**Phenotypic Characterization of Reproduction in Female Mice Lacking Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)**

by

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B.Sc., University of Victoria, 2004

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in the Department of Biology

We accept this thesis as conforming
to the required standard

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## Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a ubiquitous peptide hormone involved in the regulation of several physiological processes. This hormone is a member of the glucagon superfamily of hormones, which are primarily involved in regulation of metabolism and growth. PACAP shows the highest sequence conservation of this superfamily during evolution, suggesting a critical role for PACAP in species survival. Mice lacking PACAP (Adcyap1) show high mortality during the postnatal period, as well as impaired reproduction in females. In this thesis I have characterized the reproductive phenotype in female mice lacking PACAP due to disruption (knockout) of the single copy *pacap* gene (*Adcyap1*). Previous experiments in other laboratories have described reduced fertility in mice lacking PACAP or one of its receptors, the $PAC_1$ receptor. However, the mechanism by which PACAP acts to enhance reproduction has not yet been elucidated. The purpose of this study was to determine the site(s) of action of PACAP in the cascade of reproductive events from puberty to implantation. I hypothesized that PACAP was an important regulator of an early phase of reproduction, and that lack of PACAP would result in impaired reproductive success.

Initially I examined puberty onset, estrous cycles, mating behaviour, and delivery of live offspring. Estrous cycles were evaluated through vaginal cytology, both in juvenile

mice, to confirm that puberty had occurred, and in adult females, to determine if they maintained a normal 4-5 day estrous cycle. Mating behaviour was evaluated through a natural mating strategy, with daily assessment of the presence of a copulatory plug. The reproductive outcome of delivering live pups was characterized, and pup survival was followed. I then characterized PACAP null mice as to ovulation, ovarian histology and fertilization of eggs *in vivo*. Finally, I measured implantation rates in PACAP null females, as well as levels of the hormone prolactin, an essential regulator of early pregnancy.

In the present study I found a number of reproductive functions that were normal without PACAP. Puberty onset was unaffected in knockout mice and they displayed regular 4-5 day estrous cycles. Also, PACAP null females mated when paired with a male of proven fertility. However, mating behaviour follows an unusual pattern in PACAP null mice, where 33% mate on more than one day during a week-long pairing unlike wild-type mice that would normally mate only once. Also, significantly fewer PACAP null females than wild-type females gave birth following mating: 21% and 100%, respectively. Ovulation and ovarian histology were normal in PACAP null females, as was fertilization of released eggs. However, only 13% of PACAP null females had implanted embryos 6.5 days after mating. The mechanism of impaired implantation may be a defect in prolactin secretion. Prolactin levels were significantly lower in PACAP null females than in wild-types following mating. Prolactin is an essential hormone for the support of early pregnancy. These results support the conclusion that PACAP acts as an important regulator of prolactin in reproduction. It is possible that PACAP in the hypothalamus is the dominant prolactin-releasing factor, which has been sought many years but never definitively identified.

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# List of Abbreviations

aa: amino acid

AC: adenylyl cyclase

*ADCYAP1*: human PACAP gene

ADCYAP1: human PACAP protein

*Adcyap1*: mouse PACAP gene

Adcyap1: mouse PACAP protein

bp: base pairs

BSA: bovine serum albumin

$Ca^{2+}$: calcium

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

cm: centimeter

CNS: central nervous system

DA: dopamine

$ddH_2O$: distilled deionized water

DNA: deoxyribonucleic acid

dNTP: deoxynucleotidetriphosphate

dpc: days post coitus

DTT: dithiothreitol

$E_2$: estrogen

*E. coli*: *Escherichia coli*

EDTA: ethylenediaminetetraacetic acid

FSH: follicle-stimulating hormone

GnRH: gonadotropin-releasing hormone

h: hour

$IP_3$: inositol-1,4,5-triphosphate

IPTG: isopropyl-beta-D-thiogalactopyranoside

kb: kilobase pairs

kDa: kiloDalton

LH: luteinizing hormone

mA: milliampere

mg: milligram

$MgCl_2$: magnesium chloride

ml: milliliters

mM: millimolar

mRNA: messenger ribonucleic acid

N: sample size

ng: nanogram

nm: nanometre

NMDA: N-methyl-D-aspartate

No.: number

OD: optical density

P: progesterone

$PAC_1$: PACAP specific receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

PACAP-27: C-terminally truncated form of PACAP

PACAP-38: full length PACAP

PCR: polymerase chain reaction

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PRF: prolactin-releasing factor

PRP: PACAP-related peptide

PVDF: polyvinylidene difluoride

qPCR: quantitative real time polymerase chain reaction

RIA: radioimmunoassay

RT-PCR: reverse transcription-polymerase chain reaction

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SNS: sympathetic nervous system

$T_M$: melting temperature

TBS: Tris buffered saline

TRH: thyroid-releasing hormone

TSH: thyroid-stimulating hormone

UTR: untranslated region

UV: ultraviolet

VIP: vasoactive intestinal peptide

VMN: ventromedial nucleus of the hypothalamus

$VPAC_1$: VIP and PACAP shared receptor type I

$VPAC_2$: VIP and PACAP shared receptor type II

WAP: whey acidic protein

X-Gal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

x g: times gravity

µl: microlitre

# ACKNOWLEDGEMENTS

This thesis would not have been possible without the support and help of many people and I would like to thank them.

First and foremost I would like to thank Dr. Nancy Sherwood. Her guidance and support throughout my research have been incredible. Also, her dedication to science and keen mind has set a high example of what a scientist can and should be. I would like to acknowledge members of the Sherwood lab, past and present, for their encouragement and assistance, especially Graeme Roch and Bruce Adams. Both have been instrumental in guiding me through the rough patches and helping me to enjoy the success.

Dr. Patrick Von Aderkas and Dr. Francis Choy guided me down this path and encouraged me to pursue science as a career. My father taught me the scientist's gaze from the time I was a small child. Thank you, dad, for teaching me to love nature, to always look for the answer. Finally to my mother, for always believing in me: for sharing in my successes and comforting me in my disappointments. None of this would have been possible without your unconditional love and support.

# DEDICATION

For my brother Thomas

# 1 Introduction

## 1.1 Hormones and the endocrine system

The term hormone, first used by Starling in 1905, defines chemical signals secreted by a cell or cluster of cells into the bloodstream where they are circulated to target cells (Henderson, 2005). These chemical messengers act in three ways: on nearby cells, in a paracrine manner, on the cells that secrete them in an autocrine way, or on distant cells in an endocrine manner. The endocrine system consists of ductless glands that secrete hormones into the bloodstream, although nearly every organ and tissue in the body contains cells that secrete hormones. All multicellular animals produce hormones whose actions are determined by their pattern of secretion and signal transduction. Hormones regulate many physiological functions: most notably metabolism, reproduction, growth and development. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic hormone and is ubiquitously expressed. Current nomenclature for PACAP is Adcyap1 in mouse, although I will continue to use PACAP. This thesis will explore the role of PACAP in reproduction in female mice.

## 1.2 PACAP overview

### *1.2.1 PACAP: discovery and characterization*

PACAP was first isolated from the hypothalamus of ewes, based on its ability to stimulate adenylyl cyclase in anterior pituitary cultured cells. It was found to have a sequence of 38 amino acids in length, and the ability to stimulate the release of growth hormone, prolactin and luteinizing hormone from the anterior pituitary of rats (Miyata *et. al.,* 1989). Subsequently, a C-terminally truncated form, PACAP-27, was characterized (Miyata *et. al.,* 1990). PACAP is ubiquitously expressed throughout the central and peripheral nervous systems, with highest levels found centrally in the hypothalamus and posterior pituitary, and high peripheral expression in the testes and adrenal glands (Arimura *et. al.,* 1991).

### *1.2.2 PACAP: evolution and structure*

PACAP is a member of the glucagon superfamily of hormones, which consists of 9 functional peptide hormones including growth hormone-releasing hormone (GHRH), glucagon, secretin and vasoactive intestinal peptide (VIP) among others. These hormones are structurally related, with 68% sequence identity between PACAP and VIP (Arimura *et. al.,* 1992, Campbell *et al.*, 1992). The members of this superfamily show similar precursor molecules with each containing a signal peptide, cryptic peptide, and C-terminal peptide. PACAP is the most highly conserved member, both in terms of length and sequence. Human, mouse, and sheep PACAP molecules are all structurally identical, and PACAP can be found as far back in evolution as tunicates with 89-96% amino acid sequence identity. PACAP and GHRH are found together on the same gene in many vertebrates, including birds and fish, as well as in tunicates, but they are found encoded

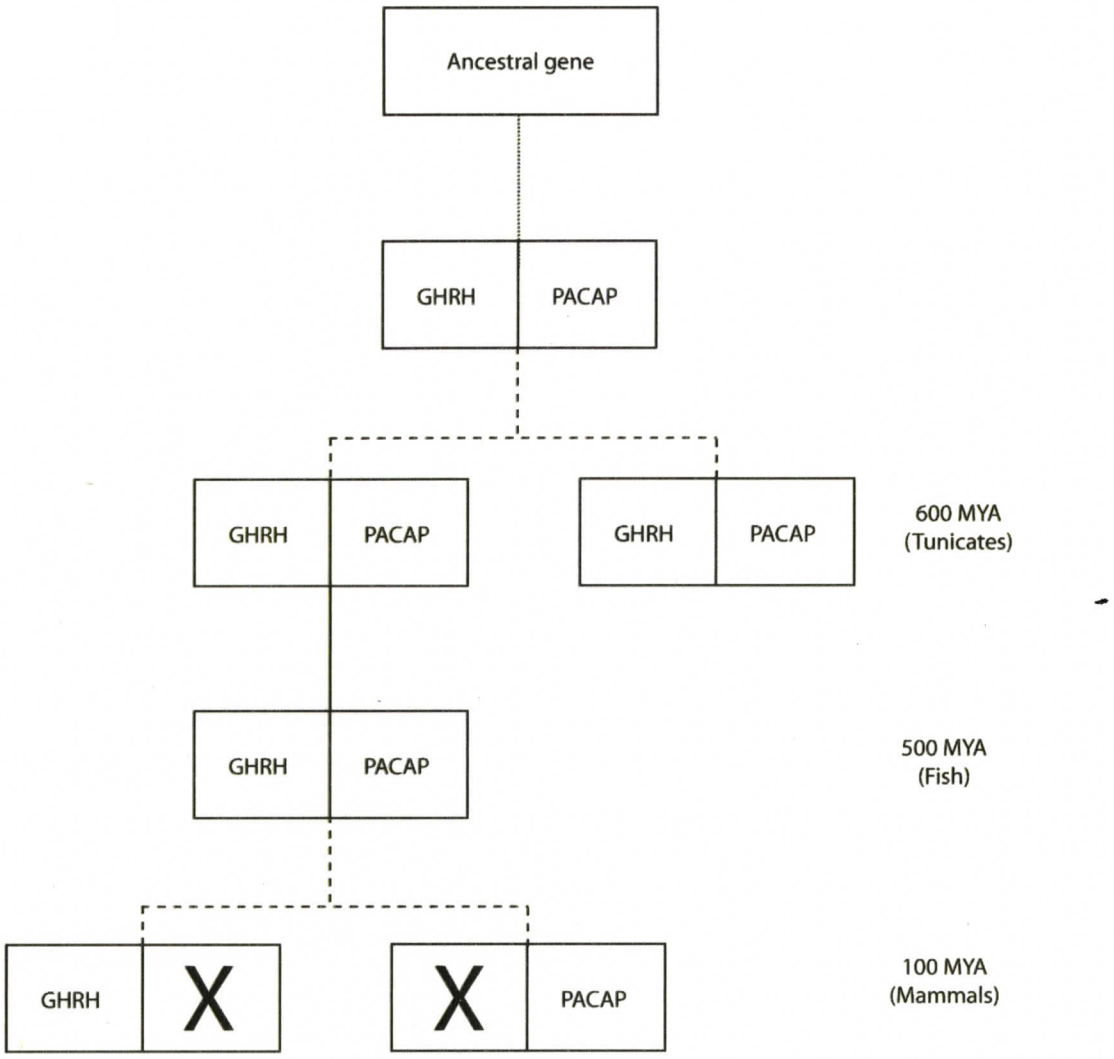



**Figure 1.1.** Proposed evolution of the PACAP gene. Exons and genes are represented by boxes. Dotted lines indicate exon duplication and dashed lines represent gene duplication. Genes no longer active are shown with an X; the PACAP gene still contains a GHRH-like gene, although no remnants of PACAP are present on the GHRH gene of mammals. Modified from Sherwood *et al.* 2000. Abbreviations: PACAP- pituitary adenylate cyclase-activating polypeptide, VIP- vasoactive intestinal peptide, GHRH- growth hormone-releasing hormone, PHM- peptide histidine methionine, MYA- million years ago.

on two separate genes in mammals. This appears to be the result of gene duplication and rearrangements (Sherwood *et. al.,* 2000). Figure 1.1 shows a schematic for the hypothesized evolution of the PACAP gene.

The PACAP gene has been found in every vertebrate examined to date, and the cDNA has been cloned in many organisms including: human (Hosoya *et. al.,* 1992), mouse (Cummings *et. al.,* 2002; Miyata *et. al.,* 2000), chicken (McRory *et. al.,* 1997), salmon (Parker *et. al.,* 1993), amphibians (Alexandre *et. al.,* 2000), and tunicate (McRory and Sherwood, 1997). The human PACAP gene (*ADCYAP1*) is located on chromosome 18p11 (Hosoya *et. al.,* 1992) and the mouse PACAP gene is present on chromosome 17 E5 (Cummings *et. al.,* 2002; Miyata *et. al.,* 2000). The mouse and human PACAP genes are encoded on five exons, containing on exon 1 the 5' untranslated region, on exon 2 the signal peptide, on exon 3 the cryptic peptide, on exon 4 the PACAP related peptide and on exon 5 both PACAP and the 3' untranslated region (Figure 1.2). The gene encodes a preproPACAP whose PACAP sequence is in the amide-rich C-terminal domain of the precursor and is 176 amino acids in length in humans. PreproPACAP contains PACAP-38, PACAP-27 and PACAP-related peptide (PRP). Eight different transcripts formed by alternate splicing and start sites have been identified in the mouse (Cummings *et. al.,* 2002; Tabuchi *et. al.,* 2001; Yamamoto *et. al.,* 1998). PACAP-related peptide has no known receptors or functions, although its expression has been observed in the rat hypothalamus (Hannibal *et. al.,* 1995; Mikkelsen *et. al.,* 1995).

The high degree of sequence conservation of PACAP over 600 million years of evolution indicates strong selective pressure and an important physiological role.

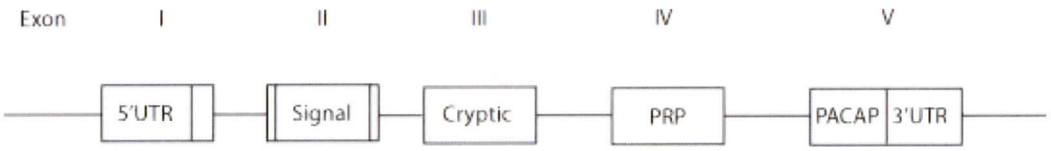



**Figure 1.2** Schematic of PACAP gene structure in mouse. Lines represent introns and boxes indicate exons (drawing not to scale). UTR- untranslated region, Signal- signal peptide, Cryptic- cryptic peptide, PRP- PACAP-related peptide, PACAP- pituitary adenylate cyclase-activating polypeptide.

### *1.2.3 PACAP: receptors and signalling*

PACAP acts on three receptors, encoded on three separate genes. They are members of a family of receptors, which include the secretin, glucagon and GHRH receptors. They are all seven transmembrane G-protein coupled receptors that stimulate second messenger activation within target cells. The PACAP receptors include the $PAC_1$ receptor, which is specific for PACAP, and the $VPAC_1$ and $VPAC_2$ receptors, which it shares with VIP (Harmar *et. al.,* 1998; Vaudry *et. al.,* 2000). All three receptors stimulate adenylyl cyclase and the $PAC_1$ receptor also stimulates phospholipase C and calcium increase within cells (Daniel *et. al.,* 2001). These three receptor types show varying activation responses to PACAP-38, PACAP-27 and VIP, as well as different tissue distributions and second messenger activation, which allows for diverse actions of these hormones. This allows for pleiotropic actions of PACAP in development, metabolism and reproduction.

The $PAC_1$ receptor was first isolated and sequenced in 1993 by various groups (Hashimoto *et. al.,* 1993; Hosoya *et. al.,* 1993; Spengler *et. al.,* 1993). It is located on chromosome 7 in humans and multiple splice variants are expressed. It shows equal activation by both PACAP-38 and PACAP-27, but 1000 fold lower activation by VIP; therefore no response to VIP is present at physiological concentrations. Ten splice variants for the $PAC_1$ receptor have been identified to date. Many variants involve the third intracellular loop, which is involved in G protein binding to the receptor (Figure 1.3). Two cassettes in the third intracellular loop, termed hip and hop, are variants expressed depending on differential splicing. The hop cassette has two different moieties, $hop_1$ and $hop_2$, which differ by one amino acid.

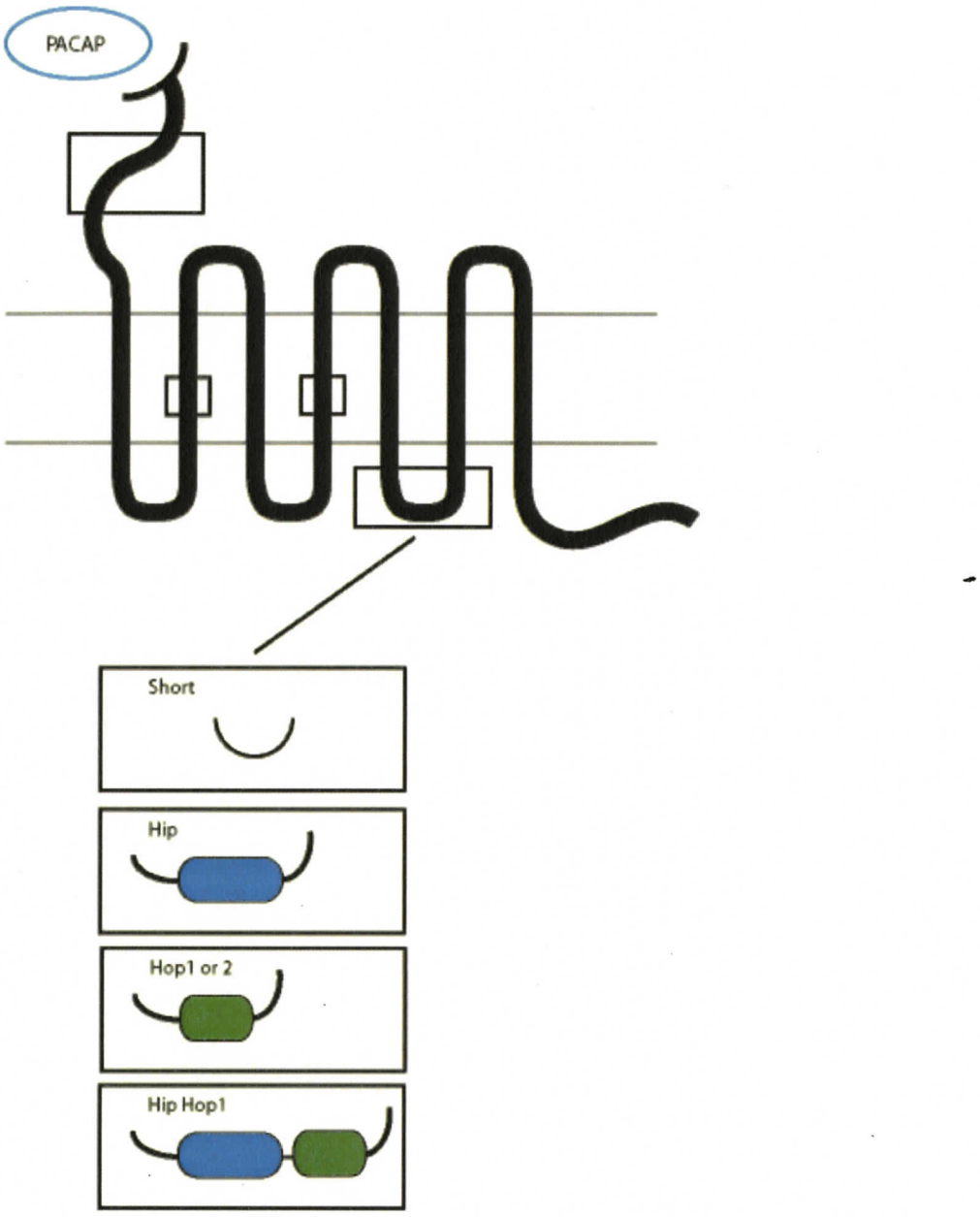



**Figure 1.3** Schematic of $PAC_1$ receptor structure detailing different isoforms. Open boxes on the receptor indicate sites at which isoforms have been characterized, including five at the third intracellular loop with alternative splicing of hip, $hop_1$ and $hop_2$ cassettes. Two splice variants result from 21 or 57 amino acid deletions and one splice variant results from a 24 amino acid insertion in the extracellular region indicated with an open box. Variants have also been found with substitutions and deletions in transmembrane domains II and IV.

