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Targeted Disruption of the Gene for Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) in Mouse Results in Metabolic Dysfunction.

Sarah Louise Gray
B.Sc, University of Victoria, 1997

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Biology

We accept this dissertation as conforming to the required standard

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ABSTRACT

A recently discovered peptide hormone, pituitary adenylate cyclase-activating polypeptide (PACAP) regulates several endocrine systems affecting essential physiological processes such as metabolism, growth, reproduction, and the stress response. PACAP acts as a hypophysiotropic factor, is a potent secretogogue of insulin, regulates production and release of catecholamines from the adrenal medulla and acts as a neuromodulator in the sympathetic and parasympathetic nervous system. The primary structure of PACAP has been highly conserved during the evolution of chordates suggesting it plays an important physiological role. The objective of my thesis was to identify PACAP's primary physiological function and to determine if it is essential for survival by generating a mouse line deficient in PACAP through targeted disruption of the PACAP gene locus.

Postnatal PACAP expression was examined to determine sites of peripheral PACAP production. In addition, several splice variants of the PACAP gene with alternate 5'untranslated regions were identified suggesting a complex system for regulating expression of the mouse PACAP gene.

A targeting vector that allows tissue specific or developmental stage specific knockout of the PACAP gene was constructed in the event that PACAP gene deletion resulted in embryonic lethality. PACAP null mice were generated from homologously recombined embryonic stem cells. Initial characterization of the PACAP null mice determined that in the absence of PACAP, mice died within the first two postnatal weeks with abnormal lipid metabolism. Lipid accumulation was present in liver, heart and

skeletal muscle and serum lipids were high. Mitochondrial dysfunction in the liver was not the cause of the lipid accumulation, as β-oxidative function was normal. I conclude that PACAP null mice are unable to regulate lipid release from white adipose tissue stores, resulting in a flood of lipids to non-adipose tissues.

The abnormal distribution of lipids observed in the PACAP null mice is characteristic of diabetes type 2, yet classical insulin resistance is not observed. Thus, elevated insulin levels were accompanied by low blood glucose levels and the response to a glucose challenge was normal. The uncontrolled release of free fatty acids may result if glucose that is taken up by cells can not be utilized and an alternate energy source is required or if white adipocytes only are insulin resistant.

The PACAP null mice were temperature sensitive, in that when raised at 21°C they exhibited metabolic dysfunction and died by two weeks of age. At 24°C most (85%) of the mice survived to adulthood with no obvious signs of metabolic dysfunction. We have determined that the inability of the PACAP null pups to thermoregulate normally when exposed to a lower environmental temperature may be associated with decreased norepinephrine levels to the brown adipose tissue. PACAP may be important for the production and release of catecholamines in the adrenal gland or within the sympathetic nervous system in times of prolonged stress.

A mechanistic connection between the lipid abnormalities and the temperature sensitivity in the PACAP null pups has yet to be made. Catecholamines affect a wide range of tissues and the problems associated with insulin regulation within the PACAP null mice may be due to the imbalance in catecholamine production. As one of two main stress response systems, the sympathetic nervous system elicits a vital coping mechanism

in times of stress and PACAP's ability to regulate this system may explain why the primary structure of PACAP has remained so highly conserved. PACAP is a wide acting hormone and therefore the metabolic problems seen in the PACAP null mice may result from altered regulation of several endocrine systems at once. Targeted disruption of the PACAP gene in mouse has revealed a role for PACAP in the regulation of lipid metabolism and in the sympathetic control of thermoregulation.

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List of Abbreviations

AC: adenylate cyclase

Ach: acetylcholine

ACTH: adrenocorticotropic hormone

BAT: brown adipose tissue

bp: base pairs

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CNS: central nervous system

CRE: cAMP response element

CRF: corticotropin-releasing factor

DBH: dopamine ß-hydroxylase

ELISA: enzyme linked immunosorbent assay

EPI: epinephrine

ES cell: embryonic stem cell

GH: growth hormone

GHRH: growth hormone-releasing hormone

GIP: glucose-dependent insulinotropic polypeptide

GLUT: glucose transporter

HDL: high density lipoprotein

HPLC: high performance liquid chromatography

HS lipase: hormone sensitive lipase

HSV-TK: herpes simplex virus thymidine kinase gene

IL-6: interleukin 6

IP_3: inositol-1,4,5-trisphosphate

kb: kilobase pairs

MOPS: morpholinopropanesulfonic acid

mRNA: messenger ribonucleic acid

NE: norepinephrine

neo: neomycin resistance gene

PAC_1: PACAP specific receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

pEGFP: enhanced green fluorescent protein

PHM: peptide histidine methionine

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PNMT: phenylethanolamine N-methyltransferase

PRP: PACAP-related peptide

RIA: radioimmunoassay

RT-PCR: reverse transcription-polymerase chain reaction

SDS: sodium doecyl sulphate

SNS: sympathetic nervous system

SSC: salt sodium citrate

TH: tyrosine hydroxylase

TSH: thyroid stimulating hormone

UCP: uncoupling protein

UTR: untranslated region

VIP: vasoactive intestinal polypeptide

VLDL: very low density lipoprotein

$VPAC_1$: VIP and PACAP shared receptor 1

$VPAC_2$: VIP and PACAP shared receptor 2

ACKNOWLEDGEMENTS

A huge amount of support, teaching and advice has been given to me throughout the compilation of this thesis and it is here that thanks will be given to those who have helped me complete this goal.

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A Ph.D. does not go unnoticed by those who are closest to you. I am truly blessed with a wonderful family and dear friends who believe in my abilities and support my endeavors completely. For this I thank you all. Finally to Wayne, for your unconditional support and understanding in the decisions I have made. Your love is the one constant in my life and for that I am truly grateful.

This thesis is dedicated to my Dad,
who inspired me to study science.

CHAPTER 1

INTRODUCTION

PACAP: a regulator of several endocrine systems

The endocrine system uses chemical messengers to communicate between non-adjacent cells and tissues. Chemical messengers of the endocrine system are called hormones. They are released from cells of a gland and act on distant cells via transport through the blood. Hormones can also act in a paracrine fashion, acting on adjacent cells or in an autocrine fashion, acting on the cell that released the hormone originally. Hormones initiate physiological changes by binding to a receptor, which can be specific for one or more hormones. Hormone receptors are classified by their protein structure, whether they are intracellular or extracellular and by the downstream signal transduction pathways they activate upon hormone binding. Binding of the hormone to its receptor initiates signal transduction pathways that ultimately result in physiological effects. Hormones can be peptides, modified amino acids, steroids or amines (Ganong, 2001). The endocrine system coordinates physiological processes and therefore most tissues of the body are influenced in some way by hormones. The following will review how a recently discovered peptide hormone labeled pituitary adenylate cyclase-activating polypeptide (PACAP) regulates several endocrine systems, and as such is involved in regulating essential physiological processes such as metabolism, growth, reproduction, and the stress response.

PACAP: discovery, related hormones, and structure

In 1989, a 38-amino-acid peptide identified by its ability to stimulate adenylate cyclase in pituitary cells was extracted from the ovine hypothalamus (Miyata et al., 1989). This peptide, labeled by its function as pituitary adenylate cyclase-activating polypeptide (PACAP), was later found to be present as two forms. In addition to the 38-amino-acid form (PACAP-38), a 27-amino-acid form, PACAP-27 was also present

(Miyata et al., 1990). Based on its primary structure, PACAP was classified as a member of the glucagon superfamily of hormones, which to date has 9 members in humans. These include PACAP, growth hormone-releasing hormone (GHRH), glucagon, glucagon-like peptide 1 and 2, secretin, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM) and glucose-dependent insulinotropic polypeptide (GIP). Of the nine hormones belonging to this superfamily, PACAP is most similar to VIP with which it shares two of its receptors. PACAP's ability to stimulate adenylate cyclase is 100-1000 fold greater then VIP's (Miyata et al., 1989). Since it's discovery in 1989, PACAP's structure and biological activity has been studied in organisms spanning from invertebrates to vertebrates, in adult and embryonic organisms and in cell culture lines (Sherwood et al., 2000).

In all vertebrates studied to date, except mammals, PACAP is encoded on the same gene as GHRH. After the divergence of reptiles to mammals, a gene duplication and exon rearrangement occurred such that in mammals PACAP and GHRH are encoded on separate genes (Fig. 1.1). In mouse the PACAP gene has 6 exons. The gene encodes a 5'untranslated region (5'UTR), a signal peptide high in basic amino acids, a cryptic peptide that has no known function or receptor, PACAP-related peptide (PRP) also with no known function or receptor, PACAP and a 3'untranslated region (3'UTR) (Fig 1.2) (Yamamoto et al., 1998, Miyata et al., 2000, Cummings et al., 2002).

The nine members of the glucagon superfamily have been derived by gene and exon duplications and rearrangements. PACAP is expressed throughout the vertebrates and even in an invertebrate, the tunicate (Sherwood et al., 2000). PACAP's primary structure has remained highly conserved over the course of evolution, with 97% identity

Fig. 1.1 - Proposed scheme for the evolution of the GHRH-PACAP gene. U, untranslated region; SP, signal peptide; Cryp., cryptic peptide; G, growth hormone-releasing hormone; PACAP, pituitary adenylate cyclase-activating polypeptide; PRP, PACAP-related peptide.

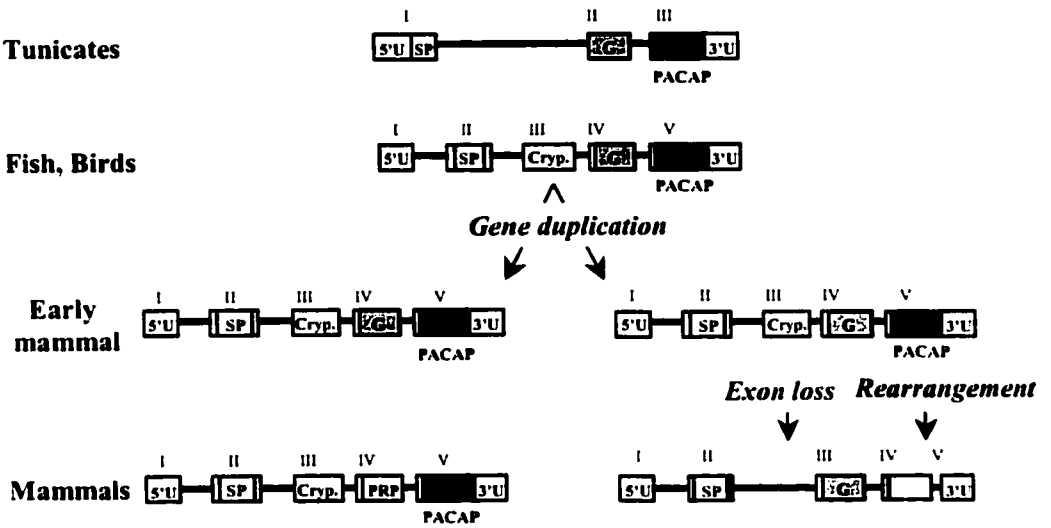
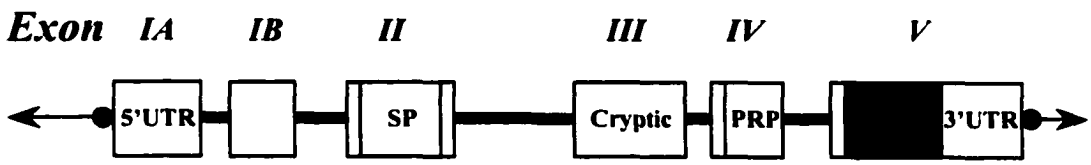

Tunicates
I
II
III
5'U
SP
3'U
PACAP
Fish, Birds
I
II
III
IV
V
5'U
SP
Cryp.
3'U
PACAP
Gene duplication
Early mammal
I
II
III
IV
V
5'U
SP
Cryp.
3'U
PACAP
Exon loss
Rearrangement
Mammals
I
II
III
IV
V
5'U
SP
Cryp.
PRP
3'U
PACAP

Fig. 1.2. Schematic representation of the mouse PACAP gene. UTR, untranslated region; SP, signal peptide; cryptic, cryptic peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; PRP, PACAP-related peptide.


Exon
IA
IB
II
III
IV
V
5'UTR
SP
Cryptic
PRP
3'UTR

in amino acid composition between tunicate and human PACAP-27 (McRory and Sherwood, 1997). PACAP's high conservation of structure and its ancient origins in an invertebrate make PACAP a candidate as the ancestral member of the glucagon superfamily (Sherwood et al., 2000).

PACAP: a functionally important molecule

Immense evolutionary pressure has kept the primary structure of PACAP essentially intact for 600 million years suggesting that PACAP plays an important physiological role. As a peptide hormone PACAP's targets are not limited to its site of synthesis. Instead, PACAP can be distributed to all tissues of the body via vascular and neural networks. PACAP is expressed throughout the brain and in several peripheral tissues. PACAP protein is present at high levels in the hypothalamus and in other brain areas. In non-neural tissue PACAP is present at high levels in testes and adrenal gland and at lower levels in the gastrointestinal tract, lung, pancreas and ovary (Arimura et al., 1991). PACAP is a ubiquitously acting peptide as its receptors are expressed in most tissues of the body.

PACAP's ability to potently stimulate cAMP production was key to its discovery in 1989, and provides a common link between the diverse functions ascribed to PACAP. In addition to signaling via adenylate cyclase (AC), PACAP receptor binding can also activate the phospholipase C (PLC)/inositol trisphosphate (IP_3) pathway and opening of calcium channels. PACAP's ability to increase intracellular concentrations of several basic signaling molecules (cAMP, Ca^{2+} and IP_3) results in activation of many downstream protein kinases (PK) (PKA, PKC, calmodulin-dependent kinases and mitogen-activated

protein kinases) and signal transduction pathways. These kinases phosphorylate (1) enzymes to increase their activity directly, (2) transcription factors that regulate transcription of genes and (3) ion channels that open to admit Ca^{2+}, which releases other hormones by exocytosis. The diverse signaling capabilities of PACAP are mediated by three different receptors, one of which uses a diverse array of splice variants that have preferential binding specificities and activate different signal transduction pathways.

PACAP receptors and signaling

PACAP's effects are mediated by seven transmembrane G-protein coupled receptors. The first receptor, PAC_1, is specific for PACAP. The other two receptors, $VPAC_1$ and $VPAC_2$, bind both PACAP and VIP with equal affinity (Harmar et al., 1998). There is a wide tissue distribution of the three receptors with expression of at least one of the three receptor subtypes in most tissues of the body (Ishihara et al., 1992, Lutz et al., 1993, Usdin et al., 1994, Pisegna and Wank, 1996, Chatterjee et al., 1996).

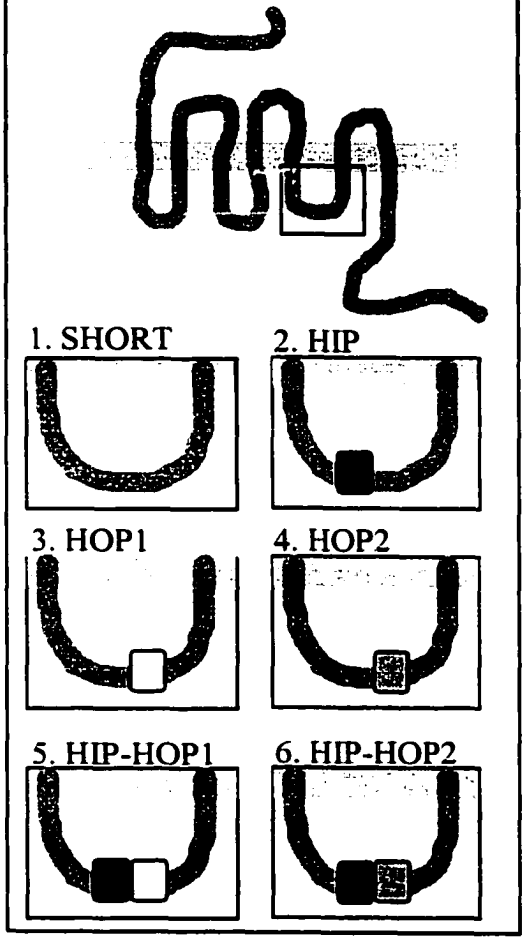
The PAC_1 receptor was first identified in rat pancreatic acinar cells (Buscail et al., 1990). Since then cDNAs have been isolated from several mammals (Pisegna and Wank, 1993, Ogi et al., 1993, Miyamoto et al., 1994, Hashimoto et al., 1996), a bird (Peeters et al. 1999), amphibians (Yon et al., 2001), and from fish (Chow et al. 1997, Wong et al., 1998, Wei et al., 1998). The single gene encoding the PAC_1 receptor (Aino et al., 1995, Chatterjee et al., 1997) is located on chromosome 7 (7p15-p14) in human (Brabet et al., 1996). The PAC_1 receptor is abundant in the CNS, particularly in the hypothalamus and is expressed in the sympathetic ganglia. Peripherally PAC_1 receptors have been identified in the anterior pituitary, pancreas, adrenal gland, heart, ovary and testis (Vaudry et al.,

2000). In mammals, the PAC_1 receptor gene undergoes alternative splicing within the coding region to produce 10 different PAC_1 receptor variants (Fig. 1.3) and within the non-coding 5'UTR producing four mRNAs with different lengths of 5'UTR (Chatterjee et al., 1997). The different 5'UTRs may contribute to tissue specific expression of the receptor or regulate stability of the mRNAs.

Six of the PAC_1 receptor variants are produced by splicing within the coding region; the variants include or exclude different combinations of two cassettes that can be inserted into the third intracellular loop of the receptor (Fig. 1.3). The hop (SV-1 in human) cassette is present in two forms (hop 1 and hop 2), and each can exist alone or with the hip (SV-2 in human) cassette. In addition, the hip cassette can be present alone, or none of the cassettes are present (Spengler et al., 1993, Journot et al., 1994, Pisegna and Wank, 1996). Two splice variants that have deletions within the N-terminal region of the PAC_1 receptor protein have been identified. One variant, identified in mouse and human, has a 21 amino acid deletion (Pantaloni et al., 1996) and another variant identified in human has a 57 amino acid deletion (Dautzenberg et al., 1999). Another PAC_1 receptor variant identified from rat testis contains a 24 amino acid addition in the N-terminal region of the PAC_1 receptor protein and has been named the $PAC_1R(3a)$ (Daniel et al., 2001) (Fig. 1.3). These receptor variants signal via adenylate cyclase (AC) or phospholipase C (PLC), except the PAC_1-hip receptor which can only act via AC (Spengler et al., 1993). Finally the PAC_1 TM4 variant was named because of substitutions and/or deletions within transmembrane domains II and IV (Fig 1.3). Unlike the other PAC_1 receptor variants that signal via AC and/or PLC, PAC_1 TM4 increases

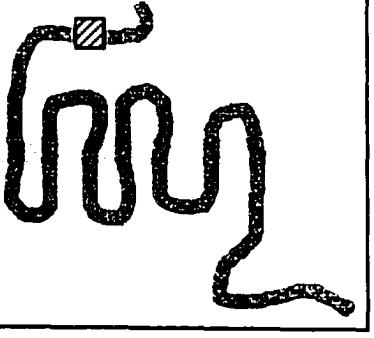
Fig 1.3. Diagrams of the ten mammalian PAC_1 receptor splice variants (modified from Moretti et al., 2002).

Variants 1-6
Insertions in the 3rd intracellular domain

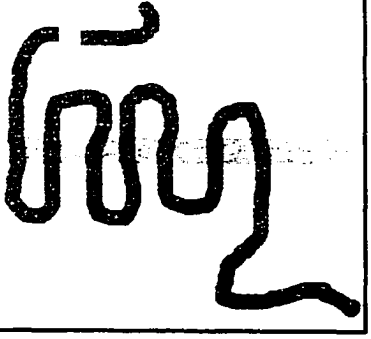


Variant 8
N-TERMINAL 57amino acid deletion

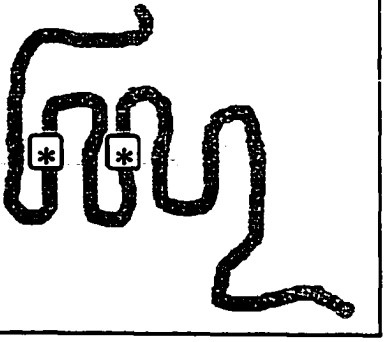
Variant 9
$PAC_1R(3a)$



Variant 7
N-TERMINAL 21amino acid deletion



Variant 10
TM4



intracellular Ca^{2+} levels by opening L-type voltage-dependent Ca^{2+} channels independent of AC or PLC (Chatterjee et al., 1996).

The $VPAC_1$ receptor was first identified from bovine brain (Ohtaki et al., 1990) and later a cDNA was isolated from the rat lung (Ishihara et al., 1992). The single gene encoding the $VPAC_1$ receptor is located on chromosome 3 (3p22) in human (Sreedharan et al., 1995). To date no splice variants of the $VPAC_1$ receptor have been characterized, but tissue expression studies using Northern analysis revealed two mRNAs for the $VPAC_1$ receptor (Sreedharan et al., 1995). The $VPAC_1$ receptor is coupled predominantly to AC (Vaudry et al., 2000), but a recent paper showed $VPAC_1$ can couple to PLC in the rat adrenal gland (Mazzocchi et al., 2002). The $VPAC_1$ receptor is expressed at high levels in the lung, and is present in a number of other peripheral tissues including liver, heart, spleen, kidney, adrenal medulla, blood vessels and pancreas (Ishihara et al., 1992, Usdin et al., 1994, Sreedharan et al., 1995, Filipsson et al., 2001). In the brain $VPAC_1$ is expressed predominantly in the cerebral cortex and the hippocampus (Ishihara et al., 1992).

A second VPAC receptor ($VPAC_2$) has been identified by isolation of cDNAs from the rat and mouse (Lutz et al., 1993, Usdin et al., 1994, Inagaki et al., 1994). To date no splice variants have been structurally characterized, yet Northern analysis of several human tissues did show the presence of two mRNAs for the $VPAC_2$ receptor subtype (Adamou et al., 1995). The $VPAC_2$ receptor is located on human chromosome 7 (7q36.3), the same chromosome that houses the PAC_1 receptor (MacKay et al., 1996). Signaling of the $VPAC_2$ receptor subtype is mediated predominantly by AC but also by PLC (Inagaki et al., 1994, MacKenzie et al., 2001). The $VPAC_2$ receptor is expressed in

the brain, predominantly in the olfactory bulb, thalamus, hippocampus and suprachiasmatic nuclei (Lutz et al., 1993). Peripherally, $VPAC_2$ is present in gastrointestinal tract, skeletal muscle, pancreas, adrenal cortex, heart, liver, kidney, testes, ovary and placenta (Usdin et al., 1994, Adamou et al., 1995).

In vertebrates other than mammals, PACAP receptor expression has been examined. In birds, two variants of the PAC_1 receptor have been identified (Peeters et al., 1999) and a partial cDNA for a VPAC receptor has been identified for two bird species (Chow et al., 1997). A partial cDNA for a VPAC receptor has been identified in a lizard (Chow et al., 1997) and all three of the PACAP receptor subtypes (PAC_1, $VPAC_1$ and $VPAC_2$) have been identified in amphibians (Yon et al., 2001). In two fish species the PAC_1 receptor and a single VPAC receptor most similar to the mammalian $VPAC_1$ receptor have been identified (Chow et al., 1997, Wong et al., 1998, Wei et al., 1998). Recently a receptor with structural similarity to the mammalian $VPAC_2$ receptor has been identified in fish, but neither PACAP-38 nor VIP activate this receptor. Instead, peptide histidine isoleucine, a peptide encoded on the same gene as goldfish VIP, and homologous to mammalian peptide histidine methionine activate the newly cloned receptor (Wong et al., 2000). Further analysis of this receptor subtype will determine if this receptor is in fact a $VPAC_2$ receptor in a fish species. It has been speculated that an ancestral receptor gene duplicated to give the $VPAC_1$ and PAC_1 receptors as represented in fish, and later the PAC_1 receptor gene duplicated to give rise to the $VPAC_2$ receptor (Vaudry et al., 2000). Because all three receptor subtypes have been identified in the amphibians the last duplication occurred at least 360 million years ago, at the divergence

of amphibians from fish. Further characterization of the PACAP receptor subtypes in fish and lower vertebrates will help to develop this evolutionary story.

PACAP, a regulator of other endocrine systems.

Since it's discovery, PACAP has been shown to affect many physiological processes. PACAP and its receptors are expressed early in the developing embryo suggesting a role in development. Fundamental processes such as metabolism, reproduction and growth are affected postnatally by PACAP. These processes are all regulated by endocrine systems, of which many are regulated by PACAP. PACAP within the brain has been shown to regulate endocrine systems such as the hypothalamic-pituitary axes and the sympathetic nervous system. In addition, PACAP is sent via neural networks to peripheral tissue to activate endocrine systems regulating processes of lipid and carbohydrate metabolism, or responses to physiological stressors. Also, PACAP is expressed locally to act on target tissues in a paracrine or autocrine manner. PACAP's ability to regulate endocrine systems accounts for the diverse functions thus far ascribed to PACAP.

PACAP as a hypophysiotrophic factor

The discovery of PACAP was a result of a search for additional hypothalamic hormones that increased cAMP in anterior pituitary cells (Miyata et al., 1989). Classicaily, a hypophysiotropic factor is made in the hypothalamus, released into the portal blood, has binding sites on anterior pituitary cells and regulates their activity (Rawlings and Hezareh, 1996). PACAP is produced at high levels within the

hypothalamus, predominantly within nerve cell bodies of the paraventricular and supraoptic nucleus (Koves et al., 1990, Arimura et al., 1991). PACAP immunoreactivity has been shown in the median eminence (Koves et al., 1990) and the level of PACAP in rat portal blood is higher than systemic levels (Dow et al., 1994). Receptors for PACAP are expressed on cells of the anterior pituitary, including normal and clonal gonadotrophs and somatotrophs, cell lines of corticotrophs and lactotrophs, adenomas and agranular cells of the anterior pituitary (Vertongen et al., 1995, Rawlings and Hezareh, 1996). In these ways PACAP could be considered a hypophysiotropic factor. PACAP is unusual in that it appears to regulate hormone release from four of the five major anterior pituitary cell types, all of which are specifically regulated by classical hypophysiotropic factors.

PACAP stimulates the release of LH and FSH from gonadotrophs. PAC_1 and $VPAC_2$ receptors are expressed on gonadotrope cells (Rawlings et al., 1995). Increased intracellular Ca^{2+} is required for gonadotropin secretion. PACAP increases intracellular Ca^{2+} in isolated rat pituitary cells by PAC_1 receptor activation and PLC mediated Ca^{2+} release from intracellular stores (Alarcon and Garcia-Sancho, 2000). PACAP is not as potent at releasing LH and FSH as gonadotropin-releasing hormone (GnRH), the classical hypophysiotropic factor responsible for gonadotropin release, yet when present together a synergistic effect occurs. This synergy occurs via cAMP dependent mechanisms (Rawlings and Hezareh, 1996).

PACAP stimulates the release of growth hormone (GH) from somatotrophs and in vivo administered PACAP has been shown to increase circulating GH in fish (Wong et al., 2000), amphibians (Yon et al., 2001) and some mammals. For example, PACAP releases GH in rat, cow and pig, but does not in sheep or human (Jarry et al., 1992,

stellate cells of the anterior pituitary; IL-6 then acts in a paracrine fashion on lactotroph cells to secrete prolactin. In isolated culture the paracrine effect of IL-6 is lost and PACAP's negative effect on prolactin secretion is seen (Murakami et al., 2001). PACAP may also induce hypothalamic VIP secretion from the hypothalamus, which in turn causes the release of prolactin from anterior pituitary cells (Yamauchi et al., 1995).

Corticotropin-releasing factor (CRF) is the classical hypophysiotropic factor controlling adrenocorticotropic hormone (ACTH) release from corticotrophs. The hypothalamic-pituitary-adrenal axis is regulated by PACAP downstream at the adrenal gland. Intravenous administration of PACAP in humans caused increased plasma ACTH concentration (Chiodera et al., 1996). In isolated corticotrophs, PACAP did stimulate ACTH release but only after a 24 hour incubation time (Hart et al., 1992). In corticotroph cell lines and in adenoma cells that secrete ACTH, PACAP receptors have been identified and a stimulatory action on ACTH observed (Rawlings and Hezareh, 1996).

Thyroid stimulating hormone (TSH), typically regulated by thyroid hormone-releasing hormone, does not appear to be regulated by PACAP (Rawlings and Hezareh, 1996). PACAP receptors are not expressed on these cells and addition of PACAP to isolated thyrotrophs does not increase intracellular Ca^{2+} levels (Alarcon and Garcia-Sancho, 2000).

PACAP's high level of expression in the hypothalamus and its regulatory role in several of the hypothalamic-pituitary axes, shows a supportive role for PACAP in regulating several neurally controlled endocrine systems.

PACAP: another neuromodulator in the sympathetic nervous system

The classical neurotransmitters of the sympathetic nervous system are acetylcholine, released from preganglionic sympathetic nerve terminals, and norepinephrine, released from postganglionic nerve terminals. Other non-cholinergic neurotransmitters in preganglionic neurons were suspected to exist because nicotinic and muscarinic blockers did not completely inhibit postganglionic neuronal activity (Ip et al., 1983). VIP was suspected as a neurotransmitter in the sympathetic nervous system, as VIP immunoreactivity is present in some preganglionic neurons. Yet, the high concentration of VIP needed to stimulate postganglionic neurons in vitro was evidence against its role as an inherent neurotransmitter. Later, PACAP became a candidate as the non-cholinergic sympathetic neuromodulator in the sympathetic nervous system due to the presence of both the peptide and its specific receptor, PAC_1 in the sympathetic nervous system. PACAP activates postganglionic neurons 1000 times more potently than VIP, suggesting that sympathetic neuron response to high concentrations of VIP was occurring through the PAC_1 receptor (Beaudet et al., 1998).

PACAP and PACAP receptor mRNA is present in the developing and adult sympathetic nervous system (Nogi et al., 1997, Nielsen et al., 1998, DiCicco-Bloom et al., 2000). In the superior cervical ganglion of rat and mouse, PACAP mRNA is present in preganglionic nerves originating from the intermediolateral cell column of the thoracic spinal cord (Chiba et al., 1996, Beaudet et al., 1998) and PACAP receptors, predominantly PAC_1 receptors, have been identified on postganglionic neurons (May and Braas, 1995, Moller et al., 1997). The preganglionic sympathetic nerves innervating the adrenal gland also contain PACAP and all three receptor subtypes are present on cells of

the adrenal medulla (Arimura, 1998, Mazzocchi et al., 2002). Receptor expression and signaling of PACAP in adrenal medullary cells, which are analogous to postganglionic neurons in the rest of the sympathetic nervous system, will be discussed in a separate section specifically dealing with PACAP and the adrenal medulla.

In rat, postganglionic nerves of the superior cervical ganglia express PAC_1 receptors only, the predominant splice variant being the PAC_1 hop1 splice variant (Lu et al., 1998, Braas and May, 1999). Although, the PAC_1 hop1 receptor variant signals via both AC and PLC pathways, PACAP-induced postganglionic nerve depolarization and secretion of neuropeptide Y and catecholamines is controlled by PLC and subsequent IP_3 activation (May and Braas, 1995, Braas and May, 1999). PACAP-induced depolarization results in Na^+ influx and K^+ efflux inhibition (Beaudet et al., 2000). $VPAC_1$ receptors are not expressed in the superior cervical ganglion (Nogi et al., 1997) and $VPAC_2$ receptors are expressed only on non-neuronal cells of the ganglion (Braas and May, 1999). Therefore, VIP does not significantly contribute to superior cervical postganglionic neuron depolarization and neurotransmitter secretion (May and Braas, 1995, Braas and May, 1999, DiCicco-Bloom et al., 2000).

PACAP is also produced in neurons of the superior cervical ganglion (Brandenburg et al., 1997). Expression of PACAP mRNA and peptide in these cells suggest PACAP can act on target tissues of the sympathetic nervous system. Brandenburg et al. (1997) showed PACAP receptor expression in several tissues targeted by postganglionic neurons originating in the superior cervical ganglion. Much of the work done showing PACAP as a neurotransmitter in the sympathetic nervous system has been done in neurons leading to or originating in the superior cervical ganglion. The

sympathetic nervous system targets tissues throughout the body. If, as in the superior cervical ganglion, PACAP is expressed in other sympathetic neurons, the effects of PACAP could be as broad as classical sympathetic neurotransmitters such as acetylcholine, norepinephrine and neuropeptide Y.

