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PACAP: A NOVEL NEUROPEPTIDE FOR PITUITARY GONADOTROPH MATURATION, FUNCTION AND REGULATION

By

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B.S., University of Sciences and Technology of China, 2005
M.S., University of Louisville, 2009

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Anatomical Sciences and Neurobiology
University of Louisville, School of Medicine
Louisville, Kentucky

May 2013

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DEDICATION

This dissertation is dedicated to my parents

Mr. Xiuzhang Yang

and

Mrs. Zhihua Shi

Who love and support me without reservation

To my dear Guoqian

Who always understands and encourages me with heart and soul

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I would like to thank my super advisor, Dr. Joseph Patrick Moore, for his support, guidance and patience. I would also like to thank another research advisor, Dr. Stephen Winters, for his kindly help and suggestions. I am indebted to my other committee members Drs. Charles Hubscher, Martha Bickford and Robin Krimm, for their comments and assistance. Many thanks to the faculty and students of Anatomical Sciences and Neurobiology who have given me knowledge, help and friendship. I would also like to thank our lab manager, Mr. Dushan Ghooray, for his help on my experiments. Also, Many thanks to my lab neighbor, Dr. Betty Villafuerte, for using her equipment.

Finally, special thanks to my wife Guoqian Yan, for her unconditional love and support, and to my parents and family members for their love.

ABSTRACT

PACAP: A NOVEL NEUROPEPTIDE FOR PITUITARY GONADOTROPH MATURATION, FUNCTION AND REGULATION

Rongqiang Yang

May 2, 2013

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino acid C-terminally α-amidated peptide that was first isolated from ovine hypothalamic extracts on the basis of its ability to stimulate cAMP production in anterior pituitary cells. As a member of the vasoactive intestinal polypeptide (VIP)/secretin/growth hormone-releasing hormone/glucagon superfamily, PACAP has been well conserved during evolution from sea squirt to humans, which suggests important biological functions. Two types of PACAP receptors have been characterized. PAC1-R is the only one PACAP-specific receptor with high affinity.

Although PACAP was first found in hypothalamus, it is also expressed in the pituitary. In the research to be presented, PACAP and PAC1-R mRNA expression in the rodent pituitary and in gonadotroph cell lines were explored using semi-quantitative PCR, laser capture micro-dissection (LCM) and single cell PCR. The level of pituitary PACAP expression is high in the fetus and declines after birth. Most of pituitary PACAP is from gonadotrophs. PAC1-R in

fetal pituitary exists as the Hop and short forms, and the level of the short form decreases after birth.

The effects of PACAP on gonadotropins synthesis and secretion were studied *in vivo* and *in vitro*. The action of PACAP on LH and FSH was investigated utilizing micro-pump implanted, containing PACAP38 or the antagonist, PACAP 6-38, in pre-pubertal male rats. Protein and mRNA analysis revealed that PACAP suppresses FSH presumably through increased follistatin, but had no significant influence on LH. The existence of PACAP, therefore, likely contributes to maintain an appropriate gonadotropin environment during sexual development.

We also studied pituitary cell cultures and gonadotroph cell lines to understand why PACAP expression in pituitary is high in the fetus, and declines at birth. PACAP was found to stimulate its own expression in that the PACAP 6-38 antagonist reduced the PACAP mRNA level in primary cell cultures, and PACAP38 induced high activity of PACAP promoter in gonadotroph cell lines. Furthermore, we found preliminary evidence to support the ideal that dopamine receptor 2 (Drd2) signaling may explain the neonatal decrease in pituitary PACAP mRNA levels. Finally, we propose that the decrease in PACAP results in a fall in pituitary follistatin, allowing for increased activin signaling which increases FSHβ, facilitates the sexual maturation of the gonads.

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CHAPTER ONE

GENERAL INTRODUCTION

1. General introduction of PACAP

1.1. Discovery of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

Each of the classical hypothalamic hypophysiotrophic hormones had been isolated and identified by the mid-1980s. Since then, each of the classical releasing hormones, TRH, LHRH, GHRH, and CRH had been demonstrated to stimulate adenylate cyclase in cultured pituitary cells (Culler et al., 1984; Labrie et al., 1979; Labrie et al., 1982). Arimura and colleagues proposed to find out whether there were undiscovered hypothalamic releasing hormones which would also stimulate adenylate cyclase. This strategy led to the identification of a peptide in ovine hypothalamus that they named Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) (Miyata et al., 1989).

Through sequence analysis, PACAP was shown to have 38-amino-acids (Miyata et al., 1989), but was also present in smaller amounts as a shorter amidated form containing only the N-terminal 27 amino acids (Miyata et al., 1990). The two forms with 38 and 27 residues were then named PACAP38 and

PACAP27. PACAP38 and PACAP27 are equivalent stimulators of adenylate cyclase (Miyata et al., 1990). However, PACAP38 and PACAP27 bind with different affinities to some PACAP receptor variants (Dautzenberg et al., 1999; Pantaloni et al., 1996).

1.2. Structure of Pituitary Adenylate Cyclase Activating Polypeptide

As a small peptide, the secondary structure of PACAP is relatively simple. Circular dichroism and nuclear magnetic resonance indicated that PACAP27 was characterized by a helical conformation of various lengths in different mediums (Inooka et al., 1992). PACAP38 has similar conformation to PACAP27 within the 1-to-27 region and is connected with a short helix (28-to-38 region) by a flexible hinge (Wray et al., 1993). The conformation of PACAP may change following binding with PACAP receptors (Inooka et al., 2001; Sun et al., 2007).

1.3. The Gene Encoding PACAP

The *PACAP* genes from different species were cloned soon after its discovery in 1989 (Inooka et al., 1992; Kimura et al., 1990; Ogi et al., 1990). The *PACAP* gene has five exons and four introns, and the introns are bounded by the consensus splicing sequences 5'-GT and 3'-AG. PACAP38 and PACAP27 are encoded by exon 5 whereas Exon 4 encodes PRP (PACAP-related peptide) and exon 1 is un-translated (Hannibal et al., 1995; Hosoya et al., 1992). In the *PACAP* gene, two CRE (cAMP-response-like element) and growth hormone transactivator factor-1 response elements, a GATA box, and a C-rich domain with GC boxes are conserved through different species (Ohkubo et al., 1992; White et al., 2000). The organization of the *PACAP* gene is similar to the that of

the *VIP* and *GHRH* genes, which are in the same superfamily (Lamperti et al., 1991; Mayo et al., 1985; Ohkubo et al., 1992).

1.4. Processing of the PACAP Precursor

The PACAP precursors contain seven mono- or dibasic residues, which are similar to VIP precursors. They can be cleaved by various prohormone convertases (PC) including PC1, PC2, PC4, PC5, PC7, Furin, and paired basic amino acid-cleaving enzyme 4 (PACE4) (Seidah and Chretien, 1999; Seidah et al., 1994; Seidah et al., 1998). In mammals, the processing of the PACAP precursors has been thoroughly characterized. The first, cleavage occurs at three dibasic sites, Arg^{79}-Arg^{80}, Lys^{129}-Arg^{130}, and Arg^{170}-Arg^{171}, to generate a large intermediate precursor of PRP (big PRP) and a glycine-extended form of PACAP38. Next, the big PRP is cleaved at a single Arg^{110} followed by hydrolysis of this C-terminal Arg residue by carboxypeptidase E, H or M to generate PRP (Eipper et al., 1992; Rouille et al., 1995). For PACAP38, the Gly169 residue allows peptidyl glycine α-amidating monooxygenase to amidate the Lys^{168} residue at the C-terminal extremity. In the final step, amidated PACAP27 is generated through the cleavage of tripeptide Gly^{158}-Lys^{159}-Arg^{160} (Okazaki et al., 1992). In sum, the processing of PACAP precursor yields three products, PACAP38, PACAP27 and PRPs.

PACAP has limited metabolic stability in the blood circulation with a half-life between 2 and 10 min after injection into mice or humans (Li et al., 2007; Zhu et al., 2003). The proteolytic enzymes dipeptidyl peptidase IV and prolyl

oligopeptidase have been reported to be involved in the degradation of PACAP (Ahren and Hughes, 2005; Tenorio-Laranga et al., 2009).

1.5. Phylogenetic Evolution of PACAP

Human, Sheep, Pig, Mouse, Rat, Guinea pig	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	100%
Chicken	-I------------------------------------	97%
Lizard	--------------------------------------	100%
Lungfish	------------------------------K-------	97%
Frog	----------------------------------I---	97%
Salmon	-------------------------------R--YR--	92%
Flounder, Halibut	----------------------------R--R---R--	89%
Sturgeon	---------------E---------------R---R--	92%
Tunicate 1	-----------------N---------	96% PACAP27
Tunicate 2	-----------------N-----IN-L-	85% PACAP27

<u>Figure 1.</u> Amino acid sequences of PACAP from different species. Percentages indicate amino acids identical between non-mammalian PACAP38 and mammalian PACAP38.

PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) superfamily, which includes glucagon, glucagon-like peptide-1 (GLP1), glucagon-like peptide-2 (GLP-2), glucose-dependent insulinotropic polypeptide (GIP), growth hormone-releasing hormone (GHRH), peptide histidine isoleucine (PHI) or peptide histidine methionine (PHM), PACAP, and VIP.

Compared to the other superfamily peptide hormones, PACAP is unique for its well-conserved primary amino acid sequence in vertebrates (Chartrel et al., 1991). The N-terminal 1 to 27 region of PACAP is responsible for the biological activity of the peptide. This region has been fully conserved in most vertebrate species, except for a few species with a single amino acid substitution (Fig. 1) (Lee et al., 2009; McRory et al., 1997; Xu and Volkoff, 2009). On the other hand, the C-terminal portion of PACAP38 is more variable and is not crucial for PACAP biological functions (Fig. 1).

1.6. PACAP Receptors

VIP and PACAP are the most closely related of these peptides because the sequence of PACAP38 shows 68% identity with VIP (Ohkubo et al., 1992). Consequently, PACAP and VIP signaling can be mediated through similar receptors. Soon after the discovery of PACAP, two classes of PACAP binding sites were characterized on the basis of their relative affinities for PACAP27 and VIP (Gottschall et al., 1990; Lam et al., 1990). Type I binding sites exhibit high affinity for PACAP and much lower affinity for VIP (Cauvin et al., 1990; Suda et al., 1992). Type II binding sites possess similar affinity for PACAP and VIP (Gottschall et al., 1990; Lam et al., 1990). From the two types of binding sites, there are three PACAP receptors cloned, which were termed PAC1, VPAC1 and VPAC2 receptors by the International Union of Pharmacology (Seidah et al., 1998). The PAC1 receptor, with 495-amino acid residues, is the type I receptor, which shows much higher affinity (100 to 1000-fold) for PACAP than VIP (Pisegna and Wank, 1993). The VPAC1 and VPAC2 receptors are type II

receptors, which have similar affinity for PACAP and VIP (Ishihara et al., 1992; Lutz et al., 1993). VPAC1, 2 and PAC1-R all belong to class B of G protein-coupled receptors families (GPCRs), which mediate many important biological functions (Hoare, 2005). There are three main mammalian GPCR families (A, B and C). The group B or secretin receptor family consists primarily of peptide hormone and neuropeptide receptors such as the secretin, calcitonin, VIP and PACAP receptors. As members of the same family, VPAC1, VPAC2 and PAC1 receptors share some common molecular properties, such as a large N-terminal extracellular domain (>120 residues), an N-terminal hydrophobic domain, and six conserved cysteine residents in the N-terminal extracellular domain and multiple consensus N-glycosylation sites (Gaudin et al., 1999; Laburthe et al., 1996).

The N-terminal extracellular domain of the PAC1 receptor is a major binding site for the central and C-terminal helical segments of PACAP (Bourgault et al., 2008; Cao et al., 1995). PAC1-R is specific for binding with PACAP but not VIP because of different sequence regions (4 -13 and 24 - 28) between PACAP27 and VIP are PAC1-R selective sites (Onoue et al., 2001; Schafer et al., 1999).

PAC1-R has nine subtypes resulting from alternative splicing after transcription (Fig. 2). Six of these subtypes are distinguished from each other by the absence or presence of two cassettes named Hip and Hop, which are located at the end of the 3rd intracellular loop of PAC1-R (Spengler et al., 1993). They are named PAC1-R-Short, PAC1-R-Hop1, PAC1-R-Hop2, PAC1-R-Hip, and PAC1-R-Hiphop1 and PAC1-R-Hiphop2. Beyond these six variants, more subtypes

were recently discovered including PAC1-R-Vs, PAC1-R-TM4 and PAC1-R-3a (Chatterjee et al., 1996; Daniel et al., 2001; Pantaloni et al., 1996). PAC1-R variants have different affinity for PACAP. Furthermore, the variants may mediate different signaling pathways in various cell types (Alexandre et al., 2002; Dickson and Finlayson, 2009; McCulloch et al., 2001; Niewiadomski et al., 2002).

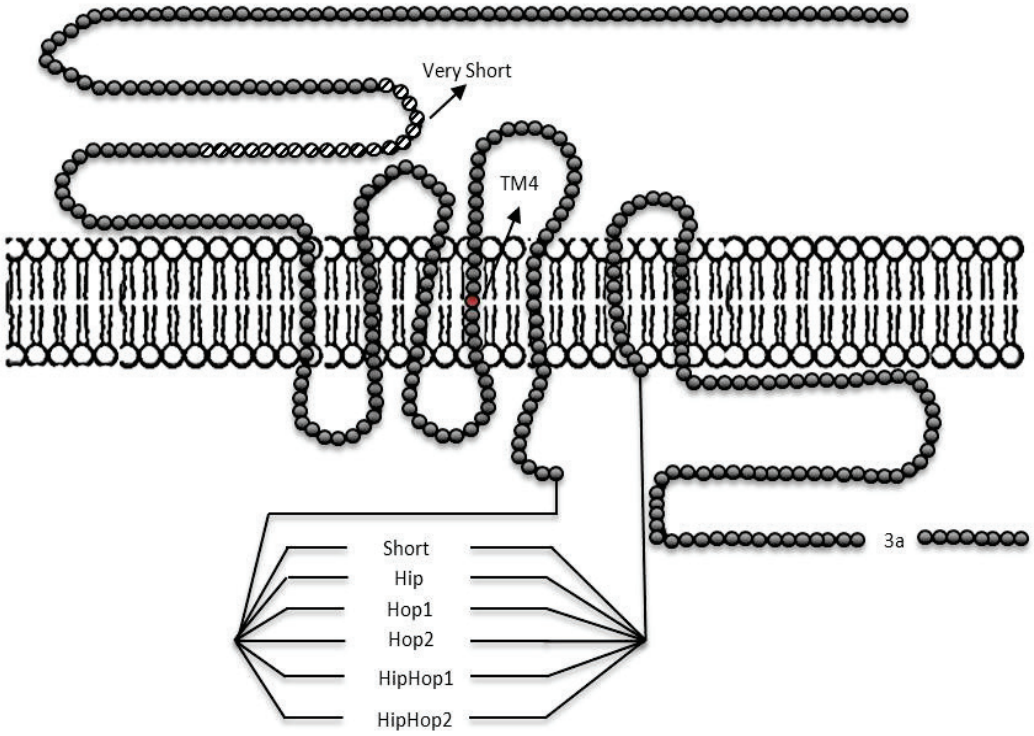


Figure 2. Schematic illustrations of the structure of the PAC1-Rs. Nine variants of the PAC1-R have been identified.

1.7. Distribution of PACAP and its Receptors

Soon after the discovery of PACAP, distribution of the polypeptide was investigated by immunoassay and PCR (Arimura et al., 1991). PACAP is widely distributed but is found in highest concentration in the Central Nervous System (CNS). PACAP is found in various brain regions, including the hypothalamic area, cerebral cortex, amygdala, hippocampus, pineal gland, substantial nigra, cerebellum, and pons, and is found in both cell bodies and fibers (Dickson and Finlayson, 2009). Although PACAP and VIP are closely related in the same superfamily, their distributions in the CNS are different (Masuo et al., 1993). For instance, in the thalamus, VIP positive fibers and PACAP fibers are localized in different regions. While VIP fibers run up the walls of the third ventricle, PACAP fibers are observed in the central thalamic nuclei (Koves et al., 1991). Similar situations occur in other brain regions, such as stria terminalis, brainstem and posterior pituitary (Koves et al., 1994; Vereczki et al., 2003). PACAP38 has been demonstrated as the major molecular form in the CNS (Arimura et al., 1991; Hannibal et al., 1995; Masuo et al., 1993; Piggins et al., 1996).

PACAP is also found in many peripheral tissues, such as most endocrine glands, the gastrointestinal tract, gonads, muscles and peripheral nervous system (Vaudry et al., 2009). PACAP38 is the predominant form in these tissues as in the CNS. In the rat testis, the concentration of PACAP is higher than any other tissues, and PACAP mRNA is only present in germ cells (Arimura et al., 1991; Hannibal and Fahrenkrug, 1995; Shioda et al., 1994). The ovary also contains PACAP but at a much lower concentration compared to testis (Steenstrup et al., 1995). PACAP is found in nerve fibers that connect with the

musculature of the gastrointestinal tracts and circulatory system (Cardell et al., 1991; Hauser-Kronberger et al., 1996; Olsson and Holmgren, 1994). In the immune system, PACAP is present in the lymphoid tissues, including the thymus, spleen and in the peritoneal macrophages (Vaudry et al., 2009). Unlike in the CNS, PACAP and VIP in peripheral tissues are often found in the same cells, and show similar expression patterns. PACAP and VIP have been co-localized in cell bodies and nerve fibers in esophageal sphincter, gut, parathyroid glands and the respiratory tract in mammals (Luts and Sundler, 1994; Sundler et al., 1992; Uddman et al., 1991). PACAP is found in the anterior pituitary and in high concentration during fetal life (Moore et al., 2009a).

VPAC and PAC1-R are widely expressed in many kinds of tissues (Vaudry et al., 2009). In the Nervous system, high concentrations of PAC1-R are found in many brain structures including olfactory bulb, cerebral cortex, septum, amygdala, cerebellum, hippocampus, thalamus, pons and hypothalamus (Vaudry et al., 2009). VPAC receptor expression levels are much lower than PAC1-R in many brain regions such as cerebellum and hypothalamic nuclei. Furthermore, the two VPAC receptors, VPAC1-R and VPAC2-R, have completely different distributions in the rat CNS (Ishihara et al., 1992; Usdin et al., 1994). Hippocampus is the only region of the CNS in which both VPAC1-R and VPAC2-R are found in (Usdin et al., 1994). In summary, the expression and density of PAC1-R is more abundant and wider compared to VPAC receptors in the CNS.

Outside the CNS, the PAC1-R has been characterized in most endocrine tissues, including the pituitary, pancreas, placenta, adrenal, ovary and testis

(Vaudry et al., 2009). In the testis, PAC1-R is found in germ cells, Leydig cells and Sertoli cells (Daniel et al., 2001; Romanelli et al., 1997; Shivers et al., 1991). In the immune system, PAC1-R is expressed in rat peritoneal macrophages but not in peritoneal lymphocytes (Delgado et al., 1996; Ganea, 1996). VPAC receptors exhibit a distinct distribution patterns in peripheral organs as well. The VPAC1-R is highly expressed in lung, intestine, pancreas and adrenal medulla. However, VPAC2-R is present within the pituitary, testis and ovary, spleen, and adrenal cortex (Vaudry et al., 2009). In summary, PACAP receptors seem to have much wider distributions than their ligand PACAP, which suggests that the biological functions of the peptide in different systems come from paracrine, endocrine and neuronal sources.

1.8. Biological and Pharmacological Effects of PACAP

The distribution of PACAP and its receptors in a variety of systems implies diverse biological functions. PACAP has been demonstrated to function as a hormone, a neurohormone, a neurotransmitter, and a trophic factor in different systems. In the hypothalamus, with the highest density of PACAP and its receptors (Arimura, 1992; Arimura and Shioda, 1995), PACAP functions as a hypophysiotropic factor, a neurohormone, a neurotransmitter and neuromodulator. There is evidence that PACAP increases *GnRH*, *somatostatin*, and *CRH* gene expression in the hypothalamus (Agarwal et al., 2005; Bredow et al., 1994; Grinevich et al., 1997; Kageyama et al., 2007). PACAP could influence food intake by transmitting leptin signals to proopiomelanocortin neurons in the ventrolateral aspect of the arcuate nucleus (ARC) (Mounien et al., 2009).

Circadian rhythms are also influenced by PACAP as evidenced by daily variations of PAC1-R mRNA expression in the suprachiasmatic nucleus (SCN) and supraoptic nucleus (Cagampang et al., 1998; Gillette and Mitchell, 2002). In the rat pineal gland, there are circadian differences as well, which suggests PACAP may regulate melatonin production (Fukuhara et al., 1998). The stimulation of PACAP on melatonin secretion has been demonstrated both *in vitro* and *in vivo*. PACAP regulates circadian rhythms indirectly through hypothalamus and neurotransmitter glutamate as well (Hannibal et al., 2001; Rekasi and Czompoly, 2002).

During the development of the CNS, PACAP may modulate neuronal cell proliferation, cell survival, cell migration and cell differentiation as a neurotropic factor. In the adult brain, PACAP protects injured neurons from apoptosis, indicating a potential target for the treatment of stroke or neurodegenerative diseases (Vaudry et al., 2009). PACAP is also involved in the regulation of non-neuronal tissues including cell proliferation, plasticity, glycogen metabolism, and release of neurotropic factors from glial cells. There is little endogenous PACAP in glial cells; therefore, these cells may be regulated by PACAP from nearby neurons or within the circulatory system (Vaudry et al., 2009).

PACAP also plays an important role in the other systems outside the CNS. The ability of PACAP to stimulate cAMP formation regulates the synthesis and secretion of many hormones in endocrine glands, like the pituitary, thyroid and adrenal glands (Arimura and Shioda, 1995; Christophe, 1993; Nussdorfer and Malendowicz, 1998). PACAP increases thyroxine secretion in the human and

porcine thyroid. The secretion of many pituitary and adrenal hormones are also influenced by PACAP exposure. The gonads have been demonstrated to contain high concentrations of PACAP and its receptors, especially the testis which contains the highest concentration of PACAP in mammals (Arimura et al., 1991). Subsequent studies demonstrate that PACAP may help sperm maturation and penile erection, and accelerate testicular aging in the male (Gozes and Fridkin, 1992; Hedlund et al., 1995; Romanelli et al., 1997). In the ovary, PACAP is involved in progesterone production, follicular apoptosis and female fertility (Shintani et al., 2002; Zhong and Kasson, 1994). In the gastrointestinal tract, PACAP is present in exocrine glands and neuronal structures (ganglia, fibers) and stimulates the secretion of saliva, gastric acid and bicarbonate directly and also increases the release of some regulatory peptides including gastrin, somatostatin, atrial natriuretic factor and PYY to regulate the system indirectly. In addition, PACAP regulates smooth muscles of the gastrointestinal tract with tissue specific effects on motility (Vaudry et al., 2009). PACAP and its receptors are also involved in regulation of smooth muscles within the respiratory and cardiovascular systems. PACAP is also detected in immune cells, where it has been shown to exert protective anti-inflammatory actions in many different autoimmune models (Abad et al., 2001; Gomariz et al., 2006). Both endogenous and exogenous PACAP affect T cell responses through direct or indirect actions suggesting PACAP could be a target for immune system drug therapy (Delgado et al., 1999; Gonzalez-Rey et al., 2007; Tan et al., 2009). PACAP and its receptors are also detected in pathological tissues including various kinds of

tumors, and many studies demonstrate either stimulatory or inhibitory effects on tumor cells. Therefore, PACAP agonists and/or antagonists may aid in the treatment of tumors depending on the specific type (Fruhwald et al., 1999).

2. General introduction of HPG axis

In the classical view, the HPG axis is organized in three levels including GnRH neurons in hypothalamus, gonadotrophs in the pituitary and the gonads including the testis and ovary (Fig. 3). GnRH neurons synthesize and secrete a peptide hormone, GnRH, and GnRH receptors are localized on pituitary gonadotrophs. Therefore, GnRH, its receptor and intracellular transduction in gonadotrophs build the fundamental regulating pathway of HPG axis to synthetize and secret the gonadotropins.

