Vertebrate Development and Physiology in Response to Augmented Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

By

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B.Sc.H, University of Victoria, 2001

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

MASTER OF SCIENCE

In the Department of Biology

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a recently discovered neuropeptide classified as a member of the glucagon superfamily of hormones that are primarily involved in metabolism and growth of vertebrate species. Some members of this superfamily including PACAP have been identified even in the invertebrate tunicate (sea squirt) suggesting their role early in evolution. The fact that PACAP has remained the most highly conserved member throughout evolution implies that its role in an organism may be significant. This is supported by studies where mice completely lack the hormone (knockout mice) in their genetic makeup resulting in death shortly after birth. Also, PACAP knockout mice are sensitive to hypothermia, anaesthesia and excessive insulin levels suggesting its protective role during stress situations of environmental and/or metabolic origin.

In the present study I have used two animal models with elevated PACAP levels to investigate PACAP's physiological role in vertebrates. First, I chose the African clawed frog, *Xenopus laevis* to overexpress PACAP. Frog transgenesis provides many advantages over mouse transgenesis because large number of modified embryos can be generated readily with stable expression of the gene of interest in desired tissues. Frogs serve as an ideal model to study development since embryos develop externally. Second, I used the house mouse, *Mus musculus* to mimick overexpression by hormone infusion using micro-osmotic pumps. Pumps eliminate the time and stress of repeated injections and allow researchers to treat an animal with a desired hormone continuously for a duration of 2 weeks.

The purpose of this study was to examine the effects of PACAP on 1) survival, growth, development and metamorphosis in amphibians at an embryonic and juvenile stage, and 2) survival, growth and carbohydrate metabolism in mature mammals exposed to excess PACAP for 12 days after weaning. Based on the findings from previous experiments I hypothesized that PACAP overload would result in normal survival rates, increased growth, abnormal development, normal metamorphosis and altered carbohydrate metabolism. In the present study, transgenic frogs had lower survival rates than wild types, were significantly smaller in size but those that survived had normal development and metamorphosis. These results indicate that PACAP may be lethal to amphibians at higher than normal concentrations and causes retarded growth when present from the day of fertilization. In contrast, mature mice that received PACAP by long-term infusion did not die, grew normally and had normal insulin and glucose levels at the end of the hormone treatment. Preliminary findings also reveal that higher concentrations of PACAP in both the frog and the mouse may have promoted a reduction in PACAP receptor sensitivity. Further research will help to define the role of PACAP at the molecular and physiological level in adult and developing vertebrates.

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LIST OF ABBREVIATIONS

AADC	aromatic amino acid decarboxylase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CNS	central nervous system
DBH	dopamine β-hydroxylase
dNTP	deoxyribonucleoside triphosphate (N = any nucleoside)
EDTA	ethylenediaminetetraacetic acid
ES cell	embryonic stem cell
G protein	trimeric guanosine triphosphate-binding protein
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
GIP	glucose-dependent insulinotropic polypeptide
GLP	glucagon-like peptide
Gq	phospholipase C-associated G protein
Gs	stimulatory G protein
longGP	full length frog GHRH and PACAP transcript
MMR	Marc's modified Ringers solution
mRNA	messenger ribonucleic acid

NPB	nuclear preparation buffer
PAC_1-/-	PACAP receptor knockout mouse
PACAP	pituitary adenylate cyclase-activating polypeptide
PACAP-/-	PACAP knockout mouse
PAC_1-R	PACAP specific receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHM	peptide histidine methionine
PLC	phospholipase C
PNMT	phenylethanolamine N-methyltransferase
PRP	PACAP-related peptide
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
shortGP	truncated frog GHRH and PACAP transcript
SP	signal peptide
SSB	sperm storage buffer
SV	simian virus
TH	tyrosine hydroxylase
UTR	untranslated region
VIP	vasoactive intestinal polypeptide
$VPAC_1$-R/ $VPAC_2$-R	PACAP and VIP shared receptor
xCAR	*Xenopus laevis* endogenous cardiac actin promoter

ACKNOWLEDGEMENTS

I would like to thank the following people for their time, help, encouragement and support because without them I would not be able to complete this work and my time spent doing research would not be nearly as enjoyable. I am thankful for having Dr. Nancy Sherwood as my supervisor who guided me during my Masters project. The Sherwood lab would also not be the same without Carol Warby, Elaine Vickers, Sarah Gray, Wayne Gray, Erica Fradinger, Bruce Adams, Sheng Wu, Nola Erdhardt, Javier Tello, Greg Gillespie, Kaaren Gibb and Emma Isaac - people who really dedicated much of their time to help me out and encouraged me not to give up my hopes. I must also mention the names of Stephen O'Leary and Brett Poulis who almost felt like being part of the Sherwood lab and Dr. Karen Helbing, Dominik Domanski and Nik Veldhoen, the frog experts, who gave me some good advice.

For the time spent in Calgary at the Foothills Hospital where I made transgenic frogs, I must thank Dr. Leon Browder and Dr. Frank Jirik for letting me work in their labs and for their mentorship during my visits away from home. I also thank Jill Johnston, Suzen Lines and Tara Stapleton (lab techs) for being patient with me and teaching me new techniques. Artee Luchman, Linda Sandercock, Alice Ford-Hutchinson and Jennette Gorday have been also helpful and good friends. Finally, I thank Kevin Cummings for letting me stay at his house and for his expertise advice in transgenesis.

Outside of the labs at UVic, I was dependent on many people to complete my work. These included the Animal Care Unit and Aquatics staff: Wendy Linn, Daniel Morgado, Ralph Scheurle and Gerry Horne. I also really appreciated the help from Eleanore Floyd, Maarten Voordouw, Roderick Haesevoets, Ted Allison, Tom Gore, Heather Down and Dr. Singla. I thank my committee members, Dr. Francis Choy, Dr. Will Hintz and Dr. Juan Ausio for their time and advice.

Finally, a special thanks goes to my family, especially to my Dad, Mom, and husband for being supportive, understanding and patient.

This thesis is dedicated to my little son, Thomas.

CHAPTER 1

Introduction

PACAP and the glucagon superfamily of hormones

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide belonging to the glucagon superfamily of hormones identified in both vertebrate and invertebrate species (1). The peptide was first isolated in 1989 by Miyata *et al.* in ovine hypothalamic extracts due to its ability to stimulate cAMP in rat anterior pituitary cells (2). PACAP is the most recent peptide to be added to the superfamily, as it has similar structure, function and receptors. Some of its receptors are shared with vasoactive intestinal polypeptide (VIP), another glucagon superfamily member. Other members of the superfamily include glucagon, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), growth hormone-releasing hormone (GHRH), peptide histidine methionine (PHM), secretin, and glucose-dependent insulinotropic polypeptide (GIP) (1). The superfamily peptides exhibit some overlap in function, tissue specificity, and structural similarity. For example, eight of the peptides are classified as neuropeptides because they are found in the brain. All of the superfamily members are found directly in the gut or at least in the gut nerve endings and some members affect the release of hormones in the anterior pituitary. In addition, all peptides seem to be involved in critical metabolic processes (1).