The shortest $PAC_1$ receptor variant, lacking both the hip and hop cassettes, shows equal activation by both PACAP-38 and PACAP-27, with 1000 fold lower response in adenylyl cyclase activation, as measured by cAMP production, and no phospholipase C activation by VIP. The hip containing $PAC_1$ receptor variant has lower adenylyl cyclase activity than the short variant and no phospholipase C activity in response to PACAP. The variant containing both hip and $hop_1$ cassettes has slightly lower adenylate cyclase and PLC activity than the short variant. The $PAC_1$ receptor is strongly expressed in the brain, pituitary, and adrenal medulla where ratios of the splice variants differ, with the short variant found predominantly in the brain and the hop variant found mainly in the adrenal medulla and the testes (Spengler *et. al.,* 1993). A less responsive splice variant was found with 24 amino acids inserted into the extracellular ligand-binding domain of the receptor (Daniel *et. al.,* 2001). Another $PAC_1$ receptor variant, here with changes in the second and fourth transmembrane domains, shows different signalling transduction than the other variants, stimulating calcium influx. No activation of AC or PLC by PACAP-27 was detected with this receptor variant, although a fast and large calcium influx was observed. This receptor variant is expressed in the β islet cells of the pancreas, and no other $PAC_1$ receptor variants are present, indicating that this receptor is involved in the control of insulin release by PACAP from the pancreas (Chatterjee *et. al.,* 1996).

The $VPAC_1$ and $VPAC_2$ receptors, characterized in 1992 from the rat lung and in 1993 from the rat olfactory bulb respectively, differ in their pharmacology for activation of AC (Ishihara *et. al.,* 1992; Lutz *et. al.,* 1993). $VPAC_1$ shows equal activation by PACAP-27, PACAP-38 and VIP. $VPAC_2$ is more responsive to VIP and PACAP-38, and less responsive to PACAP-27 (Harmar *et. al.,* 1998). The $VPAC_1$ and $VPAC_2$ receptors

are found primarily in the peripheral organs, including the lung, liver, gut and the prostate (Shivers *et. al.,* 1991).

### *1.2.4 PACAP: study through mouse transgenesis*

Mice of the genus *Mus musculus* have been used to study genetics since the beginning of the twentieth century. The use of mice over other animal models is due to the genetic similarity between mice and humans, with almost every human gene having a mouse homolog (Silver, 1995). Mice are small with short generation times and high fecundity, which lends to their success as a laboratory animal. With the advent of genetic engineering and the sequencing of the mouse genome, highly powered manipulations of the mouse genome have been made possible, allowing for study of the effects of many genes on mammalian physiology. Embryonic stem cells (ES) in the mouse can be cultured and manipulated *in vitro*, with gene targeting, gene trapping and conditional mutagenesis as the major manipulations, then re-introduced into developing blastocysts, which in turn are used to generate mice lacking functional genes (Glaser *et. al.,* 2005).

Gray *et al.* generated a PACAP null mouse through homologous recombination in ES cells. The PACAP null mouse lacks exons 3-5 of the mouse PACAP gene, which encodes both the cryptic and PACAP gene (Figure 1.4). These mice show multiple phenotypic abnormalities including altered lipid accumulation within the liver, heart and skeletal muscles, as well as elevated serum triglycerides, free fatty acids and cholesterol (Gray *et. al.,* 2001). Although PACAP null mice are normal at birth and present at expected Mendelian ratios, they die during the postnatal period. This

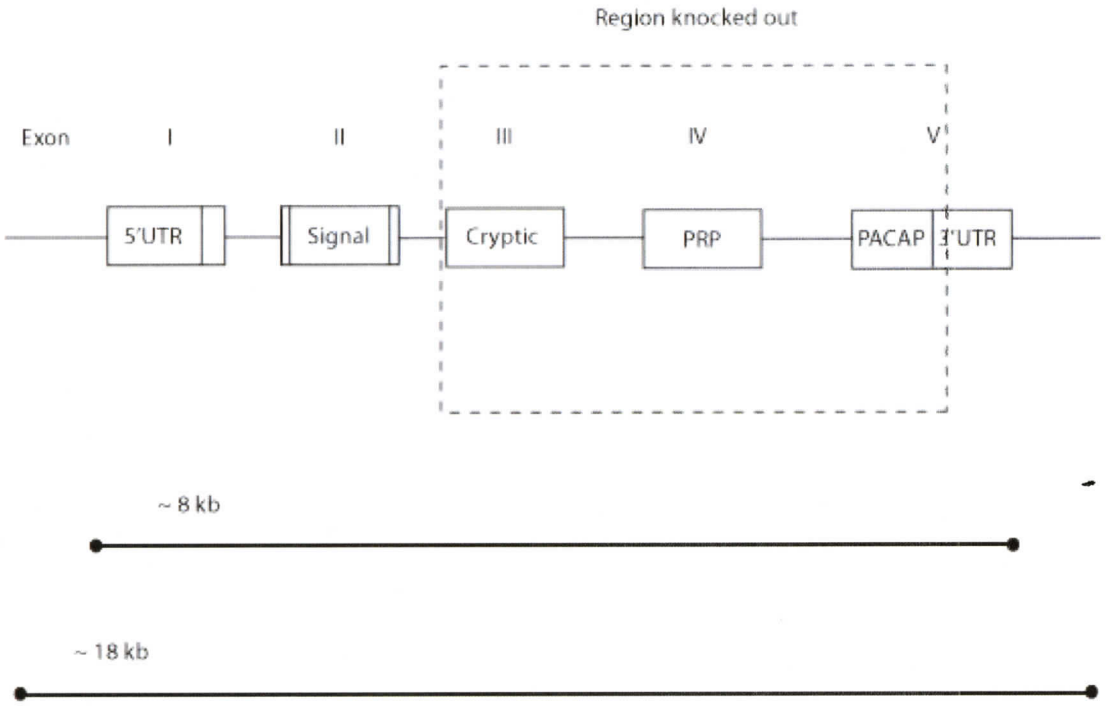



**Figure 1.4.** Schematic of the murine PACAP gene indicating region that has been knocked out in the transgenic mouse created by Gray *et al.* Lines represent introns and boxes indicate exons (drawing not to scale). UTR- untranslated region, Signal- signal peptide, Cryptic- cryptic peptide, PRP- PACAP-related peptide, PACAP- pituitary adenylate cyclase-activating polypeptide. Modified from Gray *et al.* (2001).

phenotype was later found to be temperature-sensitive and increasing room temperature to 24 °Celsius increased pup survival from 11% to 76%. Although the cause of this temperature-sensitive lethality is not fully understood, lower norepinephrine levels were found in the brown adipose tissue, which is required for non-shivering thermogenesis, suggesting a defect in adaptive thermogenesis in these mice (Gray *et. al.,* 2002). Despite survival at lower temperature once PACAP null mice are past weaning age, other laboratories have detected abnormal catecholamine stimulus and hypoglycaemia in adult PACAP null mice, indicating that they continue to have impaired thermogenesis into adulthood (Hamelink *et. al.,* 2002).

Other laboratories have generated mice lacking PACAP or its receptors to determine its many actions. Three other groups have created mice lacking the PACAP gene, all created through homologous recombination, but the phenotypes of these PACAP knockout mice differ (Colwell *et. al.,* 2004; Hamelink *et. al.,* 2002; Hashimoto *et. al.,* 2001). The strain generated by Gray *et al.* and maintained at 21 °C shows the most severe phenotype, with highest temperature sensitivity, whereas Colwell *et al.* noted 50-80% of the expected number of PACAP null mice at time of weaning and Hashimoto *et al.* observed a 90% survival in null pups, which is higher than both Gray *et al.* and Colwell *et al.* Neither Hashimoto's nor Colwell's groups supply information on the temperature at which their mice are housed, although room temperature is often 22-24 °C. Housing temperature can have a significant impact on survival of PACAP null mice and may be responsible for the difference in phenotype observed in these different transgenic lines of mice. Phenotypic differences between these different PACAP null mice may also be explained by different amounts of the gene being deleted. Some mice

lack exon 5 only (Hamelink *et. al.,* 2002; Hashimoto *et. al.,* 2001), and some lack exons three to five (Colwell *et. al.,* 2004; Gray *et. al.,* 2001).

$PAC_1$ receptor null mice have also been created and show variable phenotypes depending on the particular strain in question (Hashimoto *et. al.,* 2000; Jamen *et. al.,* 2000; Otto *et. al.,* 2001). Baba's group created their mouse line by deletion of exon 2 in the $PAC_1$ receptor gene and they found it expressed in low levels following deletion due to splicing between exons one and three. The phenotype of this mouse showed normal Mendelian ratios of offspring and no changes in growth, fertility, survival and behaviour (Hashimoto *et. al.,* 2000). Initially, they reported no behavioural abnormalities although they later found increased activity, explosive jumping and decreased anxiety in $PAC_1$ receptor null mice (Hashimoto *et. al.,* 2001). Jamen *et al.* found a 60% death rate amongst $PAC_1$ receptor null mice generated in their laboratory. The receptor was inactivated by disruption of exons 8-11 and they noted a 90% reduction in binding. The $PAC_1$ receptor deficient mouse generated by Otto *et al.* was created by deletion of exon 11 and they also detected smaller splice variants following disruption, although the splice variants were inactive due to the absence of the third intracellular loop, a region essential for G-protein coupling (Otto *et. al.,* 2001). This group did not publish any findings of decreased survival or fertility although they found altered behaviour including an impaired fear response (Otto, *et. al.,* 2001a; 2001b).

Two groups have discussed, at least in part, the reproductive phenotype of female mice lacking either PACAP (Shintani *et. al.,* 2002) or its $PAC_1$ receptor (Jamen *et. al.,* 2000). Shintani *et al.* found reduced, irregular mating frequency in PACAP null females and reduced fertility. Also, this group found differing severity of the reproductive

impairment depending on the strain in which the mutation was expressed; C57-129 PACAP null mice showed 33% fertility whereas ICR PACAP null mice produced litters with 50% frequency following a week long pairing (Shintani *et. al.,* 2002). By comparison, the Jamen group described 25% fertility, although a shorter mating period was selected (Jamen *et. al.,* 2000). As phenotypes vary from one transgenic strain to another, due in part to the strategy implemented in inactivating the gene and the strain onto which the mutation is backcrossed, it is important to fully characterize the reproductive phenotype of the mice used in our laboratory.

## 1.3 Reproduction in mammals

### *1.3.1 Female reproduction*

Female reproduction in mammals requires the successful completion of puberty, mating, fertilization, implantation, maintenance of pregnancy, parturition and lactation. All of these stages are under tight hormonal regulation and may be disrupted by changes within the hormonal milieu. Female mice are spontaneous ovulators; they ovulate with every cycle, and have a short, incomplete estrous cycle. Mice have a four to five day estrous cycle consisting of (1) diestrus, a period of slow growth, (2) proestrus, a time of anabolic growth when the body prepares for pregnancy, (3) estrus, when ovulation occurs, and (4) metestrus, during which phase catabolic changes within the uterine tract return it to a quiescent state (Rugh, 1990). Ovulation occurs during estrus, and female mice will only mate directly preceding estrus, in late proestrus, or during estrus.

Mating results in the presence of a copulatory plug, deposited by the male, and clearly visible on the outer portion of the vagina. Mating requires proreceptive behaviours from the female, which are caused by progesterone- and estrogen-induced changes in the brain (Apostolakis *et. al.,* 2004). Receptive behaviours include lordosis, which does not occur in the absence of estrogen or progesterone (Pollio *et. al.,* 1993). If mating does not occur the corpora lutea are rendered non-functional, degrade and the cycle continues. Mating, regardless of ensuing pregnancy, initiates a neuroendocrine reflex, stimulating the release of prolactin from the hypothalamus.

Prolactin, which is the major luteotrophic hormone during the first third of pregnancy, acts on luteinizing cells of the ovary, which have formed within the corpora lutea, to stimulate the release of progesterone. Prolactin protects the corpus luteum from degradation, and maintains a pseudopregnant state. Prolactin is released from the pituitary in daily biphasic surges, reaching levels 20-30 times above baseline (Terkel, 1988). Progesterone acts on progesterone receptors within the uterus to ready the organ for implantation, causing multiple changes to the epithelial tissue of the uterus.

Maturation of oocytes is caused by follicle stimulating hormone (FSH) and luteinizing hormone (LH) released from luteotrophic cells of the anterior pituitary. The maturing ovum produces estrogen which feeds back on the pituitary to increase levels of FSH and LH produced (Figure 1.5). Ovulation, the release of a mature ovum from its follicle, is caused by a surge of luteinizing hormone during proestrus.

### *1.3.2 PACAP in the hypothalamic-pituitary gonadal axis*

PACAP and its receptors are present within cells at all three levels of the hypothalamic-pituitary gonadal axis and it may play a role in regulating reproduction at any or all of these endocrine glands. PACAP expression is found widely throughout the brain, with highest levels in the hypothalamus, notably, within the median eminence, paraventricular (PVN) and ventromedial (VMN) nuclei. The latter region is associated with female reproductive behaviour (Moore *et. al.,* 2005). PACAP expression is dynamic over the estrous cycle with decreased expression in the PVN during metestrus and diestrus, followed by a doubling of PACAP immunoreactivity on the morning of proestrus (Moore *et. al.,* 2005). Receptivity in female mice can be induced in ovariectomized females by the administration of estrogen, followed 48 hours later by the administration of progesterone. PACAP can be given in lieu of progesterone and induce an equal degree of receptivity. However, this effect is not present in progesterone receptor knockout mice. Additionally, progesterone given with a PACAP antagonist does not cause receptivity in estrogen-primed mice, indicating an important role for PACAP in estrogen and progesterone-induced female receptivity in mice (Apostolakis *et. al.,* 2004). Further studies found that PACAP is acting through the $PAC_1$ receptor and cAMP second messenger to induce receptivity as administration of a $PAC_1$ receptor antagonist, but not a $VPAC_1$ or $VPAC_2$ receptor antagonist, caused a loss in female receptivity. Also, cAMP antagonists blocked receptivity (Apostolakis *et. al.,* 2005). This set of studies showed a role for PACAP in progesterone-mediated female receptivity in rats.

A large body of evidence supports a role for PACAP as a hypophysiotropic factor, a factor which acts on the pituitary gland. PACAP and its receptors are expressed in the

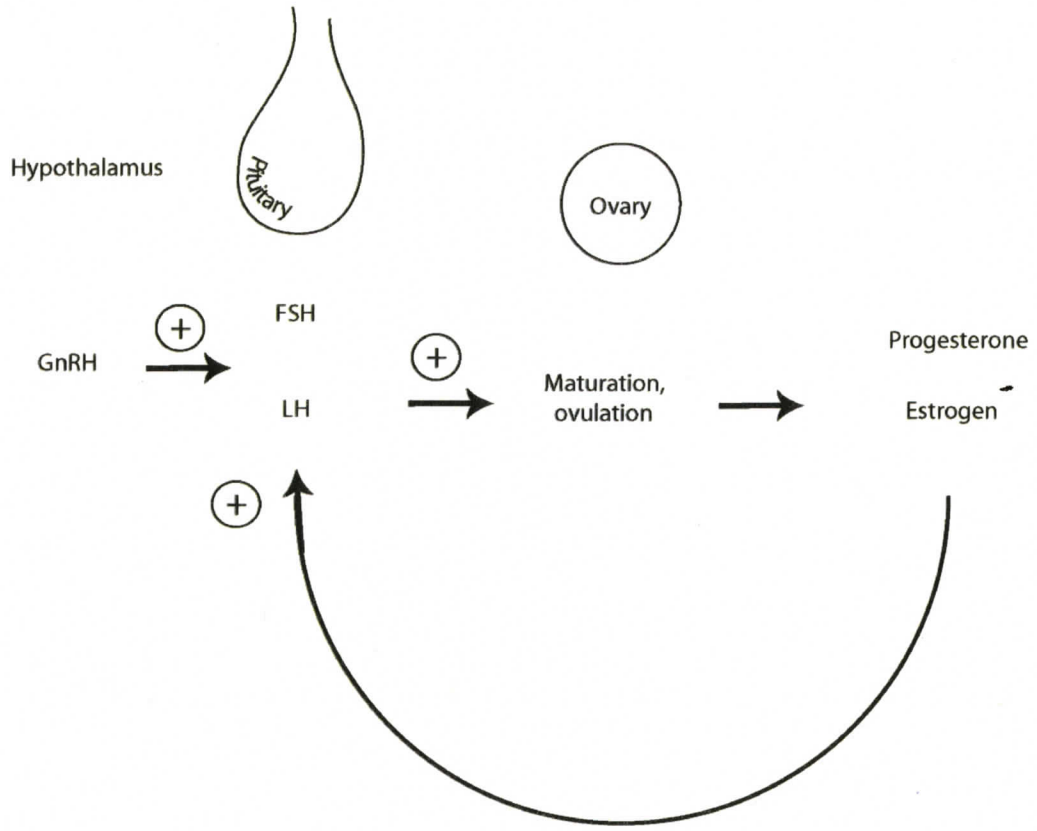



**Figure 1.5.** Representation of hormonal regulation by the hypothalamus and anterior pituitary gland of ovary development and egg release. GnRH- gonadotropin releasing hormone, LH- luteinizing hormone, FSH- follicle stimulating hormone.

anterior pituitary where they have been found in each cell type (Gottschall *et. al.,* 1990; Vigh *et. al.,* 1993). Within the portal blood of the median eminence, PACAP has been found in concentrations 2-4 times higher than levels in peripheral circulation (Tischler *et. al.,* 1995) and a dose-dependent activation of cAMP and PLC is observed in rat pituitary cell cultures following PACAP administration. PACAP has also been found to stimulate the synthesis and secretion of gonadotropins, both FSH and LH (Ortmann *et. al.,* 1999). PACAP has been shown to act synergistically with GnRH to stimulate gonadotropin secretion from rat pituitary cell cultures by stimulating GnRH receptors and by increasing responsiveness of gonadotrophs to GnRH (Ngan *et. al.,* 2001; Sadie *et. al.,* 2003). Together this evidence supports a role for PACAP in anterior pituitary function.