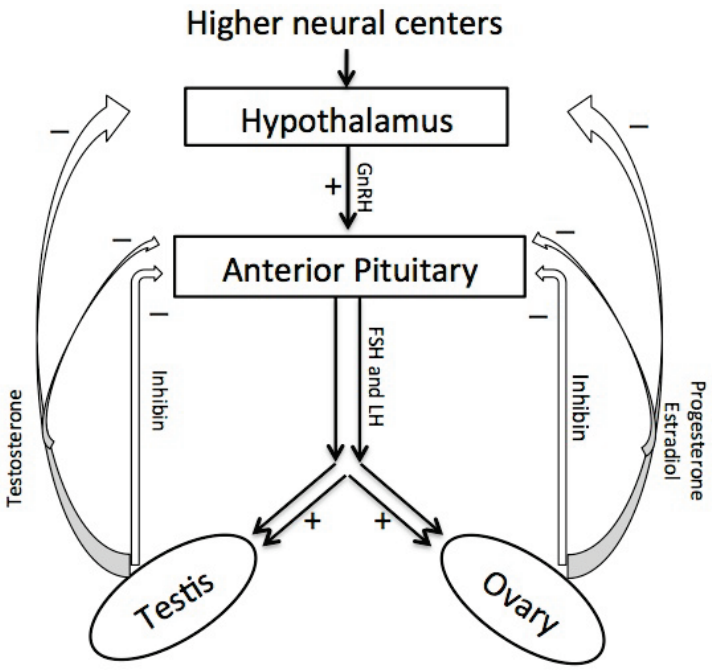


Figure 3. Schematic of the Hypothalamus-Pituitary-Gonadal axis.
GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

The gonadotropins, FSH and LH, are both dimeric glycoprotein hormones composed of a β-subunit and a α-subunit. FSH and LH share the common α-subunit whereas the β-subunits distinguish the two gonadotropins. In the male, LH regulates testicular Leydig and Sertoli cells to stimulate production of testosterone and androgen-binding protein, respectively. FSH binds to its receptors on Sertoli cells to promote spermatogonia proliferation and germ cell meiosis and postmeiotic development. In the female, LH induces ovarian thecal cells to produce androgens and the granulosa cells of the preovulatory follicle to secret progesterone during the terminal stages of follicular growth. FSH stimulates expression of aromatase enzymes that convert androgens to estradiol in ovarian granulosa cells.

Many investigators have utilized different animal models, containing both spontaneous and experimental mutations, to dissect the functional organization of HPG axis. The hypogonadal (HPG) mouse model, in which the gene encoding GnRH precursor is deleted, never enters puberty and displays a persistent hypogonadotropic-hypogonadal phenotype (Mason et al., 1986). Surgical disconnection between the hypothalamus and pituitary in sheep decreases pituitary gonadotropins and causes secondary hypogonadism (Clarke et al., 1983). These results, and others, demonstrate that GnRH is critically important for the maintenance of biological functions of the HPG axis. Male and female LHβ or LH receptor deficient mice are infertile because of marked decreases in gonadal steroid hormones, which induce defective spermatogenesis and late

follicular development (Ma et al., 2004; Zhang et al., 2001). FSHβ and FSH receptor knockout mice also have defects in reproductive system in both genders. However, there are some differences among the mutants. Female FSHβ knockout mice are infertile due to a defect in ovarian follicular maturation, whereas the males are still fertile with smaller testes and fewer sperm cells (Kumar et al., 1997). The female FSH receptor knockout mice have a similar phonotype as FSHβ deficient mice. FSH receptor deficiency induces more serious problems in males than FSHβ deficiency. FSH receptor null mice have significant decreases in Leydig cell numbers and testosterone levels (Abel et al., 2000; Kumar et al., 1997).

There are both positive feed-forward and negative feedback mechanisms in the HPG axis at different levels. Gonadal steroid hormones provide important feedback regulations of hypothalamic GnRH. In the male, testosterone exerts a negative feedback that may be mediated through the level neurons (Herbison et al., 1996; Tilbrook and Clarke, 2001). Gonadotropins also show a minor negative feedback on GnRH as well (Tilbrook and Clarke, 2001). In female, the feedback regulations from gonadal hormones (estrogen, progesterone) are more complex including negative and positive effects depending on the stage of the reproductive cycle (Glidewell-Kenney et al., 2007; Petersen et al., 1995; Petersen et al., 2003). In addition to gonadal hormones, the peptide hormones, inhibin, activin and follistatin, regulate the gonadotropins, primarily FSH, through the feedback mechanism. For instance, activin has been shown to work in a paracrine manner to induce FSHβ expression in rat pituitary cells, while gonadal

derived inhibin is a competitive inhibitor of activin. Follistatin is also produced within the pituitary and can bind with activin and blocks its activity by preventing receptor interaction. (Gregory and Kaiser, 2004; Nakamura et al., 1990).

3. PACAP in HPG axis

The previous section introduced the classical model describing the HPG axis. PACAP and its receptors are detected throughout the HPG axis suggesting that PACAP is involved in the function and regulation of the HPG axis. The actions of PACAP on HPG axis are at all three levels. In the hypothalamus, studies have revealed that PACAP stimulates synthesis and secretion of GnRH. For instance, intracerebroventricular injection (i.c.v) of PACAP increases GnRH mRNA level in rat hypothalamus while PACAP treatment increases GnRH release from mouse clonal GnRH cells (Li et al., 1996; Olcese et al., 1997). However, i.c.v administration of PACAP into the hypothalamus of ovariectomized ewes decreased LH secretion and LH pulse frequency (Anderson et al., 1996; Sawangjaroen and Curlewis, 1994). In adult female rats and mice, steroids are found to regulate the expression of PACAP mRNA in the ventromedial nucleus (Apostolakis et al., 2004), where PACAP mediates progesterone-evoked sexual behavior through PAC1-R (Apostolakis et al., 2005).

The gonadotropins, LH and FSH, are produced and secreted from gonadotroph cells in the anterior pituitary gland. In the classic view, gonadotropin secretion is predominantly regulated by GnRH as previously described (Charlton, 2008). However, gonadotropins are still present in GnRH deficient mice (Cattanach et al., 1977). This implies that GnRH is a key regulator but not the

only player in gonadotroph function. Subsequent studies have demonstrated that PACAP acts either alone or synergistically with GnRH to stimulate LH and FSH mRNA expression through direct and/or indirect mechanisms. For instance, intra-atrial injection of PACAP increases plasma LH level in the male rats (Osuga et al., 1992). Treatment of rat anterior pituitary cells with PACAP induces stimulation of gonadotropin release through calcium elevation (Rawlings et al., 1994; Tsujii et al., 1994). The effect of PACAP on gonadotropin synthesis involves the cAMP/PKA pathway, whereas its acute action on FSH/LH secretion is under the control of calcium elevation. Besides its direct action on gonadotropin release, PACAP has also been shown to increase rat GnRH receptor gene promoter activity through the cAMP/PKA pathway. Conversely GnRH can stimulate *PACAP* gene expression. Furthermore, GnRH agonist can inhibit PACAP-induced cAMP production by phosphorylation of PAC1-R through the PKC pathway, illustrating the complex interplay between GnRH and PACAP in the regulation of gonadotroph cell functions.

PACAP and its receptors are observed in the gonads and the testis has the highest level of PACAP of any of the biological systems suggesting that the peptide may operate as a local regulator of gonadal activity. Interestingly, the *PACAP* gene in the testis has a shorter promoter than in other tissues (Daniel and Habener, 2000). Testis PACAP levels are dramatically reduced by hypophysectomy and are restored by FSH administration, which indicates that the expression of PACAP in testis is regulated by pituitary gonadotropins (Romanelli et al., 1997; Shuto et al., 1995). PACAP has been demonstrated to

stimulate testosterone secretion in isolated rat Leydig cells (El-Gehani et al., 1998; Rossato et al., 1997). Compared to the testis, the ovary contains much less PACAP. However, many investigations indicate that PACAP is also involved in the reproductive function of female mice. For example, in female rats, granulosa and cumulus cells from large pre-ovulatory follicles contain PACAP mRNA, and PACAP increases progesterone production in cultured grandulosa cells (Barberi et al., 2007; Gras et al., 1996). Furthermore, PACAP deficient female mice have decreased fertility with demonstrating behavioral, ovulatory and implantation defects (Isaac and Sherwood, 2008; Sherwood et al., 2007; Shintani et al., 2002).

4. Differential regulation of gonadotropins during development

Although LH and FSH are both stimulated by GnRH secreted from hypothalamus, their expressions are not always parallel during development. At the mid gestation, serum levels of LH are nearly ten-fold higher than FSH (Debieve et al., 2000). The human fetal plasma LH levels are 25 to 100-fold higher than FSH during gestation whereas the LH:FSH ratio is only 1 to 0.5 in the adult (Siler-Khodr and Khodr, 1980). The differential regulation of the gonadotropins is also a characteristic of fetal development in male rodents. Previous studies detected LHβ as early as embryonic day 12 (E12) whereas could not observe FSHβ until E19 or E21 (Aubert et al., 1985; Nemeskeri et al., 1986; Nemeskeri et al., 1984). Similar temporal expression pattern of LHβ and FSHβ is observed in mouse pituitary development (Japon et al., 1994). The later and lower FSH levels than LH during embryonic days may be essential for an

appropriate hormonal environment of reproductive system. Why FSH expression should be repressed during embryonic days could be explained by the function of FSH during reproductive system development. Within the developing rat testis, FSH induces testosterone production from 5α-reduced steroids, which are essential for sexual differentiation of the CNS (McEwen, 1983; 1992). FSH can also suppress the production of muollerian inhibiting hormone (MIH), which is a key factor to form the male reproductive tract (Bercu et al., 1979; Kuroda et al., 1990). Furthermore, the primary effect of FSH is to stimulate Sertoli cell and prepare the testes for spermatogenesis in adulthood. Therefore, the repression of FSH during embryonic days may play a role to prevent maturation of the testes. Male mice with overexpression of FSH under the regulation of the metallothionein-1 promoter in pituitary are infertile due to disrupted sexual behavior (Gorski, 2002; Kumar et al., 1999). Lager seminal vesicles and higher circulating testosterone levels may alter sexual maturation and reproductive behavior during the perinatal period. All these evidences suggest the importance of the repressed FSH expression in the fetal pituitary. The follistatin-activin-inhibin axis is demonstrated to specifically regulate FSHβ expression in pituitary (Carroll et al., 1989). Therefore, these three peptides are the candidates for the differential regulation of FSH in the male fetus as in the adult. However, bioactive forms of inhibin are not detectable within rat testes until birth (Phillips, 2000), and expression of activin in testes and pituitary has no changes during development (Gregory and Kaiser, 2004). Thus, follistatin is suggested as a factor contributing to the selective suppression of FSH during fetal development, and our previous

studies provided evidence. Follistatin as well as PACAP expression levels are high in the embryonic male pituitary and decline significantly after birth when FSHβ mRNA levels increase dramatically (Moore et al., 2009a). Therefore, we hypothesize that FSH levels are suppressed by high concentration of follistatin induced by PACAP in embryonic male pituitary. To demonstrate the hypothesis, we designed both *in vivo* and *in vitro* experiments to answer three questions: Which cell types express PACAP and the PACAP specific receptor, PAC1-R, in the perinatal pituitary; whether PACAP stimulates follistatin expression in pituitary and if alterations in developmental pituitary PACAP expression can modify the developmental pattern of gonadotropin expression; and, Which factors may regulate pituitary PACAP expression levels.

CHAPTER TWO

PACAP AND PAC1-R EXPRESSION IN PITUITARY GONADOTROPHS

1. Introduction

PACAP was originally identified as a hypothalamic hypophysiotropic peptide with a high expression level in hypothalamus (Arimura et al., 1991; Miyata et al., 1989). PACAP mRNA was detected within the pituitary of embryonic rats by *in situ* hybridization (Jaworski and Proctor, 2000; Skoglosa et al., 1999). Furthermore, our previous studies demonstrate for the first time that pituitary expression of PACAP is very high in the fetus, and declines dramatically after birth (Moore et al., 2009a). PACAP has been demonstrated to regulate α-GSU, LHβ and FSHβ mRNA levels in the cultured pituitary cells (Tsujii and Winters, 1995b). However, very few details about PACAP expression during development in pituitary gonadotrophs have been documented. The anterior pituitary is derived from oral ectoderm and forms Rathke's pouch (Takuma et al., 1998). In the adult rat anterior pituitary, approximately 50% of the cells are somatotrophs that are primarily located in the lateral wings of the anterior lobe and producing growth hormone. Prolactin (PRL)-secreting lactotrophs represent ~15% of cells in the anterior pituitary and are randomly distributed through the lobe. Corticotrophs comprise about 15% of anterior pituitary cells (Nakane,

1970). Gonadotroph cells represent up to 10% of the human anterior pituitary cell population. Thyrotrophs are the least abundant cell type in the anterior pituitary, comprising ~5% of the total cell population, and are mostly found in the anterior-medial portion of the gland. There are many supporting and or non-neuroendocrine cells including follicular and folliculostellate cells throughout the anterior pituitary. We utilized single cell real-time PCR to determine which pituitary cell type expresses PACAP. Furthermore, we want to reveal whether there are changes of cell types with PACAP during development.

The specific receptor of PACAP, PAC1-R, is expressed in anterior pituitary as well (Vigh et al., 1993). PAC1-R binds with PACAP and initiates intracellular signaling through cAMP/PKA as well as PKC/MAPK pathways (Fowkes et al., 2001; Spengler et al., 1993). Furthermore, differential expression of the variants of PAC1-R might influence the potency of PACAP signaling. Thus, we examined the expression pattern of PAC1-R isoforms in both gonadotroph cell lines and developing rat pituitary by combined semi-quantitative PCR and restriction enzyme analysis.

2. Methods and Materials

2.1. Animals

Timed-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories, and were housed at least one week with free access to rat chow and water in accordance with the NIH Guide for the Care and Use of Laboratory Animals according to a protocol approved by the Animal Care and Use Committee of the University of Louisville.

2.2. Immortalized Cell Culture

Mouse αT3 and LβT immortalized mouse pituitary gonadotroph cells were kindly provided by Dr. Pamela Mellon (University of California, San Diego, CA). αT3 cells were grown in Eagle's Minimal Essential Medium (MEM) containing glucose (4.5g/L), NaHCO3 (2.2g/L) and HEPES (5.96g/L), with penicillin, streptomycin, and fluconazole, and supplemented with 10% fetal bovine serum (FBS). LβT2 cells were grown in Dulbecco's MEM (DMEM) containing HEPES (22.5mM), NaHCO3 (40mM), 10% charcoal-stripped FBS, penicillin, streptomycin, and fluconazole. Both cell lines were grown in 6 wells plate and used for experiments after 50%-70% confluence.

2.3. Primary cell culture

E19 pregnancy rats were sacrificed utilizing CO_2, then the pups were removed and placed into ice-cooled 1 × PBS. Pituitaries from E19 and PN1 pups were dissected and placed in HEPES buffer without BSA on ice before treatment with 0.25% trypsin in a 15 ml Falcon tube in 37° water incubator for 5 minutes, with intermittent dispersal with a siliconized pipette. Treated pituitaries were pipetted up and down till tissue fragments were invisible. The tube was centrifuged for 10 minutes at 500 × g. Suspension was discarded and replaced with 5 ml fresh DMEM with 10% FBS to wash the cells. Centrifuge again for 10 minutes at 500 × g. The cells were suspended in 5 ml DMEM then cultured in 5 cm dishes for 24 hours before the experiments. All the glass pipets used were coated overnight by SIGMACOTE® (SIGMA. CO).

2.4. RNA extraction and reverse transcription

Total RNA was prepared from cultured αT3, LβT2 cells or male rats pituitary samples using QIGEN RNAeasy Kit following the instruction of manufacturer. Sample purity was determined by Nano-drop from Thermo (Wilmington, DE). 1μg total RNA from every sample was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, CA) primed with *oligo*(dT) following the instruction from Invitrogen.

2.5. Real-time PCR

The cDNA samples were amplified in parallel by PCR with a Stratagene MX3005P Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) using the Brilliant SYBR Green QPCR Master Mix and specific primers. Accumulation of PCR product was monitored in real time, and the crossing threshold (Ct) was determined using *Mx-pro* software.

2.6. Laser Capture Microdissection

The head part tissues from E14 to PN10 rats were placed in desired orientation in cold Cryomold (Fisher Co, PA) with a thin layer of OCT on the bottom. Then, the specimen was covered with OCT then mounted to holder of microtome. Once the specimen and cryostat of microtome were temperature equilibrated, the specimens were cut to 10μm sections. The sections were stained with hematoxylin and thoroughly dehydrated using gradient concentration ethanol. Under the microscope, the tissue section is viewed through the glass slide and specific areas are identified. When the pituitary is in the field of view, a near IR laser diode integral with the microscope optics is utilized. The pulsed laser beam activates a precise spot on the transfer film immediately above the

cells of interest. At this location the film melts and fuses with the underlying cells. The film with the bonded cells is then lifted off the section, leaving all unwanted cells behind. This allows targeted dissection of pituitaries to be pooled for RT-PCR analysis. RNA from the LCM samples was isolated utilizing the PicoPure RNA isolation kit from Arcturus following the manufacturer's protocol. Briefly, transfer film caps are placed onto microfuge tubes containing RNA extraction buffer, inverted, and incubated at 42° for 30 minutes. Next, 70% ethanol is added to the extraction buffer-sample solution and passed through a pre-conditioned purification column by centrifugation. The purification membrane is washed and the RNA is eluted with buffer.

2.7. Single cell PCR

The cells from E19 and PN1 rat pituitary cell cultures were picked using glass pipettes, which were made from 0.5 mm borosilicate glass (Sutter Instrument, CA) using micropipette puller (Model P-30). The selected cell was placed into a 0.5 ml eppendorf tube and put on dry ice immediately. To each tube we added 10mM dNTP(1μl), *oligo*(dT)(1μl), RNAse inhibitor(1μl), 5×First strand buffer(4μl), ddH_2O(11μl) and Superscript III(1μl). All the cell samples were plated on a thermocycler using the standard reverse transcription protocol. After then, the mixture was ready to use as the template of PCR.

3. Results

3.1. PACAP expression in Gonadotroph cell lines

There are two immortalized gonadotroph cell lines; αT3 and LβT2 cells, each derived from pituitary gonadotroph tumors that developed at different

developmental stages in mice. The tumor from which the αT3 cell line was derived from a tumor formed during prenatal development of the donor mouse. Conversely, LβT2 cells were derived from a postnatal formed mouse pituitary tumor. The different cell lines have previously been demonstrated to represent different developmental stages in gonadotroph maturation (Turgeon et al., 1996). We collected RNA samples from both cell lines and examined PACAP mRNA expression levels. αT3 cells express a relative higher level of PACAP than LβT2 cells, in which PACAP mRNA is almost undetectable (Fig. 4). This result is similar to our previously reported findings that PACAP mRNA expression is high in the fetus, and declines substantially after birth (Moore et al., 2009a).

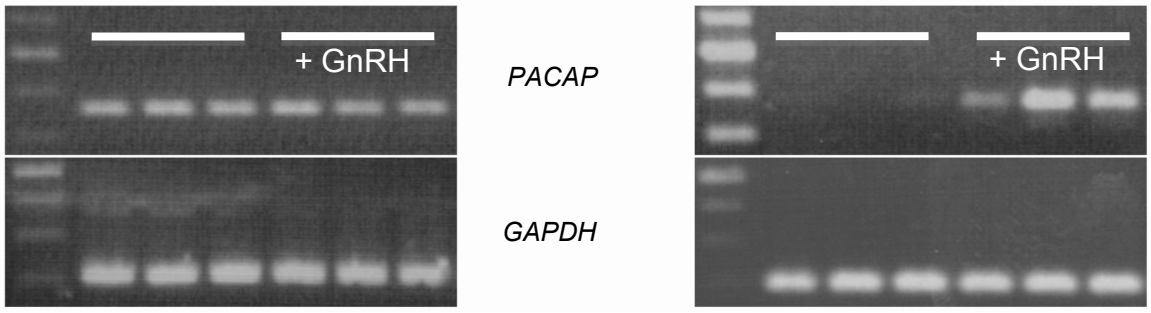
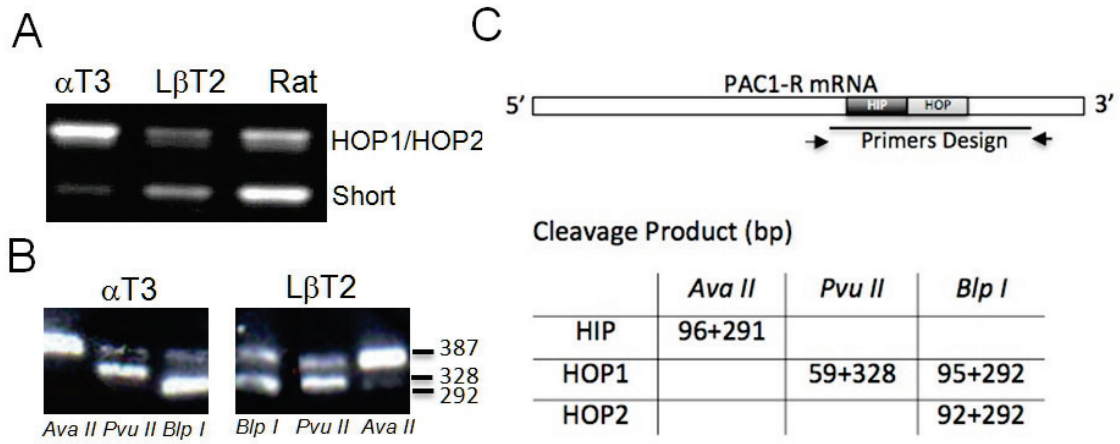


Figure 4. PACAP mRNA level in αT3 (left) and LβT2 (right) cells.

Total RNA (1μg) was isolated from cultured αT3 and LβT2 cells. PACAP mRNA expression levels were measured by semi-quantitative PCR after reverse-transcription. Housekeeping gene (*GAPDH*) mRNA levels descripted the same amount of total RNA in all samples. GnRH (10nM) treated LβT2 cell samples were the positive controls.

3.2. PAC1-R expression in Gonadotroph cell lines



	Ava II	*Pvu II*	*Blp I*
HIP	96+291		
HOP1		59+328	95+292
HOP2			92+292

Figure 5. Expression pattern of PAC1-Rs (A,B) in gonadotroph cell lines. The total PAC1-R (A) and PAC1-R variants (B) mRNA expressions were measured by semi-qauntitative PCR and restriction enzyme after RNA extraction and reverse-transcription (1μg RNA every sample). The specific PAC1-R primers and cleavage sites of enzymes are showed in C.

PACAP signaling within the gonadotrophs could also be developmentally regulated by alterations in the expression of PACAP receptors. Although there are three PACAP receptors, VPAC1, VPAC2 and PAC1R, PAC1-R is the only one specific for PACAP. PAC1-R has at least six variants resulting from alternative splicing in the third intracellular loop region (Spengler et al., 1993). The splice variants are characterized by the absence (short variant) or presence of either one or two cassettes of 28 amino acids (hip or hop1 variant) or 27 amino acids (hop2 variant) (Spengler et al., 1993). αT3 and LβT2 cells express similar

PAC1-R variants (Fig. 5A). However, they still have some differences. We found that αT3 cells contain significantly more Long form than Short form while in LβT2 cells they were in similar amount. The expression of Hip form was almost undetectable in either αT3 or LβT2 cells (Fig. 5B) whereas the Hop form dominated in both cell lines (Fig. 5B). Real-time PCR was also utilized to detect variants of PAC1-R, with similar results (data not show).