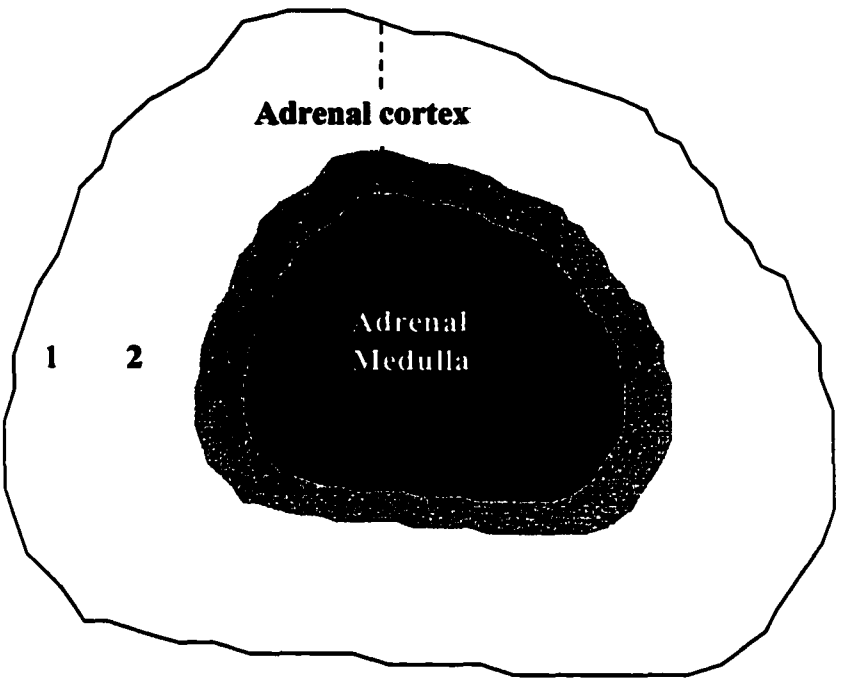
PACAP, a regulator of hormone production and release in the adrenal gland

PACAP's presence within the adrenal gland is well established. Initial studies characterizing PACAP's distribution within the body, showed high levels of PACAP-38 in the adrenal gland (Arimura et al., 1991). In addition, PACAP is present in nerves associated with the adrenal gland of amphibians (Yon et al., 1994) and with chromaffin cells of several fish species (Reid et al., 1995, Montpetit and Perry, 2000). Expression and biological activity of PACAP has been studied in both the adrenal cortex and the adrenal medulla.

PACAP and the adrenal cortex: regulation of the mineralocorticoids and glucocorticoids

It is agreed that PACAP mRNA is not expressed in cells of the adrenal cortex. Rather, PACAP receptors are present within the adrenal cortex (Mazzocchi et al., 2002). Of the three cell layers within the adrenal cortex, the zona glomerulosa, the zona fasciculata and the zona reticularis, only cells of the zona glomerulosa express a receptor for PACAP (Fig 1.4). The receptor subtypes expressed on zona glomerulosa cells and activated by PACAP are the $VPAC_1$ and $VPAC_2$ receptors (Mazzocchi et al., 2002). PACAP causes the secretion of aldosterone from the adrenal cortex. PACAP may act indirectly by stimulating catecholamine release from the adrenal medulla, which in turn

Fig 1.4. Diagram showing the regions of the mammalian adrenal gland. The adrenal cortex is divided into three layers. The outer layer, the zona glomerulosa, synthesizes aldosterone and contains PACAP receptors. The middle and inner layers, the zona fasciculata and zona reticularis respectively, synthesizes corticosteroids and do not express PACAP receptors (Ganong, 2001, Mazzocchi et al., 2002). The adrenal medulla secretes catecholamines and contains PACAP receptors.



CORTEX

1. zona glomerulosa site of ALDOSTERONE synthesis
$VPAC_1$ and $VPAC_2$ receptors expressed

2. zona fasciculata - site of CORTICOSTEROID synthesis
no PACAP receptors expressed

3. zona reticularis - site of CORTICOSTEROID synthesis
no PACAP receptors expressed

MEDULLA

adrenal medulla - site of CATECHOLAMINE synthesis
PAC_1, $VPAC_1$ and $VPAC_2$ receptors expressed

acts in a paracrine fashion to release aldosterone from cells of the zona glomerulosa (Neri et al., 1996). Or PACAP could act through the $VPAC_1$ or $VPAC_2$ receptor, directly stimulating secretion of aldosterone from zona glomerulosa cells (Mazzocchi et al., 2002).

In regards to corticosteroid release, reports in rat (Andreis et al., 1995) and calf (Edwards and Jones, 1994) suggest PACAP can elicit corticosteroid release from the adrenal cortex. Yet others state that PACAP cannot directly stimulate corticosteroid release due to a lack of PACAP receptors on cells of the zona fasciculata and the zona reticularis (Neri et al., 1996, Mazzocchi et al., 2002). In amphibians a PACAP receptor is present on cells of the adrenal cortex involved in glucocorticoid secretion and PACAP has been shown to directly induce corticosteroid secretion (Yon et al., 1994). PACAP, acting on the adrenal cortex, may originate from cells of the adrenal medulla or from nerves terminating on cells of the adrenal medulla and act in a paracrine manner on cells of the adrenal cortex (Bornstein et al., 1994, Mazzocchi et al., 2002).

PACAP and the adrenal medulla: regulation of catecholamine release

In mammals, cells of the adrenal medulla receive PACAP from two sources. PACAP is co-localized with acetylcholine in sympathetic nerve terminals innervating the adrenal medulla (Arimura, 1998, Hamelink et al., 2002) and PACAP mRNA is expressed within adrenal medullary cells producing PACAP locally and acting in a paracrine fashion (Ghatei et al., 1993, Mazzocchi et al., 2002). In rat (Watanabe et al., 1992), pig (Isobe et al., 1993), cow (O'Farrell and Marley, 1997) and dog (Lamouche et al., 1999) adrenal medullary cells and in chromaffin cells of rainbow trout (Montpetit and Perry,

2000) PACAP directly induces catecholamine release. In addition, PACAP has been shown to enhance acetylcholine-induced catecholamine secretion (Lamouche et al., 1999, Inoue et al., 2000, Fukishima et al., 2001a). Whether PACAP acts pre- or postsynaptically to enhance aceytlcholine-induced secretion has yet to be determined.

PACAP-induced secretion of catecholamines from adrenal medullary cells requires an increase in intracellular Ca^{2+}, either from intracellular stores or from an influx of Ca^{2+} from outside the cell (Isobe et al., 1993, Przywara et al., 1996, Tanaka et al., 1996, Fukushima et al., 2001b). In the perfused rat adrenal gland or in PC12 cells or in porcine adrenal chromaffin cell culture, this influx likely occurs through the opening of L-type voltage-dependent Ca^{2+} channels (Taupenot et al., 1998, 2000, Fukushima et al., 2001b). In cultured bovine adrenal chromaffin cells, L-type voltage-dependent Ca^{2+} channels (Tanaka et al., 1996), and possibly N- and Q-type Ca^{2+} channels are involved in the influx of Ca^{2+} that contributes to catecholamine secretion (O'Farrell and Marley, 1997). Ca^{2+} triggered catecholamine secretion, due to PACAP receptor activation is counteracted by the opening of Ca^{2+}- activated K^+ channels (Fukishima et al., 2002).

The PACAP specific receptor (PAC_1) (Shioda et al., 2000) and the two VPAC receptors ($VPAC_1$ and $VPAC_2$) have been identified in the adrenal medulla and, using receptor antagonists, Mazzocchi et al. (2002) have shown that all three receptor subtypes are involved in catecholamine secretion (Fig. 1.4). Most studies suggest the PAC_1 receptor is the predominant receptor type involved in PACAP-mediated catecholamine secretion (Fukishima et al., 2001a). PACAP binding to one of the three receptor subtypes initiates signaling pathways involving protein kinase A (PKA) and/or PLC. Binding of PACAP to PAC_1 receptors on chromaffin cells activates AC, increasing production of

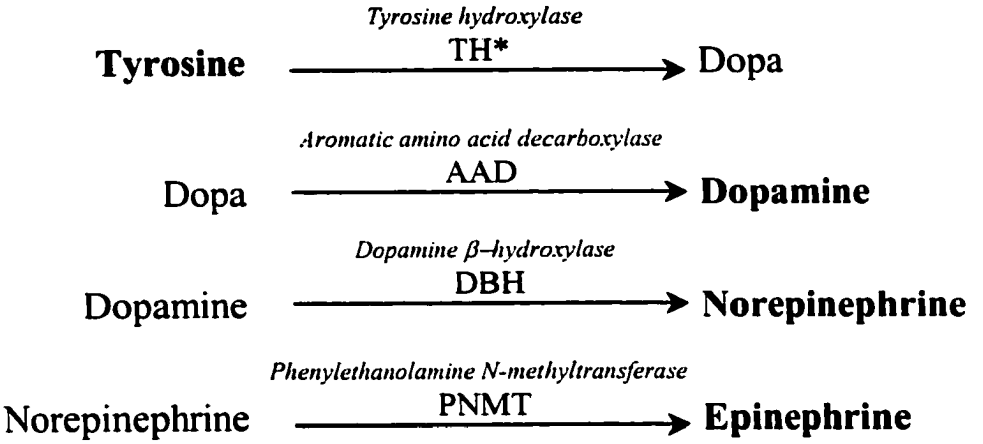
cAMP, which activates PKA (Przywara et al., 1996, Fukushima et al., 2001b, Mazzocchi et al., 2002). Fukushima et al. (2001b) propose that this signaling pathway contributes to PACAP-induced epinephrine secretion but not norepinephrine secretion. Finally, activation of PAC_1 receptors on chromaffin cells causes the release of intracellular calcium stores (Tanaka et al., 1996) via Gq protein activation of PLC (Isobe et al., 1993). The $VPAC_1$ receptor signals through AC to activate PKA or via PLC to increase intracellular IP_3 concentration or activation of PKC. Binding of PACAP to the $VPAC_2$ receptor results in activation of only the PLC-IP_3-PKC pathway (Mazzocchi et al., 2002).

PACAP and the adrenal medulla: transcriptional and posttanscriptional regulation of the catecholamine synthesizing enzymes

In addition to causing catecholamine secretion, PACAP initiates the production of catecholamines by regulating transcription and activation of the catecholamine synthesizing enzymes. The catecholamines, which include dopamine, norepinephrine and epinephrine, are the products of four sequential enzymatic reactions (Fig. 1.5). The three enzymes tyrosine hydroxylase (TH), dopamine ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) are regulated by PACAP.

TH is regulated by PACAP at a transcriptional and postranscriptional level. Increased transcription of the TH gene by PACAP results from activation of the PKA (Isobe et al., 1996, Corbitt et al., 1998, Choi et al., 1999, Park et al., 1999) and PKC pathways (Isobe et al., 1996, Choi et al., 1999). Inhibitors and stimulators of PKA have been used to show its role in PACAP induced transcription of the TH gene. The TH gene promoter contains a cAMP response element (CRE), a likely site for regulation of

Fig. 1.5 - Enzymatic reactions involved in catecholamine biosynthesis.



* rate limiting step

PACAP induced transcription of the TH gene (Kim et al., 1994, Tonshoff et al., 1997). In addition to the PKA pathway, PACAP binding to its receptors can result in activation of the PKC pathway. Using PKC inhibitors and stimulators, two groups have shown that the PKC pathway contributes to increased TH gene expression (Isobe et al., 1996, Choi et al., 1999). PACAP enhances the formation of transcription factor complexes that interact with known response elements (TRE and CRE) within the TH gene promoter. These complexes are known to be regulated by both PKA and PKC dependent pathways (Yukimasa et al., 1999). TH enzymatic activity is increased by PACAP through the PKA pathway , but not the PKC pathway (Marley et al., 1996, Moser et al., 1999). Activated PKA phosphorylates the TH protein at serine residues, increasing its activity (Moser et al., 1999).

DBH expression is also upregulated by PACAP (Isobe et al., 1996, Park et al., 1999, Choi 1999). DBH expression is increased via the PKA pathway only in bovine adrenal medullary cells (Choi et al., 1999), but by both PKA and PKC in porcine adrenal medullary cells (Isobe et al., 1996). The promoter of the DBH gene also contains a CRE, which may be the response element involved in PACAP induced expression of the DBH gene through the PKA pathway (Kim et al., 1994, Tonshoff et al., 1997). DBH enzymatic activity can also be increased by PACAP, but the signaling mechanisms involved are unknown (Choi et al., 1999).

PACAP regulation of PNMT expression does not occur by the same mechanism as the TH and DBH gene. Activation of the PKA pathway by PACAP does cause an increase in PNMT mRNA levels, but the promoter of the PNMT gene does not contain a CRE (Tonshoff et al., 1997). A report by Tonshoff et al. (1997) suggests PACAP does

not increase the rate of PNMT gene transcription. Instead, PACAP stabilizes PNMT mRNAs resulting in increased levels of PNMT mRNA compared to basal levels. In bovine adrenal medullary cells, PACAP can regulate PNMT gene expression positively through PKA and negatively through PKC (Choi et al., 1999). Thus, when PACAP acts through the PKC pathway on adrenal medullary cells, norepinephrine levels increase via increased expression of the TH and DBH genes and epinephrine levels decrease due to lowered PNMT expression. PNMT enzymatic activity is decreased by PACAP in bovine adrenal medullary cells decreasing epinephrine levels (Choi et al., 1999).

PACAP, a regulator of the endocrine pancreas

PACAP plays a role in carbohydrate and lipid metabolism through regulation of the pancreatic endocrine system. PACAP is present in nerve terminals innervating the exocrine pancreas, the blood vessels within the gland and the islet cells (Filipsson et al., 1998a, 1999). In addition, one study has identified PACAP mRNA in islet cells (Yada et al., 1994). PACAP immunoreactivity is also present in intrinsic ganglia within the pancreas (Fridolf et al., 1992, Filipsson et al., 1998a). Several studies have shown expression of PAC_1 and $VPAC_2$ receptors in cells of the exocrine and endocrine pancreas (Usdin et al., 1994, Yada et al., 1994, Filipsson et al., 1998a). Recently $VPAC_1$ receptors have also been identified on islet cells, confirming that all three PACAP receptor subtypes are present in the pancreas (Borboni et al., 1999, Jamen et al., 2002). The PAC_1 receptor was originally identified in rat pancreatic cells (Buscail et al., 1990). Several PAC_1 splice variants have been identified in the pancreas. Although individual studies have shown different sets of PAC_1 receptor variants, the predominant forms in the

pancreas appear to be the PAC_1-short and PAC_1-hop splice variants (Chatterjee et al., 1996, Borboni et al., 1999, Jamen et al., 2002). PACAP potently stimulates insulin in a glucose-dependent manner in vitro and in vivo (Filipsson et al., 2001). In two insulinoma cell lines and both mouse and rat islets, PACAP-38, PACAP-27 and VIP stimulate insulin secretion equipotently (Filipsson et al., 1998a, Jamen et al., 2002). This confirms the presence of a VPAC receptor within the pancreas. The importance of the PAC_1 receptor in PACAP-induced insulin secretion was shown in a PAC_1 receptor deficient mouse line, where PACAP-induced glucose dependent insulin secretion was reduced in the PAC_1 null mice and in PAC_1 null islets (Jamen et al., 2000, 2002).

PACAP binding to receptors on insulin producing cells, results in activation of AC and increased cAMP. PKA is activated and stimulates the opening of calcium channels, likely L-type Ca^{2+} channels, within the membrane of the ß-cells, which increases intracellular Ca^{2+} resulting in insulin secretion by exocytosis (Yada et al., 1994, Filipsson et al., 2001). The PLC signaling pathway, known to be activated by PACAP binding in other cell systems, does not play a role in PACAP-induced insulin secretion (Borboni et al., 1999, Filipsson et al., 2001, Jamen et al., 2002). PACAP may also have long term effects on insulin secretion by upregulating transcription of the insulin gene, a glucose transporter (GLUT 1) gene, a glucokinase (HK1) gene and other genes of the glucose sensing system by cAMP dependent mechanisms (Borboni et al., 1999).

PACAP also stimulates the release of two hormones that counteract insulin's glucose reducing effects. PACAP releases glucagon from α-islet cells of the pancreas of mouse, rat and human (Fridolf et al., 1992, Yokota et al., 1993, Filipsson et al., 1997), but not in the presence of glucose (Filipsson et al., 1998b). A recent study in PAC_1 deficient

mice, shows PACAP is involved in the glucagon response to insulin-induced hypoglycemia via the PAC_1 receptor (Persson and Ahren, 2002).

PACAP can also counteract insulin's action by regulating the synthesis and release of epinephrine from the adrenal medulla. Epinephrine increases blood glucose levels in times of stress. In vivo, PACAP administration results in increased insulin without increased glucose disposal. This is explained by the simultaneous increase in epinephrine as well as insulin (Filipsson et al., 1998b). This effect was also shown in vivo when mice deficient in PACAP showed impaired recovery from insulin-induced hypoglycemia due to an insufficient epinephrine response (Hamelink et al., 2002). The ability of PACAP to regulate counteracting processes, may arise from the presence of different PACAP receptor subtypes and PAC_1 receptor splice variants on the cells involved in insulin, glucagon and epinephrine release, resulting in activation of different signaling pathways under different physiological conditions.

Linkage analysis has shown chromosome 18p11 to be associated with diabetes type 2 (Parker et al., 2001). This corresponds to the chromosomal location of PACAP. As an agent that increases cAMP in β-islet cells producing a potent release of insulin, PACAP could become a new target for diabetes type 2 therapy. A recent paper showed PACAP has the ability to protect β-cells against glucose insensitivity (Yanagida et al., 2002) and this would be helpful to patients with insulin insensitivity. Yet, the potential use of PACAP as a drug target for diabetes type 2 is complicated by the fact that PACAP's effects are not limited to β-islet cells of the pancreas. PACAP receptors are present on almost all tissues and thus general administration of PACAP can affect many systems of the body. In humans, a study showing PACAP's ability to increase insulin

and glucagon secretion when administered intravenously also resulted in facial flushing and peripheral paleness, a result of PACAP's ability to act as a vasodilator (Filipsson et al., 1997). And in mice, PACAP-induced insulin release did not aid in glucose disposal, as the simultaneous release of epinephrine counteracted insulin's effects (Filipsson et al., 1998b).

History of the knockout mouse

Just prior to the discovery of PACAP, the methodology for gene targeting was being developed and successfully applied for gene inactivation in mouse (Evans and Kaufman, 1981, Bradley et al., 1984, Smithies et al., 1985, Thomas and Capecchi, 1987). Random disruption of genes in *Drosophila* and *C. elegans* had been successful at matching phenotypic traits with genes. In the more complex mouse, targeted disruption was necessary to pinpoint gene function. Since the first targeted gene inactivations in mouse (Thomas and Capecchi, 1987, Monsour et al., 1988), approximately 7000 knockout mouse models have been made (Capecchi, 2001).

Inducible gene knockouts allow for disruption of the desired gene in a specific tissue or at a specific developmental stage (Gu et al., 1994). This technique has been useful for gene disruptions that cause embryonic lethality. The disruption can be delayed past the developmental stage that causes death. The Cre-lox P targeting system is one strategy used to create inducible knockouts (Sauer and Henderson, 1988). It uses a bacteriophage recombination strategy. In the presence of two lox sites (34 bp sequences), the enzyme cre removes the intervening DNA sequence. The lox sites are introduced into the mouse genome along with the targeting construct. Later the addition of cre

recombinase to the culture medium, causes recombination of the targeted allele, producing some embryonic stem (ES) cell clones with a deletion of the gene and some with the coding sequence of interest flanked by lox sites for inducible knockout at a later time. Transgenic cre mouse lines with cre recombinase expressed under a variety of promoters for tissue specific or developmental stage knockout of the gene have been created (Cre transgenic database-http://www.mshri.on.ca/develop/nagy/cre.html). Cre transgenics are bred to mice homozygous for the lox flanked gene.

Knockout mouse models provide an in vivo mammalian system to study gene function. Because it is an in vivo system, compensation by other proteins can occur. Compensation, although difficult to address, shows how redundancy within the genetic makeup of the organism can be advantageous to the organism.

Objectives: uncovering PACAP's functions by gene disruption in mouse

The evolutionary story of PACAP, the most highly conserved member of the glucagon superfamily, suggests it has an important physiological function. In addition, the wide expression of PACAP and its binding sites has identified a diverse array of tissue and cells types that PACAP is able to regulate. The idea that one peptide could influence many physiological systems prompted us to ask what is PACAP's main physiological function and is it essential for life? We have begun to answer these questions by creating a mouse line with a targeted disruption of the PACAP gene. In mouse, PACAP is an ideal candidate for gene disruption because it is encoded on its own gene and is a single copy gene. Because PACAP can act through three receptor types, a PACAP receptor knockout would not inactivate PACAP's physiological effect entirely.

As such we chose to disrupt the mouse PACAP gene, thereby eliminating the physiological effects of the PACAP peptide within mouse. A targeting strategy that would circumvent the consequences of an embryonic lethal phenotype was used, as evidence of a role for PACAP in early brain development is substantial. Analysis of the phenotype of the PACAP knockout mouse has focused on how PACAP acts to regulate other endocrine systems. Such endocrine systems include the hormones that regulate carbohydrate and lipid metabolism and the catecholamines produced in the sympathetic nervous system and adrenal medulla, responsible for the fight or flight response to stress.

In this thesis, generation of a PACAP null mouse line has shown that PACAP is essential for survival and is important in mammalian physiology. Specifically it has identified a role for PACAP in lipid and carbohydrate metabolism and has shown its importance in regulation of the sympathetic nervous system. Under stress, such as environmental stress, the sympathetic nervous system is not regulated sufficiently to maintain catecholamine levels, which in turn affects the regulation of lipid and carbohydrate metabolism and thermoregulation.

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CHAPTER 2

Postnatal expression and novel splicing of the mouse pituitary adenylate cyclase-activating polypeptide (PACAP) gene.

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INTRODUCTION

PACAP and another glucagon superfamily member growth hormone releasing hormone (GHRH) are encoded on a single gene in some vertebrates (birds, amphibians and fish) as well as in the invertebrate tunicate (Sherwood et al., 2000). However, in mammals PACAP and GHRH are encoded on separate genes due to a gene duplication followed by substitutions or rearrangement.

PACAP expression studies have focussed on adult tissues, whereas expression in the embryonic, postnatal or juvenile periods are essentially lacking except for the brain. In adults, cell types expressing PACAP are limited, with primary sources being nerve cells including adrenal medullary cells (Ghatei et al., 1993, Miyata et al., 2000). Tissues of non-neural origin that have been identified as PACAP positive by immunostaining are cells residing in the gonads (Ghatei et al., 1993, Gras et al., 1996), small, lymphocyte-like cells in the immune tissues of the rat (Gaytan et al., 1994) and β-cells of the pancreas (Yada et al., 1997).

The PACAP gene and/or cDNA have been cloned from human (Kimura et al., 1990; Ohkubo et al., 1992; Hosoya et al., 1992), sheep (Kimura et al., 1990), rat (Ogi et al., 1990), mouse (Okazaki et al., 1995; Yamamoto et al., 1998, Miyata et al., 2000, Cummings et al., 2002), chicken (McRory et al., 1997), frog (Alexandre et al., 2000; Hu et al., 2000), salmon (Parker et al., 1993; 1997), catfish (McRory et al., 1995), goldfish (Wong et al. 2000), zebrafish (Fradinger and Sherwood, 2000) and from a tunicate, which is an invertebrate (McRory and Sherwood, 1997). PACAP has been highly conserved during evolution, with the tunicate PACAP-27 having 96% amino acid conservation with human PACAP-27 (McRory and Sherwood, 1997). This is the highest degree of

conservation among family members. Therefore, PACAP is likely to play a critical physiological role.

The mouse PACAP gene (*Adcyap 1*) was first sequenced in 1998. The sequence has been confirmed twice (Miyata et al., 2000, Cummings et al., 2002) and additions to the original promoter sequence (1707 bp) have been made (Cummings et al., 2002). The mouse PACAP genomic structure encodes the 5'UTR on exons 1A, 1B and 2, a signal peptide on exon 2, a cryptic peptide on exons 2 and 3, PACAP-related peptide (PRP) on exons 4 and 5, and the coding sequence for PACAP-38, a stop codon and 3'UTR on exon 5.

Alternative splicing that results in exon deletion, intron sliding or 5' UTR splicing has been reported for the PACAP gene and for genes of other superfamily members in several species (reviewed in Sherwood et al., 2000). In chicken (McRory et al., 1997), frog (Alexandre et al, 2000), salmon (Parker et al., 1997, Krueckl and Sherwood, 2001), and catfish (Small and Nonneman, 2001), alternative splicing produces two distinct mRNAs: a full length transcript encoding both GHRH and PACAP, as well as a shorter transcript that is missing exon 4, the coding region for the bioactive core of GHRH. The occurrence of the two transcripts varies in different tissues, and it has been proposed that this alternative splicing serves as a tissue-specific regulator of GHRH and PACAP expression. Exon deletion has not been reported for mammals as the two bioactive peptides are on separate genes with separate regulation.

PACAP transcripts with different 5'UTRs have been identified, resulting from a combination of alternative upstream promoters and alternative splicing within the 5'UTR. Daniel and Habener (2000) have described in rat a unique, testes-specific first exon that

is located 13.5 kb upstream from the first coding exon in the gene. In mouse, six PACAP mRNAs with alternative 5'UTRs have been identified (Yamamoto et al., 1998, Tabuchi et al., 2001, Cummings et al., 2002) and a transcript with an additional 19 amino acids between the cryptic peptide and PRP has been shown in T-cells (Cummings et al., 2002).

The present study shows the expression pattern of the PACAP gene in 12 tissues at four times during the postnatal and juvenile periods; expression is compared to that in the adult mouse. Alternative splicing within the murine PACAP gene is examined using RT-PCR, resulting in identification of six transcripts, two of which are unique.

MATERIALS AND METHODS

PACAP gene: isolation and sequencing

The mouse PACAP gene was isolated and sequenced in our laboratory by Kevin Cummings; I assisted in preparing samples for sequencing. I have included the sequence data for the mouse PACAP gene as a reference for primers that I used and transcripts identified in this study (Fig 2.1).

PACAP expression in nine tissues of adult mice

Nine tissues (brain, lung, heart, liver, small intestine, spleen, kidney, testis and ovary) were collected by dissection from male and female adult (>2months old) C57BL/6 mice. The tissue was frozen immediately in liquid nitrogen and stored at –80°C. Each tissue was ground into a fine powder using a chilled mortar and pestle. mRNA was isolated using Ambion's MicroPoly (A)Pure mRNA purification kit (Ambion, Austin, Tx). mRNA (5 μg) was reverse transcribed with Superscript II reverse transcriptase

Figure 2.1. Nucleotide sequence encoding mouse PACAP gene. Non-coding regions are shown in lower-case letters. Coding regions are shown in upper-case letters with one letter amino acid designations listed below in bold. The consensus TATA box, as well as upstream TATA-like elements (uTATA) are overlined. The signal peptide is underlined with a dashed line, PRP is underlined with a solid line and the region encoding PACAP in exon 5 is underlined with double lines (Cummings et al., 2002).

