketoprofen once post-surgery and returned to their home cages where they received ad libitum food access and water. Two weeks following virus injections, rats were transcardially perfused with saline and fixed with 4% paraformaldehyde solution. Rat brains were harvested and cryosectioned at 30 μm. Image capture was conducted on a fluorescent microscope (Axioskop-2, Zeiss; Thornwood, NY) and Axiovision image analysis software (Zeiss; Thornwood, NY).

In vivo PAC1R knockdown in hypothalamic VMN

Lentivirus particles containing scrambled or PAC1R-targeting shRNA plasmids with green fluorescent protein were bilaterally microinjected in VMN cells at 0.25μl/side over a 30-minute period. Animals were given 2 mg/kg ketoprofen once post-surgery and returned to their home cages where they received ad libitum food access and water. Two weeks following virus injections, rats were euthanized by rapid decapitation and VMN tissue was collected for total RNA extraction and quantitative PCR. PAC1R primers were used to measure changes in PAC1R mRNA expression relative to GAPDH.

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean. Statistical analyses were performed using SigmaPlot 11 (Systat Software, Inc, San Jose, CA). P values less than 0.05 were considered statistically significant.

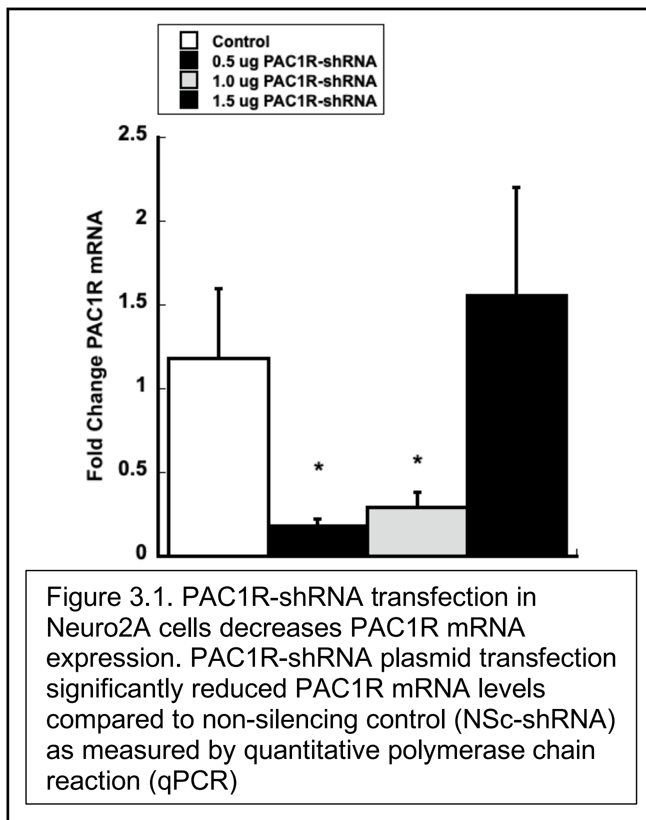


Figure 3.1. PAC1R-shRNA transfection in Neuro2A cells decreases PAC1R mRNA expression. PAC1R-shRNA plasmid transfection significantly reduced PAC1R mRNA levels compared to non-silencing control (NSc-shRNA) as measured by quantitative polymerase chain reaction (qPCR)

Results

shRNA-mediated PAC1R knockdown in Neuro2A cells

PAC1R-shRNA plasmid transfection of Neuro2A cells decreases PAC1R mRNA expression in a concentration-dependent manner (Fig 3.1). PAC1R-plasmid concentrations at 0.5 μg and 1.0 μg induced significant decreases (85% and 75%, respectively) in PAC1R mRNA expression when compared to the control plasmid. However, a higher concentration of 1.5 μg PAC1R-shRNA plasmid did not alter PAC1R mRNA levels from control treatment.

In vivo virus transduction (GFP only) in hypothalamic VMN tissue

Fluorescent and confocal microscopy image analyses show that 0.25 μl injections of AAV serotypes 8, 9 and lentivirus particles produce successful transduction of VMN cells as illustrated by GFP expression in rat VMN tissue. Lentivirus-shRNA-mediated PAC1R knockdown in VMN tissue.

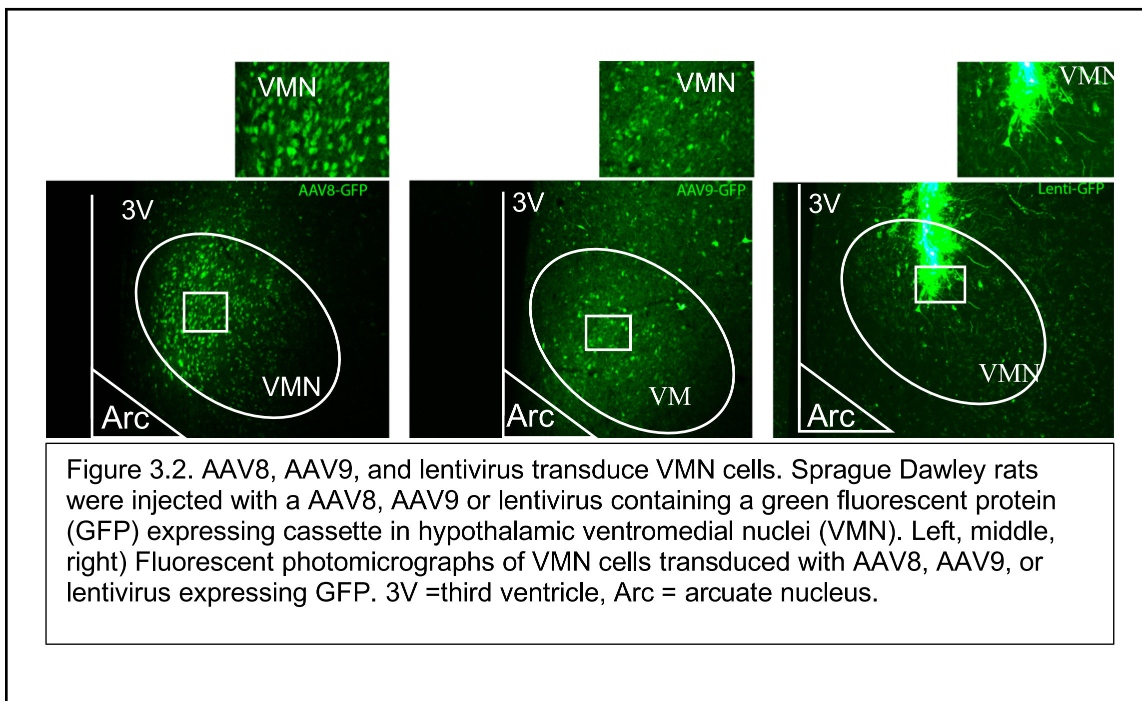


Figure 3.2. AAV8, AAV9, and lentivirus transduce VMN cells. Sprague Dawley rats were injected with a AAV8, AAV9 or lentivirus containing a green fluorescent protein (GFP) expressing cassette in hypothalamic ventromedial nuclei (VMN). Left, middle, right) Fluorescent photomicrographs of VMN cells transduced with AAV8, AAV9, or lentivirus expressing GFP. 3V =third ventricle, Arc = arcuate nucleus.

Microscopy analysis validated rat brains injected with lentivirus particles-containing PAC1R-shRNA plasmid transduce a large population of VMN cells and suppressed PAC1R mRNA levels by 20% (Fig. 3.3) compared to non-silencing control (Nsc)-shRNA.

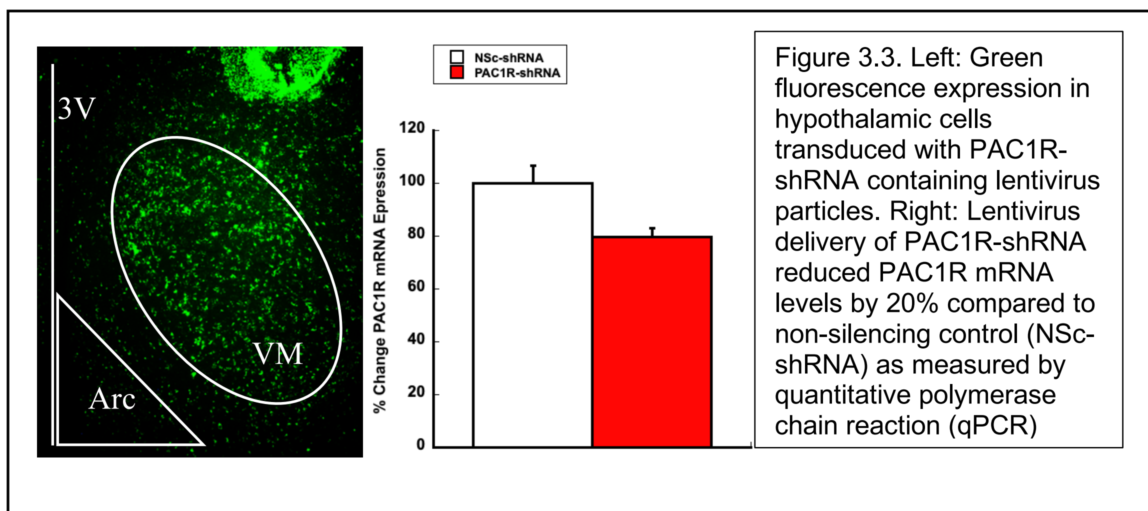


Figure 3.3. Left: Green fluorescence expression in hypothalamic cells transduced with PAC1R-shRNA containing lentivirus particles. Right: Lentivirus delivery of PAC1R-shRNA reduced PAC1R mRNA levels by 20% compared to non-silencing control (NSc-shRNA) as measured by quantitative polymerase chain reaction (qPCR)

Discussion

Dysregulation of energy homeostasis is complex as it involves multiple signaling systems that regulate food intake, metabolism, and body weight. The emergence of PACAP and its pleiotropic functions in regulating energy homeostasis has drawn massive interest in the peptide and its receptors as a potential solution for mitigating feeding related disorders and obesity (Marzagalli, Scuderi, Drago, Waschek, & Castorina, 2015; Miles, May, & Hammack, 2019; Rubio-Beltrán et al., 2018). Although we have gained tremendous insight into the contributions of PACAP and its receptors in energy homeostasis regulations, much of this effort has relied on global gene knockout and pharmacological antagonist studies (Adams et al., 2008; Bozadjieva-Kramer et al., 2021; Filatov et al., 2021; Gray, Cummings, Jirik, & Sherwood, 2001; Gray, Yamaguchi, Vencová, & Sherwood, 2002; Vu et al., 2015). While gene knockout and pharmacological manipulations are powerful and informative tools, interpretations gained from use of these tools are limited. For example, gene knockouts and pharmacological antagonist fail to account for the spatial and temporal physiological conditions under which peptide signals function.

To address some of these limitations, we have developed and validated genetic ablation tools for interrogating region-specific roles of endogenous PACAP and PAC1R signaling in the control of food intake. Using Neuro2A cells, we demonstrated that the Dharmacon PAC1R-targeting shRNA was sufficient to significantly decrease PAC1R expression and that lentivirus-mediated delivery of

PAC1R shRNA to regionally suppress PAC1 receptor mRNA is a critical strategy to target PAC1 receptors and make inferences about endogenous localized PACAP signaling. To confirm the appropriate viral vehicle, we demonstrated that lentivirus and adeno-associated virus (AAV) serotypes 8 and 9 transduce VMN cells whereas, AAV serotype 5 did not. Our results confirm the successful use of AAV serotypes to limit our interrogation of PAC1R function to the hypothalamus and subsequently the VMN. Furthermore, we confirmed that our PAC1R shRNA not only reduced PAC1 receptor mRNA in vitro, but also in vivo involving hypothalamic VMN cells. Taken together, this tool will allow us to build on our previous work on PACAP's role in hypothalamic regulation of food intake and energy expenditure (Hurley et al., 2020, 2016; Maunze et al., 2022; Resch et al., 2011, 2013, 2014). Because of AAV8's limited tropism in rat brain, we will used this for the remainder of our studies.

CHAPTER IV

HYPOTHALAMIC VENTROMEDIAL NUCLEI (VMN) PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTORS (PAC1Rs) ARE NECESSARY FOR FEEDING AND BODY WEIGHT REGULATION

Introduction

Obesity is a major public health concern affecting more than 1.5 billion people worldwide (Beck, 2000; Boughton & Murphy, 2012; Rodrigues et al., 2017). It is now apparent that obesity is a consequence of genetic, and environmental factors leading to an imbalance of energy intake and energy expenditure. Regulation of energy homeostasis involves a combination of neurotransmitters and neuropeptide systems found in the peripheral and central nervous system including the hypothalamus (Beck, 2000; Gastelum et al., 2021; Wilding, 2002). Earlier studies by Hetherington and Ranson among others established that the hypothalamus is an organized group of neuropeptide-containing cells that regulates energy expenditure, and damage to the hypothalamus and VMN leads to obesity (Hetherington & Ranson, 1942; King, 2006).

One fascinating hypothalamic peptide system that has emerged is pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor PAC1R and its role in feeding and metabolic regulation (Hurley et al., 2020; Resch et al., 2011, 2013, 2014). PACAP and PAC1 receptors are highly expressed in the hypothalamus, with the highest expression found in the VMN. PACAP stimulation of PAC1 receptors decreases food intake, and body weight with concomitant

increases in energy expenditure (Bozadjieva-Kramer et al., 2021; Hawke et al., 2009; Resch et al., 2011, 2013). Although exogenous administration of PACAP in the VMN generates discrete homeostatic responses suggesting an endogenous role in these physiological and behavioral systems, we have still yet to delineate specific anatomical circuitry to fully understand PACAP's functional purpose.

In the current study, we tested the necessity for VMN PAC1R in normal daily food intake and body weight regulation. We knocked down VMN PAC1 receptors using our adeno-associated virus (AAV) system to deliver PAC1R-targeting shRNA in male Sprague-Dawley rats. Decreasing PAC1Rs in the VMN increased meal sizes, reduced total number of meals, and induced body weight gain. In addition, PAC1R knockdown decreased BDNF and SOCS3 mRNA expression. To confirm the sufficiency of magnitude of our PAC1 receptor knock-down, we administered exogenous PACAP and observed no change in VMN food intake and body weight regulation. As an added measure, we used slice electrophysiology to confirm loss of PAC1R response to exogenous PACAP. We found that knocking down PAC1Rs prevents PACAP-dependent increase in action potential firing frequency. Our studies demonstrated that in the VMN endogenous PACAP signaling significantly contributes to normal daily food intake and body weight regulation, and this is mediated specifically through PAC1Rs.

Materials & Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. Food intake measurements were collected using the BioDAQ Food Intake Monitor for 24-hours daily from the onset of the experiments. For BioDAQ meal pattern analysis, 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; Resch et al., 2011, 2013). All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). 26-gauge stainless steel bilateral guide-cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted 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 sites 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 & Watson, 2007). Microinjectors 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 -3.3 mm below horizontal zero. Animals were given 2 mg/kg ketoprofen once post-surgery and allowed to recover from surgery for 1 week before they are included in experiments, during which time the animals were handled daily to acclimate them to the necessary physical manipulations during experiments. Correct cannulae placements were confirmed at the conclusion of each experiment by microscopic examination of fluorescent protein markers and Nissl-stained sections. Image capture was conducted on a confocal microscope using 10X and 20X magnification (Nikon-confocal; Nyquist sampling) with Nikon NIS Elements software (Nikon, Melville, NY, USA). On average, the included studies produced 85% accurate stereotaxic placement. Only those with correct placement were included in the studies when experimental design allowed.

Western Blot analysis

Bilateral dissections of the ventromedial hypothalamus (VMH; includes the VMN and surrounding areas) were collected following rapid decapitation. VMH tissue was homogenized by hand 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 (#78447; Thermo Scientific; Rockford, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4°C and the resulting supernatant was further centrifuged at 10,000 X g for 30 minutes at 4°C to remove crude membrane protein. The resulting supernatant containing crude cytoplasmic protein was saved for further processing. Protein quantification of samples was determined using a bicinchoninic (BCA) assay (#23252; Pierce). Protein (30 μg) was run on an 8% gel by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (#LC2002; Thermo Fisher Scientific; Rockford, IL). Membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) or 5% non-fat milk in tris-buffered saline containing 0.1 % Tween-20 (TBS-T). Blots were then probed with either rabbit anti-PAC1R antibody alone (#AV-R003; 1:1000; Alomone labs; Jerusalem, Israel) or dually probed with β-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) overnight at 4°C, followed by washes with TBS-T and incubation with an HRP-linked anti-rabbit secondary antibody (#7074P2; 1:3000; Cell Signaling Technologies; Danvers, MA) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA) at room temperature for 2 hours. Band intensities were developed using SuperSignal West Atto Chemiluminescent Substrate (#A38556; Thermo Scientific; Rockford, IL) and visualized using the Odyssey Fc Dual Mode Imaging System (LI-COR; Lincoln, NE). Band densities were measured and quantified using Odyssey Fc Dual-Mode Imaging System software. PAC1R antibody specificity was validated

by preincubating rabbit anti-PAC1R with PAC1R blocking peptide (#BLP-VR003; Alomone labs; Jerusalem, Israel). Following visualization of PAC1R signal alone, blots were stripped and re-probed in an identical fashion for β-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA).

RNA extraction and quantitative reverse transcription

Total RNA was extracted from brain tissue by the Trizol extraction (Invitrogen; Carlsbad, CA) method. Total RNA was treated with DNase (Life Technologies, Carlsbad, CA) to remove contaminating genomic DNA. Subsequently, 1μg total RNA was reverse transcribed into complementary DNA (cDNA) using a mixture of random primers, oligodT primers and reverse transcriptase (Reverse Transcription System, Promega; Madison, WI). Quantitative PCR was performed using a StepOne Real-Time PCR System (AppliedBiosystems; Carlsbad, CA), and PerfeCTa SYBR Green FastMix with ROX (QuantaBiosciences; Gaithersberg, MD) according to the manufacturer’s protocol. Quantification of PAC1R, BDNF, SOCS3 mRNA 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%. Melt curve analysis of experiments confirmed a single product for each reaction.

Slice electrophysiology

Coronal slices (250µm) containing the VMN of the hypothalamus were obtained from adult male rats and prepared using a vibratome (Leica VT1000S). Slices were prepared in ice-cold solution containing 229mM sucrose, 1.9mM KCl, 1.2mM NaH2PO4, 33mM NaHCO3, 10mM glucose, 0.4mM ascorbic acid, 6mM MgCl2, and 0.5mM CaCl2 oxygenated using 95% O2 5% CO2. Slices were then incubated at 31°C for ten minutes in a solution containing 119mM NaCl, 2.5mM KCl, 1mM NaH2PO4, 26.2mM NaHCO3, 11mM glucose, 0.4mM ascorbic acid, 4mM MgCl2, and 1mM CaCl2 and further incubated a minimum of 35 minutes at room temperature. Whole-cell recordings were performed in slices continuously perfused with aerated aCSF (125mM NaCl, 2.5mM KCl, 25mM NaHCO3, 10mM glucose, 0.4mM ascorbic acid, 1.3mM MgCl2, and 2mM CaCl2) at a temperature of 29°- 33°C using a gravity-fed perfusion system with a flow rate of ~2 ml/min oxygenated. Borosilicate (2.5–4.5MΩ) glass pipettes were filled with 140mM K-Gluconate, 5.0mM HEPES, 1.1mM EGTA, 2.0mM MgCl2, 2.0mM Na2-ATP, 0.3mM Na- GTP, and 5.0mM phosphocreatine (pH 7.3, 290mOsm). Action potential (AP) firing was filtered at 1kHz, sampled at 5kHz, and measured using a Sutter Integrated Patch Amplifier (IPA) with Igor Pro (Wave Metrics, Inc.) data acquisition software.

Experiments

Design and construction of shRNA

Hairpin RNA was designed to target specific regions of PAC1R mRNA (CGGAATCCACTACACAGTATT) or a non-silencing control (NSc; scrambled nucleotides) mRNA. The nucleotide sequences were individually inserted into an AAV8 vector after the H1 promoter and a red fluorescent protein reporter sequence (tdTomato) under the CMV enhancer. The resulting plasmids, AAV8-H1-shPAC1R-CMV-tdTomato and AAV8-H1-shNSC-CMV-tdTomato were made into an infectious virus with concentrations of $1.05x10^{13}$ vg/mL and $5.25x10^{12}$ vg/mL, respectively.

Virus Delivery

Approximately 1 week following cannulae surgery, rats (n=8/group) were anesthetized and placed in a stereotaxic apparatus. 0.25 μl of AAV8-H1-shPAC1R-CMV- tdTomato $1.05x10^{13}$ vg/mL or AAV8-H1-NSC-CMV-tdTomato $5.25x10^{13}$ vg/mL was microinjected through bilateral guide cannulae over 10 minutes. Following each injection an additional 20 minutes elapsed before removing injectors to minimize backflow of delivered material. Animals were allowed to recover from anesthesia and returned to their home-cage. Food intake was measured using the BioDAQ Food Intake Monitor and body weights were collected manually. Food intake and body weight measures were collected for 21 days following virus injections.

AAV Transduction and shRNA-mediated PAC1R Knockdown

21 days following AAV injection, rats were either euthanized and brains were collected for microscopic examination of fluorescent protein, quantitative PCR, and western blot analyses or they received microinjections of vehicle or pituitary adenylate cyclase activating polypeptide (#350-35; PACAP; 50 pmol/0.25μl/side; California Peptide Research; Napa, CA) followed by food intake measurements for 24-hours and a final body weight measurement. To assess fluorescent markers, rat brains were sectioned coronally at 14μm using a cryostat, thaw-mounted onto electrostatically clean sides, and stored at -80°C until postfixed. Prior to microscopic examination of fluorescent protein markers, brain sections were postfixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH7.4), and cover-slipped with mounting medium (Vector Labs, Burlingame, CA). Image capture was performed using a confocal microscope (Nikon Inc, Tokyo, Japan).

PAC1R knockdown validation: Ex vivo slice-electrophysiology

14 days following AAV8 injections, control and PAC1 receptor knockdown rats were euthanized for slice electrophysiology. Current-clamp recordings were performed in spontaneously-firing neurons in the VMN with adequate whole-cell access (Ra<20 MΩ) and capacitance verified at the beginning of the recording. Picrotoxin (100μM) and NBQX (10μM) were added to the recording aCSF to better isolate postsynaptic effects of PACAP. Following completion of a 2–5 minute baseline with <20% variation in spike firing, either PACAP (100nM), or

PACAP6–38 (100nM) was added to the recording solution. For recordings measuring PACAP-dependent spike firing after prior application of PACAP6–38, a stable baseline was first acquired in the presence of PACAP6–38 (<20% variation).

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean. Statistical analyses were performed using SigmaPlot 11 (Systat Software, Inc, San Jose, CA). P values less than 0.05 were considered statistically significant.

Results

AAV transduction and shRNA-mediated PAC1R knockdown

Confocal fluorescent microscopy analysis (10x and 20x Nikon-confocal; Nyquist sampling with Nikon NIS Elements software, Nikon, Melville, NY) validated rat brains injected with AAV8-shPAC1R-tdTomato resulted in transduction of a large population VMN cells (Fig. 4.1). Note that tdTomato fluorescent images were converted to binary black and white images. Microinjections of virus were contained within the borders of the VMN and shown

to be expressed in a large percentage of VMN cells. Western blot analysis of VMN homogenates (Fig. 4.1) confirmed that AAV8-shPAC1R-tdTomato decreased VMN PAC1R expression by approximately 50-60% compared to the scrambled shRNA non-silencing control (NSc; $F_{(1,14)}$=11.276, p<0.01).

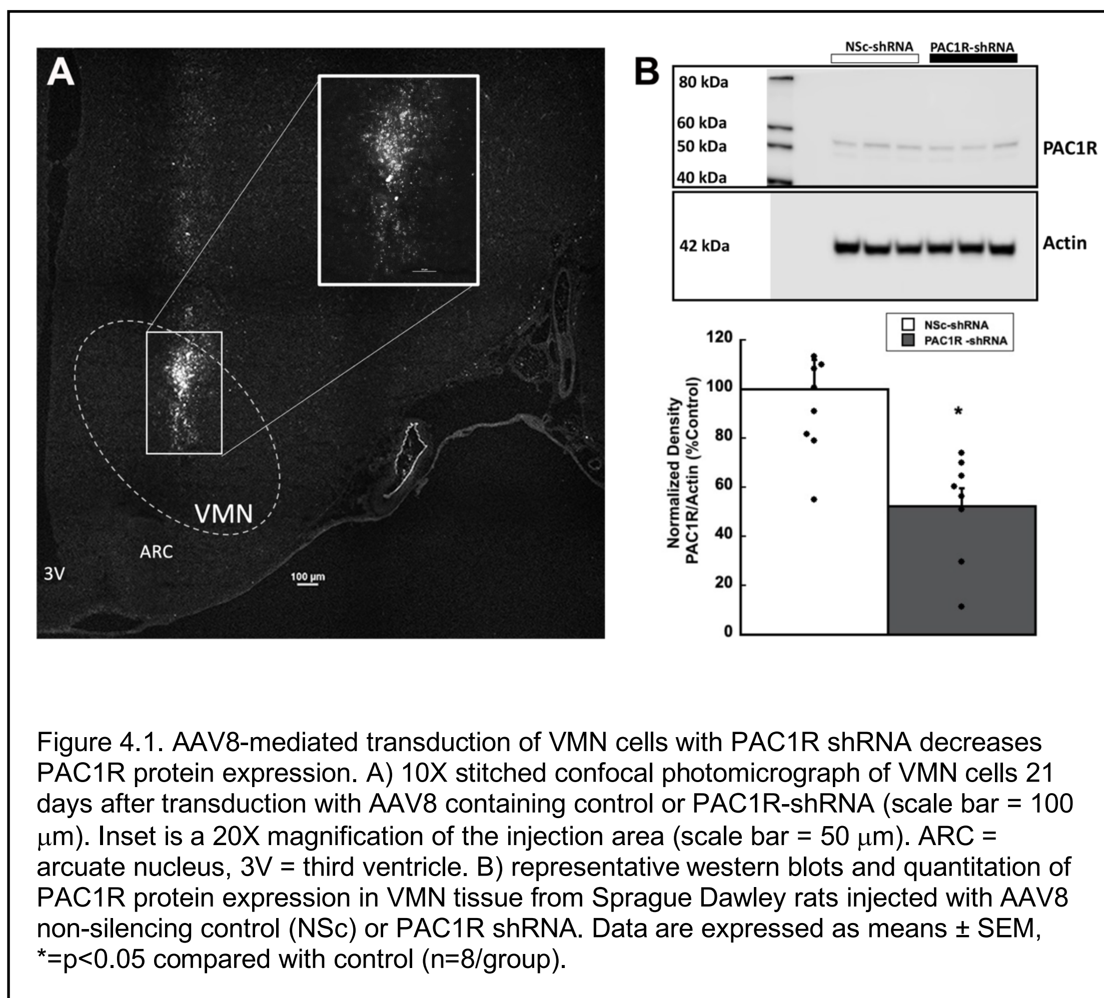


Figure 4.1. AAV8-mediated transduction of VMN cells with PAC1R shRNA decreases PAC1R protein expression. A) 10X stitched confocal photomicrograph of VMN cells 21 days after transduction with AAV8 containing control or PAC1R-shRNA (scale bar = 100 μm). Inset is a 20X magnification of the injection area (scale bar = 50 μm). ARC = arcuate nucleus, 3V = third ventricle. B) representative western blots and quantitation of PAC1R protein expression in VMN tissue from Sprague Dawley rats injected with AAV8 non-silencing control (NSc) or PAC1R shRNA. Data are expressed as means ± SEM, *=p<0.05 compared with control (n=8/group).