3.3. PACAP expression in Pituitary cells

We analyzed PACAP mRNA expression during development, and a significant decline happened after birth in male rat pituitary (Moore et al., 2009a). We cultured E19 and PN1 rat pituitary cells and picked single cells for qualitative PCR analyses. We designed specific primers for every cell type, and analyzed PACAP distribution in these cells. The results were summarized in following table. Although PACAP mRNA level in PN1 pituitary was less than E19, the pattern of expressing cell types didn't change much. PACAP mRNA in pituitary was mainly produced by gonadotroph cells.

	E19	PN1
PACAP	14 (9.5%) of 146 cells	17(14.3%) of 119 cells
Of These	8 (57.1%) cells LH+	8 (47.1%) cells LH+
	1 (7.1%) cells S100+	1 (5.9%) cells S100+
	0 cells PRL+	2 (11.8%) cells PRL+
	0 cells ACTH+	2 (11.8%) cells ACTH+
	1 (7.1%) cells GH+	1 (5.9%) cells GH+
	2 (14.2%) cells TSH+	1 (5.9%) cells TSH+

Table 1. Distribution of PACAP in different type cells of rat pituitary gland. The cell types of PACAP positive cells were determined by real-time PCR from single cell samples. E19 and PN1 rat pituitary cells were cultured for 24 hours before experiment.

3.4. PAC1-R mRNA expression in rat pituitary

We also looked at PAC1-R expression during development of male rat pituitary gland. Because pituitary glands are tiny in embryos, LCM was utilized to collect E14-PN10 tissues, and then total RNA was extracted from these samples following the manufacturer's instructions. Through semi-quantitative results, we observed a decline of PAC1-R-Short form after birth (Fig. 6A). Furthermore, we analyzed the Long form of PAC1-R in these tissues. Hop1/Hop2 form was always the main variant during development (Fig. 6B). To confirm the results, we chose the time point with most of our attentions, E19 and PN1. The pituitary gland samples in E19 and PN1 male rats, which were collected by traditional method, showed the same results (Fig. 6C).

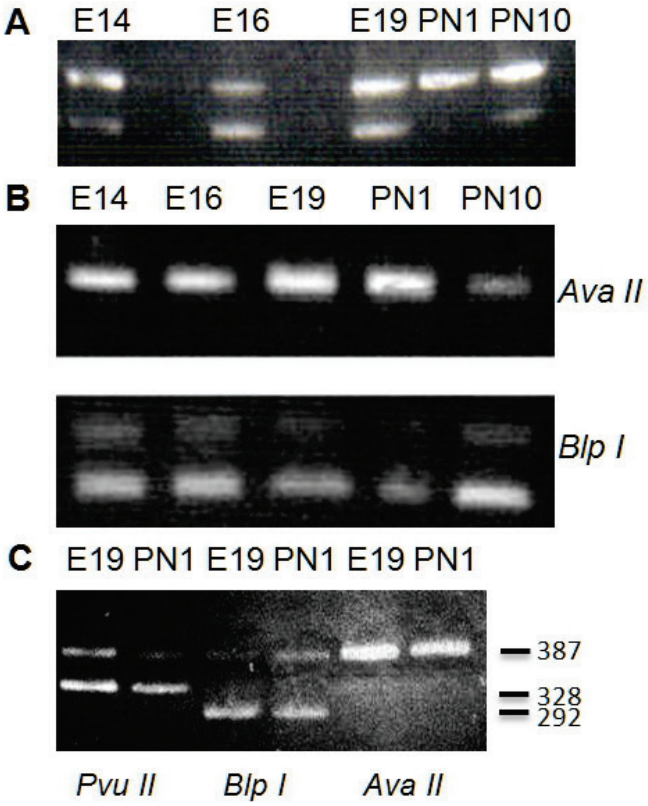


Figure 6. PAC1-R mRNA expression pattern during rat pituitary development. PAC1-R (A) and its variants (B.C) were analyzed by semi-quantitative PCR after reverse-transcription.

4. Discussion and Future Investigations

PACAP is a novel factor found to regulate synthesis and secretion of gonadotropins in cultured pituitary cells. However, very little was known about pituitary PACAP expression during development before our group first discovered dramatic decrease of pituitary PACAP levels after birth (Moore et al., 2009a). To find further evidences to support functions of PACAP in pituitary

gonadotrophs, we examined expression patterns of PACAP and its specific receptor, PAC1-R, in both gonadotroph cells and developing rat pituitary.

In the two different gonadotroph cell lines, both PACAP and PAC1-Rs mRNA expressions were much higher in αT3 cells than in LβT2 cells. At the same time, our previous studies revealed the dramatic decline of PACAP mRNA after birth (Moore et al., 2009a). All the data suggested that two gonadotroph cell lines represented different developing periods of gonadotrophs. Thus, the αT3 and LβT2 cell lines are useful *in vitro* models for studies on developing gonadotrophs. In future experiments, we could choose either more mature LβT2 or immature αT3 cells for *in vitro* researches depend on our hypotheses and targeting development stages.

After PAC1-R had been cloned in many species, six variants resulting from alternative splicing in the third intracellular loop region were identified in rats (Journot et al., 1994; Spengler et al., 1993). Different variants may be temporal and/or spatially regulated in different cell types and mediate different pathways. For instance, the Hip cassette impairs AC stimulation and abolishes phospholipase C activation, which was in opposite of observed PACAP functions (Dickson and Finlayson, 2009). We demonstrated that Hop form and Short form were the dominant variants in both gonadotroph cell lines and rat pituitary gland, which suggests the most prominent PACAP pathways involved in the pituitary gland. Thus, in the following experimental design we focused on cAMP/PKA/CREB, cAMP/MAPK and PLC/PKC pathway analysis.

Although PACAP expression in pituitary was well known for a while, the type of PACAP positive cell is still less documented. The RT-PCR on single pituitary cell indicated that most of PACAP positive cells were gonadotroph cells in both E19 and PN1 rats. The data supported that the decline in PACAP mRNA in pituitary gland after birth partially reflects a decline in PACAP expression in pituitary gonadotrophs.

The expression of PACAP and PAC1-R in developing pituitary suggests that PACAP may affect pituitary gland development. Furthermore, the result that PACAP are demonstrated to mainly exist in gonadotrophs of pituitary particularly implies that regulating gonadotrophs may be the main effect of PACAP. Different with the dramatic decline of pituitary PACAP levels at birth, its receptor, PAC1-R, only shows a decreased short subtype in postnatal period. The result suggests possible different pathways mediating the differential regulation of gonadotropins by PACAP.

Based on recent data, future investigations on PACAP and PAC1-R expression in pituitary will still focus on single cell level. All endocrine cell types in the pituitary express PAC1-Rs (Gottschall et al., 1990; Lam et al., 1990). However, there are no data about the PAC1-R variants detail in different cell types. The detailed developmental expression pattern and future specific agonists or antagonists for variants could help drug design on pituitary related endocrine-disorders.

CHAPTER THREE

FUNCTION OF PACAP ON PITUITARY GONADOTROPHS

1. Introduction

The name pituitary adenylate cyclase-activating polypeptide (PACAP) was derived from the ability of this peptide to increase cAMP production in cultures of rat pituitary cells (Miyata et al., 1989). In addition of this observation, subsequent research found that PACAP stimulates the release of most pituitary hormones from rat pituitary cells (Counis et al., 2007). Therefore, PACAP may be a novel and important hypophysiotropic factor.

Mammalian gonadotropins, Luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play a critical role in the regulation of reproductive development and function. Production of gonadotropins by pituitary gonadotrophs is mainly under the control of the hypothalamic factor GnRH as well as peripheral feedback mechanisms of gonadal hormones (Charlton, 2008). However, gonadotropins exist in GnRH deficient mice, which show us that there must be other important factors regulating the ontogeny and differentiation of gonadotrophs.

The effects of PACAP on gonadotropin secretion have been studied both *in vivo* and *in vitro*. PACAP has a consistent stimulatory action *in vitro* whereas effects *in vivo* are variable. For LH, i.v or i.c.v administration of PACAP38 in rats showed some differences between male and female. PACAP38 increased LH plasma levels in male (Osuga et al., 1992); however, it suppressed the preovulatory LH surge in female (Koves et al., 1996). The function of PACAP on the female rat has evidences demonstrating PAC1-R as a mediator (Choi et al., 2000). The interest is that PACAP27 causes opposite effects on female rat, in which PACAP27 increased circulating LH levels instead of inhibiting LH surge (Kantora et al., 2000).

On the other side, PACAP also regulates gonadotropin gene expression not just hormone release. In cultured rat pituitary cells, continuous PACAP treatment combined with pulses of GnRH increases α-subunit mRNA and LHβ mRNA transcript length, which presumably prolongs its half-life. However, FSHβ transcription was found to be reduced (Tsujii et al., 1994). On the other hand, the pulsatile treatment of PACAP alone increased α-subunit mRNA and LHβ mRNA but no effects on FSHβ mRNA (Tsujii and Winters, 1995b).

In gonadotrophs, effect of PACAP is exerted through activation of PACAP specific receptor, PAC1-R (Hezareh et al., 1996a). In Chapter two, the gondotroph cell lines, αT3 and LβT2 cells, express PAC1-R. In particular, PAC1-R Hop and Short form exist in gonadotroph cells, which indicated effect of PACAP through PAC1-R have several different pathways, PKA, PLC and [Ca^{2+}] etc. (Bresson-Bepoldin et al., 1998; Hezareh et al., 1996b; Rawlings and

Hezareh, 1996). Although these pathways all involved in gonadotropins release and mRNA transcript, PACAP induced cAMP production appears to be more important in the synthesis than release (Sherwood et al., 2000).

In addition to the direct effects of PACAP on gonadotroph cells, sometimes the actions of the peptide on gonadotropins could be mediated through indirect mechanisms. In the cultured pituitary cells and mouse clonal gonadotroph cells, exposure to PACAP reduced FSHβ mRNA levels may relate to the increase in follistatin expression (Katayama et al., 2000; Winters et al., 1997).

In spite of all that is known about PACAP actions in gonadotrophs, we still lack the information about PACAP in the *in vivo* regulation of gonadotrophs and reproductive function. During sexual maturation in the male rat, expression and secretion of LH and FSH is not parallel. LH keeps a relatively constant level in the circulation from birth to adults. However, FSH levels remain at a low level until PN15 then begin to climb to a peak value at PN38. To investigate the role of PACAP signaling in the regulation of gonadotropin synthesis and secretion during male rat sexual maturation, we implanted micro-pumps containing PACAP antagonist (6-38) or PACAP38 into PN16 male rats and evaluated serum and pituitary gonadotropins levels after 3, 5 and 7 days of treatment. If PACAP is involved in regulation of FSH during sexual maturation, the treatment of pre-pubertal male rats with PACAP or the PACAP antagonist should change the FSH levels in serum and/or pituitary. In addition, to demonstrate the hypothesis that PACAP regulates FSH through a follistatin-activin mediated mechanism, we

evaluated the follistatin levels in the pituitary of experimental animals. Finally, we utilized a mouse model of pituitary PACAP overexpression and performed gene-chip analyses to screen the pituitary genes regulated by PACAP.

2. Methods and Materials

2.1. Animals

Timed-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories, and were housed with free access to rat chow and water in accordance with the NIH Guide for the Care and Use of Laboratory Animals according to a protocol approved by the Animal Care and Use Committee of the University of Louisville.

2.2. Immortalized Cell Culture

Mouse LβT2 immortalized mouse pituitary gonadotroph cells were kindly provided by Dr. Pamela Mellon (University of California, San Diego, CA). LβT2 cells were grown in Dulbecco's MEM (DMEM) containing HEPES (22.5mM), NaHCO3 (40mM), 10% charcoal-stripped FBS, penicillin, streptomycin, and fluconazole. The cell lines were grown in 6 wells plate and used for experiments after 50%-70% confluence.

2.3. Micro-Osmotic Pumps implantation

The ALZET micro-osmotic pumps were used to implant intraperitoneal into five PN16 male rats. There were three groups of pumps, which were filled with 100μl 1-38/BSA, 6-38/BSA and BSA only respectively and final concentration was 2μg/ml. For intraperitoneal placement, we made a small midline incision in the skin below the rib cage of an animal and another small incision in the

abdominal muscle. The pump was inserted, flow moderator first, into the peritoneal cavity. The muscle incision was closed with sutures, and then the skin incision was closed with a wound clip. After three, five or seven days, animals were sacrificed for serum and pituitary samples.

2.4. Cell Transfection

LβT2 cells were plated in 6-well plates, and grow to 50%-70% confluence. The mixture was incubated at room temperature for more than 30 minutes before adding 120μl to every well. The plates were gently swirled and continue incubated at 37℃ for 20 -24 hours. LβT2 cells were transfected with Fugene 6 (Roche Applied Science, Indianapolis, IN). The transfection mixture was containing DMEM without serum, 3μl Fugene 6 and 1μg plasmid DNA every well, and incubated for 20 minutes at room temperature. 100μl of mixture was added to every well in a drop-wise manner. The plates were continuing incubated for 20 - 24 hours.

2.5. FSH and LH ELISA

The blood samples from experiment animals were collected into the dry tubes. The serum separated from the red blood cells was assayed immediately.

a. Dispense 25μl of each calibrator into the appropriate wells.

b. Dispense 25μl of samples or controls into appropriate wells.

c. Add 200μl of conjugate (CONJ HRP) into each well.

d. Incubate for 180 minutes at room temperature without shaking.

e. Flick out the contents of the wells over a basin containing bleaching water or aspirate with an automated plate washer.

f. Wash the wells 7 times with an automated system set to 250µl per well, or by adding 250µl to each well, flicking out over a basin and blotting the wells on absorbent paper to remove any residual liquid after each washing.

g. Dispense 200µl of chromogenic substrate (SUBS TMB) solution into each well, ensuring that it is initially pale colored.

h. Incubate for 30 minutes at room temperature without shaking.

i. Stop the reaction by adding 50µl of stop solution (STOP SOLN) to each well.

j. Place the plate on a flat surface, swirl gently to mix contents.

k. Measure the absorbance at 450 nm on a 96 well micro-plate reader.

2.6. Genotype of TG-mice

Genotyping was performed by real-time PCR using DNA isolated from the tails of 2- to 4-week-old mice using heat-shock method. The 5' primer is derived from the mouse αGSU-subunit promoter, and the 3' primer is derived from rabbit β-globin cDNA sequences: 5' primer, 5'- AAATCCAGAGACATTGTTCCC -3'; and 3' primer, 5'- AATCAAGGGTCCCCAAACTC -3' Using real-time PCR to identify transgenic mice.

2.7. 2 $^{-\Delta\Delta C}{}_{T}$ Method for analyzing Real-time PCR results

The 2 $^{-\Delta\Delta C}{}_{T}$ Method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

2.7.1. Derivation of the 2 $^{-\Delta\Delta T}$ Method

Exponential amplification of PCR: $X_n = X_0 \times (1+E_x)^n$ X_n is the Number of target molecules. n is the cycle number. E_x is the efficient of target amplification.

X_T is the threshold number of target molecules. $C_{T,X}$ is the threshold cycle for target amplification, and K_X is a constant. Similar, we have R_T, $C_{T,R}$ and K_R for reference molecules. Thus, we have following two equations:

$$X_T = X_0 \times (1+E_X)^{C_{T,X}} = K_X$$

$$R_T = R_0 \times (1+E_R)^{C_{T,R}} = K_R$$

Dividing X_T by R_T gives the expression

$$\frac{X_T}{R_T} = \frac{X_0 \times (1+E_X)^{C_{T,X}}}{R_0 \times (1+E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K.$$

We used the same reagent and run all the groups in the same machine at the same time. Thus, we assumed efficiencies of the target and the reference are the same. $E_X = E_R = E$,

$$\frac{X_0}{R_0} \times (1+E)^{C_{T,X} - C_{T,R}} = K, \text{ or } X_N \times (1+E)^{\Delta C_T} = K, \text{ then } X_N = K \times (1+E)^{-\Delta C_T}$$

The Final step is to divide the XN for any sample q by the XN for the calibrator:

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1+E)^{-\Delta C_{T,q}}}{K \times (1+E)^{-\Delta C_{T,cb}}} = (1+E)^{-\Delta\Delta C_T}$$

For amplifications designed to be less than 150 bp and for which the primer and Mg^{2+} have been properly optimized, E is close to 1. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

2.7.2. Following is a sample table of data analysis using the method.

Treat	PACAP average C_T	GAPDH average C_T	ΔC_T	$\Delta\Delta C_T$	Fold $2^{-\Delta\Delta C_T}$
VEH	28.04±0.30	16.09±0.08	11.95±0.31	0±0.31	1 (0.83-1.21)
0.1μM BIM	28.15±0.44	15.55±0.05	12.61±0.44	0.66±0.44	0.64 (0.52-0.77)
1μM BIM	30.00±0.40	16.46±0.25	13.54±0.47	1.60±0.47	0.33 (0.31-0.36)

2.8. Microarray analysis

The microarray analysis was performed at the University of Louisville Microarray Core Facility according to instructions from Affymetrix (Santa Clara, CA). mRNA was converted into double stranded cDNA using a T7-oligo (dT) promoter primer sequence. The double-stranded cDNA was purified and served as a template in the subsequent in vitro transcription reactions. The in vitro transcription reactions were carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for cRNA amplification. The biotinylated cRNA was purified, fragmented, and used in the hybridization cocktail containing control oligonucleotide B2 and four control bacterial and phage cDNA (BioB, BioC, BioD, cre). The labeled cRNA was hybridized to the Mouse Genome 430 2.0 Array (Affymetrix, CA), using the protocol provided by Affymetrix. The Mouse Genome 430 2.0 Array is comprised of over 45,000 probe sets representing over 21,000 well-substantiated mouse genes. The sequence clusters were created from the UniGene database (Build 107, June 2002) and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute for Genome Research (MGSC, April 2002). Alterations in RNA transcript levels were analyzed using Partek Genomics Suite 6.2 (Partek Inc., St. Louis, MO). Three different

experiments were performed for statistical analysis. Data analysis was performed using Partek Genomics Suite 6.2 (Partek Inc., St. Louis, MO). The Affymetrix probe level signal values were summarized using the RMA algorithm. Statistically significant changed genes were identified by analysis of variance (ANOVA) with FDR-corrected p-values < 0.05. The contrast between wild type and αGSU-PACAP mice yielded no significantly different in genes based on these parameters. Two-way ANOVA tests were carried out to identify differentially expressed genes in the comparison of wild type and αGSU-PACAP mice, taking treatment and batch effect for the triplicate sample processing into account. The genes that showed 2-fold induction or 2-fold suppression were transferred to separate up and down lists, respectively. The gene sets with an FDR corrected p-value of less than 0.05 were identified in these lists and Ingenuity Pathway Analysis software (Ingenuity Systems Inc., Redwood City, CA) was used to interpret the interactive pathway networks between the selected genes from the microarray data.

2.9. Statistical Analysis

All the luciferase assays and real-time PCR data were performed with triplicate samples and repeated at least three times. Values were expressed as mean ±SEM. Statistical analysis was performed using ANOVA and post Tukey's test if necessary. $P<0.05$ was considered statistically significant.

3. Results

3.1. FSH and LH levels in developing immature male rats

In male rats serum FSH levels are low at PN17 and increase significantly beginning at PN19 (Fig. 7A). By PN29, the serum level of FSH was two-fold higher than the PN17 value. A similar increase was observed in the pituitary FSHβ mRNA levels during the same period (Fig. 7B). However, the serum LH levels remained relatively constant (Fig. 7A) but showed a small increase at PN29. Likewise, no significant increase happened till PN29 for LHβ mRNA levels (Fig. 7B). The results are consistent with previous research showing that FSH and LH levels are not parallel with each other during this period of development.

3.2. PACAP and Fst-288 mRNA levels in developing immature male rats

To evaluate whether a relationship exists between expression of PACAP and follistatin and gonadotropins during the period, qRT-PCR was performed using pituitary RNA isolated from PN17 to PN29 immature male rats. PACAP mRNA concentrations decreased significantly by 4-fold (Fig. 8A) during the period. On the other hand, qRT-PCR analysis also revealed an 80% drop in Fst-288 mRNA (Fig. 8B) from PN17 to PN29. Thus PACAP mRNA is decreased in parallel with pituitary Fst-288 expression, and is reciprocal to the rise in FSH levels.

3.3. Effects of PACAP38 and PACAP antagonist 6-38 on gonadotropins in immature male rats

Follistatin was known to inhibit FSH synthesis by binding activin and limiting activin signaling to gonadotrophs (Katayama et al., 2000). Furthermore, follistatin mRNA expression followed the same expression pattern as PACAP from PN17-PN29. To further demonstrate the role of PACAP in the synthesis and

secretion of gonadotropins in immature male rats, we utilized micro-pumps filled with 2μg/ml PACAP 6-38 or PACAP38 to implant intraperitoneal in PN16 male rats. Analysis of gonadotropins levels of experimental animals was processed at 3, 5 and 7 days after surgeries.

3.3.1. Effect of 3 days i.p. administration of PACAP 6-38 on gonadotropins in immature male rats

We implanted intraperitoneal osmotic micro-pumps containing PACAP antagonist PACAP 6-38 (2μg/ml) into PN16 male rats. After three days, both serum gonadotropins (Fig. 9) and pituitary gonadotropins mRNA (data not show) from surgical animals were analyzed and compared with vehicle and non-surgical control rats. Neither LH nor FSH was affected by 3 days administration of PACAP 6-38.

3.3.2. Effect of 5 and 7 days i.p. administration of PACAP 6-38 and PACAP38 on gonadotropins in immature male rats

However, 5 and 7 days treatments in immature male rats cause significant differences. Rats treated with 6-38 for 5 and 7 days had significantly higher levels of both serum FSH and FSHβ mRNA than vehicle and nonsurgical control rats (Fig. 10 and 11). On the other hand, PACAP38 treatment for 5 and 7 days reduced levels of serum FSH and FSHβ mRNA compared to vehicles and controls (not statistic significant). Based on these results, PACAP expression in pituitaries of immature male rats contributes to suppression of FSH, and that an endogenous decline in PACAP around PN17 allows for FSH expression to

increase. Neither serum LH nor LHβ mRNA was affected by PACAP 6-38 or PACAP38 (Fig.10C and D).

3.3.3. Effect PACAP 6-38 on Fst-288 and PACAP mRNA in immature male rats

The pituitary samples collected from PACAP 6-38 treated immature male rats were analyzed for Fst-288 mRNA levels. PACAP 6-38 treated rats have much lower (40-60%) follistatin levels than vehicles and nonsurgical controls. At the same time, PACAP38 treatment increased Fst-288 level by about 50% (Fig. 12A). The data suggest that effect of PACAP on gonadotropins works through follistatin-activin signaling pathway. PACAP mRNA in pituitary samples are also affected by PACAP 6-38 and PACAP38, which PACAP 6-38 decreases the PACAP mRNA level whereas PACAP 38 increases it (Fig. 12B). However, the differences are statistically significant compared to vehicle animals but not control animals, which may be induced by surgery procedure.

3.4. Effect of PACAP38 on follistatin promoter in LβT2 cells

The *in vivo* experiments suggested that PACAP might induce follistatin expression in immature male rats. To examine how PACAP could affect follistatin expression, we investigated the activities of follistatin promoter utilizing a luciferase-reporter construct under PACAP38 treatments. We chose LβT2 cells because they have similar low level of PACAP with our *in vivo* experimental animals.

3.4.1. PACAP stimulates follistatin promoter in LβT2 cells

Increasing doses of PACAP38 treatment proved that follistatin promoter activity was stimulated more than 2-fold than medium alone at 10nM

concentration (Fig. 13A). After PAC1-R variants, Short and Hop1 form, were overexpressed in LβT2 cells, the activity of follistatin promoter stimulated by PACAP climbed to 2-fold higher than wild type in both short and hop1 overexpressed cells (Fig. 13B).