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   1  cagtcctgctggattatctttcctgttcactgcaagctctttgtgtgagggccaggaaaggtggacgctg
                          SREBP1
  71  aatatgtagttcagggatgtagtcaccccacttttcataacaaacttgtccaaaactattgcctacagtg
 141  gttctcctgacttttgagtcaaaggtgaactccattaaaattcccacttttgcctcccacaataatctca
                                  IK2
 211  atttctctactgagtttttcactctactgaatgggattgacttcaagttgtatgattttggggtacccag
 281  gccagggagtaagagtgtcaacaaaccccagatactcccatgaactggacgcaagctaggcttttgaagg
 351  cttgcttacttttctttcttccttcctttctttcttccttcctttctttctttctttctttctgtctgtc
 421  tgtctgtctgtctttctttctttctttctttctttctttctttctttctttctttctttctttctttcgt
 491  gttcaccacaactcctagctacatttatttcttgcttatttttcttgcttgtgggttttgccctttaacag
 561  accttgaagtcaaccaaactccccacatggtggctctcttgactaaaaagttgggcattgaattgtaaat
 631  tttaaatgcgtagtcctggagatttttaaatgtctttccagatggaactcatttcagaattcaaacctaa
              GATA3
 701  gatagcagacagatataatataacacaaagagaaacatcccctgtgtagaagtagtttgttgatagtatt
                      NFAT
 771  caatctaaagctaggaggaaactaagaggagacaggcagctttggatcattcaaagtcaggaaataagga
      NFAT    GATA1  MZF1                          GATA1
 841  aaatttttgataattggggaaagtattaaacctcaaacaaaggtgataatagcattaaaagtgagaaata
              NFAT                                              GFI1
 911  ctgtggcttggaaaaagtactcaggactaacagcttatttaaacttattaaacccaaatcacagtatagg
 981  cagcagcactcgtacttaattctctctctctctctctctctctctctctctctctctctctctctctctc
                                                  GATA1
1051  actgtgtgtgtgtgtgagagagagagagagagagagagagcttcagatagtaaaaatgcacactaact
1121  gttattgatgcttctatttttcccttgaaaagaaaggctctcgagatttcctagggaccagaaatttctga
1191  atcttctgaaattactcaggtgcttagaaggtccccagtgaatgtgatctttttctcattgctttcttctt
1261  ttcattttatttttagttaaaaatattaactatcccccagggaagggttcctggtagcaacctttatcaa
1331  gacaacttgtgcagggaatcctcctgcattccgagtctatatttccctaaacagaattcaacatgtaaga
                                                  S8     NFAT
1401  gaaattcaagagacacacgtcacaccctcacaatcttaaattttcaattattgcagaaaacacagtgaca
1471  tggtttcaatttttaaaactagtaagagccacggagagtgtgaaagtgtgtagacaggaaaggtaaagat
1541  ccatctgaatactaggactaactcagaagaaaaagctttgcactgaggcagggattaagcaggttctgag
1611  cactgggacattcgtggacacagaatccaagggaagattaatatgaacagcggggtgatttagacaatga
1681  actcccacagtaagagcaccactgccaaagcttcaaatttagaggctgtggtgaaaattaaaccagtggc
1751  aaatttcaacatttgcagcatctgcgcccaaatagttcagcaccaagagcctggacagcaccacaggctc
1821  tcatcctagtctcatccatcaatctattcagagacagactgtcaacccagccagactcattagatattta
1891  ctgaaaatccttataattctttccttaaaacacaaaacgacttccatgtttagtagcctatttgaaaaa
                          GATA3
1961  gcatatgcaaggaattgagagatcaaaattaaaattattaataggagatcttgatggtgcttaaatctag
      GATA3       TCF11                                          uTATA
2031  agatcagagttgtcattggtgggggttgagtgaaaattaagaaaaattagggactcaataaaaacatgac
                                                  GFI1
2101  ttcaccattctctaaattctacgagttctttacttgtctttgagaaatcagtgaaatcgaaaaccatcaa
                                                                  AP1
2171  aataattggacttcttaaaaattggattgtgtgagtgaaaggtgtttatcagaagcggatgactccggat
2241  cttatcatcctggaggactgcacagaatagttaatatgttccttgagggactaggatgctgacgtctttt
        GATA1                 MZF1                TCF11       uTATA
2311  actgataccggatcattacgtgactgggggagaaaaaaaaggaagtcatatcatgaataaaaatCGGAGT
2381  GCAACAGTGCAACCAAAATATTCTGTACTTGAAGGCAGAAAGATGTTGACAAAGAGGTGTCTCCTGAAAC
                                  TATA
2451  CACGTTCGGACAGCTTATTTTGTTAACTGCATATATAAAAACGAGCAGAAGGCCAGTCACCTCTGTCAGC
2521  AGCAGAAGCAGCAGCATCTTCAGACGCAGCCTGAGACCTCAGAGCAGAGAAGgtgcctccgactcccgac
2591 ttccgcaagctgccagatagtctcccacagaggccatcttccctctcccccccccccccactgacttttc
2661 ctcttttcttacccattagcttcttctcccggtggacttaagtccactctgctcccccctccctttggctg
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2731   ctcgcctactctctcttctacatctctctcctcgcccacttcCTTCGGTGTCACGCTCCCTCCTAGTTTT
2801   GTGCGTCTACAAAGTTTTGAGCGGAATTTTAGCCTCGGCAAACAAGTCTCCCCAGCTCCTTCAGCTAATT
2871   CCCGCGGCTTCTCTCCAGACACCAGCTCCAGACAGTGACTGATGCTTCTCGGGTTGTGATTCCAGCGCAG
2941   AAACTCGAAGGAGCCTTTGCCCGCTGTCCTACTTAGTCAACCCTTTCCTAGACACCgtaagagtctaata
3011   ataaggggtttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgttgtgtgtgtgttg
3081   ggattcttagcatactaaccgattgattgactacaggctccctccttgtgctgccagcagcatcAGTGTA
3151   TTAGTTAGCCTTCTTTAGTTCAAGGTCTGGCTAGTTACTGGGTGCTGGGCACACACAAAGCTATTTTTTC
3221   GAACTATACCAAGAGAGAAGTAGTTGGACGCCCCAGCGTGGCTAACTGGTGTTTAACACAGGGAGTCACA
3291   GTTTCACTGGCTCCCCAGCTCCCAAGAGTTTCTTCGGGTTAGAAGGTAGAAAGCCAGGAGCGATTTCACT
3361   CACTGCATACCCTCTTTCCTCTCCTGCGCAGAATGACCATGTGTAGCGGAGCAAGGCTGGCCCTGCTGGT
1                                          M  T  M  C  S  G  A  R  L  A  L  L  V
3431   GTATGGGATAATAATGCATAGCAGTGTCTCCTGTTCACCTGCCGCCGGACTCAGCTTCCCTGGGATCAGg
14     Y  G  I  I  M  H  S  S  V  S  C  S  P  A  A  G  L  S  F  P  G  I  R
       INTRON 2 (3501-5600)
5601   tacccgcagACCAGAAGACGAGGCTTACGACCAGGACGGAAACCCGCTGCAAGACTTCTATGACTGGGAC
37                P  E  D  E  A  Y  D  Q  D  G  N  P  L  Q  D  F  Y  D  W  D
5671   CCTCCCGGCGTGGGGAGCCCCGCCTCCGCGCTGCGTGACGCTTACGCCCTTTACTATCCAGCGGACAGGA
58     P  P  G  V  G  S  P  A  S  A  L  R  D  A  Y  A  L  Y  Y  P  A  D  R
5741   Ggtacaacccgcccggcttccgcctgtggcttgggcctaacctggcagaagggaccgggtaggggctcta
       R
5811   aaagtagggctttttctgttaaaagaagggtctttaagcctcccttcccatgagagctacagaaaattag
5881   cagtcagatccctgtggagcccaagccccaccacgggactgaaagctctgaggccagggaggtgtcagtg
5951   agtgtgcgactctgtaggtggggagcttttgggccacactaaataccaccttcgattcacccctggggaa
6021   cttccctaagttactcccaggtcccgtgtcactagtggctgttgggtcatccttctgacagacccaacag
6091   tgtgagccttagctatcatttgaagggcttgaggcagtgagtgacccacgtcgcattctttgtttccccca
6161   ttagAGATGTCGCCCACGAAATCCTTAACGAAGCCTATCGAAAAGTCTTGGACCAGCTGTCCGCCAGGAA
81           D  V  A  H  E  I  L  N  E  A  Y  R  K  V  L  D  Q  L  S  A  R  K
6231   GTACCTGCAGTCGGTCGTGGCCAGGGGCGCGGGgtaagagtttgtgaaagagttaaccttcgaatgatgc
103      Y  L  Q  S  V  V  A  R  G  A  G
       INTRON 4 (6301-7280)
7281   accgccccgcccccactctccgccccctctccttgcagGGAGAACCTAGGCGGCAGCGCGGTGGACGACC
114                                                E  N  L  G  G  S  A  V  D  D
7351   CGGCGCCCCTTACCAAACGCCACTCGGACGGCATCTTCACAGATAGCTACAGCCGCTACCGAAAACAAAT
125    P  A  P  L  T  K  R  H  S  D  G  I  F  T  D  S  Y  S  R  Y  R  K  Q  M
                                  ================================================
7421   GGCTGTCAAGAAATACTTGGCGGCCGTGCTAGGGAAAAGGTATAAACAGAGGGTTAAAAACAAAGGACGC
148      A  V  K  K  Y  L  A  A  V  L  G  K  R  Y  K  Q  R  V  K  N  K  G  R
         ============================================================
7491   CGAATAGCATACTTGTAGcagatcagctgccggctaccttgtgtataaaatgaaaagtcgttttccaaat
172     R  I  A  Y  L
7561   tgactgaccagtcatcactcgtgttctttccaaacatgtatttatgtatcaagtaaagccattaaatgac
7631   tattttgataataatattgttttttcttttttatgaagcactagagaatgcacagatatactttgtggacca
7701   attattgatatatattataagtatatatattaagaatatatataagtataacagagagcaattaagatgg
7771   gtgcacaaggattgaaaattcgcctgagctgtttatgtttttatataaagtaaatagagaaaatagacaa
7841   ccattgttttgaatattactcctatttttgtaaactggaattaaaggatagtatttttatccacaaccgt
7911   cttgaagataccaataatggccatttgtacaaaaaacaatgatgccctgctccaggggaattctgaggta
7981   atgacttggggaattgctgaagggctttctttccctctgagtctggggcaggctgcttgaaccccagcct
8051   aactaactcaagtgggcattgtcccactggttgcaggggcaattccaacaatttcagtttctttgattat
8121   gtgtatttgtctctcctcagactctcagcccagaaggaaattctaattaaacaacagctctatccaaatt
8191   gtgcttctccccagaactcatgtcattccctgatagaagagttgaggaactgtacagaagagacaggctt
8261   ggagagagagctctcttttctgtacttcctgattctccagggaacagactattctaaggctagggcaatt
8331   ggaacaaagtgaaagatatataagggattggtaaaggcagaacatggggatttgagatttgagagttgcc
8401   tcaggtctgagaatctgggggcaagtctagctcctctgtaggttccactgcctgacagatcaggtgctgg
8471   tgttggaatgaatgcaaagtacaatgtgtttttctccagtgctgttcatgcttttcatgttgtgaaatgg
8541   ccaggatcc
```

(Gibco BRL, Burlington, ON) using 5 µl of 20 mM oligo dT. cDNA (1µl) generated from the above reaction was added to a 50 µl reaction containing 2.5 U Taq polymerase (Gibco BRL), 1x Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$, 200 mM dNTPs (Gibco BRL) and 20 pmol of primer G made against the 5'end of the region encoding the signal peptide and primer J made against the 3' UTR (Table 2.1). PCR was carried out for 32 cycles under the following conditions: 94°C for 30 sec; 56°C for 30 sec; 72°C for 45 sec followed by a long extension of 7 min. Nested PCR was carried out on this PCR product (1 µl) using nested primers H and I (Table 2.1) with the same conditions as the first round. The 529 bp product was separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The PCR product was cloned into vector T (Promega) and transfected into XL-2 blue competent cells. Plasmids were purified using Qiagen's miniprep kit and sequenced, using two clones per amplified product.

PACAP expression in neonatal and juvenile mice

Twelve tissues (brain, eye, lung, thymus, heart, liver, small intestine, pancreas, spleen, kidney, testes and ovary) were collected from 2 littermate C57BL/6 mice, one of each sex, at four postnatal ages: 3 days (pancreas was not collected), 2 weeks, 4 weeks and 6 weeks. The tissue was frozen immediately in liquid nitrogen and stored at –80°C. mRNA was extracted and cDNA generated and amplified, as for the adult tissue expression study above.

Characterization of PACAP transcripts using RT-PCR.

Adult tissues that were positive for PACAP in the above expression study (brain,

Table 2.1 - Sequence and location of primers preA and A-J. The locations of the primers are in reference to the PACAP gene sequence in Fig. 2.1.

Primer	Sequence	Gene Location
preA	5' GAGGGACTAGGATGCTGACGTC 3'	2285-2306
A	5' CGGAGTGCAACAGTGCAACC 3'	2375-23924
B	5' CACCTCTGTCAGCAGCAGAAG 3'	2508-2528
C	5' GCCTGAGACCTCAGAGCAGAG 3'	2549-2569
D	5' GCTCCCTCCTAGTTTTGTGCG 3'	2785-2805
E	5' AGCCTCGGCAAACAAGTCTCCCC 3'	2831-2853
F	5' GTCTGGCTAGTTACTGGGTGCTG 3'	3175-3197
G	5' ATGTGTAGCGGAGCAAGGCTGG 3'	3399-3420
H	5' AGCAGTGTCTCCTGTTCACCTG 3'	3450-3471
I	5' CTGCTACAAGTATGCTATTCGGCG 3'	7511-7488
J	5' GAACACGAGTGATGACTGGTCAGTC 3'	7586-7562

PACAP mRNA expression in adult mice

RT-PCR of the nine tissues collected from adult mice, revealed that PACAP mRNA is expressed in brain, gastrointestinal tract, ovary and testis. In ovarian tissues, expression of PACAP mRNA was variable when repeated in different mice.

PACAP Expression in neonatal and juvenile mice

RT-PCR of the 12 tissues at each of the 4 postnatal development stages using primers that amplified exons 2-5 of the PACAP gene, revealed the presence of PACAP mRNA in the day 3 neonate brain, eye and thymus. By two weeks of age, expression also occurred in the testis and ovary. At 4 weeks of age PACAP mRNA expression was detected in brain, eye, thymus and testis. By 6 weeks, expression of PACAP mRNA in the thymus and testis had disappeared, but was present in the brain, eye and ovary. Hence, expression in ovarian tissue appeared to be sporadic, as PACAP mRNA was not detected in the 4-week ovary, but was present in 2- and 6-week ovary (Fig. 2.2).

Alternative splicing in the 5'UTR produces multiple PACAP transcripts

Several different 5'UTRs for the PACAP transcripts were found in the present study. RT-PCR on PACAP-positive adult tissues and 4-week-old thymic tissues using four sets of primers revealed the presence of six transcripts, each of which differed in the structure of the 5'UTR (Fig. 2.3, Table 2.2). Five of these transcripts were present in brain, two in testis, and one each in ovary, thymus and GI tract (Table 2.3). Our results confirm four transcripts from previous reports and reveal two unique transcripts with alternative splicing in the 5'UTR. The novel transcripts that we isolated are shown in

Figure 2.2. Postnatal expression of PACAP mRNA by RT-PCR in 3-day, 2-week, 4-week and 6-week-old mice. The ~450bp product represents PACAP mRNA from the signal peptide to the end of the PACAP coding region. Lanes with negative controls for PCR are labeled –ve . Primers specific for mouse β-actin were used as a control. Spaces between neighboring bands show PCR products were run on separate agarose gels.

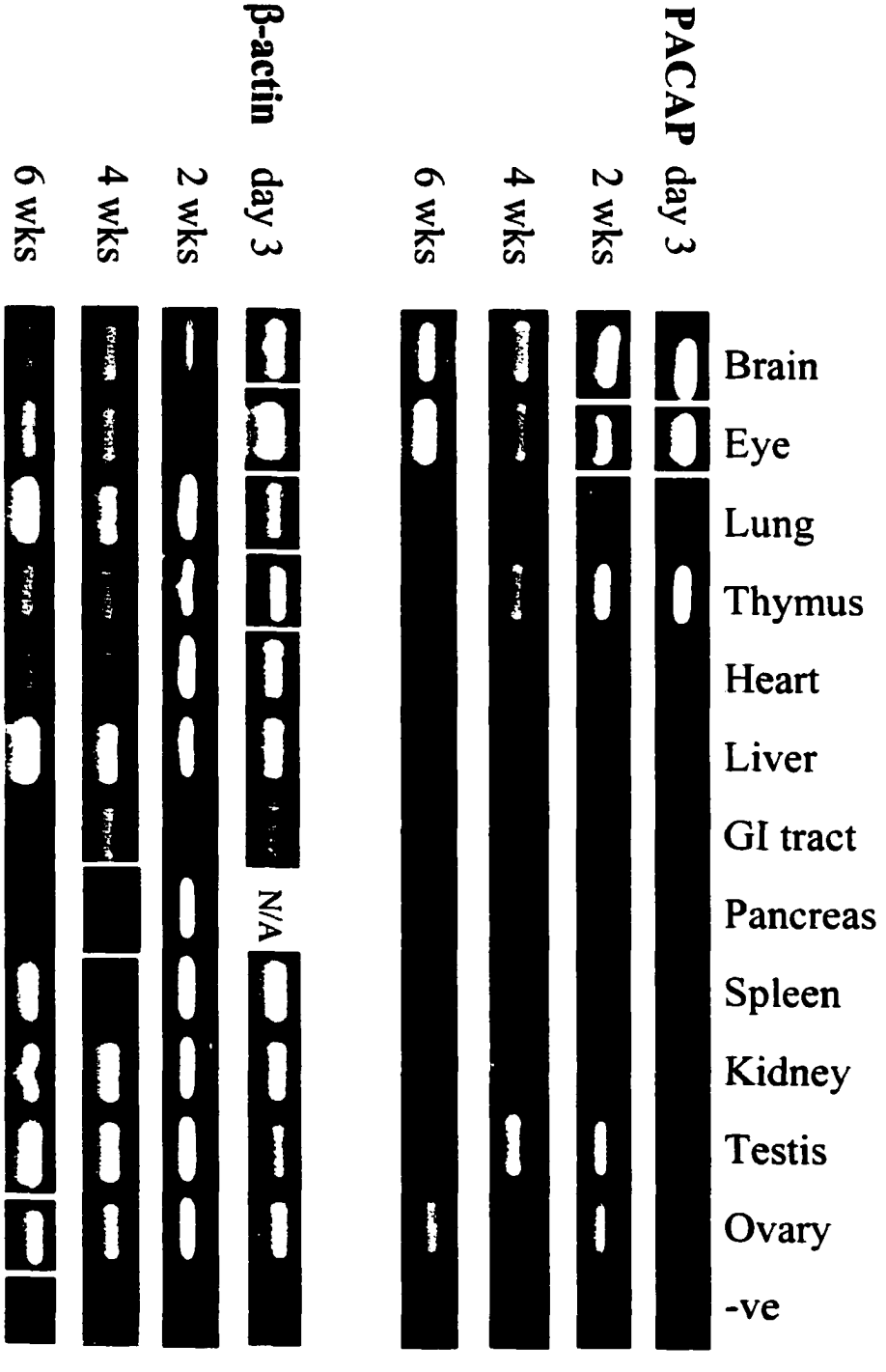
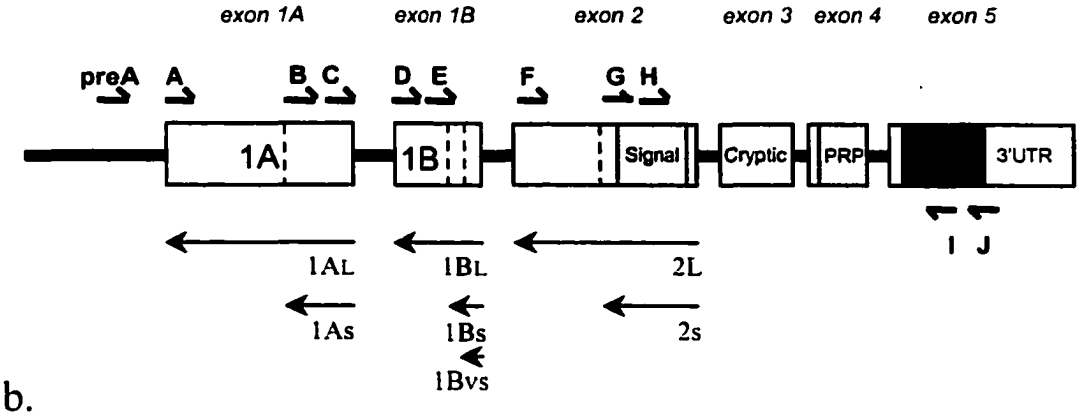

PACAP
β-actin
day 3
2 wks
4 wks
6 wks
Brain
Eye
Lung
Thymus
Heart
Liver
GI tract
Pancreas
Spleen
Kidney
Testis
Ovary
-ve
N/A

Figure 2.3. PACAP transcripts with alternate 5'untranslated regions (5'UTRs). a.) Representation of the PACAP gene with the location of the primers used to amplify the alternate transcripts by RT-PCR. b.) Seven PACAP transcripts identified by RT-PCR or 5'RACE. An asterisk next to a transcript represents a novel transcript. Cryp = cryptic peptide, PRP = PACAP-related peptide.

a.


exon 1A
exon 1B
exon 2
exon 3
exon 4
exon 5
preA
A
B
C
D
E
F
G
H
1A
1B
Signal
Cryptic
PRP
3'UTR
I
J
1AL
1BL
2L
1As
1Bs
2s
1Bvs

b.

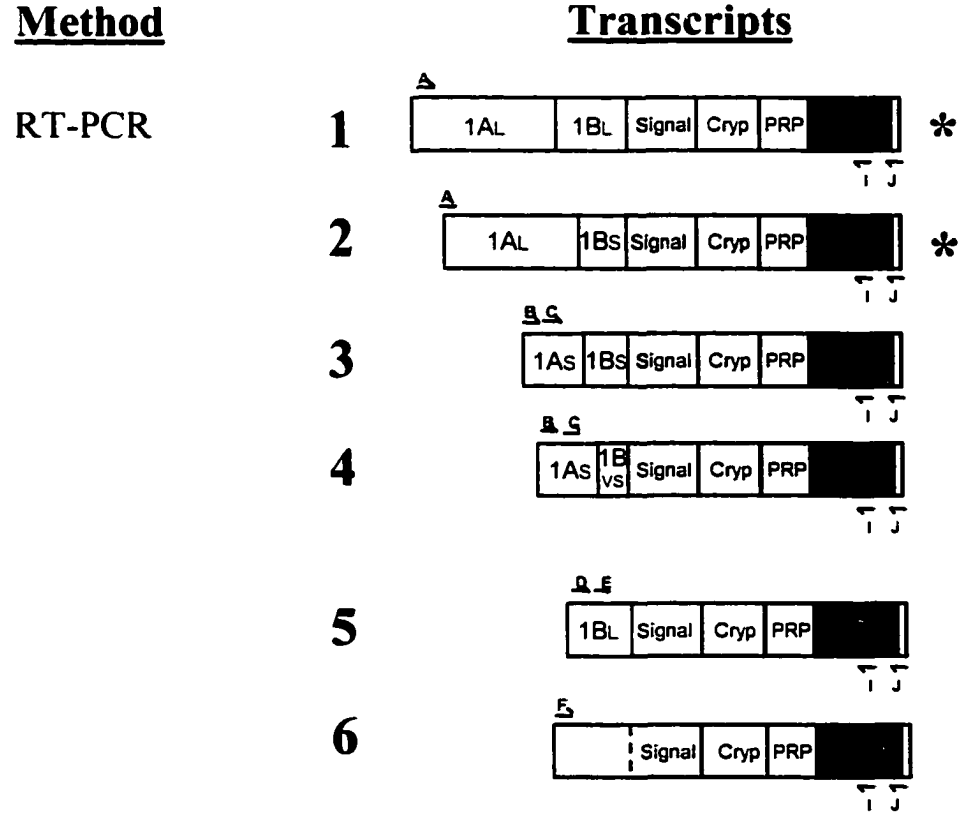

Method
Transcripts
RT-PCR
1
2
3
4
5
6
1AL
1BL
1BS
1AS
1B vs
Signal
Cryp
PRP
*

Table 2.2 - Location of the alternative forms of exons 1A, 1B and 2 in reference to the gene sequence in Fig. 2.1.

Exon		Gene location
1A	L	2375-2572
	S	2508-2572
1B	L	2772-2996
	S	2889-2996
	vs	2936-2996
2	L	3175-3499
	S	3392-3499

Table 2.3 - Tissue distribution of the six PACAP transcripts with alternate 5'UTRs.

	Brain	Ovary	Testis	GI tract	Thymus
Transcript 1	X				
Transcript 2	X				
Transcript 3	X	X	X		X
Transcript 4				X	
Transcript 5	X		X		
Transcript 6	X				

Fig. 2.3 as transcripts 1 and 2. Both were isolated only from the brain. Transcripts 1 and 2 differ from the others by the presence of a long exon 1A, which is derived from the 5'end of exon 1(Table 2.2). Transcripts 1 and 2 were obtained by using a primer directed towards a region upstream of the TATA-element making these transcripts 138 bp longer than the previously identified short (S) exon 1A (1As). Exon 1A long (L) is spliced to either a short or long exon 1B and then to an internal acceptor site in exon 2. We did not detect transcripts 1 or 2 in the thymus, ovary, testis or GI tract. The PCR reaction using the sense primer preA, which is located 90 bp upstream of primer A, did not reveal any products. The alternative internal splice sites (3' acceptor sites) include one possible site within exon 1A, two sites within exon 1B and one site within exon 2.

DISCUSSION

Postnatal expression of the PACAP mRNA

In mouse, six tissues (brain, eye, thymus, gastrointestinal tract, ovary and testis) expressed PACAP mRNA either in the adult or at various times throughout postnatal development. In 3-day-old pups, postnatal PACAP mRNA expression occurred in only three tissues (brain, eye, thymus) of the 12 tissues that were tested in our study. By 2 weeks of age, the brain, eye and thymus continued to express PACAP mRNA and, in addition, PACAP mRNA was detected in the ovary and testis. At 4 weeks of age, the pattern was the same except that the ovary no longer expressed PACAP mRNA and at six weeks, the thymus and testis did not express PACAP mRNA. Expression of PACAP mRNA was detected in the GI tract only in adults.

In the present study, PACAP mRNA was expressed in the brain from postnatal day 3 to adulthood and was used as a positive control for all expression studies. PACAP mRNA is first expressed in mouse at embryonic day (E) 9.5, continues to be expressed throughout embryonic development peaking at birth, and continues throughout postnatal development to adulthood (Shuto et al., 1996). Both PACAP immunoreactivity and mRNA expression have been shown in the central nervous system of embryonic, postnatal and adult mammals (Shuto et al., 1996; Sheward et al., 1998; Nielsen et al., 1998; Waschek et al., 1998; Sherwood et al., 2000 and Vaudry et al., 2000).

The data presented here provide the first report of PACAP mRNA expression in eye tissue in mouse. Expression was detected at 3 days after birth and in postnatal (2 weeks) and juvenile (4 and 6 weeks) mice. Olianas et al. (1997) showed expression of PACAP mRNA in the retina of the human fetus and immunoreactive PACAP has been detected in the retina of the adult rat (Hannibal et al., 1997; Seki et al., 2000). PAC_1 receptor immunoreactivity and mRNA expression have been identified in the retina of the rabbit and rat (Cavallero et al., 1996; Seki et al., 1997). These findings along with our result of early and continuous expression, suggest PACAP has a role in the eye.

Thymic tissue of mice at 3 days, 2 weeks and 4 weeks of age expressed PACAP mRNA. The expression pattern of PACAP mRNA in the thymus is interesting as it correlates with the activity of the thymus in the postnatal mice. Atrophy of the thymus occurs in mice so that lack of PACAP expression beginning at 6 weeks of age suggests PACAP plays a role in maturation of T cells. Because the thymus is a site of significant apoptosis, PACAP expression in this tissue may be related to its role as a survival factor (Gonzalez et al., 1997; Oka et al., 1999; Delgado and Ganea, 2000; Vaudry et al., 2000).

Indeed, PACAP has been shown to protect rat thymocytes from glucocorticoid-induced apoptosis (Delgado et al., 1996). Although immunoreactive PACAP in the thymus was reported for adult (90 days old) rats (Gaytan et al., 1994), our study is the first to report PACAP mRNA in the thymus of early postnatal and juvenile animals.

PACAP mRNA was not expressed in the gastrointestinal tract of postnatally developing mice (from 3 days to 6 weeks old), but PACAP mRNA was detected in the adult GI tract. PACAP immunoreactive nerve fibers have been identified in the gastrointestinal tract of many mammals including mice. PACAP appears to have a relaxant effect on gastric smooth muscle directly via PACAP receptors and indirectly through stimulation of the CNS (Sherwood et al., 2000). PACAP mRNA is expressed at low copy number in the GI tract fibers and may not be detectable until the adult stage is reached.

In adult gonads PACAP mRNA was expressed in both ovary and testis, although expression was variable in the ovary when repeated in several animals. Gras et al. (1996) showed PACAP mRNA was expressed in the rat ovary during estrus, but not at other stages of the cycle. The variable PACAP mRNA expression in the adult ovary suggests PACAP expression in this tissue is affected by the estrus cycle. PACAP mRNA expression was detected in the 2-week-old prepubescent ovary suggesting a role in maturation of this reproductive organ. PACAP has been shown to be involved in meiotic maturation of rat oocytes (Apa et al., 1997). We found that PACAP mRNA was absent in the 4-week-old ovary, reappeared by 6 weeks, and was variably present in adult samples supporting the idea that PACAP is expressed only in certain stages of ovarian development and in a cyclical manner in adults.

In testis PACAP mRNA was not expressed in 3-day-old mice, but was expressed in 2- and 4-week-old and adult mice. This is the earliest that PACAP mRNA expression has been reported for testis. PACAP mRNA was not expressed in testis of 6-week-old mice. PACAP mRNA is known to be expressed in human and rat adult testis (Ghatei et al., 1993; Shioda et al., 1994; Kononen et al., 1994; Daniel and Habener, 2000). In rat seminiferous tubules, PACAP mRNA expression has been shown to be stage specific (Kononen et al., 1994; Daniel and Habener, 2000) and thus may explain the lack of expression in the 6-week-old testis.

There is speculation regarding the presence of PACAP mRNA within the pancreas. One group has reported PACAP mRNA within isolated islet cells from rat (Yada et al., 1997). Other studies suggest PACAP acting on the pancreas is released from nerve terminals innervating the tissue (Filipsson et al., 2001). We did not identify PACAP mRNA in the pancreas of mouse at any postnatal age.

Novel transcripts of the mouse PACAP gene

This study used RT-PCR to identify 2 novel transcripts of the PACAP gene, both that extend the 5'UTR in the 5' direction (transcripts 1 and 2, Fig. 2.3). Transcripts 1 and 2 were found by using a primer (A) upstream of the TATA box. The start site is predicted to be within 90 bp upstream as no products were generated by PCR using a primer 90 bp upstream of primer A (preA) (Table 2.1, Fig. 2.3). This transcription start site does not appear to be in a position that would be associated with any of the three putative TATA boxes shown in Fig. 2.1. Previous reports have identified 5 alternate transcripts of the PACAP gene with 5'UTRs of various lengths. In 1998, Yamamoto et

al. screened a cDNA library and used 5' RACE on RNA isolated from male mouse brains to identify 3 transcripts and later Tabuchi et al. (2001) identified two additional transcripts using RT-PCR on mRNA from cultured cerebellar granule cells. This study confirms these previous results by identifying two of the three transcripts found by Yamamoto et al. (transcripts 5 and 6) and both of the transcripts identified by Tabuchi et al (transcripts 3 and 4) (Fig. 2.3). In addition we report two novel transcripts and their tissue distribution.

Our laboratory identified two other novel PACAP mRNAs. One from brain starts with a shortened exon 1B and was identified using 5'RACE and the other from T cells has a 19 amino acid addition at the start of the coding region for the PACAP-related peptide (PRP) (Cummings et al., 2002). The alternatively spliced PACAP precursor mRNA in T-cells may be a cell-specific post-transcriptional mechanism to control PACAP production. This is the first example of alternate splicing occurring in a protein coding region of the PACAP gene.

Tissue-specific transcription for the PACAP gene

Expression of the PACAP transcripts is unique within each tissue type. Transcripts 1 and 2, both with exon 1A extended upstream beyond the TATA region, were identified only in brain tissue. It is unclear whether these transcripts result from an alternative, brain-specific promoter or from other TATA-like elements in the PACAP promoter (Fig. 2.1). Alternative promoters have been identified for PACAP in the testes (Daniel and Habener, 2000) and for GHRH in the placenta (Gonzalez-Crespo and

Boronat, 1991) and testes (Srivastava et al., 1995). Each of these distal promoters was >10 kb upsteam from the proximal promoter.

Transcript 3 had the widest tissue distribution, being expressed in brain, ovary, testis and 4-week-old thymus. Transcript 4, which differs from transcript 3 by only a 54 bp deletion in exon 1B, was found solely in the GI tract. Tabuchi et al. (2001) isolated this transcript from cultured cerebellar granule cells, but we did not detect this form in brain tissue. Transcript 5 was identified in the adult mouse brain by Yamamoto et al. (1998). We too found this transcript to be expressed in the brain, but additionally in the testes. Transcript 6 was found only in the brain.

Functional implications of alternate splicing

Using RT-PCR we have identified 6 transcripts with different 5'UTRs and another study from our laboratory using 5'RACE has identified a transcript with yet another 5'UTR. The identification of 7 different PACAP mRNAs provides evidence for a complicated splicing regime of the PACAP RNA in mouse (Fig. 2.2). Alternate splicing of transcripts has been shown to be important in the tissue-specific or development-specific expression of genes (Gray and Wickens, 1998). In the PACAP gene in rat, a testis-specific transcript (Hurley et al., 1995) is produced through the use of a testis-specific promoter located approximately 13.5 kb upstream of the hypothalamic-specific promoter (Daniel and Habener, 2000). This transcript provides evidence for tissue-specific processing in the PACAP gene. The alternate splicing that generates PACAP transcripts with variable 5'UTRs as identified in this study may be important in tissue-specific or developmental expression of PACAP. Also, there are examples of