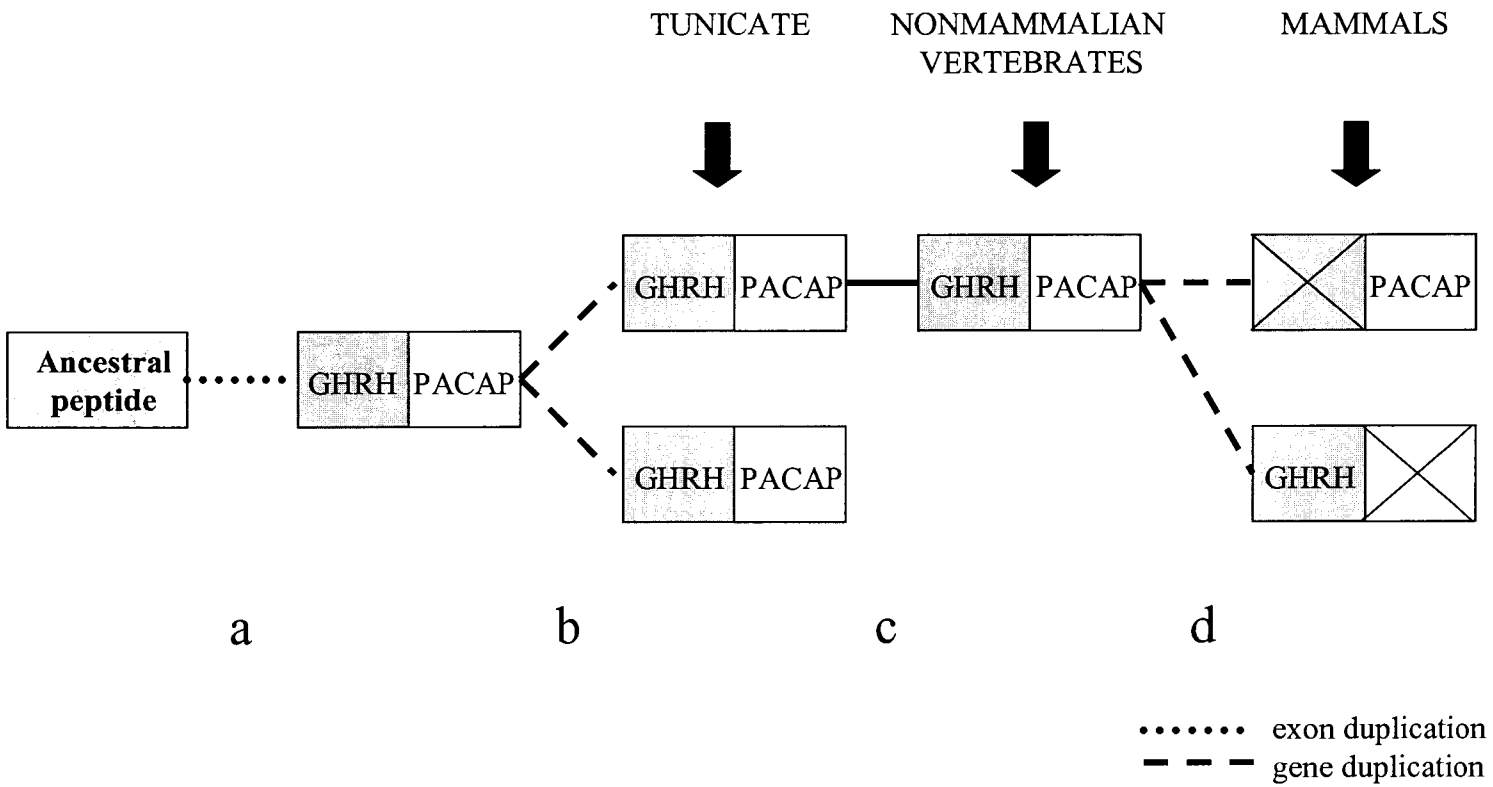
Evolution of PACAP and GHRH

PACAP has been identified in species of fish, amphibians, reptiles, birds and mammals as well as in the ancestral protochordate, tunicate (3). In 1997, McRory and Sherwood discovered two ancient forms of PACAP in the tunicate species, *Chelyosoma productum* (4). The more conserved isoform, tunicate PACAP-1 of 27 amino acids, has a

striking amino acid identity of 96% with the phylogenetically advanced mammals including mouse, rat, and human. When comparing all glucagon superfamily hormones, PACAP is the most conserved in terms of peptide length and sequence identity of either amino acids or cDNA. Because the sequence of PACAP has been extremely well preserved over an evolutionary period of approximately 700 million years, it has been suggested that PACAP may be essential for survival and is an ancestral molecule to the glucagon superfamily (1). As a comparison, the sequence of GHRH has been poorly conserved during evolution and even among closely related species such as mammals (3). Taken together, these evolutionary findings alone point to PACAP's role as a functionally important neuropeptide in the vertebrate species.

Peptides with similar sequences are grouped in a superfamily that is thought to have originated from one ancestral gene. Based on molecular evidence, it has been proposed that PACAP is the ancestral molecule that gave rise to some of the glucagon superfamily hormones through the process of exon duplications, gene duplications, and exon losses (5) (Figure 1.1). All nonmammalian species studied to date have both GHRH and PACAP encoded on one gene, giving rise to a polycistronic mRNA transcript (3). In the tunicate, two GHRH/PACAP genes exist as mentioned (4). It has been hypothesized that initially an ancestral exon duplicated to resemble the GHRH/PACAP arrangement, followed by a gene duplication event to result in two GHRH/PACAP gene copies as found in the tunicate. It is thought that the more highly conserved gene, GHRH/PACAP-1, remained conserved over a long evolutionary period and is now present in fish, amphibians, birds and presumably early mammals. The less conserved GHRH/PACAP-2 likely gave rise to other glucagon members such as VIP and PHM. Finally a gene

Figure 1.1. A hypothetical scheme for changes of the GHRH/PACAP gene throughout evolution. The tunicate gene is the most ancient form of PACAP isolated to date. Initially an exon duplication is thought to have taken place **(a)**, followed by a gene duplication **(b)**. It has been proposed that one of the genes evolved into PHM-VIP not shown **(c)**, whereas the second GHRH/PACAP gene remained conserved and duplicated again in early mammals **(d)**. Each gene lost the function of an exon indicated by ☒. Therefore, GHRH and PACAP are encoded by distinct genes in mammals. Exons and genes are represented by boxes.


TUNICATE
NONMAMMALIAN VERTEBRATES
MAMMALS
Ancestral peptide
GHRH
PACAP
GHRH
PACAP
GHRH
PACAP
GHRH
PACAP
GHRH
PACAP
a
b
c
d
exon duplication
gene duplication

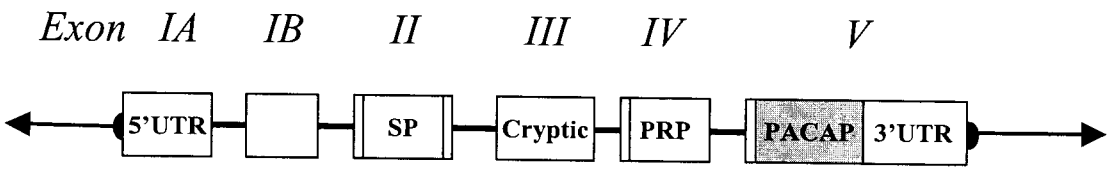
duplication event and loss of exon function resulted in separate genes for PACAP and GHRH as found in modern mammals (1, 3, 5).

The PACAP gene, mRNA, and protein

A) Mammals

The PACAP gene and/or cDNA have been cloned from human, mouse, rat, sheep, chicken, lizard, frog, fish, and tunicate (1, 6, 7). In human, the PACAP gene has been localized to chromosome 18p11 (8). The overall structure of the human and mouse genes is similar except that the mouse gene has an additional exon, IB, encoding 5' untranslated region (UTR) (Figure 1.2). Exons one to five encode 5' UTR, signal peptide, cryptic peptide, PACAP-related peptide (PRP), and PACAP/3' UTR, respectively. The murine exons IA and IB both encode 5'UTR and can be alternatively utilized to produce different transcripts (9). Subsequently, it was found that the RNA can undergo further alternative splicing to yield more mRNA variants (6, 9, 10). Multiple mRNA forms have been classified to date. Whereas some forms are found in several tissues, others are specific to only one tissue type. The alternative splicing does not affect the PACAP peptide sequence and is thought to be involved in mRNA stability (6). The mRNA transcript encodes a peptide precursor, prepro-PACAP, that undergoes post-translational modification before it is a mature bioactive peptide. After cleavage of the signal peptide, the propeptide is proteolytically processed by prohormone convertases at dibasic recognition sites to release the cryptic peptide, PRP and PACAP 38 (5, 11). The cryptic peptide and PRP have no known receptors or function so far (1). PACAP 38 may be further cleaved near the C-terminal end at a recognition site (Gly^{28}-Lys^{29}-Arg^{30}) to

Figure 1.2. Schematic representation of the murine PACAP gene. Lines represent introns and boxes represent exons (not to scale). UTR = untranslated region, SP = signal peptide, PRP = PACAP-related peptide, PACAP = pituitary adenylate cyclase-activating polypeptide.


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

generate the PACAP 27 isoform. The two isotypes are not a result of mRNA alternative splicing since they are encoded by a single exon. Finally, both PACAP 38 and 27 undergo C-terminal amidation by alpha amidating monooxygenase (11).

B) Amphibians

Two years after PACAP was discovered (2), its homolog was isolated in amphibians (12). To date, PACAP has been characterized in the green European frog, *Rana ridibunda* (12), and the South African clawed frog, *Xenopus laevis* (13). Both *R. ridibunda* and *X. laevis* PACAP38 primary structure differs only by one or two amino acid residues when compared to human or mouse PACAP peptide. In addition, a second PACAP variant has been identified in *X. laevis* where two amino acid residue differences are present (Figure 1.3). Due to higher conservation of the N-terminal region of the peptide, the primary structure of PACAP27 is identical in amphibians and mammals (14).

In *R. ridibunda*, two PACAP precursors can be generated by alternative splicing of the primary transcript. A long precursor consists of both full-length GHRH-like peptide and PACAP (longGP), whereas a shorter precursor contains truncated GHRH-like peptide and full length PACAP (shortGP) (Figure 1.4). The truncation of the shortGP removes residues 1-32 of the GHRH-like peptide. In addition, the splicing process results in the deletion of Arg^{78} and introduces a Ser residue at the splice site. It is speculated that the two precursor forms are transcribed from a single gene followed by alternative splicing (15). This is supported by evidence from a previous study of fish PACAP/GHRH-like gene, where residues 1-32 of GHRH-like peptide were found to be encoded by an individual exon that can be included or spliced out to form the final

Figure 1.3. Comparison of amino acid sequence of PACAP in mammals and amphibians. The first 27 amino acids are identical as indicated by boxed area. The complete sequence of 38 amino acids has one residue difference in *R. ridibunda* and *X. laevis* when compared to mouse, human or rat, making it 97% identical with the mammalian sequence **(a)**. In addition, a second PACAP variant was identified in *X. laevis* where two amino acid differences are present **(b)**.