3.4.2 PACAP stimulates follistatin promoter through PKA and MAPK pathway

By sequence homology and previous research, we identified some putative transcription factor binding sites on the follistatin promoter. Among these sites, AP1 and CRE binding sites were important in MAPK and PKA pathway. AP1, CRE and double mutant constructs were transfected into cells with or without PAC1-R expression vectors. AP1 mutation didn't significantly reduce the activity stimulated by PACAP compared to wild type vector (Fig. 14A). PAC1-R overexpression still induced PACAP stimulated activity. On the other hand, PACAP stimulated activity decreased after CRE mutation, and overexpression of PAC1-R got the similar pattern with native receptor types (Fig. 14B). Significant decrease in PACAP stimulated activity happened after we mutated both AP1 and CRE sites even when we overexpressed PAC1-R (Fig. 14C). All these data suggest that PAC1-R-PKA pathway through CREB was the most important for effect of PACAP on follistatin whereas MAPK pathway may also be involved in.

3.5. Microarray analysis revealed function of PACAP in gonadotrophs

We created a transgenic mouse model, in which we use αGSU subunit promoter to drive PACAP expression to maintain lifelong high pituitary PACAP levels. To evaluate genes that could be either up- or down-regulated by pituitary overexpression of PACAP, we performed gene chip microarray analysis

comparing pituitary gene expression in wild type and αGSU-PACAP mice. Pituitaries were collected from postnatal day 35 mice, an age when the difference in FSHβ and follistatin mRNA levels was maximal. Of the 45, 102 probe sets present on the gene chip, 516 were at least ±1.5 fold different (357+, 159-) in the αGSU-PACAP mice ($p<0.05$). Adjusting to a more stringent criterion of at least ± 2-fold difference and consolidation of multiple probe sets for individual genes, 108 genes (73+, 35-) were significantly ($p<0.05$) altered by chronic pituitary PACAP overexpression (Table 2). Among these genes, we found similar results with our previous experiments. Follistatin was strongly stimulated by PACAP with a 4-fold change. On the contrary, FSHβ was suppressed with a 3-fold decrease (Table 2, bold letter). We did find some novel genes, which were regulated by PACAP change. Two new genes that were affected the most by pituitary PACAP over-expression were *gastrin releasing peptide* (*GRP*, +14.97-fold) and "*phosphate regulating gene with homologies to endopeptidases on the X chromosome*" (*PHEX*, -12.2 fold).

To gain biological insight into the changes in gene expression in the pituitaries of αGSU-PACAP mice, we utilized Pathway Analysis software from Ingenuity Systems, Inc. (Table 3). These analyses revealed significant changes in the expression of genes involved in established biological networks including reproductive system disease, cancer, lipid metabolism, development and cellular growth and proliferation. The top biological functions of the genes with altered expression included neurological, endocrine, reproductive, genetic, and skeletal disorders; cellular functions including lipid metabolism, morphology, and

development; and physiological system functions including organ morphology and development of the nervous and reproductive systems. Not surprisingly, the top canonical pathway altered to the greatest extent was the GnRH signaling pathway. Through the analysis, we also listed genes which were known to be involved in pituitary function (Table 3). Furthermore, all the changes of selected genes were confirmed by real-time PCR.

4. Discussion and Future Investigations

Previous studies demonstrated that PACAP and PAC1-Rs were present in gonadotrophs during development. Subsequence investigations revealed some functions of PACAP on gonadotrophs, like stimulating gonadotrophins secretion, increasing subunit gene expression and lengthening LHβ mRNA transcripts (Rawlings et al., 1994; Tsujii et al., 1994; Tsujii and Winters, 1995b). However, most of these data were from *in vitro* studies, including pituitary cell culture and LβT2 and αT3 cell lines. Very little information has come from *in vivo* experiments. Previous studies documented that PACAP could suppress FSHβ mRNA levels in pituitary cell cultures from adult rats (Winters et al., 1997). Furthermore, PACAP was demonstrated to increase follistatin expression in rat pituitary cell cultures and clonal FS cell line (Winters et al., 1997). As follistatin is an activin-binding protein, high levels of follistatin within the pituitary would block activin stimulation of FSHβ transcription in gonadotrophs (Katayama et al., 2000). Therefore, PACAP may indirectly regulate FSHβ through follistatin then activin in gonadotrophs.

Our previous data revealed the decline of PACAP and follistatin mRNA levels happened around birth in male rats (Moore et al., 2009a). At the same time, follistatin mRNA levels begin to rise. In this dissertation, we could find another period from PN17 to PN29, during which PACAP and follistatin were decreasing whereas FSHβ was increasing (Fig. 7 and 8). Together, we noticed that PACAP and follistatin mRNA expression were parallel and reciprocal with FSHβ during the male rat sexual development. This evidence supports the hypothesis that PACAP *in vivo* should have similar function on FSHβ as observed in cell cultures. From PN16, we treated male rats with 0.5%BSA, PACAP 6-38 or PACAP38 then collected serum and pituitary samples at PN19, PN21 and PN23. After 3 day treatment, neither PACAP 6-38 nor PACAP38 showed the significant effect on FSH and LH levels in serum or pituitary (Fig. 9). On the other hand, FSH serum levels in PN21 and PN29 after PACAP 6-38 treatment were significantly higher than controls and vehicles. PACAP38 treatment did show some decreasing trends but no statistically significant differences were detected (Fig. 10 and 11). LH levels were not influenced by either treatment.

Two alternatively spliced mRNAs are produced from *follistatin* gene, with Fst-288 having greater activin-neutralizing activity (Hashimoto et al., 1997; Sugino et al., 1997), and our experiments revealed that PACAP 6-38 treatment decreased Fst-288 mRNA level in pituitary (Fig. 12A). These data provide the first *in vivo* evidence in rats demonstrating a suppressive effect of PACAP on FSH through regulating follistatin. In addition, we observed that PACAP regulates

mRNA expression of itself, which is increased by PACAP38 treatment and decreased by PACAP 6-38 (Fig. 12B).

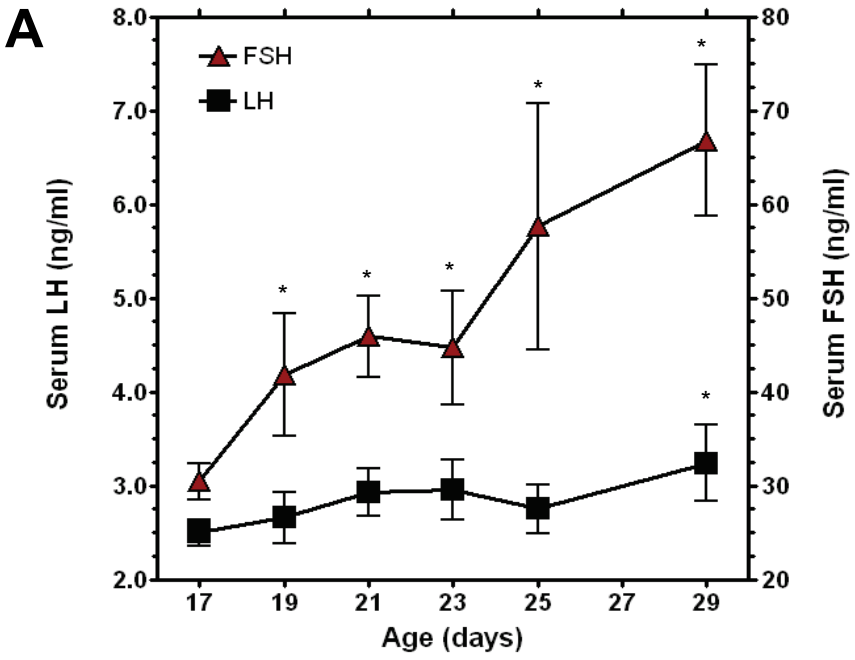
Chapter two showed that the Short and Hop forms PAC1-R dominated in developing rat pituitary and gonadotrph cell lines. In the present experiments, overexpression the Short or Hop1 form in LβT2 cells strongly increased PACAP induced follistatin promoter activity (Fig. 13). Furthermore, AP1 and CRE site mutations totally eliminated follistatin promoter activity induced by PACAP in wild type LβT2 cells. Single mutation of the CRE site showed significant reduction of follistatin promoter activity in wild type LβT2 cells but not in PAC1-R overexpression cells whereas AP1 mutation result no significant decrease in wild type cells and no effect in overexpression cells. All these data provide support that PACAP regulates follistatin mRNA mainly through PKA pathway with both Short and Hop1 forms involved. This result was not a surprise considering that the main function of PACAP is to stimulate production of cAMP, which a key factor inducing the PKA pathway.

Our lab also built a transgenic mouse line with high level pituitary PACAP in both prenatal and postnatal period. Lifelong pituitary PACAP over-expression in male mice was associated with decreased gonadotropin subunit mRNA levels, lower circulating FSH and testosterone levels, which gave us additional *in vivo* evidence about PACAP suppressing FSH. Gene chip analysis of PN35 male wild-type mice and TG-mice confirmed a significant decline in FSHβ and an increase in follistatin. PACAP overexpression in pituitary also modified the expression of some novel genes not previously shown to be PACAP dependent.

Some of these genes are known to affect pituitary function (Table 3). EGR1 (-3.99 fold) was reported to activate LHβ and other gene promoters in gonadotrophs. Gamma-aminobutyric acid (GABA) may either stimulate or inhibit gonadotropin secretion depending on the physiological state of the gonadotrophs. In PN35 PACAP overexpressing mice, we saw that *GABA A receptor, beta 2* isoform was significantly decreased (-2.56 fold). *Neurod1* (+2.51 fold) has been shown to increase *GnRH-R* expression in immortalized mouse gonadotroph cells. The gene with the highest fold change (+14.97) was *Grp*. In rats, intravenous injection of GRP increases circulating LH levels and decreases TSH concentrations. GRP also stimulates ACTH release from rat pituitary cells in vitro and GRP peptide levels within the pituitary increase following repeated stress in rats. GRP receptor is also found in the rat pituitary. Therefore, GRP may play a role in pituitary gonadotroph function, and may be regulated by PACAP. Other genes in the list have been demonstrated to be involved in pituitary development, like *Fgfr2* (+2.40 fold) and *Tgfbi* (+4.51 fold).

In summary, these data suggest that like the perinatal period, PACAP plays a role in the suppression of FSH during the infantile period of development. A significant decline in pituitary PACAP expression prior to puberty causes a decline in follistatin expression allowing for stimulation of FSH expression by locally derived activin. Advancing the decline in pituitary PACAP expression by endogenous treatments of day 16 rats with a PACAP antagonist causes a time-advanced and significant decline in pituitary follistatin and a reciprocal rise in FSH expression.

Future investigations are planned to evaluate the effect of pituitary PACAP on the developing testis. In addition to PACAP overexpressing mice, we plan to use conditional knockout mice, which could delete PACAP in specific tissue and time point. Knockout of PACAP in pituitary after birth and around PN16 would reveal more details about effect of PACAP in the postnatal pituitary gonadotrophs.


A
FSH
LH
Serum LH (ng/ml)
Serum FSH (ng/ml)
Age (days)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
80
70
60
50
40
30
20
17
19
21
23
25
27
29

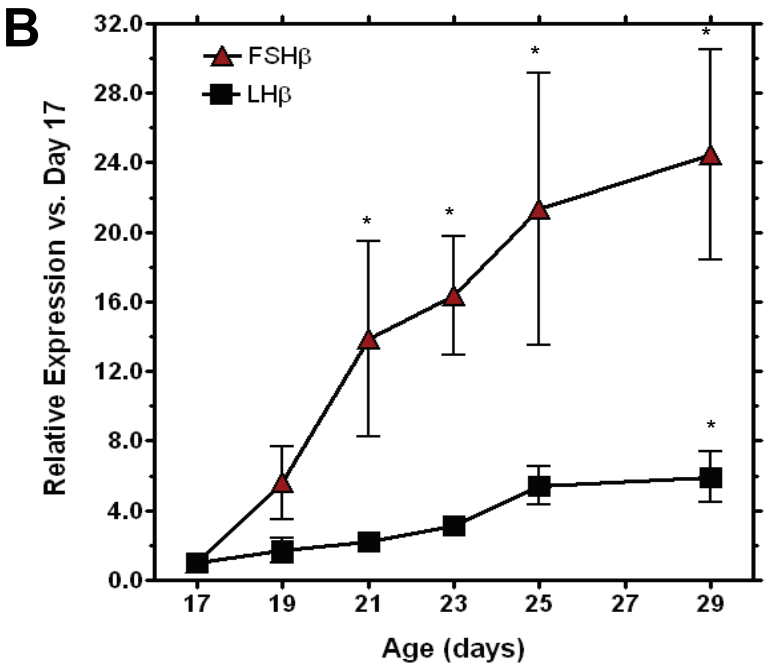

B
FSHβ
LHβ
Relative Expression vs. Day 17
Age (days)
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0.0
17
19
21
23
25
27
29

Figure 7. LH and FSH levels during male sexual development.

Serum LH and FSH (A) concentration in infant male rats were determined by ELISA. The pituitary LHβ and FSHβ mRNA (B) levels of these animals were expressed as levels relative to PN17 values. Each value represents the mean ±SEM of 6 rats per groups. * Significantly ($P<0.05$) different compared to PN17 levels.

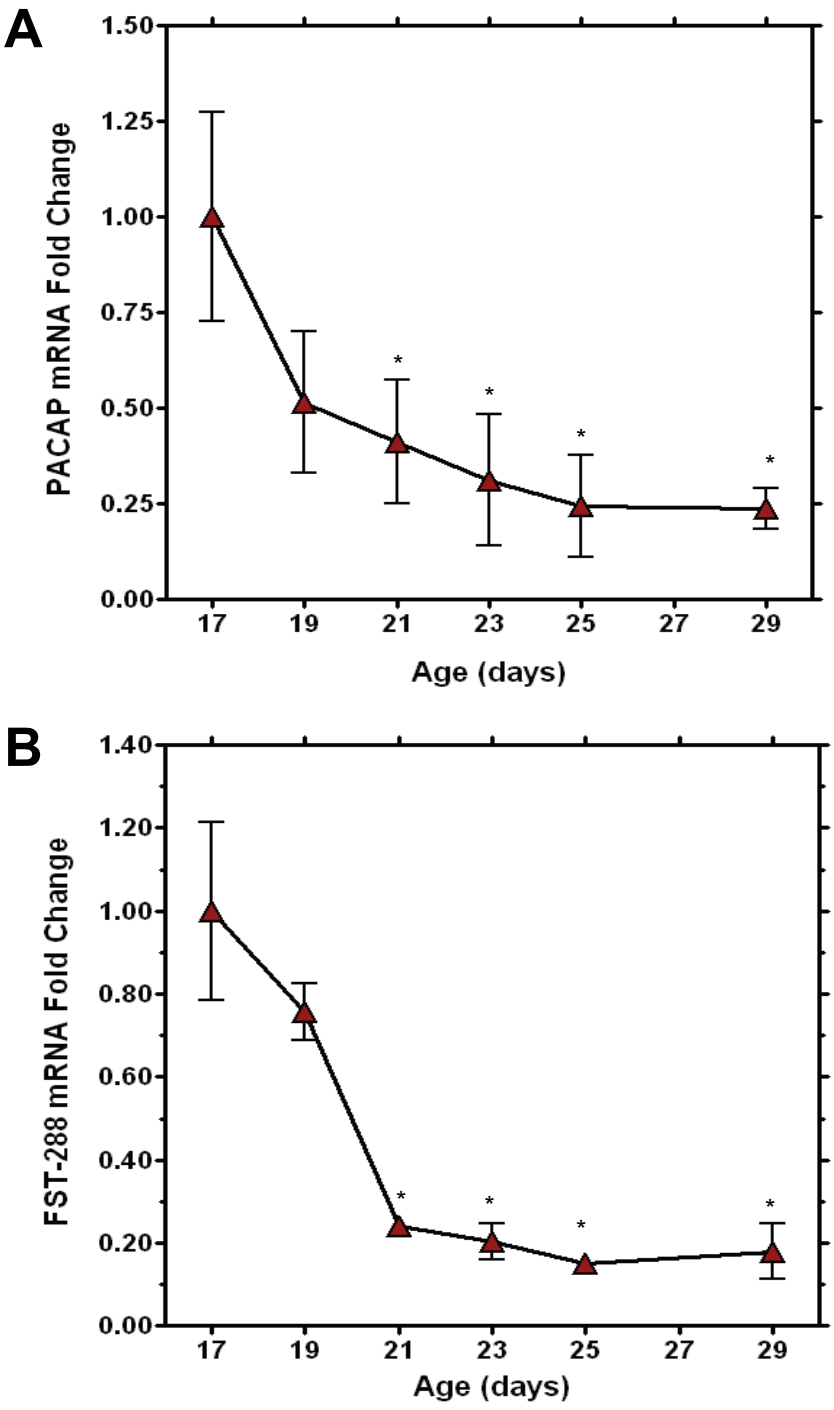

A
PACAP mRNA Fold Change
1.50
1.25
1.00
0.75
0.50
0.25
0.00
17
19
21
23
25
27
29
Age (days)
B
FST-288 mRNA Fold Change
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
17
19
21
23
25
27
29
Age (days)

Figure 8. PACAP and Fst-288 mRNA levels during male rat sexual development. PACAP mRNA (A) and Fst-288 (B) mRNA levels were determined by real-time PCR after reverse-transcription and were expressed as levels relative to PN17 values. Each value represents the mean ±SEM of 6 rats per group. * Significantly ($P<0.05$) different compared to PN17 levels.

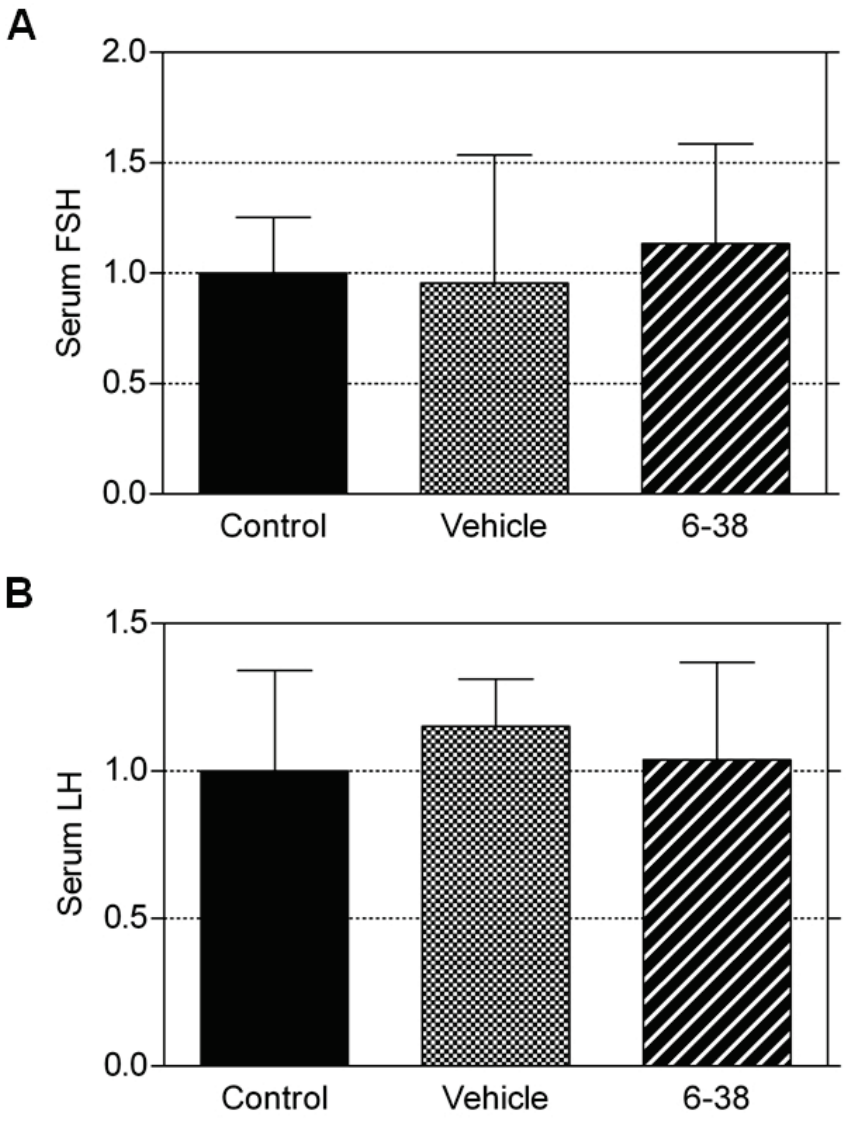

A
2.0
1.5
1.0
0.5
0.0
Serum FSH
Control
Vehicle
6-38
B
1.5
1.0
0.5
0.0
Serum LH
Control
Vehicle
6-38

Figure 9. Serum FSH and LH levels after 3 days treatment of PACAP 6-38. Micro-Osmotic pumps with vehicle (0.5% BSA) or 2μg/ml PACAP 6-38 antagonist were surgically implanted (i.p.) in PN16 male rats, and 3 days later, serum collected for determination of serum FSH (A) and LH (B) levels by ELISA. Each value represents the mean ±SEM of 5 rats per group. * Significantly ($P<0.05$) different with controls and vehicles.

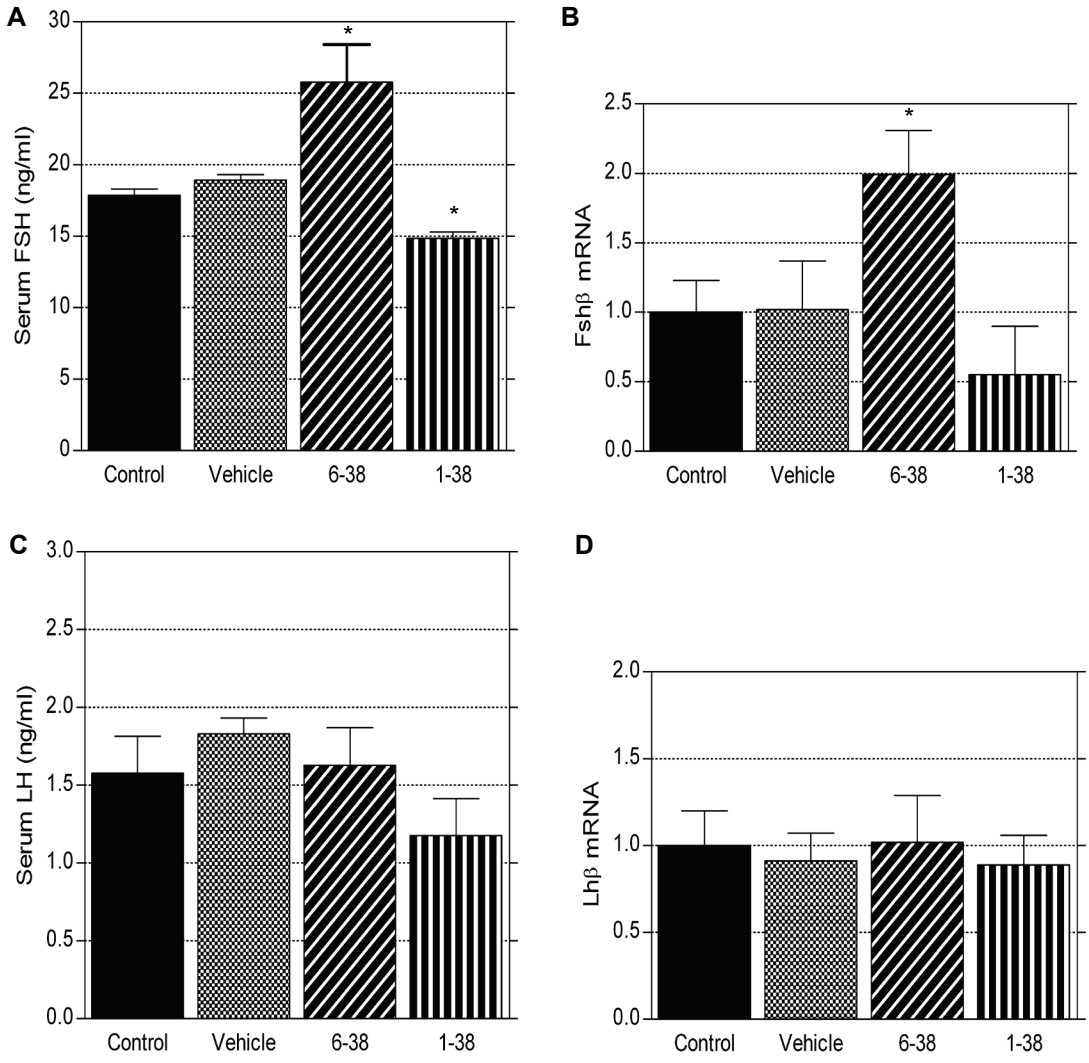

A
Serum FSH (ng/ml)
30
25
20
15
10
5
0
*
*
Control
Vehicle
6-38
1-38
B
Fshβ mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Vehicle
6-38
1-38
C
Serum LH (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Vehicle
6-38
1-38
D
Lhβ mRNA
2.0
1.5
1.0
0.5
0.0
Control
Vehicle
6-38
1-38

Figure 10. FSH and LH levels after 5 days PACAP 6-38 and 1-38 treatment. Micro-Osmotic pumps with vehicle (0.5% BSA) or 2μg/ml PACAP 6-38 antagonist or 2μg/ml PACAP 1-38 were surgically implanted (i.p.) in PN16 male rats, and 5 days later, RNA from Pituitary glands were isolated for determination of FSHβ (A) and LHβ (C) mRNA levels, while blood was collected for determination of serum FSH (B) and LH (D) levels by ELISA. mRNA data were normalized to controls and each ELISA value represents the mean ±SEM of 5 rats per group. * Significantly ($P<0.05$) different with control and vehicles.