PACAP is present within the ovary, with mRNA and protein expression detected within the granulosa cells, corpora lutea and theca cells. Also, the $PAC_1$ receptor is found in these same cell types (Moretti *et. al.,* 2002). Both PACAP and the $PAC_1$ receptor are transiently expressed by pre-ovulatory follicles and PACAP expression within granulosa cells is increased by administration of LH and FSH *in vitro*, indicating a role for PACAP before and during ovulation (Lee *et. al.,* 1999; Scaldaferri *et. al.,* 1996). PACAP stimulates cAMP production and steroidogenesis in ovarian cells and PACAP administration to cultured follicles resulted in smaller follicles and inhibition of antrum formation (Cecconi *et. al.,* 2004; Heindel *et. al.,* 1996; Zhong and Kasson, 1994). As many cells within the ovary are avascular, or are separated from general circulation by physical boundaries, it is thought that PACAP may act as a local factor within the ovary through the $PAC_1$ receptor. PACAP also causes a dose-dependent increase in progesterone accumulation in granulosa cells *in vitro*, leading to luteinization of

granulosa cells (Gras *et. al.,* 1999). Together these findings indicate a complex and multifaceted role for PACAP at the level of the ovary in regulating and facilitating reproductive potential.

As PACAP is present and found to regulate function at all three levels of the hypothalamic-pituitary-gonadal axis, it may play a complex role in the regulation of female reproduction in mice. PACAP null mice and $PAC_1$ receptor deficient mice both show some level of reproductive impairment in females, although the cause is yet to be elucidated.

### *1.3.3 PACAP and lactation*

Lactation is considered the final phase of the reproductive cycle, and is essential for the survival of neonates. Lactation is dependent upon a dynamic interplay of endocrine and local factors at the mammary gland. Hormonal changes during pregnancy contribute to the growth and differentiation of mammary tissue. The drop in progesterone and estrogen at parturition permits milk development and secretion. Suckling triggers prolactin release in the anterior pituitary, which results in the milk synthesis. PACAP inhibits prolactin and LH secretion in ewes (Anderson *et. al.,* 1996), but can stimulate prolactin release from the pituitary in rats (Murakami *et. al.,* 2001). Also, immunohistochemistry and RIA have shown PACAP to be highly expressed within fibres of the rat nipple, and increased expression of PACAP-38 has been observed in mammary glands of lactating rat (Skakkebaek *et. al.,* 1999). The role of PACAP in mammary gland function has not been examined and it may prove to be an important factor in lactation,

either through modulation of luteotrophic hormones released from the anterior pituitary, or through local innervation of the nipple.

## 1.4 Purpose

The aim of this study was to investigate the role of PACAP in female reproduction through characterization of the reproductive phenotype in PACAP null mice. This was performed with studies of mating, fertility rates, ovarian function and physiology, mammary gland function, and blastocyst implantation. Through the use of a mouse model, I have tested the hypothesis that PACAP is a critical regulator of female reproduction.

# 2 Phenotypic Characterization of reproduction in the female PACAP null mouse.

## 2.1 Introduction

Reproduction in mammals is under tight hormonal regulation and it requires the female to be competent, both to have progressed through puberty and to be in a receptive state, in proestrus or estrus for rodents. PACAP and PAC1-receptor knockouts both exhibit impaired fertility (Jamen *et. al.,* 2000; Shintani *et. al.,* 2002). However, the reasons underlying reproductive loss have not been fully investigated, in part due to difficulties in maintaining a sufficient number of PACAP null mice to reproductive age.

One site of PACAP expression is the ventromedial nucleus of the hypothalamus, where PACAP was found to regulate progesterone-mediated female receptivity in mice and rats (Apostolakis *et. al.,* 2004). PACAP stimulates the release of LH and prolactin *in vitro* from anterior pituitary cells of rats (Miyata *et. al.,* 1989). Both prolactin receptor-deficient mice and progesterone receptor-null female mice display a severe phenotype of complete infertility, and never become pregnant (Bole-Feysot *et. al.,* 1998; Lydon *et. al.,* 1995). Also, PACAP expression has been described in the anterior pituitary and the ovary; PACAP is found and may influence fertility at all three levels of the hypothalamic-pituitary-gonadal axis.

This study describes the initial phase of evaluating the role of PACAP in reproduction by determining if the female PACAP null mice generated in our laboratory have impaired reproductive performance. Subsequent chapters examine the sites in the reproductive cascade that are affected by PACAP and the mechanisms of PACAP's

action. Reproductive competency was assessed in several ways: live births, puberty onset and estrous cycles. First, the number of PACAP null females that give birth following pairing with a fertile male gives an overview of whether a defective process, including mating, is present. Second, the time of puberty onset indicates if developmental timing is affected by PACAP. Finally, the estrous cycles of PACAP null mice indicate if a disruption of the hormonal signalling required for pregnancy or pseudopregnancy has occurred.

## 2.2 Materials & methods

### *2.2.1 Animal Use and Care*

All procedures used in these studies were approved by the University of Victoria Animal Care Committee. The generation of the PACAP transgenic knockout mouse prepared in our laboratory is described earlier (Gray *et. al.,* 2001). Mice backcrossed 10 times to a C57BL/6 background were used in all experiments. Animals were housed under a 12-h light/dark cycle and had food and water *ad libitum*. Room temperature was maintained at 28 °C to maximize survival rates, due to the temperature sensitivity in the PACAP-knockout mouse.

### *2.2.2 Genotyping for Adcyap1 Allele*

Genotyping for the *pacap* (*Adcyap1*) allele with PCR amplification was used both prior to pairing and at specific time points. Tissue samples were taken from the ear of the

pup and genomic DNA was extracted using a 5% Chelex solution containing 2mM proteinase K (Gibco BRL, Burlington, ON) and 0.15 % Tween 20. Samples were incubated at 50 °C for 45 minutes, then for 15 minutes at 94 °C to inactivate the proteinase K. Polymerase chain reaction (PCR) was performed in a 50 µl reaction. A DNA sample of 2 µL was added to 2.5 U Taq polymerase, 1 X Taq buffer, 2.5 mM $MgCl_2$, 200 mM dNTPs and 20 pmol each of three primers: MP1 5'-ATGTGTAGCGGAGCAAGGCTG-3', the forward primer that binds to the signal peptide portion of the *pacap* gene, PA1 5'-CACTCGGACGGCATCTTCAC A-3', a forward primer that amplifies from within the *pacap* gene and 3'UTR 5'-GGCCATTATTGGTATCTTCAAG-3', a reverse primer that amplifies from the 3'UTR region of the *pacap* gene (Invitrogen, Burlington, ON). The reaction condition was as follows: 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 seconds, 67 °C for 30 seconds and 72 °C for 45 seconds, followed by 72 °C for 7 minutes. Amplified PCR products were resolved on a 1.5% agarose gel and imaged under UV light.

### *2.2.3 Reproductive Performance*

Primiparous female mice that were homozygous for the PACAP mutation were paired at 6-8 weeks of age with tested wild-type males after 1500 h, and the pairings were maintained for 7 days. Wild-type females were paired similarly, but with a PACAP-/- male. This created all heterozygous litters for both groups of mice. Females were checked in the morning daily for seminal plugs. For all pregnancy experiments, day 0 of pregnancy was considered the day on which the seminal plug was found; if multiple plugs occurred then dates on which all plugs were found were noted and the latter one was considered the day on which the female became pregnant. Females were then

weighed weekly, and checked daily for signs of pregnancy, which include increased weight, swollen abdomen and palpable masses within the abdomen. Gestation length in mice ranges from 18 to 21 days. Therefore females were tracked for 21 days following removal of the male to determine if pregnancy had occurred. Upon parturition, litters were counted, weighed and tracked for 14 days to assess survival and maternal competence.

### *2.2.4 Puberty Onset*

Juvenile female mice were examined daily from the time of weaning at 21 days of age for vaginal opening (N = 5). Puberty usually occurs by six weeks of age in female mice, and is visible externally as female mice are born with a membrane occluding the vagina. This membrane disappears at time of puberty and first estrus occurs within a few days. Puberty onset was considered the day of vaginal opening, when the membrane opens. Once this occurred, vaginal cytology and the examination of external cells of the vulva were performed daily for two weeks: cells from the vagina were collected by pipette after introduction of 200 µl of 0.9% sodium chloride solution into the vagina. Fluid was then transferred to a glass slide, dried and stained with methylene blue for increased contrast. Examination of cell type was carried out under magnification. Vaginal cells undergo changes that are diagnostic of the status of the whole reproductive tract. In proestrus, the majority of cells present are nucleated epithelia, with few cornified (non-nucleated) epithelia and leukocytes. In estrus, smears contain no leukocytes but both nucleated and cornified epithelia are present. In metestrus smears, mice show large quantities of cells with an abundance of clumped cornified epithelia. In

diestrus smears, fewer cells, nucleated epithelia and leukocytes, as well as mucous are present. Figure 2.1 illustrates the normal 4-5 day estrous cycle pattern in mice.

### *2.2.5 Estrous cycle evaluation*

Vaginal cytology was monitored for three weeks in the cohort of PACAP null virgin female mice of 6-8 weeks of age (N = 9), which were subsequently mated for the fertility study (See *2.2.3*). Estrous cycle length was defined as the interval between onset of one estrous event and onset of the next estrous event.

### *2.2.6 Statistical analysis*

Data were analyzed with GraphPad InStat for Windows software (GraphPad Software Inc., San Diego, CA), using independent samples *t* test. Significance was inferred at $P < 0.05$. Data are mean ± SEM unless specified otherwise.

## 2.3 Results

### *2.3.1 Genotyping*

Genotyping of mice was performed by PCR amplification. Genotype was determined from banding pattern. One band at ~550 base pairs (bp) indicates a PACAP wild-type (+/+) genotype: one band at ~950 bp represents a PACAP null (-/-) genotype;

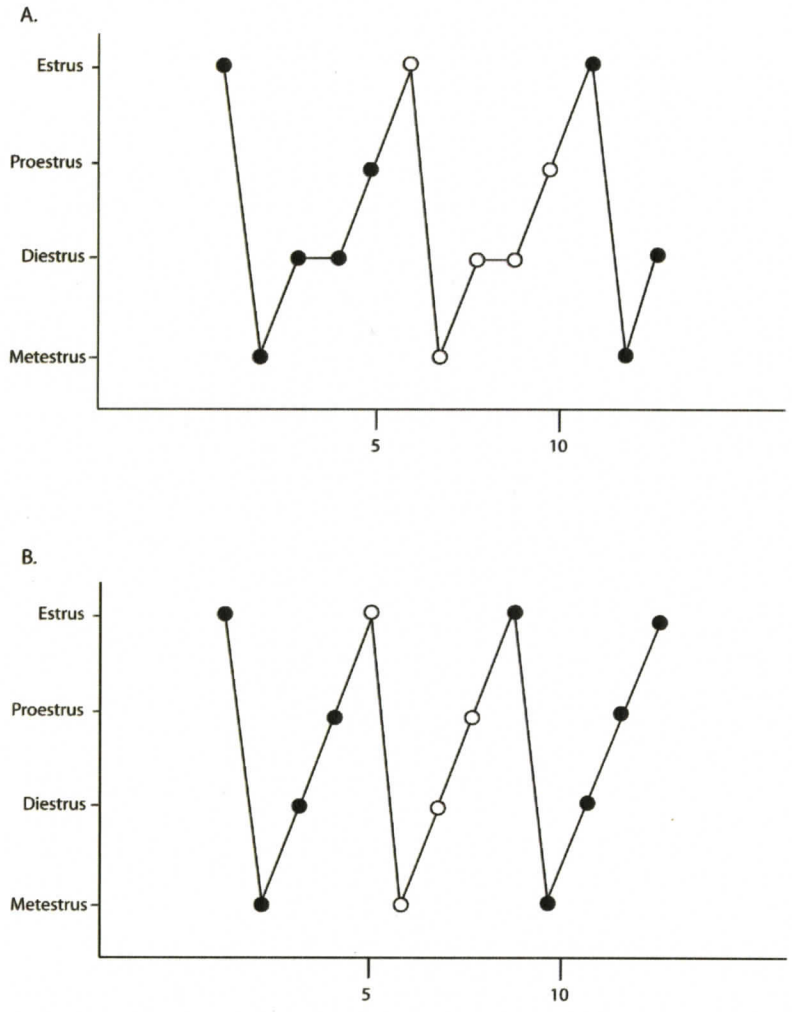


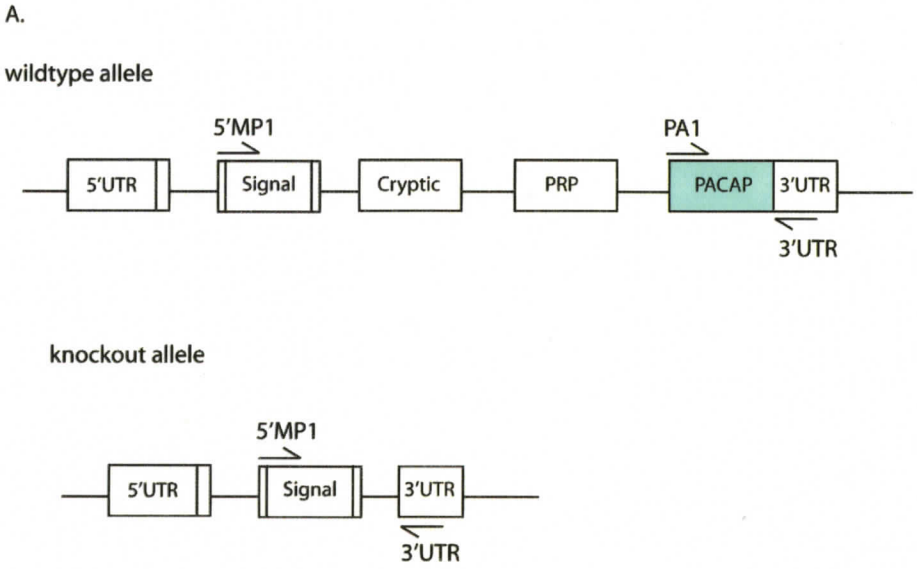

**Figure 2.1.** Illustration of typical 5 day (A) and 4 day (B) estrous cycles found in mice. Closed circles indicate first and third estrous cycles and open circles denote second estrous cycle.

and two bands, one at each of these positions, indicate a PACAP heterozygous (+/-) genotype (Figure 2.2)

### *2.3.2 Mating, pseudopregnancy and pregnancy*

To determine the effect of PACAP null mutation on the capacity of female mice to initiate and maintain pregnancy, females were placed with PACAP-/- males (for wild-type females) or wild-type males (for PACAP-/- females) of proven fertility for seven days and examined daily for the presence of copulatory plugs and subsequent birth of litters. All PACAP-/- females mated when paired with a male, although mating behaviour was abnormal. Wild-type females normally only mate once when paired with a male, then enter a state of pseudopregnancy, which lasts for 12 days, and during which time they will not mate again. Table 2.1 shows the pattern of mating of both PACAP null and wild-type females, where 33% of PACAP null females displayed plugs on multiple days in the time paired (PACAP-/- mice had plugs between one and three times in one week) but wild-type mice have plugs only once. Despite mating when paired, the incidence of establishing successful pregnancy was substantially reduced with only 21% of PACAP–/– mice producing live progeny at term compared with 100% in PACAP+/+ females (Figure 2.3). The litter size in PACAP–/– females was (mean +/- SEM) 7.17 ± 0.27 pups (N = 6 litters) compared with 7.07 ± 0.83 pups (N = 20 litters) in PACAP+/+ females (not significant, $P > 0.05$). However, pup mortality was higher for litters with PACAP-/- mothers. The mean pup mortality for litters birthed by PACAP-/- females was 0.83 +/- 0.54 pups per litter (N = 6 litters) whereas litters with PACAP+/+ mothers had mean mortality rates of 0.06 +/- 0.06 pups per litter (N = 20 litters, $P < 0.05$). The

A.




B.

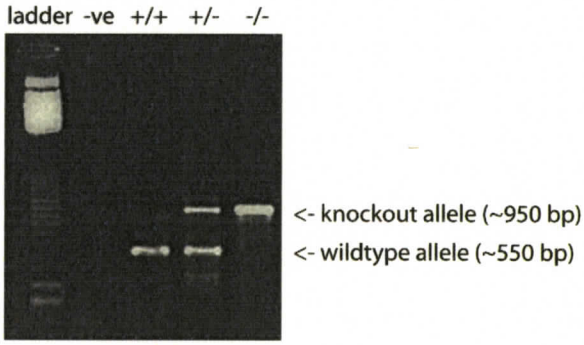



**Figure 2.2.** Genotyping of pups born to heterozygous breeding pairs. A, Representation of PACAP wild-type and PACAP knockout alleles. Location of primer annealing for PCR genotyping denoted by arrows. B, PCR amplification of genomic DNA from PACAP +/+, PACAP +/- and PACAP -/- mice using the primers 5'MP1, PA1 and 3'UTR1. Distinct banding pattern is provided for each of the three genotypes as the knockout allele provides a band of approximately 950 bp, and the wild-type allele produces a band of approximately 550 bp. Abbreviations: bp – base pairs, PCR – polymerase chain reaction.