activity-induced splicing for the PACAP gene. First, novel transcripts (see transcript 3 and 4 in Fig. 2.2) in mouse cerebellar granule neurons were identified after depolarization and calcium influx via L-type voltage-dependent calcium channels (Tabuchi et al., 2001). Second, depolarization was shown to induce a shorter PACAP transcript in sympathetic neurons due to alternative cleavage and polyadenylation at the 3' end of the molecule (Harakall et al., 1998). These studies imply that the expression of specific PACAP 5'UTRs is influenced by the activity of the cell. The authors suggest that this alternative usage of 5'UTRs may regulate PACAP functions in these cell types by altering the amount of PACAP protein produced.

This study concludes that a diverse array of PACAP transcripts are produced through alternative splicing and alternative start-site usage in the 5'UTR. Considering the multi-functional nature of PACAP, different 5'UTRs may act to regulate translation so that different quantities of PACAP protein are produced in relation to the developmental stage or physiological status.

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CHAPTER 3

Targeted disruption of the pituitary adenylate cyclase-activating polypeptide (PACAP) gene in mouse.

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INTRODUCTION

As the most highly conserved member of the glucagon superfamily of hormones, PACAP likely plays an important physiological role (Sherwood et al., 2000). The hormones of the glucagon superfamily are located primarily in the brain and gut and play important roles in growth and metabolism. PACAP is found in two forms, PACAP-27 (Miyata et al., 1990) and PACAP-38 (Miyata et al., 1989); both are able to stimulate cAMP accumulation in many tissues. PACAP has been reported to release a number of hormones from different endocrine glands. In the pancreas, PACAP releases both insulin (Yada et al., 1994, Arimura, 1998, Filipsson et al., 1998a,b, 1999, Borboni et al., 1999) and glucagon (Fridolf et al., 1992). In the adrenal gland, PACAP releases catecholamines (Arimura, 1998) and, depending on the species, glucocorticoids (Breault et al., 1998, Andreis et al., 1995, Yon et al., 1994). In the pituitary gland, the release of several hormones is enhanced, which makes PACAP distinct from most other neuropeptide-releasing factors at physiological levels (Rawlings and Hezareh, 1996). In other functions PACAP has been shown to be a vasodilator (Sherwood et al., 2000), to regulate the cell cycle in tissue culture (Gonzales et al., 1997, Lu and DiCicco-Bloom, 1997) and to have immunoregulatory properties (Arimura, 1998, Delgado et al., 1998, Delgado and Ganea, 1999). Brain development is thought to be affected by PACAP and early expression of the genes for both PACAP and its specific receptor (PAC_1-R) occurs at embryonic day 9.5 in mice (Sheward et al., 1998). However, it is not clear whether the physiological effects of PACAP are a coordinated response to one specific stimulus or to many different types of stimuli. Whether in vitro results are relevant to in vivo functions remains to be shown.

The single gene encoding PACAP in mice is a suitable candidate for targeted deletion. In contrast, complete deletion of the receptors would be more difficult because at least three genes encode the receptors for PACAP and two of the receptor types are shared with another hormone. The three receptor types are a subset of the G-protein coupled 7-transmembrane family (Laburthe et al., 1996). One receptor is a PACAP-specific receptor (PAC_1) with 10 isoforms (Spengler et al., 1993, Journot et al., 1994, Pantaloni et al., 1996, Chatterjee et al., 1996, Dautzenberg et al., 1999, Daniel et al., 2001). The other two types of receptors ($VPAC_1$ and $VPAC_2$) are shared between PACAP and vasoactive intestinal peptide (VIP) (Usdin et al., 1994). VIP, like PACAP, is also a member of the glucagon superfamily and the two peptides have some overlap of function. Thus, to separate the functions of PACAP and VIP it is important to disrupt the single copy PACAP gene.

The objective of this study is to determine whether PACAP is essential for survival and which functions can be ascribed only to PACAP. In evolution, PACAP's importance is shown by its high degree of conservation (McRory et al., 1997, Chartrel et al., 1991, Parker et al., 1997): across 600 million years of evolution, PACAP-27 has remained 96% identical to human PACAP (McRory and Sherwood, 1997). By use of gene targeting to generate mice lacking the PACAP gene, we analyze the physiological role of PACAP in an in vivo mammalian model.

MATERIALS AND METHODS

Gene Targeting

Homologous recombination

A clone containing 18 kb of the PACAP gene was isolated from a 129SvJ mouse genomic library (Stratagene, LaJolla, CA), restriction mapped and partially sequenced (Fig. 2.1, 3.1). A Cre-loxP targeting vector (Flox) was constructed (Kühn et al., 1995, Gu et al., 1994) containing a 5'arm of ~10 kb, a 3'arm of 1 kb and a region of ~4 kb which contained the coding region for PACAP to be deleted (Fig. 3.2).

The Flox vector containing three pieces of the PACAP clone was linearized and electroporated (240 V) into RI 129SvJ embryonic stem cells (ES cells) (Nagy et al., 1993) which were growing in log phase (1 μg of construct per 1 million cells was used). The electroporated cells were plated at a density of ~2 million cells /plate and 24 hrs post electroporation were exposed to G418 at a concentration of 180 μg/ml. The Flox vector contains two selection genes, the neomycin resistance gene and the herpes simplex virus thymidine kinase gene (HSV-TK) (Fig. 3.2). Therefore, only cells that had incorporated the construct would be able to grow in the presence of G418. The cells were grown for 10 days under these selection conditions and ~1000 clones were picked and grown up individually. DNA was extracted from an aliquot of each clone allowing clones containing homologous recombination events to be identified by polymerase chain reaction (PCR) (Fig. 3.3).

Fig. 3.1. A clone (18 kb) containing the mouse PACAP gene isolated from a 129SvJ mouse genomic library (Stratagene). The clone is contained within lambda phage (λ). 5'UTR, 5'untranslated region; PRP, PACAP-related peptide; 3'UTR, 3'untranslated region.

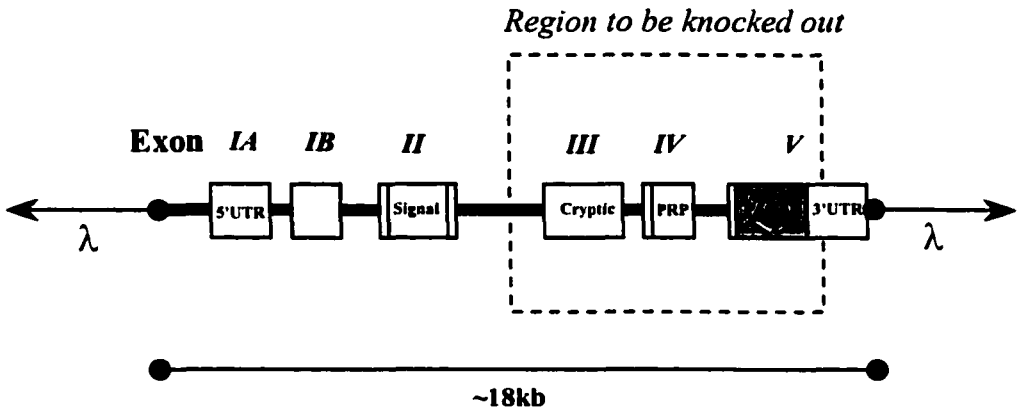

Region to be knocked out
Exon IA IB II III IV V
5'UTR
Signal
Cryptic
PRP
3'UTR
λ
λ
~18kb

Fig. 3.2. Representation of the cre-lox targeting vector (Flox) used for targeted disruption of the mouse PACAP gene. The vector contains approximately 10 kb of 5'flanking region and 1 kb of 3' flanking region. The region to be knocked out contains the cryptic peptide, PACAP-related peptide (PRP) and PACAP (shown by a black box). The selection region contains a neomycin resistance gene (Neo) and the herpes simplex virus-thymidine kinase gene (TK). The vector was linearized for transformation and the site of linearization is shown by an arrow. Lox sites are represented by black triangles.

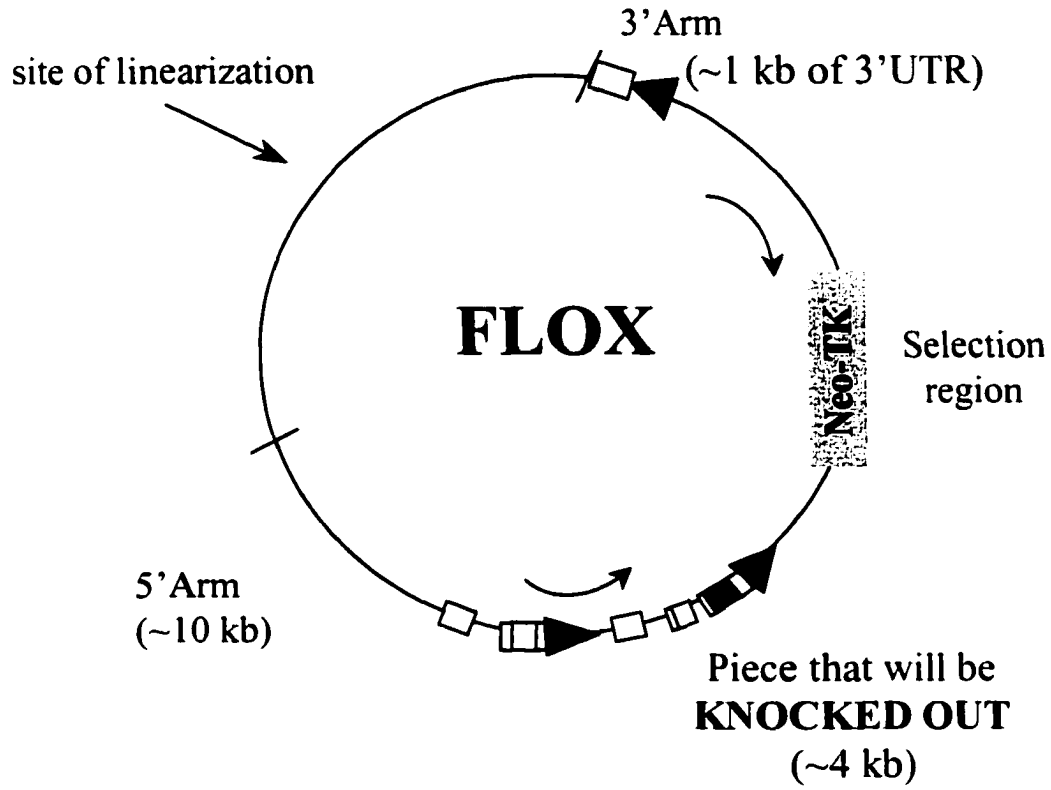
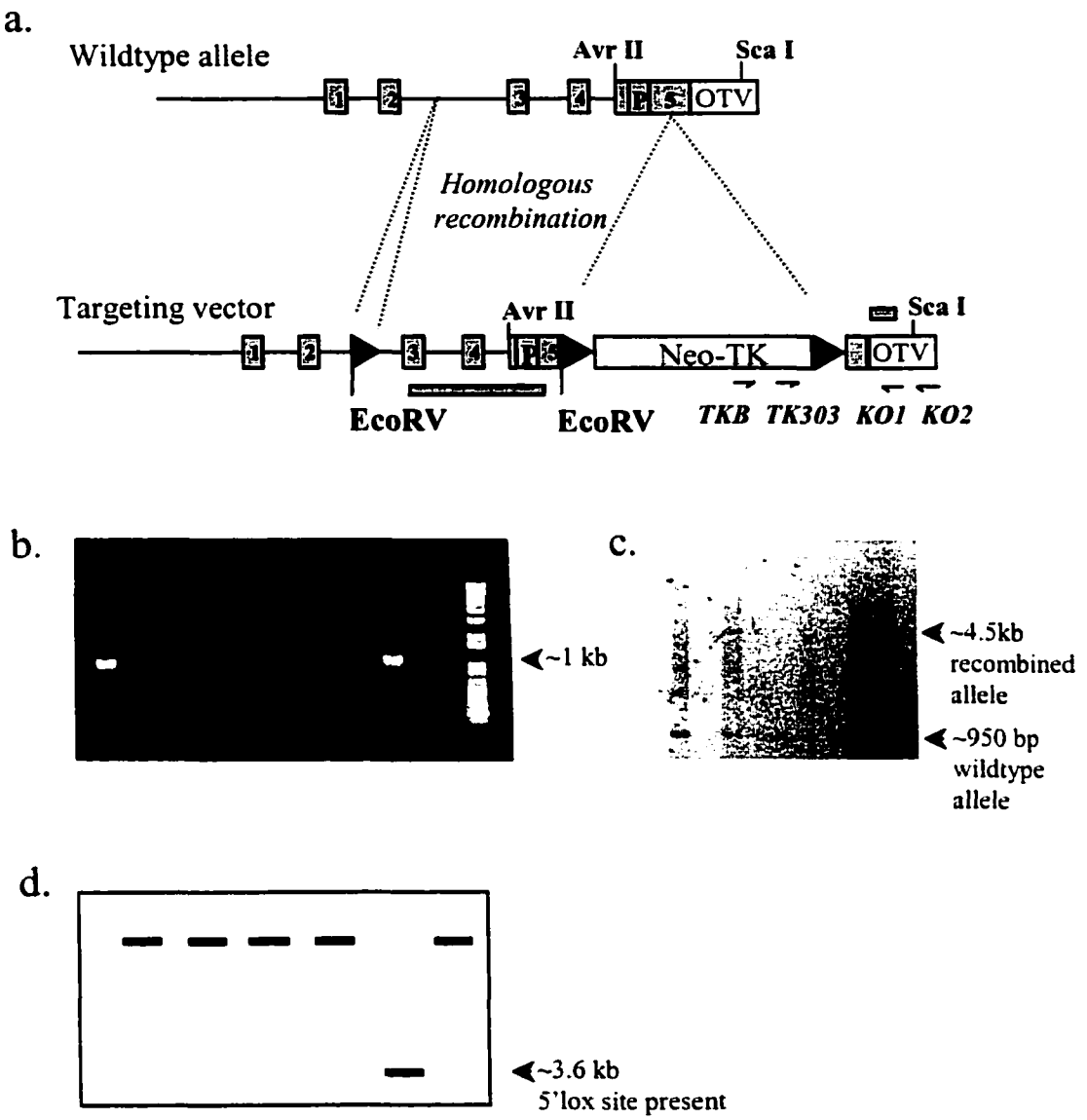

3'Arm
(~1 kb of 3'UTR)
site of linearization
FLOX
Neo TK
Selection
region
5'Arm
(~10 kb)
Piece that will be
KNOCKED OUT
(~4 kb)

Fig. 3.3. Targeting strategy used for homologous recombination at the PACAP locus in mouse embryonic stem (ES) cells. a.) Homologous recombination of the targeting vector with the wildtype PACAP allele. PCR primers (TKB, TK303, KO1 and KO2) used to identify potential positives are shown by arrows. The restriction enzyme (Avr II and Sca I) cut sites and probe (grey rectangle) location for Southern blot analysis to identify positive recombinants is shown. b.) A representative PCR of ES cell genomic DNA. A band of approximately 1 kb represents potential positives for homologous recombination of the targeting vector. c.) Southern blot analysis of Avr II and Sca I cut genomic DNA from potential positive ES cell clones identified by PCR using a probe (grey rectangle) to the 3'UTR outside of the targeting vector. d.) Diagram of a Southern blot analysis of recombined ES cell clones to determine if the 5'lox site of the targeting vector was intact. Only ES cell clones that contain the complete targeting vector produce a band of 3.6 kb when genomic DNA is cut with Eco RV. P, coding region for PACAP; Neo-TK, selection region of the targeting vector containing the neomycin resistance gene and the herpes simplex thymidine kinase gene; OTV, outside targeting vector.


a.
Wildtype allele
Avr II
Sca I
OTV
Homologous recombination
Targeting vector
Avr II
Sca I
Neo-TK
OTV
EcoRV
EcoRV
TKB
TK303
KO1
KO2
b.
~1 kb
c.
~4.5kb recombined allele
~950 bp wildtype allele
d.
~3.6 kb
5'lox site present

Screening for homologous recombinants

The 50 µl PCR reaction contained 2.5 U Taq polymerase (Gibco BRL), 1x Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$, 200 mM dNTPs, and 20 pmol of primers TKB and KO2 (Fig. 3.3) (Table 3.1). This reaction was carried out under the following conditions: denaturation at 94°C for 5 minutes, 94°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 72°C for 30 seconds for 32 cycles and a long extension of 7 minutes. Nested PCR was then performed under the same conditions as above using the primers TK303 and KO1. Products produced by the PCR reaction were separated on a 1% agarose gel and visualized by ethidium bromide staining. A band of ~1 kb was produced from homologous recombinants (Fig. 3.3b). Of approximately 1000 clones screened, 11 ES cell clones were identified as positive for homologous recombination by PCR.

Southern blot analysis was done for all PCR positive clones, as PCR can give false positives. Genomic DNA from ES cell clones was extracted and digested with the restriction endonucleases Avr II and Sca I (Fig. 3.3a). Digested DNA was run on a 1.5% agarose gel and transferred to a Hybond nylon membrane (Amersham, Baie d'Urfe, Quebec) by capillary transfer with 0.4 M NaOH. A 400 bp probe that would hybridize with a region of the 3'UTR outside of the targeting vector was made by digesting an expressed sequence tag (EST) clone obtained from American Type Culture Collection (ATCC) that contained the 3'UTR outside of the targeting vector. The probe was labeled with ^{32}P-dCTP by random priming (Gibco BRL, ON). The membrane was prehybridized in 6X SSC, 5X Denhardts and 0.5% SDS. Hybridization of the probe to the membrane was performed at 55°C overnight with 300 µg of sea urchin sperm DNA as blocking

Table 3.1 - Sequence of primers used in the generation and phenotype analysis of PACAP null mice.

Primer	Direction	Sequence
TKB	sense	5' GGGAGTTTCACGCCACCAAG 3'
KO2	antisense	5' GCTGGCTTCTGCAGAACAGTG 3'
TK303	sense	5' TGAAAACCACACTGCTCGATCCG 3'
KO1	antisense	5' AGAGCTGGCTTCTGCAGAACAGTG 3'
5'MP1	sense	5' ATGTGTAGCGGAGCAAGGCTGG 3'
MP1	sense	5' AGCAGTGTCTCCTGTTCACCTG 3'
3'UTR1	antisense	5' GGCCATTATTGGTATCTTCAAGACGG 3'
3'MP2	antisense	5' GAACACGAGTGATGACTGGTCAGTC 3'
3'lox2	antisense	5' GAGGGTTTCTGGAGCTGCGACAGGG 3'
lox2	antisense	5' GCTCCAACTCCAGTTGAAACAGGG 3'
PA-1	sense	5' CACTCGGACGGCATCTTCACAGATAG 3'

DNA. The membrane was washed in 2X SSC and 0.1% SDS for 30 min at 55°C, in 1X SSC and 0.1% SDS for 30 min at 55°C, in 0.5X SSC and 0.1% SDS for 30 min at 55°C and finally in 0.1X SSC and 0.1% SDS for 30 min at 55°C. The membrane was then exposed to Kodak diagnostic film (Rochester, NY) at –80°C for 7 days. The homologous recombinants produced a band of ~4500 bp while the wildtype allele produced a band of ~950 bp (Fig. 3.3c). Southern analysis of the 11 PCR positive clones revealed six were recombined with the targeting vector at the PACAP locus.

A second Southern Blot was performed under the same conditions on the six positive clones determined by Southern blot to confirm that the 5'lox site had been recombined (Fig. 3.3d). Genomic DNA from ES cell clones identified as positive by the previous Southern analysis was cut with EcoRV. EcoRV cut sites are not present in the wildtype PACAP gene. EcoRV sites are present within the targeting vector and therefore can be used to determine if the entire construct had been recombined (Fig. 3.3a). Digested DNA was electrophoresed and transferred to a membrane as above. A ^{32}P labeled probe that spanned exons 3 and 5 of the mouse PACAP cDNA generated by PCR and labeled by random priming was used (Gibco, BRL). The probe was hybridized to the membrane under the same conditions as above. Clones that had incorporated the 5'distal lox site were seen as a band of 3.6 kb on the Southern blot. Results of the Southern analysis of the six potential positive clones identified above showed that only one ES cell clone contained the complete targeting vector at the PACAP loci (Fig. 3.3d).

Cre recombination

Homologously recombined ES cells (13.5 million) from the first electroporation were transformed at 240 V with 1.4 μg of a construct containing cre recombinase linked to enhanced green fluorescent protein (pEGFP-Cre) under the control of the CMV promoter. The electroporated cells were plated at a density of 250,000 cells/plate in ES cell media. Four days post electroporation, cells were exposed to 1 μM gancyclovir, a guanidine residue that in the presence of thymidine kinase is phosphorylated, thus becoming a DNA synthesis inhibitor. Because the recombined cells express the HSV-TK gene, any cells escaping cre transformation or cells that are type III recombinants retaining the TK region of the targeting construct, will be unable to survive in the presence of gancyclovir (Fig. 3.4). Clones were picked 9 to 14 days post electroporation and grown up individually. An aliquot of cells was taken and DNA extracted (using a proteinase K digestion) for PCR analysis.

Nested PCR was used to identify potential type I and II recombinants. The PCR reaction mixtures and conditions were the same as above. To identify type I recombinants, the two sets of primers 5'MP1/ 3'UTR I and MP1/ 3'MP2 were used (Fig. 3.5a) (Table 3.1). To identify type II recombinants the primers 5MP1/3'lox2 and MP1/lox2 were used (Fig. 3.6a, Table 3.1). PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Type I recombinants produced one band of 743 bp (Fig. 3.5b). Type II recombinants produced two bands, one representing the wildtype allele (610 bp) and another representing the type II cre recombined allele (734 bp) (Fig. 3.6b).

Fig. 3.4. Possible recombination by cre recombinase due to the presence of three lox sites. Type I recombinants are used to generate complete knockout of the PACAP gene. Type II recombinants can be used to generate inducible PACAP knockout mice. Type III recombinants are selected against in the presence of gancyclovir due to the presence of the herpes simplex virus-thymidine kinase (HSV-TK) gene. The coding region for PACAP is shown by a black box, lox sites are represented by black triangles.

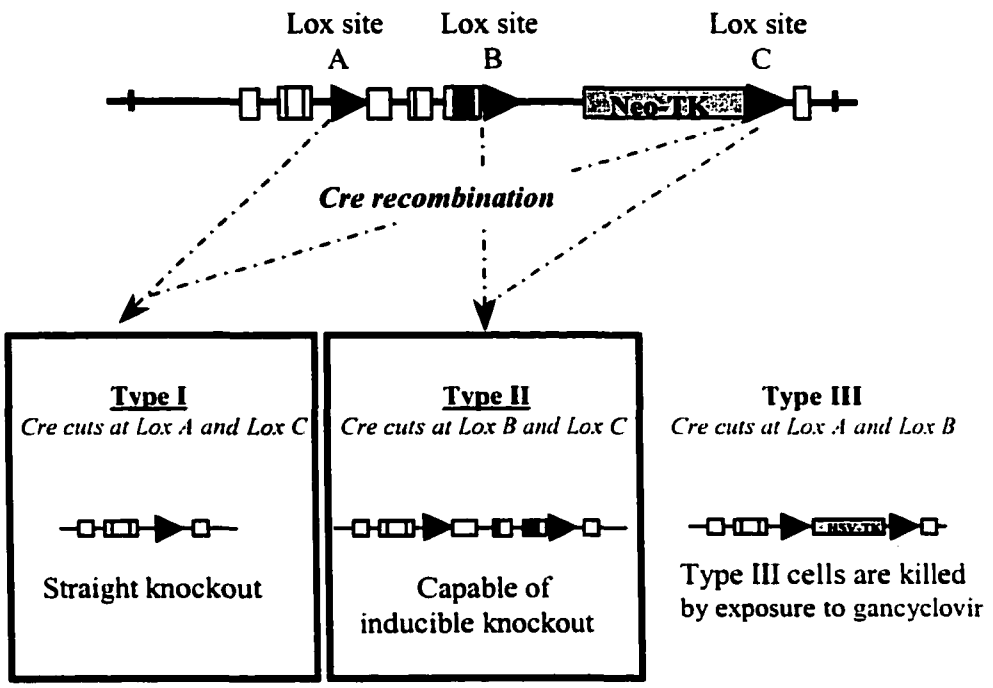
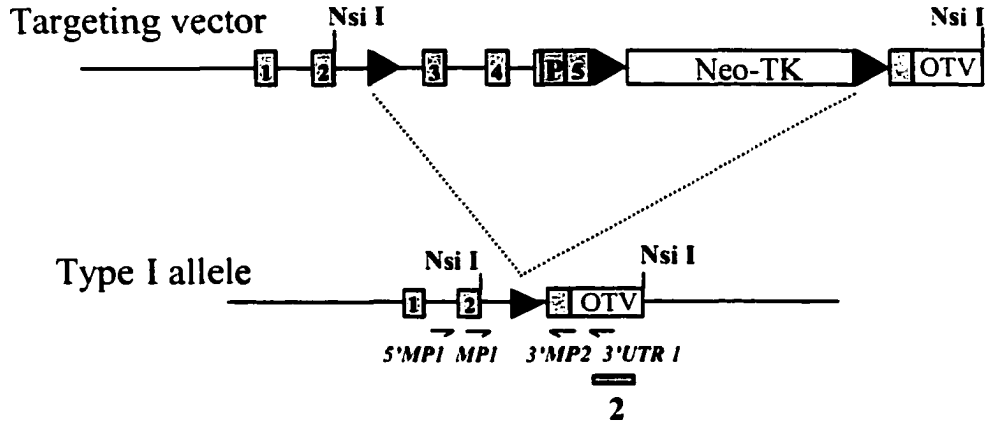

Lox site
A
Lox site
B
Lox site
C
Neo-TK
Cre recombination
Type I
Cre cuts at Lox A and Lox C
Straight knockout
Type II
Cre cuts at Lox B and Lox C
Capable of
inducible knockout
Type III
Cre cuts at Lox A and Lox B
HSV-TK
Type III cells are killed
by exposure to gancyclovir

Fig. 3.5. Cre recombination of the embryonic stem (ES) cells containing the homologously recombined targeting vector. P is the region encoding PACAP, TK-Neo is the selection region of targeting vector and OTV stands for outside targeting vector. a.) Representation of type I cre recombination. The position of the primers used for PCR and the restriction enzyme (Nsi I) cut sites and probe location used for Southern analysis are shown. b.) PCR results identifying a potential type I recombinant. The single 743 bp band represents the type I allele. A band from the wildtype allele is not generated as the product is too big to ampliy by PCR. c.) Results from a Southern blot using the strategy shown above. Type I recombinants show two bands, one representing the wildtype allele (5.1 kb) and another representing the type I recombined allele (1.7 kb).

a.


Targeting vector
Nsi I
Nsi I
1
2
3
4
5
Neo-TK
OTV
Type I allele
Nsi I
Nsi I
1
2
OTV
5'MP1
MP1
3'MP2
3'UTR I
2

b.


type I allele (743 bp)

c.

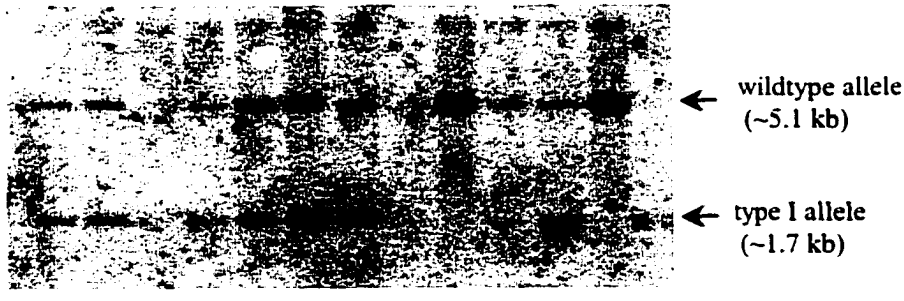
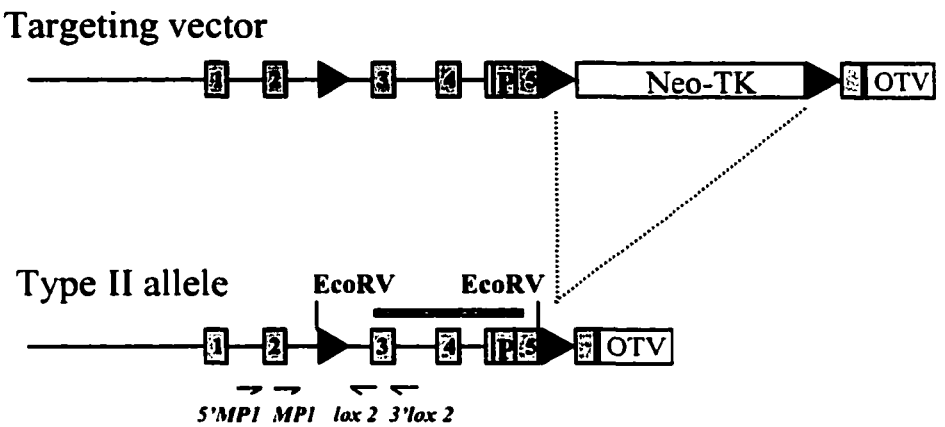
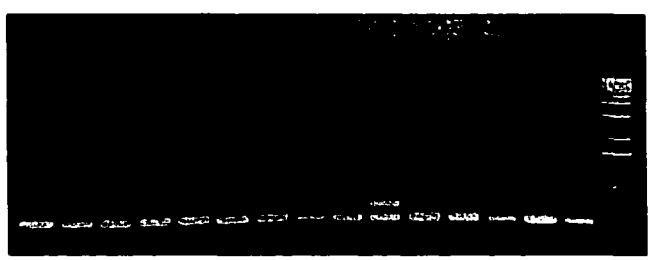

wildtype allele
(~5.1 kb)
type I allele
(~1.7 kb)

Fig. 3.6. Type II cre recombination of embryonic stem (ES) cells containing the homologously recombined targeting vector. P is the region encoding PACAP, TK-Neo is the selection region of the targeting vector and OTV stands for outside targeting vector. a.) Representation of type II cre recombination. The position of the primers used for PCR, the Eco RV cut sites and the location of the probe (grey rectangle) used for Southern analysis are shown. b.) PCR results identifying a potential type II recombinant. The smaller band (610 bp) represents the wildtype allele and the larger band (734 bp) represents the type II allele. c.) Diagram of a Southern blot using the strategy shown above. The wildtype allele is not cut by Eco RV whereas the type II allele containing the lox sites is cut twice. A 3.6 kb band on the gel represents the presence of a type II recombined allele.

a.

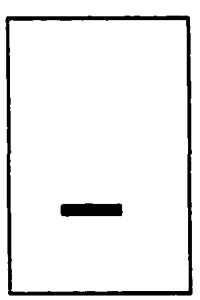


b.



type II allele (734 bp)
wildtype allele (610 bp)

c.



type II allele
(~3.6 kb)

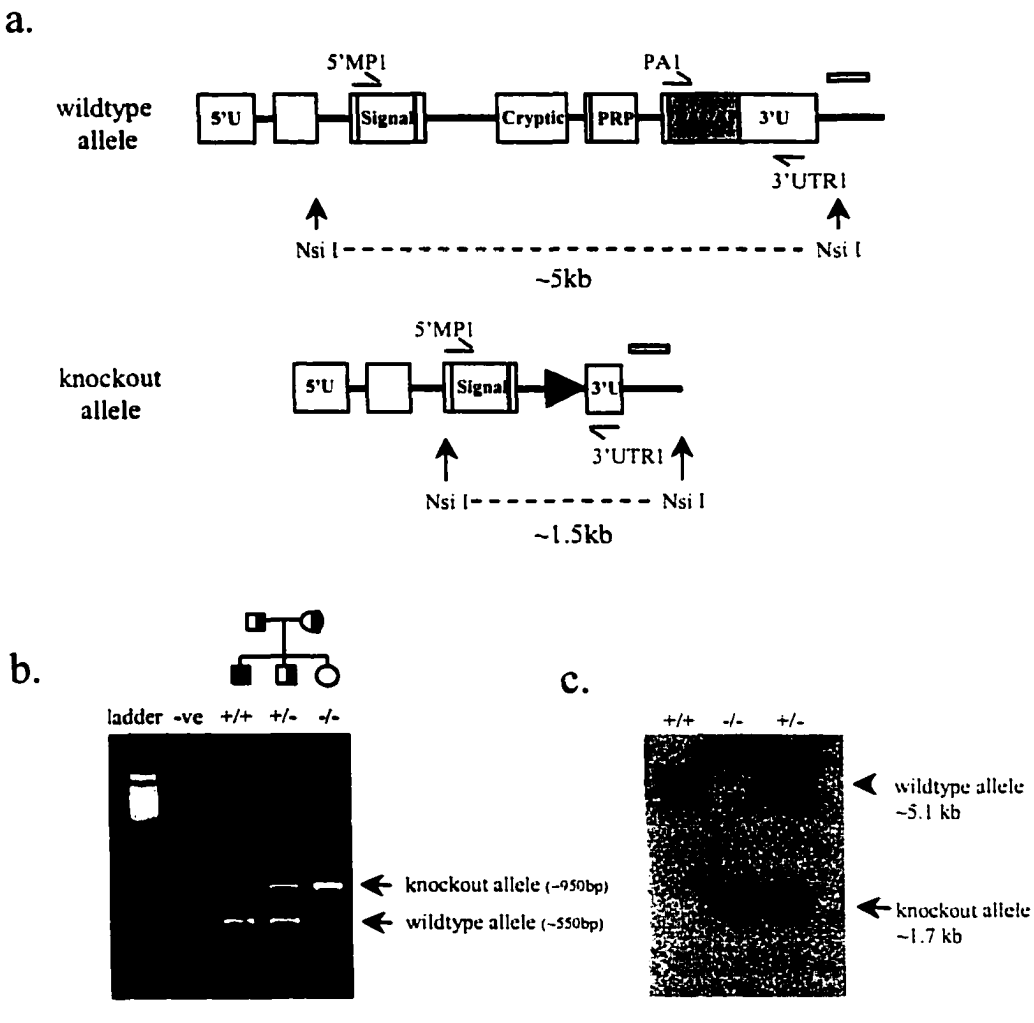
All type I and type II PCR positives were confirmed by Southern blot analysis. Genomic DNA from the potential type I ES cell clones was extracted and digested with Nsi I. A probe (400 bp) made to the 3'UTR outside of the targeting vector was labeled with ^{32}P by random priming. Hybridization occurred at 55°C overnight. Washes were performed as above. The type I allele produced a band of ~1.7 kb and the wildtype allele produced a band of ~5.1 kb (Fig. 3.5c). To confirm type II postive clones with Southern analysis, the same strategy and conditions as used to determine if the 5'lox site was intact was used. As such type II clones were represented by a 3.6 kb band on the Southern blot (Fig. 3.6c). Type II recombinant ES cell clones were expanded and are stored in liquid nitrogen for future generation of an inducible PACAP knockout mouse.

Generation of PACAP null mice

ES cells heterozygous for the PACAP deletion (type I) were expanded and microinjected into C57/Bl6 blastocysts by Anita Borowoski. Germline transmission of the PACAP deletion was achieved from two male chimeras. Heterozygous littermates were crossed to produce mice homozygous for the PACAP deletion. Mice for all studies were genotyped using a PCR strategy that had been confirmed by Southern blot (Fig. 3.7).

The PCR strategy used for genotyping was a 50 µl reaction that contained 2.5 U Taq polymerase (Gibco BRL), 1x Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$, 200 mM dNTPs, and 20 pmol of the three primers 5'MP1, PA1 and 3'UTR 1 (Fig. 3.7a) (Table 3.1). The reaction conditions were: denaturation at 94°C for 5 minutes, 94°C for 30 seconds; annealing at 67°C for 30 seconds; extension at 72°C for 32 seconds for 30 cycles

Fig. 3.7. Genotyping of the mice born to heterozygous breeding pairs. a.) Representation of the mouse PACAP wildtype and PACAP knockout alleles. Location is shown for primers used for PCR of genomic DNA, Nsi I restriction enzyme cut sites and probe (grey rectangle) for Southern analysis. b.) PCR amplified genomic DNA from PACAP+/+, PACAP+/- and PACAP-/- mice using the primers 5'MP1, PA-1 and 3'UTR 1 in a single reaction. The knockout allele produces a band of approximately 950 bp and the wildtype allele produces a band of 550 bp, providing distinct banding patterns for each of the three genotypes. c.) Southern blot analysis of PACAP+/+, PACAP+/- and PACAP-/- mice genomic DNA using a probe to the 3'UTR of the PACAP gene. 5'U, 5'untranslated region; 3'U, 3'untranslated region; PRP, PACAP-related peptide.


a.
wildtype allele
5'MP1
PA1
5'U
Signal
Cryptic
PRP
3'U
3'UTR1
Nsi I
Nsi I
~5kb
knockout allele
5'MP1
5'U
Signal
3'U
3'UTR1
Nsi I
Nsi I
~1.5kb
b.
ladder -ve +/+ +/- -/-
knockout allele (~950bp)
wildtype allele (~550bp)
c.
+/+ -/- +/-
wildtype allele ~5.1 kb
knockout allele ~1.7 kb

and a long extension of 7 minutes. Products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light (Fig. 3.7b). The Southern blot used to confirm the genotyping results obtained from the above PCR strategy was the same as the one used to identify type I recombinants (Fig. 3.7c).

The procedures used in all studies were approved by the University of Victoria's Animal Care Committee and by the University of British Columbia's Animal Ethics Committee.

Detection of mRNA in Brain and Liver Tissue

PACAP+/+, PACAP+/- and PACAP-/- mice were euthanized using isofluorane. Liver and brain tissues were collected from all three genotypes and frozen immediately in liquid nitrogen. Tissue was ground into a fine powder using a chilled mortar and pestle. RNA was isolated using TRIzol (Gibco BRL). mRNA (5 μg) was reverse transcribed with Superscript II reverse transcriptase using oligo dT. The RNA was incubated with 0.2 μM oligo dT and heated to 70°C for 10 min to linearize the RNA. The remaining components of the reaction mix were added to produce a 50 μl reaction containing 1X single strand buffer, 2 mM dNTPs, 10 mM dithiothreitol (DTT) and 100 units Superscript II reverse transcriptase (Gibco, BRL). The reaction was incubated at 42°C for 90 min and at 90°C for 10 min to denature the reverse transcriptase. The cDNA generated from the above reaction was added to a 50 μl reaction containing 2.5 U Taq polymerase (Gibco BRL), 1x Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$, 200 mM dNTPs, and 20 pmol of primer 5'MP1 and primer 3'MP2 (Table 3.1). PCR was carried out under the following conditions: denaturation at 94°C for 30 sec; annealing at 61°C for 30 sec; extension at

72°C for 45 sec for 33 cycles and a long extension of 7 min. Products produced by the PCR reaction were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Western Blot

Protein was extracted from PACAP+/+, PACAP+/- and PACAP-/- mice brain using NP-40 lysis buffer with protease inhibitors. Protein (100 μg) was run through a 16.5% Tris-tricine gel (BioRad Laboratories Inc., Hercules, CA). The protein was transferred onto a PVDF membrane (Bio-Rad Laboratories Inc.) and blocked overnight at 4°C in Tris-buffered saline with Tween 20 (TBST) and 5% BSA. The membrane was then probed with rabbit anti-PACAP-38 (Peninsula Laboratories, Belmont, CA), diluted 1:2000 in TBST with 1% BSA overnight at 4°C, washed 3 times with TBST and incubated with secondary goat anti-rabbit antibody (Dako Corp., Carpenteria, CA) diluted 1:10,000 in TBST with 1% BSA for 45 min at room temperature. The blot was washed 4 times with TBST and antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England) and exposed to film.

Light Microscopy

Tissues (brain, heart, lung, thymus, stomach, liver, pancreas, spleen, intestine, kidney, adrenal gland, skin, skeletal muscle and bone) from 8-10 day old PACAP+/+, PACAP+/- and PACAP-/- mice were collected and fixed in 4% paraformaldehyde in

PBS. Routine processing, paraffin embedding and sectioning (10 μm) were performed. All sections were stained with hematoxylin and eosin.

RESULTS

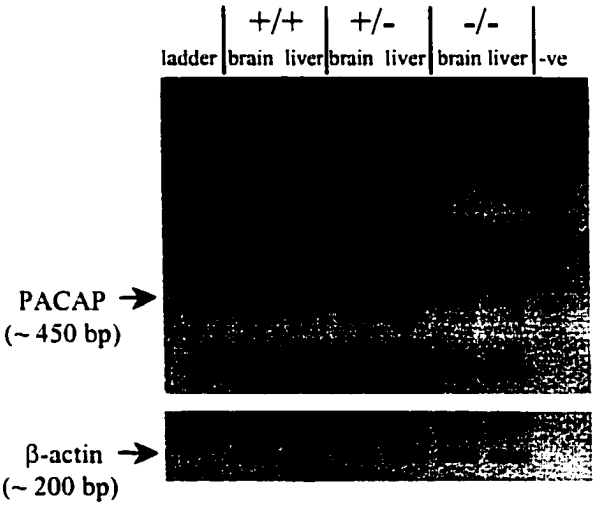
Generation of PACAP$^{-/-}$ Mice