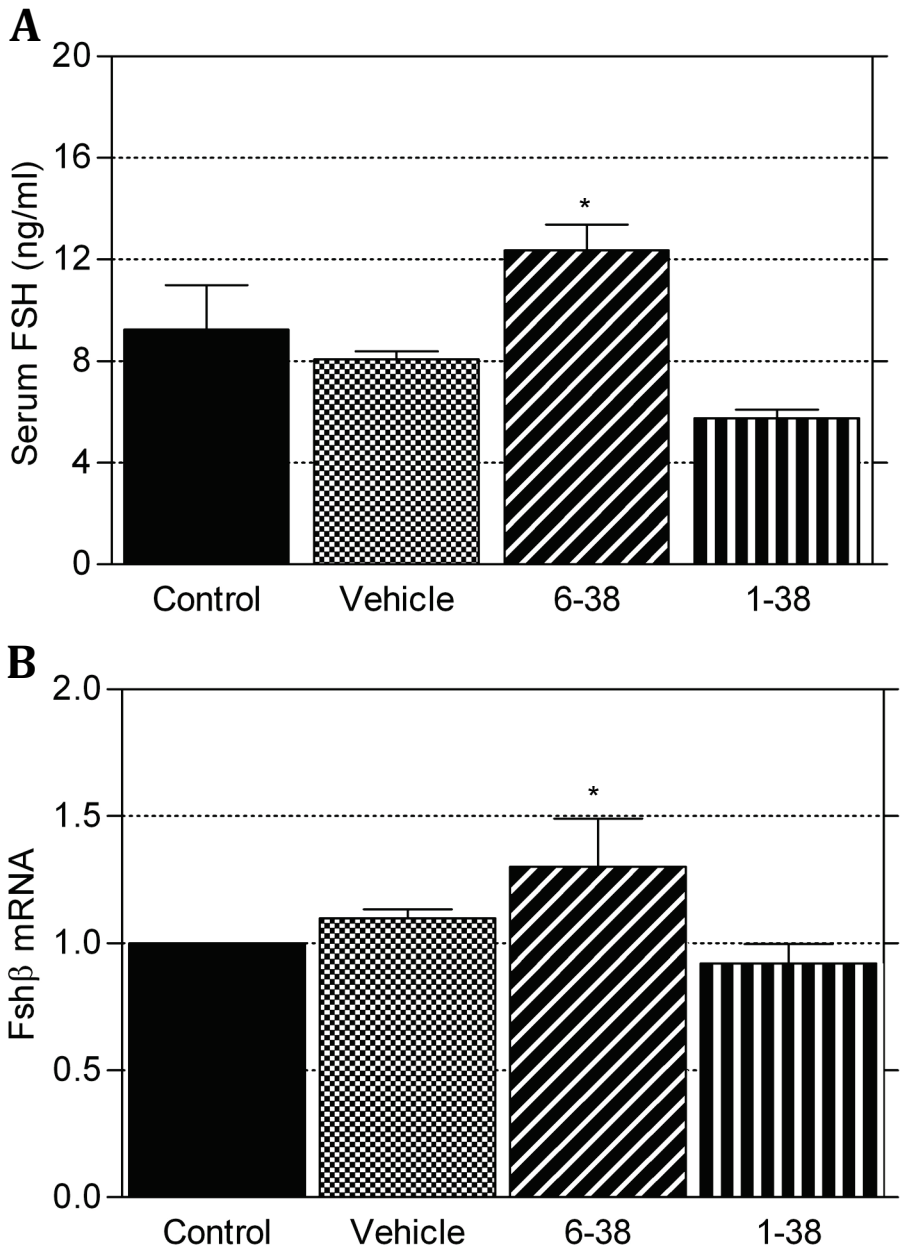

A
20
16
12
8
4
0
Serum FSH (ng/ml)
*
Control
Vehicle
6-38
1-38
B
2.0
1.5
1.0
0.5
0.0
Fshβ mRNA
*
Control
Vehicle
6-38
1-38

Figure 11. FSH levels after 7 days PACAP 6-38 and 1-38 treatment. Micro-Osmotic pumps with vehicle (0.5% BSA) or 2μg/ml PACAP 6-38 antagonist or 2μg/ml PACAP 1-38 were surgically implanted (i.p.) in PN16 male rats, and 7 days later, RNA from Pituitary glands were isolated for determination of FSHβ (A) mRNA levels, while blood was collected for determination of serum FSH (B) levels by ELISA. mRNA data were normalized to controls and each ELISA value represents the mean ±SEM of 5 rats per group. * Significantly ($P<0.05$) different with controls and vehicles.

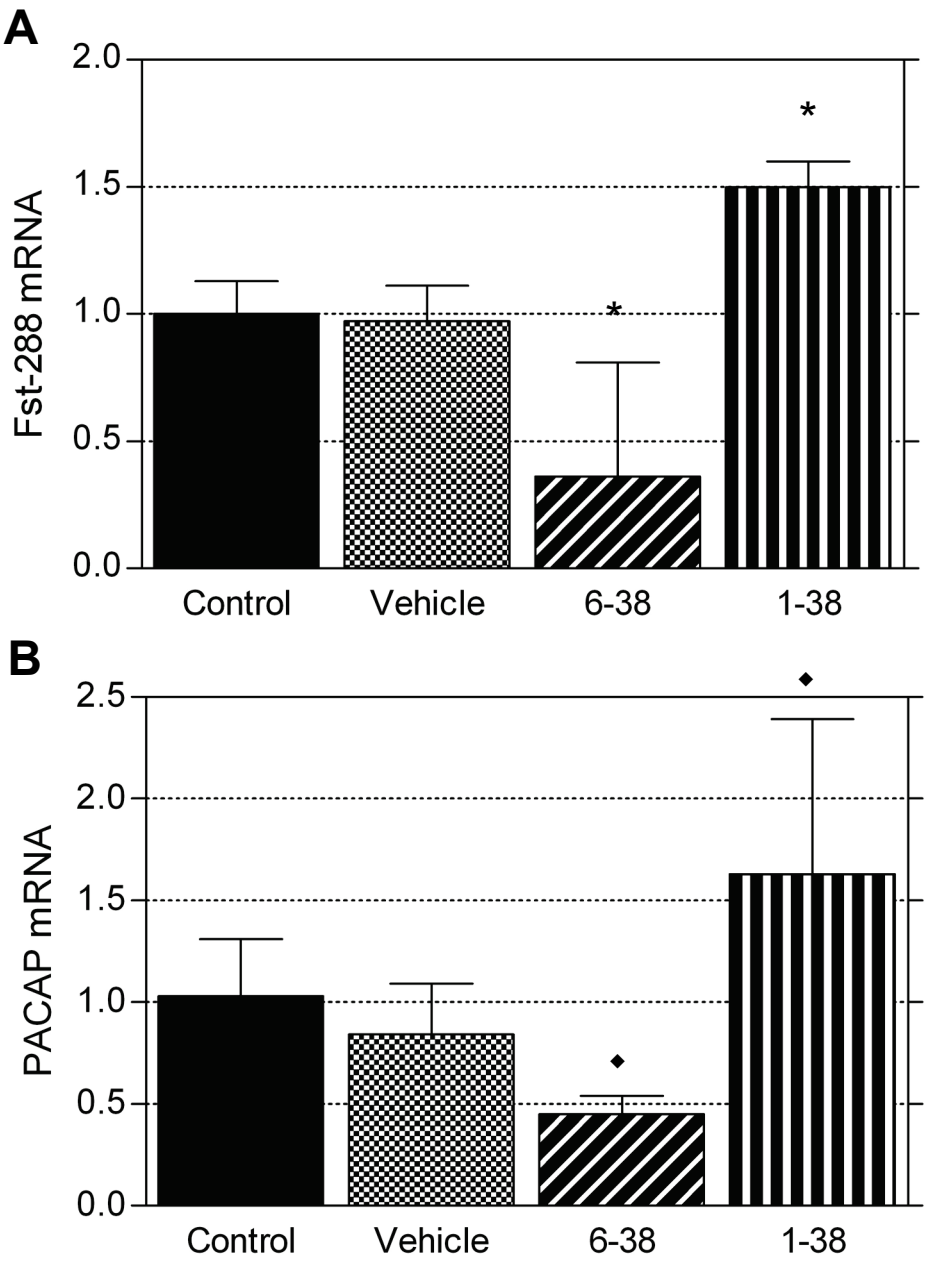
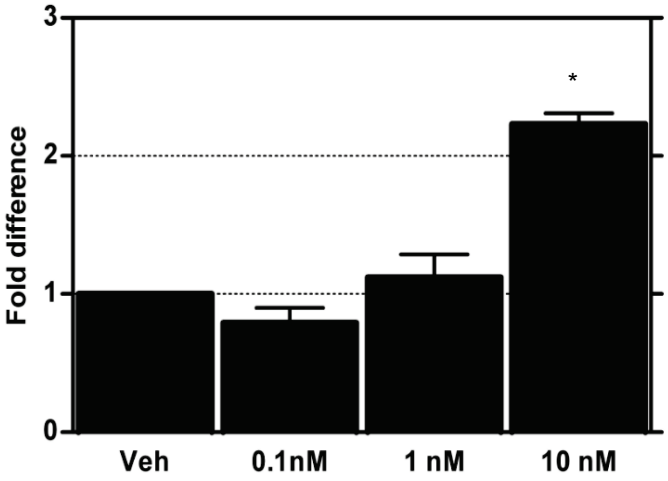

A
2.0
1.5
1.0
0.5
0.0
Fst-288 mRNA
*
*
Control
Vehicle
6-38
1-38
B
2.5
2.0
1.5
1.0
0.5
0.0
PACAP mRNA
◆
◆
Control
Vehicle
6-38
1-38

Figure 12. Fst-288 and PACAP mRNA level after PACAP 6-38 and 1-38 treatment. Micro-Osmotic pumps with vehicle (0.5% BSA) or 2 μg/ml PACAP 6-38 antagonist or 2μg/ml PACAP 1-38 were surgically implanted (i.p.) in PN16 male rats, and 5 days later, RNA from Pituitary glands were isolated for determination of Fst-288 (A) and PACAP (B) mRNA levels, mRNA data were normalized to controls. * Significantly ($P<0.05$) different with controls and vehicles. ◆ Significantly ($P<0.05$) different with vehicles only.

A.


Fold difference
3
2
1
0
*
Veh
0.1nM
1 nM
10 nM

B.

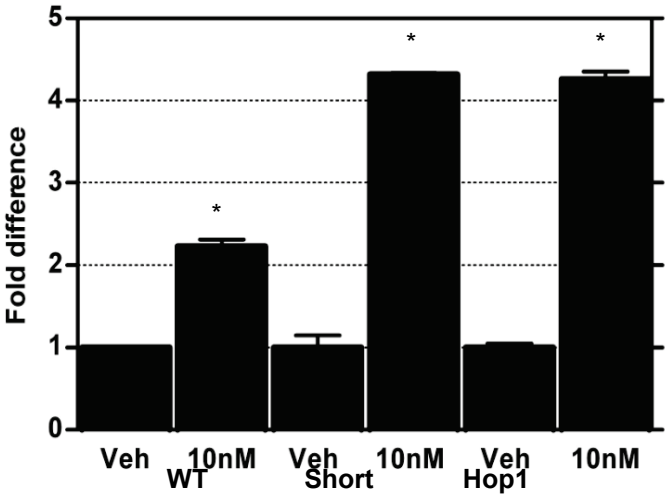

Fold difference
5
4
3
2
1
0
*
*
*
Veh
10nM
Veh
10nM
Veh
10nM
WT
Short
Hop1

Figure 13. Effect of PACAP on follistatin promoter in LβT2 cells.

LβT2 cells were transfected with the mouse the Fst-luc promoter reporter construct only (A) and with PAC1-R overexpression (Short and Hop1) (B), then treated for six hours with increasing doses (A) or 10nM (B) of PACAP38. Data are expressed as fold difference normalized to treatment with media alone. * Significantly different than medium alone ($p<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

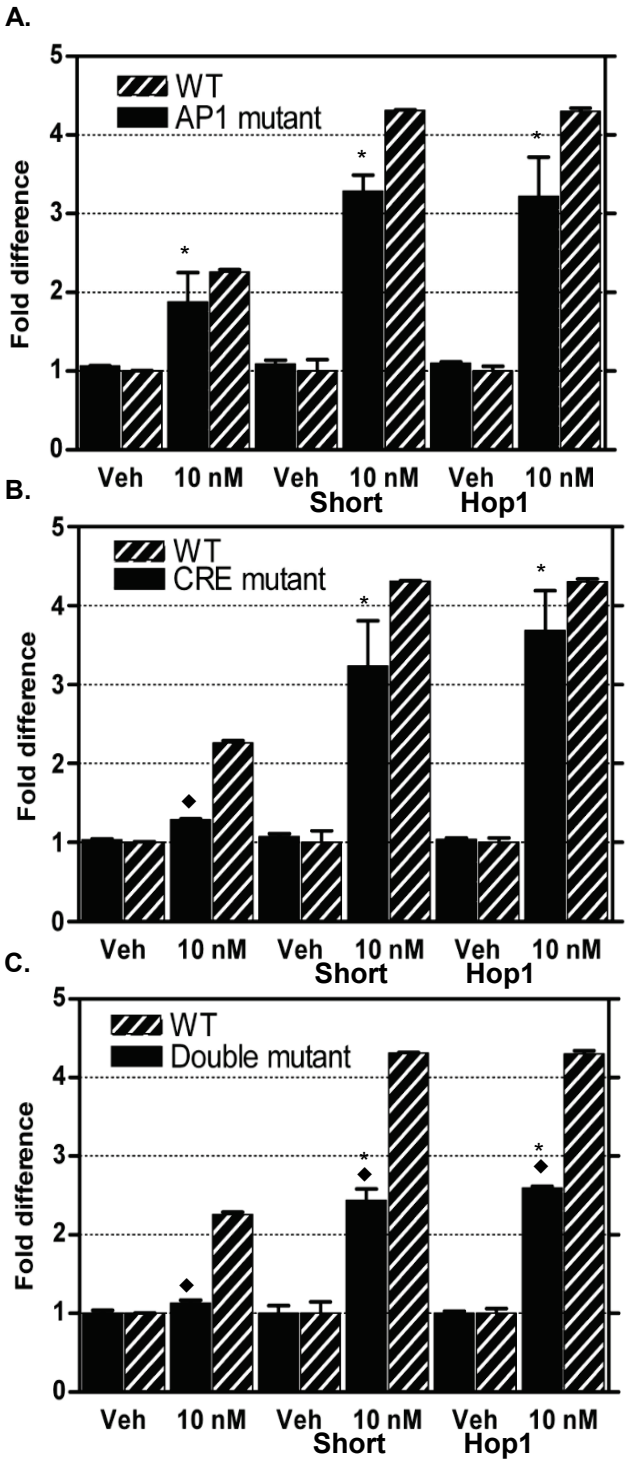

A.
WT
AP1 mutant
Fold difference
0
1
2
3
4
5
Veh
10 nM
Veh
10 nM
Veh
10 nM
Short
Hop1
B.
WT
CRE mutant
Fold difference
Veh
10 nM
Veh
10 nM
Veh
10 nM
Short
Hop1
C.
WT
Double mutant
Fold difference
Veh
10 nM
Veh
10 nM
Veh
10 nM
Short
Hop1

Figure 14. Effect of PACAP on mutant follistatin promoters in LβT2 cells. LβT2 cells were transfected with three types mutant Fst-luc alone, AP-1 (A) CRE (B) and Double mutation (C), or together with PAC1-R-Short or Hop1 expression vectors, then treated for six hours with medium alone or 10nM PACAP38. Data are expressed as fold difference normalized to FBS-free media treatment of transfected cells. * Significantly different than media alone ($p<0.05$) by ANOVA. ◆ Significantly different than wild type by Tukey's test. Results were from three experiments with triplicate wells.

Top Molecules (Change > ±2 fold, p<0.01)					
Gene Title	p-value	Fold-Change	Gene Title	p-value	Fold-Change
phosp. Reg. gene homol. to endopep. on the X chror	5.98E-06	-12.201	serine (or cysteine) peptidase inhibitor, clade F, 1	5.23E-04	2.291
gene regulated by estrogen in breast cancer	7.07E-04	-7.184	gap junction protein, delta 2	1.08E-03	2.292
early growth response 1	7.39E-03	-3.998	scavenger receptor class B, member 1	2.24E-03	2.294
hyaluronan and proteoglycan link protein 1	1.49E-03	-3.964	neuropeptide Y receptor Y1	1.72E-03	2.308
contactin associated protein-like 4	3.91E-05	-3.750	CART prepropeptide	4.94E-03	2.311
follicle stimulating hormone beta	6.57E-05	-3.214	ectonucleotide pyrophosphatase/phosphodiesterase 2	4.39E-05	2.363
serine (or cysteine) peptidase inhibitor, clade B, 1a	2.13E-05	-3.148	lumican	4.96E-04	2.381
angiotensin II receptor, type 1a	4.08E-03	-3.023	collagen, type V, alpha 1	2.80E-03	2.401
interleukin 23, alpha subunit p19	1.45E-05	-3.020	fibroblast growth factor receptor 2	2.83E-04	2.401
Rho GDP dissociation inhibitor (GDI) gamma (Arhg	4.16E-04	-2.810	solute carrier family 6, member 20A	7.98E-03	2.414
myosin, light chain 10, regulatory	1.82E-05	-2.693	RIKEN cDNA 5830443L24 gene	1.25E-03	2.431
gamma-aminobutyric acid (GABA) A receptor, beta	3.96E-03	-2.559	collagen and calcium binding EGF domains 1	3.74E-04	2.451
dynein, axonemal, heavy chain 9	6.71E-03	-2.517	seizure related 6 homolog like	1.05E-03	2.513
vasoactive intestinal polypeptide	3.48E-03	-2.509	odd Oz/ten-m homolog 2 (Drosophila)	7.97E-05	2.557
RIKEN cDNA 2810039B14 gene	4.18E-04	-2.316	periostin, osteoblast specific factor	2.80E-05	2.568
angiopoietin 1	2.24E-04	-2.309	EF hand domain containing 1	2.44E-03	2.612
EGF-like repeats and discoidin I-like domains 3	6.30E-03	-2.302	calcium channel, voltage-dependent, alpha2/delta 3	8.51E-04	2.710
gonadotropin releasing hormone receptor	1.35E-04	-2.267	protein C receptor, endothelial	1.29E-03	2.730
serine (or cysteine) peptidase inhibitor, clade E, 2	2.98E-05	-2.207	tachykinin 4	8.03E-03	2.815
G protein-coupled receptor 101	6.67E-03	-2.156	mitogen-activated protein kinase 4	1.69E-04	2.828
luteinizing hormone beta	5.86E-05	-2.124	sphingomyelin phosphodiesterase, acid-like 3B	8.56E-04	3.001
mitogen-activated protein kinase kinase 7	2.40E-04	-2.107	natriuretic peptide receptor 3	7.67E-04	3.196
oligodendrocyte myelin glycoprotein	2.28E-03	-2.063	predicted gene 626	2.63E-03	3.284
laminin, alpha 1	3.14E-03	-2.028	RIKEN cDNA 1810009J06 gene	2.79E-04	3.310
tweety homolog 1 (Drosophila)	2.14E-04	2.008	protease, serine, 28	1.15E-03	3.497
cyclin I	3.60E-04	2.022	kelch repeat and BTB (POZ) domain containing 5	2.29E-03	3.580
neurexin III	8.58E-03	2.025	pregnancy specific glycoprotein 18	1.06E-03	3.583
mannosidase 1, alpha	2.99E-03	2.035	fibronectin type III domain containing 1	6.75E-05	4.042
purinergic receptor P2Y, G-protein coupled, 14	7.61E-03	2.044	**follistatin**	1.73E-05	4.113
G protein-coupled receptor kinase 5	1.08E-03	2.093	UDP glucuronosyltransferase 1	1.45E-04	4.123
neurogenic differentiation 1	8.72E-04	2.098	growth associated protein 43	7.67E-05	4.126
melanoregulin	2.75E-03	2.104	cytochrome P450	1.45E-04	4.505
protein phosphatase 1, inhibitor subunit 3B	1.38E-03	2.133	transforming growth factor, beta induced	3.65E-07	4.513
suppression of tumorigenicity 18	6.07E-03	2.164	gremlin 2 homolog	1.29E-04	4.538
calcium/calmodulin-dependent protein kinase IV	2.86E-03	2.181	cartilage acidic protein 1	1.64E-05	4.754
sema domain, Ig domain , short basic domain	4.75E-03	2.214	neuropeptide Y receptor Y5	2.59E-05	4.951
collagen, type V, alpha 2	3.28E-03	2.215	nescient helix loop helix 2	5.66E-04	5.309
contactin associated protein-like 2	1.90E-03	2.224	diacylglycerol kinase kappa	1.01E-03	5.344
SH2B adaptor protein 2	9.41E-04	2.272	RIKEN cDNA 2900052N01 gene	1.32E-03	5.580
ribosomal modification protein rimK-like family	7.70E-03	2.276	pregnancy-specific glycoprotein 28	1.09E-04	7.965
complement component 1, q subcomponent-like 2	4.04E-03	2.278	doublecortin domain containing 2a	7.91E-06	8.277
			gastrin releasing peptide	4.8614E-07	14.9652

Table 2. List of genes mostly regulated by PACAP overexpression.