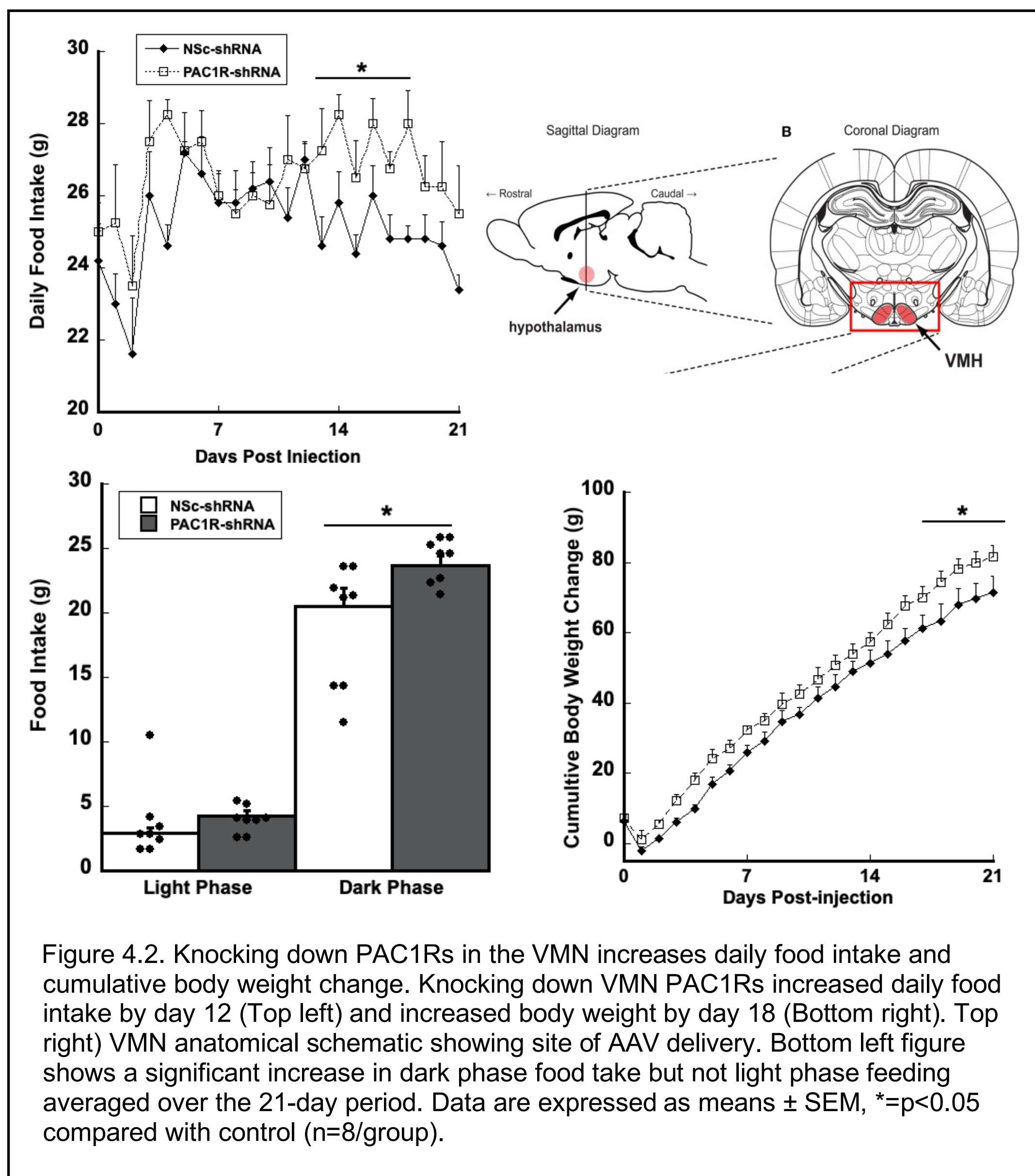


Figure 4.2. Knocking down PAC1Rs in the VMN increases daily food intake and cumulative body weight change. Knocking down VMN PAC1Rs increased daily food intake by day 12 (Top left) and increased body weight by day 18 (Bottom right). Top right) VMN anatomical schematic showing site of AAV delivery. Bottom left figure shows a significant increase in dark phase food take but not light phase feeding averaged over the 21-day period. Data are expressed as means ± SEM, *=p<0.05 compared with control (n=8/group).

PAC1R knockdown: food intake and body weight

RNA interference of PAC1 receptors by AAV8-shPAC1R-tdTomato injections in the VMN increased both food intake and body weight compared to the scrambled shRNA non-silencing control (Fig. 4.2). Specifically, decreasing PAC1R expression in the VMN reduced the number of meals consumed while

increasing overall meal size (Table 1). In addition, significant increased food intake occurred only during the dark phase starting at day 4 and consistently between days 13 to 21. The lower left graph in Fig 4.2, illustrates significantly increased dark phase food intake averaged over the 21-day period but not light phase feeding. Consistent and significant differences were detected in feeding by day 13, whereas body weight changes were significant by day 18. Analysis of variance (ANOVA) showed a significant main effect of treatment for food intake $F_{(1,25)}=4.916$, $p<0.04$, and body weight $F_{(1,21)}=3.534$, $p<0.001$. Post-hoc all pairwise multiple comparison procedures (Fisher LSD method) analysis showed that rats injected with AAV8-shPAC1R-tdTomato significantly increased body weight after day 18 ($p<0.03$) compared with non-silencing control shRNA (Fig. 42. Lower right)

Table 1. *Meal pattern analysis following PAC1R knockdown*

	Nsc-shRNA	PAC1R-shRNA
Average number of meals	8.381 ± 0.257	7.333 ± 0.268*
Average Meal size (g)	2.486 ± 0.08	2.883 ± 0.08*

Data are means ± SEM. Meal patterns were analyzed for 21 days post-injection. Nsc, non-silencing control shRNA.
*P<0.05 vs Nsc-shRNA

To test the functional degree to which PACAP receptors in the VMN were suppressed, exogenous PACAP was administered to stimulate hypophagia and changes in body weight in both virus and non-silencing shRNA treated animals. PAC1R-shRNA treatment prevented exogenous PACAP-induced hypophagia and ameliorated the decrease in body weight (Fig. 4.3). Thus, a 50-60% PAC1R knockdown was sufficient to disrupt PAC1R regulation of feeding and body

weight in the VMN. Analysis of food intake data show a significant effect of treatment ($F_{(3,37)}$=6.499, p<0.001), time ($F_{(11,33)}$=361.500, p<0.001), and treatment x time interaction ($F_{(33,460)}$=2.460, p<0.001; Figure 4.3, left), whereas body weight changes showed a significant main effect of treatment $F_{(3,37)}$=2.964, p<0.05 when compared to the control group (Figure 4.3, right).

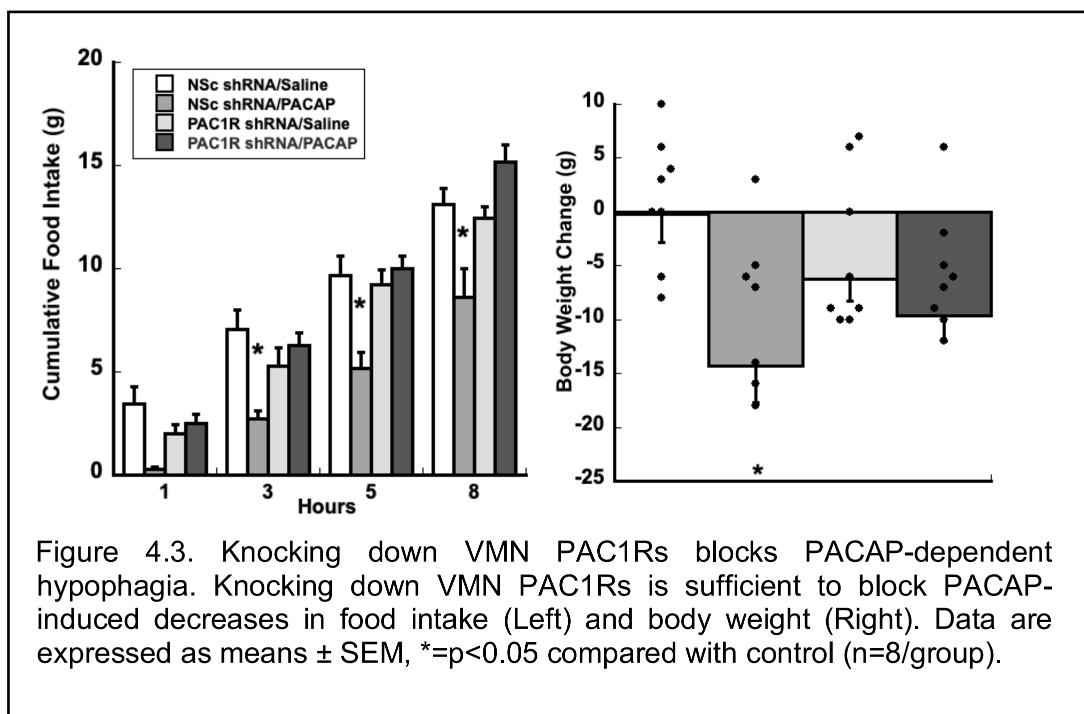


Figure 4.3. Knocking down VMN PAC1Rs blocks PACAP-dependent hypophagia. Knocking down VMN PAC1Rs is sufficient to block PACAP-induced decreases in food intake (Left) and body weight (Right). Data are expressed as means ± SEM, *=p<0.05 compared with control (n=8/group).

PAC1R-shRNA expression in VMN cells and PACAP-dependent action potential firing frequency

Our lab in collaboration with the Hearing lab (Marquette University, Wisconsin) and others have previously demonstrated that PACAP-dependent increases in VMN cell action potential firing frequency is mediated through PAC1 receptors (Fig. 4.4). We used this prior knowledge to test if our knockdown of PAC1R using shRNA was sufficient to prevent PACAP-induced increases in

action potential firing frequency. Bath application of PACAP (100nM) to ex vivo VMN slices increased action potential firing frequency which can be blocked by $PACAP_{6\text{-}38}$, a PAC1 receptor-specific antagonist (Fig. 4.4). Similarly, PACAP application to VMN cells expressing PAC1-shRNA does not show any change in action potential firing frequency (Fig. 4.4).

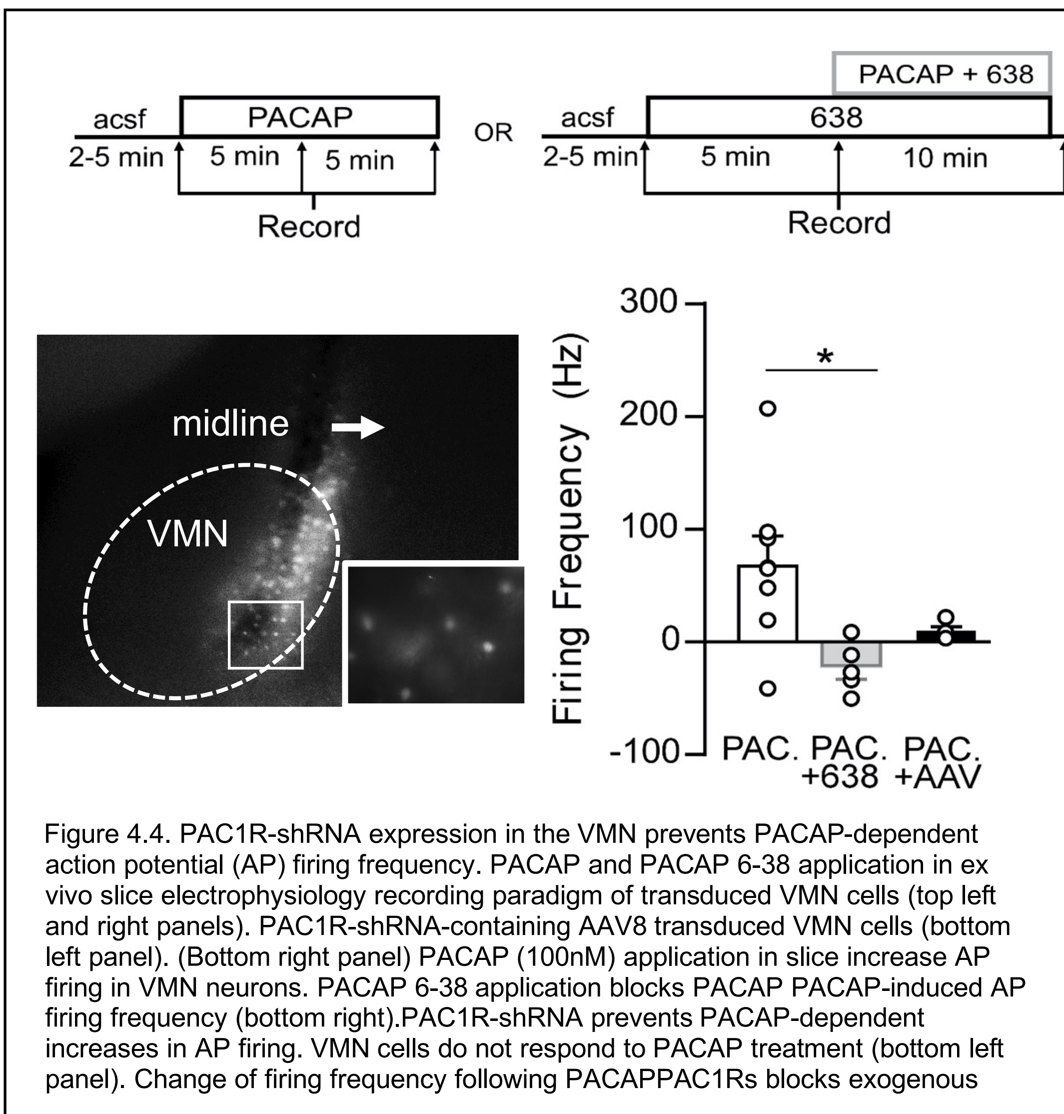


Figure 4.4. PAC1R-shRNA expression in the VMN prevents PACAP-dependent action potential (AP) firing frequency. PACAP and PACAP 6-38 application in ex vivo slice electrophysiology recording paradigm of transduced VMN cells (top left and right panels). PAC1R-shRNA-containing AAV8 transduced VMN cells (bottom left panel). (Bottom right panel) PACAP (100nM) application in slice increase AP firing in VMN neurons. PACAP 6-38 application blocks PACAP PACAP-induced AP firing frequency (bottom right).PAC1R-shRNA prevents PACAP-dependent increases in AP firing. VMN cells do not respond to PACAP treatment (bottom left panel). Change of firing frequency following PACAPPAC1Rs blocks exogenous

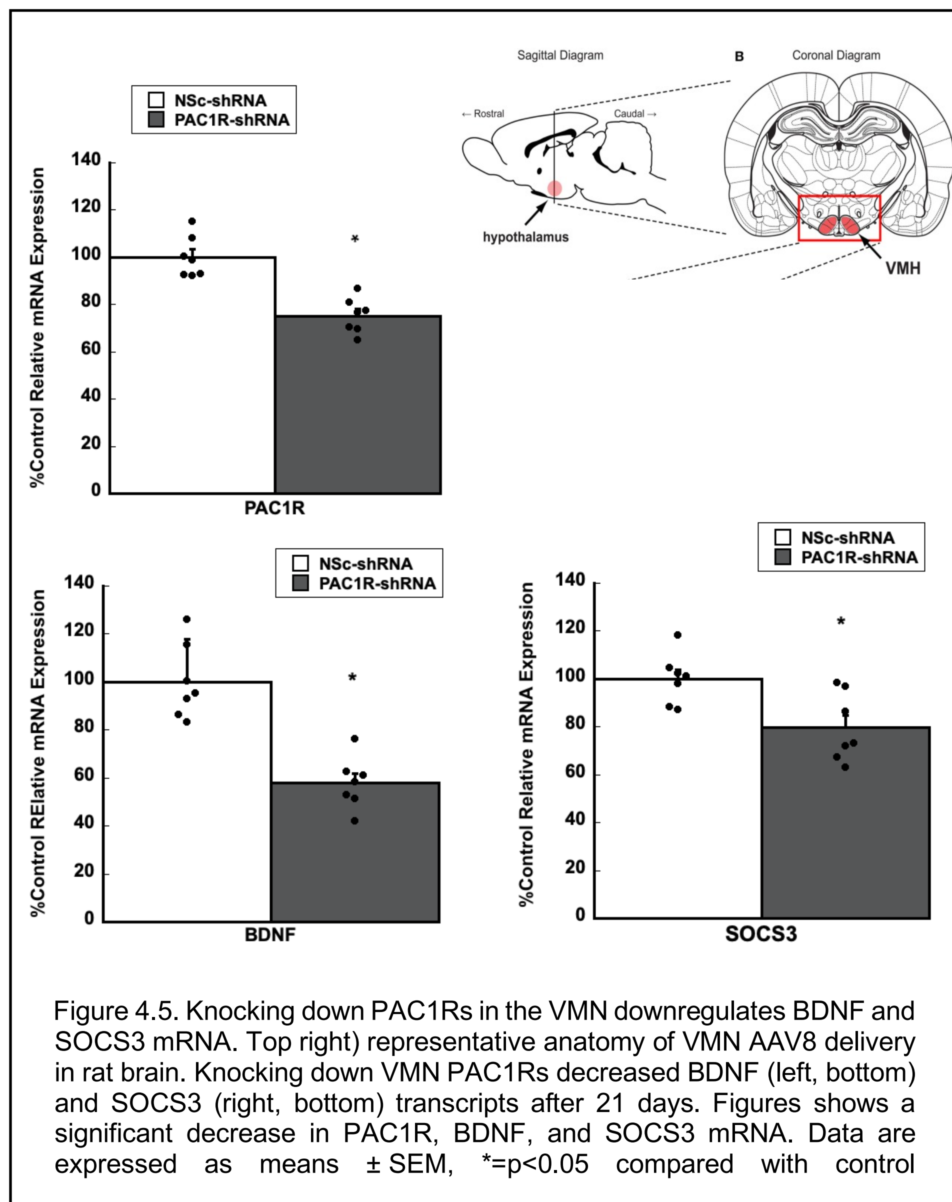


Figure 4.5. Knocking down PAC1Rs in the VMN downregulates BDNF and SOCS3 mRNA. Top right) representative anatomy of VMN AAV8 delivery in rat brain. Knocking down VMN PAC1Rs decreased BDNF (left, bottom) and SOCS3 (right, bottom) transcripts after 21 days. Figures shows a significant decrease in PAC1R, BDNF, and SOCS3 mRNA. Data are expressed as means ± SEM, *=p<0.05 compared with control

Discussion

In these experiments, we demonstrate that PACAP's type I receptor (PAC1R) signaling in the VMN is endogenously relevant for feeding and body weight control. shRNA-mediated PAC1R knockdown in the VMN, resulting in a 50-60% reduction in PAC1R protein expression, functionally increased daily food consumption and body weight gain. Specifically, diminishing PAC1R expression to this magnitude significantly decreased the number of meals while increasing the size of each meal (Table 1). This suggest that perhaps PACAP serves as a satiety signal working to counterbalance hunger inducing factors. As such, rats eat larger meals in the absence of endogenous PACAP signaling. This is consistent with our data in which the largest impact of PACAP-induced anorexia is observed almost exclusively in the dark phase when rats eat approximately 90% of their daily food intake. Moreover, the ability for exogenous PACAP administration to potently induce hypophagia and body weight loss was blocked following VMN PAC1R knockdown indicating that this magnitude of PAC1R knockdown $F_{(1,12)}=35.752$, $p<0.001$ was sufficient to disrupt endogenous VMN PACAP signaling. The effectiveness of inhibition is further supported by our ex vivo electrophysiology results showing that PACAP fails to increase action potential firing frequency in VMN cells expressing PAC1-shRNA. Thus, there is a clear role for endogenous PACAP signaling in the VMN for the control of feeding behavior and body weight regulation.

The changes in food consumption and body weight gain following VMN-specific PAC1R knockdown is similar to that observed in postnatal steroidogenic

factor-1 (SF1) knockout mice (Kim et al., 2011). In the CNS, not only is SF1 exclusively expressed in the VMN but VMN SF1 positive neurons co-express both PACAP and PAC1Rs (Gonçalves et al., 2021; Hurley et al., 2020; Kim et al., 2011). While VMN SF1 knockout animals exhibit dysregulated temperature regulation and locomotion, we did not evaluate the metabolic consequences of shRNA induced PAC1R knockdown. Although, we might predict that animals lacking VMN PAC1Rs would exhibit decreased spontaneous locomotion and core body temperature since the PAC1R antagonist in the VMN reverses exogenous PACAP's thermogenic and locomotor effects in addition to preventing hypophagia (Hurley et al., 2020; Resch et al., 2013), it is also possible that we would not detect any significant changes in core body temperature and locomotor activity based on our previous work illustrating that PACAP6-38 alone did not produce significant changes in these metabolic indices compared to control (Resch et al., 2013). Overall, there are salient similarities between our findings and those in mice with genetic mutations in PAC1R. Congenital PAC1R mutations result in phenotypes that can be replicated by local genetic or pharmacological PAC1R inhibition such as increased feeding suggesting that the hypothalamic VMN could be a primary control site (H. Hashimoto et al., 2000).

In addition to the overt behavioral and physiological responses to VMN PACAP manipulation, there are a cohort of PACAP regulated genes whose basal expression is significantly decrease following PAC1R knockdown and whose response to physiological challenge is altered in the absence of PAC1R expression (Fig. 4.5). These genes include brain derived neurotrophic factor

(BDNF) $F_{(1,12)}=35.033$, $p<0.001$, and suppressor of cytokine signaling 3 (SOCS3) $F_{(1,12)}=9.627$, $p=0.01$ (Hammack et al., 2009; Hurley et al., 2020; Macdonald et al., 2005; Resch et al., 2013; Yaka et al., 2003). BDNF and SOCS3 transcripts normally increase in response to PACAP. Our laboratory (Resch et al., 2011) and others (Macdonald et al., 2005; Yaka et al., 2003) have consistently shown that PACAP promotes BDNF transcription in multiple brain regions, therefore, a decrease in these genes following PAC1R knockdown suggest PACAP signaling is required for their expression. Earlier reports in mice lacking PAC1 receptors show reduced BDNF expression (Zink et al., 2004). The relationship between PACAP and BDNF may be significant since BDNF has been shown to amplify excitatory signaling as well as regulate energy balance (Crozier, Black, & Plummer, 1999; Martin & Finsterwald, 2011). Global BDNF deficient mice or regional deletion of BDNF in the VMN produces hyperphagia, weight gain, and metabolic dysregulation (C. Gao & Wolf, 2007). Moreover, BDNF expression in the VMN is the most abundant among hypothalamic cell groups (Xu et al., 2003). Specifically, these studies show that PAC1R-deficiency decreases expression of the exon-III and exon-IV transcripts (Zink et al., 2004). Interestingly, the promoter sequence of exon III contains cAMP response elements, which suggests the possibility of regulation by one of PACAP's signaling arms, the Gs/cAMP/PKA pathway (Yaka et al., 2003). To that end, PAC1R activation has been shown to increase CREB phosphorylation with concomitant enhancement of BDNF transcription (Solés-Tarrés, Cabezas-Llobet, Vaudry, & Xifró, 2020). Therefore, BDNF transcription may be a mechanism by which PACAP signaling regulates

energy homeostasis in the VMN. Alternatively, PACAP signaling could influence BDNF transcripts indirectly via NMDA receptor (NMDAR) signaling. Previous reports demonstrate that PACAP directly modulates NMDARs which, in turn, increase BDNF transcript following activation, see Vaudry et al for a review (Solés-Tarrés et al., 2020).

In addition to changes in BDNF mRNA expression, PACAP signaling in the VMN also increases SOCS3 transcription with concomitant decreased food intake and body weight (Hurley et al., 2020). Although we were not surprised that SOCS3 mRNA transcript significantly decreased in the absence of PACAP signaling $F_{(1,12)}=9.627$, $p=0.01$ (Fig. 4.5), we were surprised that low SOCS3 mRNA was accompanied by increased in food intake and body weight. SOCS3 deficiency in the mediobasal hypothalamus has been shown to reduce food intake and prevent weight gain (Matarazzo et al., 2012). Canonically, SOCS3 mRNA increases in response to leptin receptor activation as a mechanism to negatively regulate leptin signaling (Buchanan, Mahesh, Zamorano, & Brann, 1998; Hawke et al., 2009; Ronghua Yang & Barouch, 2007a). We would expect that endogenous leptin would chronically suppress feeding and weight gain when SOCS3 transcription is low as seen following PAC1R knockdown. One explanation could be that although PAC1 and leptin receptors have been shown to co-localize in a majority of VMN cells, there may be PAC1 and leptin receptors that do not reside in the same cell. Therefore, the SOCS3 mRNA transcript decrease observed may be accounted for by VMN cells expressing PAC1 receptors alone, and that in this cell population SOCS3 mRNA transcript might

not be necessary for leptin signaling and the comprehensive VMN-dependent changes in food intake. In chapter VIII, we present data that demonstrate that PAC1 and leptin receptors form an immunocomplex, therefore, it is possible that PAC1R knockdown may structurally disrupt the leptin receptor and render it unresponsive to their cognate ligand. In support of this idea, we administered leptin in the VMN following PAC1R knockdown and found leptin to no longer suppress food intake and decrease body weight. This could suggest that there are transcriptional and physical effects of PAC1R deficiency on the physiological function and behaviors of rats that may explain or reveal alternative mechanisms of action and functions of neuropeptides in energy homeostasis regulation. The results from these experiments showed two distinct and interesting outcomes: 1) PAC1R knockdown decreases expression of genes that tend to suppress food intake when their ligands are administered in the VMN, and 2) PACAP-dependent SOCS3 mRNA expression might have multiple explanations including independent cytokine receptor signaling in non-leptin receptor expressing cells or that leptin receptors physically depend on PACAP receptors to maintain functional integrity.

Collectively, the approximate 16% increase in feeding and 20% increase in body weight following PAC1R knockdown demonstrate that PACAP signaling in the VMN has a wide influence over energy balance. Additional studies will be required to better understand the relationship between leptin, PACAP, and BDNF in the VMN.

CHAPTER V

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTOR (PAC1R) ACTIVATION IN THE HYPOTHALAMIC VENTROMEDIAL NUCLEI DIFFERENTIALLY REGULATES DIURNAL HYPOPHAGIA AND METABOLISM BY RECRUITING PROTEIN KINASE A.

Introduction

Dysregulation of energy homeostasis causes obesity and health complications such as cancer and diabetes resulting in shortened life expectancy (reviewed in 11, 88). Understanding the mechanisms regulating energy homeostasis is key to treating obesity-related diseases. The central nervous system (CNS), and hypothalamic nuclei play indispensable roles in energy intake and energy expenditure regulation through the reciprocal transfer of neurotransmitter and neuropeptide signals to and from the peripheral milieu. The neuropeptide pituitary adenylate-cyclase activating polypeptide (PACAP) has emerged as an important and potent regulator of energy intake and expenditure in the peripheral and central nervous systems (Filatov et al., 2021; Harmar et al., 2012; Hurley et al., 2016; Maunze et al., 2022; Resch et al., 2013).

PACAP injections in hypothalamic ventromedial nuclei (VMN) decrease food intake and body weight including increases in thermogenesis and locomotion (Hawke et al., 2009; Resch et al., 2011, 2013). Although our laboratory has previously shown that PACAP’s effects in the VMN are mediated through the PAC1 receptors, the specific intracellular signaling mechanisms responsible for PACAP’s pleiotropic effects remain to be fully explored.

PAC1 receptors belong to the class B family of G-protein coupled receptors (GPCRs) that can couple to Gs/cAMP/PKA-related signaling and has been extensively reviewed (Denes, Geck, Mester, & Gabriel, 2019; Vaudry et al., 2009). Cyclic adenosine monophosphate AMP (cAMP) is generated from adenosine triphosphate (ATP) catalyzed by adenylyl cyclase (AC), which is activated by Gs-coupled GPCRs such as PAC1R. Previous studies have demonstrated that cyclic adenosine monophosphate (cAMP) and protein kinase A (cAMP/PKA) signaling cascades act downstream of G-protein coupled receptors to mediate neuropeptide regulation of feeding and metabolism involving GPCRs in the hypothalamus and VMN (London, Bloyd, & Stratakis, 2020; London, Nesterova, & Stratakis, 2017; Sheriff et al., 2003; L. Yang, 2018). For example, orexigenic peptides decrease cAMP/PKA whereas anorexigenic peptides like PACAP increase cAMP/PKA activity (Sheriff et al., 2003). Earlier studies demonstrate that hypothalamic cAMP/PKA activity and regulation of scheduled feeding share an inverse relationship; cAMP/PKA activity levels are low during the dark cycle, when rats normally eat, and peak during the light cycle, when rats eat little (Murakami & Takahashi, 1983; Sheriff et al., 2003; Valases, Wright, & Catravas, 1980). Moreover, administration of a membrane permeable cAMP agonist (Sp-cAMP) in the perifornical hypothalamus results in a significant increase in VMN PKA activity and suppression of food intake (Sheriff et al., 2003). Taken together, hypothalamic circadian regulation of cAMP/PKA activity appears to be correlated with the regulation of feeding. Although PAC1Rs are known to couple with Gαs/cAMP/PKA signaling, no study has directly assessed

the potential for PKA signaling to comprehensively mediate PACAP's pleiotropic effects on feeding and metabolism in the VMN. In the current study we examined if PKA was necessary for PACAP's pleiotropic functions in the VMN feeding and metabolism regulation. To that end, we blocked VMN PKA prior to PACAP injections and measured PACAP's hypophagic, thermogenic and locomotion effects during lights off and lights on.

Materials and Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. On experiment days, animals were weighed, and food intake measurements were collected using the BioDAQ Food Intake Monitor for up to 24-hours after the onset of each experiment. For BioDAQ meal pattern analysis the data were analyzed over the first 12 hours. 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; Resch et al., 2011, 2013). All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). 26-gauge stainless steel bilateral guide-cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted 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 sites 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 & Watson, 2007). Microinjectors 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 -3.3 mm below horizontal zero. Animals were given 2 mg/kg ketoprofen once post-surgery and allowed to recover from surgery for 1 week before they are included in experiments, during which time the animals were handled daily to acclimate them to the necessary physical manipulations during experiments. Correct cannulae placements were confirmed when possible at the conclusion of each experiment by microscopic examination of fluorescent protein markers and Nissl-stained sections. Image capture was conducted on a confocal microscope using 10X and 20X magnification (Nikon-confocal; Nyquist sampling) with Nikon NIS Elements software (Nikon, Melville, NY, USA). On average, the included studies produced

85% accurate stereotaxic placement. Only those with correct placement were included in the studies when experimental design allowed.