Targeted disruption of the PACAP gene was generated through homologous recombination in mouse embryonic stem (ES) cells (Fig. 3.3a). The PACAP knockout mouse line was generated from two individual type I Cre recombinant clones, which were derived from one homologously recombined ES cell clone. A C57/Bl6 backcrossed line (7 backcrosses) of PACAP null mice has been generated and these PACAP null mice exhibit the same phenotype as the original genetically mixed PACAP null mice. This evidence supports the conclusion that the PACAP null mouse is free of non-linked mutations.

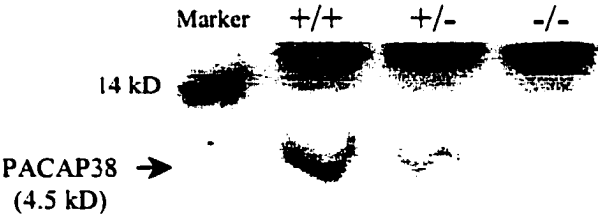
RT-PCR for PACAP mRNA (Fig. 3.8a) and Western blot for PACAP protein (Fig. 3.8b) showed that PACAP was not expressed in the brain of the PACAP-/- mice but was present in wildtype and heterozygous littermate controls. Genotyping of 270 offspring from heterozygous crosses showed the expected mendelian ratio of 1:2:1 (21% wild type, 52% heterozygotes and 27% homozygotes). PACAP null mice appeared normal at birth with no obvious signs of neuropathology or overt developmental abnormalities. In the second postnatal week, most PACAP-/- mice died. In the first group of homozygotes (n=73) the pups died or were used for the collection of serum samples after they became moribund. In the second group of homozygotes (n=86), 9% lived.

Fig. 3.8. a.) RT-PCR amplification of PACAP mRNA in brain and liver of PACAP+/+, PACAP+/- and PACAP-/- mice. mRNA is expressed in both PACAP+/+ and PACAP+/- brains whereas the PACAP-/- mouse does not express PACAP mRNA in the brain. b.) Western blot analysis of protein isolated from the brains of PACAP +/+, PACAP+/- and PACAP-/- mice. PACAP protein is present in PACAP+/+ and PACAP+/- mice but not in the brain of the PACAP-/- mouse.

a.


+/+
+/-
-/-
ladder
brain
liver
brain
liver
brain
liver
-ve
PACAP
(~ 450 bp)
β-actin
(~ 200 bp)

b.


Marker
+/+
+/-
-/-
14 kD
PACAP38
(4.5 kD)

Initial characterization of the PACAP null phenotype

PACAP null mice were born the same size as heterozygous and wildtype littermates and the three genotypes remained matched in weight for several days. Thereafter, a few of the PACAP-/- pups died suddenly without losing weight, whereas most died following several days in which they failed to gain weight and finally lost weight (Fig. 3.9a,b). All pups continued to nurse and had milk in their stomachs at or near the time of death.

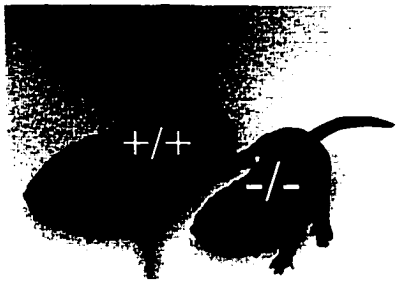
Necropsies, done at 6-8 days of age or at death, revealed that the livers of PACAP-/- mice were buff colored. Histological examination of 15 tissues stained with hemotoxylin and eosin showed microvesicular steatosis was present in hepatocytes of PACAP null mice (Fig. 3.10). In addition, subcutaneous white fat deposits were depleted totally in wasted PACAP-/- mice at the time of death. All other tissue did not show abnormalities.

DISCUSSION

Our interest in generating a mouse that is devoid of PACAP was to determine whether PACAP is critical to development and/or function of the neural, endocrine and peripheral organ systems. In addition, the PACAP-/- mice are useful to tease apart overlapping functions for PACAP and VIP, which are both pleiotropic and share a network of target sites. This unusual situation stems from the sharing of two receptors by PACAP and VIP (Usdin et al., 1994). In addition PACAP uses a specific 7-transmembrane receptor (Spengler et al., 1993). The other question is whether the responses initiated by PACAP are triggered by a specific physiological event(s) as occurs

Fig. 3.9. Weight differences among genotypes. a.) Difference between PACAP+/+ and wasted PACAP-/- littermates at postnatal day 10. b.) Mean weights of PACAP+/+ (n=3), PACAP+/- (n=11) and PACAP-/- (n=9) mice from two litters.

a.


+/+
-/-

b.

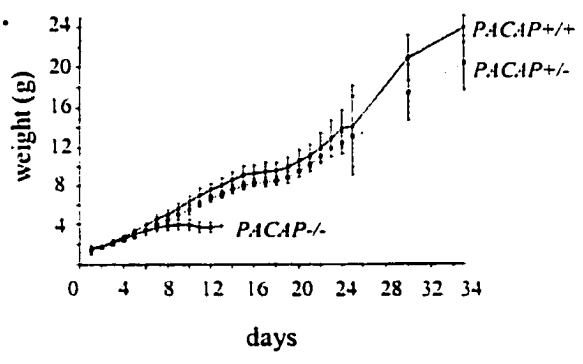
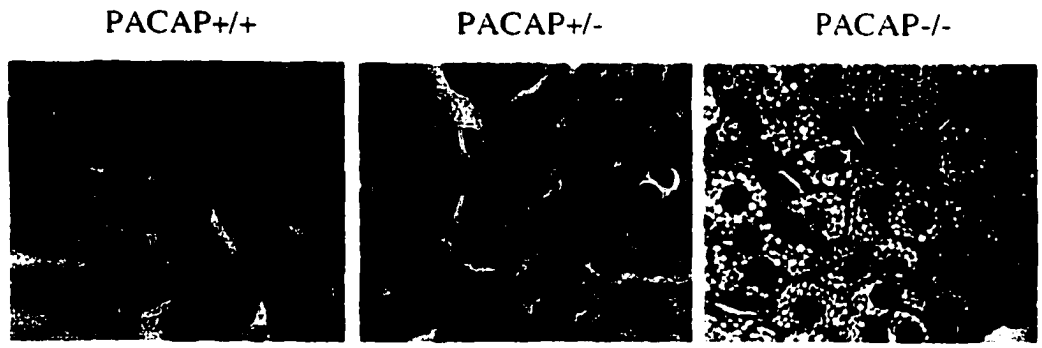

weight (g)
24
20
16
12
8
4
0
4
8
12
16
20
24
28
32
34
days
PACAP+/+
PACAP+/-
PACAP-/-

Fig. 3.10. Hemotoxylin and eosin stained sections of liver tissue from seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice, showing the morphology of the hepatocytes. The hepatocytes of PACAP-/- mice are filled with lipid droplets.


PACAP+/+
PACAP+/-
PACAP-/-

for many hormones. For example, high blood glucose is important for insulin release; high blood calcium for calcitonin release; and excitement or stress for adrenaline release.

Early expression of PACAP in the mouse embryo (E9.5) (Sheward et al., 1998) and its suggested role in patterning of the neural tube (Waschek et al., 1998) predicted PACAP null mice may die in utero or present with developmental abnormalities. Therefore the Cre-lox gene targeting strategy was used so that in the event that PACAP null mice did not survive to birth, an inducible PACAP knockout mouse could be made. Offspring of PACAP+/- breeding pairs produce PACAP+/+, PACAP+/- and PACAP-/- mice at the expected mendelian ratio indicating that PACAP-/- mice did not die in utero. PACAP null mice born to heterozygous parents appeared normal at birth with no obvious defects in neuropathology, despite a large body of literature suggesting a role for PACAP in early brain development (Sherwood et al., 2000).

Although PACAP null mice appear normal at birth, most of the PACAP-/- mice died in the second postnatal week in a wasted state. PACAP is a releaser of growth hormone (Rawlings and Hezareh, 1996), but the rapid weight loss in the PACAP null mice suggests that wasting rather than lack of growth hormone is involved as it is known that growth hormone deficiency does not obviously affect growth until 2 weeks of age in mice (Baker et al., 1993). It would be difficult to assess the role of growth hormone in the PACAP null mice because of lethality. The PACAP-/- mice show lipid accumulation in liver. In addition, white adipose tissue of wasted PACAP null mice was reduced or absent at death. The accumulation of lipids in hepatocytes and the reduction in white adipose tissue suggests lipid metabolism of PACAP null mice is affected.

Preliminary analysis of the PACAP null phenotype has identified a role for PACAP in lipid metabolism and has shown that PACAP is essential for survival. The ability of PACAP null mice to survive in utero and a lack of obvious developmental abnormalities suggests that PACAP is not essential for fetal development. Rather PACAP may regulate developmental processes in concert with other peptide hormones, such as VIP, and these hormones may compensate for PACAP's absence.

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CHAPTER 4

Altered carbohydrate and lipid metabolism in PACAP null mice.

A version of this chapter has been published as:

Gray SL, Cummings KJ, Jirik FR, Sherwood NM. 2001 Targeted disruption of the pituitary adenylate cyclase-activating polypeptide (PACAP) gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism. Molecular Endocrinology 15:1739-1747.

INTRODUCTION

Lipids are stored as triglycerides and are released from storage when carbohydrates are low. Triglycerides are broken down into free fatty acids, which circulate to metabolically active tissues (predominantly the liver and muscle tissue) where they undergo oxidative phosphorylation to produce energy. The storage and release of lipids is controlled by hormones that respond to chemical cues within the body. This balance appears to be altered in PACAP null mice, as high levels of lipid are present in the blood and metabolically active tissues (Gray et al., 2001). A direct role for PACAP in lipid metabolism has not previously been described, yet PACAP is capable of regulating several endocrine systems involved in the balance of carbohydrate and lipid metabolism.

A role for PACAP in regulating hormone release from the pancreas has been identified. Nerve fibers innervating the exocrine and endocrine pancreas release PACAP, which has a potent secretogogue effect on β-cells of the islet, releasing insulin in a glucose-dependent manner (Filipsson et al., 2001). This was confirmed in PAC_1 receptor-deficient islets and a mouse line as PACAP-induced glucose dependent insulin secretion was reduced (Jamen et al., 2000, 2002). In addition, PACAP can release glucagon from α-islet cells of the pancreas when blood glucose levels are low (Fridolf et al., 1992, Filipsson et al., 1998). Insulin and glucagon act in opposition regulating carbohydrate and lipid metabolism to maintain homeostasis. Insulin decreases blood glucose levels by transporting glucose into cells, stimulating glycogen synthesis and inhibiting gluconeogenesis, whereas glucagon increases blood glucose levels by stimulating glycogen breakdown and glucose production in the liver (Cryer and

Polonsky, 1998). Insulin and glucagon release are controlled chemically by the concentration of glucose within the blood and hormones can potentiate insulin and glucagon release. In addition to regulating carbohydrate metabolism, insulin and glucose regulate lipid storage and release. Insulin prevents the breakdown of triglycerides into free fatty acids by suppressing hormone sensitive lipase within the white adipocytes. However, glucagon has the opposite effect, increasing triglyceride breakdown to increase circulating free fatty acids by activating hormone sensitive lipase (Ganong, 2001, Saltiel and Kahn, 2001).

Recently white adipocytes were shown to synthesize and release a hormone named leptin (Zhang et al., 1994) that acts on the hypothalamus to suppress appetite and increase energy expenditure (Baskin et al., 1999). This discovery identified white adipocytes as endocrine cells, not merely storage containers for triglycerides. A direct association between leptin and PACAP has yet to be made. However, PACAP is present in the hypothalamus, within neurons associated with appetite control (Koves et al., 1990, Arimura et al., 1991). Also, centrally administered PACAP has been shown to reduce food intake likely in a cAMP-dependent manner (Chance et al., 1995, Mizuno et al., 1998). The anorexic effects of PACAP will likely make it a target for anti-obesity drugs (Christophe, 1998).

A role for PACAP in regulating hormone release from the adrenal gland has been shown. Several hormones released from the adrenal gland including epinephrine, norepinephrine and corticosteroids, affect carbohydrate and lipid metabolism. The catecholamines released from the adrenal medulla initiate alertness within the organism, metabolically mobilizing glucose from glycogen stores and fatty acids from triglyceride

stores. PACAP has an established role in the synthesis and release of catecholamines from the adrenal medulla (Watanabe et al., 1992, Ghatei et al., 1993, Park et al., 1999). From the adrenal cortex, corticosteroids also affect metabolic processes: they increase blood glucose levels resulting in increased insulin secretion, they activate protein catabolism, which under prolonged elevation results in increased blood glucose as amino acids are converted into glucose, and corticosteriods mediate the ability of catecholamines to mobilize free fatty acids (Ganong, 2001). In mammals, PACAP does not directly stimulate corticosteroid release (Mazzocchi et al., 2002), but PACAP can act to release ACTH from corticotrope cells of the anterior pituitary (Rawlings and Hezareh, 1996) and catecholamines from the adrenal medulla (Watanabe et al., 1992), both of which would indirectly result in increased corticosteroid release.

This chapter describes a study to determine if the absence of PACAP disrupts other hormones involved in regulating lipid metabolism, such as insulin, corticosterone or leptin. Analysis of lipid distribution within peripheral tissues and serum of the PACAP null mice shows that PACAP null mice cannot regulate lipid metabolism and have symptoms similar to those seen in diabetes. One possibility is that the PACAP null mice are insulin resistant; another possibility is that the adrenal cortex and corticosteroid secretion are deficient. We hypothesize that lack of PACAP results in uncontrolled release of free fatty acids from white adipocyte storage by disrupting other endocrine systems involved in fat metabolism.

MATERIALS AND METHODS

Breeding and genotyping of mice

All mice used in the following procedures were obtained by breeding male and female mice heterozygous for the PACAP deletion. Mice were housed at 21°C and fed *ad libitum*. Pups born to heterozygous breeding pairs were genotyped and identified by earclipping on postnatal day 4 or 5. Genomic DNA was extracted and PCR performed as described previously in Chapter 3. PCR results were confirmed at sampling when an additional piece of tissue was taken for DNA extraction and PCR. All experiments were approved by the University of Victoria's Animal Care Committee.

Oil Red O staining of liver, heart and skeletal muscle sections

Tissues were collected from euthanized PACAP+/+, PACAP+/- and PACAP-/- mice, fixed in 4% paraformaldehyde and cryoprotected by infiltrating with a 20% sucrose solution. The tissues were then frozen at –80°C, sectioned using a cryostat and stained with Oil Red-O to identify the presence of lipids within the cells of these three tissues by staining the lipids red.

Electron Microscopy

Livers of wasted PACAP-/- mice and their littermates (PACAP+/+ and PACAP+/-) were fixed with a 2.5% glutaraldehyde primary fixative by cardiac perfusion. The tissue was rinsed with phosphate buffer and post-fixed in osmium tetroxide for 1 hr at 4°C. The sections were rinsed in ddH_20, then dehydrated in an ethanol series and embedded in epon. Ultra thin sections were cut, collected on grids and stained with lead

citrate. Sections were examined using a transmission electron microscope (Hitachi H-7000).

Serum collection from mice.

Mice were anesthetized with isoflurane. Blood was collected from PACAP+/+, PACAP+/- and PACAP-/- mice by cardiac puncture. The blood was kept at 4°C for approximately 1 hour and spun down at 3,000 g for 5 minutes. The serum was removed to a fresh tube and stored at –80°C.

Assessment of ß-oxidative function in PACAP null mice

Gas chromatography-mass spectrometry was used to analyze the presence of 3'hydroxy (3'OH) fatty acids of various chain lengths in serum of PACAP+/+, PACAP+/- and PACAP-/- mice to determine if there was a block in the ß-oxidation of fatty acids in the mitochondria. This procedure was performed by Dr. M. Bennett and Dr. P. Jones at the Southwestern Medical Center in Dallas Texas.

Measurement of serum lipids in PACAP null pups compared to controls

Serum lipids were measured in PACAP+/+, PACAP+/-, PACAP-/- mice. Free fatty acids were measured using a kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. In the protocol, free fatty acids are broken down first by acyl-coenzyme A synthase and then acyl-coenzyme A oxidase to enoyl-coenzyme A and hydrogen peroxide. The hydrogen peroxide converts a substrate into a red dye that is visible at a wavelength of 546 nm. Serum cholesterol was measured with a kit from Sigma (St. Louis, MO). Also, levels of the ketone, ß- hydroxybutyrate were

measured (Sigma). Serum triglycerides were measured at the Vancouver General Hospital core laboratory facility. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

Plasma lipids in adult PACAP null mice compared to controls

PACAP+/+ and PACAP-/- mice that survived to adulthood (approximately 6 months old) were fasted overnight. Blood was collected by cardiac puncture into heparinized syringes to obtain plasma instead of serum. Data was recorded for female (n=10) and male (n=10) PACAP+/+ mice and female (n=11) and male (n=11) PACAP-/- mice. Plasma levels of triglycerides, total cholesterol and high density lipoprotein were measured by enzymatic color reactions (MDS Metro Laboratories, Victoria, B.C.). Significance ($p>0.05$) of the values was determined using a Student's two-tailed T-test.

Levels of glycogen in the liver

PACAP+/+ (n=4), PACAP+/- (n=4) and PACAP-/- (n=4) mice (postnatal day 8-10) were euthanized with isofluorane. Livers were dissected from the mice, frozen immediately in liquid nitrogen and stored at $-80^{\circ}C$. Frozen livers were ground into a fine powder using a chilled mortar and pestle. Glycogen content in the liver was determined using a starch assay kit (Sigma). Liver tissue (50- 100 mg) was extracted with ethanol to remove glucose. The glycogen in the tissue homogenate was digested to glucose with α-amylase and amyloglucosidase (from *Aspergillus niger*) according to the manufacturer's protocol. The resulting glucose was oxidized by glucose oxidase to gluconic acid and hydrogen peroxide, which changes to a pink colour when exposed to o-dianisidine in the

presence of peroxidase and sulfuric acid (Sigma). The colour formed from this reaction was detected by a spectrophotometer at a wavelength of 540 nm. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

Blood glucose levels in PACAP null mice

Blood glucose levels were measured in fasted (4 hours) and fed PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day 5 and 7 using a glucometer (Glucometer Elite, Bayer, Toronto, ON). Blood (2 μl) was taken from the femoral vein of the leg. The mice were not anesthetized as isoflurane can affect blood glucose levels. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

Serum insulin level in PACAP null mice

Serum insulin levels were measured in fasted (4 hours) and fed PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day 5 and 7 using a radioimmunoassay (Sensitive Rat Insulin RIA Kit, Linco Research Inc., St. Charles, MO). Mice were anesthetized with isofluorane and blood was collected by cardiac puncture. Serum was stored at –80°C until assayed. In the insulin radioimmunoassay, several samples (7 out of 86) contained very low levels of insulin and were undetectable by the assay. For statistical purposes a conservative value of 0.02 ng/ml, the lowest detectable concentration for Linco's Sensitive Rat Insulin Radioimmunoassay Kit, was assigned to these samples. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

Glucose tolerance test in adult mice

Adult PACAP+/+, PACAP+/- and PACAP-/- mice raised at 21°C were fasted overnight. A blood sample from the femoral vein in the leg was taken prior to glucose administration. D-Glucose (150 mg in 500 µl) was given by gavage tube directly into the stomach. Blood samples were taken at 10, 30, 60 and 120 minutes post gavage. Blood glucose levels at each of the time points were recorded using a glucometer (Glucometer Elite, Bayer). After blood glucose levels were measured, blood samples collected at the pre bleed and at the final bleed were spun down and serum was removed to a new tube. The serum was stored at –80°C. Serum insulin concentration was determined in these samples by a radioimmunoassay (Rat Sensitive Insulin RIA Kit, Linco Research Inc.). Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison's test and Student's two-tailed t-tests.

Measurement of leptin in serum using radioimmunoassay

Serum leptin levels were measured in seven day old PACAP+/+, PACAP+/- and PACAP-/- mice using radioimmunoassay (Mouse Leptin Radioimmunoassay Kit, Linco Research Inc.). Serum was stored at –80°C until assayed. Several of the PACAP-/- serum samples contained leptin levels greater than the upper limit of the leptin radioimmunoassay kit, and were conservatively assigned a value of 20 ng/ml (before the dilution factor), the highest detectable concentration for Linco's Mouse Leptin Radioimmunoassay Kit. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

Histology of adrenal glands of PACAP+/+, PACAP+/- and PACAP-/- mice

Seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice were euthanized using isoflurane. The adrenal glands were dissected from the mice and fixed in 4% paraformaldehyde in PBS overnight. The adrenals were washed two times in sterile double distilled H_20 for 20 min and dehydrated through a graded ethanol series. The tissue was embedded in glycol methacrylate (Technovit 700) as described by the manufacturer (Heraeus Kulzer GmbH & Co., Wehrheim, Germany). Once embedded in the plastic, 7 μm sections were cut using a glass knife and a JB-4 microtome (Sorvall, Newtown, CT). Sections were stained with Delafield's Hemotoxylin and Eosin Y and viewed on a Universal microscope (Zeiss, Germany).

Measurement of corticosterone in serum using radioimmunoassay

Serum corticosterone levels were measured in seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice using radioimmunoassay (ICN diagnostics, Orangburn, NY). Blood collection was done at the same time on each sampling day, as corticosterone levels are circadian. Serum was stored at –80°C until assayed. Significance ($p>0.05$) of the values was determined using Tukey-Kramer Multiple Comparison Test.

RESULTS

Lipid accumulation in liver, heart and skeletal muscle of PACAP-/- mice and depletion of white adipose tissue

The pale liver observed in the PACAP null pups upon necropsy occurred from lipid accumulation within the hepatocytes. This was shown using Oil Red O staining of cryostat sectioned tissue (Fig. 4.1a) and electron microscopy (Fig. 4.1b). The lipid was present within microvesicles, a condition known as hepatic microvesicular steatosis. In addition, both heart (Fig. 4.2a) and skeletal muscle (Fig. 4.2b) showed intracellular fat accumulation in Oil Red O-stained sections. In contrast, subcutaneous white fat deposits were depleted totally in wasted PACAP-/- mice at the time of death.

Normal β-oxidative phosphorylation in PACAP null mice

Microvesicular accumulation of lipids in hepatocytes is characteristic of mitochondrial dysfunction specifically due to a block in the β-oxidation pathway (Jones et al., 2000). However, electron micrographs of hepatocytes of PACAP+/+, PACAP+/- and PACAP-/- mice showed mitochondria of PACAP-/- mice to be morphologically normal (Fig. 4.1b). There was an even distribution of 3'OH-fatty acids (C6 to C16) in the serum of PACAP+/+, PACAP+/- and PACAP-/- mice showing that the ß-oxidation pathway in mitochondria was not blocked due to an enzymatic defect (Table 4.1).

Elevated triglycerides, cholesterol, free fatty acids and ketone bodies in PACAP-/- mice at postnatal day 7

Significantly elevated serum levels of triglycerides (Fig. 4.3a) and cholesterol (Fig. 4.3b) were present in PACAP-/- mice compared to wildtype and heterozygotic mice at postnatal day 7. Free fatty acids in the serum of the PACAP null mice were significantly higher than those of wildtype mice at postnatal day 7 (but not significantly

Fig. 4.1. Morphology of hepatocytes from seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice. a.) Hemotoxylin and eosin staining (i.), and oil red-O staining (ii.) showing microvesicular lipid deposits in the hepatocytes of PACAP-/- mice but not in the PACAP+/+ and PACAP+/- hepatocytes. b.) Electron micrographs of hepatocytes from PACAP+/+, PACAP+/- and PACAP-/- mice showing morphologically normal mitochondria and lipid accumulation in hepatocytes of PACAP-/- mice.

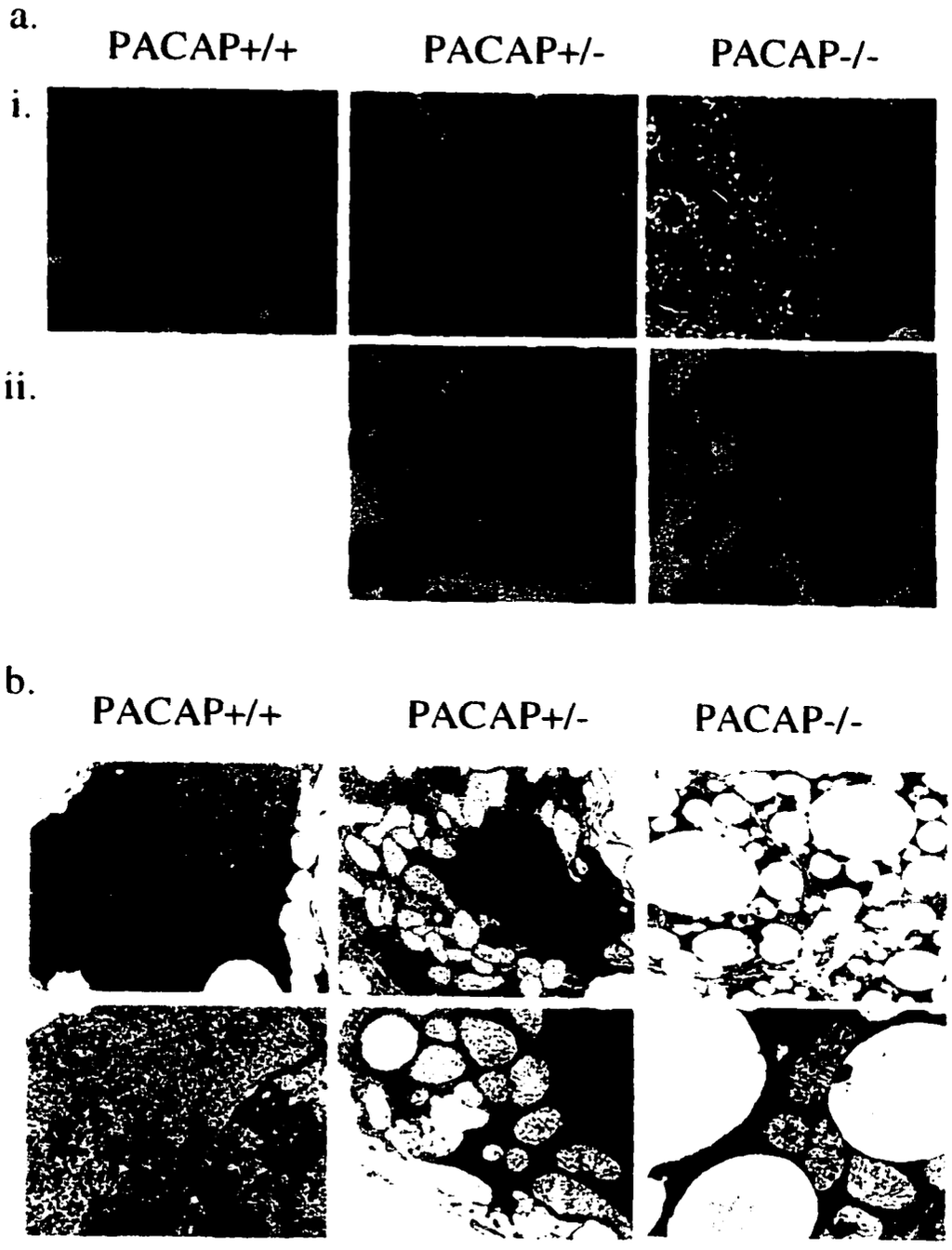

a.
PACAP+/+
PACAP+/-
PACAP-/-
i.
ii.
b.
PACAP+/+
PACAP+/-
PACAP-/-

Fig. 4.2. Histological sections of heart and skeletal muscle from seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice stained with oil red-O. a.) Microvesicular lipid accumulation in PACAP-/- heart cells. b.) Microvesicular lipid accumulation in PACAP-/- skeletal muscle cells. Lipid accumulation was not observed in PACAP+/+ and PACAP+/- heart or skeletal muscle cells.

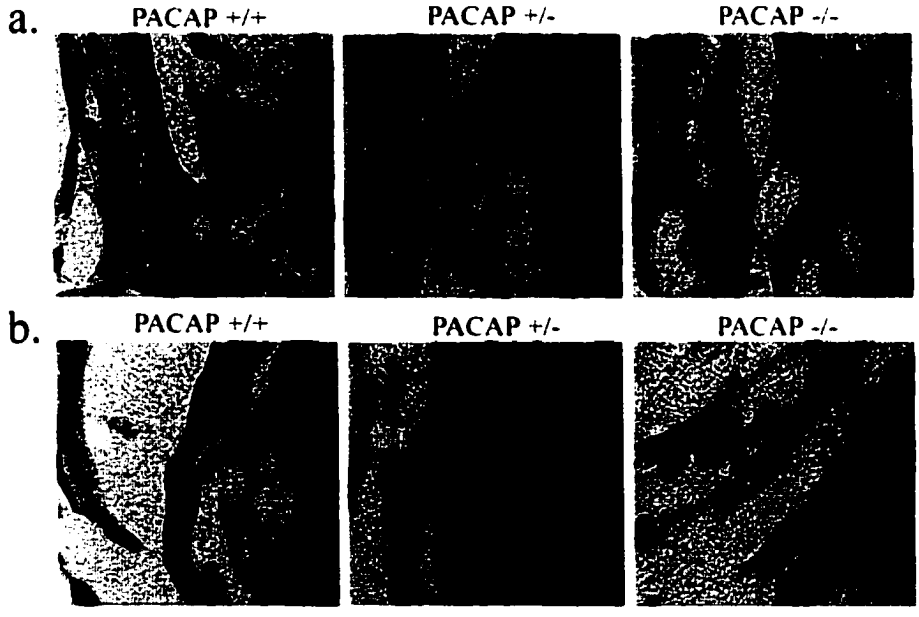
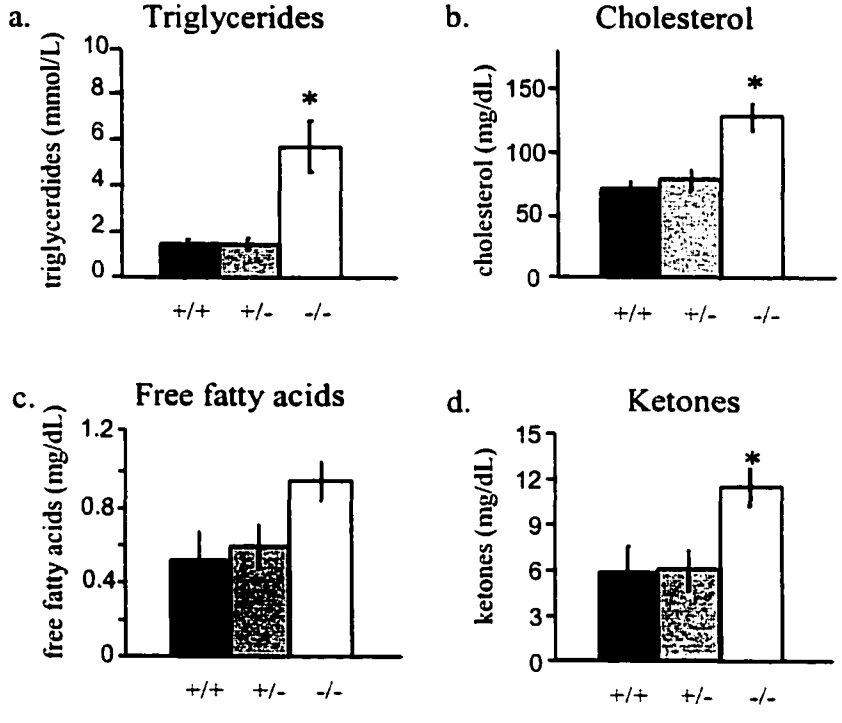

a.
PACAP +/+
PACAP +/-
PACAP -/-
b.
PACAP +/+
PACAP +/-
PACAP -/-