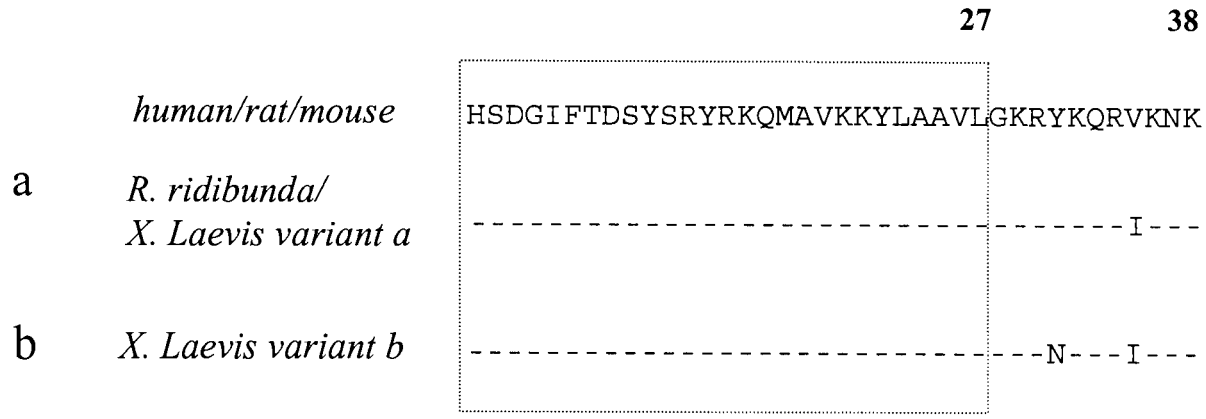
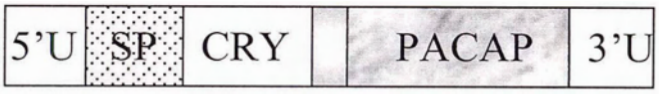

27
38
human/rat/mouse
HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
a
R. ridibunda/
X. Laevis variant a
--------I---
b
X. Laevis variant b
---N---I---

Figure 1.4. A schematic diagram of frog GHRH/PACAP cDNA structure. A long precursor (longGP) consists of both GHRH and PACAP encoded on one gene **(a)**. A 32 amino acid shorter precursor (shortGP) encodes truncated GHRH and full length PACAP **(b)**. U = untranslated region, SP = signal peptide, CRY = cryptic peptide, GHRH = growth hormone-releasing hormone, PACAP = pituitary adenylate cyclase-activating polypeptide.

a

b



transcript (16,17). Similarly, as in the European green frog, *X. laevis* possesses two PACAP precursors. The longGP is identical to that of *R. ridibunda*. However, the shortGP has one additional residue difference within PACAP, one within GHRH-like peptide, and 9 others within the signal peptide. Based on this dissimilarity, Hu *et al.* hypothesized that two different PACAP genes are present in *X. laevis,* each giving rise to a different transcript (13). This is plausible because *X. laevis* frogs are tetraploid as a consequence of their genome duplication approximately 30 million years ago (14).

PACAP receptors

A) Overview

To date, two types of PACAP receptors have been identified in vertebrates. Type I receptors are PACAP specific (PAC_1-R), as they bind PACAP with much higher affinity than VIP. Type II receptors (VPAC-R) are shared and bound equally by both PACAP and VIP. These receptors can further be divided into two subtypes ($VPAC_1$-R and $VPAC_2$-R), distinguished by differential binding affinities to secretin and helodermin (18). Both type I and II receptors belong to the secretin/glucagon subfamily of receptors and are seven-transmembrane G protein coupled.

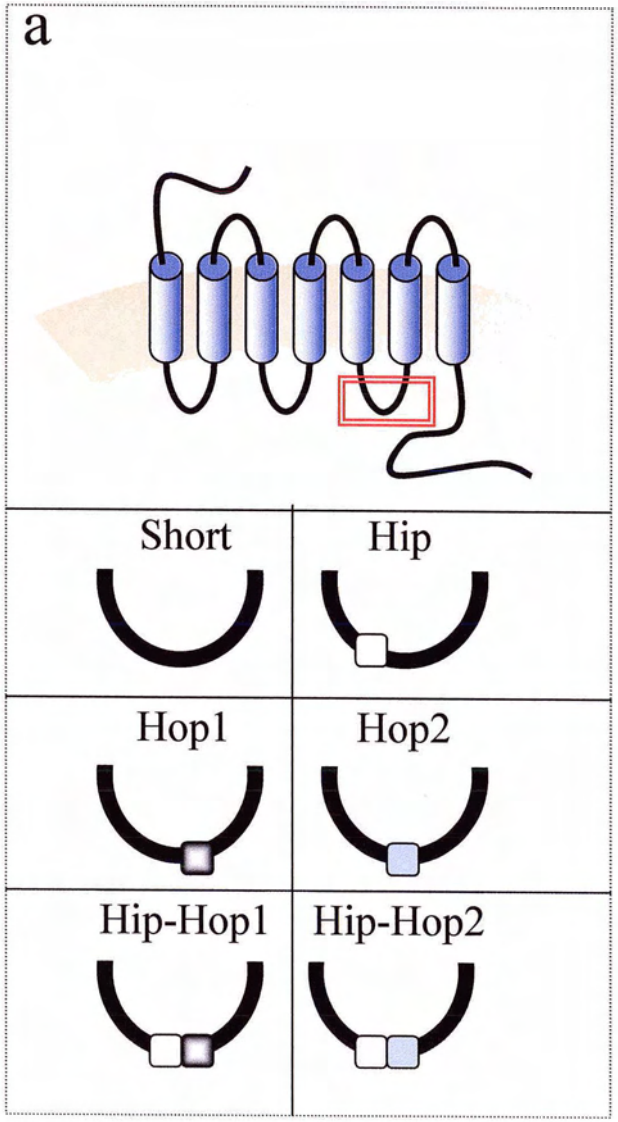
Multiple variants of PAC_1-R can be produced from alternative splicing of the transcript or as a result of substitutions or deletions within receptor domains (19). Tissue distribution, signal pathway coupling, and preferential binding to either PACAP27 or PACAP38 isoforms differs among the receptor variants (1). Receptor variants were identified in the extracellular domain, transmembrane domains and within the third intracellular loop of the receptor. Six PAC_1-R variants can be generated by splicing of

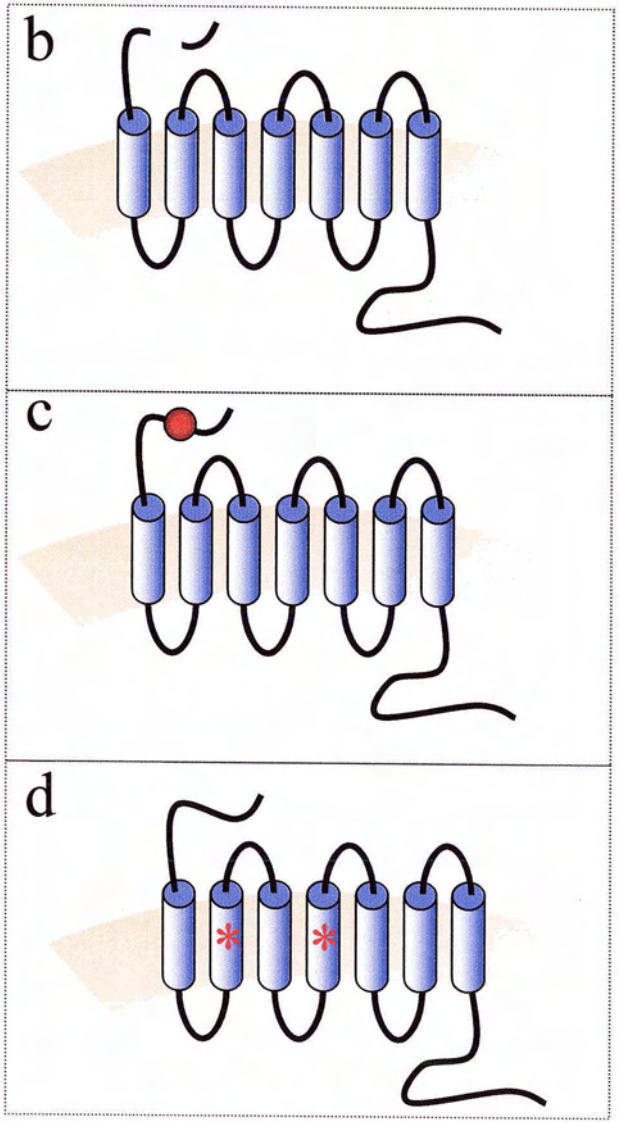
Hip, Hop1 and Hop2 cassettes either alone or in combination (Figure 1.5) (20). Splice variants with a deletion of either 21 amino acids (21) or 57 amino acids (22) have been identified in the N-terminal region of the receptor. Another variant with a 24 amino acid addition was also identified in the N-terminal region (23). Finally, a variant with substitutions and deletions within transmembrane domains II and IV, differs from all of the other variants due to its ability to couple to L-type voltage sensitive Ca^{2+} channels (Figure 1.5) (24). The majority of PAC_1-R variants are coupled to adenylyl cyclase via G_s proteins to activate adenylate cyclase and downstream accumulation of cAMP. Some variants are also known to trigger the phospholipase C (PLC) pathway via G_q. The VPAC receptors also stimulate cAMP turnover or Ca^{2+} mobilization. All of these pathways can eventually affect gene transcription and result in physiological changes. In summary, PACAP can elicit a variety of effects in target tissues because it acts on so many different receptor types and signaling pathways (11).