Microinjections

In all experiments, a microinjection pump was used to inject 0.25 μl/side of vehicle or treatment through bilateral guide-cannulae in awake animals over a 2-minute period. After injection delivery was complete, an additional minute elapsed before removing injectors to minimize backflow. Control and treatment groups were reversed after a two-day washout period and normal food intake levels were confirmed. Microinjections were completed approximately 30 minutes before lights off for experiments assessing nocturnal food intake. For experiments assessing core body temperature and locomotor activity and subsequent food intake, microinjections were initiated 2 hours after the onset of the lights on and lasted approximately for 1 hour.

Experiments

PKA Inhibitor: Dark phase food intake

Thirty minutes before the onset of lights off, microinjections of vehicle, and two doses of KT5720 (#1288/100U; protein kinase A inhibitor; 2.5nM/0.25μl/side and 10nM/0.25μl/side; Bio-Techne Corporation, Minneapolis, MN) were administered 15 minutes before rats (n=6/group) received a second bilateral injection of either saline containing 1% DMSO or (PACAP; 50pmol/0.25μl/side).

Approximately 5 minutes after injections, animals were returned to their home-cage and food intake was measured for the next 24 hours followed by a final measurement of body weight. KT5720 was prepared as DMSO stocks, diluted and injected in animals. The final concentration of DMSO was <1%. PACAP was prepared as a stock in 0.9% saline and diluted just before use.

PKA Inhibitor: Light phase food intake

Two hours after the onset of lights on, microinjections of vehicle, KT5720 (#1288/100U; protein kinase A inhibitor; 10nM/0.25μl/side; Bio-Techne Corporation, Minneapolis, MN) were administered 15 minutes before rats (n=6/group) received a second bilateral injection of either saline containing 1% DMSO or PACAP (50pmol/0.25μl/side). Approximately 5 minutes after injections, animals were returned to their home-cage. Food intake and body weight measures were taken at the beginning of the experiment and again 24 hours following pharmacological manipulations. KT5720 was prepared as DMSO stocks, diluted and injected in animals. The final concentration of DMSO was <1%. PACAP was prepared as a stock in 0.9% saline and diluted just before use.

PKA Inhibitor: Thermogenesis and spontaneous locomotor activity

At the time of cannulation surgery, telemetry probes (Mini-Mitter, Sunriver, OR) were implanted in the intraperitoneal cavity of rats to record core body temperature and spontaneous locomotor activity. On the experiment day, 2 hours after the onset of the light phase, rats (n=6/group) received bilateral injections of KT5720 (10nM/0.25μl/side) 10-15 minutes prior to an injection of either saline or PACAP (50pmol/0.25μl/side). Animals were returned to their home cage and telemetric data for core body temperature and spontaneous locomotor activity were collected remotely as previously described (Resch et al., 2011, 2013). In brief, spontaneous locomotor activity was collected remotely every 5 minutes and then summed to give cumulative activity every hour. Core body temperature data were averaged by the hour, and spontaneous locomotor activity data were summated to give cumulative activity over a specified amount of time.

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean.

Statistical analyses were performed using SigmaPlot 11 (Systat Software, Inc, San Jose, CA). P values less than 0.05 were considered statistically significant.

Results

PKA inhibitor (KT5720): ***Dark phase*** *PACAP and food intake*

Bilateral VMN injections of KT5720 prior to PACAP dose-dependently blocked PACAP-induced decrease in *dark phase* food intake (Fig. 5.1). Specifically, KT5720 (2.5nM and 10nM) injections significantly attenuated PACAP-induced hypophagia, $p = 0.006$ and $p < 0.001$ respectively. KT5720 doses of 2.5nM and 10nM alone did not significantly influence food intake when compared to saline controls (Fig. 5.1).

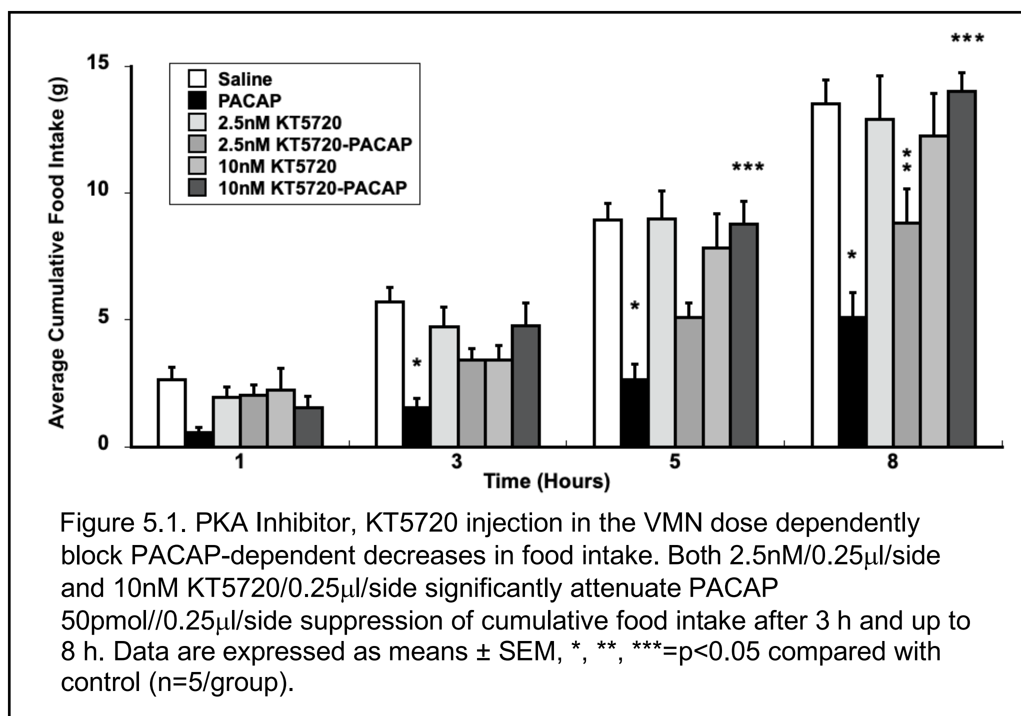


Figure 5.1. PKA Inhibitor, KT5720 injection in the VMN dose dependently block PACAP-dependent decreases in food intake. Both 2.5nM/0.25μl/side and 10nM KT5720/0.25μl/side significantly attenuate PACAP 50pmol//0.25μl/side suppression of cumulative food intake after 3 h and up to 8 h. Data are expressed as means ± SEM, *, **, ***=p<0.05 compared with control (n=5/group).

Bilateral VMN injections of KT5720 (10nM), a PKA-specific inhibitor just prior to PACAP injections attenuates PACAP-induced decreases in *dark phase* food intake for up to 8 hours and body weight at 24 hours compared to controls. Food intake analyses show a significant interaction of treatment x time ($F_{(33,264)}$ =9.820, p<0.001) and a significant main effect of treatment ($F_{(3,24)}$=20.536, p<0.001) when compared to saline controls (Fig. 5.2, Left). Significant inhibition of PACAP-induced hypophagia by KT5720 is observed by 5h and up to 8h p<0001(PACAP vs KT5720+PACAP), which was similar to feeding levels observed in animals that received saline or KT5720 alone (Fig. 5.2, Left).

Analyses of body weight change (Fig. 5.2, Right) revealed that pretreatment of KT5720 significantly attenuated but did not completely block PACAP-induced decreases in body weight (saline controls vs PACAP; $F_{(3,18)}$=40.996, p<0.001) and (PACAP vs KT5720+PACAP; p=0.009).

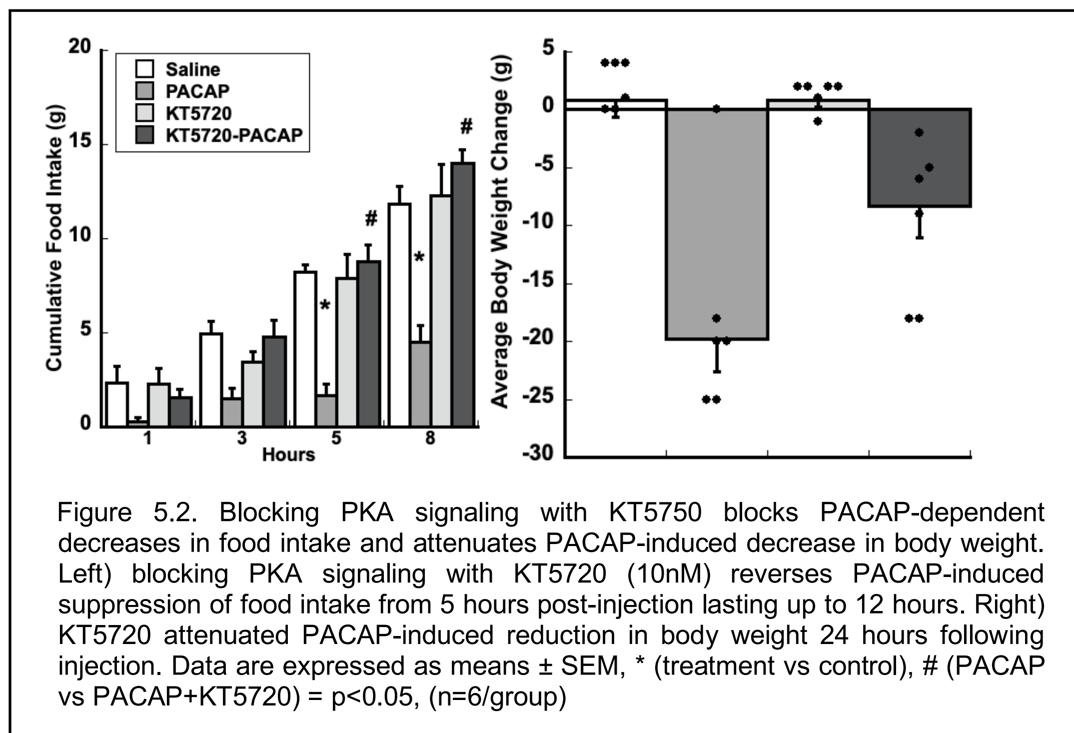


Figure 5.2. Blocking PKA signaling with KT5750 blocks PACAP-dependent decreases in food intake and attenuates PACAP-induced decrease in body weight. Left) blocking PKA signaling with KT5720 (10nM) reverses PACAP-induced suppression of food intake from 5 hours post-injection lasting up to 12 hours. Right) KT5720 attenuated PACAP-induced reduction in body weight 24 hours following injection. Data are expressed as means ± SEM, * (treatment vs control), # (PACAP vs PACAP+KT5720) = p<0.05, (n=6/group)

PKA inhibitor (KT5720): **Light phase** PACAP and hypophagia

Because it is still not clear whether endogenous PACAP exerts its action on VMN regulation of feeding and energy expenditure during the dark and/or **light phases**, we blocked PKA and measured PACAP-induced hypophagia during lights on. We found that an acute dose of PACAP during lights on does not influence food intake in ad lib fed animals over a 24 h period. This was not surprising because our previous results show that PACAP's hypophagic effects only last up to 8 h following an acute dose of PACAP (Maunze et al., 2022; Resch et al., 2011, 2013), and rats normally eat in the dark while consuming a mere 10% of their daily food intake during the light phase. The 10% of light phase food intake amount to approximately 2.5 g (data not shown). Interestingly, blocking PKA prior to PACAP during lights on seems to influence 24 h food intake (Fig. 5.3). One-way ANOVA of 24 h food intake data shows that blocking

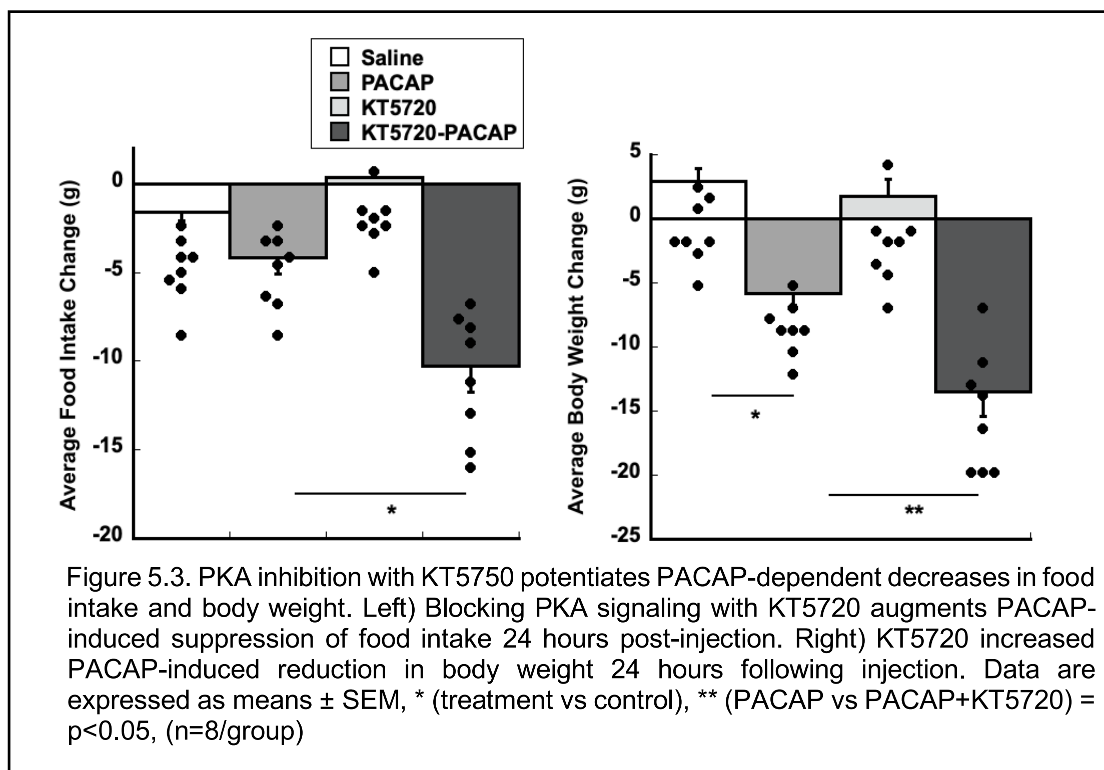


Figure 5.3. PKA inhibition with KT5750 potentiates PACAP-dependent decreases in food intake and body weight. Left) Blocking PKA signaling with KT5720 augments PACAP-induced suppression of food intake 24 hours post-injection. Right) KT5720 increased PACAP-induced reduction in body weight 24 hours following injection. Data are expressed as means ± SEM, * (treatment vs control), ** (PACAP vs PACAP+KT5720) = $p<0.05$, (n=8/group)

lights-on PKA activity significantly augmented PACAP-dependent decreases in 24 h feeding $F_{(3,28)}$ =18.88, p<0.001, and p <0.003 when compared to PACAP treatment alone (Fig. 5.4). PACAP treatment alone trends toward suppressing food intake although it was not significant. PACAP's effects on feeding are usually short-lived, lasting up to 8 hours (Maunze et al., 2022; Resch et al., 2011, 2013). Analysis of bodyweight change shows that blocking PKA during lights on potentiates PACAP induced decrease in body weight when compared to PACAP treatment alone ($F_{(3,28)}$ = 31.385, p<0.001).

PKA inhibitor (KT5720): Light phase PACAP and energy expenditure

Consequences of blocking PKA on PACAP-induced increases in core body temperature and spontaneous locomotor activity were assessed at the onset of the light phase when both are at their nadir. As previously shown (Resch et al., 2013), PACAP infusions in the VMN increase both core body temperature

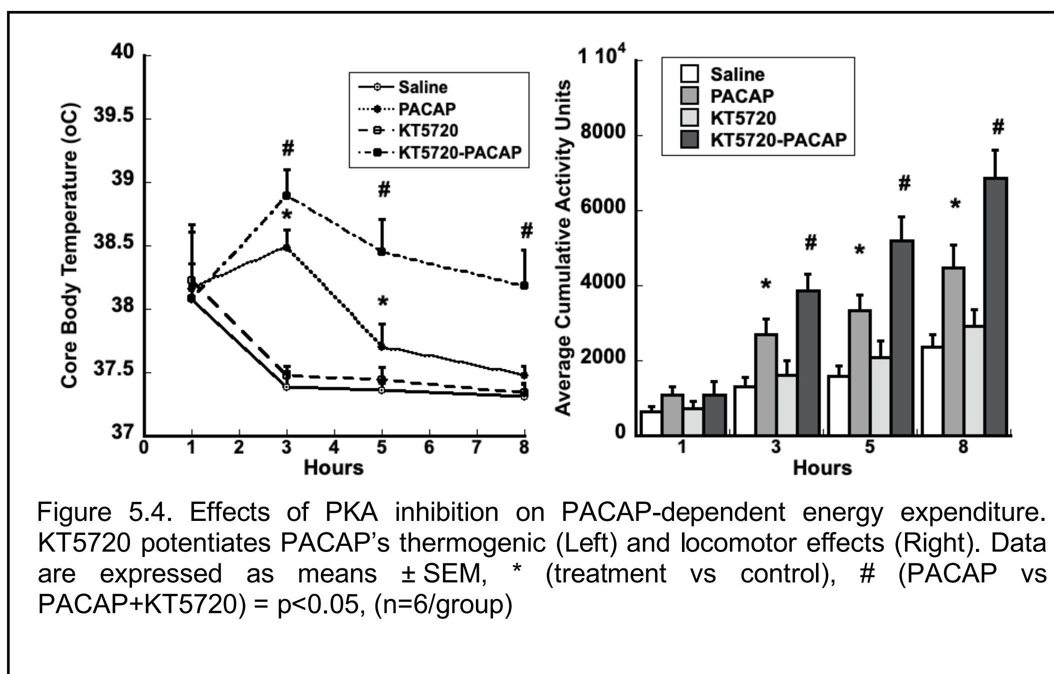


Figure 5.4. Effects of PKA inhibition on PACAP-dependent energy expenditure. KT5720 potentiates PACAP's thermogenic (Left) and locomotor effects (Right). Data are expressed as means ± SEM, * (treatment vs control), # (PACAP vs PACAP+KT5720) = p<0.05, (n=6/group)

and locomotor activity. However, blocking PKA activity with KT5720 prior to PACAP infusions did not suppress PACAP-induced increases in core body temperature or locomotor activity. Interestingly, blocking PKA activity with KT5720 appeared to potentiate PACAP-induced increases in both core body temperature and locomotor activity (Fig. 5.4, Left, Right). ANOVA of core body temperature and locomotor activity show a significant treatment x time interaction ($F_{(24,120)}$=13.527, $p<0.001$; $F_{(24,127)}$=10.152, $p<0.001$), respectively when compared to controls. Post-hoc analysis of treatment on core body temperature and locomotor activity following a two-way repeated measures assessment shows that blocking PKA prior to PACAP infusions potentiates both PACAP-induced increase in core body temperature and locomotor activity from 5h up to 8h $p<0.01$ (PACAP vs KT5720+PACAP; Figs. 5.4, Left, Right).

Discussion

It is now well established that PAC1Rs are GPCRs that couple to and stimulate Gs/cAMP/PKA-related signaling that influence various physiological systems (Blechman et al., 2013; Hardwick et al., 2017; Macdonald et al., 2005; Zhou et al., 2001). However, it is yet to be determined whether PACAP signaling through PKA might explain PACAP’s pleiotropic effects in the VMN as well as in the hypothalamus since cAMP and PKA activity are implicated in neuronal regulation of feeding and metabolism (Dorfman et al., 2017; London et al., 2017; Ross et al., 2008; Sheriff et al., 2003; L. Yang & McKnight, 2015).

In this study, we recapitulate previous observations that PACAP in the VMN reduces specifically nocturnal food consumption and subsequent decreases in body weight 24 hours following injection (Chang et al., 2021; Hawke et al., 2009; Resch et al., 2011, 2013, 2014) in addition to increasing core body temperature and spontaneous locomotor activity when administered in the VMN during the light phase (Adams et al., 2008; Resch et al., 2011). Presently, we demonstrate that directly inhibiting PKA had significant effects on PACAP signaling in the VMN. PKA inhibition at the onset of the dark phase completely blocked PACAP-induced hypophagia, whereas PKA inhibition during lights on had no influence on PACAP's actions on feeding. This is not surprising considering that rats only consume 10% of their food during the light period. By contrast, PACAP injections during the light phase seems able to influence feeding occurring later in the dark phase following PKA inhibition (Fig 5.4).

It is worthwhile to note that, during the light phase, PACAP injections alone in the VMN do not appear to significantly affect 24 h food consumption, perhaps because feeding levels are at their lowest point and PACAP's effects have probably warn off. However, blocking PKA prior to PACAP during the light phase seems to extend PACAP's action into the dark phase and influence overall nocturnal feeding levels. This is not surprising as studies show that cAMP and PKA levels change throughout the day as energy demand changes potentially impacting other orexigenic and anorexigenic peptide systems that utilize PKA in the VMN. Interestingly, inhibiting PKA appears to facilitate PACAP-induced thermogenesis and locomotor activity during lights on, suggesting that PACAP

dependency on PKA signaling is behavior specific. Studies in other brain regions show that leptin dependent signaling blocks PKA activity as a mechanisms to prevent transcription of SOCS3, a negative regulator of leptin signaling (L. Yang & McKnight, 2015). VMN cells express leptin receptors and respond to leptin by inducing hyperthermia (Hawke et al., 2009; Hurley et al., 2020). Blocking PKA alone had no effects on feeding or energy expenditure, however, there is a possibility that PKA inhibition may be influencing other peptide systems that may impact metabolism. Alternatively, we cannot rule out the fact that PACAP signaling may still be occurring through the Gq/PLC/PKC arm of PAC1R. While we can be more certain of the signaling mechanisms PACAP employs when we can demonstrate they are discretely inhibited by our chemical interventions, we are less confident when our treatment facilitates PACAP's effects. Such is the case with the enhanced hypophagic, thermogenic and locomotor activity responses to the light phase administration of the PKA inhibitor and PACAP. In this case, the observed effects on food consumption, core body temperature and locomotor activity following inhibition of PKA signaling may or may not involve PACAP. One possible alternative explanation is that adenosine monophosphate-activated protein kinase (AMPK) may be driving enhanced energy expenditure. VMN AMPK inhibition suppresses appetite and enhances energy expenditure (J. Wang et al., 2020). Interestingly, PKA regulates AMPK's activity by decreasing AMPK phosphorylation at threonine residue 172 (Thr172), a residue required for its activation (Przygrodzka et al., 2021). This suggests that in the absence of PKA, AMPK is inactive thereby enhancing energy expenditure.

This combination of both positive and negative influences of PKA on energy regulation creates a conundrum as to when PACAP might engage PKA. PKA inhibition influences the directionality of PACAP's hypophagic effects depending on the time of day. In the hypothalamus, cAMP/PKA is a second messenger target of several neuropeptides and hormones such as NPY and has been shown to influence leptin signaling, both of which have been implicated in the neuronal regulation of feeding and metabolism (Sheriff et al., 2003; L. Yang & McKnight, 2015). Earlier reports find that cAMP and PKA activity increase in the hypothalamus including the VMN when animals are fasting and asleep whereas, cAMP and PKA levels diminish during nocturnal feeding when rats are active (Murakami & Takahashi, 1983; Sheriff et al., 2003; Valases et al., 1980). In VMN cells, genetic ablation of CREB binding protein, a downstream target of PKA binding protein causes hyperphagia, and thermogenic dysregulation with concomitant decreases in BDNF and POMC mRNA (L. Yang & McKnight, 2015).

The selectivity of PKA involvement suggests that other signaling mechanisms may be primary for the metabolic features of PACAP signaling that contribute to its overall effects on body weight. This raises important future questions of how downstream PAC1R signaling differentially regulates indices of homeostasis. In the hypothalamic arcuate nuclei for example, impaired PKA signaling exaggerates leptin's catabolic and molecular effects (L. Yang & McKnight, 2015). In the absence of functional PKA, low leptin doses potently decrease feeding with concomitant increases in energy expenditure suggesting there exists a PKA-regulating signaling system that may dictate the magnitude of

leptin's influence on metabolism (L. Yang & McKnight, 2015). Considering that accumulating evidence in the VMN is establishing a relationship between PAC1 and leptin receptor signaling, it is possible that VMN PKA may be involved in the gating of leptin signaling although this remains to be tested (Hawke et al., 2009; Hurley et al., 2020).

The failure of PKA inhibition to only attenuate nocturnal PACAP-induced body weight loss may suggest that signaling mechanisms independent of PKA inhibition continue to engage metabolic systems and reduce body weight following PACAP administration. Taken together, these findings position PKA as a key signaling cascade in the VMN regulation of energy balance. Further experiments that combine mechanisms to inhibit PACAP signaling (PACAP6-38 or PAC1 shRNA) would need to be incorporated into similar studies to determine whether our results are solely related to VMN PACAP signaling.

CHAPTER VI

VENTROMEDIAL NUCLEI OF THE HYPOTHALAMUS UNIQUELY ENGAGE PROTEIN KINASE C TO REGULATE FEEDING AND ENERGY EXPENDITURE

Introduction

The imbalance between energy intake and energy expenditure leads to obesity and health complications such as diabetes and cancer (Nijhawan, Behl, & Arora, 2020; Roh, Song, & Kim, 2016; Timper & Brüning, 2017). The pursuit and discovery of therapeutic targets for treating energy homeostasis-related ailments will alleviate a wide array of diseases while also improving the quality of life. A prominent role is emerging for pituitary adenylate cyclase-activating polypeptide (PACAP) in the regulation of energy homeostasis. PACAP and its receptors are highly expressed in the peripheral and central nervous system where they potently regulate food intake and energy expenditure (Gottschall et al., 1990; Resch et al., 2011, 2013). This positions PACAP signaling to regulate multiple aspects of energy homeostasis throughout the body.

In the hypothalamic ventromedial nuclei (VMN), PACAP suppresses food intake and increases energy expenditure (Maunze et al., 2022; Resch et al., 2011, 2013). PACAP's hypophagic and thermogenic effects are mediated through its PAC1 receptor, which belongs to the class B family of G-protein coupled receptors (GPCRs). PAC1 receptors can dually couple to Gs/cAMP/PKA and Gq/PLC/PKC to regulate a plethora of cell signaling mechanisms implicated in energy homeostasis (Murakami & Takahashi, 1983; Planas, Cummings,

Idzerda, & McKnight, 1999; Ross et al., 2008). As described earlier, the PAC1 receptor gene (ADCYAP1) transcript is highly spliced to generate PAC1 receptors isoforms, which adds an additional layer of complexity to PACAP signaling (Blechman et al., 2013). These PAC1R isoforms display different efficacies and potency to phospholipase C (PLC), an inducer of PKC. A link between PKC signaling has now been recognized in peripheral and central control of different aspects of energy homeostasis (Dewing, Christensen, Bondar, & Micevych, 2008a; Z. Gao et al., 2007; Nijhawan et al., 2020; Raddatz et al., 2011; Ross et al., 2008; Luoying Zhang et al., 2012). However, despite the wealth of information on PKC mediated control of peripheral and central control of energy homeostasis, the understanding of PKC's contributions on PACAP's influence in feeding and energy expenditure remains limited. The pleiotropic effects of peripheral and central PKC regulation of energy homeostasis make this family of kinases a potential contributor to PACAP's regulation of multiple aspects of energy homeostasis.

Protein kinase C is a family of serine/threonine kinases that belong to the cAMP-dependent, cGMP, and protein kinase C superfamily of protein kinases involved in numerous signaling processes important for cell physiology (Farese, Sajan, & Haley, 2010; Mehta, 2014). The PKC family is composed of different lipid-dependent kinases that activate in response to diacylglycerol (DAG) and calcium (Dewing, Christensen, Bondar, & Micevych, 2008b; Kolczynska, Loza-Valdes, Hawro, & Sumara, 2020). These include the classical conventional PKCs (PKCα, PKCβII, PKCβII, and PKCγ) and the novel PKCs (PKCδ, PKCε, PKCθ,

and PKCη). Additionally, there is the atypical PKC (PKCζ, and PKCι/λ) whose function are regulated by protein-protein interactions (Black & Black, 2012).

Despite limitations in our knowledge of specific PKC isozyme contributions to PACAP's role in energy homeostasis, we examined the broad role of PKCs in PACAP's role in the VMN. We used a broad PKC inhibitor, GF109203X to examine PKC contributions to PACAP-induced hypophagia and energy expenditure. We found that broad PKC inhibition in awake male Sprague Dawley rats attenuates PACAP's hypophagic and metabolism effects during the dark phase whereas these effects are potentiated when PKC is inhibited during the lights phase. Assessment of energy expenditure during lights on showed that PKC inhibition potentiated only PACAP's thermogenic and not spontaneous locomotion effects.

Materials and methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. On experiment days, animals were weighed, and food intake measurements were collected using the BioDAQ

Food Intake Monitor before and up to 24-hours after the onset of the experiments. For BioDAQ meal pattern analysis the data were analyzed over the first 12 hours. 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; Resch et al., 2011, 2013). All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). 26-gauge stainless steel bilateral guide-cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted 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 sites 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 & Watson, 2007). Microinjectors 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 -3.3 mm below horizontal zero. Animals were given 2 mg/kg ketoprofen once post-surgery and allowed to recover from surgery for 1 week before they are included in experiments, during which time the animals were handled daily to acclimate them to the necessary

physical manipulations during experiments. Correct cannulae placements were confirmed by microscopic examination of Nissl-stained sections.