Table 4.1. Total 3'OH fatty acids (μmol/L) measured in blood of PACAP+/+, PACAP+/- and PACAP-/- mice by gas chromatography-mass spectrometry.

Genotype	3'OH-C6	3'OH-C8	3'OH-C10	3'OH-C12	3'OH-C14	3'OH-C16
PACAP+/+	4.10	2.60	0.60	0.60	0.50	1.10
PACAP+/-	3.70	3.00	0.80	0.60	0.30	0.70
PACAP-/-	5.60	2.50	0.80	0.60	0.60	1.20

Fig 4.3. Serum lipid concentrations in seven day-old PACAP+/+, PACAP+/- and PACAP-/- mice. Error bars represent standard error of the mean. * shows significance at $p<0.05$. a.) Triglyceride serum levels in the PACAP+/+ (n=4), PACAP+/- (n=5) and PACAP-/- (n=5) mice. b.) Cholesterol concentration in the serum of PACAP+/+ (n=11), PACAP+/- (n=12) and PACAP-/- (n=16) mice. c.) Levels of free fatty acids in the serum of PACAP+/+ (n=4), PACAP+/- (n=4) and PACAP-/- (n=4) mice. d.) Levels of β-hydroxybutyrate (a ketone) in the serum of PACAP+/+ (n=11), PACAP+/- (n=13) and PACAP-/- (n=16) mice.


a.
Triglycerides
triglycerdides (mmol/L)
10
8
6
4
2
0
*
+/+ +/- -/-
b.
Cholesterol
cholesterol (mg/dL)
150
100
50
0
*
+/+ +/- -/-
c.
Free fatty acids
free fatty acids (mg/dL)
1.2
0.8
0.4
0
+/+ +/- -/-
d.
Ketones
ketones (mg/dL)
15
12
9
6
3
0
*
+/+ +/- -/-

higher than heterozygotes) (Fig. 4.3c). Serum levels of ß-hydroxybutyrate (a ketone) were significantly elevated in PACAP null mice compared to wildtype and heterozygotic mice (Fig. 4.3d).

Normal levels of serum triglycerides, cholesterol and high density lipoprotein in adult PACAP null mice

Serum levels of cholesterol, triglycerides and high density lipoprotein were not significantly higher in adult PACAP null mice compared to adult wildtype controls ($p>0.05$) (Fig. 4.4a). The adult PACAP null mice did not show a similar serum lipid profile to the PACAP null pups. Male and female serum lipid levels were compared and no significant difference between the sexes was observed ($p>0.05$) (Fig. 4.4b).

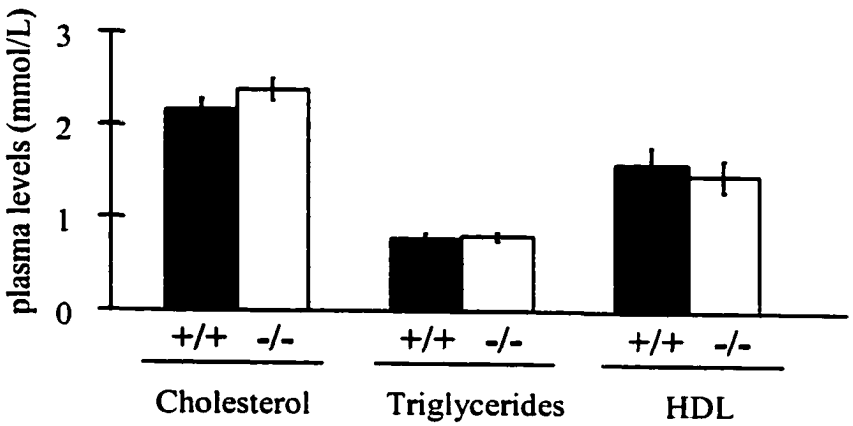
High Insulin levels with low glucose in fasted mice at postnatal day 5.

Fasted PACAP null mice at postnatal day 5 had significantly lower blood glucose compared to littermates, yet in the fed state, blood glucose levels of PACAP null mice were no different than littermate controls (Fig. 4.5a). At postnatal day 7 blood glucose levels were normal in both the fasted and fed state (Fig. 4.5a).

The concentration of insulin was measured in serum of fed and fasted wildtype, heterozygous and null mice at postnatal day 5 and day 7 (Fig. 4.6). In the fasted state, PACAP null mice at postnatal day 5 had higher levels of serum insulin than PACAP+/- and PACAP+/+ mice (Fig. 4.6). Yet at postnatal day 7 (when most PACAP null mice are showing signs of wasting) there was no significant difference in serum insulin concentration among the three genotypes (Fig. 4.6). In the fed state insulin levels of

Figure 4.4. Plasma cholesterol, triglycerides and high density lipoprotein (HDL) levels in PACAP+/+ (n=20) and PACAP-/- (n=22) that survive to adulthood at 21°C (approximately 6 months old). b.) Comparison of plasma lipid levels in female versus male PACAP+/+ (n=10) and PACAP-/- mice (n=10). Error bars represent standard error of the mean.

a.


plasma levels (mmol/L)
3
2
1
0
+/+ -/-
+/+ -/-
+/+ -/-
Cholesterol
Triglycerides
HDL

b.

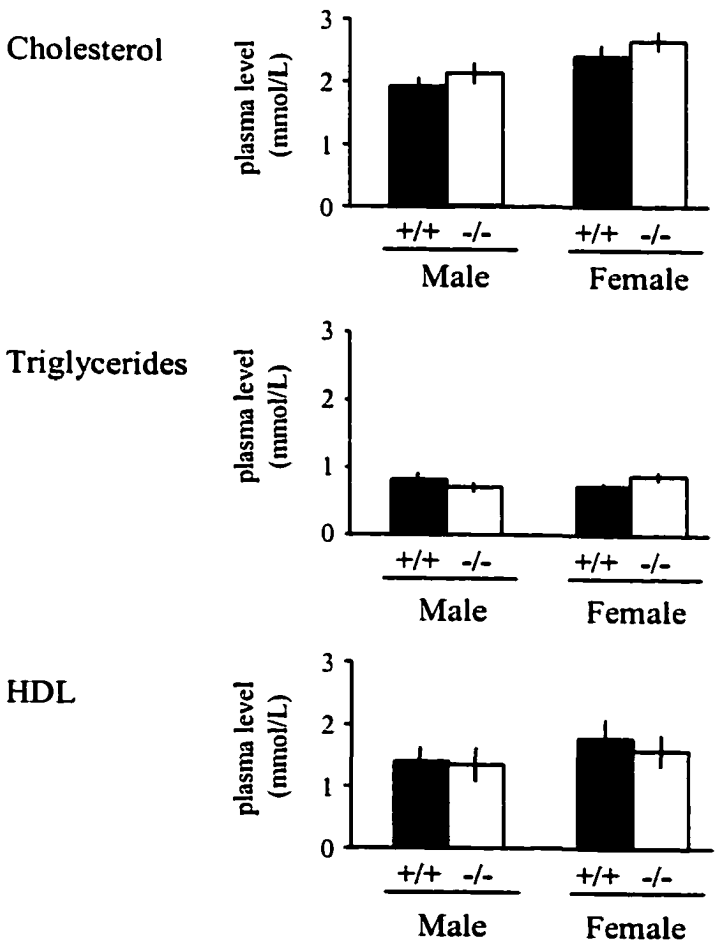
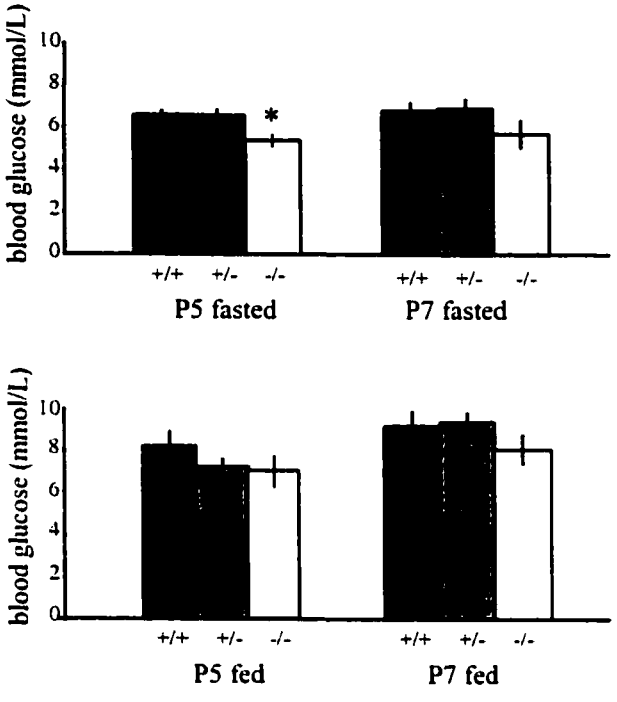
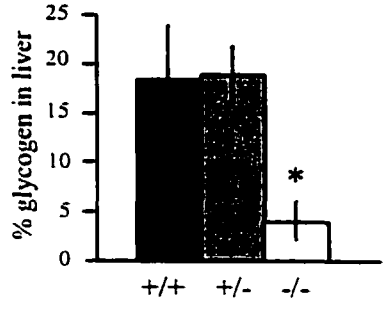

Cholesterol
plasma level (mmol/L)
3
2
1
0
+/+ -/-
+/+ -/-
Male
Female
Triglycerides
plasma level (mmol/L)
3
2
1
0
+/+ -/-
+/+ -/-
Male
Female
HDL
plasma level (mmol/L)
3
2
1
0
+/+ -/-
+/+ -/-
Male
Female

Figure 4.5. a.) Blood glucose levels in fasted PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day 5 (P5) and at postnatal day 7 (P7) (n=10 in each of the six groups). Blood glucose levels in fed PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day 5 (n=8, n=15 and n=6, respectively) and postnatal day 7 (n=5, n=19 and n=11, respectively) b.) Percent glycogen in the liver of PACAP+/+ (n=4), PACAP+/- (n=4) and PACAP-/- (n=4) mice. Error bars represent standard error of the mean. * shows significance at $p<0.05$.

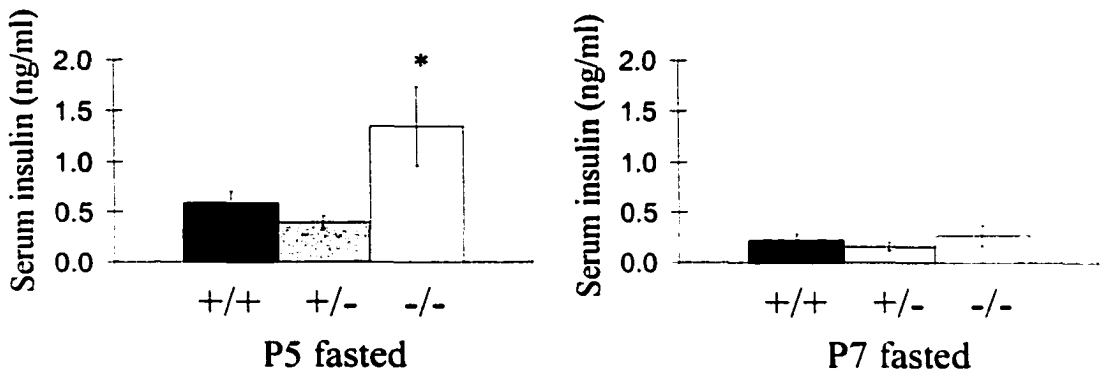
a.


blood glucose (mmol/L)
10
8
6
4
2
0
*
+/+ +/- -/-
P5 fasted
+/+ +/- -/-
P7 fasted
blood glucose (mmol/L)
10
8
6
4
2
0
+/+ +/- -/-
P5 fed
+/+ +/- -/-
P7 fed

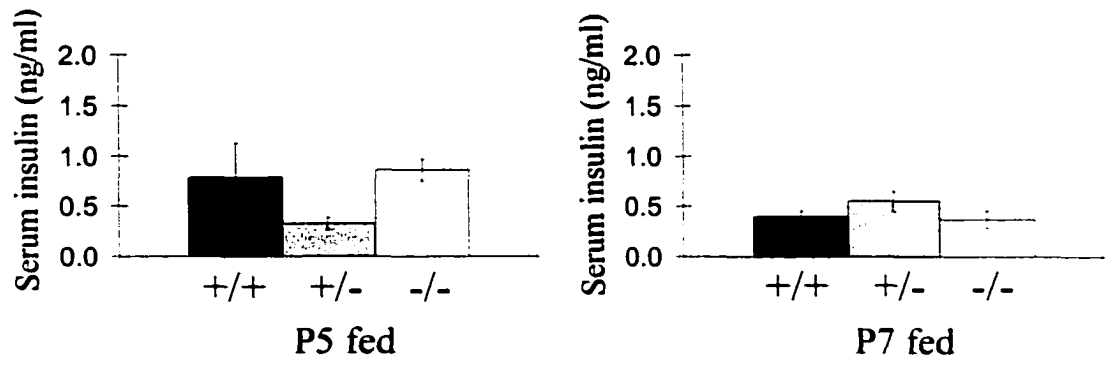
b.


% glycogen in liver
25
20
15
10
5
0
*
+/+ +/- -/-

a.


Serum insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
*
+/+ +/- -/-
P5 fasted
Serum insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
+/+ +/- -/-
P7 fasted

b.


Serum insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
+/+ +/- -/-
P5 fed
Serum insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
+/+ +/- -/-
P7 fed

PACAP null mice were not significantly different from levels measured in wildtype and heterozygous mice at postnatal days 5 and 7.

Reduced glycogen in liver of PACAP null mice near death

Significantly reduced levels of glycogen in the liver of PACAP null mice were measured at day 7 compared to PACAP+/+ and PACAP+/- mice (Fig. 4.5b).

Normal glucose disposal in adult PACAP null mice

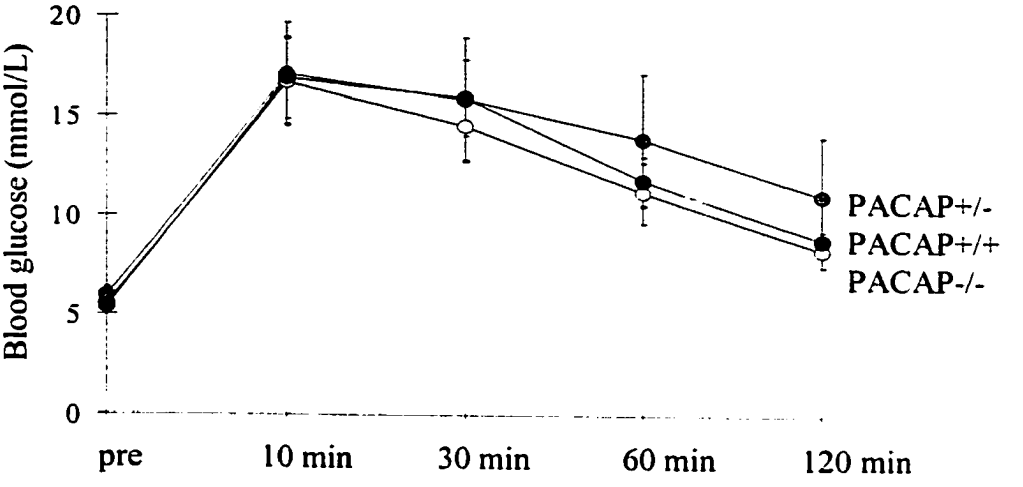
Adult PACAP-/- mice were able to lower blood glucose levels after gastric glucose administration as efficiently as PACAP+/+ and PACAP+/- mice (Fig. 4.7a). Serum was collected to measure insulin levels by radioimmunoassay for the pre gavage and 120 minute post gavage sample time, and levels of serum insulin at both time points were not significantly different ($p>0.05$) in PACAP null mice compared to wildtype and heterozygote mice (Fig. 4.7b). At 10, 30 or 60 minutes post gavage a sufficient amount of blood could not be collected for radioimmunoassay of serum insulin concentration.

Normal adrenal morphology and high serum corticosterone levels in PACAP null mice

Histological sections of the adrenal gland showed no gross morphological abnormalities. The medulla and cortex were completely differentiated and identifiable (Fig. 4.8a). Mean corticosterone levels were significantly higher in the PACAP-/- mice than PACAP+/- and PACAP+/+ mice (Fig. 4.8b). The concentration of corticosterone varied greatly in PACAP null mice with some mice showing extremely high levels of serum corticosterone and other mice with normal levels (Fig. 4.8b).

Figure 4.7. Glucose tolerance test in adult PACAP+/+, PACAP+/- and PACAP-/- mice. a.) Blood glucose levels in fasted, adult PACAP+/+ (n=7), PACAP+/- (n=7) and PACAP-/- (n=7) mice prior to glucose gavage (pre) and at 10 min, 30 min, 60 min and 120 min after glucose gavage. b.) Serum insulin levels in PACAP+/+ (n=6), PACAP+/- (n=4) and PACAP-/- (n=6) adult mice (same mice as above but performed at a different time). Mice were fasted overnight. A blood sample was taken prior to glucose gavage (pre) and 120 min after glucose gavauge. Error bars represent standard error of the mean.

a.


Blood glucose (mmol/L)
20
15
10
5
0
pre
10 min
30 min
60 min
120 min
PACAP+/-
PACAP+/+
PACAP-/-

b.

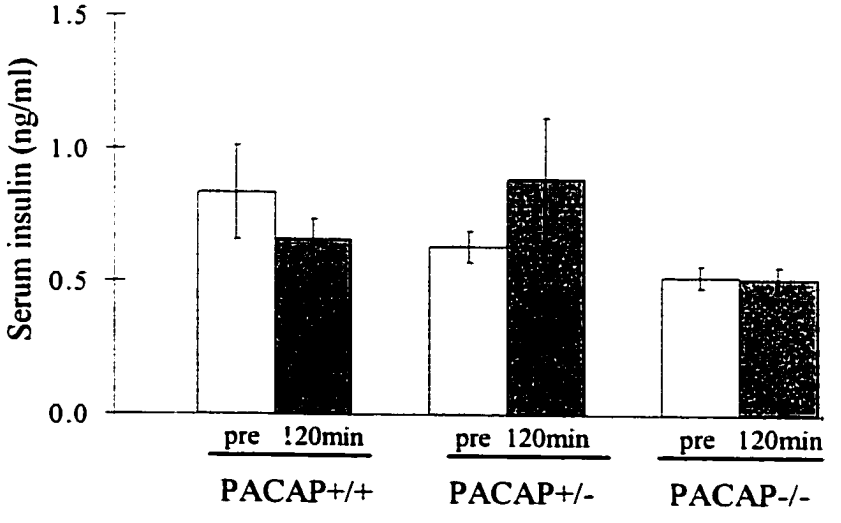
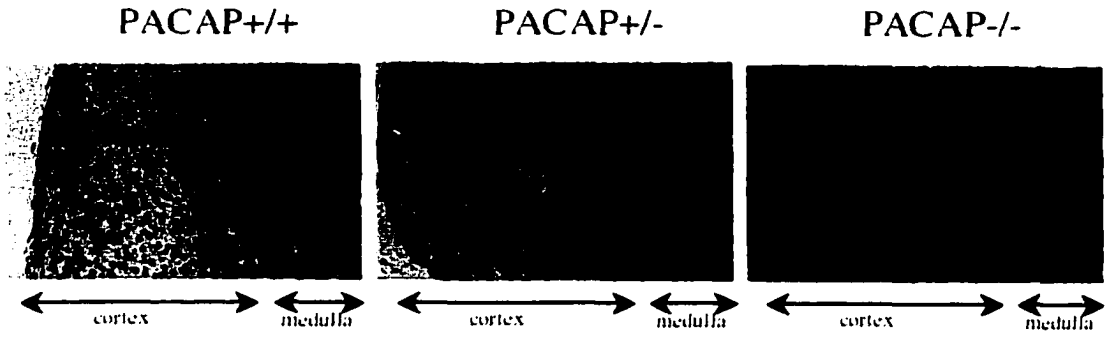
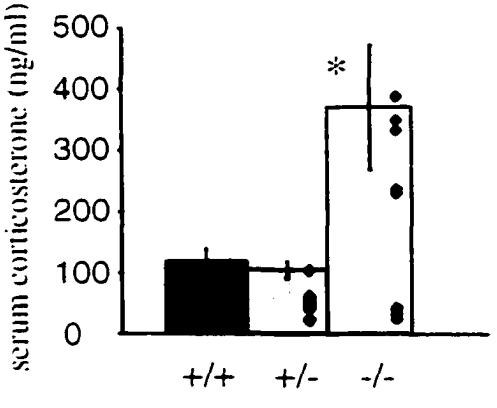

Serum insulin (ng/ml)
1.5
1.0
0.5
0.0
pre !20min
pre 120min
pre 120min
PACAP+/+
PACAP+/-
PACAP-/-

Figure 4.8. a.) Hemotoxylin/eosin stained sections of the adrenal gland of a seven day old PACAP+/+, PACAP+/- and PACAP-/- mouse. b.) Serum corticosterone levels in seven day-old PACAP+/+ (n=9), PACAP+/- (n=10) and PACAP-/- (n=9) mice. The histogram shows the mean corticosterone concentration for each genotype whereas the scatter plot shows the individual corticosterone serum levels. Error bars represent standard error of the mean. * shows significance at $p<0.05$.

a.


PACAP+/+
PACAP+/-
PACAP-/-
cortex
medulla
cortex
medulla
cortex
medulla

b.


serum corticosterone (ng/ml)
500
400
300
200
100
0
*
+/+
+/-
-/-

Elevated levels of leptin in the PACAP null mice at postnatal day 7

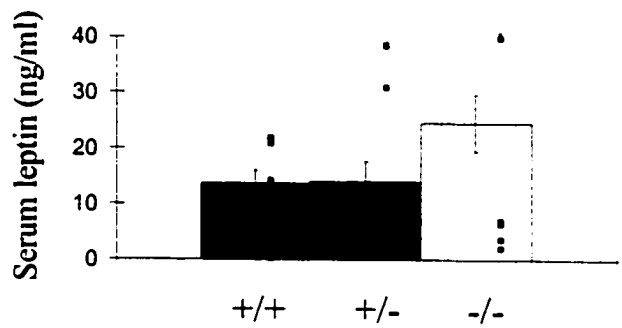
Serum leptin levels in PACAP null mice were elevated but not significantly different ($p>0.05$) from the PACAP+/+ and PACAP+/- mice. As with the corticosterone data, the level of leptin in the serum of PACAP null mice at postnatal day 7 was variable, with normal levels in some mice and extremely high levels in others (Fig. 4.9).

DISCUSSION

We found that PACAP null mice die early in postnatal life in a wasted condition (Gray et al., 2001). Upon necropsy, livers were extremely pale, which results from lipid accumulation in hepatocytes. Histological examination of other tissues, revealed that the lipid accumulation was also present in heart and skeletal muscle. The lipids are stored in microvesicles, a condition that can occur from a defect in hepatic fat metabolism associated with mitochondrial dysfunction (Hautekeete et al., 1990, Rinaldo et al., 1999).

However, our evidence suggests that PACAP null mice do not have mitochondrial dysfunction. Mitochondria of PACAP null mice were morphologically normal. The similar distribution of 3'OH-fatty acids of different chain lengths (Jones et al., 2000) among the mice genotypes confirmed that an enzymatic defect in fatty acid ß-oxidation in mitochondria was not present in the PACAP null mice and lipid accumulation in hepatocyctes was not a result of a blocked oxidative phosphorylation pathway. Despite abnormal fat storage in hepatocytes, entry of fatty acids into the mitochondria was unlikely to be reduced because ketosis was present in the PACAP null mice. This suggests that metabolic flux in the mitochondria was increased, in turn resulting in the formation of ketone bodies that are released into the blood.

Figure 4.9. a.) Serum leptin levels in PACAP+/+ (n=10), PACAP+/- (n=10) and PACAP-/- (n=10) mice at postnatal day 7 (P7). Serum leptin concentration was determined by radioimmunoassay. The histogram shows the mean serum leptin concentration for each genotype whereas the scatter plot shows the individual leptin serum levels. Error bars represent standard error of the mean. The PACAP-/- values were not significantly different ($p>0.05$).


Serum leptin (ng/ml)
40
30
20
10
0
+/+
+/-
-/-

Our evidence indicates that the defect seen in the PACAP null mice is not likely to be attributable to hepatic dysfunction. Elevated serum triglycerides and cholesterol suggest that PACAP null mice are capable of synthesizing and releasing very low density lipoprotein (VLDL) particles from the liver into the blood. The high free fatty acid serum concentration indicates increased mobilization of fatty acids from adipose tissue in the PACAP null mice compared to wildtype controls. The elevated serum levels of triglycerides, free fatty acids and ketone bodies, along with the even distribution of 3'OH fatty acids in the serum suggests that a hepatic dysfunction is not the primary defect in the PACAP null mice. Instead, the liver, heart and skeletal muscle may simply be responding to an increased mobilization of fatty acids from adipose tissue in PACAP null mice.

Another possible mechanism resulting in the PACAP deficient syndrome is the lack of insulin release. PACAP is known to stimulate insulin release in a glucose-dependent manner (Filipsson et al., 2001); thus, hyperglycemia was predicted in the PACAP null mice due to reduced insulin levels. However, PACAP-/- pups (postnatal days 5 and 7) had low to normal blood glucose in the fasted and fed state. Similar to our findings, adult PAC_1 receptor null mice (with intact PACAP/VIP-shared receptors) had normal basal blood glucose levels in the fed and fasted state (Jamen et al., 2000) and in another PACAP knockout mouse line, fasted adult PACAP null mice had lower blood glucose than wildtype controls (Hamelink et al., 2002). Basal levels of serum insulin in PACAP null mice varied depending on age and feeding state, being elevated only in fasted PACAP null mice at postnatal day 5. This high insulin level was in parallel with low glucose in fasted mice of the same age. Thus, lack of insulin release did not appear

to trigger the PACAP deficient syndrome. PACAP null mice at day 7 had a significantly reduced level of glycogen in the liver. Reduced hepatic glycogen suggests an increased demand for glucose, an impairment of glycogen synthesis and/or gluconeogenesis or displacement of glycogen by the lipids accumulated in the hepatocytes (Ganong, 2001).

The ability of the PACAP null mice to handle high levels of glucose was tested with a glucose tolerance test. Continuous assessment of insulin/glucose levels and glucose/insulin tolerance tests could not be performed in neonatal mice pups because they have a small blood volume; only one serum sample of less than 100 μl could be obtained from an individual by cardiac puncture. Instead a glucose tolerance test was performed in adult PACAP-/- mice that had survived to adulthood. We predicted that PACAP null mice might respond abnormally in the test because PAC_1 receptor null mice were not able to reduce serum glucose or increase insulin as quickly as normal mice after a gastric or intravenous glucose tolerance test (Jamen et al., 2000). However, in our adult PACAP null mice, giucose disposal was normal after gastric glucose administration, with normal insulin levels prior to and 2 hours after glucose administration. I conclude that PACAP null mice can maintain normal glucose serum levels in the fed state or in a glucose overload situation, presumably by use of other hormones that compensate for PACAP in insulin release such as glucose-dependent insulinotropic polypeptide and/or glucagon-like peptide-1.

The PACAP null mice display some symptoms characteristic of diabetes. Diabetes occurs when insulin cannot be produced (type 1) or when insulin's effects can not be mediated by its receptor (type 2). In PACAP null mice, high serum free fatty acids and triglycerides, deposition of lipid in liver and cardiac cells, excess ketones produced

from increased β-oxidative phosphorylation and reduced glycogen in the liver are all symptoms observed in diabetics (Lewis et al., 2002, Ganong, 2001, Regan et al., 1977). Contrary to these symptoms, classical insulin resistance is not observed in the PACAP null mice. The variation in the level of insulin in the PACAP null mice at different ages and in the fed or fasted state is puzzling. If insulin resistance were occurring, one would expect high insulin levels to be constant and accompanied by high glucose. The PACAP null pups do not have high blood glucose and adult PACAP null mice are able to reduce blood glucose and return serum insulin to normal levels 2 hours after glucose administration. This suggests insulin is able to mediate glucose disposal in PACAP null mice. PACAP null mice may be insulin resistant only at the level of the white adipocyte or may be incapable of using intracellular glucose resulting in the release of free fatty acids to compensate as an energy source.

PACAP null mice have a number of characteristics similar to the insulin receptor knockouts. Complete knockout of the insulin receptor in mouse results in early postnatal death (on postnatal days 3-7). The phenotype of insulin receptor null pups is similar to the PACAP null pups in that at birth they appear normal, yet by postnatal day 7 are smaller than littermate controls, have elevated serum triglycerides, decreased liver glycogen, lipid accumulation within hepatocytes and exhibit severe ketosis. In contrast to the PACAP null pups, the insulin receptor null mice are clearly insulin resistant with high plasma glucose and insulin levels compared to wildtype and heterozygous mice (Joshi et al., 1996, Accili et al., 1996). Several tissue-specific insulin receptor knockouts (skeletal muscle, pancreatic β-cell, hepatocyte, brain and brown adipose tissue) have been generated and show milder phenotypes (Bruning et al., 1998, Kulkarni et al., 1999,

Michael et al., 2000, Bruning et al., 2000, Guerra et al., 2001). The muscle specific insulin receptor knockout shows an interesting phenotype with increased serum lipids and body fat mass, decreased glucose transport and glycogen synthesis yet normal blood glucose and glucose tolerance (Bruning et al., 1998, Kim et al., 2000). Aside from the increase in body fat this is similar to the phenotype of the PACAP null mice. Knockout of the insulin receptor in the β-cell has revealed a role for the insulin receptor in controlling glucose induced insulin secretion (Kulkarni et al., 1999). The absence of insulin receptors in hepatocytes only causes hyperglycemia (Micheal et al., 2000). Mice which have brown adipose tissue that do not express insulin receptors exhibit a diabetic phenotype with elevated fasting blood glucose, impaired glucose tolerance, a decrease in the number of β-cells in the pancreas and lower basal plasma insulin (Guerra et al., 2001). The problems associated with lipid and carbohydrate balance in the PACAP null mice may result from impaired expression or function of the insulin receptor or one of its downstream effectors.

The mechanism for the wasting syndrome in PACAP null mice is not due to adrenal cortical insufficiency. PACAP acts as a hypophysiotrophic factor and may contribute to ACTH release (Rawlings and Hezareh, 1996). Addison's syndrome, resulting from a lack of corticosteroids due to insufficient ACTH release or adrenal cortex damage, results in a wasted condition. The high to normal levels of corticosterone measured in the serum of seven-day-old PACAP null pups shows that the wasting syndrome of the PACAP null mice is not Addison's syndrome. The variability in serum corticosterone concentration between PACAP null pups may result from the variable health of the PACAP null mice at postnatal day 7.

The wasted condition of the PACAP null mice would predict low serum leptin levels due to reduced fat storage; reduced leptin normally results in increased appetite and decreased energy consumption. Although the mean is not significantly different, the PACAP null mice pups show elevated serum leptin concentration at postnatal day 7, suggesting that hypothalamic control of lipid storage may be altered in the PACAP null mice near death. Also, there is evidence that leptin protects peripheral tissues from lipid accumulation by increasing fatty acid oxidation and decreasing lipogenesis (Lewis et al., 2002).

This is the first report that PACAP has a role in lipid metabolism. The early death after birth associated with wasting and the accumulation of lipid in tissue and serum were striking results that were not predicted from previous literature. Our data show that the absence of PACAP disrupts lipid homeostasis within the body, causing uncontrolled release of free fatty acids from storage, flooding metabolically active tissue and resulting in ketosis. The precise mechanism by which the absence of PACAP is causing the disruption of lipid metabolism is unknown. We can conclude that the wasting syndrome of the PACAP null mice is not Addison's syndrome. In addition we know that insulin is secreted in the PACAP null mice and is able to mediate glucose disposal. The diabetes-like condition seen in the PACAP null pups may result from insulin resistance at the white adipose tissue only or from an inability of cells to utilize glucose.

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CHAPTER 5

Temperature sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP)

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INTRODUCTION

The phenotype of the PACAP null mouse in which pups die if raised at 21°C rather than 24°C suggests a role for PACAP in thermoregulation. The mechanism of action of PACAP in thermoregulation could depend on norepinephrine and epinephrine that are released from the adrenal medulla or on norepinephrine released from nerve endings in brown adipose tissue. PACAP is present in preganglionic nerve terminals that innervate the adrenal gland (Arimura, 1998, Hamelink et al., 2002). PACAP regulates activity and mRNA expression of the catecholamine synthesizing enzymes tyrosine hydroxylase, dopamine ß-hydroxylase and phenylethanolamine N-methyltransferase (Isobe et al., 1996, Marley et al., 1996, Choi et al., 1999) and is a potent secretogogue of catecholamines from the adrenal medulla (Vaudry et al., 2000).

Within the sympathetic nervous system, PACAP is present with acetylcholine in preganglionic neurons that synapse in the sympathetic ganglia or synapse onto adrenal medullary cells (Braas and May, 1999, Hamelink et al., 2002). In addition, PACAP is produced in postganglionic nerves of the superior cervical ganglia (Brandenburg et al., 1997). The PACAP specific (PAC_1) receptor is expressed on postganglionic nerves of the superior cervical ganglion (Braas and May, 1999) and cells of the adrenal medulla (Spengler et al., 1993). Multiple signaling pathways through the PAC_1 receptor (Shioda et al., 2000a, Lamouche and Yamaguchi, 2001) are implicated in PACAP-induced catecholamine secretion.

Norepinephrine is the major regulator of adaptive thermogenesis, although thyroid hormones are known to regulate obligatory thermogenesis, (Ricquier et al., 2000). The importance of norepinephrine in adaptive thermogenesis was reiterated when mice unable to produce norepinephrine and epinephrine (dopamine ß-hydroxylase null mice) were severely cold sensitive (Thomas and Palmiter, 1997). Leptin is also involved in adaptive thermogenesis, but its effects are mediated by norepinephrine (Commins et al., 1999, Takekoshi et al., 2001).

The role of norepinephrine in thermogenesis occurs in brown adipose tissue. In adult humans and other large mammals, the major site of thermoregulation is skeletal muscle (Ricquier et al., 2000). In neonates and rodents, brown adipose tissue is the major source of heat production through non-shivering or adaptive thermogenesis (Ricquier et al., 2000). Cold is detected and adaptive thermogenesis is activated by the brain, likely in the hypothalamus. Neurons in the hindbrain (raphe pallidus) are activated to stimulate preganglionic sympathetic nerves in the spinal cord (Morrison et al., 1999), which in turn synapse onto postganglionic neurons in the middle and inferior cervical ganglia and in the first 5 thoracic ganglia (Girardier and Seydoux, 1986). These postganglionic nerves innervate brown adipocytes and the blood vessels within brown adipose tissue to activate heat production and distribution, respectively (Derry and Daniel, 1969). Norepinephrine is released from sympathetic nerve endings and binds to β_3-adrenergic receptors on brown adipocytes activating hormone sensitive lipase (HS lipase) and uncoupling protein 1 (UCP 1) (Lowell and Spiegelman, 2000). HS lipase is activated by cAMP/PKA and causes the breakdown of stored triglycerides into free fatty acids (Ganong, 2001). Brown adipose tissue produces heat by expressing UCP 1, a specialized protein that uncouples

mitochondrial respiration from ATP production such that energy is released in the form of heat (Nedergaard et al., 2001).

Two structurally homologous uncoupling proteins, UCP 2 and UCP 3, have been identified but their role in non-shivering thermogenesis remains unclear (Lowell and Spieglman, 2000). When expressed in yeast cells, UCP 2 and UCP 3 have uncoupling properties (Fleury et al., 1997, Gong et al., 1997). However, it is argued that UCP 1 is the only protein able to mediate uncoupling of ATP formation and that UCP 2 and 3 share homology in structure with UCP 1, but do not contribute directly to non-shivering thermogenesis (Negergaard et al., 2001, Golozoubova et al., 2001). In UCP 1 null mice, UCP 2 mRNA was upregulated in brown adipose tissue suggesting a compensatory mechanism (Enerbäck et al., 1997). However, the UCP 1 -/- mice were cold sensitive supporting the conclusion that UCP 2 does not contribute to uncoupling of oxidative phosphorylation (Nedergaard et al., 2001). UCP 3 has been shown to be regulated by thermogenic signals (Gong et al., 1997), but knockout and overexpression studies suggest that the primary role of UCP 3 is in the regulation of fatty acid metabolism (Harper et al., 2001).