**Table 2.1.** Copulatory plug presence in PACAP null female mice and wild-type females over a one week period while paired with a fertile male. The table summarizes the occurrence of no plugs, a single plug, or multiple plugs during the mating period.

| | Wild-type Females (N=14) | PACAP Null Female (N=27) |
|---|---|---|
| No Plug | 0 | 0 |
| Single Plug | 14 | 18 |
| Multiple Plugs | 0 | 9 |
| Parturition (successful Live births) | 14 | 6 |

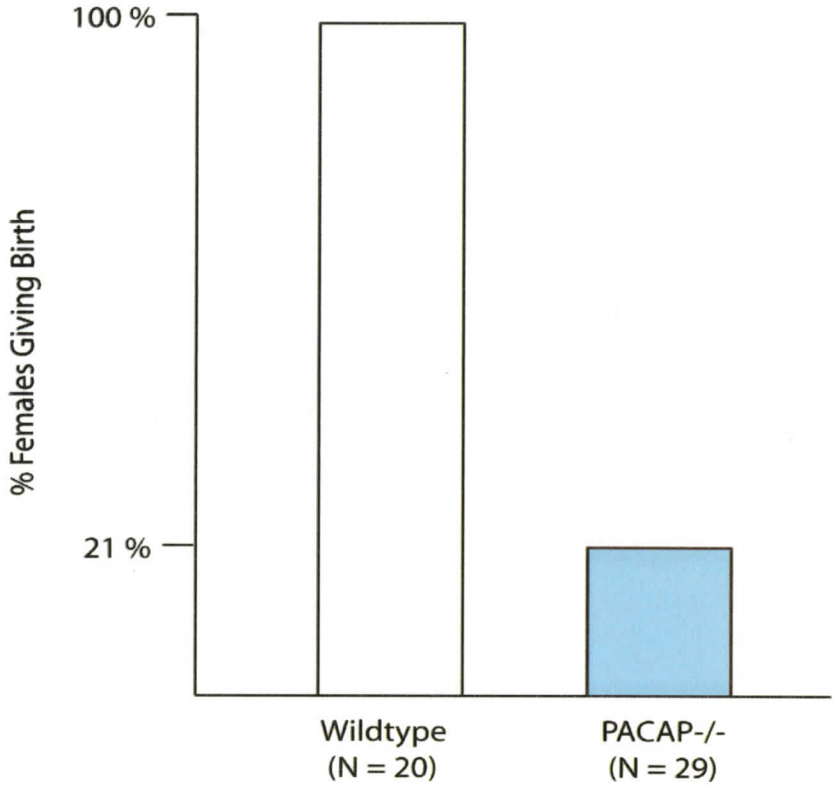



**Figure 2.3.** Percentage of wild-type and PACAP null females giving birth to litters following a week-long pairing with a fertile male. The graph shows females that gave birth within 30 days of pairing with a male.

PACAP–/– females that did not give birth to pups did not show visible signs of pregnancy.

### *2.3.3 Puberty onset*

The failure to become pregnant when paired with a male indicated a disruption in PACAP null mice at six weeks of age. To determine whether this impairment was due to a delay in puberty, PACAP-/- females were assessed daily following weaning at 21 days of age. Sexual maturity as measured by age at appearance of vaginal opening and age at first estrus was normal in PACAP-/- mice. In both groups, all mice achieved vaginal opening by 35 days of age (PACAP-/- 29.0 +/- 0.95 days, N = 5; PACAP+/+ 29.2 +/- 0.97 days, N = 5; P>0.05; Figure 2.4). PACAP null mutants were the same age at the time of first estrus after vaginal opening (1.6 +/- 0.68 days after vaginal opening in PACAP+/+ females (N = 5) *vs.* 1.8 +/- 0.8 days in PACAP-/– females (N = 5); $P > 0.05$). External indicators include a pink and swollen vulva, striations appearing on the ventral portion of the vulva and gaping of the vulva. Smears were prepared daily for two weeks and confirmed the normal estrous cycles of the mice.

### *2.3.4 Estrous cyclicity*

The failure of some PACAP null mice to become pregnant, despite mating, and the abnormal mating behaviour in many of these mice, indicated a possible disruption in the estrous cycling in these mice. To investigate the role of PACAP in estrous cyclicity, the stage of estrous cycle was tracked by vaginal cytology over three weeks. All mice examined showed normal estrous cycles with estrus occurring either 4 or 5 days apart.

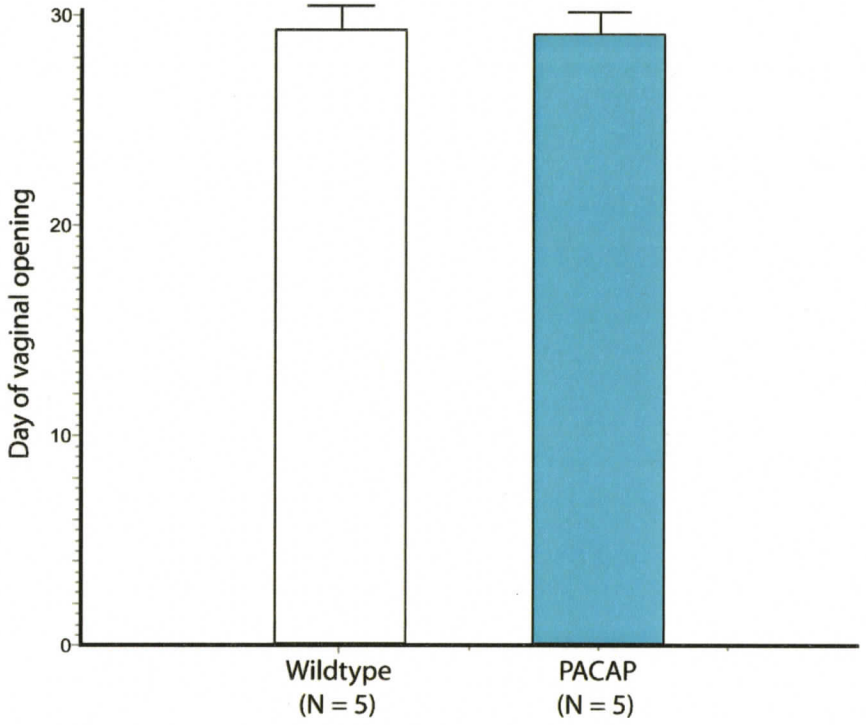



**Figure 2.4.** The effect of PACAP gene knockout on puberty onset in juvenile female mice. Puberty onset was defined as day of vaginal opening, and is not affected in PACAP null (-/-) mice. Data are mean $\pm$ SEM, $P > 0.05$.

The mean estrous cycle length in PACAP-/- mice was 4.43 days +/- 0.10 days, which was not significantly different from the cycle length found in wild-type females, 4.77 +/-0.10 days. One PACAP null female exhibited a single cycle that was three days in length, although all her other cycles were of normal length. Also, one wild-type female had a single six day estrous cycle and all other cycles for this mouse were five days in length. These two discrepancies are most likely due to variations in time of day the mice were examined, or may have resulted from the slightly ambiguous transitional stages between cycle phases. Female mice enter estrus during the dark phase of the dark-light cycle. Estrus is detected the following morning but the transition to metestrus can be rapid.

## 2.4 Discussion

The aim of this study was to characterize the reproductive phenotype of the PACAP null mice generated in our laboratory. Based on previous studies, I expected a reduced fertility rate as assessed by the number of live births following pairing with a male of proven fertility. However, the investigation of the fecundity of our particular PACAP knockout model was warranted, as fertility rates may depend on the environmental temperature and the strain of mice on which the mutation is expressed (Shintani *et. al.,* 2002). Both previous studies compared to the present study found higher pregnancy rates in their PACAP and PAC1 receptor null mice, 33% or 50% depending on background by Shintani *et al.* for PACAP null mice, and 30% by Jamen *et al.* in PAC1 receptor null mice. The lower incidence of pregnancy in the PACAP null mice in our laboratory may

be due to the genetic background on which the mutation is expressed. The 33% pregnancy rate found by Shintani's group was for mutants with a C57BL/6-129/Ola mixed background. All mice used for my experiments are backcrossed 10 times to a C57BL/6 background. The observation that between 21 and 50% of mice lacking functional PACAP or $PAC_1$ receptor are able to reproduce normally indicates that other factors or circumstances must attenuate the severity of effects caused by absence of this hormone. There may be some compensatory factor within the genes of other strains of mice that afford some amelioration of the severity of the reproductive deficit.

Shintani *et al.* have reported reduced mating frequency in PACAP null females when paired with vasectomized mice (Shintani *et. al.,* 2002). Based on these findings, I examined mating frequency in PACAP null females when paired with males of proven fertility. I found increased mating frequency in PACAP null females compared to wild-types with an abnormal pattern of repeated copulation within a week-long pairing. In a 12 day period, rodents normally mate only once after which they then enter a state of pseudopregnancy and are no longer receptive. This is due to their short, incomplete estrous cycle. In humans, who have a long complete menstrual cycle, the corpus luteum secretes progesterone for several days following ovulation, which allows implantation to follow. In mice, which lack a spontaneous luteal phase, cervical stimulation that occurs during mating triggers hormonal secretions that maintain the new corpora lutea and prevents degradation. This reproductive strategy in mice allows for a shortened estrous cycle length and increases their fecundity by allowing for a rapid subsequent ovulation. Pseudopregnancy is initiated in the hypothalamus, which triggers the release of prolactin from the anterior pituitary. Prolactin then acts on the ovary where it maintains the

corpora lutea (Terkel, 1988). As mating behaviour is abnormal in PACAP null mice I hypothesized that PACAP affects the initiation of pseudopregnancy. The disruption of prolactin secretion would prevent pregnancy. Therefore, further examination into prolactin secretion, progesterone levels and occurrence of implantation events is required, and will follow in the subsequent chapter.

To further characterize the reproductive phenotype of the PACAP null mouse, endpoints including number of live offspring resulting from successful pregnancy, and survival of those offspring were assessed. A normal litter size was found in PACAP null females, indicating that the reproductive deficit is not a reduction in viability of the embryo. If reduced viability were the problem, smaller litter sizes would be observed, as only some of the embryos would be capable of implanting. Instead, the problem in reproduction is all-or-none. If the female does manage to overcome the effect caused by the lack of PACAP, the pregnancy results in a normal number of offspring.

A significant number of pups died (approximately one per litter) when the maternal genotype was PACAP-/-. The difference in survival of pups was significant despite the small sample size of litters born to PACAP null females, due to their reduced fertility. This high mortality was not the result of pup genotype, as all pups were heterozygous for both the PACAP null females and wild-type controls. Survival of pups born to mice lacking either PACAP or its specific receptor, the PAC1 receptor, was not discussed in either the Jamen or Shintani papers. However, Shintani *et al.* reported decreased crouching behaviour, an aspect of maternal behaviour, by PACAP null females. However, all other aspects of maternal care were found to be normal. The mortality observed in litters born to PACAP null females may be the result of decreased

maternal care. Alternately, the possibility of impaired lactation in PACAP deficient mice exists. Prolactin is the hormone involved in milk secretion in mice. PACAP inhibits prolactin and LH secretion in ewes (Anderson *et. al.,* 1996), but can stimulate prolactin release from the pituitary in rats (Murakami *et. al.,* 2001). Also, immunohistochemistry and RIA have shown PACAP to be highly expressed within fibres of the rat nipple, and increased expression of PACAP-38 has been observed in mammary glands of lactating rat (Skakkebaek *et. al.,* 1999). The role of PACAP in mammary gland function has not been examined and it may prove to be an important factor in lactation, either through modulation of luteotrophic hormones released from the anterior pituitary, or through local innervation of the nipple. A defect in lactation in PACAP null mice may explain the increased mortality among heterozygous offspring, as the pups may receive insufficient nutrients to thrive.

Based on findings of impaired fertility, further studies into the precise mechanism by which PACAP acts to enhance reproduction were of interest. The actions of PACAP on the control of gonadotropin hormone secretion are well documented (For a review, see Sherwood *et. al.,* 2000). PACAP has been reported to stimulate LH and FSH release from anterior pituitary cells *in vitro* (Miyata *et. al.,* 1989) and to stimulate LH-β mRNA levels *in vivo* (Tsujii *et. al.,* 1994).

Administration of PACAP to neonatal rats was found to delay puberty onset (Koves *et. al.,* 1998). This study indicates a role for PACAP in the establishment and temporal regulation of puberty; therefore it was of interest to assess puberty in PACAP null mice. Puberty is easily detected in female mice, as the occurrence of vaginal opening is a visible external indicator of puberty onset. I hypothesized that mice lacking

PACAP would have disrupted puberty onset possibly not followed by normal estrous cycles. This study found no effect of PACAP deficiency on puberty. Puberty occurred at the same age in PACAP null females, and was closely followed by first estrus in both PACAP deficient and wild-type groups. Therefore, PACAP is not an essential regulator of puberty onset in female mice.

As female mice deficient in PACAP have reduced fertility, but have undergone puberty by six weeks of age, I expanded endpoints of interest to include the cycling status of PACAP null females. A defect in the estrous cycle, where females possess a long diestrous phase or do not cycle at all, would reduce the fertility rate of mice when paired for only a week. It was possible that an irregular estrous cycle was responsible for the reduced fertility in mice deficient in PACAP. Previous findings on the estrous cycle in mice deficient in PACAP or the $PAC_1$ receptor were contradictory. Mice lacking PACAP were reported to have normal four to five day estrous cycles, whereas mice lacking the $PAC_1$ receptor were found to have a long, irregular diestrous phase. The results here show normal cycles in PACAP null mice.

In conclusion, some aspect of reproduction other than puberty onset or normal estrous cyclicity is responsible for the reduced fertility rate in mice lacking PACAP. A clue to the mechanism underlying the low incidence of pregnancy found in this study may be the impairment in the formation of pseudopregnancy following mating. Abnormal mating behaviour shown by repeated mating in a subset of the PACAP null females supports the defect in establishing pseudopregnancy. The next chapter in this thesis will explore the levels of prolactin, as this hormone is required for pseudopregnancy. Alternatively, the reduction in fecundity in mice lacking PACAP may

be the result of impaired follicle development and ovulation. PACAP null females may cycle normally but not generate follicles normally. If follicular development were disrupted in a portion of PACAP null mice, they may appear to cycle normally but not release viable eggs for fertilization. This thesis will continue with an examination of ovulation, egg release, egg fertilization and implantation.

# 3 Site of PACAP's action in the cascade of events from ovulation to implantation.

## 3.1 Introduction

Reproductive competency in female mammals requires the ovary to release eggs in response to hormonal signals and the uterus to be receptive to implantation of blastulas. Both ovulation and implantation are controlled by a complex milieu of hormones that regulate: development of follicles within the ovary, ovulation, decidualization of the uterus, and implantation. Decidualization is the proliferation and differentiation of stromal cells into large binucleated decidual cells, an essential change that readies the uterus for implantation. Decidualization is required to convert the uterus into a secretory tissue, in which endometrial glands synthesize and release proteins and histotroph factors that are necessary for embryo implantation.

PACAP is widely distributed throughout central and peripheral organs, including areas essential for reproduction, notably the hypothalamus, anterior pituitary and ovaries, where it may function as a neurotransmitter or neuromodulator (for a review, see Sherwood *et. al.,* 2000). PACAP has been reported to stimulate the synthesis and secretion of the gonadotropins, FSH and LH, which are released from the anterior pituitary in a cycle-dependent manner and cause follicular development and ovulation in the ovary (Ortmann *et. al.,* 1999). However, some controversy surrounds these findings as they involve concentrations of PACAP above physiological relevance. Also, PACAP has been found to have no effect on LH secretion in some studies, or to decrease LH secretion in yet another (Rawlings and Hezareh., 1996).

PACAP may act as a local regulator of oocyte development in the ovary. PACAP expression has been found in granulosa and cumulus cells, as well as within the corpora lutea (Moretti *et. al.,* 2002). PACAP levels in the ovary are dynamic, with increased expression occurring after the preovulatory LH surge (Gras *et. al.,* 1999). PACAP stimulates steroidogenesis in ovarian cells and has been found to modulate differentiation and proliferation of ovarian cells (Zhong and Kasson, 1994). Notably, PACAP stimulates oocyte maturation and proliferation of mouse primordial granule cells but decreases apoptosis of pre-ovulatory follicles *in vitro* (Apa *et. al.,* 1997; Lee *et. al.,* 1999). Together these findings indicate a role for PACAP in the development of follicles in the ovary. PACAP may be necessary for the coordinated development of follicles in the ovary and the loss of a functional PACAP gene, as in PACAP null mice, could result in impaired egg development. I hypothesized that lack of PACAP would result in disrupted follicle development with fewer follicles developing to the preantral stage.

Implantation requires decidualization of the uterine tissue. Decidualization is progesterone-dependent and can be triggered by cervical stimulation (Bell, 1983). Prolactin, the primary luteotrophic hormone in early pregnancy in mice, is also released by cervical stimulation. Prolactin maintains the corpus luteum, which produces progesterone and estrogen. PACAP is localized in neuronal cells of the uterus, and may therefore be involved in signal transduction of cervical stimulation to the central nervous system to trigger pseudopregnancy (Fahrenkrug and Hannibal, 1998).

Alternatively, increased expression of PACAP has been found with increased decidualization and it has been proposed that PACAP may act as a local factor increasing blood flow to facilitate decidualization (Spencer *et. al.,* 2001). Loss of PACAP in null

mice may result in a decrease in circulation to the uterine tissue and impair decidualization.

PACAP has been shown to increase progesterone accumulation in granulosa cells of the ovary in a dose-dependent manner, with levels of progesterone more than twenty times higher than controls *in vitro* (Gras *et. al.,* 1999). As PACAP regulates progesterone accumulation and luteinization of granulosa and lutein cells, PACAP null mice may show impaired implantation resulting from decreased progesterone levels and a resulting reduction in decidualization of the uterus.

Previous studies have suggested a role for PACAP in local regulation of egg development in the ovary, gonadotropin secretion from the anterior pituitary and local regulation of blood flow during decidualization of the uterus. I hypothesized that PACAP affects reproduction through establishment of pregnancy or pseudopregnancy and is not required for ovulation and gonadotropin secretion. PACAP's actions on gonadotropin secretion are controversial but the findings of altered mating in mice lacking PACAP are indicative of impaired pseudopregnancy. This study investigates the role of PACAP in ovulation, egg fertilization, establishment of pregnancy and implantation.

## 3.2 Materials and methods

### *3.2.1 Assessment of ovulatory performance*

To analyze ovulation and egg fertilization *in* vivo, female mice two to three months of age with either a PACAP +/+ or PACAP -/- background were placed individually with one PACAP +/+ male of proven fertility after 1500 h. They were checked daily for the presence of a copulatory plug, and sacrificed between 1000 and 11:00 h on the day the plug was found; the oviducts and ovaries were removed from the female; the oviducts were placed in HEPES-buffered FHM media (Specialty Media, NJ, USA) and the ovaries were placed in formalin solution at 4 °C. Cumulus-enclosed zygotes were removed from the ampulla of the oviduct by manual dissection. The cumulus mass containing ovulated eggs and cumulus cells was placed in hyaluronidase (0.3mg/ml; Sigma, Oakville, ON) in a HEPES-buffered FHM medium for less than 5 minutes, which released the ovulated eggs from the cumulus mass. Embryos were washed in HEPES-buffered FHM media and transferred into culture dishes. The embryos were assessed for fertilization 12 hours of culture and analysis under a light microscope at 50X magnification.

### *3.2.2 Histological analysis of ovaries*

Ovaries were stored in formalin solution at 4 °C until processed. Left or right ovaries were randomly selected to be dehydrated through graded alcohol solutions to 100% ethanol, then embedded in JB-4 plastic (Electron Microscopy Sciences, Hatfield, PA, USA). Three ovaries per genotype were serially sectioned at 5 μm thickness with a JB-4 microtome and stained with a regressive hematoxylin and eosin staining method (N = 3). Sections were photographed with a Nikon digital E990 camera (Nikon Corp., Japan) using a Universal microscope (Zeiss, Germany) with a green filter, wavelength

550 nm, to increase resolution. Image brightness and contrast were adjusted with Adobe Photoshop. Due to the heterogenous nature of the ovary, every tenth section was viewed throughout the entire ovary, and the number of follicles in different stages of development was quantified, as well as the number of corpora lutea present (Figure 3.1).