Microinjections

In all experiments, a microinjection pump was used to inject 0.25 μl/side of vehicle or treatment through bilateral guide-cannulae in awake animals over a 2-minute period. After injection delivery was complete, an additional minute elapsed before removing injectors to minimize backflow. Control and treatment groups were reversed after a two-day washout period and normal food intake levels were confirmed. Microinjections were completed approximately 30 minutes before lights off for experiments assessing nocturnal food intake. For experiments assessing core body temperature and locomotor activity and subsequent food intake, microinjections were initiated 2 hours after the onset of the lights on and lasted approximately for 1 hour.

Experiments

*PKC Inhibitor: **Dark phase** food intake*

Thirty minutes before the onset of lights off, microinjections of vehicle or protein kinase C inhibitor (GF109203X; 0.1mM/0.25μl/side) were administered 15 minutes before rats (n=7/group) received a second bilateral injection of either saline containing 1% DMSO or PACAP (50pmol/0.25μl/side). Approximately 5 minutes after injections, animals were returned to their home-cage and food

intake was measured for the next 24 hours followed by a final measurement of body weight. GF109203X was prepared as DMSO stocks, diluted and injected in animals. The final concentration of DMSO was <1%. PACAP was prepared as a stock in 0.9% saline and diluted just before use.

PKC Inhibitor: ***Light phase*** food intake

Two hours after the onset of lights on, microinjections of vehicle, GF109203X (#0741/1; protein kinase C inhibitor; 0.1mM/0.25µl/side; Bio-Techne Corporation, Minneapolis, MN), were administered 15 minutes before rats (n=7/group) received a second bilateral injection of either saline containing 1% DMSO or PACAP (50pmol/0.25µl/side). Approximately 5 minutes after injections, animals were returned to their home-cage. Food intake and body weight measures were taken at the beginning of the experiment and again 24 hours following pharmacological manipulations. GF109203X was prepared as DMSO stocks, diluted and injected in animals. The final concentration of DMSO was <1%. PACAP was prepared as a stock in 0.9% saline and diluted just before use.

PKC Inhibitor: Light phase PACAP, temperature and activity

At the time of cannulation surgery, telemetry probes (Mini-Mitter, Sunriver, OR) were implanted in the intraperitoneal cavity of rats to record core body temperature and spontaneous locomotor activity. On the experiment day, 2 hours after the onset of the lights on cycle, rats (n=6/group) received bilateral injections of GF109203X (0.1mM/0.25µl/side) 10-15 minutes prior to an injection of either

saline or PACAP (50pmol/0.25µl/side). Animals were returned to their home cage and telemetric data for core body temperature and spontaneous locomotor activity were collected remotely as previously described (Resch et al., 2011, 2013). In brief, spontaneous locomotor activity was collected remotely every 5 minutes and then summed to give cumulative activity every hour. Core body temperature data were averaged by the hour, and spontaneous locomotor activity data were summated to give cumulative activity over a specified amount of time.

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean. Statistical analyses were performed using SigmaPlot 11 (Systat Software, Inc, San Jose, CA). P values less than 0.05 were considered statistically significant.

Results

PKC inhibitor (GF109203X): ***Dark phase*** *PACAP and food intake*

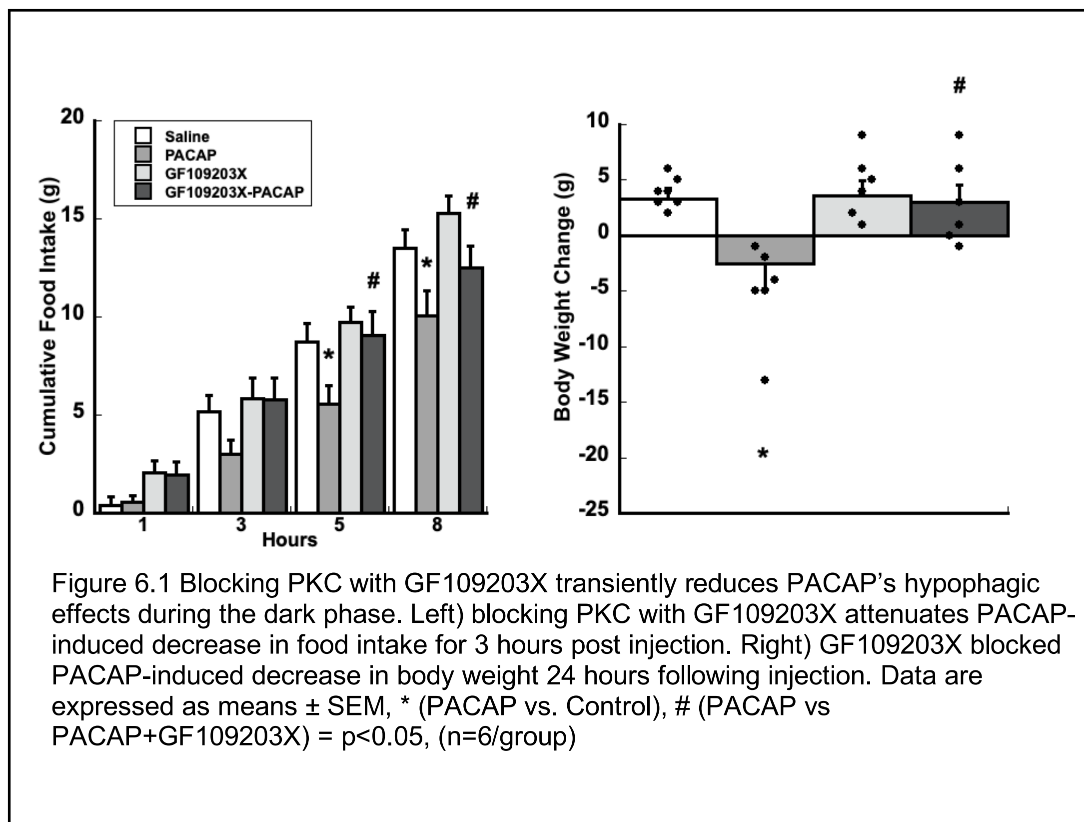


Figure 6.1 Blocking PKC with GF109203X transiently reduces PACAP's hypophagic effects during the dark phase. Left) blocking PKC with GF109203X attenuates PACAP-induced decrease in food intake for 3 hours post injection. Right) GF109203X blocked PACAP-induced decrease in body weight 24 hours following injection. Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+GF109203X) = $p<0.05$, (n=6/group)

Activation of PKC is a downstream response of PAC1R activation through the Gαq /PLC pathway. Direct injections of GF109203X, a PKC-specific inhibitor in the VMN, preceding PACAP injections significantly reversed PACAP's anorexigenic effects in rats during the first eight hours of the dark phase when rats normally eat. Food intake data show a significant interaction between treatment and time ($F_{(33,231)} = 2.242$, $p<0.001$), whereas body weight data reveal a significant main effect of treatment ($F_{(3,21)}=3.821$, $p<0.03$; Figs. Left, Right).

Post hoc analyses show that PACAP's capacity to suppress food intake and decrease body weight is dependent on time. PACAP significantly inhibits food intake from 5h ($p=0.028$) up to 8h ($p=0.016$) and decreases body weight after 24h ($p=0.019$) respectively when compared to saline controls (Figs. 6.1, Left, Right). PACAP's hypophagic and anorexic effects are reversed by GF109203X at 5h ($p<0.015$), 8h ($p<0.019$) and 24h ($p=0.023$) respectively when compared to PACAP alone (PACAP vs GF109203X+PACAP; Figs.6.1 Left, Right). However, after five hours PACAP's hypophagic actions persisted and food intake begins to decrease although not significantly different from saline controls. This suggests that PACAP-dependent regulation of feeding and body weight in the VMN likely involves induction of PKC-related signaling.

PKC inhibitor (GF109203X): ***Light phase*** *PACAP and food intake*

Several organisms including rodents exhibit circadian rhythms in a myriad of cellular, physiological and behavioral processes (Luoying Zhang et al., 2012). PKC has been shown to influence food entrainment and overall activity in rodents (Luoying Zhang et al., 2012). Because it is still not clear what time of day PACAP exerts its action on VMN regulation of feeding and energy expenditure, we blocked PKC and measured PACAP-induced hypophagia during the lights phase. Light phase PKC inhibition facilitates PACAP-dependent decreases in food intake occurring during the dark phase, and not during the light phase. One-way ANOVA of food intake data shows that blocking PKC significantly intensified PACAP-dependent decreases of food intake $F_{(3,26)}=4.36$, $p=0.020$, when

compared to PACAP treatment alone. PKC inhibition alone did not affect food intake or body weight. Analysis of bodyweight change shows that GF109203X injections also seem to extend PACAP induced decrease in body weight when administered during the light phase. This is opposite to what we observed when PKC inhibition during the dark phase reversed PACAP-dependent decreases in body weight $_{(3,26)}$ = 16.89, p<0.001 when compared to PACAP treatment alone. PACAP treatment alone during lights on did not significantly influence body weight loss.

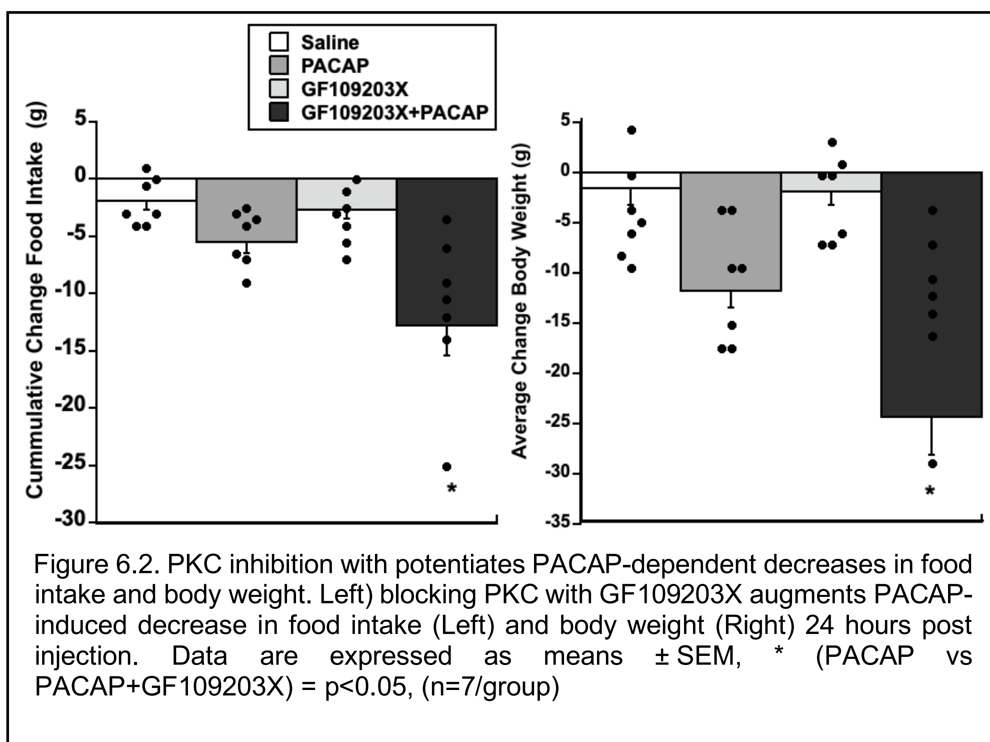


Figure 6.2. PKC inhibition with potentiates PACAP-dependent decreases in food intake and body weight. Left) blocking PKC with GF109203X augments PACAP-induced decrease in food intake (Left) and body weight (Right) 24 hours post injection. Data are expressed as means ± SEM, * (PACAP vs PACAP+GF109203X) = p<0.05, (n=7/group)

PKC inhibitor (GF109203X): Light phase PACAP and energy expenditure

During the light phase, however, when rodent core body temperature and locomotor activity are at their nadir, GF109203X injections in the VMN prior to PACAP injections were not sufficient to prevent PACAP-induced increases in temperature and locomotor activity. In fact, GF109203X-mediated inhibition of PKC enhanced PACAP-induced increase in core body temperature. Core body temperature showed a significant interaction between treatment and time ($F_{(24,96)}=3.405$, $p<0.001$.) Post hoc pairwise comparisons show that PACAP increased both core body temperature and locomotor activity from 3h lasting up to 8h post injection $p<0.03$ when compared to saline controls. GF109203X enhanced PACAP's potentiation of core body temperature at 3h but not locomotor activity $p<0.25$ when compared PACAP treatment alone (Figs.6.3 Left, Right).

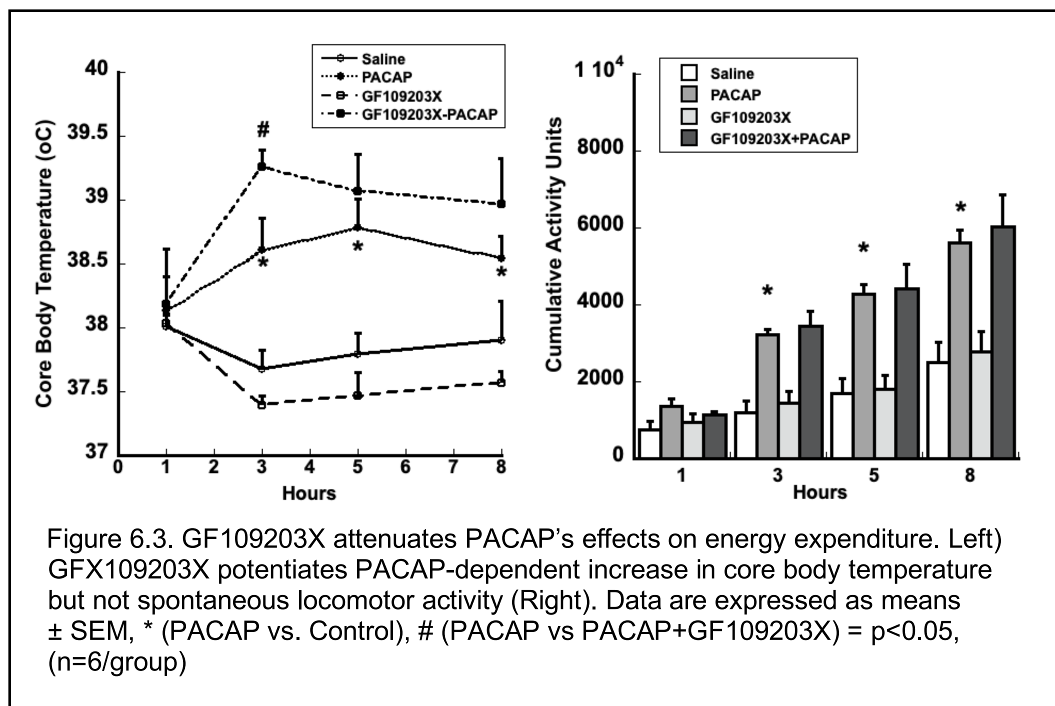


Figure 6.3. GF109203X attenuates PACAP's effects on energy expenditure. Left) GFX109203X potentiates PACAP-dependent increase in core body temperature but not spontaneous locomotor activity (Right). Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+GF109203X) = p<0.05, (n=6/group)

Discussion

It is now well recognized that PAC1Rs couple to Gq/PLC/PKC second messenger signaling, however, the direct consequences of this signaling on VMN regulation of food intake and metabolism remains to be thoroughly investigated. Our data suggest that PKC plays an important role in VMN PACAP-mediated energy balance. We found that PKC is required for PACAP signaling in the VMN since PKC inhibition abrogated nocturnal PACAP-induced hypophagia whereas, it facilitated PACAP-stimulated hypophagia when injected during the light phase. The effects of PKC inhibition on PACAP's effect during the dark phase suggest that the PAC1R arms that remain intact in the absence of PKC signaling are insufficient to regulate feeding and body weight alone. What is interesting is that the effects of PKC inhibition during the dark phase were short-lived suggesting that either GF109203X had worn off, or other PAC1R signaling may have taken over to suppress feeding. For example, we recently showed that PACAP signaling through Gs/cAMP/PKA was also required for PACAP's hypophagic effects. This suggest that PACAP signaling may coordinate these two kinases PKA and PKA to regulate VMN control of hypophagia. PKC activity also plays an important role in PACAP's role in energy expenditure. PKC inhibition potentiated PACAP's thermogenic effects and had no effect on PACAP-induced locomotor activity. Previous studies have shown that VMN PKC is involved in regulating other physiological and behavioral outcomes in rodents including sexual behaviors and glucose metabolism (Dewing et al., 2008a; Irani et al., 2010; Ross

et al., 2008; Thaler et al., 2009). It cannot be overlooked that PAC1R signaling may also utilize PKC activity to regulate energy balance. One recent study showed that isoforms of PKC are highly expressed in hypothalamic nuclei including the VMN, where they have been shown to influence feeding and metabolism in response to leptin and insulin (Thaler et al., 2009). Interestingly, a recent study demonstrated that POMC-neuron-specific deletion of PKCλ, a PKC isoform disrupts leptin signaling and renders high fat diet fed mice susceptible to obesity (Dorfman et al., 2017). VMN neurons also regulate glucose and lipid metabolism and are significant targets of PACAP and leptin signaling (Flak et al., 2020; Ross et al., 2008). In addition, emerging evidence suggest a dependent relationship between PAC1 and leptin receptor signaling, positioning PKC as a possible link. Taken together, however, these findings suggest a putative role for VMN PACAP receptors to engage PKC-related signaling to regulate different aspects of energy homeostasis. A complete understanding of PKC's contribution to PACAP signaling in VMN control of energy homeostasis will require examining the specific contributions of individual PKC isozymes. Findings from this and future studies may aid our understanding of the etiologies of energy imbalances and design of nuanced therapies with minimal side effects.

CHAPTER VII

PACAP'S HYPOHAGIC EFFECTS IN THE VMN ARE MEDIATED THROUGH PAC1 RECEPTOR TRAFFICKING

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional 38 amino-acid peptide that regulates a number of physiological responses including energy intake and energy expenditure (Hurley et al., 2016; Miyata et al., 1989; Resch et al., 2013) by binding to PAC1 receptors (PAC1R) in the hypothalamic ventromedial nuclei (VMN) with high affinity (Hawke et al., 2009; Resch et al., 2013). Identifying which specific PAC1R-mediated second messenger signaling is activated or whether PACAP-induced PAC1R endocytosis in the VMN affect feeding and metabolism are still unknown. The PACAP-preferring PAC1 receptor is a G-protein coupled receptor transcribed from the ADCYAP1R1 gene (Gottschall et al., 1990; H. Hashimoto et al., 1993; Miyata et al., 1990). The transcribed PAC1R protein possesses amino acid motifs within the C-terminal domain that determine the ability not only for G-protein signal transduction, but also for receptor trafficking (Lyu et al., 2000).

G-protein coupled receptors (GPCRs) are cell surface transmembrane proteins in eukaryotes that transduce and integrate a broad range of cell signaling and physiological processes (Eichel & Von Zastrow, 2018). GPCRs interact with multiple proteins, some that facilitate receptor coupling to

heterotrimeric G-proteins while others attenuate receptor signaling. It has emerged recently that in addition to plasma membrane signaling, PAC1 receptors are endocytosed and trafficked as part of several necessary processes that are integral to neuronal action potential generation (May & Parsons, 2017). Canonically, GPCRs are serine/threonine (Ser/Thr)-phosphorylated at specific intracellular motifs by GPCR kinases following activation (Ferguson, 2007). Subsequently, this phosphorylation facilitates the recruitment of endocytosis machinery, and signaling molecules such as kinases, small GTPases, and β-arrestins (Hupfeld & Olefsky, 2007). β-arrestin 1 and β-arrestin 2 are some of the key components in the mechanisms regulating G-protein coupled receptor signaling termination and trafficking. In addition to GPCR desensitization and endocytosis, β-arrestins' recruitment to phosphorylated GPCRs promotes non-canonical signaling (Broca et al., 2009; DeWire et al., 2007). Recent findings demonstrate that PACAP-stimulation of PAC1Rs induces PAC1R association with β-arrestins 1 and 2 leading to differential β-arrestin 1 or β-arrestin 2 subcellular co-localization with PAC1R (Shintani et al., 2018). PAC1Rs associated with β-arrestin 1 localize to the plasma membrane, whereas PAC1R associated with β-arrestin 2 appear to be internalized into the cytosol (Shintani et al., 2018). Earlier studies show that β-arrestins influence energy homeostasis and prevent diet induced obesity suggesting a putative mechanism by which PACAP signaling may influence energy homeostasis (Pydi et al., 2020; Rodgers & Puigserver, 2009; Zhuang et al., 2011).

In the current work, we examined PACAP-induced changes in PAC1R association with β-arrestin, PAC1R subcellular localization, and the consequences of blocking PACAP-induced PAC1R trafficking on VMN control of food intake. Our data show that VMN PAC1 receptors co-immunoprecipitate with β-arrestin 2, and PACAP injections in the VMN dose-dependently increase PAC1R association with β-arrestin 2. To demonstrate functional trafficking of PAC1 receptors, we tested whether VMN PACAP ligand binding leads to a reduction in PAC1R expression in the plasma membrane while increasing PAC1R expression in the cytosol, which can be blocked using endocytosis inhibitors Pitstop 2 and Dyngo 4a. Furthermore, blocking endocytosis by targeting clathrin and dynamin protein in the VMN prior to PACAP injection prevented PACAP-induced decreases in food intake, whereas blocking β-arrestin association with adaptor-protein 2 with Barbadin does not.

Materials and Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. On experiment days, animals were weighed, and food intake measurements were collected using the BioDAQ

Food Intake Monitor before and up to 24-hours after the onset of the experiments. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with 2% isoflurane using a SomnoSuite low-flow anesthesia system (Kent Scientific, Torrington, CT) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). 26-gauge stainless steel bilateral guide-cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted 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 sites 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 & Watson, 2007). Microinjectors 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 -3.3 mm below horizontal zero. Animals were given 2 mg/kg ketoprofen once post-surgery and allowed to recover from surgery for 1 week before they are included in experiments, during which time the animals were handled daily to acclimate them to the necessary physical manipulations during experiments.

Microinjections

In all experiments, a microinjection pump was used to inject 0.25 µl/side of vehicle or treatment through bilateral guide-cannulae in awake animals over a 2-minute period. After injection delivery was complete, an additional minute elapsed before removing injectors to minimize backflow. Control and treatment groups were reversed after a two-day washout period and normal food intake levels were confirmed. Microinjections were completed approximately 30 minutes before lights off for experiments assessing nocturnal food intake.

Western Blot analysis

Protein analysis using western blot was conducted once for each animal in the study. Bilateral dissections of the ventromedial hypothalamus (VMH; including the VMN and surrounding areas) were collected following rapid decapitation. VMH tissue was homogenized by hand (10 strokes) 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 (#78447; Thermo Scientific; Rockford, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4°C and the resulting supernatant was further centrifuged at 10,000 X g for 30 minutes at 4°C to remove crude membrane protein. The resulting supernatant rich in crude cytoplasmic protein was saved for further processing. Protein quantification of samples was determined using a bicinchoninic (BCA) assay (#23252; Pierce). Protein (30 µg) was run on an 8% gel by SDS-PAGE and transferred to a

polyvinylidene fluoride (PVDF) membrane (#LC2002; Thermo Fisher Scientific; Rockford, IL). Membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) or 5% non-fat milk in tris-buffered saline containing 0.1 % Tween-20 (TBS-T). Blots were then probed with either rabbit anti-PAC1R antibody alone (#AV-R003; 1:1000; Alomone labs; Jerusalem, Israel) or dually probed with β-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) overnight at 4°C, followed by washes with TBS-T and incubation with an HRP-linked anti-rabbit secondary antibody (#7074P2; 1:3000; Cell Signaling Technologies; Danvers, MA) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA) at room temperature for 2 hours. Band intensities were developed using SuperSignal West Atto Chemiluminescent Substrate (#A38556; Thermo Scientific; Rockford, IL) and visualized using the Odyssey Fc Dual Mode Imaging System (LI-COR; Lincoln, NE). Band densities were measured and quantified using Odyssey Fc Dual-Mode Imaging System software. PAC1R antibody specificity was validated by preincubating rabbit anti-PAC1R with PAC1R blocking peptide (#BLP-VR003; Alomone labs; Jerusalem, Israel). Following visualization of PAC1R signal alone, blots were stripped and re-probed in an identical fashion for β-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA).

Crosslinking and immunoprecipitation

VMH tissue was homogenized by hand (10 strokes) in ice-cold immunoprecipitation lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 10 μM CaCl2, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mM AEBSF, 1% Triton X-100) containing Halt protease and phosphatase inhibitor cocktail (#78447; Thermo Scientific; Rockford, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4°C and the resulting whole-cell lysate supernatant was saved for further processing. Protein quantification of samples was determined using a bicinchoninic (BCA) assay (#23252; Pierce). Chemical crosslinking and co-immunoprecipitation for quantitating PACAP-induced association of PAC1 and β-arrestin1 or β-arrestin2, covalent protein cross-linking with a chemical crosslinker, Dithiobis succinimidylpropionate (DSP; Thermo Fisher Scientific). 100 mg/sample VMN whole-cell lysate, crude membrane, and cytosolic fractions were incubated with 2.5 mM DSP for 30 mins at room temperature and the crosslinking reaction was quenched with 0.1 ml of1 M Tris (pH 7.5). To prevent co-elution of IgG fragments, the immobilized antibodies were covalently cross-linked to protein G-Sepharose beads. Antibody-coupled protein G-Sepharose beads were resuspended in 5 mM BS3 (Thermo Fisher Scientific) and incubated for 30 min at room temperature. Crosslinking was quenched by the addition of 0.1 ml of1 M Tris (pH 7.5). VMN lysates were incubated with either PAC1R antibody (1:500), β-arrestin1-, or β-arrestin2-coupled protein G-Sepharose beads for 2 h at 4˚C while shaking. Following incubation, the beads were collected by magnet and

washed three times with wash buffer (0.1M phosphate buffered saline). Proteins bound to the beads were eluted and the crosslink was reversed with 2X Sample buffer containing 3% (v/v) 2-mercaptoethanol and analyzed by performing immunoblotting with specific antibodies as indicated.

Experiments

PACAP induces PAC1R co-immunoprecipitation with β-arrestin

Pituitary adenylate cyclase-activating polypeptide (PACAP 50pmol/0.25µl/side in the VMN; California Peptide Research; Napa, CA), or saline were microinjected over a two-minute period in awake animals followed by an additional minute to prevent backflow. Rats were returned to their home cages and euthanized at 30, 60, or 90 minutes after PACAP and saline injections. VMN tissue was harvested for immunoprecipitation and western blot analyses.

VMN PACAP Microinjections and PAC1R trafficking

Pituitary adenylate cyclase-activating polypeptide (PACAP 50pmol/0.25µl/side in the VMN; California Peptide Research; Napa, CA), or saline were microinjected over a two-minute period in awake animals followed by an additional minute to prevent backflow. Rats were returned to their home cages and euthanized by rapid decapitation 30 minutes after PACAP and saline injections. VMN tissue was collected, and crude subcellular fractionations were collected for western blot analysis.

Trafficking and Endocytosis Inhibitors

Microinjections of vehicle, endocytosis inhibitors Pitstop 2 (#ab120687; clathrin inhibitor; 6mM/0.25µl/side; Abcam, Cambridge, MA), Hydroxy-Dynasore (#HY-13863; dynamin inhibitor; Dyngo-4a; 0.38mM/0.25µl/side; MedChem Express LLC; Monmouth Junction, NJ) and Barbadin (#AOB37364; β-arrestin/β2-adaptin interaction inhibitor; 15μM/0.25ul/side; AOBIOUS INC; Gloucester, MA) were administered 15 minutes before rats (n=6/group) received a second bilateral injection of either saline containing 1% DMSO or (PACAP; 50pmol/0.25µl/side). Approximately 5 minutes after injections, animals were returned to their home-cage and food intake was measured for the next 24 hours followed by a final measurement of body weight. Barbadin, Pitstop 2 and Dyngo-4a were prepared as DMSO stocks, diluted and injected in animals. In order to avoid potential VMN cell toxicity, low concentrations of Pitstop 2 and Dyngo-4a were combined and administered as an endocytosis inhibitor cocktail (Kuriyan et al., 2017). The final concentration of DMSO was <1%. PACAP was prepared as a stock in 0.9% saline and diluted just before use.

Results

PAC1Rs undergo endocytosis following stimulation by their cognate ligand, PACAP (Tompkins et al., 2018a). We measured changes in PAC1R subcellular expression from rat VMN tissue injected with PACAP. Western blot analysis of VMN tissue homogenates confirmed that exogenous PACAP promotes PAC1R trafficking from the membrane to the cytosol. Crude subcellular fractionation analyses show PACAP significantly increases cytosolic PAC1Rs

Figure 7.1. PACAP induces surface PAC1R endocytosis. Top and middle) representative western blot analysis showing PAC1R expression the membrane to the cytosol. Bottom) experimental design for pharmacological treatments of PACAP and endocytosis inhibitors, Dyngo-4a/Pitstop 2.

and decreases membrane PAC1Rs $F_{(3,12)}=4.084$, $p=0.03$, $F_{(3,12)}=4.619$, $p<0.03$ respectively (Fig. 7.1, and 7.2) This PACAP-induced shift in PAC1R subcellular localization was blocked using a cocktail of dynamin and clathrin inhibitors, Dyngo-4a and Pitstop 2 $p=0.025$ (membrane), and $p=0.023$ (cytosolic) when compared to PACAP treatment alone (Figs. 7.1, and 7.2).