This study identifies the phenotype of PACAP null mice as temperature sensitive. To determine the mechanism by which PACAP is regulating thermogenesis, brown adipose tissue and hormones regulating brown adipose tissue within the PACAP null mice are compared to PACAP+/- and PACAP+/+ mice. The paper examines brown adipose tissue as to histology and expression of HS lipase and UCP 1. Tyrosine hydroxylase (TH) expression is assessed in adrenal tissue. Levels of catecholamines in plasma, interscapular brown adipose tissue and adrenal tissue are measured. In times of

stress, such as cold stress, lack of PACAP may prevent sufficient production of catecholamines from the sympathetic nerves innervating brown adipose tissue, resulting in insufficient levels of norepinephrine to activate brown adipocyte non-shivering thermogenesis.

MATERIALS AND METHODS

Temperature sensitive mortality of PACAP-/- mice

PACAP+/- breeding pairs were housed at 21°C and 24°C. Pups born at the different temperatures were monitored and mortality was recorded. The procedures used in this study were approved by the University of Victoria's Animal Care Committee.

Temperature challenge

Seven-day-old PACAP+/+ and PACAP-/- mice were removed from their mothers and placed individually in a cage with corncob bedding at 21°C. The body temperature of the mice was measured using a chromel-alumel thermocouple (diameter 0.006 mm) as a rectal probe (Omega, Stamford, CT) and recorded by a CR10 data logger (Campbell Scientific Inc., USA). Body temperature was recorded at the start of the experiment and at 5, 10, 15, 20, 30, 40 and 50 minutes after removal from the mother. The animals were euthanized once their body temperature had dropped 10°C. Temperature readings at each time point were averaged for each group of mice, PACAP+/+ (n=7) and PACAP-/- (n=9), and the standard error calculated. Significance ($p<0.05$ or $p<0.01$) of the values at each time point was determined using Tukey-Kramer's multiple comparison test.

Histology and mass of brown adipose tissue

Seven-day-old PACAP+/+ (n= 9), PACAP+/- (n=20) and PACAP-/- (n=13) mice were weighed and then euthanized using isoflurane. The interscapular brown fat was dissected from the mice, weighed and then fixed in 4% paraformaldehyde in PBS overnight or frozen rapidly using liquid nitrogen. Interscapular brown fat mass to body mass ratio was calculated for each fresh brown fat sample. The masses for each genotype were averaged and the standard error was calculated. Significance ($p<0.05$) was determined using Tukey-Kramer's multiple comparison test.

Interscapular brown fat that had been fixed was trimmed to a cube approximately 2 mm^3. It was washed two times in sterile double distilled H_20 for 20 min and dehydrated through a graded ethanol series. The tissue was embedded in glycol methacrylate (Technovit 700) as described by the manufacturer (Heraeus Kulzer GmbH & Co., Wehrheim, Germany). Once embedded in the plastic, 7 μm sections were cut using a glass knife and a JB-4 microtome (Sorvall, Newtown, CT). Sections were stained with Delafield's Hemotoxylin and Eosin Y and viewed on a Universal microscope (Zeiss, Germany).

Preparation of probes for UCP1 and HS lipase

Interscapular brown adipose tissue was collected from seven-day-old PACAP+/+, PACAP+/-, PACAP-/- littermates, frozen immediately in liquid nitrogen and stored at –80°C. Tissue was ground into a fine powder using a chilled mortar and pestle. mRNA was isolated using Ambion's MicroPoly (A)Pure kit (Ambion, Austin, TX).

cDNAs for UCP1 and HS lipase were isolated by RT-PCR to act as template to generate probes for Northern analysis. The mRNA (1 μg) was reverse transcribed in a 50

μl reaction that contained 2 mM oligo dT, 2 mM dNTPs, 1x first strand buffer, 0.01 M DTT, 5 U RNase inhibitor and 100 U superscript II reverse transcriptase (Life Technologies, Burlington, ON). The reaction was incubated at 42°C for 90 minutes and the enzyme was heat inactivated at 90°C for 10 minutes.

cDNA (1μl) generated from the above reaction was added to two 50 μl PCR reactions containing 2.5 U Taq polymerase, 1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Life Technologies) and 20 pmol of sense primer and antisense primer. To isolate a fragment of the UCP 1 cDNA (318 bp), a sense primer (5'UCP 1) and an antisense primer (3'UCP 1) were used (Table 5.1) (Soumano et al., 2000). For HS lipase, a 477 bp fragment was amplified using a sense primer (5'HSL) and an antisense primer (3'HSL) (Table 5.1) (Plee-Gautier et al., 1996). PCR was carried out under the following conditions: denaturation at 94°C for 45 sec; annealing at 60°C for 45 sec; extension at 72°C for 1 min for 32 cycles and a long extension of 5 minutes. PCR products were separated on a 1.5% agarose gel and visualized under ultraviolet light using the Eagle Eye still video system (Stratagene, San Diego, CA). The UCP 1 and HS lipase PCR products were cloned into the pGEM-T vector system (Promega, Madison, WI) and transfected into XL-2 blue competent cells (Stratagene). Plasmids were purified using a miniprep kit (Qiagen, Mississauga, ON) and sequenced. cDNA probes for UCP 1 and HS lipase were labeled with ^{32}P using a random priming DNA labeling system according to the manufacturer (Life Technologies). The labeled probes were purified on a NAP 5 Sephadex column (Amersham Pharmacia, Uppsala, Sweden), boiled for 7 minutes and iced immediately.

Table 5.1 - Sequence of primers used to generate probes for hormone sensitive lipase, uncoupling protein 1 and tyrosine hydroxylase northern blots.

Primer	Direction	Sequence
5'UCP 1	sense	5' AAGGCCAGGCTTCCAGTACTATTAGGT 3'
3'UCP 1	antisense	5' GGTTTGATCCCATGCAGATGGCTCTG 3'
5'HSL	sense	5' ATGGATTTACGCACGATGACACAG 3'
3'HSL	antisense	5' TAGCGTGACATACTCTTGCAGGAA 3'
5'TH	sense	5' GGTATTCGAGGAGAGGGATGGA 3'
3'TH	antisense	5' ACCCGACGCACAGAACTGAG 3'

Northern blot of HS lipase and UCP 1

Interscapular brown adipose tissue collected from seven-day-old PACAP+/+, PACAP+/-, PACAP-/- littermates was frozen immediately in liquid nitrogen and stored at –80°C. Each tissue was ground into a fine powder using a chilled mortar and pestle. Total RNA was isolated using TRIzol as described by the manufacturer (Life Technologies). Total RNA from each genotype was pooled to obtain enough RNA for Northern analysis.

A formaldehyde agarose gel was prepared consisting of 210 mls 2% agarose in DEPC treated H_20, 60 mls 12.3 M formaldehyde and 66 mls 5x formaldehyde gel running buffer (0.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA). The gel was pre-run at 50 V for 5 min. For each genotype 15 μg of total RNA, 3.5 μl formaldehyde, 10 μl formamide and 2 μl of 10x formaldehyde gel loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was loaded onto the gel and run at 60 V for 5 h. Once the dye front had migrated 8 cm, the gel was stopped; marker lanes were cut off and stained with ethidium bromide and visualized under UV light using a still video system (Eagle Eye, Stratagene). The remaining part of the gel was soaked in DEPC H_20 for 15 min and then the RNA was transferred to a positively charged nylon membrane (Ambion) via capillary transfer overnight in 10 mM NaOH. The membrane was soaked in 2x SSC and 0.1% SDS for 5 min at room temperature, dried and baked at 65°C for 30 min. The membrane was prehybridized in ULTRAhybe hybridization buffer (Ambion) for 2 h at 55°C.

Both the UCP 1 and HS lipase probes and 50 μl of sea urchin sperm DNA (300 mg/ml) were added to the hybridization buffer and incubated at 55°C with shaking

overnight. The membrane was washed with 2x SSC and 0.2% SDS at 55°C for 15 min two times, and with 0.2x SSC and 0.2% SDS at 55°C for 30 min two times, wrapped and exposed to a Phosphor screen (Molecular Dynamics, Sunnyvale, CA) overnight. The image was developed on the STORM phosphoimager (Molecular Dynamics) and data were analyzed using ImageQuant software (Molecular Dynamics). The membrane was re-probed with a ^{32}P labeled cDNA made against mouse β-actin, under the same conditions as above to standardize the amount of RNA present.

Preparation of TH probe

Seven-day-old PACAP +/+, PACAP+/- and PACAP-/- mice were euthanized using isoflurane and adrenal glands were collected. mRNA from pooled adrenal glands was isolated using the MicroPoly (A) Pure mRNA isolation kit (Ambion). mRNA (1 μg) was used as template in a reverse transcription reaction as above. cDNA (1 μl) generated from the reverse transcription reaction was used in a 50 μl PCR reaction containing reagents as above, except that the sense primer (5'TH) and the antisense primer (3'TH) were substituted (Table 5.1) (Ichikawa et al., 1991). The primers were used to generate a 240 bp fragment of the tyrosine hydroxylase cDNA. The products generated from the PCR reaction were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light using a still video system (Eagle Eye, Stratagene). PCR products were cloned into the pGEM-T vector system (Promega) and sequenced to confirm their identity. The TH cDNA generated by the above RT-PCR was used as probe in a Northern blot analysis. The TH cDNA was labeled and purified as above.

Northern Blot for TH in adrenal gland

Adrenal glands were collected from seven-day-old PACAP+/+, PACAP+/-, PACAP-/- littermates, frozen immediately in liquid nitrogen and stored at –80°C. Each tissue was ground into a fine powder using a chilled mortar and pestle. Total RNA from 12 adrenal glands for each genotype was isolated using TRIzol as described by the manufacturer (Life Technologies). Total RNA from each genotype was pooled to obtain enough RNA for Northern analysis.

A formaldehyde agarose gel was prepared and pre-run as above but in a total volume of 50 ml. For each genotype 12 μg of adrenal gland total RNA, 3.5 μl formaldehyde, 10 μl formamide and 2 μl of 10x formaldehyde gel loading buffer was loaded onto the gel and run at 40 V for 4 h. Once the dye front had migrated 6.5 cm, the gel was stopped and the RNA was transferred and fixed to a positively charged nylon membrane (Ambion) as above. The prehybridization and hybribization reactions were run under the same conditions as above, except the hybridization solution contained a probe specific to TH. The membrane was washed twice with 2x SSC and 0.2% SDS at 55°C for 10 min, wrapped and exposed to a Phosphor screen (Molecular Dynamics) overnight. The image was developed, analyzed and standardized as above.

Plasma catecholamines

Plasma and tissue catecholamine levels were measured in samples collected from seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice at the University of Victoria. The plasma and tissue samples were extracted and then sent to Dr. Nobuharu Yamaguchi

at McGill University in Montreal for HPLC analysis and electrochemical detection of catecholamines.

Plasma catecholamine concentrations were determined in arterial blood obtained by cardiac puncture with a heparinized needle and syringe from mice anesthetized with isofluorane. Blood (200 µl) was transferred to a centrifuge tube containing 4 µl of preservative solution (pH 6.5) consisting of ethylene glycol-bis (β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (95 mg/ml) and glutathione (60 mg/ml). Blood samples were immediately centrifuged for 5 min at 12,000 revolutions/min. Plasma was then transferred to another tube and stored at –80°C until assayed. Plasma concentrations of adrenaline, noradrenaline and dopamine were quantified by means of an isocratic high-performance liquid chromatographic system (HPLC, Gilson, Villiers-Le-Bel, France) coupled with an electrochemical detector "Coulochem II" (model 5200; ESA, Bedford, MA) according to the methods previously published in detail (Yamaguchi, 1993).

Tissue catecholamines

Tissue catecholamine contents were determined in the adrenal glands and interscapular brown adipose tissue obtained from anesthetized mice. The tissue was excised and immediately frozen in liquid nitrogen. Frozen tissue was ground into a fine powder using a chilled mortar and pestle and weighed. The powdered tissue was then added to 500 µl of 0.2 N acetic acid containing 3 mM sodium metabisulfite and 5 mM EDTA and mixed vigorously (modified from Vollmer et al., 1995). The homogenate was centrifuged at 12,000 ×g for 15 min. The supernatant was transferred to a new tube and stored at –80°C until assayed. Catecholamines were extracted from the supernatant

according to the methods described for plasma catecholamine extraction (Yamaguchi, 1993) with slight modifications as follows. To 300 µl of the supernatant in a 15 ml glass tube with a screw cap, 20 µl of an aqueous solution containing dihydroxybenzylamine (10 ng/ml prepared with 0.08 M acetic acid; served as internal standard) and 1 ml of 2 M NH_4OH-NH_4Cl buffer (pH 8.7) containing 0.1% diphenylborate-ethanolamine and 0.5% EDTA were added. After the addition of 5 ml of n-heptane containing 1% n-octanol and 0.25% tetraoctylammonium bromide, the sample solution was mixed with a rotating mixer (Reax 2, Caframo, Wiarton, Ontario) for 5 min and centrifuged at 2,500 revolutions/min for 5 min. Then 4 ml of the organic phase were transferred to a conic tube, mixed with 2 ml of n-octanol and 600 µl of 0.08 M acetic acid for 5 min, and centrifuged at 2,500 revolutions/min for 5 min. The organic phase was discarded, and the aqueous phase was transferred to an amber microtube for the HPLC coupled with the electrochemical detector "Coulochem II" (ESA). The supernatant extracts were analyzed by injecting 20 µl of the aqueous phase into the HPLC column (CSC-Vitess, 3 µm, 5 × 0.46 cm, CSC Sciences, Montreal, Quebec) through a complete filling with an aliquot of 100 µl by means of an autosampling injector (model 231-401, Gilson). Pump flow of the mobile phase was 0.8 ml/min. The effluent was monitored at the following potentials: +300 mV for the first electrode, +60 mV for the second and screen electrode, and –300 mV for the third and quantifying electrode. Full scale sensitivity was 50 nA and 1 µA for brown adipose tissue and adrenals, respectively.

Significance ($p<0.05$) of catecholamine levels in the three genotypes in plasma, brown adipose tissue and adrenal tissue was determined by a one-way ANOVA and

significance between genotypes was determined using the Tukey-Kramer multiple comparison test.

RESULTS

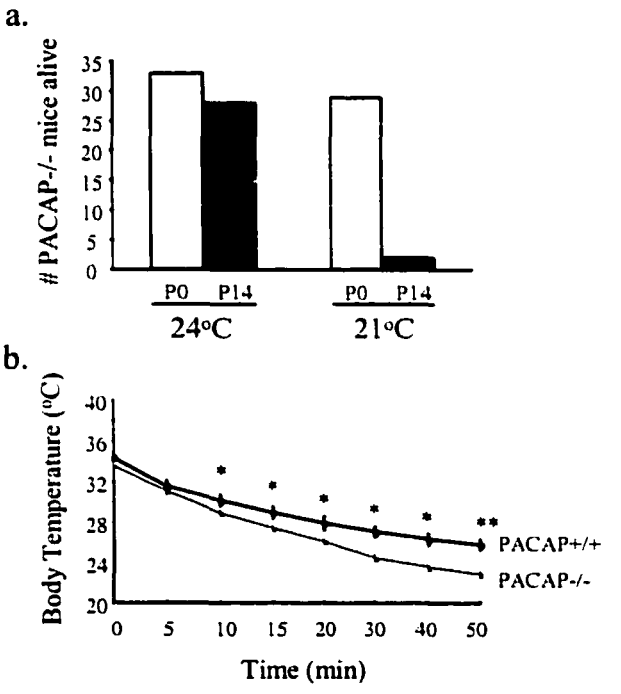
PACAP-/- mice survive at 24°C, but not 21°C

Previously we have shown that PACAP null mice born to heterozygous breeding pairs housed at 21°C die in their second postnatal week in a wasted state (Gray et al., 2001). Here we show that PACAP null mice born to heterozygous breeding pairs housed at 24°C survive with no signs of the previously reported phenotype. At 24°C, 28 out of 33 (85%) PACAP-/- mice were alive at postnatal day 14 compared to only 2 out of 29 (7%) mice at 21°C (Fig. 5.1a).

Temperature challenge severely affects PACAP null mice

A temperature challenge at 21°C shows that the body temperature of PACAP null mice drops faster than their wildtype littermates (Fig. 5.1b). The mean values for body temperature were tightly grouped for each genotype, with little variation. After 10 min separation from the mother, the knockout mice had a significantly ($p<0.05$) lower body temperature than the wildtype controls. After 50 min the body temperature of the PACAP null pups had dropped 10°C, whereas the body temperature of the wildtype littermates had only dropped 7°C.

Fig. 5.1. Temperature sensitive phenotype of PACAP null mice. a.) Postnatal survival of PACAP+/+ and PACAP-/- mice when raised at 24 °C vs 21°C. b.) Loss of core body temperature in seven-day-old PACAP+/+ (n=7) and PACAP-/- (n=9) mice when exposed to a temperature challenge (removed from mother) at 21°C. P0, postnatal day 0; P14, postnatal 14; * $p<0.05$; ** $p<0.01$.


a.
PACAP-/- mice alive
35
30
25
20
15
10
5
0
P0
P14
P0
P14
24°C
21°C
b.
Body Temperature (°C)
40
36
32
28
24
20
*
*
*
*
*
**
PACAP+/+
PACAP-/-
0
5
10
15
20
30
40
50
Time (min)

Brown adipocytes in null mice are differentiated and of normal mass

Interscapular brown fat mass to body ratios were calculated for PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day seven. There was no significant difference ($p>0.05$) between the interscapular brown fat mass to body ratios for each genotype (Fig. 5.2a). Histological examination of brown adipose tissue from PACAP+/+, PACAP+/- and PACAP-/- mice shows no difference in the appearance of brown adipose tissue among the three genotypes (Fig. 5.2b). Brown adipocytes of PACAP null mice contain multiple, lipid filled vacuoles within the cytoplasm, a feature representative of differentiated brown adipose tissue.

UCP 1 and HS lipase are expressed in brown adipocytes of PACAP null mice.

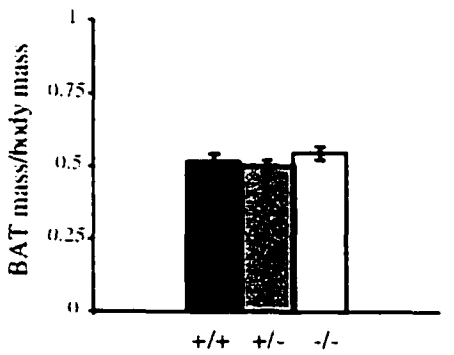
Levels of HS lipase mRNA in brown adipose tissue were measured by Northern blot. In the PACAP-/- mice, HS lipase expression was 1.6 times higher than in PACAP+/+ mice. The heterozygous PACAP mice also expressed HS lipase at levels 1.6 times higher than wildtype controls (Fig. 5.3). UCP 1 mRNA is expressed in brown adipocytes at the same level in PACAP+/+ mice and PACAP+/- mice using β-actin mRNA levels as a reference. In PACAP-/- mice, UCP 1 mRNA levels were 1.6 times higher than PACAP+/+ and PACAP+/- controls (Fig. 5.3). Because pooled tissue was used statistical analysis of the data cannot be performed.

Tyrosine hydroxylase expression in the adrenal gland of PACAP null mice

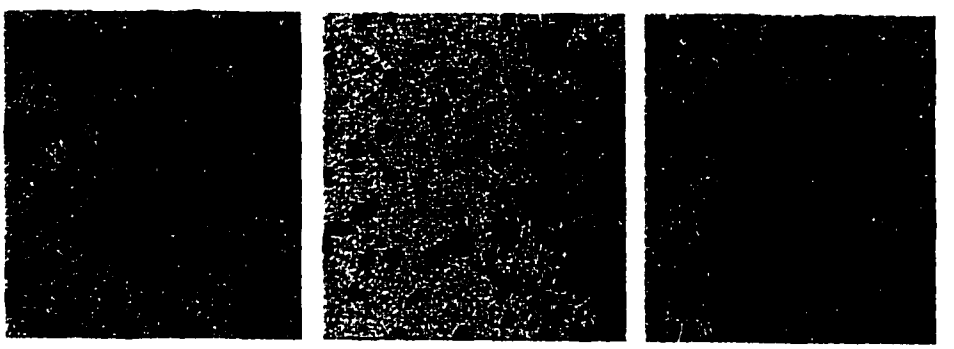
TH mRNA expression levels in the adrenal medulla of PACAP null mice is twice as high as in heterozygous and wildtype controls (Fig. 5.4). Because of the small size of

Fig. 5.2. Presence and morphology of brown adipose tissue in PACAP+/+, PACAP+/- and PACAP-/- mice. a.) Interscapular brown adipose tissue (BAT) mass to body mass ratios for PACAP+/+, PACAP+/- and PACAP-/- mice at seven days after birth. b.) Histological sections of brown adipose tissue from PACAP+/+, PACAP+/- and PACAP -/- mice shows the presence of lipid droplets in all three genotypes.

a.



b.



PACAP+/+ PACAP+/- PACAP-/-

Fig. 5.3. Northern blot analysis of brown adipose tissue showing expression of hormone sensitive lipase (HSL) and uncoupling protein 1 (UCP 1) mRNA in seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice raised at 21°C. Each value represents the amount of HSL or UCP mRNA present in each of the three genotypes. Relative band intensity resulting from the ß-actin Northern bolt was used to standardize the amount of mRNA present for each genotype.

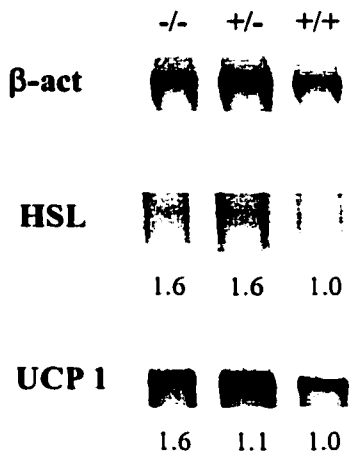
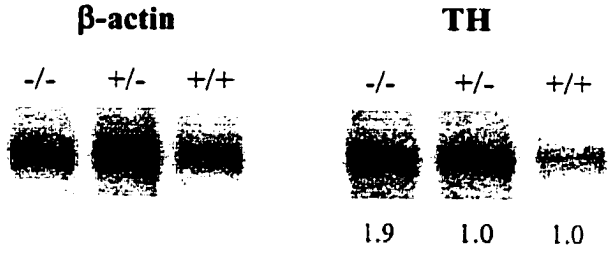

-/-
+/-
+/+
β-act
HSL
1.6
1.6
1.0
UCP 1
1.6
1.1
1.0

Fig. 5.4. Northern blot analysis of adrenal tissue showing expression of tyrosine hydroxylase (TH) mRNA in seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice raised at 21°C. Each value represents the amount of TH mRNA present in each of the three genotypes. Relative band intensity resulting from the ß-actin Northern blot was used to standardize the amount of mRNA present for each genotype.


β-actin
TH
-/-
+/-
+/+
-/-
+/-
+/+
1.9
1.0
1.0

the adrenal glands in the 7 day old mice pups, pooled tissue was used which prevents statistical analysis of the data.

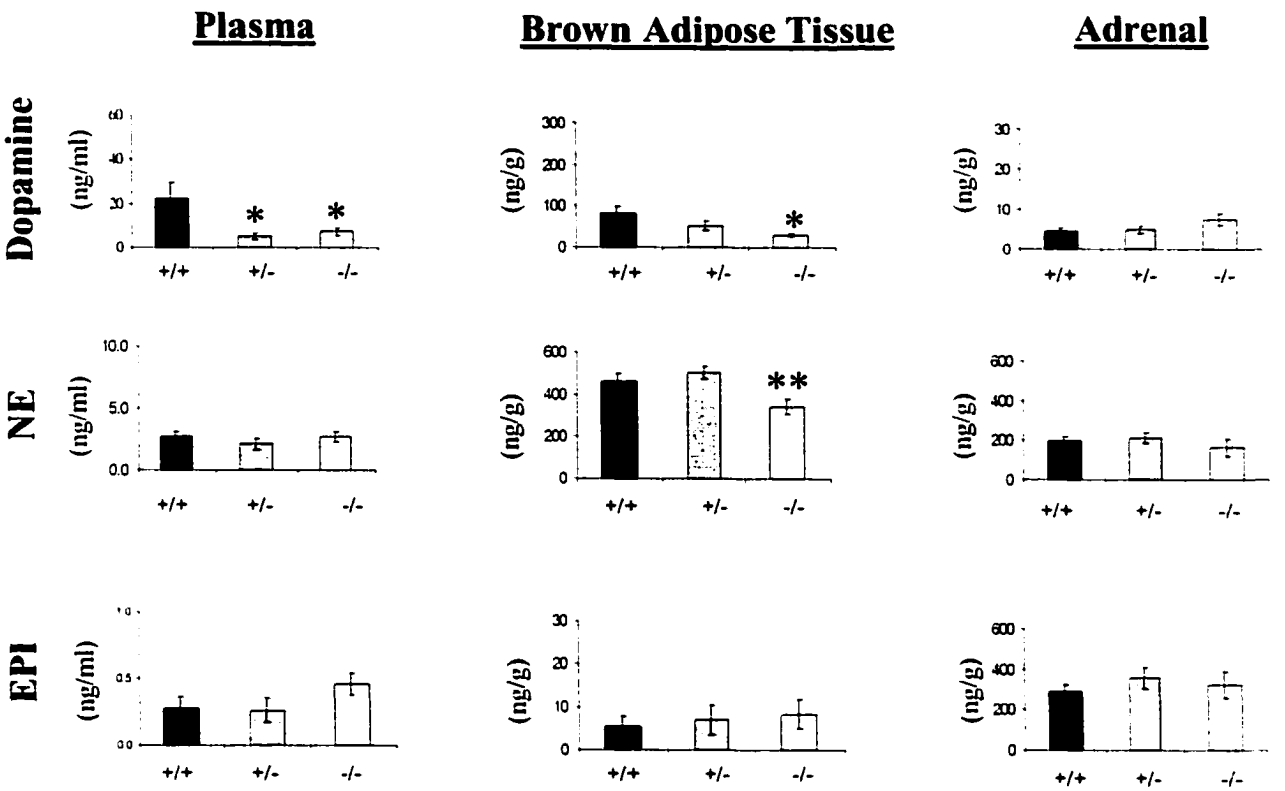
Norepinephrine and dopamine are low in brown adipose tissue of PACAP null mice.

In brown adipose tissue, norepinephrine levels were significantly ($p<0.05$) lower in PACAP-/- mice compared to both PACAP+/- and PACAP+/+ littermates (Fig. 5.5). Norepinephrine levels in plasma and adrenal tissue of seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice did not differ ($p>0.05$) among the three genotypes (Fig. 5.5). Epinephrine levels in brown adipose tissue, plasma and adrenal tissue did not differ among the genotypes (Fig. 5.5). In brown adipose tissue, PACAP null mice showed significantly lower levels of dopamine than wildtype mice, but were not different from heterozygotes (Fig. 5.5). Also, dopamine in the plasma of heterozygote and null mice was significantly lower ($p<0.05$) than that seen in wildtype mice. Dopamine levels in adrenal tissue did not differ between genotypes (Fig. 5.5).

DISCUSSION

The mortality of PACAP null mice increases greatly if they are raised at 21°C compared to 24°C suggesting PACAP plays a role in thermogenesis (Fig. 5.1a). The temperature challenge study confirms the importance of PACAP in thermoregulation as PACAP null mice lost core body temperature more readily than wildtype controls (Fig. 5.1b). Therefore, normal mechanisms to increase body temperature in times of cold stress are not functioning correctly in PACAP null mice.

Fig. 5.5. Dopamine, norepinephrine (NE) and epinephrine (EPI) levels in plasma, brown adipose tissue and adrenal tissue of seven-day-old PACAP+/+, PACAP+/- and PACAP-/- mice raised at 21°C.


Plasma
Brown Adipose Tissue
Adrenal
Dopamine
NE
EPI
(ng/ml)
(ng/g)
+/+
+/-
-/-
*
**

Previous experiments have shown a role for PACAP in thermoregulation. Reserpine-induced hypothermia in mice was reversed by the administration of PACAP-38. VIP did not elicit the same response, which suggests that the PACAP specific receptor mediates the hypothermia-reversing effects of PACAP (Masuo et al., 1995). Pataki et al. (2000, 2002) showed that cerebroventricular administration of PACAP induced hyperthermia in rats. The pathway involved in PACAP induced hyperthermia is unknown. However, PACAP is produced in the hypothalamus, a site of thermoregulatory control, and has been shown to regulate thermogenic hormones such as thyroid hormones and catecholamines (Sherwood et al., 2000).

The main mechanism for adaptive thermogenesis in neonatal rodents is activation of non-shivering thermogenesis in brown fat by UCP 1 (Ricquier et al., 2000). We found that brown adipose tissue of the PACAP-/- mice is present and fully differentiated as is the brown adipose tissue of PACAP+/- and PACAP+/+ littermates. The calculated interscapular brown fat mass to body ratios for PACAP+/+, PACAP+/- and PACAP-/- mice at postnatal day seven were not significantly different from one another (Fig. 5.2a). Histological examination of brown adipose tissue shows the presence of multiple lipid filled vacuoles in the cytoplasm of the brown adipocytes (Fig. 5.2b). In undifferentiated brown adipocytes, lipid vacuoles are absent or sparse (Casteilla et al., 2000). Therefore, we conclude that the thermoregulatory problems associated with the lack of PACAP are not due to a lack of brown adipose tissue or to an inability of brown adipose tissue to differentiate during embryogenesis in the PACAP null mice.

Brown adipocytes of PACAP-/- mice are able to express two enzymes, HS lipase and UCP 1, which function in the breakdown of stored fats for the production of heat. In

PACAP null mice, HS lipase and UCP 1 mRNAs are produced at levels at least as high as wildtype controls (Fig. 5.3). The ability of brown adipocytes from PACAP-/- mice to express both HS lipase and UCP 1 suggests the lack of PACAP is not affecting transcription of these enzymes. In fact, both HS lipase and UCP 1 mRNA are upregulated in the PACAP null mice compared to wildtype controls. Brown adipose tissue of PACAP null mice is likely able to function in non-shivering thermogenesis if a regulatory signal reaches the brown adipocytes.