Morphological classification of follicles followed guidelines proposed by Myers *et al.* (2004), where follicles were classified as primordial when surrounded by squamous granulosa cells. Primary follicles are those which show a single layer of cuboidal granulosa cells. Follicles were classed as secondary when possessing more than one layer of granulosa cells but no antrum. Antral follicles were divided into two groups: early antral, which possessed only small areas of antrum (follicular fluid), and antral, which contained a larger antral cavity. Pre-ovulatory follicles had oocytes which were surrounded by a ring of cumulus cells (Figure 3.2). Only follicles in which the nucleus of the developing oocyte was visible in the section were counted. This avoids the counting of an oocyte twice and misidentification of the follicular stage (Myers *et. al.,* 2004). Complete follicular counts were tabulated and compared between PACAP null females and their wild-type counterparts.

### *3.2.3 Implantation assessment*

PACAP null and wild-type female mice were paired at 6-8 weeks of age with a fertile wild-type male, N = 15. The morning on which a copulatory plug was observed, the male and female were separated and this was considered 0.5 days postcoitus (dpc). On the morning of 6.5 dpc, a vaginal smear was performed and the female was weighed. The female was then sacrificed 30 minutes before the dark cycle commenced by cervical

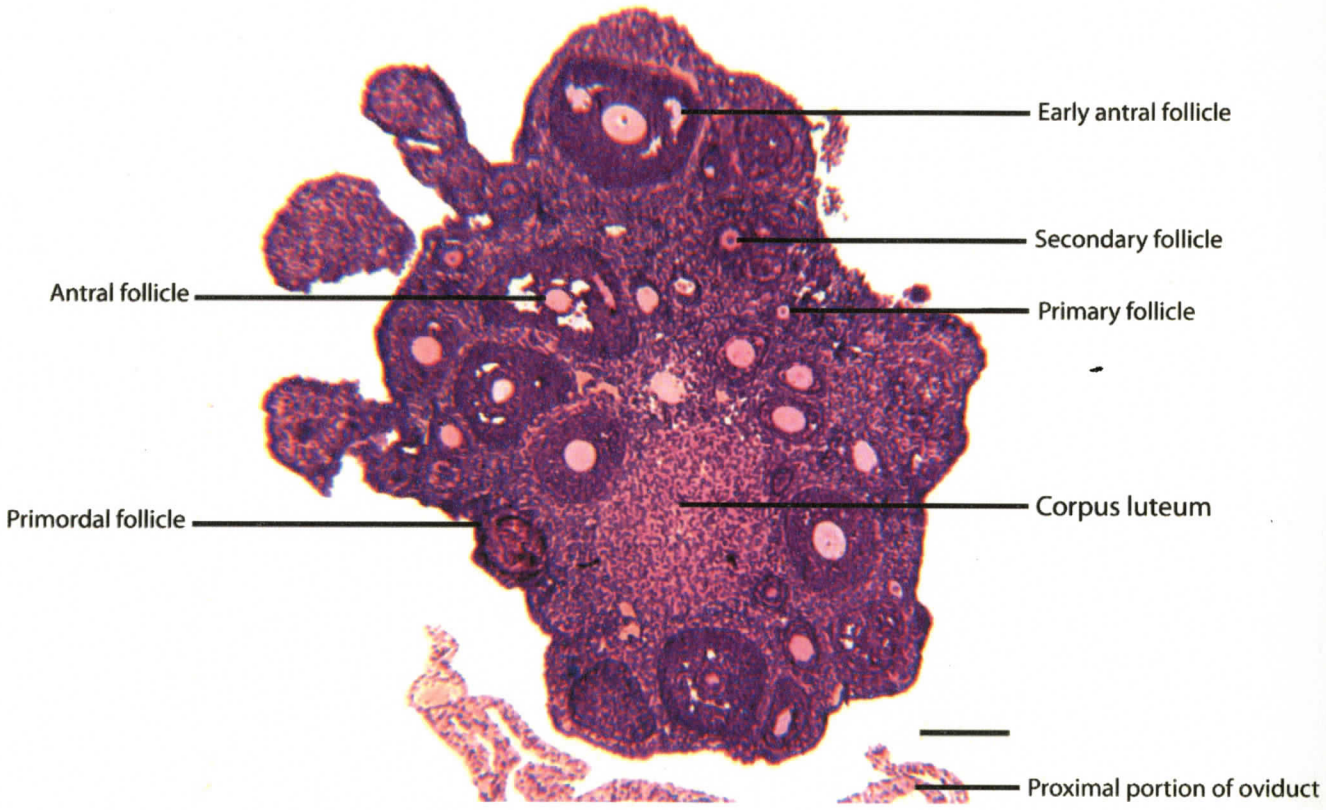



**Figure 3.1** Representative section of a wild-type mouse ovary taken on the morning following mating. Section shows the heterogenous nature of ovarian tissue with multiple follicles at different stages of development. Section is 5 μm thick, embedded in JB-4 plastic and stained with hematoxylin and eosin regressive staining. Bar = 1 mm

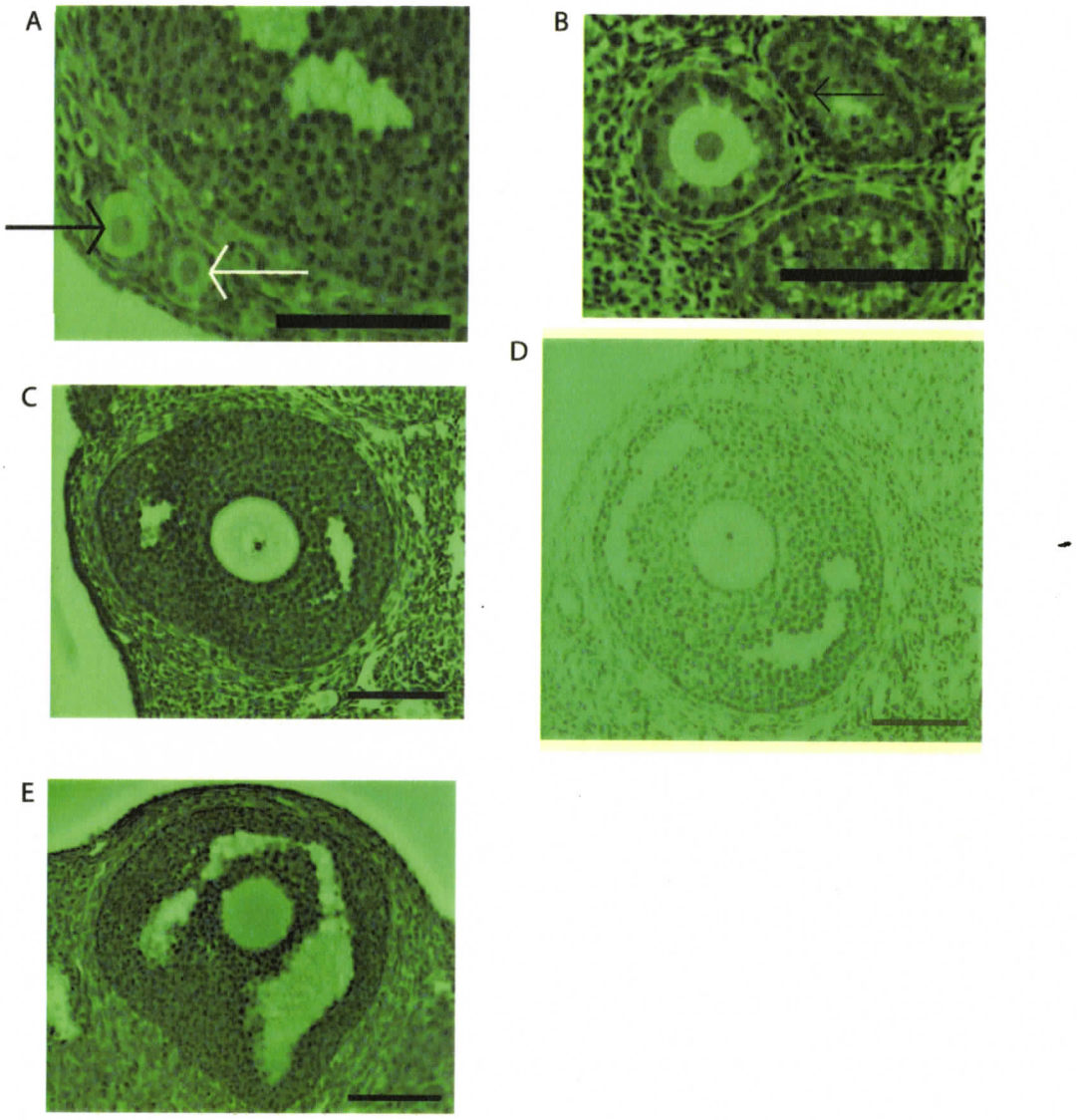



**Figure 3.2.** Follicular classification used for analysis. A) Primordial follicles, indicated by a black arrow, were defined as oocytes surrounded by a single layer of squamous epithelial cells. Primary follicles (white arrow) were surrounded by predominantly cuboidal granulosa cells. B) Secondary follicles possessed more than one layer of cuboidal granulosa cells and no antrum. C) Early antral follicles had emerging antral cavities and antral (D) had clearly defined antral spaces. E) Preovulatory follicles had a defined layer of cumulus cells surrounding the oocyte. Tissue was sectioned at 5 μm thickness, embedded in JB-4 plastic and stained with hematoxylin and eosin regressive staining. Images were taken under a green filter. Bars = 100 μm.

dislocation and blood was collected by partial decapitation within 30 seconds of initial disturbance of the cage. This method for sacrificing mice was required due to the elevation in prolactin levels that occurs in response to stress (Barkley *et. al.,* 1978). Blood was spun down for five minutes at 5000 X g and whole serum was stored at -80 °C. The uterus was then removed, weighed and examined for implantation. By day 6.5 the implanted embryos are clearly visible (Figure 3.3) and embryos were removed by manual dissection. The uterus, after removal of any embryonic tissue, was snap-frozen and stored at -80 °C until further processing.

### *3.2.4 Serum prolactin levels*

Whole serum samples were sent to Dr A.F. Parlow at the National Hormone and Pituitary Program at the Harbor-UCLA Medical Center for progesterone and prolactin analysis. Here prolactin radioimmunoassay was performed with three replicates of 20 µl of mouse serum using anti-mPRL antibody from rabbit at a 1:500,000 dilution.

### *3.2.5 Statistical analysis*

Data were analyzed with GraphPad Instat software, using independent sample *t* tests. Significance was inferred at $P < 0.05$. Data are mean $\pm$ SEM unless specified otherwise.

## 3.3 Results

### *3.3.1 Ovulation*

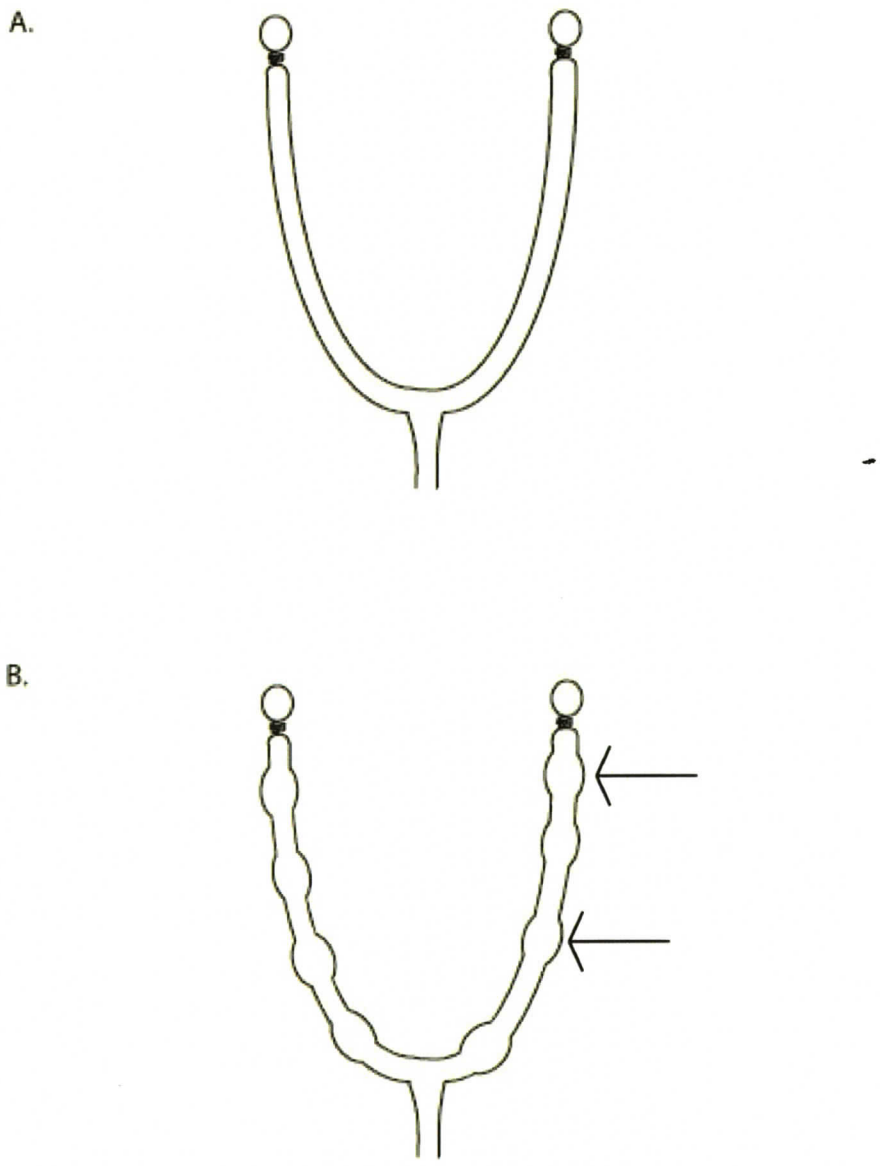



**Figure 3.3.** Schematic of a mouse uterus A) lacking implantation events on day 6.5 of pregnancy and B) showing implantation events on day 6.5 of pregnancy, indicated by arrows. Implantation is clearly visible 6.5 days following mating.

Firstly, the number of mice in which eggs were found in the oviduct following natural mating with a fertile wild-type male was assessed. No significant difference was found in number of females with eggs present in the oviduct following mating in PACAP null mice, P>0.05, See Table 3.1. Although 88.1% of wild-type females had eggs present in the ampulla and only 65% of PACAP null females had eggs present, this difference was not significant. Second, the eggs in the oviduct were recovered and counted. When eggs were found, the number of eggs was the same for PACAP null ($7.4 \pm 0.67$) and wild-type ($7.3 \pm 0.92$) mice (Figure 3.4; $P > 0.05$). Third, fertilization of released eggs was determined. Fertilization, which was measured by cleavage occurring within 24 h of culture, was not affected in PACAP null females (Figure 3.4).

### *3.3.2 Ovarian histology*

To further investigate ovarian performance, ovarian tissue was recovered from PACAP+/+ and PACAP-/- females on 0.5 dpc. Analysis of representative sections from both wild-type and PACAP null females showed follicles in all expected stages of development and mature antral follicles were present regardless of genotype. Quantitative analysis of follicle numbers showed no effect by PACAP on the relative abundance of primordial, primary, secondary, antral and preovulatory follicles (Figure 3.5). Furthermore, there was no effect of genotype on the proportion of corpora lutea in the ovary. Corpora lutea were present in both PACAP null and wild-type females.

### *3.3.3 Implantation assessment*

**Table 3.1.** Preimplantation egg retrieval in adult PACAP+/+ and PACAP-/- females on 0.5 dpc. Eggs were recovered from the oviduct of naturally cycling females paired with PACAP+/+ stud males. The number of females with eggs present and number recovered per female (mean ± SEM) is shown. dpc – days post-conception

| | PACAP+/+ | PACAP-/- |
|---|---|---|
| No. of mice with eggs present | 18 | 13 |
| No. with eggs absent | 2 | 7 |
| No. of eggs retrieved, when eggs were present | 7.3 ± 0.9 | 7.4 ± 0.7 |

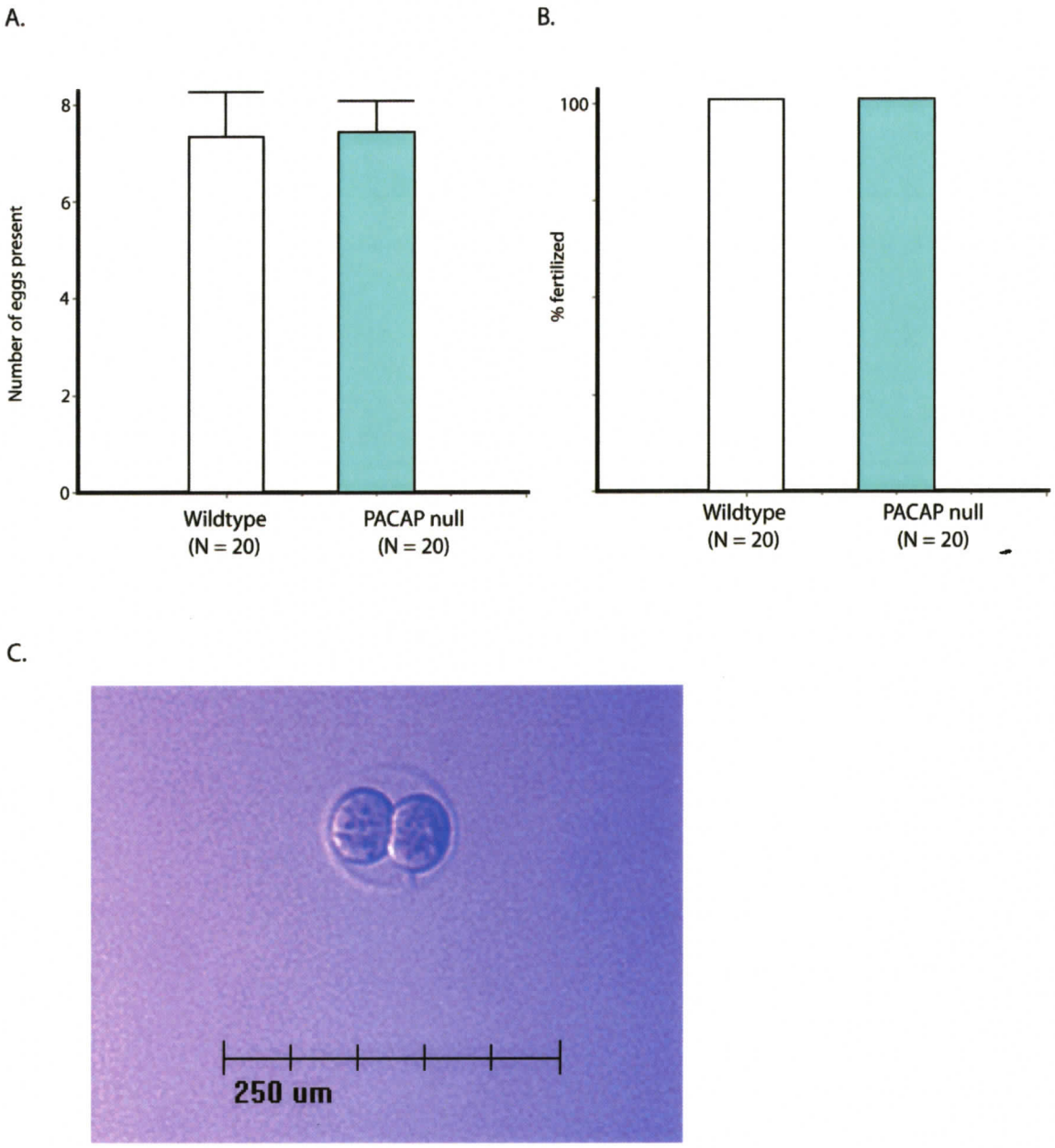



**Figure 3.4.** The effect of PACAP null mutation on A. numbers of eggs ovulated and B. number of eggs fertilized. Bars represent number of embryos recovered from females with eggs in the oviduct of naturally cycling females mated with a PACAP+/+ fertile male. Data are mean ± SEM. C. A representative image of cleaved eggs from a wild-type female is shown following culture for 24 h.