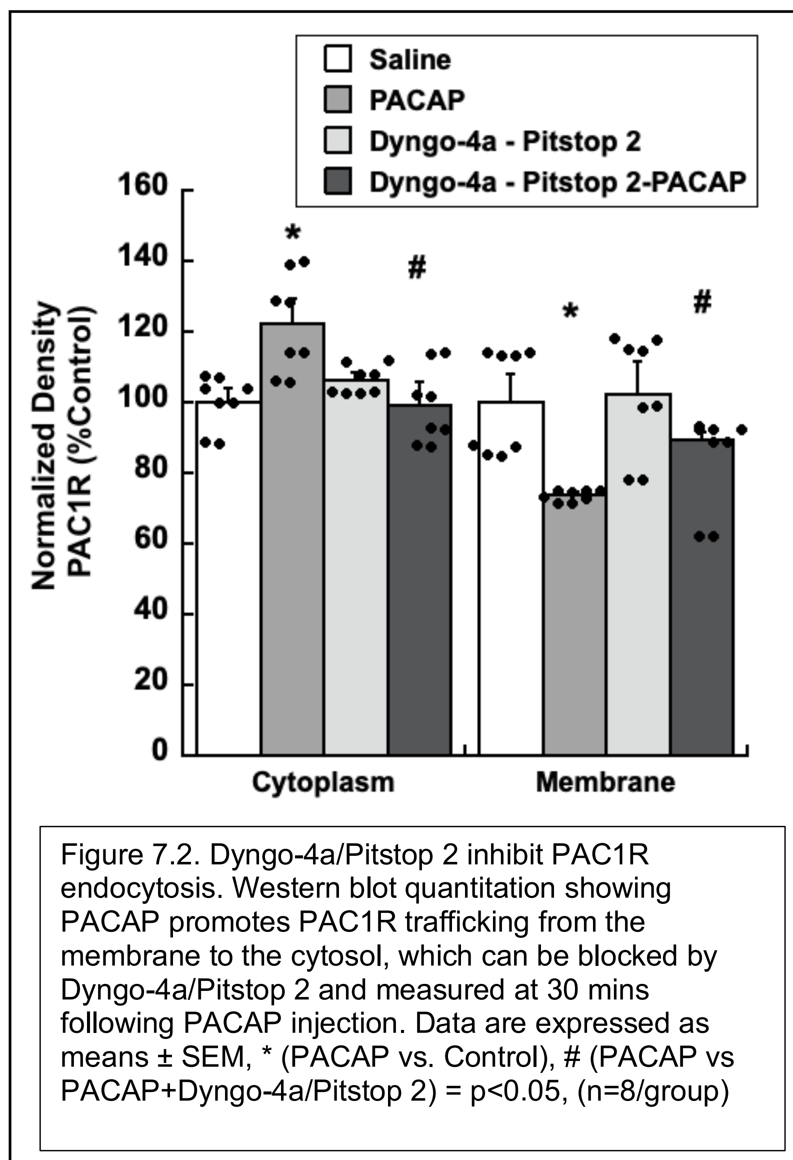


Figure 7.2. Dyngo-4a/Pitstop 2 inhibit PAC1R endocytosis. Western blot quantitation showing PACAP promotes PAC1R trafficking from the membrane to the cytosol, which can be blocked by Dyngo-4a/Pitstop 2 and measured at 30 mins following PACAP injection. Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+Dyngo-4a/Pitstop 2) = p<0.05, (n=8/group)

Canonically, G-protein coupled receptors like PAC1R are known to associate with endocytosis machinery and internalize following agonist exposure (Merriam et al., 2013). To determine whether hypothalamic ventromedial nuclei (VMN) cells express β-arrestins that may facilitate PAC1R trafficking, we used western blot analysis of VMN homogenates to detect β-arrestin 1 and β-arrestin 2 expression. We found that VMN cells express β-arrestin 1 and β-arrestin 2 (Fig. 7.3). Next, we used co immunoprecipitation (coIP) to determine if PAC1Rs associate with β-arrestins following stimulation. We found that PACAP promotes PAC1R association with β-arrestin.

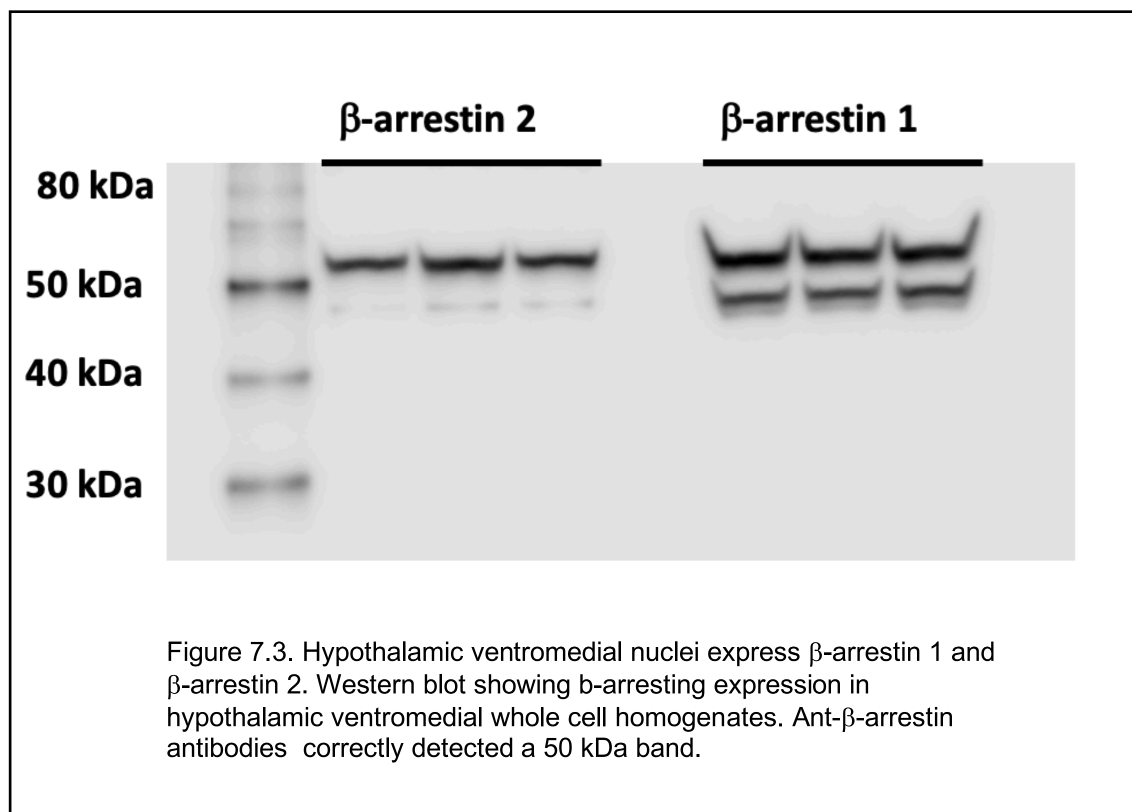
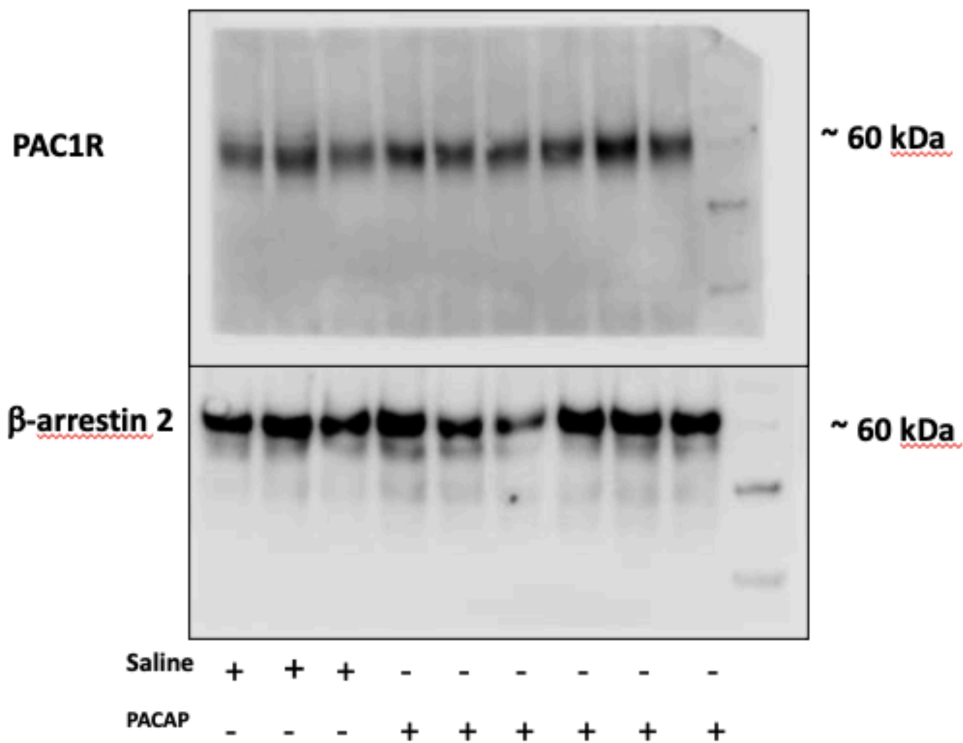


Figure 7.3. Hypothalamic ventromedial nuclei express β-arrestin 1 and β-arrestin 2. Western blot showing b-arresting expression in hypothalamic ventromedial whole cell homogenates. Ant-β-arrestin antibodies correctly detected a 50 kDa band.



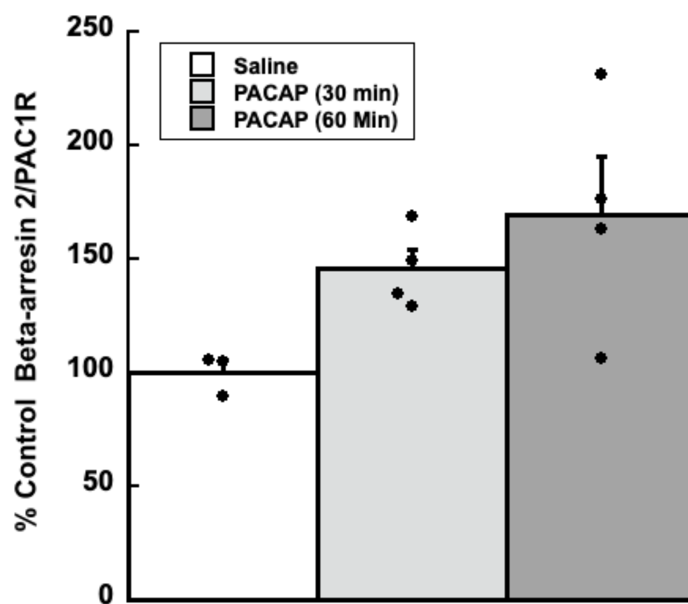


Figure 7.4. PACAP promotes PAC1R association with β-arrestin 2. Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+Barbadin) = p<0.05, (n=4group)

To determine if β-arrestin and PAC1R trafficking contributed to PACAP's hypophagic effects in the VMN, we used barbadin, a novel selective β-arrestin-β2-adaptin association inhibitor that can prevent agonist-induced endocytosis of GPCRs. We injected barbadin just prior to PACAP injection in the VMN and measured changes in food intake and body weight and observed that barbadin did not block PACAP's hypophagic effects in the VMN. Repeated measures analysis of variance shows that PACAP is able to significantly suppress food consumption in the presents of barbadin ($F_{(3,29)}$=677, $p<0.003$; Fig. 7.5, Left). There is no difference in food consumption in rats that received PACAP alone or combined barbadin and PACAP. Analysis of body weight change show that blocking β-arrestin and adaptor proteins interaction may influence some

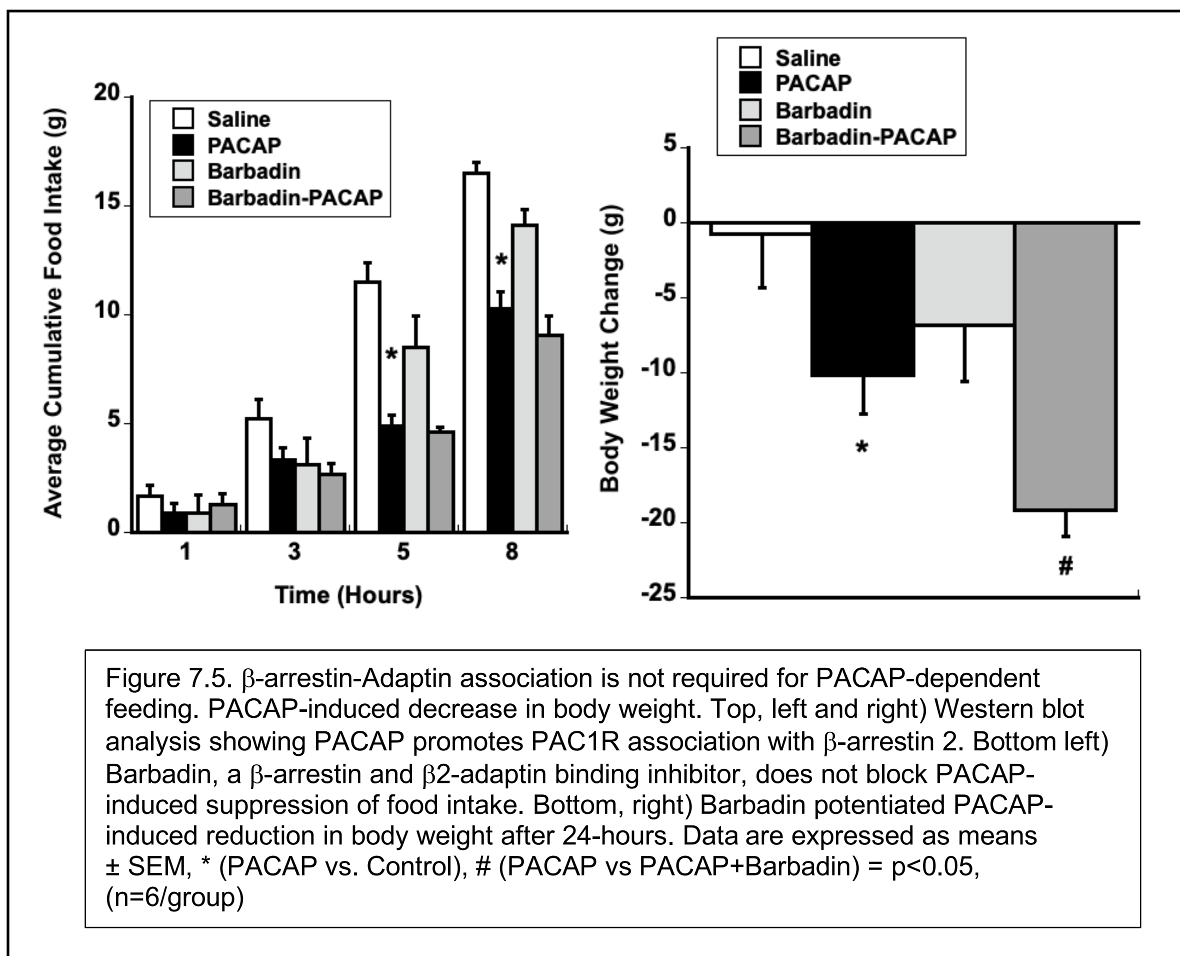


Figure 7.5. β-arrestin-Adaptin association is not required for PACAP-dependent feeding. PACAP-induced decrease in body weight. Top, left and right) Western blot analysis showing PACAP promotes PAC1R association with β-arrestin 2. Bottom left) Barbadin, a β-arrestin and β2-adaptin binding inhibitor, does not block PACAP-induced suppression of food intake. Bottom, right) Barbadin potentiated PACAP-induced reduction in body weight after 24-hours. Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+Barbadin) = p<0.05, (n=6/group)

metabolic effect. One-way ANOVA showed that barbadin enhanced PACAP's effect on decreased body weight, however, a close assessment suggests that barbadin alone may decrease body weight. The results indicate that blocking β-arrestin binding to adaptin is not sufficient to block PACAP's suppression of feeding, however, body weight results are unclear.

GPCRs use other nucleating proteins during endocytosis.

Because barbadin targets the association between β-arrestin with β2-adaptin, we could not rule out other avenues for PAC1R trafficking. For example, adaptin is able to interact directly with GPCRs through which it may facilitate clathrin-mediated endocytosis independently of arresting (Chen, Paing, & Trejo, 2004; Diviani, Lattion, Abuin, Staub, & Cotecchia, 2003; Moo, Van Senten, Bräuner-Osborne, & Møller, 2021). In addition our earlier results showed that blocking clathrin and dynamin using Dyngo-4a and Pitstop 2 prevents PAC1R endocytosis. To that end, we used a Dyngo-4a/pitstop 2 cocktail to determine whether PACAP-induced PAC1R endocytosis was required for PACAP's anorexigenic effects in the VMN. In rats, combined Dyngo-4a and Pitstop 2 infusions in the VMN prior to PACAP administration briefly blocked PACAP-induced decreases in food intake (~ 3 hours) but did not block body weight change (Fig. 7.6). Analyses of food intake data show that blocking endocytosis transiently reversed PACAP induced hypophagia at 2h and 4 hours, $p<0.03$, and $p=0.023$ respectively when compared to PACAP treatment (Fig. 7.6). There is a significant interaction between treatment and time $F_{(12,80)}=2.320$, $p<0.02$.

Comparably, blocking endocytosis prior to PACAP injection did not prevent PACAP-induced decreases in body weight, it appears that PACAP continued to suppress body weight at 24 hours following injections. There is a significant main effect of treatment $F_{(3,20)}=7.267$, $p<0.003$ (Fig. 7.6, Left).

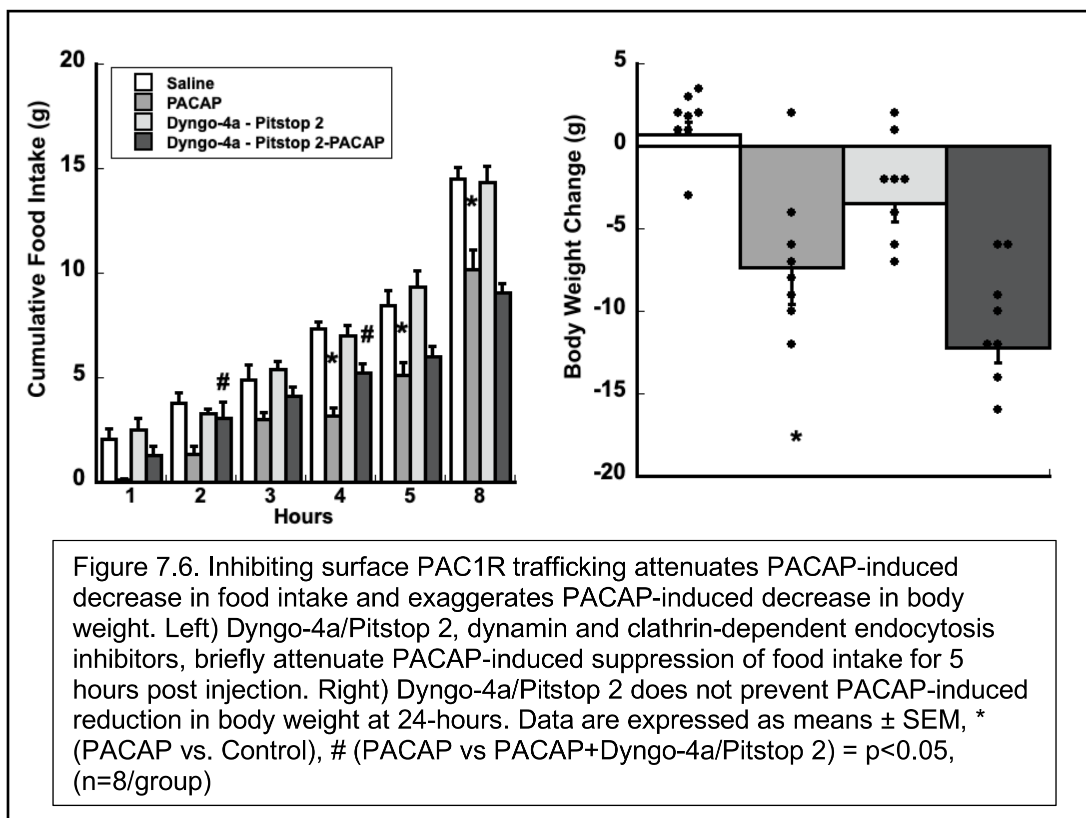


Figure 7.6. Inhibiting surface PAC1R trafficking attenuates PACAP-induced decrease in food intake and exaggerates PACAP-induced decrease in body weight. Left) Dyngo-4a/Pitstop 2, dynamin and clathrin-dependent endocytosis inhibitors, briefly attenuate PACAP-induced suppression of food intake for 5 hours post injection. Right) Dyngo-4a/Pitstop 2 does not prevent PACAP-induced reduction in body weight at 24-hours. Data are expressed as means ± SEM, * (PACAP vs. Control), # (PACAP vs PACAP+Dyngo-4a/Pitstop 2) = $p<0.05$, (n=8/group)

Previously, our laboratory showed that PACAP increases VMN STAT3 phosphorylation, and STAT3 has been shown to influence food intake and energy expenditure (Hurley et al., 2020). In this study, we also measured STAT3 phosphorylation to determine if PAC1R endocytosis may be a mechanism by which PACAP regulates STAT3. Although we corroborated that PACAP increases STAT3 phosphorylation (Fig. 7.7), we were not able to assess the contributions of blocking PAC1R endocytosis on STAT3 phosphorylation

because the same dose that blocked PACAP's hypophagic effects, influenced STAT3 phosphorylation alone (Figs. 7.7, 7.8). PACAP and endocytosis inhibitors alone did not influence STAT3 endogenous expression (Figs. 7.7, 7.8).

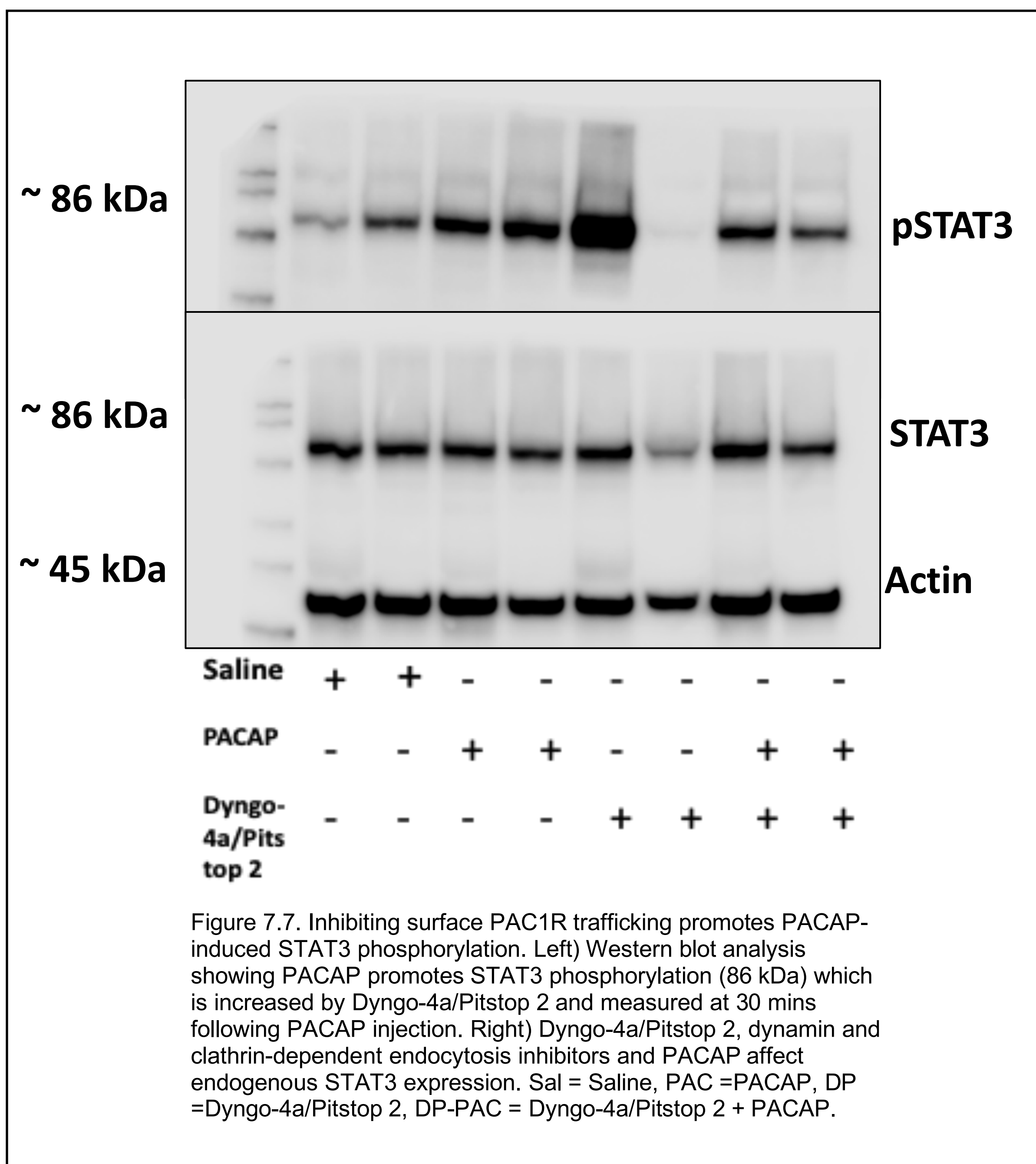


Figure 7.7. Inhibiting surface PAC1R trafficking promotes PACAP-induced STAT3 phosphorylation. Left) Western blot analysis showing PACAP promotes STAT3 phosphorylation (86 kDa) which is increased by Dyngo-4a/Pitstop 2 and measured at 30 mins following PACAP injection. Right) Dyngo-4a/Pitstop 2, dynamin and clathrin-dependent endocytosis inhibitors and PACAP affect endogenous STAT3 expression. Sal = Saline, PAC =PACAP, DP =Dyngo-4a/Pitstop 2, DP-PAC = Dyngo-4a/Pitstop 2 + PACAP.

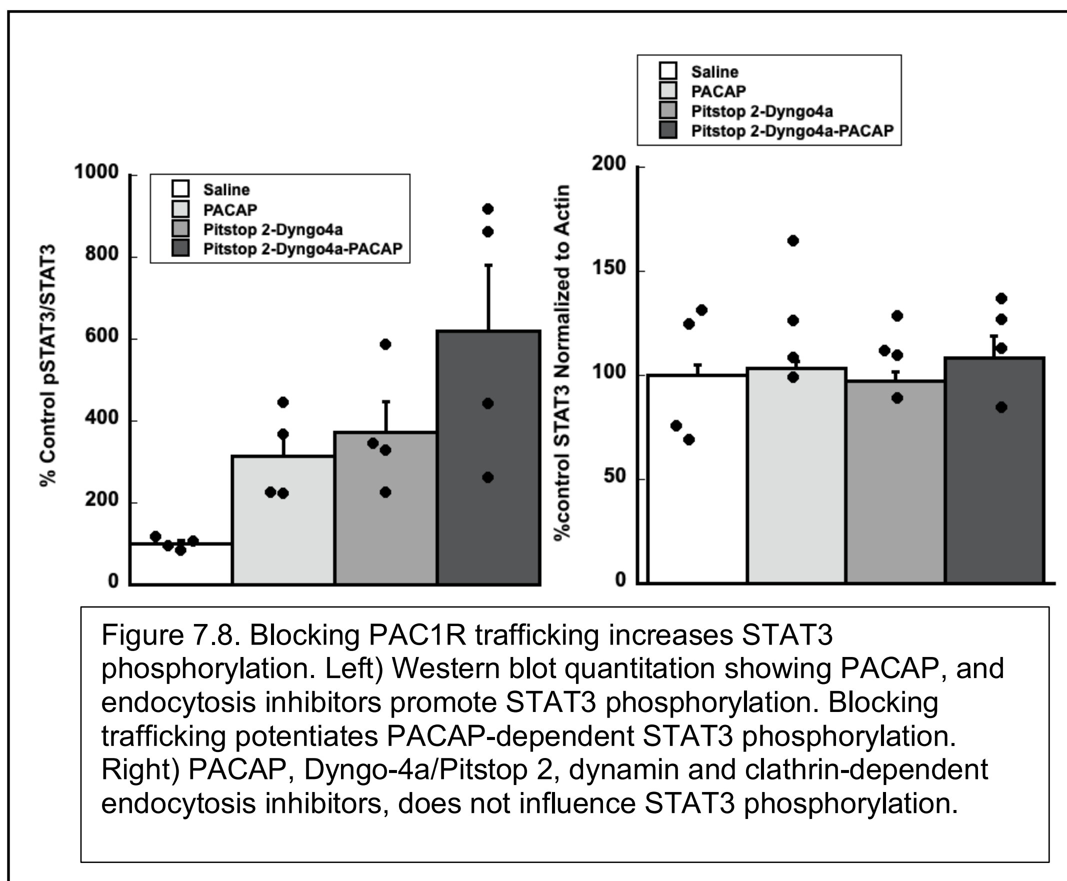


Figure 7.8. Blocking PAC1R trafficking increases STAT3 phosphorylation. Left) Western blot quantitation showing PACAP, and endocytosis inhibitors promote STAT3 phosphorylation. Blocking trafficking potentiates PACAP-dependent STAT3 phosphorylation. Right) PACAP, Dyngo-4a/Pitstop 2, dynamin and clathrin-dependent endocytosis inhibitors, does not influence STAT3 phosphorylation.