Top Biological Fuctions		
Diseases and Disorders		
Name	p-value	# molecules
Genetic Disorder	1.72E-04 - 1.95E-02	160
Neurological Disease	1.85E-05 - 1.95E-02	109
Skeletal and Muscular Disorders	2.26E-04 - 1.95E-02	83
Endocrine System Disorders	6.12E-05 - 1.95E-02	72
Reproductive System Disease	9.83E-05 - 1.95E-02	53
Molecular and Cellular Functions		
Name	p-value	# molecules
Cellular Development	1.51E-04 - 1.95E-02	72
Cell-To-Cell Signaling and Interaction	1.41E-04 - 1.95E-02	45
Cell Morphology	1.05E-04 - 1.95E-02	43
Small Molecule Biochemistry	6.60E-07 - 1.95E-02	40
Lipid Metabolism	6.60E-07 - 1.95E-02	29
Physiological System		
Name	p-value	# molecules
Tissue Development	4.02E-04 - 1.95E-02	42
Nervous System Development and Function	1.35E-04 - 1.95E-02	31
Reproductive System Development and Function	3.32E-05 - 1.95E-02	25
Tissue Morphology	2.14E-04 - 1.95E-02	21
Organ Morphology	3.32E-05 - 1.95E-02	16
Top Networks		
Associated Network Functions		Score
Small Molecule Biochemistry, Cancer, Reproductive System Disease		42
Lipid Metabolism, Small Molecule Biochemistry, Cell Cycle		37
Cell Morphology, Cell Movement, Skeletal and Muscular Development and Function		33
Amino Acid Metabolism, Cancer, Cell Morphology		33
Cellular Development, Cellular Growth and Proliferation, Gene		28
Top Canonical Pathways		
Name	p-value	ratio
GNRH Signaling	7.63E-03	7/144 (0.049)
PPARα/RXRα Activation	1.07E-02	8/182 (0.044)
Factors Promoting Cardiogenesis in Vertebrates	2.00E-02	5/89 (0.056)
Induction of Apoptosis by HIV1	2.50E-02	4/65 (0.062)
Human Embryonic Stem Cell Pluripotency	2.80E-02	6/148 (0.041)
Top Genes related to Pituitary		
Name	p-value	fold-change
Gastrin releasing peptide	4.86E-07	14.9652
Tansfroming growth factor, beta induced	3.65E-07	4.5133
Fibroblast growth factor receptor 2	2.83E-04	2.4007
CART prepropeptide	4.94E-03	2.3106
Neurogenic differentiation 1	8.72E-04	2.0981
Vasoactive intestinal polypeptide	3.48E-03	-2.5091
GABA-A receptor, subunit beta 2	3.96E-03	-2.5594
Angiotensin II receptor, type 1a	4.08E-03	-3.0232
Early growth response 1	7.38E-03	-3.9981

Table 3. IPA software analysis results

CHAPTER FOUR

REGULATORY FACTORS OF PITUITARY PACAP EXPRESSION

1. Introduction

The synthesis and secretion of LH does not always parallel FSH as we described in chapter three. The first physiological instance of differential gonadotropin regulation in males appears during fetal development with the predominance of LH over FSH (Chiappa and Fink, 1977; Chowdhury and Steinberger, 1976; Ketelslegers et al., 1978; Moore et al., 2003). The reason for this divergence is not known. Pituitary adenylate cyclase activating polypeptide (PACAP) has been demonstrated to differentially regulate gonadotropin subunit gene expression. PACAP stimulates α-subunit transcription, lengthens LHβ mRNA and presumably prolongs its half-life in rat pituitary cell cultures. However, PACAP inhibits the synthesis of FSHβ by stimulating follistatin transcription in gonadotrophs and folliculostellate cells (Carroll et al., 1989; Fujii et al., 2002; Tsujii and Winters, 1995b; Winters et al., 1997). We reported previously that PACAP and follistatin expression levels are high in the embryonic male pituitary, and decline significantly and in parallel at birth at which time FSHβ mRNA levels increase dramatically (Moore et al., 2009a). From these results, we hypothesize

that continuous exposure of the embryonic anterior pituitary to PACAP facilitates the early appearance of α- and LHβ-subunits but delays ontogeny of FSHβ by stimulating follistatin production and this hypothesis has been supported with our *in vivo* data from the male rats and the transgenic mice in chapter three.

However, little is known about the mechanisms that regulate PACAP gene expression in the pituitary. Treatment of rats with PACAP-38 increases pituitary PACAP mRNA levels (Radleff-Schlimme et al., 1998). The PACAP promoter contains sequence homologous to the CRE, and is activated by forskolin and by PACAP itself in neuroblastoma cells and in PC12 cells (Kozawa et al., 1995; Suzuki et al., 1994).

In the present study, we analyzed additional factors that could potentially regulate PACAP expression, both through stimulation and suppression. We first examined whether gonadal hormones have feedback on PACAP expression in gonadotrophs. In addition, activation of Dopamine-2 Receptor (Drd2) was tested as a potential down-regulation factor. The dramatic decline in pituitary PACAP expression that occurs around the time of birth provides an excellent model to examine developmental PACAP gene regulation in the pituitary. We propose that developmental changes in both pituitary-derived and endocrine factors regulate pituitary expression of PACAP, and the present studies were designed to begin to understand the factors that regulate pituitary PACAP expression. We utilized mouse PACAP promoter-reporter constructs (1.2 kb) transfected into αT3 and LβT2 gonadotroph cells, as models of immature and mature gonadotrophs, respectively. We measured basal and stimulated promoter activity in the two cell

lines in the absence or presence of PACAP and demonstrate the involvement of the PKA, PKC and MEKI signaling pathways in the activation of the PACAP promoter. Furthermore, we identified potential promoter regions, important for the regulation of *PACAP* gene transcription.

2. Materials and Methods

2.1. Cell Culture

αT3 and LβT2 immortalized mouse pituitary gonadotroph cells were kindly provided by Dr. Pamela Mellon (University of California, San Diego, CA). αT3 cells were grown in Eagle's Minimal Essential Medium (MEM) containing glucose (4.5g/L), $NaHCO_3$ (2.2g/L) and HEPES (5.96g/L), with penicillin, streptomycin, and fluconazole, and supplemented with 10% fetal bovine serum (FBS). LβT2 cells were grown in Dulbecco's MEM (DMEM) containing HEPES (22.5mM), $NaHCO_3$ (40mM), 10% charcoal-stripped FBS, penicillin, streptomycin, and fluconazole. Primary cell culture is the same as described in Chapter one.

2.2. Mouse PACpro-luc reporter constructs

A PCR generated cDNA fragment, including the promoter (-1218 - + 36) region of mouse PACAP, was cloned separately into the pSTBlue-1 vector by TA cloning. The cDNA were sequenced and found to be identical to published sequences. The cDNA was excised with restriction enzymes and cloned into the pGL3-Basic vector (Promega Corp., WI). Truncated promoter sequences were produced utilizing the restriction enzyme *Kpn I* and one of four other enzymes (-1018 by *Spe I*, -700 by *Bst I*, -541 by *Nde I*, -200 by *AaT II*) followed by re-

ligation. CRE site mutation (-200) was constructed utilizing the restriction enzymes *Aat II*, which broken the CRE site sequence.

2.3. Expression vectors

A dominant-negative inhibitor protein expression vector of CREB, termed A-CREB, was obtained from Dr. Vinson (Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, USA).

A rat PAC1-R-Short expression vector was obtained from Dr. Laurent Journot (National Center of Scientific Research, Montpellier, France). A human PAC1-R-Hop1 expression vector was obtained from Dr. Eve Lutz (Royal College, Glasgow, UK)

2.4. Cell Transfections

For transfections, αT3 and LβT2 cells were plated in 6-well plates at 1–2×10^6 cells/well and used within 1–2 days at 50–80% confluency. Approximately 3h prior to transfection, the media were replaced with fresh media (MEM+10% dextran-coated-charcoal-stripped FBS). Cells were transfected with Fugene-6 or GeneJammer Transfection Reagent (Stratagene, CA) according to the manufacturers' protocols. For each plasmid, phRL-TK vector (Promega, WI) was co-transfected into the cells (0.2mM) for use of Renilla luciferase expression to monitor transfection efficiency. After 24 hours, cells were washed and incubated with fresh media. After 48 hours, cells were treated with test substances for 6-8 hours; cells were then lysed and assayed for luciferase activity.

2.5. RNA extraction and reverse transcription

Total RNA was prepared from cultured αT3 and LβT2 cells using QIAGEN RNAeasy Kit following the instruction of manufacturer. 1μg total RNA from every sample was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, CA) primed with *oligo* (dT) following the instructions from Invitrogen.

2.6. Cell Count

0.5 ml of primary cell suspension was placed in a 1.5ml tube, then 0.1ml of 0.4% Trypin Blue (by GIBCO) was added with cells. Cells were staining for 5 minutes at room temperature. Metallized Hemacytometer (by Hausser) was filled with stained cell suspension (about 100μl). Numbers were counted under the 20 × Microscope then calculated using the following formula:

The number of cells per milliliter = Number of cells counted per square millimeter × dilution × 10,000

2.7. $2^{-\Delta\Delta C_T}$ Method for analyzing Real-time PCR results

Same procedure was used as described in Chapter three.

2.8. Microarray Analysis

Samples from wild type and PAC1-R-Hop1 overexpressed LβT2 cells treated with PACAP or medium alone are analyzed following the procedure stated in Chapter three.

2.9. Statistical Analysis

All the luciferase assays and real-time PCR data were performed with at least double samples and repeated at least three times. Values were expressed as mean ± SEM. Statistical analysis was performed using ANOVA ant post Tukey's test if necessary. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of Gonadal Hormones on PACAP expression

PACAP regulates gonadotropins synthesis and secretion and influence gonadal hormones through modulating gonadal responses to gonadotropins. To examine whether there are feedback mechanisms from gonadal hormones, we treated αT3 cells with increasing dose of hormones and analyzed PACAP mRNA expression (data not show) and promoter activity (Fig. 15). No changes in PACAP promoter activity were observed following various concentrations of testosterone, estradiol, or progesterone exposure. These data suggest that gonadal hormones within the circulatory system do not influence PACAP expression in gonadotrophs.

3.2. Self-stimulation of PACAP

3.2.1. Effects of PACAP on mouse PACAP promoter activity in gonadotroph cell lines

The *in vivo* results of the effects of PACAP and PACAP antagonist on pituitary PACAP expression suggest that PACAP can self-regulate its own expression. To examine how PACAP affects activation of the PACAP promoter in gonadotrophs, we utilized a luciferase-reporter construct containing the mouse PACAP promoter (-1218 - +36). Figure 16 shows that the PACAP promoter is activated by PACAP in both αT3 and LβT2 cells but is more sensitive to stimulation by PACAP in LβT2 cells than in αT3 cells (Fig. 16A and B).

3.2.2. Effects of PACAP on mouse PACAP promoter activity with PAC1-R receptors overexpressed in gonadotroph cell lines

The PAC1-R specific receptor has several splice subtypes (Spengler et al., 1993). Figure 17A confirms that the level of expression of Hop1 exceeds PAC1-R short in αT3 cells (Rawlings et al., 1995), while both subtypes are expressed at similar low levels in LβT2 cells. We overexpressed the PAC1-R-Short or Hop1 in αT3 and LβT2 cells (Fig. 17A). When these cell lines were also transfected with the PACpro-luc reporter construct, 10nM PACAP significantly increased PACAP stimulated promoter activation more strongly in PAC1-R-Short and PAC1-R-Hop1 cells than in wild type (WT) αT3 or LβT2 cells. Compared to WT LβT2 cells, PAC1-R Short overexpression induced a 3-fold increase in PACAP promoter activity, while PAC1-R-Hop1-overexpression resulted in a 4-fold increase in PACAP activation of the PACAP promoter compared to stimulation in WT cells (Fig. 17C). Based on the level of expression following transfection (Fig, 17A), the Hop1 form appears to be more effective than the short form in increasing PACAP stimulated PACAP promoter activity in LβT2 cells. In αT3 cells, on the other hand, only PAC1-R-Short overexpression significantly increased PACAP stimulation of the PACAP promoter. Thus the PAC1-R-Hop1 is more effective in transducing the PACAP signal in mature LβT2 than in immature αT3 cells (Fig. 17B).

3.2.3. Evaluation of second messenger cascades involved in stimulation of the PACAP promoter

PACAP has been demonstrated to increase cAMP production, PKC activity and Ca^{2+} mobilization (Miyata et al., 1989; Niewiadomski et al., 2002; Rawlings et al., 1993; Taupenot et al., 1999). However, little is known about the signaling pathways by which PACAP stimulates the PACAP promoter. To

determine the important pathways that regulate PACAP promoter activity, we used the pharmacological inhibitors BIM (PKC inhibitor), H-89 (PKA inhibitor) and PD98059 (MAPK inhibitor) in cultures of αT3 and LβT2 cells transfected with the mPACpro-luc. Basal promoter activity in αT3 cells was decreased when the PKA (50%) or MAPK (30%) pathways (Fig. 18A) were inhibited but not when PKC signaling was blocked with BIM at doses previously shown to inhibit forskolin-stimulated follistatin promoter activity (Winters et al., 2007). In contrast, basal promoter activity was reduced by H89 but was unaffected by PKC or MAPK inhibitors in LβT2 cells (Fig. 18B). In αT3 cells stimulated with PACAP, H-89 and PD98059 markedly decreased PACAP promoter activity while BIM was less effective (Fig. 18C). These results demonstrate that the PKC, PKA and MAPK pathways are all involved in PACAP induced promoter activity and suggest that the PKA and MAPK pathways are more critical mediators of PACAP promoter activity in αT3 cells.

3.2.4. Regional analysis of basal and stimulated mouse PACAP promoter activity in αT3 and LβT2 cells

By sequence homology and previous research, we identified a series of putative binding sites for transcription factors on the PACAP promoter that share at least 80% homology to their consensus sequences and are present in pituitary (Fig. 19) (Ohkubo et al., 1992; White et al., 2000). Based on these findings, we constructed five mouse PACAP promoter reporter constructs, each with a sequential truncation of approximately 200 *bp*, and treated αT3 and LβT2 cells for 6 hours with 10nM PACAP or control media.

Fig. 19A and B showed the basal activity changes after sequential cutting. We found two regions that may mediate an inhibitory signal to the PACAP promoter. One element is located between position -700 and -541 and is effective both in αT3 and LβT2 cells. The second region, between -1218 and -1018, appears to possess inhibitory binding sites but only in αT3 cells. In figure 19C and D, in which cells were treated with PACAP, a second element (-1218 to -1018) was essential for maximum PACAP induced activity in αT3 but not in LβT2 cells. The promoter region from -541 to -200 contains elements that mediate the effects of PACAP because deletion of this region resulted in a pronounced loss of promoter activation in both cell lines (Fig. 19C and D). However, the element (-700 to -541) does not influence PACAP induced promoter activity.

3.2.5. PACAP promoter activity required CRE site

PKA and MAPK signaling mediate PACAP expression in gonadotrophs, and serial deletions of the PACAP promoter identified the -541 to -200 region to be critical for promoter activity. This region of the mouse PACAP promoter contains regions with similarity to the consensus CRE element, a well-described mediator of PKA signaling. Furthermore, we have evidence that PACAP induces CRE activities utilizing a luciferase reporter construct with a CRE element in both cell lines. Therefore, αT3 and LβT2 cells were transfected with the mPACpro-luc construct and with increasing concentrations of an expression plasmid encoding a dominant negative inhibitor of CREB (A-CREB) and a filler plasmid. A-CREB

decreased both basal (Fig. 20A) and PACAP-induced (Fig. 20C) promoter activity dose-dependently to a similar extent in αT3 and LβT2 cells.

We also designed a CRE site mutant (around -200) mPACpro-luc construct and transfected it into both αT3 and LβT2 cells. In αT3 cells, CRE site mutation almost totally blocked PACAP induced promoter activity compared to WT construct, whereas in LβT2 cells PACAP induced a little activity with mutant construct but not statistic significant (Fig. 21A and B). Furthermore, overexpression of the PAC1-R-Short or Hop1 forms in LβT2 cells could not totally abrogate this inhibition but caused a significant increase compared to medium alone (Fig. 21C).

3.2.6. Gene-array analysis LβT2 cells overexpressed PAC1-R and treated with 10nm PACAP

To evaluate novel genes that could be either up- or down-regulated by PACAP signaling, we performed gene chip microarray analysis comparing untreated or 10 nM PACAP treated LβT2 cells and PACAP treated LβT2 cells with Hop1 overexpression. As depicted in Table 4, six hour PACAP38 treatment of LβT2 cells transfected with control vector resulted in significant ($p<0.01$, change > 20%) alterations in 223 genes (106 increased, 117 decreased). PACAP38 treatment of PAC1-R-Hop1 transfected LβT2 cells resulted in significant alterations of 393 genes (171 increased, 222 decreased), of which, 113 (44 increased, 69 decreased) were common to PACAP treated control cells. Ingenuity Systems Pathway Analysis revealed significant changes in gene expression of putative molecules related to specific canonical pathways (Table 4).

PACAP treatment of control and PAC1-R-Hop1 transfected LβT2 cells resulted in significant changes in molecules in pathways known to regulate gonadotroph function such as, increases in the PKA (PRKACB, CREM) and PI3K/AKT (*cRAF, Bcl-XL, p21cip1, eNOS*) signaling pathways while decreasing molecules in the ERK/MAPK (*Myc, PIK3R1, TLN2*) and BMP (*BMPR2, Runx2*) pathways. Overexpression of the PAC1-R-Hop1 receptor leads to additional changes in gonadotroph regulatory pathways including significant increases in molecules in the JAK/STAT (*Bcl-XL, p21cip1, RAF1, CDKN1A*), TGF-β (*INHα, RAF1, HNF*) and Interleukin (*BAX1, RAF1, GNB3, CDH1, CXCL 10/11, I-TAC*) signaling pathways and significant decreases in molecules in the GnRH (*EGR1, GnRHr*), insulin and IGF-1 (*FOX01, SOCS2*), EGF (*NRAS, PIK3R1, MAP3K*), Wnt/β-catenin *(β-catenin, ACVR1, ACVR2A, Frizzled family receptor*), estrogen and androgen (*HSP90, SRY*) signaling pathways. Many other molecules related to signaling pathways not previously associated with gonadotroph functioning were also significantly altered.

3.3. Dopamine-2 Receptor activation Decreases PACAP expression in rat pituitary gland

Last section tells us that PACAP stimulated itself through PKA pathways as one of the positive regulators in addition to GnRH (Grafer et al., 2009a). We proposed one potential negative regulator (dopamine) that might interrupt PACAP expression in gonadotrophs. The dopamine-2 receptor, Drd2, has been demonstrated to be expressed in αT3 and LβT2 gonadotroph cell lines (Kanasaki et al., 2002; Mutiara et al., 2006). Drd2 couples to Gαi to inhibit adenyl cyclase

activity, and reduce cAMP levels (Missale et al., 1998) whereas PACAP increases the level of cAMP in gonadotroph cells. Furthermore, Dopamine production increases near birth in the rat hypothalamus, the main control center of pituitary (Hooghe-Peters et al., 1988). It is possible that dopamine activates gonadotroph Drd2 to suppress cAMP production, which can induce a decreased PACAP expression.

To prove the possibility that Drd2 activity is involved in regulating pituitary PACAP expression, pituitary cell cultures were prepared from E19 rats, and were treated for 24 hours with the dopamine receptor agonists (Bromocriptine and BIM53097) or with PACAP antagonist 6-38. Both PACAP 6-38 and dopamine receptor agonists lowered PACAP expression (Fig. 22).

4. Discussion and Future Investigations

The mammalian gonadotropins, Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) play a critical role in the regulation of reproductive development and function. In females, an acute rise in circulating LH triggers ovulation and development of the corpus luteum while FSH stimulates the maturation of follicles in the ovary. In males, LH stimulates Leydig cell production of testosterone, and FSH stimulates primary spermatocytes to undergo the first division of meiosis to form secondary spermatocytes (Dorrington and Armstrong, 1979; Leung and Armstrong, 1980; Louvet et al., 1975). Interestingly, LH and FSH expression are not always parallel during important physiologic functions. Compared to LH, FSH is more prominent in mature mammals whereas during fetal life, a lower level of expression of FSHβ may prevent the early maturation of

the reproductive system. Since PACAP was discovered as a hypothalamic-releasing factor, and is known to be expressed in the gonadotrophs of the anterior pituitary, subsequent research has revealed its potential functions in reproduction. PACAP contributes to the regulation of gonadotrope function through several mechanisms: directly on gonadotropes, either alone or by an interaction with GnRH signaling, indirectly, by modifying GnRH release or GnRH receptor expression, and through paracrine/autocrine actions (Counis et al., 2007; Culler and Paschall, 1991; McArdle et al., 1994; Ortmann and Diedrich, 1999; Tsujii and Winters, 1995a). For example, there is evidence that PACAP suppresses FSH expression before birth through stimulation of follistatin. Most importantly, in Chapter three, we proved that PACAP suppresses FSH expression during male rat sexual development through stimulation of follistatin. Follistatin binds with Activin to prevent stimulation of FSHβ transcription (Fujii et al., 2002; Moore et al., 2009b). Therefore, the PACAP expression pattern is important for reproductive development, and it is important to evaluate the factors involved in the regulation of PACAP expression. Compared to the number of studies of PACAP functions, however, only a few papers have described regulation of PACAP expression. PACAP has been shown to up-regulate its own level of expression in several cell lines, and injection of PACAP (10μg bolus) increased PACAP mRNA expression in the pituitary of adult rats (Radleff-Schlimme A, 1998). GnRH was found to increase PACAP expression in LβT2 cells through PKA and PKC pathways (Grafer et al., 2009b). Within the HPG axis, gonadal hormones have some feedback to the hypothalamus GnRH expression.

However, only estrogens and progesterone are found to stimulate PACAP in the rat hypothalamus (Apostolakis et al., 2004; Ha et al., 2000).

Our experiments revealed that gonad hormones could not stimulate or suppress PACAP promoter activity in gonadotroph cell lines (Fig. 15). Therefore, there is no direct feedback connection between gonadal hormones and gonadotroph PACAP expression. But we cannot ignore the possibility that gonadal hormones regulate PACAP indirectly through other factors, like GnRH.

PACAP increase its own level of expression in several cell lines and in the rat pituitary. The results from Chapter three tell us that PACAP treatment increases its pituitary PACAP mRNA levels in immature male rats whereas antagonist decreases the level. PACAP antagonist decreases PACAP mRNA expression in E19 primary pituitary cell cultures as well. To gain more information about PACAP self-stimulatory function, we designed experiments to study the mouse PACAP promoter. The -1280 to +36 region of the mouse PACAP promoter was cloned into a luciferase-reporter plasmid. The promoter was active in both gonadotroph cell lines, and was stimulated 4-fold by PACAP even though the level of expression of PAC1-R is much lower in LβT2 cells. Furthermore, dose response curves revealed that LβT2 cells are more sensitive to PACAP treatment than αT3 cells. These differences may be due to the much higher level of PACAP expression in αT3 cells. Overexpression of the PAC1-R-Short or Hop1 forms substantially increased PACAP stimulated PACAP promoter activity in LβT2 cells with a much smaller increase in αT3 cells. The Hop1 form was also more effective than the Short form in LβT2 cells even though the level of

overexpression of the two receptors was similar. The various cassettes of PAC1-R are couple to different second messengers, and while both isoforms stimulate cAMP production Hop1 also stimulates PLD (McCulloch et al., 2000). The much lower level of expression of PAC1-R in LβT2 than αT3 cells may also partly explain this finding. In αT3 cells, there already exists a high level of endogenous PACAP expression, which may mask the effect of exogenous PACAP treatment.

PAC1-R is known to activate several signaling pathways including the PKA, PKC and MAPK-pathways (Dickson and Finlayson, 2009; Holighaus et al., 2011; May et al., 2010). BIM, H-89 and PD98059 were used to disrupt the PKC PKA, and MAPK pathways, respectively, in un-stimulated or PACAP-stimulated αT3 and LβT2 cells. The results imply that the PKA pathway plays a key role in PACAP promoter activity both in αT3 and LβT2 cells. Blocking the MAPK pathway also markedly inhibited basal and PACAP stimulated PACAP expression in αT3 cells providing the first evidence for MAPK regulation of PACAP in gonadotrophs. On the other hand, MAPK signaling plays a lesser role in PACAP expression in the more mature LβT2 cell line.

In a regional analysis of the mouse PACAP promoter, we found three potential regions, which could play a role in pituitary PACAP expression. The region between -1280 and -1080 appears essential for maximal basal and PACAP stimulated activity in αT3 whereas -541 to -200 is important for basal and stimulated activity in both cell lines. In addition, we found that the putative CREB, Jun and AP1 sites, which are important in PKA and MAPK pathways, are essential for maximal PACAP expression. The region from -700 to -541 is more

critical for promoter activity in LβT2 cells. It may mediate an inhibiting signal for PACAP regulation in both cell lines. In this region, there is an important transcript factor binding site for pituitary function, SMAD3. SMAD3 has been demonstrated to be essential for Activin and GnRH active FSHβ expression (Coss et al., 2007; Coss et al., 2010). Through Chapter three, PACAP might inhibit FSHβ through follistatin binding with activin. During pituitary development, it is possible that some factors connected with SMAD3 pathway could suppress PACAP expression after birth. Similar interactions could exist for other factors in this region such as STAT, GATA3. The differences between LβT2 and αT3 cells may be caused by different maturity levels and imply that signaling pathways are changing during development.