B) PACAP receptors: mammals

The PAC_1-R has been isolated in mammals from human, rat, cow and mouse. The receptor distribution of the PAC_1-R has been studied most extensively in mammals, especially rats. So far, the receptor has been located in many tissues and most endocrine organs; the tissues include the brain, spinal cord, lung, liver, thymus, spleen, pancreas, adrenal medulla, placenta, ovary and testis (1, 18). Both VPAC receptors have been cloned and characterized in human, rat, and mouse (1, 25). Globally, VPAC receptor transcripts are less abundant and their distribution is more restricted when compared to PAC_1-R (26, 27). VPAC receptors are found in the central nervous system (CNS).

Figure 1.5. Mammalian PAC_1 receptor variants. Six splice variants are generated by alternative splicing of hip, hop1, and/or hop2 cassettes in the third intracellular loop **(a)**. Two splice variants result from a 21 or 57 amino acid deletion in the N-terminal domain **(b)**. A splice variant can have 24 amino acid addition in the N-terminal domain **(c)**. A variant can also have substitutions and deletions within transmembrane domains II and IV **(d)**. Red circle = amino acid addition; red asterisk = amino acid substitution/deletion. Adapted from Moretti *et al.* (20).


a
Short
Hip
Hop1
Hop2
Hip-Hop1
Hip-Hop2


b
c
d

Peripherally, $VPAC_1$-R is present mainly in the lung, pancreas, liver, kidney, adrenal gland, heart, intestine, uterus and thymus. $VPAC_2$-R is also found at relatively low levels in several tissues (18) including ovary, testis, uterus, spleen, kidney, thymus, adrenal gland, heart, lung, and pancreas (1). The wide tissue distribution of PACAP receptors points to PACAP as a neuropeptide of many functions.

C) PACAP receptors: amphibians

In amphibians, extensive research has characterized PACAP receptors. In 1999, Jeandel *et al.* identified PACAP specific binding sites in the brain of *R. ridibunda* using radioactively labeled PACAP. The distribution of PACAP receptors in the brain was in part similar to rat receptor distribution with some species specific differences (28). Recent analysis of *R. ridibunda* brain cDNA library revealed the existence of several frog PAC_1-R variants. Northern blot analysis revealed that PAC_1-R mRNA was predominant in the CNS with moderate expression in the distal lobe of the pituitary, spleen, testis and lung (29). Similarly, in *X. laevis*, a PAC_1-R homologue has been characterized, but variants still remain to be identified (30). Binding site distribution resembled those of *R. ridibunda* with the strongest abundance in the CNS. Additionally, a VPAC-R has also been cloned from a *R. ridibunda* pituitary cDNA library (31). The receptor shares the highest sequence identity with the human $VPAC_1$-R, however, its pharmacological profile resembles mammalian $VPAC_2$-R. The receptor was widely distributed as it was found in 11 different tissues. It is clear that PACAP probably acts as a hypophysiotropic neurohormone in amphibians as in mammals because 1) PACAP is located in frog CNS,

2) PACAP has the ability to stimulate adenylate cyclase, and 3) PACAP has specific receptors in the pituitary (14).

A pleiotropic neuropeptide

A) An overview of function

PACAP is termed a hypophysiotropic neuropeptide because it is synthesized in hypothalamic neurons and is transported to the pituitary gland by the portal capillary bed in tetrapods. Receptors specific for PACAP are present in the pituitary through which PACAP has been shown to stimulate the release of several hormones including growth hormone, prolactin, adrenocorticotropic hormone, follicle-stimulating hormone and luteinizing hormone (32). Another common feature of hypophysiotropic neurohormones is their wide distribution in the CNS and in peripheral organs where they induce diverse biological events in addition to their hypophysiotropic actions. In support of this, PACAP is highly expressed in the CNS but to a lesser extent in a wide number of peripheral organs (11). When comparing PACAP isoforms, PACAP 38 is predominant in the CNS and peripheral tissues. Because PACAP also acts as a neurotransmitter in the CNS (33), nearly all organs and tissues contain detectable levels of PACAP-like immunoreactivity due to its localization in nerve fibers that innervate the tissues (34). Furthermore, it has been shown that the concentration of the peptide in the rat portal blood is significantly higher than in peripheral blood (32). From these studies it can be deduced that PACAP plays an important role in mammals as a hypophysiotropic factor in the brain as well as a neurotransmitter from axons that innervate peripheral target organs.

In adult frogs of *R. ridibunda*, expression of the PACAP/GHRH-like peptide gene was investigated in several tissues using RT-PCR and Northern blot analysis. Intense mRNA signals were present in the brain and spinal cord whereas a faint signal was detected in the pituitary neurointermediate lobe. A weaker signal was discovered in the adrenal gland, skeletal muscle, and colon, whereas mRNA was not detected in the distal pituitary lobe, liver, spleen and testis (15). Also, in *X. laevis*, a high abundance of mRNA was present in CNS. Tissues that did not express PACAP were muscle, lung, intestine and the liver (13). Brain PACAP/GHRH-like peptide mRNA localization using in situ hybridization has been extensively examined in both frog species. Studies revealed a wide distribution of mRNA throughout the brain that is somewhat similar to that in mammals. Also, when compared to mammals, the overall distribution of PACAP immunoreactivity in the central nervous system of the frog is similar (14).

Previously, PACAP has been shown to be involved in the nervous, endocrine, cardiovascular, muscular and immune systems of mammals (1). More recently, PACAP has been ascribed a function in lipid metabolism, carbohydrate metabolism (35), thermoregulation (36) and behavior (37). Such a wide array of functions may be explained in several ways. First, PACAP can act on multiple receptor types and subtypes coupled to three major signaling pathways (19). Second, one of the more common pathways that PACAP is coupled to involves adenylate cyclase, which converts ATP molecules to cAMP. As a ubiquitous molecule, cAMP acts as a secondary messenger to induce a wide range of downstream signaling pathways that eventually lead to different physiological effects. Third, PACAP exists in two isoforms, each having a different affinity for receptor types and a different abundance in tissues. Fourth, PACAP receptors

and PACAP itself are widely distributed in several tissues, being mainly in the nervous system and to a lesser extent in some peripheral tissues. In mammals, both PACAP isoforms have been detected in most endocrine glands and other organs (11). Finally, PACAP is known to release hormones from the pituitary broadening the spectrum of downstream physiological effects (38). In conclusion, it has been difficult to determine the primary role of PACAP *in vivo*.

In mammals, PACAP is located and involved in physiological systems that are nonexistent in tunicates that seem to lack a pituitary gland. Instead, tunicates possess a neural ganglion where PACAP-1 mRNA is expressed. Therefore, the ancestral role of PACAP in the protochordates was unlikely as a hypophysiotropic factor, or a regulator of lipid and/or carbohydrate metabolism as is currently hypothesized for mammals. McRory and Sherwood speculated that the peptide's most ancient role was as a growth or cellular proliferation factor (4). This function is still applicable to mammals during early development of the brain (34), but is not likely to be its primary role, as many other compensatory growth factors exist due to redundancy in the vertebrate genome (5). Protochordate gene families are a simplified version of those found in the vertebrate species (39). Therefore, a functional growth factor may be critical for survival in the protochordates. However, it is not yet known if PACAP was critical for survival in the ancient tunicate.