Discussion

We explored whether endocytosis of PAC1R was essential to PACAP's behavioral and physiological effects. Canonically, cell surface GPCRs are endocytosed following stimulation, in part, as a desensitization mechanism. However, more recent reports show that ligand-activated PAC1R endocytosis is important for neuronal action potential firing for PAC1R in hippocampal and cardiac neurons (Liao et al., 2021; Parsons & May, 2018; Tompkins et al., 2018b). Considering that our lab recently reported that PACAP increases action potential firing frequency in ex vivo VMN slices, it is possible that blocking VMN PAC1R endocytosis would alter action potential generation and subsequently food intake or metabolic indices such as thermogenesis and/or locomotor activity.

In the VMN, PACAP promotes PAC1R endocytosis from the membrane to the cytosol, which was subsequently blocked by the inhibitors of endocytosis, clathrin and dynamin. Inhibition of PAC1R trafficking temporarily attenuated PACAP-induced hypophagia but did not prevent its anorexic effects after 5 hours. The short-lived effects of endocytosis inhibitors are in line with data demonstrating that endogenous endocytosis can be restored within approximately one hour following washout (McCluskey et al., 2013). This experiment demonstrates that PACAP's hypophagic effects in the VMN could be mediated, in part, by PAC1R endocytosis. Conversely, temporal blockade of PAC1R trafficking and food intake produced a more pronounced body weight loss suggesting that transient inhibition of trafficking is not sufficient to attenuate PACAP's decrease in body weight. The fact that we observe partial potentiation of PACAP's effects on body weight may indicate a delayed effect of PAC1R endocytosis on other metabolic factors that may contribute to body weight change, however this is only speculation at this point. Interestingly, targeting the β-arrestin dependent endocytosis mechanisms did not influence PACAP's effects on food intake in the VMN. Suggesting that GPCR trafficking is a complicated process which requires the coordination of multiple proteins. In our case, PAC1R association with β-arrestin seems dispensable in PACAP's hypophagic effects. However, we show that barbadin is sufficient to influence PACAP's effects on body weight, suggesting that barbadin may be influencing aspects of energy homeostasis in addition to PACAP. Alternatively, It has been demonstrated that GPCRs can internalize independent of β-arrestin by directly binding adaptin and recruiting

clathrins, suggesting that PAC1Rs may have internalized and induced hypohagia. β-arrestin is also know to engage other signaling cascades in addition to GPCR trafficking (DeWire et al., 2007). For example, β-arresting induce extracellular regulated kinase (Erk) (Rahmouni, Sigmund, Haynes, & Mark, 2009)which has directly been implicated in decreasing feeding in the brain. Other studies show that β-arrestin are involved in energy regulation. An extensive review (Pydi et al., 2021, 2020) on β-arrestins and their role in metabolism highlight the fact that β-arresin 1 and β-arrestin knock out in the peripheral or central nervous system of mice cause severe metabolic disorders. Future studies will need to assess whether blocking PAC1R trafficking delays the onset of thermogenesis and increase in locomotor activity as well as the need to determine the consequences of blocking PAC1R endocytosis on PACAP-induced increase in firing frequency.

CHAPTER VIII

VMN CELLS INTEGRATE MULTIPLE COMPLEX SIGNALS TO REGULATE FEEDING AND ENERGY HOMEOSTASIS: PACAP AND LEPTIN SIGNALING

Introduction

The balance between energy intake and energy expenditure is tightly regulated by several brain structures including the hypothalamic ventromedial nuclei (VMN) (Andermann & Lowell, 2017; Timper & Brüning, 2017). Factors that cause extreme swings in energy balance result in health complications including obesity and anorexia (Andermann & Lowell, 2017; Gastelum et al., 2021; Wilding, 2002). The VMN is thought to regulate food intake by stimulating satiety and energy expenditure by integrating short-term and long-term feedback from the gut and adipose tissue (Andermann & Lowell, 2017; Hawke et al., 2009; Roh et al., 2016) via several anorexigenic and orexigenic neuropeptides including pituitary adenylate cyclase-activating polypeptide (PACAP), leptin, and their cognate receptors, PAC1 and leptin receptor (Bozadjieva-Kramer et al., 2021; King, 2006).

Decades of research have now identified and implicated leptin signaling malfunction as one of the leading causes of obesity (Boughton & Murphy, 2012; Wada et al., 2014; Ronghua Yang & Barouch, 2007c). Despite the breadth of knowledge on leptin signaling, several attempts to target this signaling system as a therapeutic approach have fallen short of treating obesity (Boughton & Murphy, 2012). Recent findings in our lab and others have identified a novel signaling

partner, PACAP, that may contribute to understanding and treating obesity stemming from leptin signaling malfunction (Hawke et al., 2009; Hurley et al., 2020).

Leptin is an adipose tissue-derived peripheral hormone encoded by the LEP gene and it plays an important role in several central nervous system structures including the VMN that regulate food intake and energy expenditure (Buchanan et al., 1998; Hawke et al., 2009; Wada et al., 2014). PACAP is a neuronally derived peptide whose actions in the VMN mirror that of leptin wherein both ligands induce hypophagia and increase energy expenditure (Hawke et al., 2009; Hurley et al., 2020; Resch et al., 2011, 2013). PACAP and leptin share other striking similarities in their expression and signaling mechanisms. For example, in situ hybridization analysis shows that leptin and PACAP receptor transcripts co-localize within the same VMN cells (Hawke et al., 2009; Hurley et al., 2020). Leptin and PACAP both stimulate their cognate receptors to induce signal transducer and activator of transcription 3 (STAT3) phosphorylation (pSTAT3), and subsequently silencer of cytokine signaling 3 (SOCS3) transcription (Hawke et al., 2009; Hurley et al., 2020). More importantly, blocking PAC1 receptors prevents leptin's ability to engage its downstream signaling and influence feeding and energy expenditure (Hawke et al., 2009; Hurley et al., 2020). Collectively, these findings suggest that elucidating the signaling mechanisms underlying the cross talk between PACAP and leptin signaling may help us understand the etiologies of obesity, while providing potential therapeutic avenues to treat obesity.

In this study, we not only corroborated that rat VMN PAC1 and leptin receptors are expressed in the same cell, we also demonstrated that leptin and PAC1Rs co-immunoprecipitated, suggesting they are in close proximity and may share a physical connection. Using a newly developed immortalized rat hypothalamic in vitro model, rHypoE-19 (Belsham et al., 2009; Gingerich et al., 2009; Nazarians-Armavil, Menchella, & Belsham, 2013), we found that PACAP induces Janus kinase 2 phosphorylation and that blocking protein kinase C, a downstream effector of PACAP signaling, attenuates leptin induced STAT3 phosphorylation.

Materials & Methods

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 225-300 g were individually housed in either standard cages or BioDAQ feeding cages (Research Diets, New Brunswick, NJ) in a climate-controlled room under a 12-hr light/dark cycle (lights on at 0200h). Animals were weighed daily and provided ad libitum access to standard chow (Teklad global 18% protein diet 2018 formulation, Envigo, Indianapolis, IN) and water. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

In situ Hybridization

Rat brains were sectioned coronally at 12 μm and then postfixed 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 PAC1R, and long-form leptin receptor (LepRb) transcripts (Choi, Milwaukee, WI), which were subsequently diluted in hybridization cocktail (Amresco, Solon, OH) with tRNA. Sections were hybridized overnight at 60°C with either digoxigenin (DIG) or fluorescein (FITC)-labeled riboprobes. After hybridization, slides were treated with RNase A and stringently washed in 0.3X SSC at 65°C (PAC1R) for 30 min. Slides were incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche) overnight at 4°C. Riboprobe signal was amplified using the TSA-Plus fluorophore system with either fluorescein or Cy3 (PerkinElmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2; Zeiss, Thornwood, NY) and Axiovision image analysis software (Zeiss, Thornwood, NY).

Cell culture and reagents

rHypoE-19 neurons (Cedarlane CELLutions Biosystems; Burlington, NC), generated as described previously were cultured in monolayer in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific; Rockford, IL) supplemented with 8% fetal bovine serum (# 26140; GIBCO), and grown in 5% CO2 at 37°C.

Mouse recombinant leptin (Bio-Techne Corporation, Minneapolis, MN), PACAP (25nM; California Peptide Research, Napa, CA), $PACAP_{6-38}$ (100nM; Anaspec, Fremont, CA) were reconstituted in saline. KT5720 (#1288/100U; protein kinase A inhibitor; 10μM; Bio-Techne Corporation, Minneapolis, MN), and GF109203X (#0741/1; protein kinase C inhibitor; 10μM; Bio-Techne Corporation, Minneapolis, MN) were diluted in dimethyl sulfoxide (DMSO; #116070; Thermo Fisher Scientific; Rockford, IL). Anti-STAT3, phospho-specific STAT3 (Tyr705), anti-JAK2, phospho-specific JAK2 (Tyr1007/1008), anti-Src, phospho-specific antibodies were obtained from (Cell Signaling Technologies; Danvers, MA).

Immunohistochemistry

Immortalized rat hypothalamic embryonic day 19 neurons (rHypoE-19) were grown and fixed in 12-well plates. Prior to immunohistochemical staining, cells were fixed in 4% PFA for 10 minutes on ice and rinsed in 0.1 M PBS (pH 7.4). Cells were incubated in blocking buffer (0.05M KPBS, 3% normal donkey serum, 0.1% Triton X-100) for 1h at room temperature followed by an overnight incubation at 4°C in blocking buffer containing either primary antibody against PAC1R (rabbit anti-PAC1R, (#AV-R003; Alomone; Israel), leptin receptor (mouse anti-LepR; Santa Cruz Biotechnology), or rabbit anti-phospho-specific STAT3 (Tyr705) (Cell Signaling Technologies; Danvers, MA). After several rinses in 0.05M KPBS, sections were incubated in blocking buffer containing donkey anti-rabbit Alexafluor 594 or Alexafluor 488 conjugated secondary antibody (1:250;

Life Technologies; Grand Island, NY). Upon completion of fluorescent staining, cells labelled with PAC1R and lepRb were imaged.

Measurement of forskolin-induced cAMP release.

cAMP release was determined using mouse/rat cAMP assay kit (catalog no. KGE012B) from R&D Systems (Minneapolis, MN). rHypoE19 cells were cultured in a monolayer in DMEM. Cell lysates were prepared using the lysis buffer provided in the kit. A representative sample was taken from each condition and subject to the competitive immunoassay. After addition of stop solution, optical density readings were performed using a microplate reader system that was set to 450 nm and 540 nm wavelengths. Wavelength correction was made by subtracting 450-nm readings from 540-nm readings. A cAMP standard was prepared as per the manufacturer's protocol. The mean absorbance of the samples was normalized to the standard, then multiplied by the dilution factor.

Western Blot analysis

VMH tissue or rHypoE-19 cells were homogenized in ice-cold homogenization buffer (320 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10mM EDTA, 10mM EGTA) or RIPPA buffer containing Halt protease and phosphatase inhibitor cocktail (#78447; Thermo Scientific; Rockford, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4°C and the resulting supernatant was saved for further processing. Protein quantification of samples was determined using a bicinchoninic (BCA) assay

(#23252; Pierce). Protein (30 µg) was run on an 8% gel by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (#LC2002; Thermo Fisher Scientific; Rockford, IL). Membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) or 5% non-fat milk in tris-buffered saline containing 0.1 % Tween-20 (TBS-T). Blots were then probed with rabbit anti-PAC1R antibody alone (#AV-R003; 1:1000; Alomone labs; Jerusalem, Israel) or dually probed with b-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) overnight at 4°C, followed by washes with TBS-T and incubation with an HRP-linked anti-rabbit secondary antibody (#7074P2; 1:3000; Cell Signaling Technologies; Danvers, MA) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA) at room temperature for 2 hours. Band intensities were developed using SuperSignal West Atto Chemiluminescent Substrate (#A38556; Thermo Scientific; Rockford, IL) and visualized using the Odyssey Fc Dual Mode Imaging System (LI-COR; Lincoln, NE). Band densities were measured and quantified using Odyssey Fc Dual-Mode Imaging System software. PAC1R antibody specificity was validated by preincubating rabbit anti-PAC1R with PAC1R blocking peptide (#BLP-VR003; Alomone labs; Jerusalem, Israel). Following visualization of PAC1R signal alone, blots were stripped and re-probed in an identical fashion for b-actin using a mouse anti-β-actin (#MA5-15452; 1:2000; Thermo Scientific; Rockford, IL) and HRP conjugated anti-mouse (#7076P2; 1:5000; Cell Signaling Technologies; Danvers, MA).

Crosslinking and immunoprecipitation

VMH tissue was homogenized by hand (10 strokes) in ice-cold immunoprecipitation lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 10 μM CaCl2, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mM AEBSF, 1% Triton X-100) containing Halt protease and phosphatase inhibitor cocktail (#78447; Thermo Scientific; Rockford, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 2 minutes at 4°C and the resulting whole-cell lysate supernatant was saved for further processing. Protein quantification of samples was determined using a bicinchoninic (BCA) assay (#23252; Pierce). Chemical crosslinking and co-immunoprecipitation for detecting association of PAC1-leptin receptors, covalent protein cross-linking with a chemical crosslinker, dithiobis(succinimidyl) propionate (DPS; Thermo Fisher Scientific). 100 mg/sample VMN whole-cell lysates were incubated with 2.5 mM DSP for 30 mins at room temperature and the crosslinking reaction was quenched with 0.1 ml of 1 M Tris (pH 7.5). To prevent co-elution of IgG fragments, the immobilized antibodies were covalently cross-linked to protein G-Sepharose beads. Antibody-coupled protein G-Sepharose beads were resuspended in 5 mM BS3 (Thermo Fisher Scientific) and incubated for 30 min at room temperature. Crosslinking was quenched by the addition of 0.1 ml of 1 M Tris (pH 7.5). VMN lysates were incubated with either PAC1R antibody (1:500), or leptin receptor antibody-coupled protein G-Sepharose beads for 2 h at 4˚C while shaking. Following incubation, the beads were collected by magnet and washed three times with wash buffer (0.1M phosphate buffered saline). Proteins

bound to the beads were eluted and the crosslink was reversed with 2x sample buffer containing 3 % (v/v) 2-mercaptoethanol and analyzed by performing immunoblotting with specific antibodies as indicated.

RNA extraction and quantitative reverse transcription

Total RNA was extracted from brain tissue by the Trizol extraction (Invitrogen; Carlsbad, CA) method. Total RNA was treated with DNase (Life Technologies, Carlsbad, CA) to remove contaminating genomic DNA. Subsequently, 1μg total RNA was reverse transcribed into complementary DNA (cDNA) using a mixture of random primers, oligodT primers and reverse transcriptase (Reverse Transcription System, Promega; Madison, WI). Quantitative PCR was performed using a StepOne Real-Time PCR System (AppliedBiosystems; Carlsbad, CA), and PerfeCTa SYBR Green FastMix with ROX (QuantaBiosciences; Gaithersberg, MD) according to the manufacturer's protocol. Quantification of PAC1R isoforms, BDNF, SOCS3, xCT, and lepRb mRNA 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%. Melt curve analysis of experiments confirmed a single product for each reaction.

Data analysis

Data are presented as means ± standard errors of the mean and were analyzed statistically by analysis of variance (with repeated measures when appropriate). Fisher LSD, and Tukey HSD analyses were used for post-hoc group comparisons. Group sizes were designed to detect a 0.05 significance level with a power of 0.8 assuming a standard deviation of 10% of the mean.

Results

PAC1 and the long-form leptin receptor form an immunocomplex.

Given that our lab and others previously demonstrated that VMN PAC1 and leptin receptors exist in the same cell, we investigated whether the overlapping expression pattern of PAC1R and lepRb might allow for potential binding interactions. To that end, we used immunoprecipitation with antibodies specific for the long-form leptin receptor and PAC1R. We found that when the PAC1R antibody was used to isolate PAC1 receptors from VMN tissue homogenates, we were able to detect leptin receptor expression in the immunocomplex, and the opposite was true (Fig. 8.1). When we used the leptin receptor antibody to isolate the leptin receptor from VMN tissue homogenates, PAC1R protein was present in complex with the leptin receptor (Fig. 8.1). As a control measure, we pre-absorbed PAC1R or leptin receptor antibodies and subsequently failed to detect their cognate receptors. This finding suggests that the PAC1R and leptin receptor immunocomplex is not coincidental and PAC1

and leptin receptors could potentially interact physically or are in very close proximity.

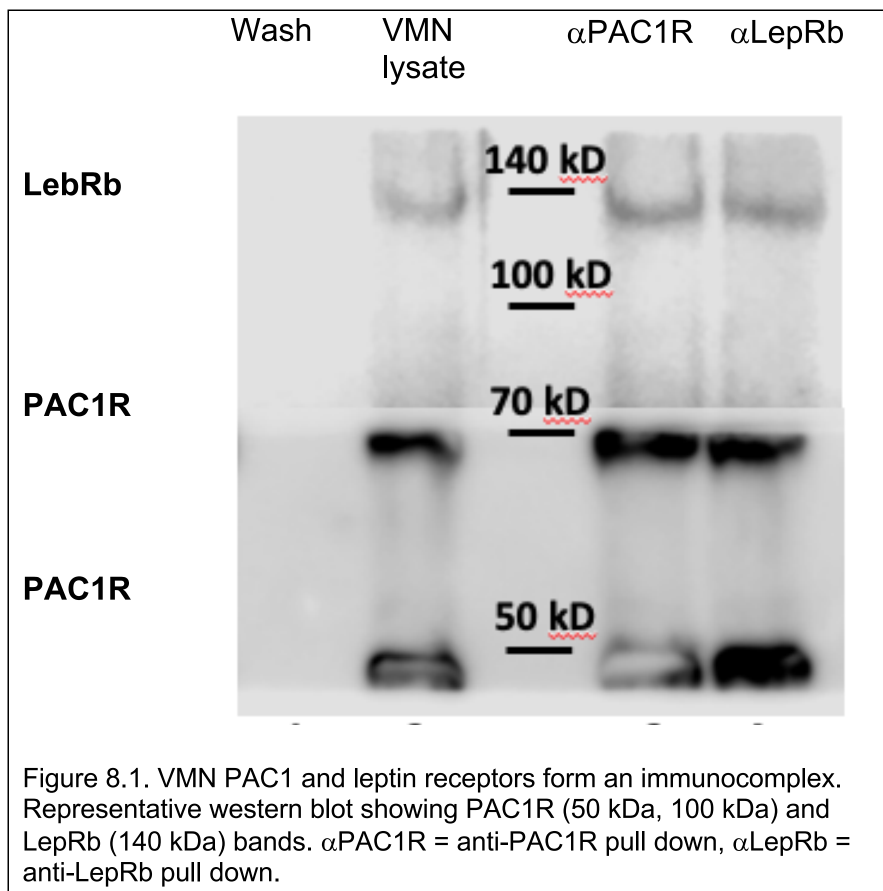


Figure 8.1. VMN PAC1 and leptin receptors form an immunocomplex. Representative western blot showing PAC1R (50 kDa, 100 kDa) and LepRb (140 kDa) bands. αPAC1R = anti-PAC1R pull down, αLepRb = anti-LepRb pull down.

VMN and rHypoE19 cells express PAC1 and long-form leptin receptors

Accumulating evidence suggests PAC1R, a G-protein coupled receptor may be involved in regulating a cytokine receptor in leptin, which becomes more likely considering emerging evidence of crosstalk between the two receptor families (Moody et al., 2020; Swift et al., 2011; W. Wang et al., 2018). Here, we sought to evaluate the overlapping PACAP signaling mechanisms that may influence leptin receptor function. To closely examine shared PAC1 and leptin receptor signaling, we employed a newly developed immortalized rat hypothalamic neuronal cell line, rHypoE-19 (Belsham et al., 2009; Gingerich et al., 2009; Nazarians-Armavil et al., 2013). First, we confirmed the presence of both PAC1R and leptin receptors within rHypoE-19 neurons, using immunohistochemical (IHC) staining for both the PAC1 and leptin receptor proteins (Fig 8.2; bottom row). PAC1 receptor immunoreactivity localized perinuclear and extensively to the periphery whereas leptin receptor immunoreactivity was seen throughout the cell. An image overlay of PAC1 and leptin receptor immunoreactivity show similar co-localization of PAC1 and leptin receptors to that seen in the VMN (Fig 8.2; top row).

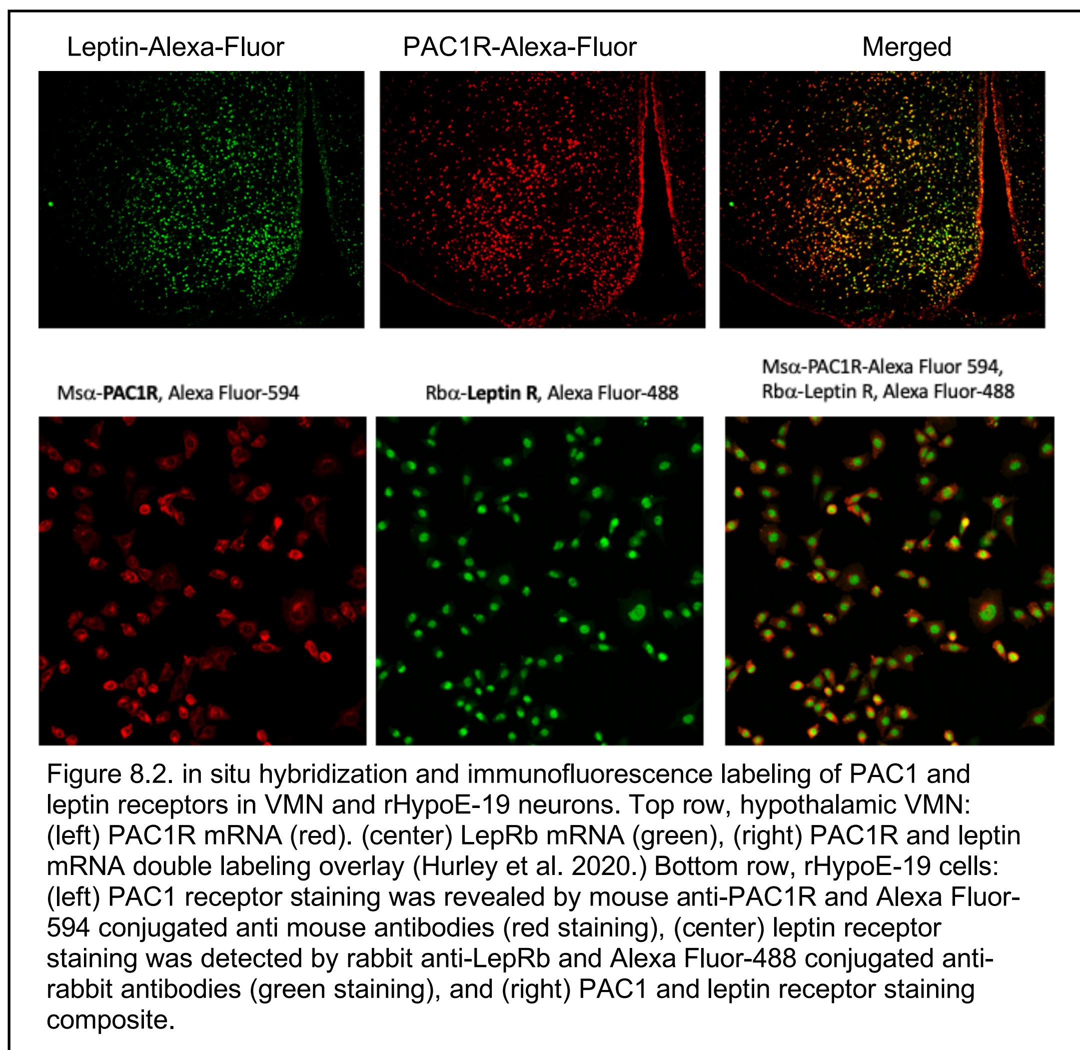


Figure 8.2. in situ hybridization and immunofluorescence labeling of PAC1 and leptin receptors in VMN and rHypoE-19 neurons. Top row, hypothalamic VMN: (left) PAC1R mRNA (red). (center) LepRb mRNA (green), (right) PAC1R and leptin mRNA double labeling overlay (Hurley et al. 2020.) Bottom row, rHypoE-19 cells: (left) PAC1 receptor staining was revealed by mouse anti-PAC1R and Alexa Fluor-594 conjugated anti mouse antibodies (red staining), (center) leptin receptor staining was detected by rabbit anti-LepRb and Alexa Fluor-488 conjugated anti-rabbit antibodies (green staining), and (right) PAC1 and leptin receptor staining composite.

rHypoE-19 neurons express functional PAC1 and Leptin receptors

In rat hypothalamic ventromedial nuclei (VMN), PAC1R activation stimulates cAMP accumulation with concomitant induction of BDNF, SOCS3, and xCT transcription. We begin by investigating the effects of PACAP treatment on rHypoE-19 cells on cAMP accumulation and gene transcription. PACAP treatment in rhypoE-19 cells caused an increase in cAMP accumulation (Fig. 8.3, Right), which was confirmed using forskolin, a well-known direct stimulator of cAMP generation, as a positive control (Fig. 8.3). rHypoE-19 exposure to PACAP

treatment increases BDNF and SOCS3 mRNA expression as well as the PAC1 receptors isoforms PAC1null, and PAC1hop, as previously identified in the VMN. PACAP's hypophagic effects in the VMN have been shown to be mediated by Src kinase phosphorylation (pSrc), which can be reversed using protein phosphatase 1 (PP1) (Resch et al., 2014). Similarly, PACAP responses in rHypoE19 cells also revealed that PACAP significantly induces pSrc $F_{(3,30)}=13.121$, $p<0.001$ when compared to control, which was blocked using PP1 pretreatment (Fig. 8.4, Right).

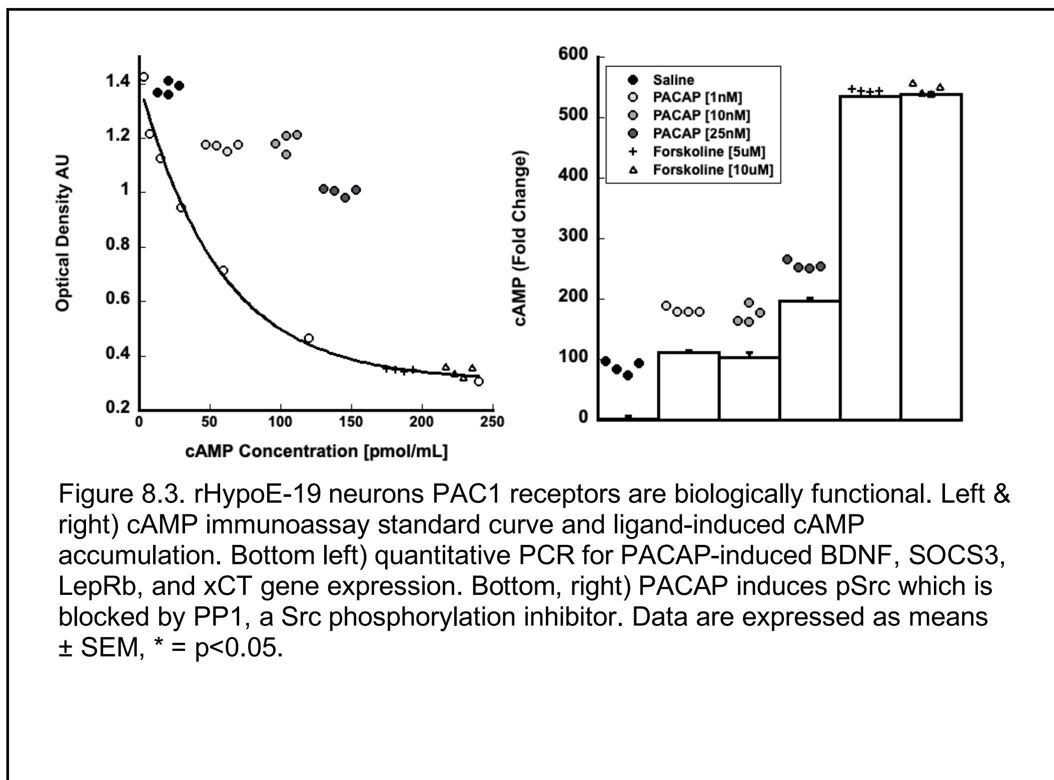


Figure 8.3. rHypoE-19 neurons PAC1 receptors are biologically functional. Left & right) cAMP immunoassay standard curve and ligand-induced cAMP accumulation. Bottom left) quantitative PCR for PACAP-induced BDNF, SOCS3, LepRb, and xCT gene expression. Bottom, right) PACAP induces pSrc which is blocked by PP1, a Src phosphorylation inhibitor. Data are expressed as means ± SEM, * = p<0.05.