Norepinephrine, which affects brown adipocyte function is produced and secreted from two sources. The first source is in sympathetic nerve endings terminating on brown adipocytes; the second is in the adrenal medulla where norepinephrine is released into the circulation. As the main hormone involved in adaptive thermogenesis, norepinephrine production in PACAP null pups that are unable to thermoregulate was assessed. Because the majority of norepinephrine supplied to brown adipocytes is released directly from the postganglionic nerve terminals innervating brown adipose tissue, we measured levels of catecholamines in extracted interscapular brown adipose tissue. Levels of norepinephrine in interscapular brown adipose tissue (463 ng/g) was 171 times higher than levels of norepinephrine in plasma (2.7 ng/ml) and 2.4 times higher than levels in adrenal tissue (194 ng/g). PACAP null mice had significantly decreased levels of norepinephrine and its precursor dopamine in postganglionic nerve terminals innervating brown adipose tissue compared to wildtype and heterozygous controls, suggesting that in times of prolonged cold stress, lack of PACAP inhibits production of dopamine and norepinephrine. Another study in PACAP null mice showed abnormal catecholamine synthesis in adult PACAP null mice after an insulin challenge, where the lack of PACAP

resulted in prolonged hypoglycemia and increased mortality from an inability to maintain sustained epinephrine release from the adrenal medulla (Hamelink et al., 2002).

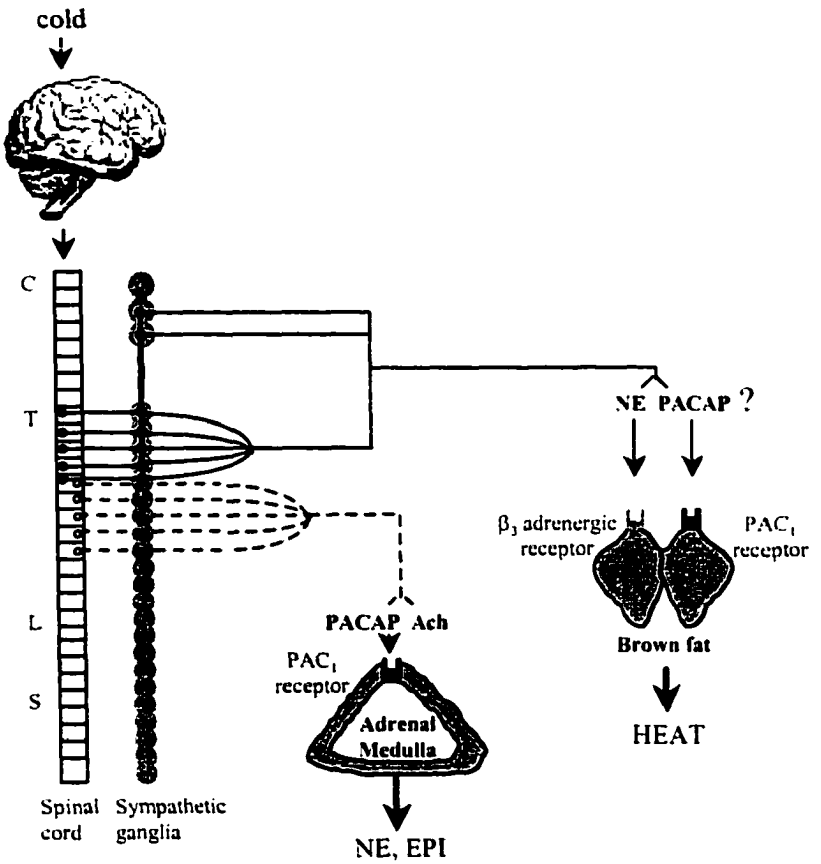
Colocalization of PACAP and norepinephrine in sympathetic nerves that terminate in brown adipose tissue has not been studied, but the two hormones are colocalized in nerve terminals in the supraoptic nuclei of the hypothalamus that synapse on arginine vasopressin neurons. Together, PACAP and norepinephrine have a synergistic effect on vasopressin release (Shioda et al., 2000b). If PACAP and norepinephrine were colocalized in the terminals of postganglionic nerves innervating brown adipose tissue, the absence of PACAP in the null mice may prevent synergistic effects of PACAP and norepinephrine on brown adipocytes (Fig. 5.6).

Although PACAP causes secretion of the catecholamines, norepinephrine and epinephrine, directly from the adrenal medulla via multiple signaling pathways, catecholamines in the adrenal gland of seven-day-old PACAP null mice raised at 21°C were not significantly different from wildtype or heterozygous littermates. Plasma norepinephrine and epinephrine levels were not significantly different in the cold stressed PACAP null mice compared to controls. Unexpectedly, dopamine levels in the plasma of PACAP+/- and PACAP-/- mice were significantly lower than in wildtype mice. Production and distribution of heat from brown adipose tissue is activated by norepinephrine from sympathetic neurons. Therefore, normal levels of epinephrine in all tissues examined and norepinephrine in the adrenal gland and plasma of cold stressed PACAP null mice is expected.

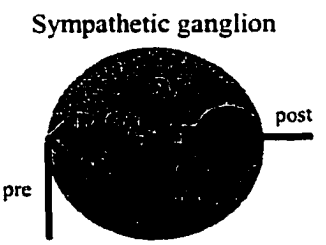
Catecholamines are produced in an enzymatic reaction involving three main enzymes (TH, dopamine β-hydroxylase and phenylethanolamine N-methyltransferase).

Fig. 5.6. Hypothesized role of pituitary adenylate cyclase-activating polypeptide (PACAP) in the sympathetic control of adaptive thermogenesis. a.) One group of preganglionic nerves originate in thoracic (T) segments (T_1-T_5) of the spinal cord and synapse in the sympathetic ganglia; postganglionic nerves synapse on brown adipose tissue. A second group of sympathetic preganglionic nerves synapse directly on adrenal medullary cells. PACAP is known to be colocalized with acetylcholine (Ach) in the preganglionic neurons that synapse in the adrenal medulla. b.) It is not yet elucidated whether there is colocalization of PACAP with Ach in preganglionic fibers that synapse in the sympathetic ganglia or whether there is colocalization of PACAP with norepinephrine (NE) in postganglionic neurons for sympathetic control of brown adipose tissue. EPI, epinephrine, PAC_1, PACAP-specific receptor, C, cervical, L, lumbar, S, sacral spinal cord.

a.


cold
C
T
L
S
Spinal cord
Sympathetic ganglia
NE PACAP ?
β3 adrenergic receptor
PAC1 receptor
Brown fat
HEAT
PACAP Ach
PAC1 receptor
Adrenal Medulla
NE, EPI

b.


Sympathetic ganglion
post
pre

PACAP has been shown to regulate transcription and/or activity of all three enzymes in adrenal medullary cells (Isobe et al., 1996, Tonshoff et al., 1997, Choi et al., 1999, Park et al., 1999). As the rate limiting enzyme of catecholamine synthesis, TH mRNA expression levels in adrenal medulla of PACAP null mice was assessed compared to controls. Normally, PACAP activates transcription of the TH gene, so decreased transcription of the TH gene in PACAP null mice was expected. However, in adrenal tissue of PACAP-/- mice TH was expressed at levels twice as high as controls (Fig. 5.4). The upregulation of TH mRNA expression in the adrenal medulla of PACAP null mice suggests an increased need for TH in the adrenal medulla of cold stressed PACAP null mice. Therefore, the thermoregulatory problems of the PACAP null mice are not associated with decreased transcription of the TH gene. PACAP can regulate TH activity by phosphorylating serine residues of the TH protein through the cAMP, PKA pathway (Marley et al., 1996, Moser et al., 1999). Decreased TH activity after an insulin challenge was shown in adult PACAP-/- mice that were unable to maintain prolonged epinephrine release (Hamelink et al., 2002).

The present study suggests a role for PACAP in sustained activation of the sympathetic nervous system in times of prolonged physiological stress, such as cold stress. When raised at 21°C, PACAP null mice cannot supply appropriate levels of norepinephrine to brown adipocytes and therefore adaptive thermogenesis of PACAP null mice is decreased. If PACAP also regulates other stress responses in which norepinephrine and epinephrine are released, this could explain why the structure of the PACAP peptide has remained highly conserved (Sherwood et al., 2000).

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CHAPTER 6

CONCLUSIONS

Consequences of PACAP and PACAP receptor gene knockout.

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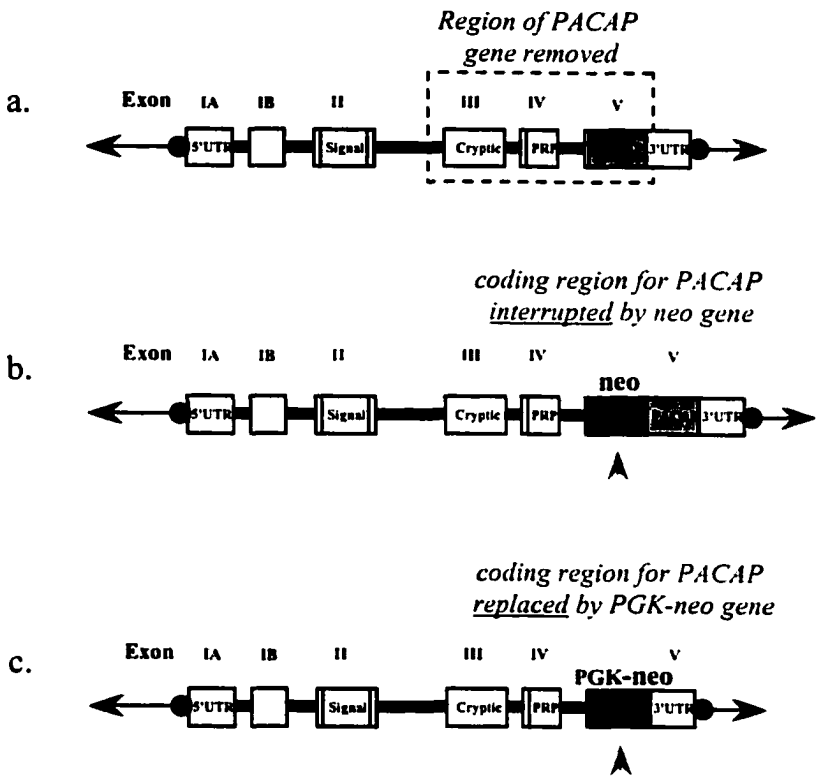
Our understanding of the role of PACAP in the nervous and endocrine systems has been expanded by analyzing mouse lines with a targeted gene disruption of the PACAP peptide and of the PACAP-specific receptor (PAC_1 receptor). Using a PAC_1 receptor knockout, several groups have been successful in uncovering a role for PACAP in behavior (Otto et al, 2001a,b, Sauvage et al., 2000) and glucose homeostasis (Jamen et al, 2000a). Knocking out the PACAP gene has shown that PACAP has a role in the metabolic balancing act that controls the handling of lipids and carbohydrates (Gray et al, 2001), in the sympathetic control of thermoregulation (Gray et al., in press) and glucose homeostasis (Hamelink et al., 2002). Behavioural abnormalities have also been shown in mice lacking PACAP (Hashimoto et al., 2001). The mechanism by which PACAP exerts these effects remains unclear. We do not know if the effects are direct or are mediated by changes in production, release or action of other hormones such as epinephrine, norepinephrine, glucocorticoids or insulin. PACAP's high degree of conservation implies a crucial role in normal physiology or in the physiological response to changing environments. Comparison of PACAP and PACAP-receptor gene knockout phenotypes will contribute to our understanding of PACAP's physiological role.

Similarities and differences between three strains of PACAP null mice.

Different targeting strategies used to disrupt the PACAP gene in mouse

We recently created a line of PACAP null mice (Gray et al, 2001) by deletion of the coding region for PACAP and two nonfunctional peptides, the cryptic peptide and PACAP-related peptide (Fig. 6.1a). Since then two additional PACAP null mouse lines have been generated using different targeting strategies. A second mouse line deficient in

Fig. 6.1. Targeting strategies for the PACAP knockout mice lines. a. The coding region for the cryptic peptide, PACAP related peptide (PRP) and PACAP are removed preventing production of all three peptides (Gray et al, 2001). b. The coding region for PACAP is interrupted by the neomycin-resistance (neo) gene, preventing the production of PACAP (Hashimoto et al, 2001). c. The coding region for PACAP is replaced by the phosphoglycerate kinase (PGK) promoter-neomycin-resistance (neo) gene, preventing the production of PACAP (Hamelink et al, 2002).


a.
Region of PACAP gene removed
Exon
IA
IB
II
III
IV
V
5'UTR
Signal
Cryptic
PRP
3'UTR
b.
coding region for PACAP interrupted by neo gene
neo
c.
coding region for PACAP replaced by PGK-neo gene
PGK-neo

PACAP was generated (Hashimoto et al, 2001) through inactivation of the peptide-coding region of exon 5 (Fig. 6.1b). A neomycin resistance cassette was inserted in front of exon 5 of the PACAP gene, which prevented expression of PACAP in mice homozygous for the mutated allele. This mouse line was generated in 129/SvJ embryonic stem cells and backcrossed to both a C57Bl/6 and an ICR background. In a third mouse line, part of exon 5 including the PACAP-encoding region was replaced with the neomycin resistance gene (Fig. 6.1c) (Hamelink et al, 2002).

The litter sizes and ratio of the genotypes from heterozygous breeding pairs support the conclusion that pups do not die in utero. However, our PACAP null mice showed a striking phenotype in which most of the pups died during the second postnatal week. The mouse line generated by Hashimoto et al. (2001) had a mendelian ratio of genotypes in litters from heterozygous matings at birth, but by weaning there was a significant loss of PACAP null pups. Although a high mortality of PACAP null pups was reported in the first two studies (Gray et al, 2001; Hashimoto et al, 2001) mortality was not examined in the third study (Hamelink et al, 2002).

Temperature sensitive phenotype

Although high mortality of PACAP null pups prior to weaning has been reported in another PACAP null mouse line (Hashimoto et al., 2001), temperature sensitivity has not been identified as the cause of mortality. Our observation of a dramatic decline in PACAP null mouse survivability when raised at 21°C compared to 24°C (Fig. 5.1) has not been reported in other PACAP null mouse lines thus far.

Altered metabolism in PACAP null mice

Metabolic abnormalities have been shown in two of the three PACAP null mouse lines (Gray et al., 2001, Hamelink et al., 2002). Our PACAP null mice have a striking phenotype of early postnatal death accompanied by wasting and lipid accumulation in tissue and serum. This phenotype is temperature sensitive and when animals are raised at 24°C, the mortality of the pups is greatly reduced (Fig 5.1). Surviving PACAP null mice show no signs of wasting. Carbohydrate metabolism appears to be abnormal in fasted PACAP null pups at 5 days of age as serum insulin levels are higher compared to controls. No comment was made on postnatal mortality for the PACAP null mouse line generated by Hamelink et al. (2002). The temperature at which the animals are being raised was not reported but may account for this discrepancy. Lowered glucose in fasted, adult PACAP null mice compared to controls was observed (Hamelink et al., 2002). In addition, severe hypoglycemia and increased mortality ensued in response to an insulin challenge, due to a lack of sustained epinephrine secretion from the adrenal medulla (Hamelink et al., 2002). This abnormal catecholamine response due to insulin stress suggests the absence of PACAP is preventing sustained secretion of epinephrine from the adrenal medulla. In spite of the abnormal catecholamine response, adrenal morphology in 7 day-old (Gray et al., 2001) and adult (Hamelink et al., 2002) PACAP null mice is normal.

Our PACAP null mice also exhibit a decreased catecholamine response to environmental stress. We have shown that under cold stress, lower levels of norepinephrine are present in the brown adipose tissue of PACAP null mice. This suggests that in the absence of PACAP, insufficient norepinephrine is secreted from

postganglionic sympathetic nerve terminals or that innervation of sympathetic nerves to the brown adipose tissue is reduced.

Behavioural abnormalities in the PACAP null mouse

The morphology of the nervous system of 6-8 day old pups is not grossly changed (Gray et al, 2001). However, in the peptide knockout mice generated by Hashimoto et al (2001), behavioral abnormalities have been observed. PACAP null mice have increased locomotor and exploratory behavior and a decrease in anxiety- related behaviours compared to wild type controls. In addition, the PACAP null mice showed explosive jumping behaviour when placed in an open field. These observations show abnormal psychomotor behaviour in the PACAP deficient mice (Hashimoto et al, 2001).

Phenotype of PACAP hormone versus PAC_1 receptor knockout mice.

To uncover PACAP's basic functions using gene manipulation, at least two approaches have been tried. The first is to disrupt the single-copy PACAP gene prior to conception so that the PACAP protein product is completely eliminated (Gray et al., 2001, Hashimoto et al., 2001, Hamelink et al., 2002). This approach revealed that PACAP is important for postnatal survival and identifies a number of PACAP functions, although it does not show if the effects are primary or secondary. The second strategy is to disrupt, one at a time, each of the PACAP receptor subtypes. It is understood that deletion of the shared receptors ($VPAC_1$ and $VPAC_2$) would not show whether PACAP or VIP was the critical peptide. However, deletion of the PACAP-specific receptor (PAC_1-R) reveals a subset of PACAP's actions. Eventually, crossing of the individual

PACAP receptor knockouts would result in multiple receptor knockouts in one mouse line.

Early postnatal death, seen in two of the PACAP null lines (Gray et al, 2001, Hashimoto et al, 2001), is also seen in PAC_1 receptor knockout mice where high mortality of PAC_1 receptor null pups occurs in the 4 weeks between birth and weaning (Jamen et al, 2000a, Otto et al, 2001b).

As in PACAP null mice, abnormal carbohydrate metabolism was seen in PAC_1 receptor knockout mice. Although PAC_1 receptor null mice also show normal blood glucose in the fed and fasted state, insulin levels are high in the fed state suggesting a possibility of insulin resistance. In contrast to the PACAP null mice, PAC_1 receptor null mice show an impaired insulin response and glucose intolerance after intravenous or gastric glucose administration (Jamen et al., 2000a). These studies are of considerable interest in that insulin in the receptor knockout is high in the fed state, yet in the peptide gene knockout insulin is high in the fasted state. One difference is that insulin was measured in the receptor knockout mice as adults, but in the PACAP knockouts as 5-day-old pups.

As in the PACAP knockout mouse line generated by Hashimoto et al. (2001), the PAC_1 receptor knockout mouse exhibits increased locomoter behaviour and decreased anxiety (Otto et al., 2001a,b). In addition, the PAC_1 receptor mice display a decrease in learned fear (contextual fear conditioning) and hippocampus-dependent learning (Otto et al., 2001a,b). A forebrain-specific PAC_1 receptor knockout, where the PAC_1 receptor is inactivated postnatally in the hippocampus and cortical forebrain has been generated (Otto et al., 2001a). The forebrain PACAP-deficient strain showed neither increased

locomotor behaviour nor a decrease in anxiety-related behaviours suggesting the action of PACAP in the hippocampus is not involved in locomotor behaviour and anxiety (Otto et al, 2001a). The decrease in learned fear (contextual fear conditioning) and hippocampus-dependent learning was present in both the PAC_1 receptor knockout and the forebrain specific PAC_1 receptor knockout. Development of conditional knockouts will be important in assessing the role of PACAP in the CNS. The PAC_1 receptor knockout model has been used to assess PACAP's role in other neuronally controlled processes. PAC_1 receptor knockout mice showed a reduced pain response in chronic inflammation (Jongsma et al, 2001) and circadian rhythms were altered in the PAC_1 receptor null mouse (Hannibal et al, 2001). Histological examination did not show pathological abnormalities in the brain and neurological tests did not reveal any deficits in sensory or motor abilities (Otto et al, 2001b). These studies provide evidence that PACAP acts directly or indirectly to control behaviour.

To date, heterozygous mating pairs reproduce as efficiently as wildtype breeding pairs. PACAP null crosses are able to mate and care for their young (personal observation, Hashimoto et al, 2001; Hamelink et al, 2002). However, there are fewer pups than with heterozygous crosses due to fewer pregnancies (personal observation, Hashimoto et al, 2001). Reproductive efficiency has also been assessed in PAC_1 receptor knockouts. One study reports a long estrous cycle in PAC_1 receptor null females associated with decreased fertility (Jamen et al, 2000b). A mouse line lacking the $VPAC_2$ receptor had normal fertility (West et al, 1998).

Future directions

Generation of the PACAP knockout mouse has provided an in vivo mammalian system to study PACAP's function. We have identified a role for PACAP in lipid metabolism and thermoregulation. Determining the mechanism by which PACAP's absence is causing these abnormalities may involve complex signaling systems. So far we have identified problems in the sympathetic nervous system and in the pancreatic endocrine system. Further work to determine the mechanism by which the lack of PACAP causes these changes continues.

Studies in our laboratory have been performed on mixed strain mice. We have backcrossed the PACAP null mutation onto a C57Bl/6 mouse line and currently have mice that have been backcrossed seven times. The postnatal mortality with wasting and abnormal lipid accumulation is present in the backcrossed PACAP null mice confirming the PACAP null phenotype is not a result of mouse strain characteristics. Further studies in our lab and in other laboratories should be performed on inbred lines of PACAP null mice to provide consistency of results among the various laboratories.

To examine the thermoregulatory problems associated with the PACAP null mice, I chose to focus on the sympathetic nervous system that activates adaptive thermogenesis. We have shown abnormal levels of norepinephrine in the brown adipose tissue of PACAP null mice. In addition to sympathetic control of thermoregulation, the thyroid hormones are important in obligatory thermogenesis. A study is underway in our laboratory examining the thyroid hormone system in the PACAP null mice. The results of this study will allow us to determine if one or both hormonal systems regulating thermogenesis are affected by the absence of PACAP.

The temperature sensitive phenotype of the PACAP null mice provides a unique opportunity to study the effects of the absence of PACAP in both neonatal mice under temperature stress and adult mice without temperature stress. Mice raised at a temperature of 24°C survive to adulthood. We can assess the effect of other stressors on these adult mice, and examine the effects of the lack of PACAP on other physiological systems that may be masked in the ailing PACAP null pups raised at 21°C.

Initial studies examining carbohydrate and lipid metabolism in the PACAP null mouse presented here have shown that the absence of PACAP may be affecting how insulin is regulated. Further work to characterize the mechanism behind the diabetic-like symptoms seen in the PACAP null mice is needed. These studies should be performed in both PACAP null pups and in PACAP null mice that survive to adulthood. An assay that allows measurement of insulin in a very small amount of plasma or serum is needed so that glucose tolerance or insulin challenge tests can be performed in the PACAP null pups affected by temperature. ELISA kits that only require 5 µl of serum or plasma are available, but need to be optimized in our laboratory. Examination of insulin signaling in the PACAP null mice compared to controls would confirm whether the PACAP null mice are insulin resistant. These studies could include expression of insulin receptor mRNA in different tissues, phosphorylation of the receptor, levels of the glucose transporter molecule GLUT-4 and levels of other components in the insulin receptor signaling pathways in PACAP null mice compared to controls. We plan to use microarrays to identify abnormal expression of genes within the adipocytes of PACAP null mice compared to wildtype controls.

Examination of the reproductive integrity of the PACAP null pups has begun. Preliminary data suggests the PACAP null mice are not able to reproduce normally. Studies examining the breeding capabilities of both male and female PACAP null mice, morphological examination of the reproductive organs and the measurement of reproductive hormones that may be affected by PACAP's absence will be examined.

The PACAP null mouse provides us with a whole animal model to study gene function. In addition, primary cell culture from tissue of the PACAP null mouse would provide a cell system that cannot produce PACAP compared to those that can. Because PACAP is a hormone, the site of synthesis and release may not be the site of action, yet paracrine and autocrine effects of locally synthesized PACAP could be studied in a cell culture system.

The targeting strategy we used to generate the PACAP null mouse used the cre-lox P targeting system. Embryonic stem cells that house a "floxed" PACAP gene have been generated and could be used to generate mouse lines with a conditional PACAP knockout.

Conclusions

We chose to delete the single copy PACAP gene whose product activates both PACAP-specific and VIP-shared receptors. We found that the PACAP null mice had an unusual phenotype with major changes in lipid distribution, some impairment in carbohydrate metabolism and impaired thermogenesis that results in early postnatal death in most of the PACAP-/- mice raised at 21°C. These results may be primary or secondary, as PACAP has a role in the regulation of other hormones that affect

metabolism and thermoregulation, such as insulin, corticosteroids, acetylcholine and the catecholamines. PACAP can activate several intracellular pathways to produce its diverse actions, but we don't know whether the actions are coordinated in response to a physiological challenge. PACAP was discovered 13 years ago and already it has been associated with a diverse array of physiological functions (Sherwood et al., 2000). Generation of the PACAP null mouse has identified the importance of PACAP in mammalian physiology. Specifically it has identified a role for PACAP in lipid and carbohydrate metabolism and has exemplified its role in regulating the sympathetic nervous system. The knockout study has confirmed that PACAP, which has remained structurally conserved for 700 million years, is a physiologically important molecule.

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