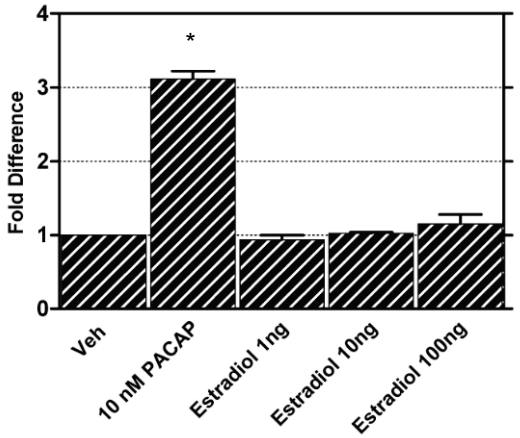
Through the PACAP promoter regional analysis, -541 to -200 is critical for promoter activities in both cell lines. Sequence analysis revealed a putative CRE binding site located at around -210. Its binding protein CREB family is believed to depend on PKA pathway (Meinkoth JL, 1993). Co-transfecting the luciferase-report construct with CREB dominant-negative construct to αT3 and LβT2 cells showed CRE pathways were definitely involved in PACAP basal and stimulated promoter activity. The same result was received when we used CRE-site mutant mPACpro-luc construct. Furthermore, overexpression of PAC1-R receptors did not have any influence on these results. So stimulation of PACAP promoter needs participation of CRE- family. And the results proved that PKA signaling induced transcript factor CRE-family binding to PACAP promoter may be the most important regulation pathway for PACAP.

Global analysis of alterations in gene expression in LβT2 cells in response to PACAP stimulation confirmed known changes in gene expression and revealed changes in molecules previously not associated with PACAP signaling. PACAP receptors are known to stimulate the PKA and PI3K pathways, and molecules associated with these pathways were increased significantly in response to 6h PACAP exposure. Surprisingly, Ingenuity Pathway Analysis predicted a net decrease in ERK/MAPK signaling (Table 4) in response to PACAP; however, MEK1 and MEK2 were both increased by PACAP perhaps through an alternate intracellular signaling pathway. Confirmation of the importance of MAPK signaling was demonstrated by a significant decrease in PACAP promoter activity in the presence of a MEK1 inhibitor (Fig. 18). The predicted decrease in BMP signaling was strongly influenced by the significant decrease in BMP receptor type II expression. BMP has been demonstrated to selectively increase synthesis and secretion of FSH while PACAP has the opposite effect (Huang et al., 2001; Otsuka and Shimasaki, 2002; Takeda et al., 2007; Takeda et al., 2003) so that the decreases in BMP receptor expression and signaling may contribute to the selective down regulation of FSH in response to PACAP. Of particular interest were the changes observed in gene expression following overexpression of the Hop1 isoform of the PACR-1 receptor. Increased PACAP signaling through the Hop1 receptor lead to increases in molecules related to interleukin signaling including molecules in the JAK/STAT signaling cascade. The interleukins have been shown to inhibit gonadotropin release, and stimulation of various interleukin signaling pathways may have a role in the

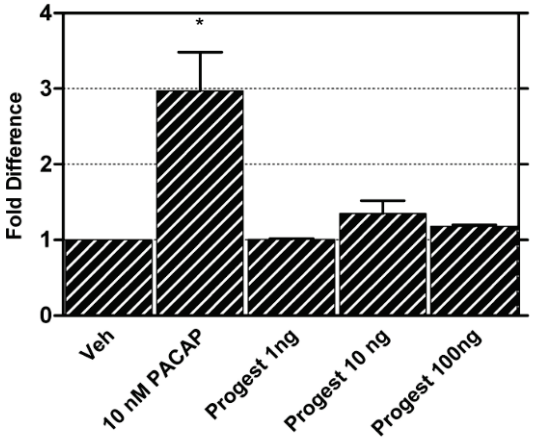
suppressive effect of PACAP on FSH production (Bilezikjian et al., 1998; Feng et al., 1991; Karanth and McCann, 1991; Murata and Ying, 1991; Yamaguchi et al., 1990). The predicted decrease in TGFβ signaling was strongly influenced by observed decreased expression of the activin receptors I and IIa. TGFβ is a potent stimulator of FSH secretion (Ying et al., 1986) and stimulates FSHβ mRNA expression (Suszko and Woodruff, 2006). This observation suggests yet another mechanism by which PACAP can inhibit the expression of FSH. The gene array results also suggest that PACAP may have a role in mediating the effects of EGF, IGF-1, insulin, Wnt/β-catenin and through each of these pathways was predicted to be reduced following PACAP exposure of cells with PAC1-R-Hop1 overexpression (Gardner et al., 2010; Gutierrez et al., 2007; Mouihate et al., 1996; Navratil et al., 2009; Weiss et al., 2003; Weiss et al., 2006; Xia et al., 2001). Of particular interest were the effects of PACAP exposure on GnRH signaling. Six hour PACAP exposure resulted in significant decreases in GnRH receptor expression and signaling, and in Egr1 expression in LβT2 cells with PAC1-R-Hop1 overexpression. A decrease in these molecules was also observed in transgenic mice that overexpress PACAP in the pituitary (Moore et al., 2012). Pituitary PACAP transgenic mice have lifelong suppression of gonadotropin secretion due in part to increased follistatin and decreased Egr1 and GnRH receptor expression. The global gene expression analysis reveals that PACAP may interact with multiple extracellular and intracellular signaling pathways to regulate gonadotroph function.

Dopamine activates five types of G-protein coupled receptors, D1-D5, and their variants. Drd2 is expressed at a high level in pituitary, and we found it in αT3 and LβT2 cell lines. Furthermore, single cell analysis demonstrated that Drd2 mRNA and PACAP mRNA co-localized in some cells. Two Drd2 agonists, Bromocriptine and BIM53097, suppressed PACAP mRNA expression in E19 rat pituitary cell cultures respectively (Fig. 22). All these data implied the suppressive effect on PACAP might be through the activation of Drd2. In addition, dopamine levels in the CNS rise dramatically during the perinatal period, while PACAP mRNA begins to decrease (Hooghe-Peters et al., 1988). In future experiments, we will utilize *in vitro* systems to examine the cAMP levels changes after treatment of Drd2 agonists and validate that activation of Drd2 reduce its levels. Furthermore, pharmacological inhibitors of different signaling pathways will be utilized to reveal which pathways are involved in this suppression function.

A.


Fold Difference
4
3
2
1
0
*
Veh
10 nM PACAP
Estradiol 1ng
Estradiol 10ng
Estradiol 100ng

B.


Fold Difference
4
3
2
1
0
*
Veh
10 nM PACAP
Progest 1ng
Progest 10 ng
Progest 100ng

C.

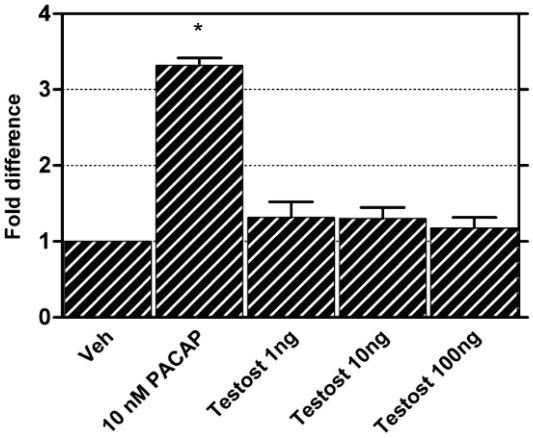

Fold difference
4
3
2
1
0
*
Veh
10 nM PACAP
Testost 1ng
Testost 10ng
Testost 100ng

Figure 15. Effect of Gonadal hormones on PACAP promoter activity.

The mPACpro-luc construct was transfected into αT3 cells, then treated cell with increasing dose of gonadal hormones for 24 hours. Data are expressed as fold difference normalized to medium alone. * Significantly different than media alone ($P<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

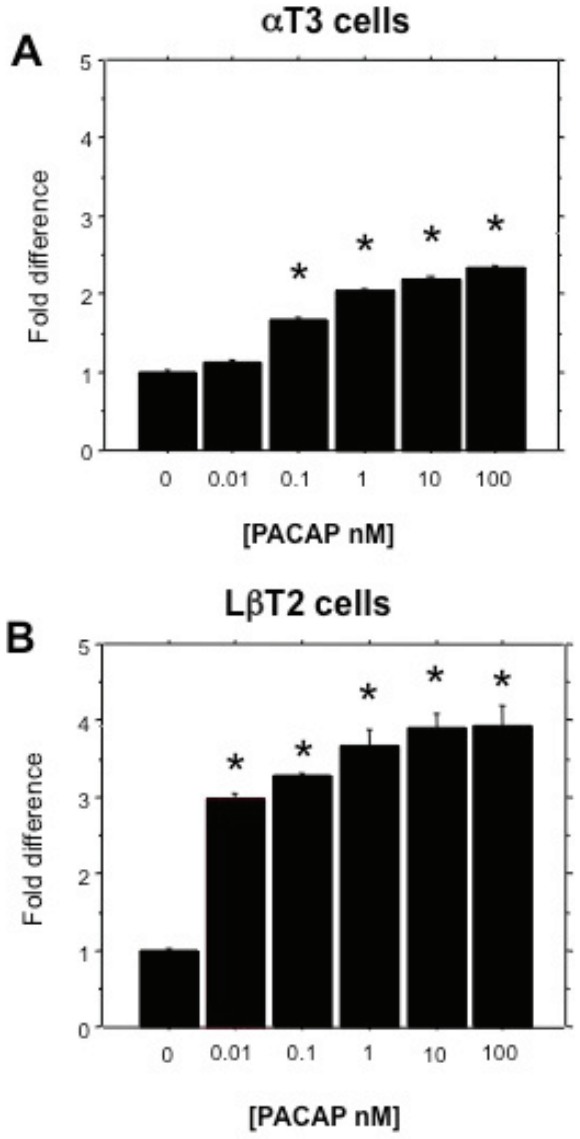
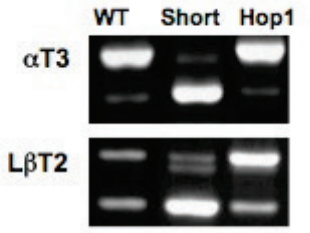

A
αT3 cells
Fold difference
0
1
2
3
4
5
*
*
*
*
0
0.01
0.1
1
10
100
[PACAP nM]
B
LβT2 cells
Fold difference
0
1
2
3
4
5
*
*
*
*
*
0
0.01
0.1
1
10
100
[PACAP nM]

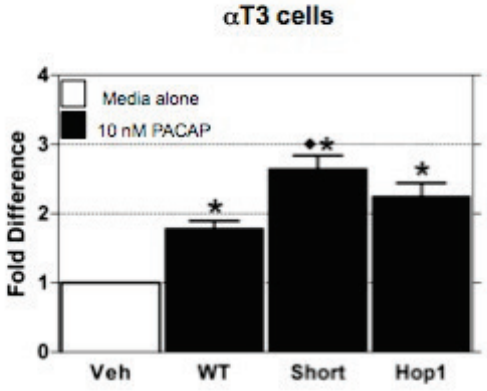
Figure 16. PACAP induced promoter activity in αT3 and LβT2 cells.

αT3 (A) and LβT2 (B) cells were transfected with the mPACpro-luc construct and treated for six hours with increasing concentrations (0-100nM) of PACAP. Data are expressed as fold difference normalized to media alone. * Significantly different than media alone ($P<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

A


WT
Short
Hop1
αT3
LβT2

B


αT3 cells
Media alone
10 nM PACAP
Fold Difference
0
1
2
3
4
Veh
WT
Short
Hop1

C

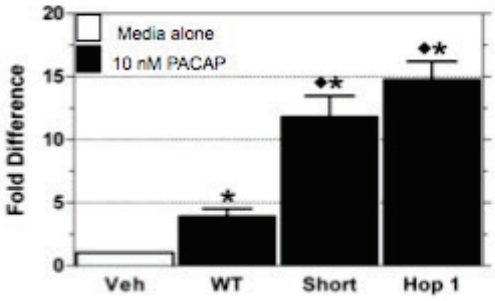

LβT2 cells
Media alone
10 nM PACAP
Fold Difference
0
5
10
15
20
Veh
WT
Short
Hop 1

Figure 17. Effect of overexpression PAC1-Rs on PACAP stimulated activity of promoter. PAC1-R mRNA isoform expression in wild type αT3 and LβT2 cells and cells transfected with PACR-1 short or PACR-1 hop expression vector (A). αT3 cells (B) and LβT2 (C) were transfected with the mPACpro-luc (2.5μg) and PAC1-R express vector (50ng) and treated for six hours with 10nM PACAP. Data are expressed as fold difference normalized to media alone. * Significantly different than media alone (P<0.05) by ANOVA. ◆ Significantly different than Wild type cells with 10nM PACAP (P<0.05) by Tukey's Test. Results were from three experiments with triplicate wells.

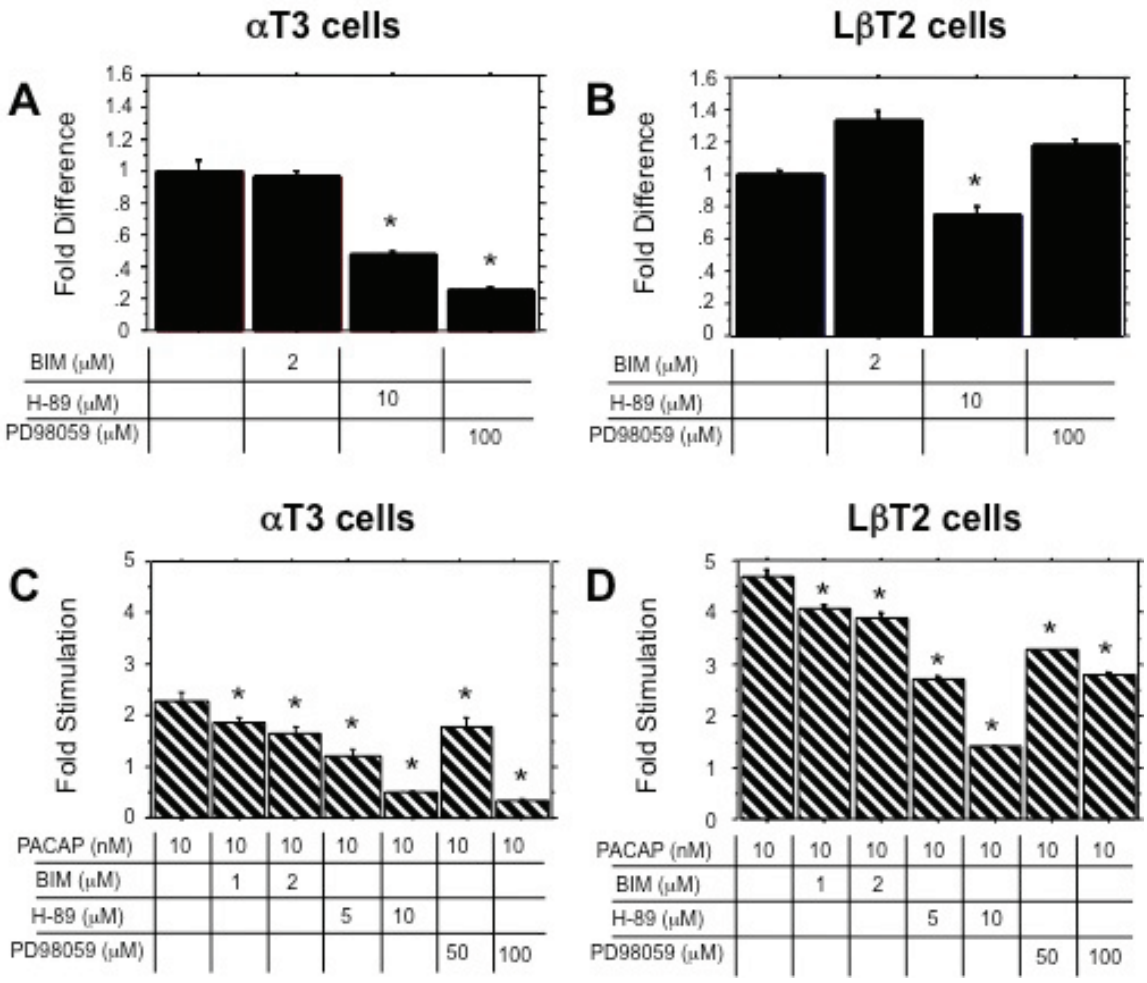
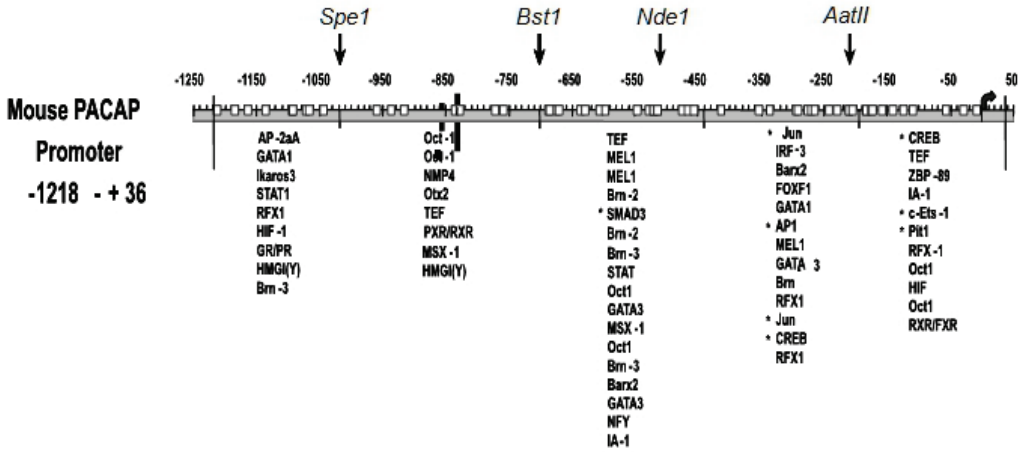

αT3 cells
LβT2 cells
A
B
Fold Difference
1.6
1.4
1.2
1
.8
.6
.4
.2
0
BIM (μM)
H-89 (μM)
PD98059 (μM)
2
10
100
αT3 cells
LβT2 cells
C
D
Fold Stimulation
5
4
3
2
1
0
PACAP (nM)
BIM (μM)
H-89 (μM)
PD98059 (μM)
10
1
2
5
10
50
100

Figure 18. Pathway analysis on PACAP promoter activity.

αT3 (A, C) and LβT2 (B, D) cells were transfected with the mPACpro-luc construct and treated for six hours with media alone (A,B) or 10nM PACAP (C,D) with or without the indicated concentrations of the PKC inhibitor, bisndolylmaleimide (BIM), the PKA inhibitor, H89, or the MEK1 inhibitor, PD98059. Media alone groups used Renilla luciferase test. Data are expressed as fold difference or stimulation normalized to media alone. * Significantly different than media alone ($p<0.05$) by ANOVA. Results were from three experiments with triplicate wells.


Spe1
Bst1
Nde1
AatII
-1250 -1150 -1050 -950 -850 -750 -650 -550 -450 -350 -250 -150 -50 50
Mouse PACAP
Promoter
-1218 - + 36
AP-2aA
GATA1
Ikaros3
STAT1
RFX1
HIF-1
GR/PR
HMGI(Y)
Brn-3
Oct-1
OCT-1
NMP4
Otx2
TEF
PXR/RXR
MSX-1
HMGI(Y)
TEF
MEL1
MEL1
Brn-2
SMAD3
Brn-2
Brn-3
STAT
Oct1
GATA3
MSX-1
Oct1
Brn-3
Barx2
GATA3
NFY
IA-1
Jun
IRF-3
Barx2
FOXF1
GATA1
AP1
MEL1
GATA 3
Brn
RFX1
Jun
CREB
RFX1
CREB
TEF
ZBP-89
IA-1
c-Ets-1
Pit1
RFX-1
Oct1
HIF
Oct1
RXR/FXR

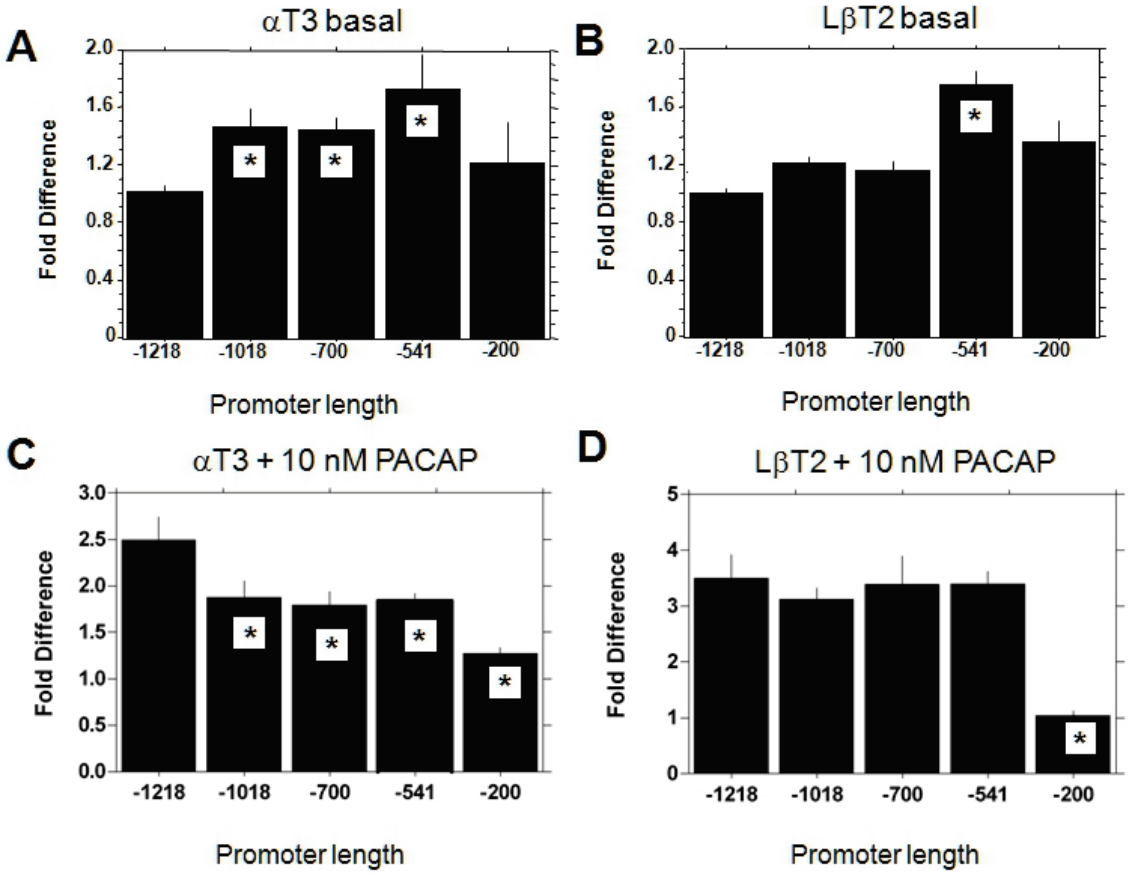

A
αT3 basal
Fold Difference
2.0
1.6
1.2
0.8
0.4
0
-1218 -1018 -700 -541 -200
Promoter length
B
LβT2 basal
Fold Difference
2.0
1.6
1.2
0.8
0.4
0
-1218 -1018 -700 -541 -200
Promoter length
C
αT3 + 10 nM PACAP
Fold Difference
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-1218 -1018 -700 -541 -200
Promoter length
D
LβT2 + 10 nM PACAP
Fold Difference
5
4
3
2
1
0
-1218 -1018 -700 -541 -200
Promoter length

Figure 19. Region analysis of PACAP promoter.