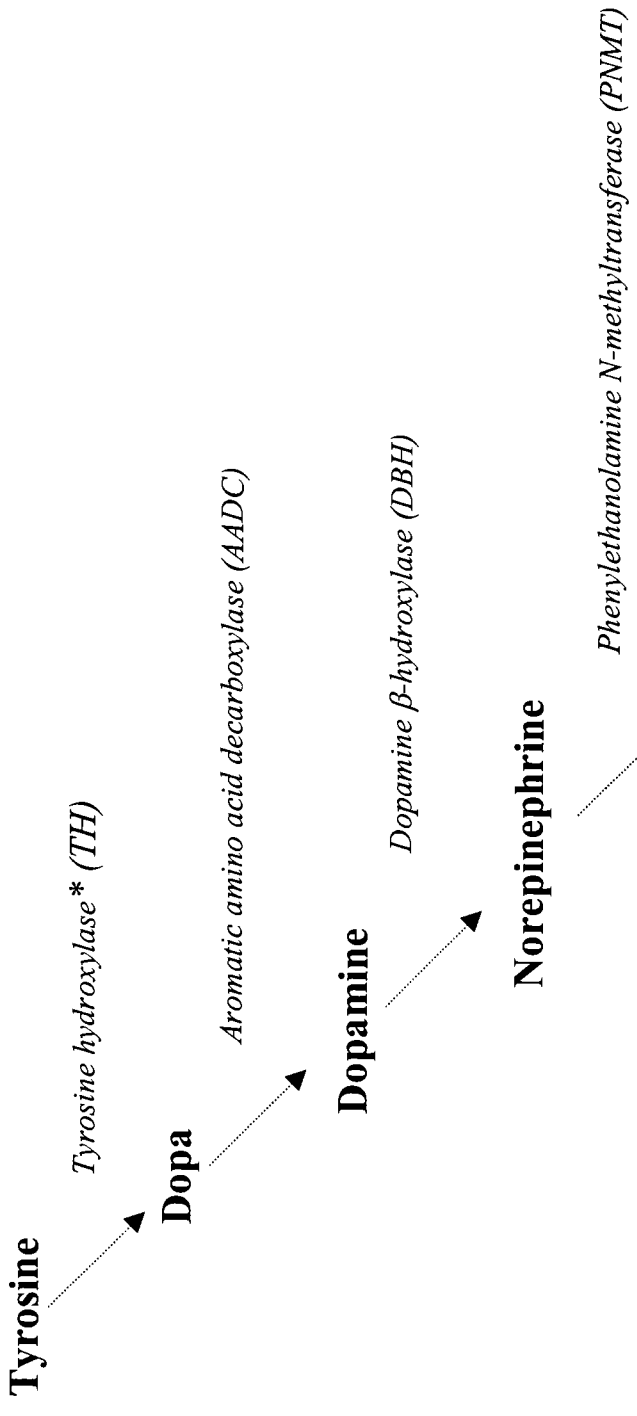
B) PACAP in the adrenal medulla

The adrenal gland is an endocrine organ involved in several different metabolic processes and can be divided into two parts that secrete different hormones: the adrenal

cortex and adrenal medulla. Although cortical hormones are as essential as hormones of the medulla, only the latter will be discussed here. Chromaffin cells of the adrenal medulla secrete two closely related hormones (catecholamines) synthesized from tyrosine, an amino acid: epinephrine (adrenalin) and norepinephrine (noradrenalin) (40, 41). The hormone synthesis involves four enzymatic reactions forming the following substances in order: L-3,4-dihydroxyphenyl-alanine (dopa), dopamine, norepinephrine, and epinephrine (Figure 1.6). The initial step where tyrosine is converted to dopa by tyrosine hydroxylase (TH) is the rate-limiting step that is the determining factor of how much end product will accumulate in the cell. The remaining enzymes of catecholamine synthesis include aromatic amino acid decarboxylase (AADC), dopamine β-hydroxylase (DBH), and phenylethanolamine N-methyltransferase (PNMT) (40). Epinephrine is the major component of chromaffin cells and is released along with norepinephrine upon stimulation from the splanchnic preganglionic axons (40). Various types of stress conditions such as cold initiate an impulse in the hypothalamus and the signal is carried to preganglionic neurons in the spinal cord via synapses (36). In response to cold, norepinephrine release from neurons seems to be preferential, however, epinephrine is released from the adrenals at times of low blood sugar (42).

Acetylcholine is the primary neurotransmitter that affects catecholamine release from the adrenal medulla. However, it has been proposed that noncholinergic neurotransmitters are also present in preganglionic neurons innervating chromaffin cells because acetylcholine or cholinergic agonists alone could not mimic a sustained secretion and biosynthesis of catecholamines (43). Accumulating neuroanatomical,

Figure 1.6. Catecholamine biosynthesis within chromaffin cells of the adrenal medulla. Italics to the right of arrows indicate enzyme names.


Tyrosine
Tyrosine hydroxylase* (TH)
Dopa
Aromatic amino acid decarboxylase (AADC)
Dopamine
Dopamine β-hydroxylase (DBH)
Norepinephrine
Phenylethanolamine N-methyltransferase (PNMT)
Epinephrine
* Rate limiting step

pharmacological, and physiological evidence points to PACAP as the noncholinergic co-transmitter in the adrenomedullary nerve endings (42). Both PACAP and PACAP receptors have been identified in the adrenals of mammals, amphibians, and fish using immunoreactivity and mRNA detection (1). In the mouse, PACAP peptide positive fibers were shown to overlap with a cholinergic marker in synapses within the adrenal medulla, indicating its colocalization with acetylcholine (43). Also, PACAP can act locally on the chromaffin cells in a paracrine manner because PACAP mRNA is expressed in the medulla. PAC_1-R, $VPAC_1$-R and $VPAC_2$-R receptors have been identified in the adrenal medulla (44, 45). The PAC_1-R is the predominant subtype expressed in adrenomedullary tissue and because PACAP is more potent than VIP in terms of mediating post-synaptic effects in chromaffin cells, it has been suggested that this action is mediated through PAC_1-R (42).

PACAP has been shown to be one of the most potent secretagogues of catecholamines *in vitro* from cultured mammalian chromaffin cells and *in vivo* when infused into several mammalian species (34, 42). In addition to catecholamine release, PACAP increases the gene transcription of three catecholamine biosynthetic enzymes: TH, DBH, and PNMT (46). Increased enzyme expression is regarded as critical in replenishing catecholamine stores at times of chronic stress (47). The most important component of the biosynthetic pathway is the rate-limiting enzyme tyrosine hydroxylase. A short-term post-transcriptional mechanism of TH activity occurs mainly by phosphorylation of the enzyme. Indeed, PACAP is a potent activator of TH as it has the ability to phoshorylate specific amino acid residues on the enzyme (48, 49). In support of these findings, PACAP has the ability to mobilize multiple second messenger pathways

that allow it to act at many levels in catecholamine regulation. For example, Ca^{2+} influx is required for catecholamine release, whereas cAMP is important in the transcription and activation of catecholamine-synthesizing enzymes (43). Furthermore, an *in vivo* genetic mouse model where PACAP expression was ablated has revealed that mice had an impaired sympathoadrenal axis as discussed below.

C) PACAP in the endocrine pancreas

The endocrine portion of the pancreas consists of closely associated cells called islets of Langerhans that include hormone-releasing alpha cells that secrete glucagon and beta cells that secrete insulin to maintain carbohydrate and lipid homeostasis. The role of glucagon is to elevate blood glucose at times of hypoglycemia via several mechanisms such as liver glycogenolysis and gluconeogenesis. In addition, glucagon stimulates the breakdown of fats into fatty acids and glycerol (50). High blood sugar serves as a negative feedback system to inhibit further glucagon release. The actions of insulin are opposite to those of glucagon with the purpose to reduce blood glucose during hyperglycemic conditions after a meal intake, for example. Insulin acts to promote glycogenesis in muscle and liver and also enhances protein and fat synthesis as well as glucose uptake into muscle and adipose tissues. As in the case of glucagon, insulin secretion is stringently regulated by blood glucose concentration (41, 50).

After food intake, pancreatic beta cells are stimulated by parasympathetic nerve impulses to release insulin in response to nutrients in the gut and other hormones in the blood (GLP-1 and GIP) (51). Vagus nerve activation specifically results in the stimulation of postganglionic nerve cells in pancreatic ganglia and release of

neurotransmitters close to the islets. Execution of pancreatic autonomic nerve stimulation is thought to be chiefly cholinergic although noncholinergic mechanisms may also play a role as recently discovered (52). Accumulating evidence indicates that the pleiotropic neuropeptide PACAP also seems to be an adequate candidate as a pancreatic neurotransmitter (53). PACAP has been located in nerves of the pancreas, within pancreatic exocrine cells, and within the actual pancreatic islets in mammals. Since islet innervation is widespread and uniform, PACAP has a potential ability to be involved in the regulation of all islet cell types including alpha and beta cells. PAC_1-R, $VPAC_1$-R, and $VPAC_2$-R subtypes are expressed in rodent pancreas and all types seem to take part in mediating the insulinotropic effect of PACAP (52). PACAP is thought to elicit short-term insulinotropic action by increasing cytoplasmic cAMP and Ca^{2+} that leads to the activation of exocytosis to release stored insulin. In addition, PACAP may have long-term effect on insulin release via cAMP which affects transcription of the insulin and other anabolic genes (54).

PACAP potently stimulates insulin release in a dose- and glucose-dependent fashion. However, PACAP does not affect plasma glucose even though it leads to insulin release as examined *in vivo* (53). Ahren and Filipsson have studied the effects of PACAP on glucose disposal in adrenalectomized mice and found that glucose disposal was altered. This finding indicates that PACAP administration also results in epinephrine release that would counteract insulin action and therefore no net glucose disposal would be noted (55). In addition to its insulinotropic action, PACAP also releases glucagon from the alpha pancreatic islets as shown *in vitro* and *in vivo*, but this has not been examined as extensively (56, 57). The dual hormone release is intriguing since insulin

and glucagon have opposing roles in carbohydrate and lipid metabolism. It seems that the physiological state, especially blood glucose concentration, is the determining factor of whether PACAP enhances either insulin or glucagon release. A study performed by Bertrand *et al.* has shown that PACAP mediated glucagon release is inversely related to blood glucose concentration, whereas PACAP mediated insulin release is directly related to blood glucose concentration (58). Clearly, these findings point to PACAP as a pancreatic neuropeptide and a regulator of carbohydrate and lipid metabolism. Valuable experiments where mice either lack PAC_1-R or PACAP support this notion as discussed below.