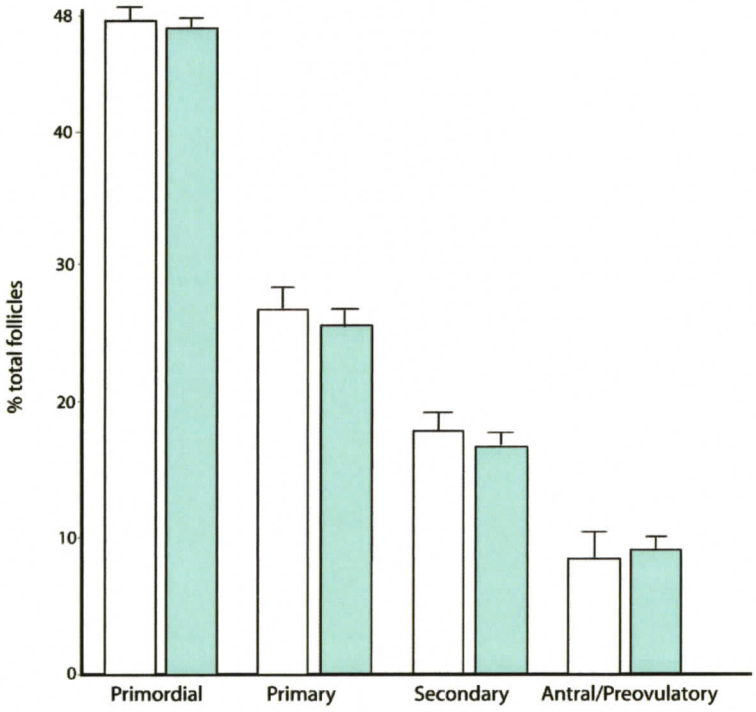



**Figure 3.5.** The distribution of ovarian follicles at different stages of development in PACAP+/+ and PACAP-/- females (N = 3). Ovaries were taken on the morning of estrus, following mating with a fertile PACAP+/+ male. Data are mean ± SEM. Follicles were classified according to Myers *et al.,* (2004).

To investigate the effect of PACAP mutation on implantation of embryos, uteri were removed 6.5 dpc and examined for implantation events. Significantly fewer PACAP null females had embryos implanted; 13.3% of PACAP null females showed implantation in comparison to 80% of wild-type females. This decreased occurrence of implantation was accompanied by a lower average uterine weight, 198 mg for wild-type uteri and 106 mg for PACAP null uteri, but no differences in female weight or number of embryos, when present, were detected.

### *3.3.4 Serum prolactin*

To investigate the cause of implantation failure in embryos from PACAP null females, serum prolactin levels were evaluated on 6.5 dpc. Regardless of pregnancy status, this hormone is normally elevated following mating and is essential for preparing the uterus for possible implantation. Prolactin is secreted in a biphasic manner for ten days following mating, with one increase, termed the diurnal surge, occurring between 14:00 and 20:00 h with a peak at 18:00 h (the onset of the dark portion of the dark-light cycle) and the other increase, the nocturnal surge, occurring between 23:00 and 8:00 h with a peak at 300 h. The diurnal surge is the larger of the two increases, with approximately two-fold higher prolactin levels than the nocturnal surge. Significantly lower prolactin levels were present in PACAP null females at 17:15 h of 6.5 dpc (Table 3.2 and Table 3.3; $P < 0.05$). Average prolactin levels for PACAP null females were 82.6 ng/ml versus 279.9 ng/ml in wild-type controls (Figure 3.6). Also, prolactin levels were below 100 ng/ml in 11 of 15 PACAP null females but below 100 ng/ml in only 2 of 15 wild-type controls (Table 3.3).

**Table 3.2.** Serum prolactin levels in PACAP gene knockout (ko) and wild-type (wt) females from 17:15 h on 6.5 dpc. Prolactin concentrations are given in ng/ml. Data are mean concentrations of prolactin detected with RIA.

| Mouse ID | Serum prolactin (ng/ml) | 95% confidence limits |
|---|---|---|
| Wt3 | **268** | 203-358 |
| Wt4 | **365** | 274-493 |
| Wt6 | **392** | 295-529 |
| Wt7 | **821** | 604-1137 |
| Wt8 | **218** | 165-290 |
| Wt9 | **68** | 52-89 |
| Wt12 | **105** | 81-138 |
| Wt13 | **85** | 65-112 |
| Wt14 | **127** | 97-167 |
| Wt16 | **324** | 244-434 |
| Wt17 | **525** | 389-719 |
| Wt18 | **366** | 276-493 |
| Wt19 | **315** | 238-421 |
| Wt20 | **126** | 96-165 |
| Wt21 | **138** | 106-182 |
| Ko1 | **255** | 193-340 |
| Ko2 | **17** | 13-23 |
| Ko3 | **<6** | * |
| Ko4 | **18** | 14-24 |
| Ko6 | **63** | 48-82 |
| Ko7 | **6** | 5-8 |
| Ko8 | **57** | 43-74 |
| Ko9 | **43** | 33-57 |
| Ko10 | **175** | 134-232 |
| Ko11 | **31** | 24-40 |
| Ko12 | **20** | 15-26 |
| Ko14 | **262** | 198-349 |
| Ko16 | **111** | 84-147 |
| Ko17 | **29** | 23-28 |
| Ko18 | **92** | 71-121 |

* Values not provided, below range.

**Table 3.3.** Distribution of serum prolactin concentrations in PACAP-/- and PACAP+/+ mice 6.5 days following mating.

| | Prolactin ≤ 100 ng/ml | Prolactin ≥ 100 ng/ml |
|---|---|---|
| PACAP+/+ | 2 | 13 |
| PACAP-/- | 11 | 4 |

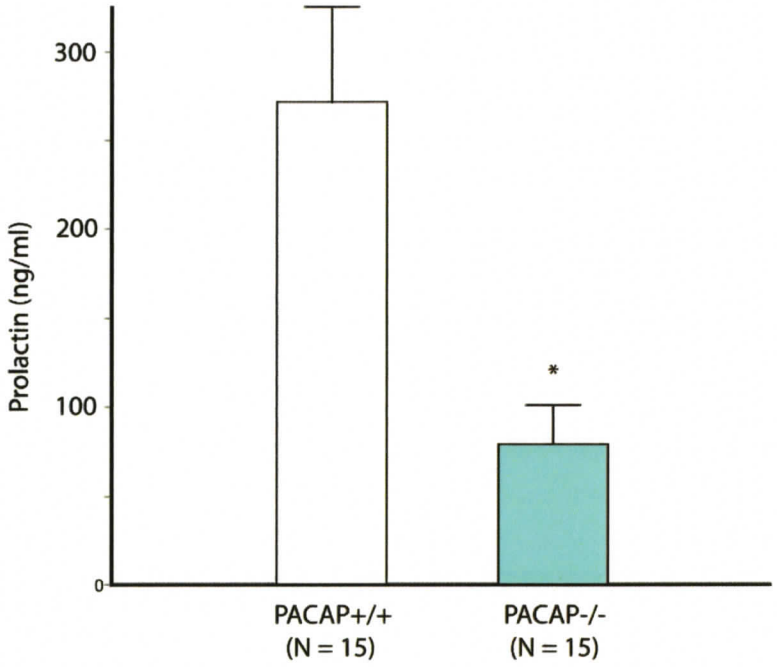



**Figure 3.6.** The effect of PACAP null mutation on prolactin hormone production following mating. The concentration of prolactin in serum recovered from PACAP+/+ (N = 15) and PACAP-/- (N = 15) females on 6.5 dpc after natural mating with PACAP+/+ fertile males is shown. Data are mean $\pm$ SEM; *, $P < 0.05$

## 3.4 Discussion

This study examined the early reproductive events in mice lacking PACAP to determine the mechanism of fertility loss. Based on previous findings, I expected an event in ovarian function or early pregnancy to be disrupted in these mice. Weight gain in naturally mated PACAP null mice did not increase following pseudopregnancy as occurs in pregnant mice. For the PACAP null mice that did not give birth following pairing with a fertile male for a week, disruption in fertility occurred between mating and mid-pregnancy. I hypothesized that ovulation or pseudopregnancy was disrupted and the females were unable to either become pregnant or be receptive to implantation of the embryo. Ovulatory function in PACAP null mice was assessed by examining the histology of the ovary in 6-8 week-old mice, as well as by determining if ovulation and fertilization occurred following mating. Findings showed normal histology of the ovary. Follicles were present in normal numbers and in all stages of development in PACAP null mice. Although PACAP is dynamically expressed in the ovary, with level of expression changing over the estrous cycle, mice lacking PACAP ovulate normally (Moretti *et. al.,* 2002). Also, oocyte maturation is stimulated by PACAP, but PACAP is not essential for follicle development to occur (Apa *et. al.,* 1997). To further characterize pre-implantation events in the PACAP null mouse, fertilization and early embryonic development were assessed. This study found that all eggs in PACAP null mice were fertilized and underwent cleavage when cultured. These findings together suggest that the mechanism of fertility loss in PACAP null mice occurs at a later stage in the establishment of pregnancy.

Based on impaired fertility but normal ovulation and fertilization, further studies into implantation and hormonal status of PACAP null mice were warranted. A disruption in the normal mating pattern indicated a potential problem with the pituitary hormones and pseudopregnancy response. Mice were mated and examined shortly after the expected time of implantation to assess implantation success. Results show a decrease in implantation in mice lacking PACAP following a single mating event with a male, as only 13% have implanted embryos at 6.5 days following mating. This decrease is large enough to account for the decreased fertility observed in PACAP null females. My previous findings showed 21% of PACAP null females gave birth following a week-long pairing. Although this number is slightly higher than the 13% with implantation, it may be explained by the longer mating time and repeated mating events in the latter study, where 33% of the PACAP null females mated more than once.

The results of this implantation study indicate an important role for PACAP in either implantation of embryos into the uterus or in preparing the mouse for early stages of pregnancy. Other studies have found PACAP localized within neurons of the uterus (Fahrenkrug and Hannibal, 1998) and increased expression of PACAP with increased decidualization (Spencer *et. al.,* 2001). To assess whether PACAP was acting indirectly on mouse receptivity to implantation, serum prolactin levels were assessed, as PACAP is synthesized in the hypothalamus and thought to influence release of prolactin from the pituitary. Prolactin levels were more than three fold higher in wild-type females when compared with PACAP-/- mice. Prolactin acts upstream of progesterone and estrogen in the establishment of a uterine environment that supports implantation and pregnancy. Figure 3.7 shows the pathway of positive prolactin regulation and prolactin's major

actions in mammals. Prolactin synthesis and release from the anterior pituitary is regulated by the hypothalamus. Prolactin is under general negative control by the hypothalamus; transplantation of the pituitary to an exogenous site in the body results in hyperprolactinaemia (Saha *et. al.,* 2002). However, the factors that control prolactin regulation are not fully understood. Dopamine is a negative regulator of prolactin synthesis, through protein kinase A (PKA); thyroid-releasing hormone is a positive regulator, through calcium levels. Also, PACAP may function as an important regulator of prolactin *in vivo*. All three PACAP receptors are expressed in the anterior pituitary, and PACAP has been reported to positively regulate prolactin gene activation *in vitro* (Coleman *et. al.,* 1996; Rawlings and Hezareh, 1996). A proposed schematic of prolactin regulation by TRH, DA and PACAP is shown in Figure 3.8. Prolactin is the only essential pituitary hormone in early pregnancy in mice (Terkel, 1988). Disruption of prolactin secretion causes immediate pregnancy loss and degradation of the corpora lutea. In contrast, exogenous prolactin administration to hypophysiectomized mice re-establishes pregnancy (MacDonald and Greep, 1968). The regulation of prolactin by PACAP in mice following mating has not previously been demonstrated. My results support an important role for PACAP in the establishment of pseudopregnancy, without which implantation cannot occur. Pseudopregnancy causes synthesis and secretion of progesterone from the ovary, which in turn causes changes in the uterus necessary to support embryonic implantation.

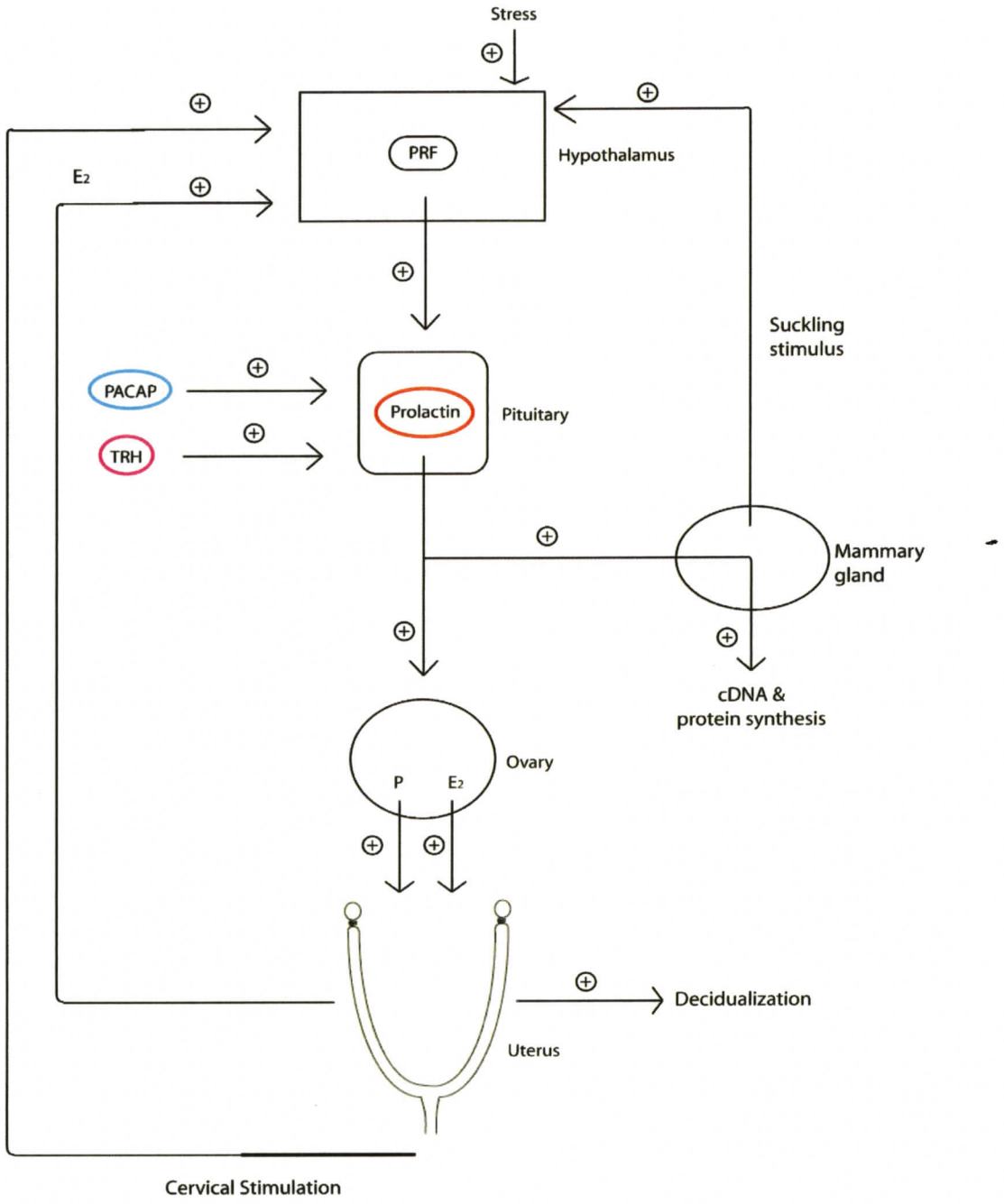



**Figure 3.7** Schematic of positive regulation of prolactin secretion and the effects of prolactin on major reproductive organs. Schema shows the hypothalamic-pituitary-gonadal axis with known positive regulatory factors, as well as the proposed regulatory hormone PACAP. PRF- prolactin releasing factor (hypothetical), PACAP- pituitary adenylate cyclase-activating polypeptide, TRH- thyroid-releasing hormone, P- progesterone, $E_2$- estrogen.

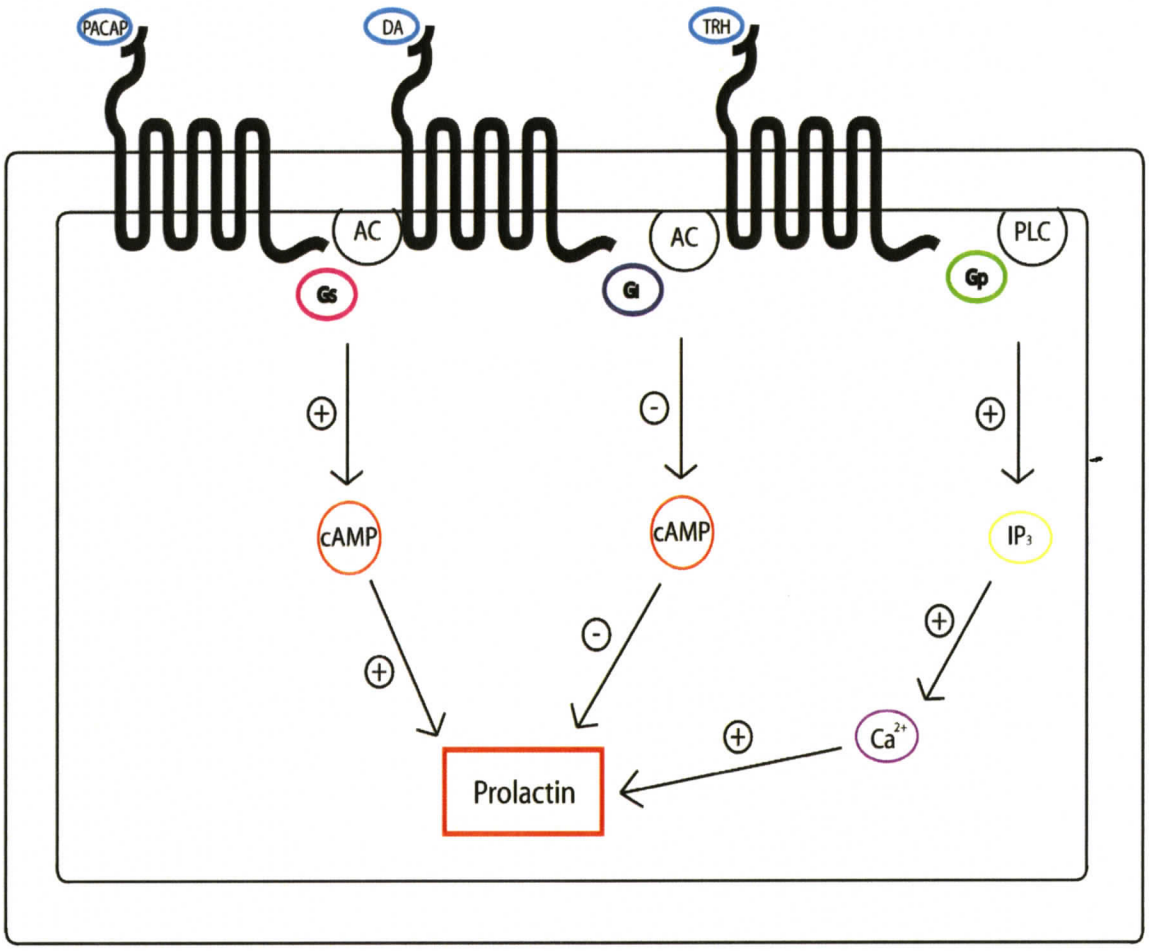



**Figure 3.8** Proposed regulation of prolactin secretion by dopamine, PACAP and TRH. Schematic shows a lactotrophic cell of the anterior pituitary expressing all three G-protein coupled receptors for the hormones DA, PACAP and TRH. Abbreviations: DA- dopamine, PACAP- pituitary adenylate cyclase-activating polypeptide, TRH- thyroid releasing hormone, AC- adenylate cyclase, Gs- stimulatory G protein, Gi- inhibitory G protein, Gp- phospholipase C-activating G protein, cAMP- cyclic adenosine monophosphate, IP3- inositol triphosphate, $Ca^{2+}$- calcium. Drawing is not to scale.