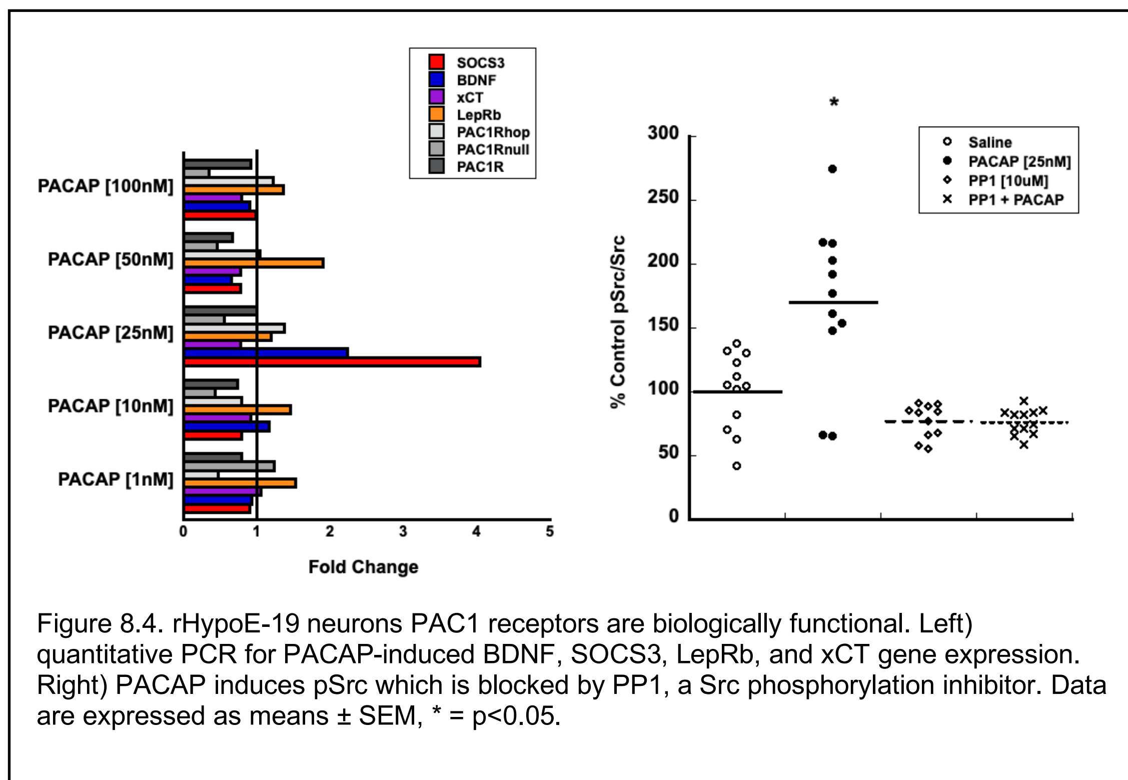


Figure 8.4. rHypoE-19 neurons PAC1 receptors are biologically functional. Left) quantitative PCR for PACAP-induced BDNF, SOCS3, LepRb, and xCT gene expression. Right) PACAP induces pSrc which is blocked by PP1, a Src phosphorylation inhibitor. Data are expressed as means ± SEM, * = p<0.05.

Canonically, leptin binding to its receptor leads to induction of pSTAT3 in the VMN and other brain regions (Hurley et al., 2020; Wada et al., 2014). As such, pSTAT3 induction has been used as the hallmark of leptin signaling. Hurley, et al (Hurley et al., 2020) recently showed that acute leptin injections in the VMN induce pSTAT3. In this study, we found that leptin treatment in rHyoE-19 cells induced pSTAT3 in a dose-dependent manner (Fig 8.5). This is consistent with a study by Belsham et al (Nazarians-Armavil et al., 2013) showing leptin treatment in rHypoE-19 cells leads to pSTAT3. With the confidence that rHypoE19 cells express functional PAC1 and leptin receptors, next we investigated if PACAP signaling influences leptin signaling. We blocked PAC1R signaling using PACAP 6-38 and asked whether leptin can induce

pSTAT3. We found that, blocking PAC1Rs prevents leptin induced pSTAT3 inductions.

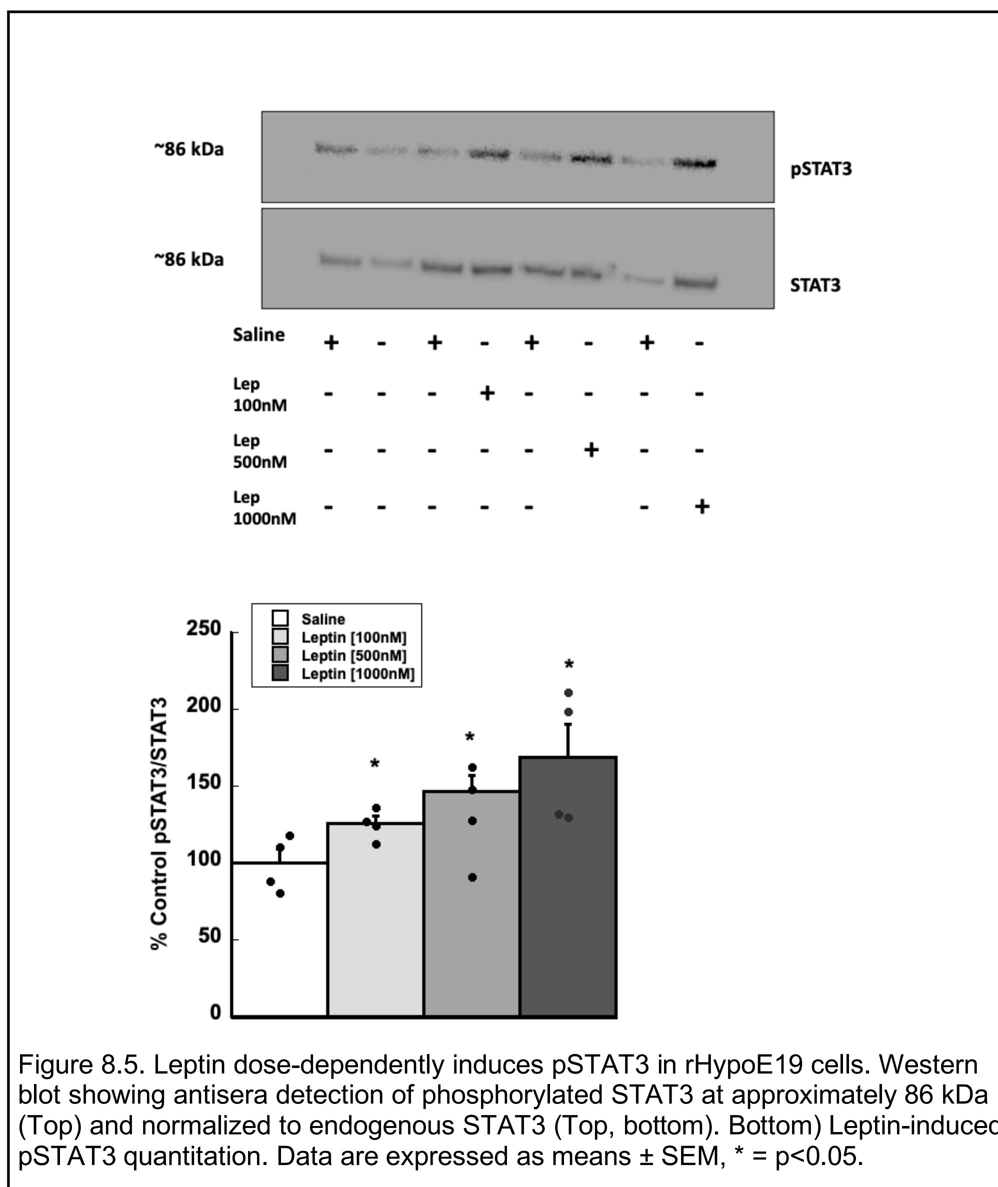


Figure 8.5. Leptin dose-dependently induces pSTAT3 in rHypoE19 cells. Western blot showing antisera detection of phosphorylated STAT3 at approximately 86 kDa (Top) and normalized to endogenous STAT3 (Top, bottom). Bottom) Leptin-induced pSTAT3 quantitation. Data are expressed as means ± SEM, * = $p<0.05$.

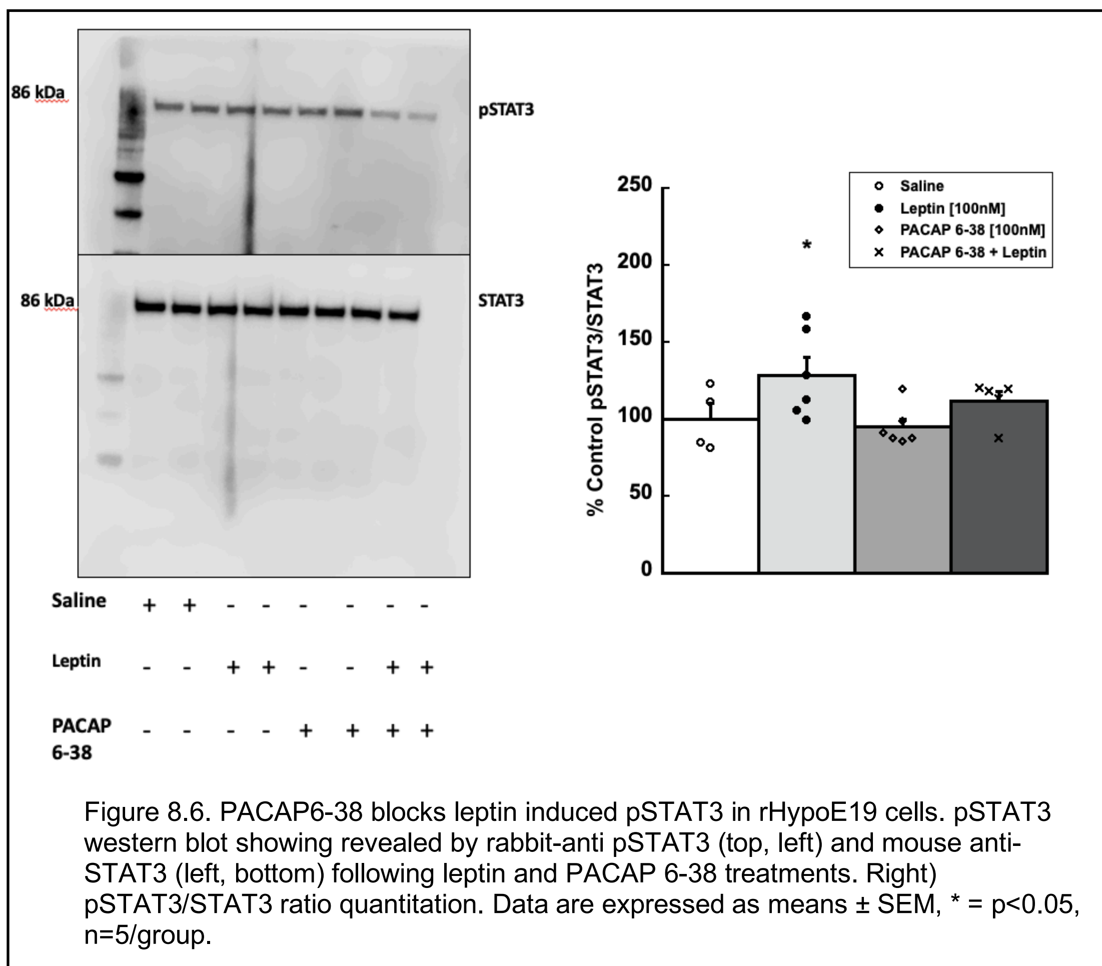


Figure 8.6. PACAP6-38 blocks leptin induced pSTAT3 in rHypoE19 cells. pSTAT3 western blot showing revealed by rabbit-anti pSTAT3 (top, left) and mouse anti-STAT3 (left, bottom) following leptin and PACAP 6-38 treatments. Right) pSTAT3/STAT3 ratio quantitation. Data are expressed as means ± SEM, * = $p<0.05$, n=5/group.

In an effort to understand how blocking PACAP signaling might influence leptin signaling, we first assessed if PAC1 receptor stimulation is capable of activating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) in rHypoE-19 neurons. Although STAT3 phosphorylation is highly associated with canonical cytokine signaling, studies have now demonstrated that GPCRs signaling pathways can also lead to STAT3 phosphorylation (Ram & Iyengar, 2001). Previous work in our lab showed PACAP injections in the VMN lead to STAT3 phosphorylation which can be blocked by PACAP6-38 (Hurley et al., 2020). Similarly, PACAP treatment in rHypoE19 cells significantly induced

STAT3 phosphorylation which could be attenuated by prior treatment with PACAP6-38 (Fig 8.7), p<0.023, when compared to control. However, saline or PACAP6-38 treatment alone did not influence STAT3 phosphorylation.

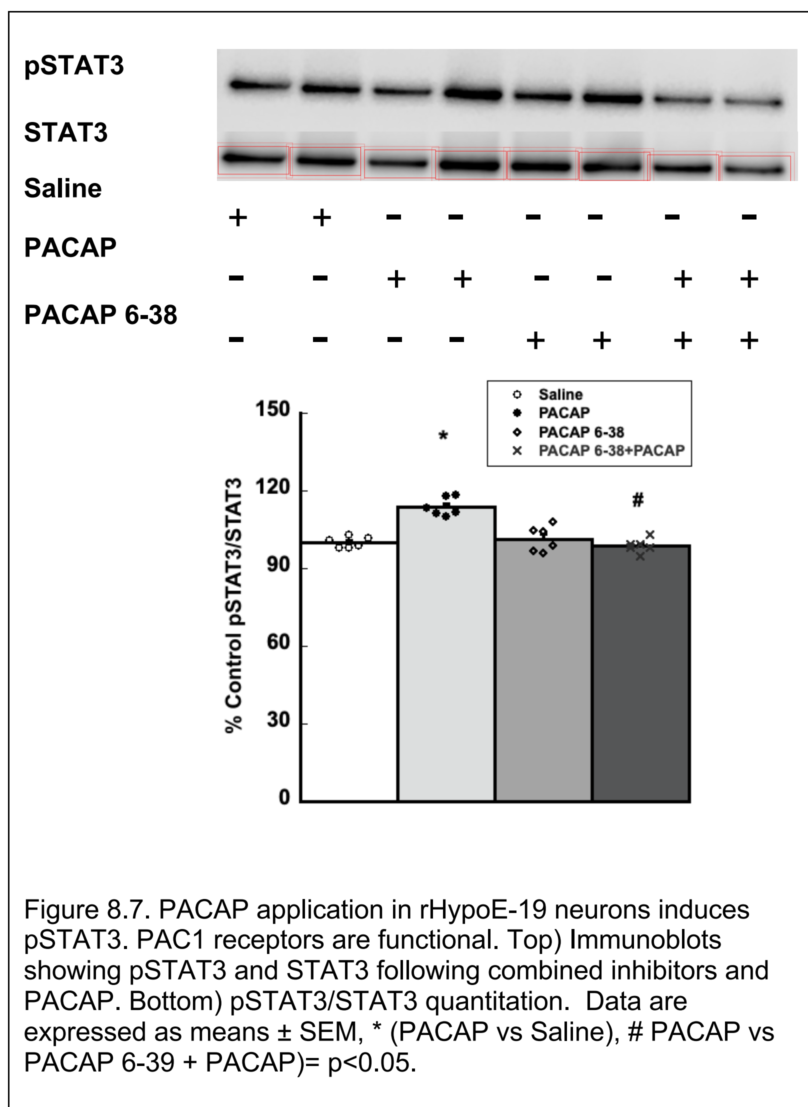


Figure 8.7. PACAP application in rHypoE-19 neurons induces pSTAT3. PAC1 receptors are functional. Top) Immunoblots showing pSTAT3 and STAT3 following combined inhibitors and PACAP. Bottom) pSTAT3/STAT3 quantitation. Data are expressed as means ± SEM, * (PACAP vs Saline), # PACAP vs PACAP 6-39 + PACAP)= p<0.05.

To determine whether PACAP stimulated the phosphorylation of STAT3 in a direct manner or at the antecedent step with the phosphorylation of the JAK kinase, PACAP-induced phosphorylation of JAK 2 (pJAK2) was measured in rhypoE19 cells. PACAP treatment increased pJAK2 significantly p<0.038, when compared to saline control (Fig 8.8) suggesting that PACAP and leptin receptor activation share similar pathways to pJAK2 and possibly pSTAT3.

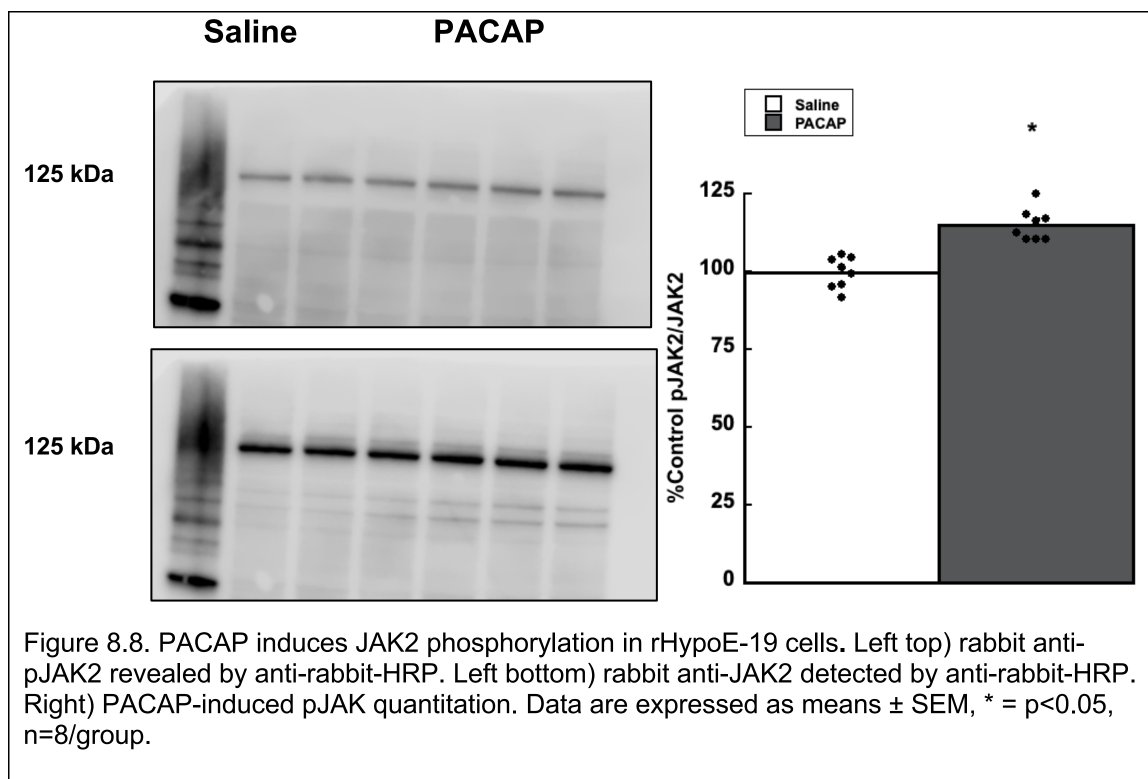


Figure 8.8. PACAP induces JAK2 phosphorylation in rHypoE-19 cells. Left top) rabbit anti-pJAK2 revealed by anti-rabbit-HRP. Left bottom) rabbit anti-JAK2 detected by anti-rabbit-HRP. Right) PACAP-induced pJAK quantitation. Data are expressed as means ± SEM, * = p<0.05, n=8/group.

The phosphorylation state and activities of enzymes that are regulated by tyrosine phosphorylation are controlled by the combined actions of protein tyrosine kinases and protein tyrosine phosphatases. To investigate potential mechanisms by which PACAP signaling may influence leptin receptor function we examined known downstream effectors of PACAP signaling. Recently, we demonstrated that PACAP's action in the VMN control of feeding are mediated

through PKA and PKC activity (Maunze et al., 2022). To determine whether PKA or PKC activity was required for leptin-induced pSTAT3, we blocked PKA and PKC activity and measured leptin-dependent pSTAT3. We found that blocking PKC prevented leptin-induced pSTAT3 in rHypoE-19 cell whereas blocking PKA did not (Fig. 8.9). Blocking PKC activity significantly reduced leptin-dependent pSTAT3, $p<0.043$ when compared to control. This result suggests the possibility that PACAP 6-38 inhibition of PACAP signaling could be perturbing endogenous protein kinase activity downstream of PACAP to provide net inhibition of leptin receptor signaling. The effects of blocking PKA on leptin-induced pSTAT3 agree with studies showing that leptin potently increases pSTAT3 in the absence of functional PKA (L. Yang & McKnight, 2015).

Discussion

Dysregulation of energy homeostasis and obesity can be caused by leptin signaling malfunction (Ronghua Yang & Barouch, 2007c). Attempts to restore normal leptin signaling have fallen short of treating obesity suggesting that our understanding of leptin signaling malfunction and consequent obesity is still lacking (Beck, 2000; Boughton & Murphy, 2012). Recent studies have suggested that PACAP signaling may be a key regulator of leptin signaling in rodents (Hawke et al., 2009; Hurley et al., 2020). The present studies extend these earlier findings by suggesting a potential physical interaction between PAC1 and leptin receptors in VMN cells. PAC1 and leptin receptors are widely distributed in hypothalamic nuclei including the VMN where they both influence feeding and

metabolism (Dhillon et al., 2006; Hawke et al., 2009; Hurley et al., 2020; Resch et al., 2011). In this study, we demonstrated that PAC1 and leptin receptors not only co-express within the same cell but isolate within the same immunocomplex, suggesting that PAC1 and leptin receptors have the capacity to influence or facilitate the other's signaling as evidenced by studies showing that lepRb and PAC1R expression overlaps in the VMN.

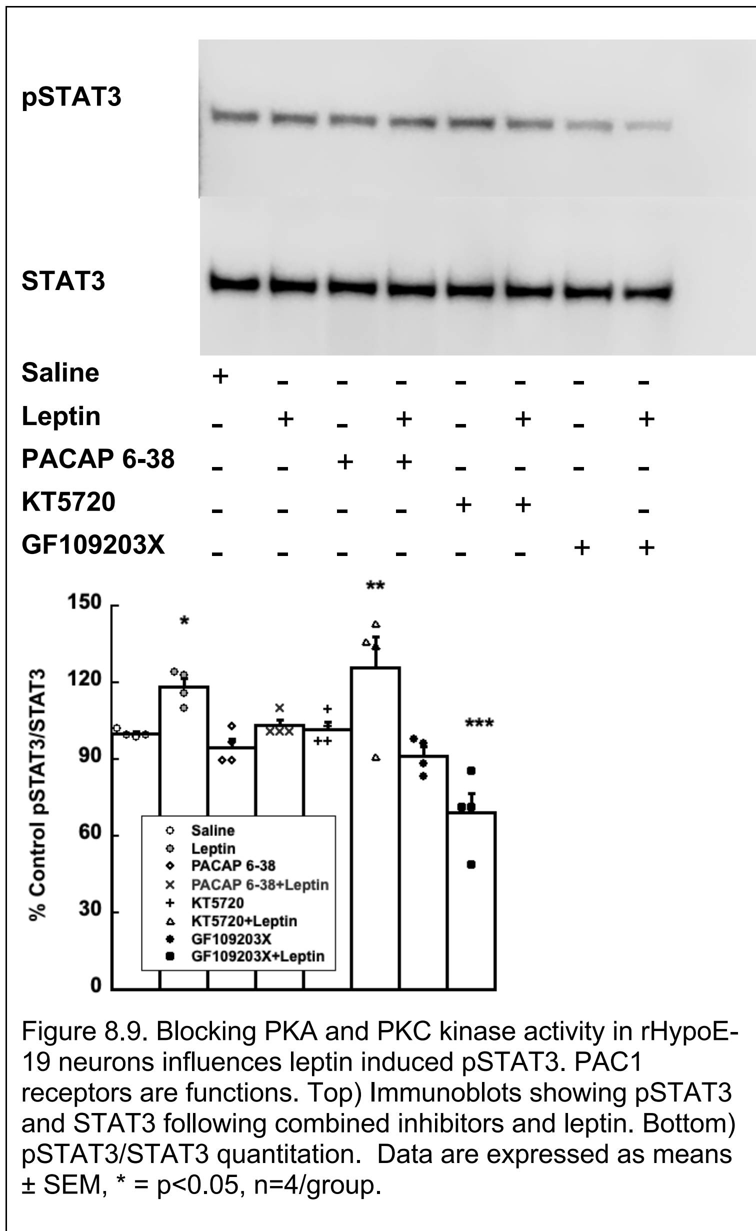


Figure 8.9. Blocking PKA and PKC kinase activity in rHypoE-19 neurons influences leptin induced pSTAT3. PAC1 receptors are functions. Top) Immunoblots showing pSTAT3 and STAT3 following combined inhibitors and leptin. Bottom) pSTAT3/STAT3 quantitation. Data are expressed as means ± SEM, * = $p<0.05$, n=4/group.

The extent to which PAC1 and leptin receptors co-localize raises the possibility that these receptors either share a physical interaction, or they exist in a shared complex that allows for very close proximity. Although the capacity for PAC1 and leptin receptors to form PAC1R-LepRb heterodimers has not yet been reported, and the two receptors are entirely dissimilar, recent advances in molecular biology suggest potential mechanisms for crosstalk. Reciprocal GPCRs and cytokine receptor regulation has been proposed to occur indirectly through transactivation review in (W. Wang et al., 2018). For example, PAC1R has been show to regulate transactivation of the receptor tyrosine kinases for the epidermal growth factor (EGFR), HER2, and HER3) by modulating receptor phosphorylation (Moody et al., 2020). Such a PAC1 and leptin receptor collaboration could provide a potential mechanism by which energy homeostasis regulation combines GPCR-related signaling with cytokine signaling. For example, one receptor can be differentially coupled to two transduction pathways depending on a specific naturally distinct ligand. Alternatively, this receptor could collaborate or interact with another receptor to modify the pharmacology of a ligand binding site and/or the molecular interactions within the receptor binding site to influence coupling. Thus, dysregulation in PAC1 or LepRb signaling could impact the intrinsic function of PACAP and leptin signaling necessary for normal energy homeostasis regulation.

While the mechanism by which PACAP blocks leptin function remains speculative, we have in this study explored possible aspects of PACAP receptor activation that may intersect with leptin signaling. For example, we found that

PACAP induces pJAK2, suggesting that the JAK/STAT3 signaling cascade is a potential link between leptin receptor cell surface activation and nuclear transcriptional events leading to changes in physiology or behavior. While JAK/STAT3 is considered a canonical cytokine signaling sequence, recent studies have revealed that JAK/STAT3 can also be activated by GPCRs (Marrero, Venema, Ju, Eaton, & Venema, 1998). GPCRs principally coupled to Gαq have been shown to associate and couple to JAK2 (Ferrand et al., 2005). PAC1 receptors are known to couple to Gαs, and Gαq-related signaling in several brain regions and recently, the VMN (H. Hashimoto et al., 1993; Maunze et al., 2022). To that end we explored whether blocking Gαs/cAMP/PKA or Gαq/PLC/PKC-related signaling influenced leptin's ability to induce pSTAT3. We found that blocking PKC, a Gαq signaling mechanisms prevents leptin induced pSTAT3. However, blocking PKA prior to leptin, potentiated leptin stimulated pSTAT3. This finding is not shocking considering earlier reports demonstrating that leptin potently stimulates pSTAT3 in the absence of functional PKA in part by preventing PKA-dependent transcription of SOCS3, a negative regulator of leptin signaling (L. Yang & McKnight, 2015). Although Gq/PKC signaling has not been directly implicated in pSTAT3 induction, Gq/PKC signaling has been shown to regulate JAK2, a known upstream regulator of pSTAT3 (Ferrand et al., 2005). In summary, the PAC1R and leptin receptor immunocomplex suggests a functional or overlapping dependence in PACAP and leptin receptor signaling that involves JAK2 and PKC.

CHAPTER IX

GENERAL DISCUSSION

The hypothalamic ventromedial nuclei (VMN) integrate information about the body's energy state and responds accordingly to maintain energy balance (Flak et al., 2020; Hawke et al., 2009; Hetherington & Ranson, 1942; King, 2006). Previously, our lab and others demonstrated that pituitary adenylate cyclase-activating polypeptide (PACAP) and the Type 1 PACAP receptor (PAC1R) are abundantly expressed in the VMN, and exogenous activation of those receptors cause hypophagia and increased metabolism (Hawke et al., 2009; Resch et al., 2011, 2013). However, the endogenous role of PACAP in the VMN remain unclear.

The findings described in this dissertation demonstrate that endogenous PACAP signaling has a role in normal VMN regulation of energy balance since knocking down VMN PAC1 receptors increased overall food intake and body weight, which is consistent with global PACAP and PAC1R knockout models (Gray et al., 2001, 2002; H. Hashimoto et al., 2000). The increase in food intake was characterized by an increase in meal size along with a decrease in meal number suggesting that endogenous PACAP may exert short-term changes in energy requirements by serving to signal satiety or meal duration. In our case, the absence of PAC1 receptors and the subsequent reduction in the satiety signal could leave a hunger drive unopposed. Previously reported in mice, PACAP signaling correlates with energy status and increases immediately

following a meal. Starved mice show decreased VMN PACAP and PAC1R mRNA expression, which is reversed following food presentation (Delgado et al., 2019; Hawke et al., 2009; Resch et al., 2011). This emergence of PACAP's influence on food intake and meal microstructure is not unusual, Resch et al showed that PACAP in the paraventricular nuclei (PVN) increases latency to engage in meals and overall food intake (Resch et al., 2013), which is not surprising considering that both VMN and PVN express PACAP and project and receive input from each other, as shown in retrograde tracing experiments (Resch et al., 2013). Such a feature can be found in other orexigenic and anorexigenic neuropeptides such as neuropeptide Y (NPY), histamine, serotonin (Leibowitz & Alexander, 1998) and dopamine that act in the hypothalamus to regulate meal structure and overall feeding, see review (King, 2006). The VMN is well positioned to regulate meal-related satiety information since it receives inputs from the nucleus tractus solitarius (NTS) (Lindberg, Chen, & Li, 2013), likely conveying gastrointestinal tract information regarding gastric distension and glucose levels (Travers, Travers, & Norgren, 1987). Future studies will have to determine if NTS neurons that project to the VMN contain PACAP.