Transgenesis

A) Transgenic Mice Overview

Previously, functional studies using *in vivo* and *in vitro* models have demonstrated that PACAP acts on several target tissues and some endocrine organs to further stimulate the release of other hormones resulting in a wide array of physiological effects. These studies included using primary cell culture, tumoral cell lines, isolated perfused organs, PACAP injection, or PACAP antibody administration. Such approaches were impractical or were restricted to the cellular level where assumptions could be made in the context of the tissue but not the whole organism (59). Therefore, the primary role of PACAP, if there was one, could not be identified due to limitations in technology. Recently, an alternative approach that allows one to examine the physiology of hormones in the context of the animal has been developed. Due to two major technological breakthroughs, mouse strains deficient of a desired gene (transgenic knockout mice)

could be generated. The function of the disrupted gene could then be analyzed by looking at the phenotype of the transgenic mice (60). For example, mortality of null mice would indicate the gene is significant and necessary for survival.

In 1981, pluripotent embryonic stem (ES) cells were isolated from cultured early mouse embryos (61, 62). Upon injection into a developing mouse blastocyst, ES cells were capable of contributing to all tissue types including the germ line, thus giving rise to a hybrid mouse, or chimera. If the transgenic ES cells were transmitted into the germ line, their genotype could be "recycled" *in vivo* and passed on to future generations (60). In 1986, Gossler *et al* and Robertson *et al* demonstrated that the ES cells could be genetically manipulated in culture by introducing a targeting vector with a transgene into the cells and that offspring carrying the genetic modification could be obtained (63, 64). This was possible due to homologous recombination between the endogenous gene sequence and the introduced DNA sequence, also referred to as gene "targeting". In this fashion, genes could be selectively knocked out as long as the transgene was made nonfunctional either by disruption or partial deletion (60). Such transgenic knockout mice serve as a valuable *in vivo* model and have a great impact on all aspects of mammalian physiology including endocrine function (65, 66).

B) PAC_1-R and PACAP knockout mice

One approach to understand the role of a hormone in mammalian physiology is to examine the effects after the complete removal of the hormone gene or its receptor gene from an organism. The first knockout mouse generated to study the function of PACAP *in vivo* lacked the PAC_1 specific receptor gene (PAC_1-/-) (67-69). The most severe

phenotype observed was mortality of PAC_1-/- pups within the first four postnatal weeks. Surviving PAC_1-/- mice had impaired glucose stimulated insulin release and glucose intolerance after administration of glucose intravenously or via gastric route. These findings indicate that the presence of the PAC_1 receptor is required to maintain mammalian carbohydrate homeostasis and may be critical for survival (67). A knockout model such as this may pose a problem since PACAP not only acts on one receptor type but also shares receptors with VIP (11). Therefore, in the PAC_1-/- mouse, PACAP can still act via VPAC receptors and result in a milder phenotype than expected. In that case only a subset of PACAP's actions may be revealed and it appears that a PACAP knockout may be of greater value and interest (18).

To determine whether PACAP is essential for survival, several groups have taken advantage of the emerging transgenic animal biotechnology, and have generated PACAP knockout (PACAP-/-) mice (35). To date, three PACAP knockout mice have been generated by different research groups (35, 37, 43). It is of interest that different phenotypic characteristics were observed in each case. This may be explained by the fact that each group used a different room temperature and the knockout mice were shown to have a temperature sensitive phenotype (36, 70).

The most severe phenotype was observed by Gray *et al.* in which most PACAP-/- mice died within two weeks of age when housed at 21C (36). Hashimoto *et al.* also observed reduced survival rate of null mice but not as severe as Gray *et al.*, perhaps because pups were housed at a higher temperature of 23C (37, 71). Also, Gray et al. observed that PACAP-/- mice had elevated plasma triglycerides, fatty acids and cholesterol. In addition, the liver, muscle and cardiac tissues were flooded with fatty

microvesicle deposits. Other metabolites including glucose and insulin were abnormal in fasted PACAP-/- pups at 5 days of age. Interestingly, the abnormal phenotype with the associated mortality was not present in most null pups when litters were housed at 24C. Gray *et al.* reported significantly reduced norepinephrene levels in brown fat and suggested this to be the cause of temperature sensitivity because norepinephrine normally stimulates the production of heat in brown adipose tissue (36). In the study done by Hamelink *et al.* where the room temperature was not reported, adult PACAP-/- mice were challenged with an insulin injection that resulted in exaggerated hypoglycemia in a dose-related manner leading to death. Because PACAP is responsible for sustained catecholamine secretion and replenishment it was hypothesized that PACAP-/- mice have an impaired long-term epinephrine secretion and/or biosynthesis in the adrenal medulla (43). In summary of the Gray *et al.* and Hamelink *et al.* studies, PACAP-/- mice have disrupted lipid and carbohydrate metabolic processes and abnormal catecholamine synthesis that may be related to environmental temperature. It has been proposed that PACAP may be critical for survival especially during environmental and/or metabolic stress situations. It is not yet clear whether these results are downstream effects of other hormones that PACAP is known to regulate (70). However, it can be stated that PACAP is a key regulator in mammalian physiology.

C) Transgenic Frogs

In vertebrates other than mice, it is not yet possible to create a gene knockout. However, other methods can be used to study hormones *in vivo* (1). One way to eliminate gene function during frog development is to inject morpholinos into early

embryos. This was successfully demonstrated in both *X. laevis* and *Xenopus tropicalis* by blocking GFP expression in specific tissues. The translational inhibition lasted throughout early development until at least stage 43 (72), when embryos are free-swimming (73). This technique is useful when studying only early development and is limited to the number of frogs injected. Another way to study the function of a gene rather than removing it, is to insert additional copies of the gene into the genome and study the effects of excess of a specific protein in the organism. In 1996, Kroll and Amaya made this possible by developing a method to generate transgenic *X. laevis* frogs that could overexpress a gene of interest (74). This method produced stable, nonmosaic expression of cloned genes in embryos. Three years later, Marsh-Armstrong *et al.*, 1999, examined the germ-line transmission of the transgenes in *Xenopus*. Transgenic animals were raised to sexual maturity and offspring were analyzed for transgene expression. The results are promising since transgenic progeny expressed the transgenes faithfully indicating that stable lines of *X. laevis* can be maintained (75).

Purpose of this study

The purpose of the present study was to further investigate the role of PACAP in vertebrate species through hormone excess using two different animal models. The type of models used in this study were 1) transgenic frogs overexpressing PACAP in most tissues and 2) mice exposed to PACAP infusion for several days at an elevated concentration to mimic gene overexpression. Transgenic frogs were used to examine the effects of PACAP on development, metamorphosis, growth and survival. Mice exposed to PACAP over prolonged periods of time were used to study physiology with respect to

carbohydrate metabolism. With these transgenic and nontransgenic model approaches as opposed to a regular PACAP knockout mouse, I have tested the general hypothesis that PACAP is critical for normal development and is a key regulator of carbohydrate metabolism.

REFERENCES

1. **Sherwood NM, Krueckl SL, McRory JE** 2000 The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocrine Reviews 21:619-670

2. **Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH** 1989 Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochemical and Biophysical Research Communications 164:567-574

3. **Montero M, Yon L, Kikuyama S, Dufour S, Vaudry H** 2000 Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. Journal of Molecular Endocrinology 25:157-168

4. **McRory J, Sherwood NM** 1997 Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. Endocrinology 138:2380-2390

5. **Holmgren S, Jensen J** 2001 Evolution of vertebrate neuropeptides. Brain Research Bulletin 55:723-735

6. **Cummings KJ, Gray SL, Simmons CJ, Kozak CA, Sherwood NM** 2002 Mouse pituitary adenylate cyclase-activating polypeptide (PACAP): gene, expression and novel splicing. Molecular and Cellular Endocrinology 192:133-145

7. **Adams BA, Lescheid DW, Vickers ED, Crim LW, Sherwood NM** 2002 Pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in sturgeon, whitefish, grayling, flounder and halibut: cDNA sequence, exon skipping and evolution. Regulatory Peptides 109:27-37

8. **Hosoya M, Kimura C, Ogi K, Ohkubo S, Miyamoto Y, Kugoh H, Shimizu M, Onda H, Oshimura M, Arimura A, et al.** 1992 Structure of the human pituitary adenylate cyclase activating polypeptide (PACAP) gene. Biochimica et Biophysica Acta 1129:199-206

9. **Yamamoto K, Hashimoto H, Hagihara N, Nishino A, Fujita T, Matsuda T, Baba A** 1998 Cloning and characterization of the mouse pituitary adenylate cyclase-activating polypeptide (PACAP) gene. Gene 211:63-69

10. **Tabuchi A, Koizumi M, Tsuda M** 2001 Novel splice variants of PACAP gene in mouse cerebellar granule cells. Neuroreport 12:1181-1186

11. **Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H** 2000 Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacological Reviews 52:269-324

12. **Chartrel N, Tonon MC, Vaudry H, Conlon JM** 1991 Primary structure of frog pituitary adenylate cyclase-activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. Endocrinology 129:3367-3371