Other mouse models lacking hormones or receptors relevant to reproduction have been generated and characterized in other laboratories. Notably, mice lacking the progesterone receptors or the prolactin receptors have been characterized. Progesterone acts through two nuclear receptors, termed A and B, that are transcribed from a single gene (Mulac-Jericevic and Conneely, 2004). Mice lacking the A form of the progesterone receptor show severely impaired fertility (Table 3.4). In these mice ovulation is significantly reduced and they have decreased decidualization of the uterus but normal mammary gland function (Conneely *et. al.,* 2003). In contrast, mice lacking the B form of the progesterone receptor exhibit normal ovulation and decidualization, but impaired mammary gland branching and development (Mulac-Jericevic *et. al.,* 2000; Mulac-Jericevic *et. al.,* 2003). Also, a knockout mouse lacking both forms of the progesterone receptor has been characterized and shows a more severe phenotype than is seen in individual progesterone receptor A or B knockouts. When both forms of the receptor are absent, females are sterile with no ovulation or implantation, and severely impaired mammary gland development (Conneely *et. al.,* 2003).

Prolactin receptor null mice have complete infertility, accompanied by impaired lactation. These mice show abnormal mating behaviour related to the lack of a pseudopregnancy response; they will mate repeatedly when paired with a male. They ovulate, but the number of eggs and fertilization of eggs is reduced. Lack of implantation and maintenance of pregnancy is the major factor in the sterility seen in this mouse model, although almost all aspects of reproduction are abnormal (Ormandy *et. al.,* 1997). Interestingly, the administration of only progesterone in prolactin receptor deficient mice has been shown to rescue the implantation defect. Implantation was reported to be

rescued in prolactin receptor null mice through exogenous administration of progesterone following mating (Binart *et. al.,* 2000).

A mouse line lacking thyroid-releasing hormone (TRH), which has been found to positively regulate prolactin secretion from the anterior pituitary, has also been generated. The resulting phenotype shows no effect of TRH knockout on serum prolactin levels, during the estrous cycle or pregnancy, although levels are reduced during lactation. Also, a decrease in prolactin mRNA was detected in the anterior pituitary. This study found normal fertility and mammary gland function, with normal growth of pups, despite the decrease in prolactin secretion during lactation (Yamada *et. al.,* 2006).

The PACAP null mouse, in comparison to these other knockout mice, has reduced, but not completely ablated, fertility. Like the prolactin receptor null mice, PACAP null mice show abnormal mating behaviour. However, egg release and fertilization are normal, unlike the progesterone receptor A and prolactin receptor null mice. Also, mice lacking PACAP share the characteristic of impaired implantation, although this defect is more severe in prolactin receptor null mice. Together, these findings show that the PACAP knockout closely resembles the prolactin receptor knockout in regards to implantation and pseudopregnancy. These results suggest that PACAP may act upstream of prolactin and may be an important regulator of prolactin secretion *in vivo*. PACAP appears to be a more important regulator of prolactin than TRH, as PACAP knockout has a more severe effect on prolactin levels and reproduction than does TRH. A comparison of the reproductive phenotypes in null mice for prolactin receptor, progesterone receptors, TRH and PACAP is shown in Table 3.4.

In conclusion, the results of this investigation have demonstrated that the decreased fertility in mice lacking PACAP is the result of impaired prolactin secretion preventing the establishment of pseudopregnancy, which results in disrupted progesterone signalling to the uterus and lack of decidualization.

**Table 3.4** Summary of the reproductive phenotypes of mice lacking progesterone receptors (PR) isoform A (PRA) or isoform B (PRB), prolactin receptor (Prl R), thyroid-releasing hormone (TRH) or PACAP hormone.

| Characteristic | PR-ko | PRA-ko | PRB-ko | Prl R-ko | TRH ko | PACAP-ko |
|---|---|---|---|---|---|---|
| Ovulation | None | Reduced | Normal | Reduced | Normal | Normal |
| Implantation | None | Impaired | Normal | None | Normal | Impaired |
| Fertility | None | Severely impaired | Normal | None | Normal | Impaired |
| Mammary (Development or lactation) | Impaired | Normal | Impaired | Impaired | Normal | Impaired |

# 4 Site of action of PACAP in events required for mammary gland function.

## 4.1 Introduction

Lactation is considered the final phase of the reproductive cycle, and is essential for the survival of neonates. Lactation is dependent upon a dynamic interplay of endocrine and local factors at the mammary gland. Hormonal changes during pregnancy contribute to the growth and differentiation of mammary tissue. The drop in progesterone and estrogen at parturition permits milk development and secretion. Suckling triggers prolactin release in the anterior pituitary, which results in the milk protein synthesis and secretion. PACAP is reported to inhibit prolactin and LH secretion in ewes (Anderson *et. al.,* 1996), but to stimulate prolactin release from the pituitary in rats (Murakami *et. al.,* 2001). Also, immunohistochemistry and RIA have shown PACAP to be highly expressed within fibres of the rat nipple, and increased expression of PACAP-38 has been observed in mammary glands of lactating rat (Skakkebaek *et. al.,* 1999). PACAP may be involved in relaying the suckling signal to the hypothalamus, which stimulates the pituitary to release prolactin.

Caseins and whey proteins are commonly used as molecular markers for mammary gland differentiation. The genes encoding these families of proteins are regulated by peptide and steroid hormones, including the lactogenic hormone prolactin. Recently, real-time PCR (qPCR) has been used for quantifying mRNA transcription levels and may be used for quantifying milk protein mRNAs. The use of this method has

become widespread due to its reproducibility, accuracy and sensitivity (Rosen *et. al.*, 1999).

The role of PACAP in mammary gland function has not been examined and it may prove to be an important factor in lactation, either through modulation of luteotrophic hormones released from the anterior pituitary, or through local innervation of the nipple. To assess the effect of PACAP on lactation, the ability of PACAP null mice to adequately nourish their pups was evaluated. This was done by analysis of litter weight gain following parturition and through an acute test of milk yield at peak lactation.

Hence, the aim of this study was twofold. Firstly, to examine mammary gland function in PACAP null mice and secondly, to compare mRNA transcription levels using qPCR of milk proteins in PACAP knockout and wild-type mice during lactation.

## 4.2 Materials & Methods

### *4.2.1 Animals*

The procedures used in this study were approved by the University of Victoria Animal Care Committee. Housing and care of animals is described earlier (Section *2.2.1*).

Primiparous PACAP null female mice were paired at 8 weeks of age with a tested wild-type male. Pairings were made after 15:00 h and were maintained for 7 days. Females were checked daily for seminal plugs while paired. For all pregnancy experiments, day 0 of pregnancy was considered the day on which the seminal plug was

found. Females were then weighed weekly, and checked daily for signs of pregnancy. Litters were normalized to six pups and litters were checked and weighed daily.

### *4.2.2 Tissue collection*

Mammary tissue was collected at day 14 of lactation. The mammary glands were snap-frozen in liquid nitrogen and stored at -80 °C. Ear clips were taken for genotyping purposes.

### *4.2.3 Milk yield determination*

Milk yield, as a measure of mammary gland function, was estimated by the weigh-suckle-weigh method (Jara-Almonte and White, 1972). Litters were separated from their dams and weighed at ten days of age, time of peak lactation in dams. They were returned four hours later and allowed to suckle for 30 minutes. Litters were then reweighed to determine quantity of milk consumed by the litters. Dams that did not retain six pups to day 10 of lactation were not included in the study.

### *4.2.4 Litter weights*

Litter weights were measured as an assessment of maternal lactation function. Litters were tracked for 14 days following birth, at which time pups begin to eat solid food in addition to suckling milk. Growth rates of litters were taken as an indirect measure of dam lactational output.

### *4.2.5 RNA isolation and cDNA generation*

Total RNA was isolated from frozen mouse mammary tissue with TRIzol reagent (Invitrogen, Burlington, ON). Approximately 50mg of mammary tissue was homogenized in 1ml of TRIzol reagent. Samples were incubated to allow for phase separation, and RNA was precipitated with isopropyl alcohol.

mRNA was isolated from frozen mammary tissue and from fresh brain tissue with MicroPoly(A)PuristTM kit (Ambion, Austin, TX). Approximately 50mg of tissue was homogenized in 600 µl of a guanidium-based lysis solution, according to manufacturer's instructions. mRNA was selectively removed with Oligo(dT) cellulose, then eluted and stored in water.

RNA concentration was quantified by spectrophotometer readings (A260/280), and by comparison with an RNA standard. cDNA was generated from isolated RNA and mRNA with SuperScriptTM II Reverse Transcriptase (Invitrogen). An aliquot containing 500ng of mRNA, or 5µg total RNA was added to 1ng of Oligo(dT) primers and 10mM dNTPs. The mixture was heated to 65 °C for 5 minutes, then 0.1M DTT and 5X buffer were added; the solution was incubated at 42 °C for 50 minutes.

### *4.2.6 β casein and whey acidic sequencing*

Primers were designed against β casein and whey acidic mRNA sequences from the BLAST database (http://www.ncbi.nlm.nih.gov/entrez/) using Primer Premiere 5 (See Table 4.1). Melting temperature ($T_M$) was calculated by the primer design software (Primer Premier 5, Palo Alta, CA)

**Table 4.1**. Oligonucleotide primers for sequencing of the *Mus musculus* whey acidic protein and β casein cDNA, designed in Primer Premier 5

| Gene | Primer Name | TM | Sequence |
|---|---|---|---|
| *Whey Acidic* | F1wap | 56.7 | 5'-CAAGTCTTCAACTCAGTTCAGTCCA-3' |
| *Protein* | R1wap | 59.4 | 5'-CGGAACACCAATGTTGACAGGAGTT-3' |
| | F2wap | 58.1 | 5'-AGACAGTGGTCTGAAGATCCCAG-3' |
| | R2wap | 59.6 | 5'-TCACTCCCGACAGGCAGG-3' |
| *β Casein* | F1bcas | 57.3 | 5'-AAGGACTGGACAGCCATGAAGG-3' |
| | R1bcas | 53.0 | 5'-TGAAATGACTGGAAAGGAAATAGG-3' |
| | F2bcas | 55.6 | 5'-CATCCTTTCAGCTTCACCTCC-3' |
| | R2bcas | 53.5 | 5'-TTGACTTGCATTTTCAACTCCAT-3' |

Polymerase chain reaction (PCR) was performed in a 50 µl reaction. Each of the primer sets (20 pmol of each primer) was added to 2 µL of the mouse mammary cDNA, 2 U Taq polymerase, 1 X Taq buffer, 1.5 mM $MgCl_2$, and 200 mM dNTPs. The reaction condition was as follows: 94 °C for 2 minutes followed by 34 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 68 °C for 2.5 minutes. These cycles were followed by a 5 minute extension at 68 °C. Products were resolved on a 1.3% agarose gel under UV transillumination.

The amplified PCR product was purified with QIAquick PCR Purification Kit (Qiagen, Mississauga, ON). Buffer PB was added to 40 µl of PCR product and the DNA was bound to a QIA quick spin column. Sample was washed with buffer PE and DNA was eluted from the spin column with 50 µl distilled water.

The purified PCR product was then ligated into a pGEM-T vector (Promega, Madison, WI) in a 10µl reaction. A 10X ligation buffer, 3U DNA ligase, 50ng pGEM-T vector, and 2 µl PCR product were incubated overnight at 4 °C. An aliquot of 2 µl of the ligation product was then combined with XL-1 blue competent cells and electroporated. Cells were incubated for 1 hour at 37 °C, plated onto LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C. White colonies were picked and incubated at 37 °C overnight in Ampicillin/LB media.

Plasmid DNA was purified from the resulting colonies using QIAprep Miniprep (Qiagen, Mississauga, ON). Bacteria were lysed under alkaline conditions. Cellular components were cleared by centrifugation. DNA was bound to a QIAprep column, while RNA, cellular components and metabolites were removed by centrifugation. DNA bound to the column was washed with buffer PB to remove endonucleases, and then

washed with buffer PE, which removes salts. DNA was eluted from the QIA prep column with distilled water. A restriction digest was performed on the purified plasmid DNA using restriction enzymes Pst1 and Sph1. The digest was incubated for five hours at 37 °C, then 65 °C for 20 minutes. The direct ligation results were visualized on a 1.5% agarose gel under UV transillumination and DNA concentration was determined by spectrophotometer (OD260/280) readings. Samples were submitted to Roderick Haesevoets in the University of Victoria DNA sequencing laboratory.

### *4.2.7 Validation of qPCR primers*

qPCR primers were designed against PACAP, whey acidic protein, β casein protein and RNA polymerase II mRNA sequences obtained from the NCBI BLAST database using Primer Premiere 5 (Table 4.2). Most primers sit on exon-exon junctions to rule out genomic contamination. The PACAP primers were not designed to sit on junctions, as the gene and mRNA sequences did not provide ideal primer attachment sites at these junctions.

PCR with wild-type mammary cDNA in a 17 µl reaction was performed to validate qPCR primers. An aliquot of 2 µl of sample was added to 1 U Platinum *Taq* polymerase, 1 X Taq buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs and 20 pmol of each primer. The reaction condition was as follows: 94 °C for 5 minutes followed by 39 cycles of 94 °C for 15 seconds, 55 °C for 15 seconds and 72 °C for 30 seconds. These cycles were followed by a 10 minute extension at 72 °C.

**Table 4.2.** Oligonucleotide primers used for quantitative RT-PCR of whey acidic protein, β casein, PACAP and RNA Polymerase II. Primers were designed in Primer Premier 5 at exon-exon junctions, when possible.

| Gene | Primer Name | TM | Sequence |
|---|---|---|---|
| *Whey acidic protein* | WAP1f241 | 56.7 | 5'-CAAGTCTTCAACTCAGTTCAGTCCA-3' |
| | WAP1r398 | 58.3 | 5'-ACACCAATGTTGACAGGAGTTTTGC-3' |
| | WAP2f369 | 59.9 | 5'-GACCCGCAAAACTCCTGTCAACATT-3' |
| | WAP2r568 | 63.2 | 5'-GAAGGGTTATCACTGGCACTGGGGG-3' |
| | WAP3f540 | 63.2 | 5'-TACACCCCCAGTGCCAGTGATAACC-3' |
| | WAP3r682 | 55.7 | 5'-AGCAGCAGATTGAAAGCATTATGTT-3' |
| *β Casein* | BCAS1f65 | 56.7 | 5'-TTCATCCTCGCCTGCCTT-3' |
| | BCAS1r166 | 55.0 | 5'-ATGTTCAACAGATTCCTCACTGG-3' |
| | BCAS2f27 | 57.0 | 5'-CTTGTCCTCCACTAAAGGACTTGA-3' |
| | BCAS2r168 | 54.8 | 5'-ATATGTTCAACAGATTCCTCACTGG-3' |
| | BCAS3f142 | 56.4 | 5'-TTCCAGTGAGGAATCTGTTGAACAT-3' |
| | BCAS3r243 | 60.1 | 5'-TGAACTTTAGCCTGGAGCACATCCT-3' |
| *Pacap* | PACAP1f198 | 61.9 | 5'-CACCAATGACCATGTGTAGCGGAGC-3' |
| | PACAP1r329 | 60.0 | 5'-CGTAAGCCTCGTCTTCTGGTCTGAT-3' |
| | PACAP2f198 | 61.9 | 5'-CACCAATGACCATGTGTAGCGGAGC-3' |
| | PACAP2r347 | 63.5 | 5'-GCGGGTTTCCGTCCTGGTCGTA-3' |
| | PACAP3f306 | 60.9 | 5'-TCAGACCAGAAGACGAGGCTTACGA-3' |
| | PACAP3r459 | 60.9 | 5'-ATTTCGTGGGCGACATCTCTCCTG-3' |
| *Rna polymerase II* | RNA PolII 1f441 | 59.4 | 5'-TAATGACTATGTGGAGCAGGA-3' |
| | RNA PolII 1r651 | 59.6 | 5'-GGAATACTCAGTGTTGGGCCA-3' |

Samples were run on a Mastercycler Gradient (Eppendorf, ON). Products were resolved on a 1.5% agarose gel under UV transillumination. This PCR was repeated using wild-type mouse brain cDNA.

### *4.2.8 qPCR analysis*

Analysis of β casein using primer set 2 and RNA Polymerase II, the housekeeping gene, was performed using the Mx3000P qPCR machine (Stratagene).
Each reaction had a 17 µl volume containing 1X PCR buffer, 3mM $MgCl_2$, 1.5 U Jumpstart Taq DNA polymerase, 0.25 mM dNTPs, 1X Syto, 1X Rox and 25 pmol of each primer. Samples were run in conjunction with reference and β casein standard dilutions. Samples were denatured at 95 °C for 5 minutes, then 40 cycles were performed of: denaturing at 95 °C for 30 seconds, annealing at 55 °C for one minute and extension at 72 °C for 30 seconds. Each sample was run in triplicate, whereas standard dilutions were run in duplicate. Rox, short for a glycine conjugate of 5-carboxy-X-rhodamine, was used as a reference dye and RNA Polymerase II was used as the reference gene. The minimum acceptable amplification efficiency based on the standard curves was set at 95%, in order to obtain accurate results. Absolute quantification of mRNA levels was determined with the equation of the line from the standard curve.

### *4.2.9 Computational analysis*

Sequences were compared with proteins in the NCBI nucleotide database using the method of Altschul *et al.* (1997).

### *4.2.10 Data analysis*

Values are expressed as means ± SEM. Litter weight gain in both experiments was compared by unpaired t test analysis using GraphPad InStat software (GraphPad Software Inc.).