Following PAC1R knock-down, the increase in food intake and weight gain could be explained mostly in part, by the subsequent decrease in brain derived neurotrophic factor (BDNF) transcripts. Previous studies show that in mice, BDNF deficiency in the VMN cause overeating and weight gain (Mou et al., 2015). Moreover, it does not come as a surprise that BDNF expression decreases following PAC1R knockdown since we and others have consistently

demonstrated that PACAP signaling stimulates BDNF transcription in hypothalamic brain regions (Macdonald et al., 2005; Resch et al., 2011; Yaka et al., 2003). Thus, PACAP-induced hypophagia may be facilitated, in part, by BDNF transcription and release either in the VMN or VMN projections.

In addition to neuropeptides, hypothalamic cell groups express numerous receptors for both intrinsic and extrinsic signaling molecules to regulate both feeding behavior and metabolism. One in particular that has been given considerable attention is the leptin receptor, which binds the adipose secreted leptin. Mutations in the obese (ob) gene encoding leptin or the diabetes (db) gene encoding the leptin receptor leads to positive energy states with increased feeding behavior, hypometabolism, hyperinsulinemia, and obesity. The hypothalamus abundantly expresses the receptors for leptin, which require active transport across the blood-brain barrier. Leptin administration into the VMN mimics the behavioral and metabolic responses that are produced by exogenous PACAP administration such as hyperphagia, elevated core body temperature, and weight gain. While the similar functional outcomes could suggest parallel but distinct pathways to coordinate energy balance, it could also suggest that these two receptors have a shared circuitry.

Rats lacking VMN PAC1 receptor expression gain a considerable amount of weight and allow for the speculation that PAC1R knockdown may dysregulate energy sensing mechanisms such as insulin or leptin signaling systems in the hypothalamus. In support of this idea, previous work in our lab demonstrated that pharmacologically inhibiting PACAP receptors in the VMN with a PAC1 receptor

antagonist prevented the canonical responses to VMN leptin. In the current studies, we injected leptin in rats following VMN PAC1R knockdown produced by RNA interference and found that exogenous leptin failed to induce canonical cytokine signaling, suppress feeding and decrease body weight, and not only did it not produce a significant increase in SOCS3 mRNA, there was a significant decrease in SOCS3 mRNA levels. With the growing evidence that PACAP and leptin signaling could be intertwined in the VMN, it becomes necessary to have a better understanding of PAC1 receptor signaling mechanisms that could interact with the leptin receptor signaling cascade. Conversely, future studies examining PACAP actions on energy balance will need to factor in body weight and tonic circulating levels of leptin since the receptors may be functionally connected. Since circulating leptin levels are typically proportional to body weight adipose tissue (Buchanan et al., 1998; Sims et al., 2020), future considerations will have to take into account basal circulating levels of leptin. Additionally, leptin signaling malfunction is typically characterized by leptin insensitivity (Farley et al., 2003; Morris & Rui, 2009), which has been implicated in the majority of all human obesity.

Genetically knocking down PAC1R or using a pharmacological inhibitor for the receptor decreases suppressor of cytokine signaling 3 (SOCS3) mRNA, which would be expected since PACAP signaling is known to increase SOCS3 transcription. However, we find it perplexing considering that rats still manage to increase food consumption and gain weight despite the fact that SOCS3 functions as a negative feedback inhibitor on the leptin receptor. Diminishing

SOSC3 concentrations should alleviate negative actions on the leptin receptor and increase the sensitivity of the receptor to leptin and subsequently decrease feeding. This is contrary to results from recent studies showing that mediobasal SOCS3 deficiency is protective of overeating and weight gain and SOCS3 deficiency improves leptin signaling and sensitivity (Matarazzo et al., 2012). In studies in which PAC1R knock down increased feeding behavior, an acute leptin injection in the VMN did not induce hypophagia suggesting that PACAP signaling through PAC1R in the VMN may have an independent or alternate path to parts of the leptin receptor cascade. In support of this possibility is the fact that PACAP can phosphorylate Janus Kinase 2 (pJAK2), which is known to phosphorylate signal transducer and activator of transcription 3 (STAT3), that in turn drives SOCS3 transcription. Moreover, this is consistent with reports that other class B G-protein coupled receptors (GPCRs) also induce pJAK2 (Marrero et al., 1998), suggesting a putative explanation for the decreased SOCS3 in the absence of PAC1R.

While it was previously unclear how PACAP induces pleiotropic effects on VMN regulation of feeding and energy expenditure, the current studies may begin to add some clarity. PAC1 receptors are class B G-protein coupled receptors that can dually couple to Gs/cAMP/PKA, and Gq/PLC, PKC (Blechman et al., 2013). In addition, the PAC1R gene (ADCYAP1R1) is highly spliced to generate several isoforms including PAC1 receptors isoforms PAC1null and PAC1hop (Braas et al., 1998; Holighaus et al., 2011; Lyu et al., 2000; Rongquiang Yang, Winters, & Moore, 2020). The experiments in chapter (II)

identified the presence of PAC1null and PAC1hop receptor isoforms in the VMN, and that PAC1hop isoform transcript increased, whereas PAC1null decreased in response to changes in energy state and stress. This finding suggests that PACAP signaling could be much more complex in the VMN control of feeding and energy expenditure than previously described. Such a dichotomy in PAC1R isoform response to physiological changes is not uncommon in the rat brain. For example, PAC1 signaling is required for physiological stress responses since PACAP activates CRH transcription in vivo (Amir-Zilberstein et al., 2012) and that PAC1R isoforms and their relative expression ratio may be involved in homeostasis in response to stress (Amir-Zilberstein et al., 2012; Blechman et al., 2013). Moreover, the activation and termination of CRH transcription, in response to stress, is dually regulated by PAC1null and PAC1hop in that PAC1hop terminates transcription and PAC1null activates transcription (Blechman et al., 2013). Similarly, PACAP's effects in the VMN may be a consequence of a collective contribution of individual PAC1R isoforms. This is exemplified in our experiment showing that injecting PACAP-conjugated saporin should ablate all VMN PAC1R isoforms.

As mentioned previously, fasted mice exhibited decreases in PAC1R mRNA expression in the VMN with a reversal following refeeding (Delgado et al., 2019; Hawke et al., 2009; Resch et al., 2011). However, in our early studies we failed to detect any changes in rat PAC1R mRNA (without consideration of isoforms) regardless of an animal's energy state (data not shown). By contrast, when we probed for specific PAC1R isoforms we found that PAC1null mRNA

decreases whereas PAC1hop increases after a meal. Therefore, comprehensive PACAP signaling in the current studies most likely entails contributions from PAC1 receptor isoforms PAC1null and PAC1hop. In addition, the ability to parse PACAP signaling in this manner may be key to explaining the pleiotropic molecular and physiological consequences of PACAP signaling. Recent work in cardiac, and dentate neurons of the hippocampus demonstrate that PAC1 receptor endocytosis following stimulation is necessary for action potential generation and physiology, and is dependent on PKC (May et al., 2014). Interestingly, the PAC1hop isoform contains a consensus sequence for PKC phosphorylation possibly contributing to fine-tuned regulation of receptor function (May et al., 2014) and would also be in line with studies showing that PACAP signaling potentiates glutamatergic signaling by modulating AMPA and NMDA receptors (Macdonald et al., 2005; Resch et al., 2014; Yaka et al., 2003).

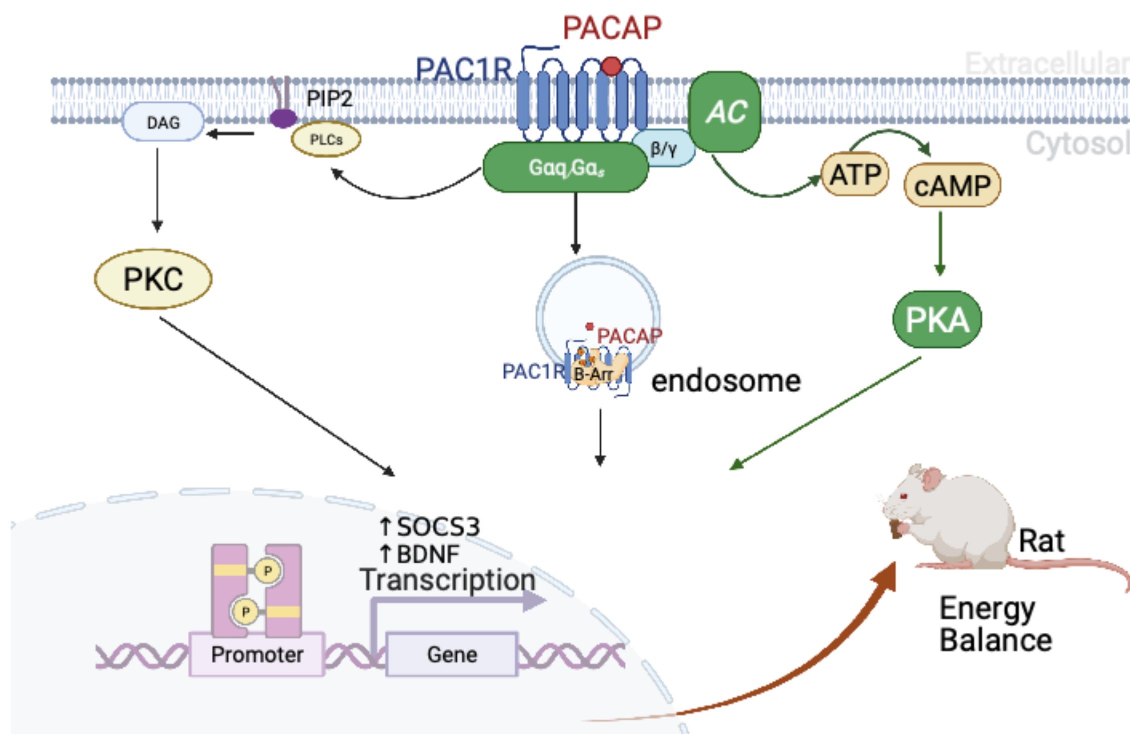


Figure 9.1. Schematic of PAC1 receptor signaling at the plasma membrane and endocytosis. PACAP/PAC1R activation can engage multiple signaling from the plasma membrane to the cytosol following internalization. PACAP/PAC1R activation can stimulate adenylyl cyclase (AC), cAMP/protein kinase A (PKA), or phospholipase c (PLC)/ diacyl glycerol (DAG) protein kinase C (PKC), and endocytosis. Created with BioRender.com

To begin to describe which PACAP signaling mechanisms may explain PACAP's effects in VMN-dependent hypophagia and hyperthermia, we examined known downstream effectors of PAC1R signaling. As previously mentioned above, PAC1 receptors are GPCRs that can dually couple to Gs/cAMP/PKA and Gq/PLC/PKC PKA, which have directly been implicated in regulating hypothalamic energy balance (Nakajima et al., 2016; L. Yang & McKnight, 2015). Blocking PKA or PKC activity prior to PACAP injections at the onset of the dark

phase attenuated PACAP-induced hypophagia, suggesting that PACAP utilizes both signaling mechanisms to regulate feeding. However, PKA or PKC inhibition during the light phase did not block but enhanced PACAP's hyperthermia, whereas only PKA inhibition enhanced spontaneous locomotor activity. One possible explanation is that blocking PKA or PKC might disinhibit other mechanisms in the VMN that positively drive energy expenditure such as leptin. A recent study in hypothalamic arcuate neurons showed that PKA deficiency impairs hypothalamic SOCS3 expression and prevents feedback inhibition of leptin signaling (L. Yang & McKnight, 2015). Adding to the possibility that blocking VMN PKA might facilitate leptin signaling to potentiate energy expenditure, which would need to be confirmed in future studies.

An alternative explanation for how PKA and PKC may influence VMN regulation of feeding and metabolism involves glutamatergic signaling. PKA and PKC have both been implicated in glutamate signaling by modulating AMPA and NMDA receptors (Ciranna & Costa, 2019; Fukuchi et al., 2015; Macdonald et al., 2005; Yaka et al., 2003) to augment glutamate signaling. Earlier studies in our lab have previously demonstrated that PACAP's hypophagic effects in the VMN are mediated by glutamatergic signaling (Resch et al., 2014). Therefore, the inhibition of PKA or PKC could prevent modulation of glutamate signaling that is important for PACAP-mediated hypophagia.

PKC is also utilized in VMN neurons and studies have demonstrated that manipulations of PKC in the VMN regulate glucose metabolism thus, connecting VMN PKC activity with regulating energy homeostasis. Administration of a PKC

agonist, OAG, suppresses glucose production (Ross et al., 2008), suggesting that PACAP signaling through PKC could facilitate glucose homeostasis. This is in line with the finding by Resch et al that PVN and VMN PACAP injections increase plasma glucose (Resch et al., 2013). Moreover, direct Gq activation in VMN PACAP-containing neurons increases glucose in a glucose tolerance test. In this study, CNO was used to activate Gq DREADS and expressed in VMN cells by virus transduction (Khodai et al., 2018; Ross et al., 2008). Taken together, aspects of PACAP signaling may engage PKC signaling to regulate glucose homeostasis and influence food intake.

New and emerging model of GPCR signaling adds another possible explanation for PACAP's numerous effects on energy balance. The generation of second messengers by GPCRs like PAC1R and its termination is currently thought to occur exclusively at the plasma membrane. However, this paradigm is not consistent with recent observations that PAC1R stimulation continues to suppress food intake and increase metabolism long after the peptide has likely been cleared from the synapse and membrane signaling has been terminated. The transcribed PAC1 receptor protein possesses amino acid motifs within the C-terminal domain that determine G-protein coupling and receptor trafficking (Lyu et al., 2000). In addition to G-protein dependent signaling at the plasma membrane, the internalization and trafficking of GPCRs are recognized to represent fundamental processes targeting complex signaling networks to different cellular compartments (May & Parsons, 2017). We found that PACAP induces PAC1R trafficking from the plasma membrane and into the cytosol,

which could be blocked using Dyngo-4a and Pitstop 2, dynamin and clathrin inhibitors. This finding suggesting that PAC1R behaves similarly to other class B GPCRs that internalize following activation. However, a recent study demonstrated that PAC1Rs are rapidly phosphorylated and internalized immediately (Lyu et al., 2000) and approximately 50% are internalized within 3 minutes after application of radiolabeled PACAP and reaching a plateau after 60 minutes (Lyu et al., 2000). While it is still unclear whether internalized receptors are degraded or recycled back to the plasma membrane, the internalization of the receptor may play a role in the enduring behavioral and physiological effects of PACAP receptor activation.

Canonically, GPCR kinases (GRKs) phosphorylate the C-terminal tail of GPCRs to attenuate G-protein signaling and allow for the binding and activation of β-arrestins which mediate receptor desensitization and internalization. However, recent work has shown that some GPCRs engage in sustained G-protein signaling from within internalized cellular compartments rather than desensitizing the complex. β-arrestins, which are classically considered signaling terminating proteins, are critical signal transducers at GPCRs in the membrane as well as downstream of numerous GPCRs through their ability to nucleate signaling complexes containing members of the MAPK and Src kinases (Broca et al., 2009; DeWire et al., 2007; Eichel & Von Zastrow, 2018; Shintani et al., 2018; Thomsen et al., 2018). Activated cardiac and dentate neurons show PAC1R forms a complex with β-arrestins and becomes critically necessary for receptor trafficking, neuronal excitability and consequent biological outcomes (Johnson et

al., 2020; May et al., 2021; May & Parsons, 2017; Parsons & May, 2018). With data directly implicating β-arrestins in mediating this phenomenon, we hypothesize that PAC1R signaling is potentially mediated by endocytosis. While it has already been demonstrated that PAC1Rs associate and internalize with β-arrestins in vitro, this mechanism remains to be elucidated in VMN control of food intake and metabolism.

Our data indicate that PACAP promotes PAC1R trafficking from the membrane to the cytosol as well as PACAP-induced PAC1R and β-arrestin association in a dose-dependent manner. Specifically, we demonstrated the VMN PAC1 receptors form a complex with β-arrestin 2 following PACAP stimulation. Others have demonstrated that PACAP stimulation induces PAC1R association with β-arrestins leading to differential β-arrestin 1 or β-arrestin 2 subcellular co-localization with PAC1R (Shintani et al., 2018). In this case, PAC1Rs associated with β-arrestin 1 localize to the plasma membrane, whereas PAC1Rs associated with β-arrestin 2 appear to be internalized into the cytosol (Shintani et al., 2018). Moreover, its been shown that β-arrestins influence energy homeostasis and prevent diet induced obesity suggesting a putative mechanism by which PACAP signaling may influence energy homeostasis (Pydi et al., 2020; Rodgers & Puigserver, 2009; Zhuang et al., 2011). In the current work, we examined whether PAC1 receptor endocytosis was required for VMN regulation of energy balance by inhibiting PAC1R endocytosis. We found that blocking β-arrestin dependent endocytosis, with barbadin was not sufficient to block PACAP induced hypophagia although, blocking clathrin and dynamin

proteins using a Dyngo-4a and Pistop 2 cocktail did prevent PACAP-induced hypophagia.

PACAP and leptin receptors are both potent regulators of food intake and metabolism in the VMN via their intracellular signaling cascades that involve G-protein and cytokine signaling, respectively (Dhillon et al., 2006; Hawke et al., 2009; Hurley et al., 2020; Resch et al., 2011). We and others have also demonstrated that PACAP and leptin induce the phosphorylation of STAT3 and transduction of SOCS3 and BDNF mRNA expression. More recently, we showed that blocking PACAP signaling (Hurley et al., 2020) or knocking down PAC1Rs using RNA interference prevents leptin receptor function in the VMN. Collectively, these findings suggest a crosstalk between PACAP and leptin signaling. To explore the means of this crosstalk, we used co-immunoprecipitation to demonstrate that PAC1R and leptin receptors form an immunocomplex, suggesting these two receptors either share a physical interaction or are in close proximity and form a signaling complex. Interestingly, we found that PACAP treatment in PAC1R expressing rHypoE-19 cells induced pJAK2, which is in line with reports that other GPCRs have the ability to phosphorylate Janus Kinase (Ferrand et al., 2005). The characteristics demonstrated here for PAC1 and leptin receptors are similar to findings that suggest the two receptors may signal through GPCR transactivation mechanisms, extensively reviewed by Wang et al (W. Wang et al., 2018). Several studies indicate that GPCRs transactivate cytokine or receptor tyrosine kinases (McCole, Truong, Bunz, & Barrett, 2007; Shi, Jin, & Andres, 2010) and, in turn, cytokine receptors can reciprocally

transactivate GPCRs. (W. Wang et al., 2018) propose that there are two possible modes of transactivation between a GPCR and a receptor tyrosine kinase: the ligand dependent and the ligand independent pathway (W. Wang et al., 2018). The ligand-dependent pathway is mediated by an active GPCR induced ligand whereas, in the ligand-independent pathway GPCRs activate Src kinases. This latter method is intriguing since PACAP not only induces Src kinase phosphorylation but Src kinase appears to be required for PACAP's hypophagic effects (Resch et al., 2014). Interestingly, Src has been shown to induce pSTAT3 which could account for the observed PACAP-induced phosphorylation of STAT3 through a pathway that is potentially independent of leptin. Even more convincing is a recent report (Shi et al., 2010) demonstrating that PAC1Rs transactivate tyrosine kinase receptors such as EGFR, HER2, and HER3 (Moody et al., 2020), and insulin like growth factor 1 (IGF1) (Delcourt et al., 2007). A recurring theme and common denominator in PACAP-dependent transactivation of RTKs and cytokine receptors is Src kinase.

Taken together we have accumulated evidence for a potential model signaling mechanism that may explain how signaling from a single peptide is amplified to regulate several aspects of cell signaling and physiology through close proximity and a shared PAC1 and leptin receptor signaling. Findings from this work revealed that endogenous PACAP signaling in the VMN has a potent regulatory influence over both energy intake in the form of feeding and energy output via thermogenesis and locomotor activity. Moreover, PACAP actions in the VMN share a nearly identical molecular and physiological consequences to

leptin administration in the same brain region suggesting that these two neuropeptides could functionally intersect. These experiments begin to explore the VMN PAC1Rs dependence on PKA, PKC, and receptor trafficking to mediate PACAP's pleiotropic effects on feeding and metabolism as well as potential intersecting points with leptin receptor signaling. By understanding the complex cell signaling complex involving PACAP and leptin we might find more complex receptor configurations such as transactivated receptors, which may open new doors to understanding the neurochemistry of behaviors and physiological regulation as well as provide better insight to disease states like obesity and their

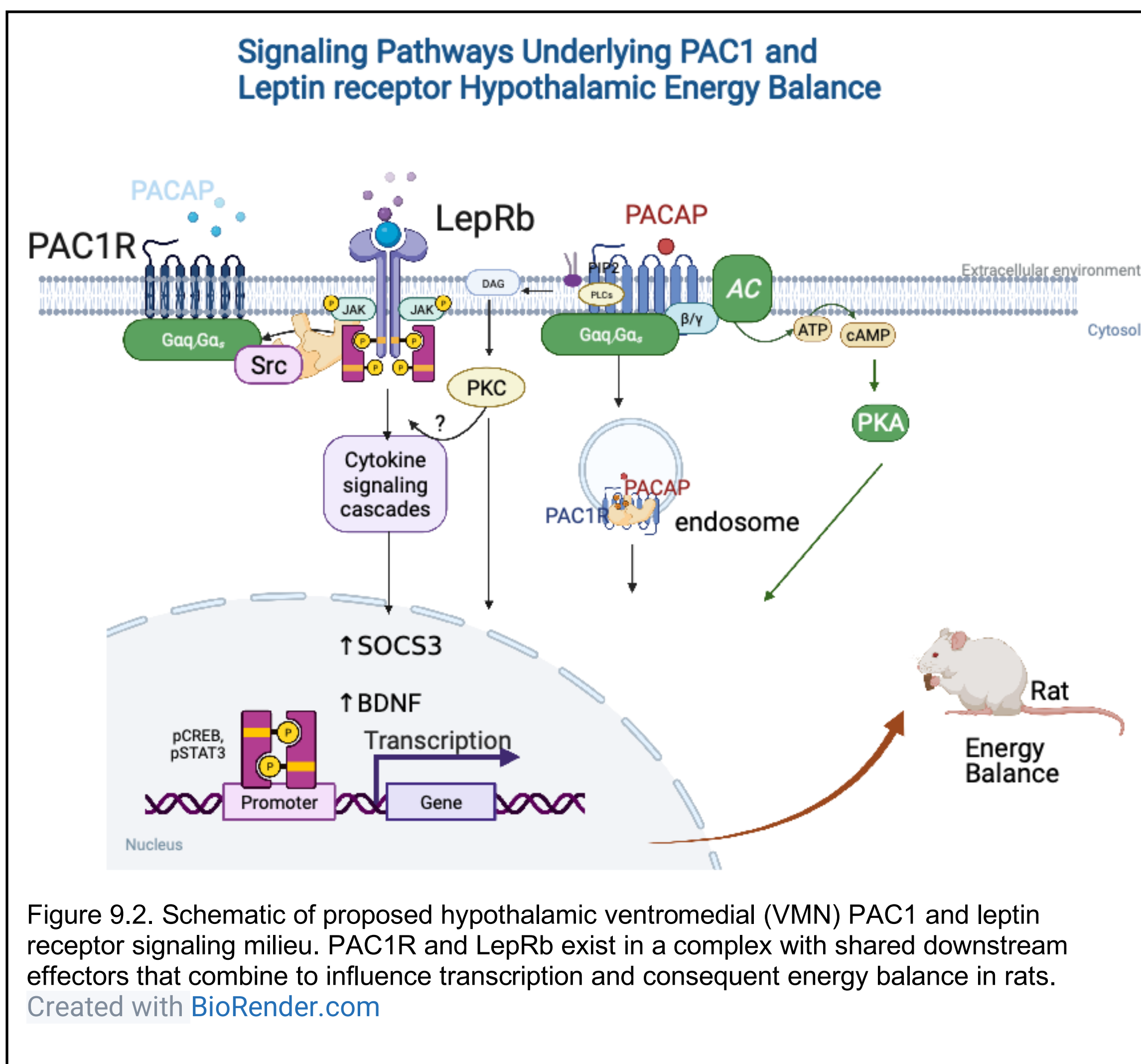


Figure 9.2. Schematic of proposed hypothalamic ventromedial (VMN) PAC1 and leptin receptor signaling milieu. PAC1R and LepRb exist in a complex with shared downstream effectors that combine to influence transcription and consequent energy balance in rats.
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therapeutic treatment. Receptor transactivation has emerged as an important aspect of the GPCR repertoire that allows GPCRs access to a broader array of highly interconnected signaling mechanisms that influence numerous physiological and pathological conditions (Fig. 9.2). Therefore, nuanced approaches to characterizing PAC1R signaling will be necessary for the development of novel drugs to treat disease states like overweight and obesity.

PERSPECTIVES ON THE RELEVANCE AND NECESSITY FOR NUANCED UNDERSTANDING OF CELL SIGNALING TRANSDUCTION GOVERNING ENERGY BALANCE.

The role of biochemical signaling transduction networks is to reliably transmit specific information about the extracellular environment to downstream effectors, allowing the cell to adjust its physiological state to changing conditions (Brennan et al., 2012; Selimkhanov et al., 2014). Understanding the complex network of information relays in the form of signal transduction is critical as evidence of aberrant signaling is emerging in diseases such as obesity (Abdelsalam et al., 2019; Dorfman et al., 2017; Z. Gao et al., 2007; Sutton et al., 2004; Zheng et al., 2013).

The stochasticity of molecular interactions that underlies various forms of competing signal transduction perturbations (noise) in biological systems can interfere and influence the directionality of biochemical signal transduction while also degrading the transmitted information (Brennan et al., 2012; Purvis & Lahav, 2013; Selimkhanov et al., 2014).

The work in this thesis has revealed an endogenous role of VMN PACAP while also shedding some light on the nature of exogenous PACAP-dependent downstream signaling on feeding and metabolism that may be perceived as stochastic at first glance. What has become apparent is that even with a knowledge of the downstream effectors of PACAP signaling, the comprehensive state of the system (VMN) cannot be precisely predicted. This includes the observations of the negative and positive influences of PKA, PKC or endocytosis inhibition on light phase or dark phase PACAP-dependent effects on feeding and metabolism, which raises the question, how do biochemical signaling transduction networks perform their core functions in the presence of a cacophony of crosstalk?

Protein kinases and subcellular cytoskeletal structures are key transducers in signaling transduction networks that determine the collective spatial resolution and dynamic properties of signaling that influences physiology (Rosse et al., 2010). Current ligand-receptor models of signaling are based on scalar measurements performed at a single time point and assume comprehensive whole cell signaling (Selimkhanov et al., 2014). However, the information on activating ligands like PACAP is likely encoded using dynamic signaling represented by a multivariate vector such as combined PACAP-induced PKA, PKC, and PAC1R trafficking and other molecular events that are contained as a single cell’s response at multiple timepoints. The effect of signal location can be as important as signal strength in determining net cell response. The localization of signaling molecules to appropriate subcellular milieu that possess

appropriate downstream targets is critical and may determine the output of a particular signaling cascade.

The relevance of kinases, PKA and PKC has long been recognized in cell biology, and subcellular location of kinase activity has important physiological importance (Gervasi et al., 2007; Igumenova, 2015; Rosse et al., 2010). For example, PKA at the membrane can modulate receptor and ion channels to influence neuronal excitability, whereas in the cytosol and nucleus, PKA affects cytoskeletal proteins, metabolic enzymes, and gene transcription (Gervasi et al., 2007). The functional compartmentalization of kinase activity has been demonstrated in cardiomyocytes and neurons that process spatially restricted signaling microdomains (Koschinski & Zaccolo, 2017; Rosse et al., 2010; Sposini et al., 2017).

Our findings in this dissertation isolated (static) VMN experiments involving PKA, PKC, and endocytosis inhibition and their influence on PACAP signaling during the circadian light and dark phases suggest the likelihood that VMN PACAP-dependent dynamic responses contain more information than static responses where PACAP influences feeding and metabolism. This relevant functional information may be encoded in the structural components of the cells and will require empirical measurements in sufficient detail. Despite the recognition of the importance of localized signaling, our ability to locally interfere with signals to prove evidence of necessity and sufficiency has been limited by a lack of appropriate experimental methodologies. However, albeit mostly descriptive, encouraging new advances is microscopy resolution and

developments in genetically engineered tools that can track and localize signaling events allowing us to nuance our interpretations. Such promising tools can be leveraged to develop future strategies that allow manipulation of spatiotemporal and dynamic physiological contexts. These strategies may employ unique cell compartmentalization markers that deliver and limit cell perturbations to subcellular compartments. Nonetheless, there is immense value that can be derived from scalar biological measures, although there is understanding that aspects of biological function cannot be discovered by simply looking at static structures.

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