Top of the figure is schematic diagram depicting the regions of the mouse PACAP promoter utilized in this investigation. αT3 (A, C) and LβT2 (B, D) cells were transfected with DNA constructs containing various lengths of the mouse PACAP promoter reporter construct (mPACpro-luc) and treated for six hours with media alone (A, B) or media containing 10nM PACAP (C, D). Media alone groups used Renilla luciferase test. Data are expressed as fold difference normalized to media alone treatment of mPACpro-luc transfected cells. * Significantly different than basal -1218 promoter activity. Results are from three experiments with triplicate wells.

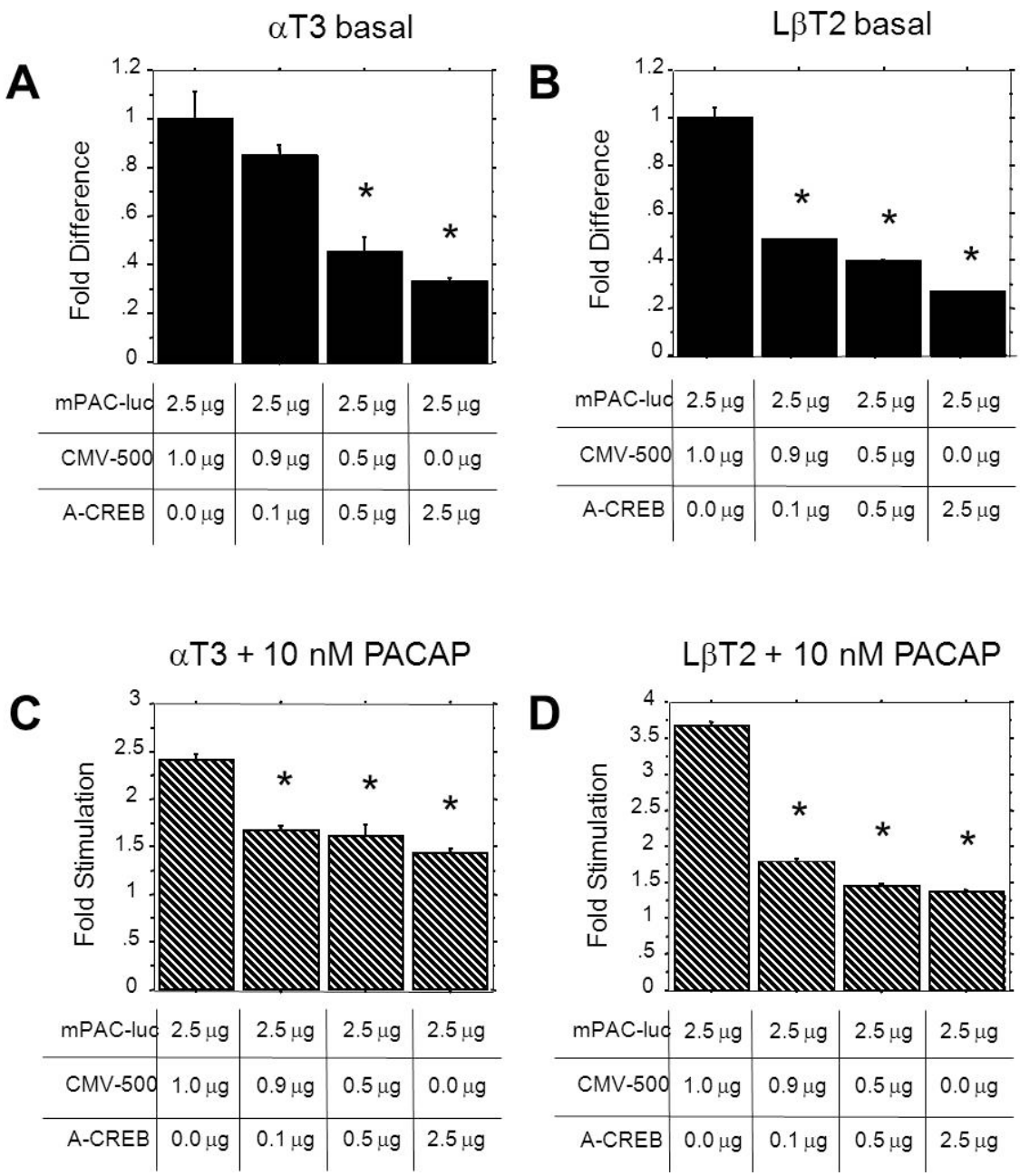

αT3 basal
A
Fold Difference
1.2
1
.8
.6
.4
.2
0
*
*
mPAC-luc 2.5 μg 2.5 μg 2.5 μg 2.5 μg
CMV-500 1.0 μg 0.9 μg 0.5 μg 0.0 μg
A-CREB 0.0 μg 0.1 μg 0.5 μg 2.5 μg
LβT2 basal
B
Fold Difference
1.2
1
.8
.6
.4
.2
0
*
*
*
mPAC-luc 2.5 μg 2.5 μg 2.5 μg 2.5 μg
CMV-500 1.0 μg 0.9 μg 0.5 μg 0.0 μg
A-CREB 0.0 μg 0.1 μg 0.5 μg 2.5 μg
αT3 + 10 nM PACAP
C
Fold Stimulation
3
2.5
2
1.5
1
.5
0
*
*
*
mPAC-luc 2.5 μg 2.5 μg 2.5 μg 2.5 μg
CMV-500 1.0 μg 0.9 μg 0.5 μg 0.0 μg
A-CREB 0.0 μg 0.1 μg 0.5 μg 2.5 μg
LβT2 + 10 nM PACAP
D
Fold Stimulation
4
3.5
3
2.5
2
1.5
1
.5
0
*
*
*
mPAC-luc 2.5 μg 2.5 μg 2.5 μg 2.5 μg
CMV-500 1.0 μg 0.9 μg 0.5 μg 0.0 μg
A-CREB 0.0 μg 0.1 μg 0.5 μg 2.5 μg

Figure 20. Effect of CREB inhibitor on PACAP promoter activity.

αT3 (A, C) and LβT2 (B, D) cells were transfected with the mPACpro-luc construct and with the indicated concentrations of expression plasmid coding for the dominant negative inhibitor of CREB (A-CREB), and a filler plasmid (CMV-500) to control for total transfected DNA amount. Transfected cells were treated for six hours with media alone (A, B) or 10nM PACAP (C, D) and the cell lysates were collected for luciferase activity determination. Data are expressed as fold difference or stimulation normalized to media alone. * Significantly different than media exposure alone ($p<0.05$) by ANOVA. Results were from three experiments with triplicate wells.

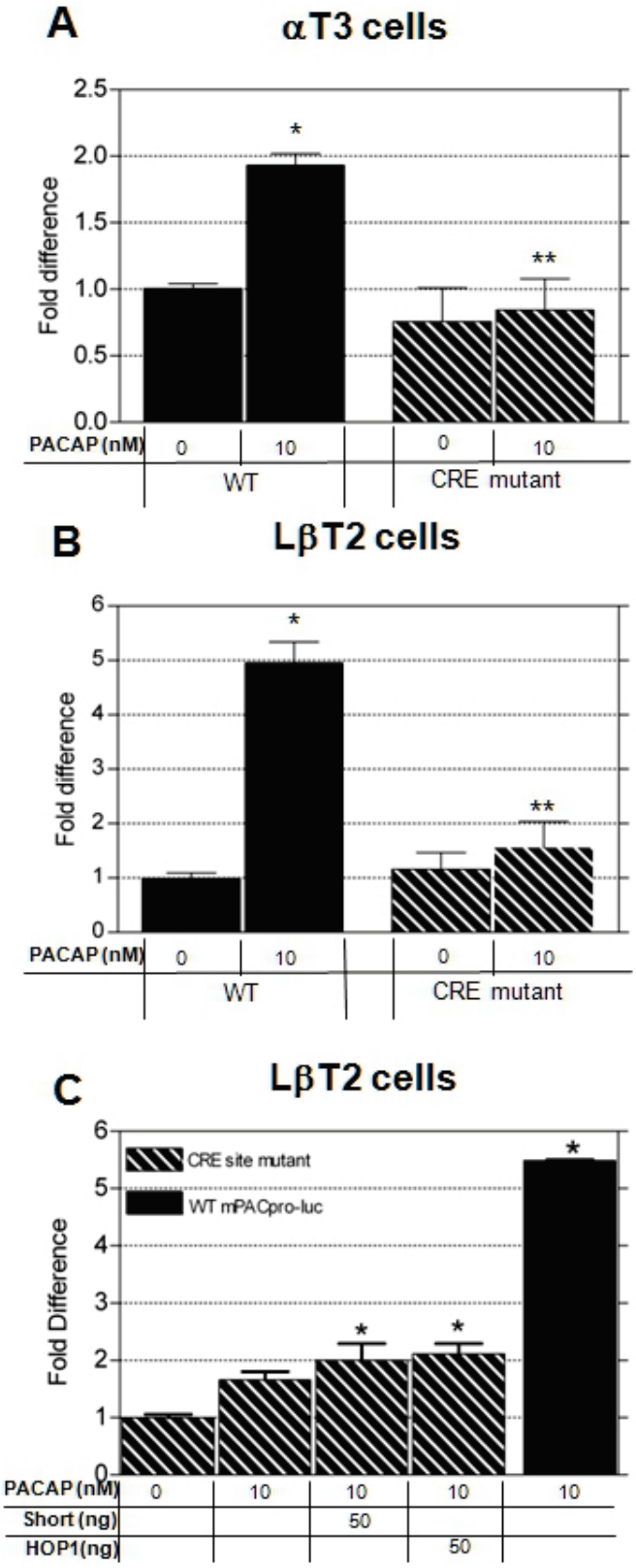
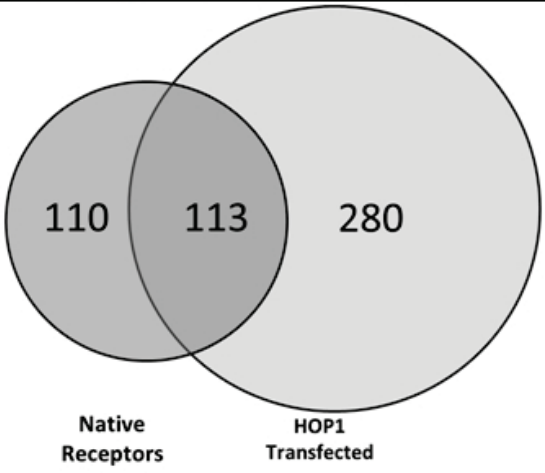

A
αT3 cells
Fold difference
2.5
2.0
1.5
1.0
0.5
0.0
*
**
PACAP (nM)
0
10
0
10
WT
CRE mutant
B
LβT2 cells
Fold difference
6
5
4
3
2
1
0
*
**
PACAP (nM)
0
10
0
10
WT
CRE mutant
C
LβT2 cells
CRE site mutant
WT mPACpro-luc
Fold Difference
6
5
4
3
2
1
0
*
*
*
PACAP (nM)
0
10
10
10
10
Short (ng)
50
HOP1(ng)
50

Figure 21. Effect of CRE-site mutation on PACAP promoter activity. αT3 (A) and LβT2 (B, C) cells were transfected with the CRE-mutant mPACpro-luc (A, B) construct and with the PAC1-R expression plasmid (C). Transfected cells were treated for six hours with media alone and 10nM PACAP and the cell lysates were collected for luciferase activity determination. Data are expressed as fold difference to media alone. * Significantly different than media exposure alone ($P<0.05$) by Tukey's test. Results were from three experiments with triplicate wells.

Altered by PACAP in both	Specific to native receptors	Specific to HOP1 transfected
Up Regulated	**Up Regulated**	**Up Regulated**
PKA Signaling ⋆	Glutamate Metabolism	Ceremide Signaling
PI3K/AKT Signaling ⋆	**Down Regulated**	Inositol Metabolism
ERK/MAPK Signaling ⋆	Mismatch Repair in Eukaryotes	Inositol Phosphate Metabolism
NGF Signaling ⋆	Agrin Interactions at NMJ	IL-2 Signaling
Neurotrophin/TRK Signaling	RAN Signaling	IL-8 Signaling
DHA Signaling	Human Stem Cell Pluripotency	IL-17 Signaling
ALS Signaling	ER Stress Pathway	VEGF Signaling
P53 Signaling		EGF Signaling
Down Regulated		Glucocorticoid Anti-inflamatory
EIF2 Signaling ⋆		Insulin Receptor Signaling
ATM Signaling ⋆		IGF-1 Signaling
BMP Signaling ⋆		Apoptosis Signaling
mTOR Signaling		Cyclins and Cell Cycle Regulation
HGF Signaling		JAK/STAT Signaling
Role of CHKs in Cell Cycle Control		PTEN Signaling
		Fc Epsilon RI Signaling
		Down Regulated
		GnRH Signaling
		Prolactin Signaling
		Androgen Signaling
		Estrogen Receptor Signaling
		TGF-β Signaling
		PDGF Signaling
		G2/M DNA Damage Regulation
		LPS-stimulated MAPK Signaling
		SAPK/JNK Signaling
		Gα 12/13 Signaling
		Melatonin Signaling
		PPARα/RxRα Activation
		AMPK Signaling
		CNTF Signaling
		Wnt/β-catenin
		Sonic Hedgehog Signaling
		PAK Signaling
		Cell Cycle Regulation by BTGs

Distribution of genes significantly altered > 20%



	Altered in Both	Native Only	HOP1 Only
Up-Regulated	44	62	127
Down-Regulated	69	48	153

Table 4. PACAP induced signaling pathways in gonadotroph cells.

Canonical pathways with molecules significantly affected by alterations in gene expression elicited by PACAP signaling. ⋆ Significantly greater in PAC1-R-Hop1 transfected cells.

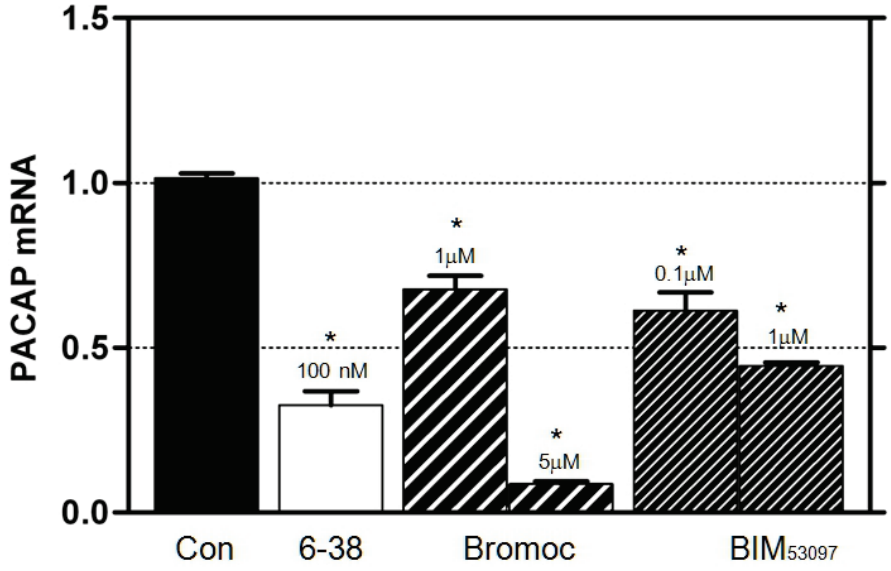

PACAP mRNA
1.5
1.0
0.5
0.0
*
100 nM
*
1μM
*
5μM
*
0.1μM
*
1μM
Con
6-38
Bromoc
BIM53097

Figure 22. Effect of PACAP 6-38, Drd2 agonists on PACAP expression in E19 pituitary cell cultures. Cells were cultured for 24 hours before treated with 100nM PACAP 6-38, 1μM and 5μM Bromocriptine, or 0.1μM and 1μM BIM53097, or control medium for 24 hours. PACAP mRNA level was tested by real-time PCR and analyzed utilizing the $\Delta\Delta C_T$ method of normalization to control value. Values are the mean ± SEM (n=3) experiments. * Significant different ($P<0.05$) than control.

CHAPTER FIVE

SUMMARY AND SIGNIFICANCE

It is already more than 20 years since PACAP was discovered in ovine hypothalamus. Previous studies have demonstrated diverse functions of PACAP in many different biological systems and animal models from the molecular level to physiological characterization. This dissertation investigates the developing rodent pituitary, with a focus on the role of PACAP in the regulation of gonadotrophs in the anterior pituitary. PACAP as well as its receptors are demonstrated to play a role in the function of pituitary gonadotrophs.

PACAP mRNA levels in the rat pituitary show a dramatic decline after birth, and a low level of expression in adults. Single cell PCR of pituitary cells reveals that PACAP is mostly present in gonadotroph cells from both E19 and PN1 rats. Therefore, the perinatal decrease of PACAP expression in the pituitary is mainly contributed by changes in gonadotroph cells. In addition, we observed significant differences in PACAP mRNA levels between two gonadotroph cell lines, αT3 and LβT2. We utilized αT3 and LβT2 gonadotroph cells, representing immature and mature gonadotroph stages for *in vitro* experiments. We evaluated PAC1-R mRNA expression levels from the E14 to PN10 rat pituitaries. Furthermore, we

demonstrate that PAC1-R-Hop and PAC1-R-Short form are predominate during rat pituitary development as well as in the αT3 and LβT2 cell lines, which suggests that actions of PACAP in pituitary gondatrophs are mediated by the two receptor subtypes.

During early sexual development of male rats, we implanted osmotic micro-pumps containing PACAP38 or the PACAP antagonist, PACAP 6-38, into the peritoneal cavities. After 5 or 7 days treatment, PACAP 6-38 causes an increase of FSH levels in both serum and pituitary whereas PACAP 1-38 decrease the FSH levels. However, neither treatments change the LH levels. In addition, Fst-288 mRNA levels in pituitary are suppressed by PACAP 6-38 and stimulated by PACAP 1-38. In summary, the results provide the first *in vivo* evidences of PACAP regulation of FSH, likely through its regulation of local follistatin levels. Furthermore, we demonstrate that PACAP induces high follistatin promoter activity through PKA pathways and mediated by PAC1-Rs in the gonadotroph cells utilizing the follistatin promoter reporter vectors. The results demonstrate the hypothesis that high level PACAP in fetus suppresses FSH expression through follistatin-activin mechanism. Therefore, the increase of FSH after birth is caused by the decreased PACAP.

Besides the studies on PACAP actions in pituitary gonadotroph, the dissertation gives more information about regulation of PACAP expression. PACAP expression in gonadotrophs is not regulated by feedback mechanism from gonadal hormones. However, we observed a decrease of PACAP mRNA after treatment of PACAP 6-38 both *in vitro* and *in vivo*, which suggests PACAP

may be stimulated by itself. Subsequent studies reveal that PACAP stimulates its promoter in the αT3 and LβT2 cell lines through PKA and MAPK pathway, and the existence of a proximal CRE binding site is required. In addition to positive regulation, a potential inhibitory factor was discovered and evaluated in this dissertation. Activation of Dopamine-2 receptor suppresses PACAP mRNA expression in cultured E19 rat pituitary cells. Therefore, the stimulation of PACAP on itself may contribute to the high level PACAP in the fetus while Drd2 activity may mediate the decline of pituitary PACAP at birth.

The importance of differential regulation of gonadotropins by PACAP during sexual development in humans is not yet known. However, as in rodents, human preterm infants have less FSH than LH (Massa et al., 1992), and PACAP increases follistatin mRNA levels in primate FS cell-enriched pituitary cultures (Kawakami et al., 2002). Thus, PACAP might also differentially regulate gonadotropins during human sexual development. Furthermore, many symptoms of the reproductive diseases relate to abnormal levels of gonadotropins in the human. For example, polycystic ovary syndrome (PCOS), which is characterized by menstrual dysfunction and hyperandrogenism in the female, shows low to normal FSH levels in the face of increased LH (Hall et al., 1998). The drugs utilized for treatment usually will regulate both FSH and LH levels through stimulating GnRH. Our investigations in this dissertation suggest that it is possible to specifically regulate FSH levels through PACAP-follistatin-activin mechanism. Therefore, PACAP and PAC1-R might be the targets of drug designs for the gonadotroph disorders.

In conclusion, PACAP is a novel regulatory factor for pituitary gonadotrophs. PACAP is involved in the differential regulation of the gonadotropins that occur during the perinatal and infantile periods in the male rat. In the fetus, high level of PACAP, increases follistatin expression thereby suppressing FSH through blocking the stimulatory function of activin. The high levels of fetal pituitary PACAP could possibly be induced by self-stimulation through high levels of pituitary or hypothalamic PACAP expression. After birth, pituitary PACAP levels decrease significantly causing a decline of local follistatin levels and allows for activin stimulation of FSH (Fig. 23). Our data suggests that the perinatal decline in pituitary PACAP expression may be the result of a significant increase in Drd2 activation through increased dopamine exposure or responsiveness. Further research is needed to determine the importance of PACAP in reproductive system development of the human.

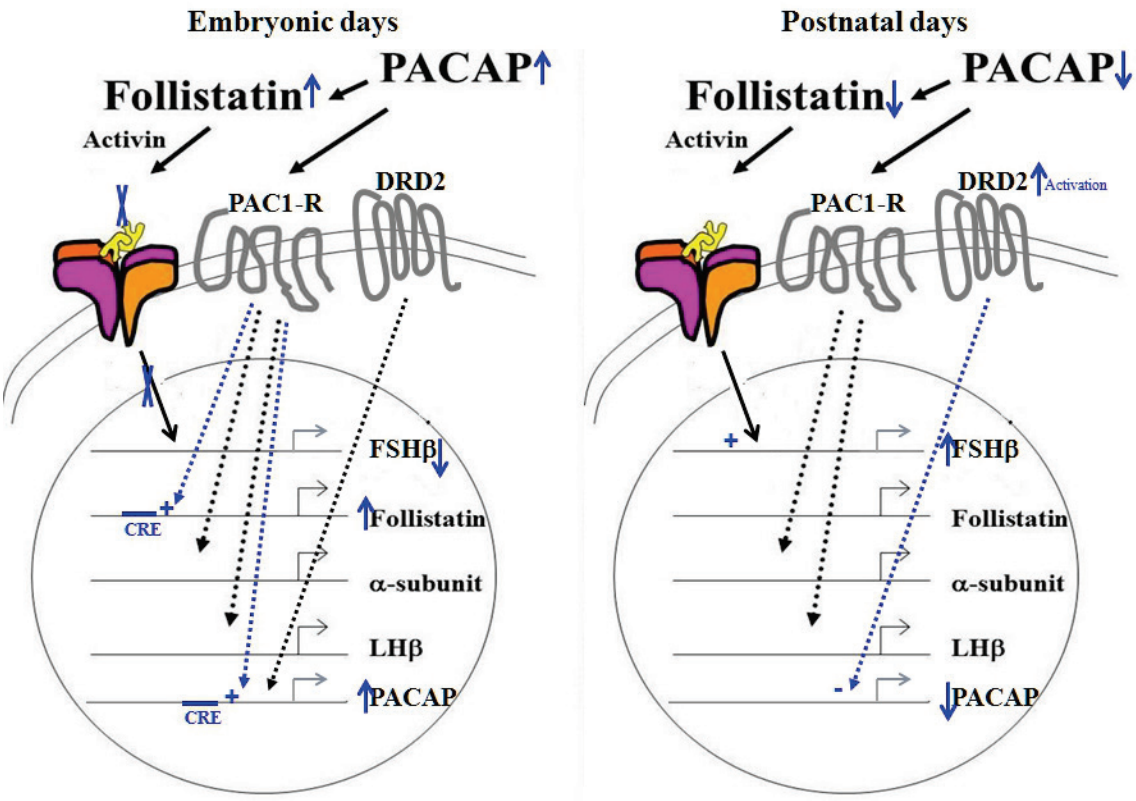


Figure 23. Schematic diagram of the dissertation summary.

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PROFESSIONAL SOCIETIES

Member, Advancing Science Serving Society, 2011 - Present