## 4.3 Results

### *4.3.1 Litter weight gain*

To assess whether PACAP null dams influenced the growth of neonatal pups, heterozygote litters were normalized to six pups and weighed daily. Litters gained an equivalent amount of weight at the start of the growth trial, but by day 3, the litters suckled by PACAP null dams weighed significantly less (12 ± 0.37 g, $P < 0.05$) than the litters of wild-type dams (13.19 ± 0.37 g, Figure 4.1). Neonates raised by PACAP null mice continued to weigh less than those raised by wild-type females until day 10 of lactation (30.3 ± 0.62 and 31.6 ± 1.9 g, respectively, $P > 0.05$), after which time weights remained equivalent for litters of PACAP null and wild-type dams. Growth rates of neonates nursed by PACAP-/- dams were normal at peak lactation, which occurs 10 days after birth. As all pups included in this study were heterozygous, the diminished growth rate observed in the early period of lactation was not caused by genotype.

### *4.3.2 Weigh-suckle-weigh*

To determine mammary gland function in PACAP null females, an acute test of milk output was performed. Milk output at peak lactation is a standard test for assessing

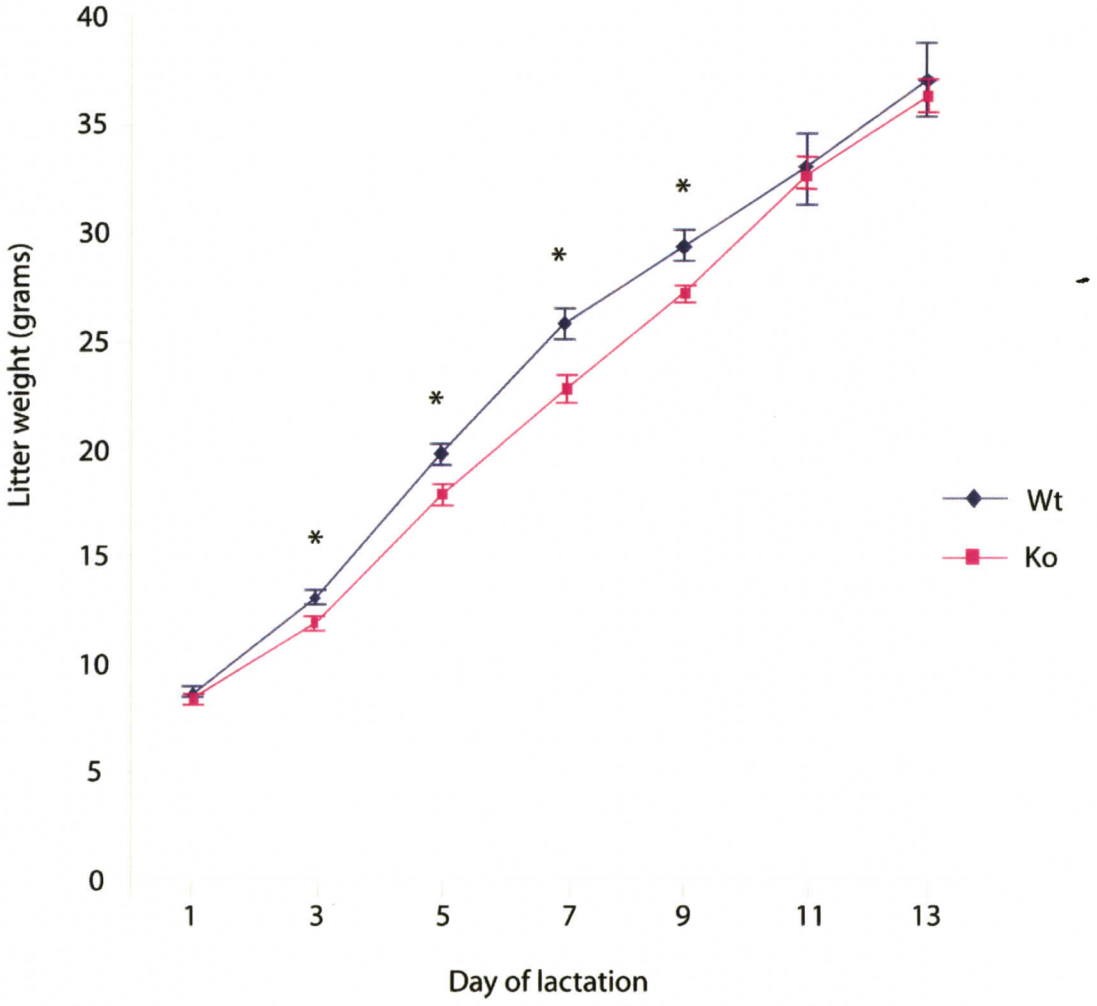



**Figure 4.1.** Milk yield of PACAP null and PACAP wild-type females. Milk yield was evaluated as litter weight gain, with litters heterozygous for the PACAP null mutation. All litters contained six pups. Pink line represents litters nursed by PACAP null dams and blue line denotes litters nursed by wild-type dams. All values are mean $\pm$ SEM. * denotes significant differences ($P < 0.05$).

mammary gland function (Jara-Almonte *et. al.,* 1972). PACAP null litters gained 880 mg ± 0.05 and wild-type litters gained 860 mg ± 0.05 (mean ± SEM, $P > 0.05$). No significant difference in mean pup weight gain was detected. Both litters contained 6 pups of heterozygous genotype and the stomachs of all pups contained milk.

### *4.3.3 β Casein and whey acidic protein sequencing*

Sequences obtained through cDNA amplification were compared to those present on the NCBI database, in order to confirm sequence conservation between the genome of mice examined with those present in the genome database. The sequences obtained for both clones created from cDNA amplified by the first whey acidic protein primer set matched mRNA for *Mus musculus* whey acidic protein in the NCBI BLAST database. Results for the amplicon from the second whey acidic protein primer set corresponded to genomic contamination and matched whey acidic protein introns. Both β casein amplicons BLASTed with perfect alignment to *Mus musculus* casein beta mRNA (accession numbers AL603787.8 and NM_009972.1).

### *4.3.4 qPCR primer selection*

All primer sets for PACAP failed to cleanly amplify product during initial primer validation. Whey acidic cDNA amplification worked with regular PCR amplification with all primer sets designed, and was cloned into vectors, but qPCR amplification failed. RNA Polymerase II primers and β casein primer set 2 both met the stringent requirements for qPCR and were analyzed in wild-type and PACAP null mammary tissue.

### *4.3.5 qPCR efficiency for standard curves*

Standard curves were generated with targets at known concentrations. A slope between -3.3 and -3.8 indicates high efficiency, where -3.32 is 100% efficienct. The efficiency of qPCR for both target genes fell within this range (Table 4.3), with similar slopes for both the target and reference genes, showing almost equivalent amplification efficiencies.

### *4.3.6 β casein levels in mammary tissue*

$C_T$, the cycle at which the amplification plot crosses the fluorescence threshold and all samples are in exponential growth, is grouped for PACAP null and wild-type samples (N=4 animals, with 3 replicates). The $\Delta C_T$, the difference between the $C_T$ value of the target and reference gene, accounts for variations in template between the samples and $\Delta\Delta C_T$ shows the difference between the $\Delta C_T$ of the PACAP null and wild-type β casein gene expression. The relative expression, the ratio of wild-type and PACAP null expression is 1.0867 (Table 4.4), virtually unchanged.

Absolute quantification, showing the number of copies present in each sample, is derived from interpolation using the slope of the standard curve and is given in Table 4.5. There is no significant difference in β casein gene expression levels between wild-type and PACAP null mammary tissue (P=0.6743, unpaired T test)

## 4.4 Discussion

This study investigates the potential influence of PACAP on mammary gland function and gene expression of milk proteins during lactation. Previous studies have

**Table 4.3.** Efficiency of β casein and RNA Polymerase II quantitative RT-PCR experiments. The $R^2$ value indicates the robustness of the curve while the efficiency is determined by the following calculation: Efficiency = $10^{(-1/m)}$, where m is the slope of the line.

| Standards | $R^2$ | Slope | Efficiency |
|---|---|---|---|
| RNA Polymerase II | 0.995 | -3.2272 | 2.0411 |
| β Casein | 0.9925 | -3.3666 | 1.9817 |

**Table 4.4.** Relative expression of β casein in mouse mammary tissue at day 14 of lactation.

| Group | Ave. $C_T$ | $\Delta C_T$ | $\Delta\Delta C_T$ | Relative Expression* |
|---|---|---|---|---|
| Wild-type | 13.685 | -11.63 | -0.12 | 1.0867 |
| PACAP null | 14.693 | -11.75 | | |

*Relative Expression = $2^{-\Delta\Delta C}T$

**Table 4.5.** Absolute expression ratios in wild-type and PACAP null mouse mammary tissue at day 14 of lactation.

| Group | Absolute Values (copies/µl) |
|---|---|
| Wild-type | $1.53 \times 10^7$ |
| PACAP Null | $7.70 \times 10^6$ |

found PACAP and its receptors in the mammary gland, as well as a regulatory role for PACAP on prolactin secretion from the anterior pituitary (Anderson *et. al.,* 1996; Skakkebaek *et. al.,*1999). The functional role of PACAP in the lactating mammary gland has not yet been studied, and so lactational function, milk protein expression and PACAP hormone and receptor expression during lactation would provide important information into some possible functions of PACAP in the mouse mammary gland and hypothalamus.

This study demonstrated a role for PACAP in mammary gland function. Growth of heterozygous neonates raised by PACAP null dams was decreased in the week following birth, despite equivalent weights of litters at the time of birth. This growth impairment was limited to seven days at the beginning of nursing, suggesting PACAP's role was confined to early lactation. At peak lactation, no difference was detected in milk consumption by neonates. Mammary gland function was equivalent for both PACAP null and wild-type dams. PACAP may act in the mammary gland to signal differentiation and ductal development; a delay in development could cause the impaired mammary function observed in the early part of lactation in PACAP null mice. Alternately, PACAP may act through innervation of the nipple to relay the suckling signal to the brain. Impairment in this signaling pathway could decrease lactation, although it would not explain the improvement in weight gain observed in litters during the latter part of lactation.

Whey acidic protein and β casein mRNA sequences were determined through RT-PCR, and were found to closely match the mRNA sequences available in the NCBI BLAST database. Few base pair differences were identified, and primers were designed from these sequences using mRNA and genomic sequences in BioEdit. These primers

were complementary to exon-exon junctions, allowing for specific amplification of mRNA regardless of possible genomic DNA contamination present in the sample. This provided a more limited range of possible primer pairs. Future real-time PCR primer generation should consider a larger range of perhaps more suitable primer pairs.

Careful selection of a reference gene is essential to a successful qPCR. The reference gene allows for normalization of templates to a housekeeping gene with consistent expression. These differences may arise from different amounts of starting tissue, the heterogenous nature of the tissue or differences in RNA preparation or cDNA synthesis. The reference gene should not be regulated by the experimental procedure and must be expressed at detectable levels in the sample. Radonic *et al.* found RNA Polymerase II to be the most suitable reference gene of thirteen candidates tested. RNA Polymerase II is a transcription-regulated gene and was found to be resistant to cellular activation and expressed with low variation in all tissues tested (Radonic *et. al.,* 2004). RNA polymerase gene expression was found at high levels in all mammary samples tested, as would be expected for transcriptionally active tissue.

Absolute quantification of β casein gene expression showed high levels of expression in all mammary tissue examined. This was expected as the lactating mouse mammary gland is highly transcriptionally active as it produces copious amounts of milk protein for the nourishment of pups. Some of the gene expression levels were higher than $10^8$ copies per µl of cDNA, which was above the range of the standards. Ideally, the amount of the target should fall within the range of the standards, but extrapolation from the standard curve is acceptable.

qPCR is a method for profiling mRNA transcription. It allows for gene expression analysis of starting amounts of template. This method utilizes fluorescent dyes, measuring fluorescence at each cycle of the PCR. The fluorescent dye Syto emits a fluorescent signal at 543 nanometres (nm) when bound to double-stranded DNA. With the increase in double-stranded PCR product there is an increase in fluorescence emitted by the fluorescent dye. The advantages of qPCR over other methods for measuring gene expression include a broad dynamic range, high accuracy and it is fast and reproducible (Bustin, 2002).

No significant difference was observed in β casein expression between PACAP null and wild-type females on day 14 of lactation. PACAP may affect other aspects of mammary gland function during lactation, such as relaying the suckling response from the nipple, or PACAP may have no role in lactation. Further studies may look at other milk proteins or at other stages of lactation, but as my previous research showed only a small difference in weight gain of litters born to PACAP null females, further studies of altered milk protein expression may show that PACAP has no effect or other factors compensate for milk protein expression within the mammary gland of lactating mice.

The reproductive phenotype in mice lacking PACAP differs from those of mice lacking other reproductive hormones. Mammary gland function is only impaired during the first half of lactation in PACAP null mice, and even then the defect is not as severe as that seen in prolactin receptor null mice and progesterone receptor null mice. However, other aspects of reproduction are significantly altered in mice lacking PACAP, notably pseudopregnancy and implantation. The unique subset of defects and the degree of impairment seen in PACAP null mice indicates an important role for PACAP in female

reproduction. PACAP may act as a prolactin releasing factor at sites in the anterior pituitary. This is a novel function of PACAP described in this thesis for the first time. Further characterization of the mechanism of action of PACAP in prolactin release is warranted, and may lead to an expansion in current understanding of prolactin control in females.

# 5 Future direction of research and a possible role for PACAP in human female reproduction.

## 5.1 General conclusions

The reproductive phenotype in mice lacking PACAP differs from those of mice lacking other reproductive hormones. Mammary gland function is only impaired during the first half of lactation in PACAP null mice, and even then the defect is not as severe as that seen in prolactin receptor null mice and progesterone receptor null mice (Lydon, et *al.*, 1995; Ormandy, *et al.*, 1997). However, other aspects of reproduction are significantly altered in mice lacking PACAP, notably pseudopregnancy and implantation. The unique subset of defects and the degree of impairment seen in PACAP null mice indicates an important role for PACAP in female reproduction. PACAP may act as a prolactin-releasing factor at sites in the anterior pituitary in response to mating. This is a novel function of PACAP described in this thesis for the first time. Further characterization of the mechanism of action of PACAP in prolactin release is warranted, and may lead to an expansion in current understanding of prolactin control during early pregnancy in rodents.

## 5.2 Future directions

Concurrent low serum progesterone levels with reduced prolactin levels would support that there is an impairment of pseudopregnancy in female mice lacking PACAP. Additional serum is available from peri-implantation females in which serum prolactin was assayed. Analysis of progesterone levels in these serum samples may provide further

evidence for impaired corpus luteum maintenance in PACAP null female mice. To further support the findings of impaired pseudopregnancy in these mice, estrous smears following mating would be of interest. Pseudopregnancy is accompanied with a constant diestrus state, which is easily detected by vaginal cytology. Diestrus smears lasting for ten days would show that pseudopregnancy had occurred, and a resumption of cycling before that would show that pseudopregnancy was either lacking or impaired in these mice.

The implantation failure that occurs in mice lacking a functioning prolactin receptor was attributed in large part to insufficient progesterone levels that are required for the support of implantation (Bole-Feysot, *et al.*, 1998). This implantation defect was rescued by Binart *et al.* 2000 in prolactin receptor-deficient mice by administration of progesterone in early pregnancy. Progesterone slow-release capsules were implanted subcutaneously in early pregnancy and embryo development and implantation resulted. If this same factor of reduced prolactin levels causing impaired progesterone production is responsible for the lack of implantation observed in female mice lacking PACAP, then a similar rescue experiment would result in implantation. Although other rescue experiments involving administration of PACAP or prolactin may be more direct, they involve methodological difficulties.

In an experiment attempting to rescue the implantation defect observed in female mice lacking PACAP with administration of exogenous PACAP, an unknown dose, administration site and pattern of administration of PACAP would be required. PACAP secretion may change with cervical stimulation, and may be dose-dependent. Hormones can have different physiological effects on hormone secretion from the

anterior pituitary with different lengths of administration. One example is estrogen, which reduces gonadotropin levels when first administered, then causes increased secretion 24 hours later (Shupnik, 1996). The way in which PACAP may be acting to control prolactin at the anterior pituitary is yet unknown, so predicting dosage amounts and patterns would be difficult. Another drawback to rescuing implantation through PACAP administration results from the route of administration. Previous experiments completed in our lab have shown that subcutaneous slow-release of PACAP-38 from micro-osmotic pumps may fail as the result of degradation of PACAP (unpublished data). From these difficulties associated with rescue by PACAP administration, I have concluded that rescue by progesterone administration is a preferred method.

Rescue of implantation in mice lacking PACAP with prolactin administration would be a more direct method than rescue with progesterone supplementation. However, prolactin secretion occurs in biphasic surges through pseudopregnancy and would be difficult to replicate. If rescue by progesterone administration is successful, a subsequent rescue experiment with prolactin may be of interest.

### 5.3 Comparison of PACAP in mouse and human reproduction

Control of female reproduction in mammals is often specialized with some mammals exhibiting higher fecundity than others. Mice have high fecundity, and produce a relatively large number of offspring in a small time period. They accomplish this by having both a short estrous cycle and an incomplete luteal phase. The estrous cycle of mice is only 4-5 days in duration; they are fertile approximately 25% of their sexually mature life, after they have progressed through puberty and are not pregnant.

An incomplete luteal phase allows for rapid cycling, as luteinization of the ovary occurs only reflexively in response to mating (Barkley, *et al.*, 1978). Mice require cervical stimulation, which occurs during mating, to trigger prolactin release from the anterior pituitary. Prolactin is the major luteotrophic hormone in rodents and without it the corpora lutea regress and pregnancy fails (Ormandy, *et al.*, 1997).

In contrast to mice, humans have a lower fecundity and produce fewer offspring in a longer time frame. Humans exhibit a longer menstrual cycle and a complete luteal phase. The menstrual cycle of women is approximately 28 days in length, and it contains a spontaneous luteal phase. The corpus luteum does not degrade shortly after ovulation, as it does in female mice that have failed to mate. Instead, it is maintained for a sufficient length of time to allow for implantation if fertilization has occurred. This difference decreases fecundity in women and increases cycle length. As females have a spontaneous luteal phase, establishment of pregnancy in humans is independent of mating stimulus and prolactin (Malassine, *et al.*, 2003).

Humans and mice differ in endocrine regulation of establishment of pregnancy. Human establishment of pregnancy does not require the pituitary gland or prolactin secretion, whereas the mouse is dependent on pituitary release of prolactin for the establishment of pregnancy. Both humans and mice require progesterone throughout gestation, but mice require the corpus luteum to produce progesterone throughout pregnancy, whereas humans switch progesterone production from the corpus luteum to the placenta in the first trimester (Malassine, *et al.*, 2003). Resulting from these differences, the putative role of PACAP in early pregnancy would not be equivalent for both humans and mice. However, the prolactin-releasing factor has not yet been

identified in humans, and PACAP may regulate prolactin secretion in humans, albeit in response to different stimuli. PACAP may play a role in prolactin synthesis and release in humans, as prolactin is important in lactation in humans. Also, prolactin is expressed dynamically over the menstrual cycle, so that PACAP may promote release of prolactin during the cycle or lactation.

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