13. **Hu Z, Lelievre V, Tam J, Cheng JW, Fuenzalida G, Zhou X, Waschek JA** 2000 Molecular cloning of growth hormone-releasing hormone/pituitary adenylyl cyclase-activating polypeptide in the frog Xenopus laevis: brain distribution and regulation after castration. Endocrinology 141:3366-3376

14. **Yon L, Alexandre D, Montero M, Chartrel N, Jeandel L, Vallarino M, Conlon JM, Kikuyama S, Fournier A, Gracia-Navarro F, et al.** 2001 Pituitary adenylate cyclase-activating polypeptide and its receptors in amphibians. Microscopy Research and Technique 54:137-157

15. **Alexandre D, Vaudry H, Jegou S, Anouar Y** 2000 Structure and distribution of the mRNAs encoding pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in the frog, Rana ridibunda. Journal of Comparative Neurology 421:234-246

16. **Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM** 1997 Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology 138:414-423

17. **Parker DB, Coe IR, Dixon GH, Sherwood NM** 1993 Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. European Journal of Biochemistry 215:439-448

18. **Chow BKC, Pang RTK, Ng SSM** 2003 Molecular Evolution of PACAP Precursor and PACAP Receptors. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 25-48

19. **Shioda S, Zhou C, Ohtaki H** 2003 PACAP receptor signaling. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 95-124

20. **Moretti C, Mencacci C, Frajese GV, Cerilli M, Frajese G** 2002 Growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide in the reproductive system. Trends in Endocrinology and Metabolism 13:428-435

21. **Pantaloni C, Brabet P, Bilanges B, Dumuis A, Houssami S, Spengler D, Bockaert J, Journot L** 1996 Alternative splicing in the N-terminal extracellular domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potencies of PACAP-27 and PACAP-38 in phospholipase C activation. Journal of Biological Chemistry 271:22146-22151

22. **Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL** 1999 N-terminal splice variants of the type I PACAP receptor: isolation, characterization and ligand binding/selectivity determinants. Journal of Neuroendocrinology 11:941-949

23. **Daniel PB, Kieffer TJ, Leech CA, Habener JF** 2001 Novel alternatively spliced exon in the extracellular ligand-binding domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1R) selectively increases ligand affinity and alters signal transduction coupling during spermatogenesis. Journal of Biological Chemistry 276:12938-12944

24. **Chatterjee TK, Sharma RV, Fisher RA** 1996 Molecular cloning of a novel variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor that stimulates calcium influx by activation of L-type calcium channels. Journal of Biological Chemistry 271:32226-32232

25. **Hashimoto H, Nishino A, Shintani N, Hagihara N, Copeland NG, Jenkins NA, Yamamoto K, Matsuda T, Ishihara T, Nagata S, et al.** 1999 Genomic organization and chromosomal location of the mouse vasoactive intestinal polypeptide 1 (VPAC1) receptor. Genomics 58:90-93

26. **Basille M, Vaudry D, Coulouarn Y, Jegou S, Lihrmann I, Fournier A, Vaudry H, Gonzalez B** 2000 Comparative distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) binding sites and PACAP receptor mRNAs in the rat brain during development. Journal of Comparative Neurology 425:495-509

27. **Basille M, Vaudry D, Coulouarn Y, Jegou S, Lihrmann I, Fournier A, Vaudry H, Gonzalez BJ** 2000 Distribution of PACAP receptor mRNAs and PACAP binding sites in the rat brain during development. Annals of the New York Academy of Sciences 921:304-307

28. **Jeandel L, Yon L, Chartrel N, Gonzalez B, Fournier A, Conlon JM, Vaudry H** 1999 Characterization and localization of pituitary adenylate cyclase-activating polypeptide (PACAP) binding sites in the brain of the frog Rana ridibunda. Journal of Comparative Neurology 412:218-228

29. **Alexandre D, Vaudry H, Grumolato L, Turquier V, Fournier A, Jegou S, Anouar Y** 2002 Novel splice variants of type I pituitary adenylate cyclase-

activating polypeptide receptor in frog exhibit altered adenylate cyclase stimulation and differential relative abundance. Endocrinology 143:2680-2692

30. **Hu Z, Lelievre V, Chao A, Zhou X, Waschek JA** 2000 Characterization and messenger ribonucleic acid distribution of a cloned pituitary adenylate cyclase-activating polypeptide type I receptor in the frog Xenopus laevis brain. Endocrinology 141:657-665

31. **Alexandre D, Anouar Y, Jegou S, Fournier A, Vaudry H** 1999 A cloned frog vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating polypeptide receptor exhibits pharmacological and tissue distribution characteristics of both VPAC1 and VPAC2 receptors in mammals. Endocrinology 140:1285-1293

32. **Rawlings SR, Hezareh M** 1996 Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. Endocrine Reviews 17:4-29

33. **Gonzalez BJ, Vaudry D, Basille M, C. R, A. F-M, Vaudry H** 2003 Function of PACAP in the Central Nervous System. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 125-152

34. **Arimura A** 1998 Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Japanese Journal of Physiology 48:301-331

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

36. **Gray SL, Yamaguchi N, Vencova P, Sherwood NM** 2002 Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 143:3946-3954

37. **Hashimoto H, Shintani N, Tanaka K, Mori W, Hirose M, Matsuda T, Sakaue M, Miyazaki J, Niwa H, Tashiro F, et al.** 2001 Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). Proceedings of the National Academy of Sciences of the United States of America 98:13355-13360

38. **Arimura A, Shioda S** 1995 Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. Frontiers in Neuroendocrinology 16:53-88

39. **Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, et al.** 2002 The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298:2157-2167

40. **Young JB, Landsberg L** 1998 Catecholamines and the adrenal medulla. **In:** Williams Textbook of Endocrinology, 9th ed. Wilson JD, Foster DW, Kvonenberg HM, and Larsen PR. W.B. Saunders Company, Toronto p. 665-728

41. **Hole J.W. J** 1987 Human anatomy and physiology, 4th ed. Brown Publishers, Dubuque, Iowa p. 470-476

42. **Hamelink C, E. W, Eiden LE** 2003 PACAP: An 'Emergency Response' Co-Transmitter in the Adrenal Medulla. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 227-250

43. **Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, Eiden LE** 2002 Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. Proceedings of the National Academy of Sciences of the United States of America 99:461-466

44. **Mazzocchi G, Malendowicz LK, Neri G, Andreis PG, Ziolkowska A, Gottardo L, Nowak KW, Nussdorfer GG** 2002 Pituitary adenylate cyclase-activating polypeptide and PACAP receptor expression and function in the rat adrenal gland. International Journal of Molecular Medicine 9:233-243

45. **Mazzocchi G, Malendowicz LK, Rebuffat P, Gottardo L, Nussdorfer GG** 2002 Expression and function of vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, and their receptors in the human adrenal gland. The Journal of Clinical Endocrinology and Metabolism 87:2575-2580

46. **Tonshoff C, Hemmick L, Evinger MJ** 1997 Pituitary adenylate cyclase activating polypeptide (PACAP) regulates expression of catecholamine biosynthetic enzyme genes in bovine adrenal chromaffin cells. Journal of Molecular Neuroscience 9:127-140

47. **Corbitt J, Hagerty T, Fernandez E, Morgan WW, Strong R** 2002 Transcriptional and post-transcriptional regulation of tyrosine hydroxylase messenger RNA in PC12 cells during persistent stimulation by VIP and PACAP38: differential regulation by protein kinase A and protein kinase C-dependent pathways. Neuropeptides 36:34-45

48. **Marley PD, Cheung CY, Thomson KA, Murphy R** 1996 Activation of tyrosine hydroxylase by pituitary adenylate cyclase-activating polypeptide (PACAP-27) in

bovine adrenal chromaffin cells. Journal of the Autonomic Nervous System 60:141-146

49. **Haycock JW** 1996 Short- and long-term regulation of tyrosine hydroxylase in chromaffin cells by VIP and PACAP. Annals of the New York Academy of Sciences 805:219-231

50. **Beaulieu E-E, Kelly AK** 1990 Hormones from molecules to disease. Hermann Publishers, New York p. 491-512

51. **Filipsson K, Holst JJ, Ahren B** 2000 PACAP contributes to insulin secretion after gastric glucose gavage in mice. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 279:R424-R432

52. **Filipsson K, Kvist-Reimer M, Ahren B** 2001 The neuropeptide pituitary adenylate cyclase-activating polypeptide and islet function. Diabetes 50:1959-1969

53. **Ahren B** 2003 PACAP in the Endocrine Pancreas. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 185-206

54. **Borboni P, Porzio O, Pierucci D, Cicconi S, Magnaterra R, Federici M, Sesti G, Lauro D, D'Agata V, Cavallaro S, et al.** 1999 Molecular and functional characterization of pituitary adenylate cyclase-activating polypeptide (PACAP-38)/vasoactive intestinal polypeptide receptors in pancreatic beta-cells and effects of PACAP-38 on components of the insulin secretory system. Endocrinology 140:5530-5537

55. **Ahren B, Filipsson K** 2000 The effects of PACAP on insulin secretion and glucose disposal are altered by adrenalectomy in mice. Annals of the New York Academy of Sciences 921:251-258

56. **Yokota C, Kawai K, Ohashi S, Watanabe Y, Suzuki S, Yamashita K** 1993 Stimulatory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on insulin and glucagon release from the isolated perfused rat pancreas. Acta Endocrinologica 129:473-479

57. **Filipsson K, Tornoe K, Holst J, Ahren B** 1997 Pituitary adenylate cyclase-activating polypeptide stimulates insulin and glucagon secretion in humans. The Journal of Clinical Endocrinology and Metabolism 82:3093-3098

58. **Bertrand G, Puech R, Maisonnasse Y, Bockaert J, Loubatieres-Mariani MM** 1996 Comparative effects of PACAP and VIP on pancreatic endocrine secretions and vascular resistance in rat. British Journal of Pharmacology 117:764-770

59. **Brabet P, Jamen F, Rodriguez-Henche N, Bertrand G, Bockaert J** 2003 PACAP receptor knockout and transgenics: what have we learnt? **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 323-346

60. **Babinet C, Cohen-Tannoudji M** 2001 Genome engineering via homologous recombination in mouse embryonic stem (ES) cells: an amazingly versatile tool for the study of mammalian biology. Anais da Academia Brasileira de Ciencias 73:365-383

61. **Martin GR** 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proceedings of the National Academy of Sciences of the United States of America 78:7634-7638

62. **Evans MJ, Kaufman MH** 1981 Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156

63. **Gossler A, Doetschman T, Korn R, Serfling E, Kemler R** 1986 Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proceedings of the National Academy of Sciences of the United States of America 83:9065-9069

64. **Robertson E, Bradley A, Kuehn M, Evans M** 1986 Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445-448

65. **Capecchi MR** 1989 Altering the genome by homologous recombination. Science 244:1288-1292

66. **Capecchi MR** 1994 Targeted gene replacement. Scientific American 270:52-59

67. **Jamen F, Persson K, Bertrand G, Rodriguez-Henche N, Puech R, Bockaert J, Ahren B, Brabet P** 2000 PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. The Journal of Clinical Investigation 105:1307-1315

68. **Hashimoto H, Shintani N, Nishino A, Okabe M, Ikawa M, Matsuyama S, Itoh K, Yamamoto K, Tomimoto S, Fujita T, et al.** 2000 Mice with markedly reduced PACAP (PAC(1)) receptor expression by targeted deletion of the signal peptide. Journal of Neurochemistry 75:1810-1817

69. **Otto C, Kovalchuk Y, Wolfer DP, Gass P, Martin M, Zuschratter W, Grone HJ, Kellendonk C, Tronche F, Maldonado R, et al.** 2001 Impairment of mossy fiber long-term potentiation and associative learning in pituitary adenylate cyclase activating polypeptide type I receptor-deficient mice. Journal of Neuroscience 21:5520-5527

70. **Sherwood NM, Gray SL, Cummings KJ** 2003 Consequences of PACAP Gene Knockout. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 347-360

71. **Arimura A** 2002 Impaired adaptive thermogenesis in pituitary adenylate cyclase-activating polypeptide-deficient mice. Endocrinology 143:3715-3716

72. **Nieuwkoop PDaF, J.** 1994 Normal Table of Xenopus laevis (Daudin). © Garland, New York, Garland.

73. **Nutt SL, Bronchain OJ, Hartley KO, Amaya E** 2001 Comparison of morpholino based translational inhibition during the development of Xenopus laevis and Xenopus tropicalis. Genesis 30:110-113

74. **Kroll KL, Amaya E** 1996 Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173-3183

75. **Marsh-Armstrong N, Huang H, Berry DL, Brown DD** 1999 Germ-line transmission of transgenes in Xenopus laevis. Proceedings of the National Academy of Sciences of the United States of America 96:14389-14393

CHAPTER 2

Overexpression of PACAP in African clawed frog (*Xenopus laevis*)

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide is a highly conserved neuropeptide belonging to the glucagon superfamily of hormones. Because PACAP and its receptors have been identified in the CNS and multiple other tissues and because PACAP exerts a large array of biological effects (1), identifying its primary role is the focus of current studies. Recently, advantage has been taken of gene targeting to generate mice with a nonfunctional PACAP gene. Findings from these experiments point to PACAP as critical for survival in mammals because the neuropeptide seems to play a key role in physiology during times of stress (2-4). To date, mice are the only vertebrate models in which a deliberate, permanent loss of gene function can be examined (1). Nevertheless, other methods such as temporary gene dysfunction have been applied in amphibians using morpholinos (5) and siRNA (6). In addition, permanent, stable and nonmosaic overexpression of a desired gene has been recently developed in *Xenopus laevis*, the African clawed frog (7). This system provides several advantages because generation of transgenic frogs is much faster and less labor intensive than mouse transgenesis. Also, many embryos can be produced at once and can be studied starting at 4 cell stage, continuing through development and metamorphosis, all the way to adulthood (7). Co-expressing a gene of interest with GFP allows easy and noninvasive identification of transgenic embryos (7, 8). Finally, transgenic lines can be established in *X. laevis*, since transgenes are transmitted faithfully to next generations (9).

Gene overexpression is an alternative approach to study gene function *in vivo* and has been successfully employed in the past. For example, the function of growth hormone (GH) has been examined in *X. laevis* using gene overexpression. Excess GH

resulted in bone deformities and increased size in transgenic frogs indicating that GH has growth promoting effects resembling those previously described in mammals (10). In the present study, gene overexpression was used to study the function of PACAP *in vivo* in amphibians. Although PACAP has been implicated in playing a protective role in metabolic and/or environmental stress conditions in mammals (11), PACAP may serve a different function in amphibians. To date, the structure and localization of PACAP has been well studied in amphibians but its primary role still remains to be discovered. To examine PACAP's function, an interesting study was conducted by Otto *et al.* where PACAP 38 was injected into the *Xenopus* embryo blastocoel. Embryos containing excess PACAP 38 were strongly anteriorized when compared to control embryos. This suggests that PACAP may be involved in secondary embryonic axis development (12). In frogs, PACAP is encoded on the same mRNA transcript along with GHRH and is expressed primarily in the CNS suggesting PACAP is involved in neurotransmission and/or neuromodulation (13). Other studies suggest that PACAP may be involved in the early embryonic development of the *Xenopus* neural tube (14). In conclusion, to determine whether PACAP alone or a combination of PACAP and GHRH are involved in amphibian growth, development, metamorphosis, or survival, transgenic frogs overexpressing the hormones have been generated and examined.

MATERIALS AND METHODS

Xenopus laevis brain PACAP cDNA extraction:

Female adult *Xenopus laevis* frogs (Xenopus-I Inc., Dexter, MI) were euthanized in clove oil anaesthetic, one part eugenol to 10 parts 95% ethanol (100ppm final volume) for approximately 20 min as approved by the University of Victoria Animal Care Committee. The whole brain was removed and frozen on liquid nitrogen. The tissue was ground with a chilled mortar and pestle. Total RNA from 50-100 mg tissue was extracted using Trizol reagent (Invitrogen Canada Inc., Burlington, ON) and 1μg RNA was used to synthesize cDNA using Superscript II reverse transcriptase (Gibco BRL, Burlington, ON). Polymerase chain reaction (PCR) was used to amplify the cDNA and to introduce chosen restriction enzyme sites via linkers designed within the primers in order to cut the cDNA where desired. The enzyme sites introduced were EcoRI in the forward primer and XbaI in the reverse primer as indicated below by underline. A 50 μl reaction consisted of 1 ng cDNA, 200mM dNTPs, 1XPCR buffer, 2.5mM $MgCl_2$, 20 pmol each primer and 2.5U Taq DNA polymerase (Invitrogen). The mixture was placed in the Perkin Elmer Cetus DNA Thermal Cycler. PCR was carried out using the following primers: A) 5' GCTAGAATTCAAAGACAATGTGTAGGAAAGC 3'and B) 5' GCATTCTAGAACATCGCTACAAATATGCTAC 3'. Reaction conditions consisted of a denaturation at 94^0C for 30 s; annealing at 57^0C for 30 s; extension at 72^0C for 45 s for 35 cycles and a 7 min extension for the last cycle. After separation on 1.5% agarose gel and ethidium bromide staining, three PCR products of 570bp, 549 bp and 453 bp were visible. The PCR products were each cloned into pGEM-T vector system II (Promega Corporation, Madison, WI), then grown up in electro-competent XL-1 Blue

Escherichia coli cells (Stratagene, La Jolla, CA) on ampicillin LB/agar plates. Plasmid DNA was extracted with the QIAprep spin miniprep kit (Qiagen Inc., Mississauga, ON). DNA sequences were verified by the dideoxy method in the Biomedical Research Centre by Roderick Haesevoets, University of Victoria. The 549 bp product corresponds to the full-length frog GHRH/PACAP transcript, whereas the 453 bp product is a truncated GHRH/PACAP transcript, where the first 32 amino acids of GHRH have been spliced out (15). Each of these products was used to make two types of construct for generating transgenic frogs. The largest band of 570 bp was cloned several times and sequenced but did not correspond to any known gene and was likely a result of non-specific priming.

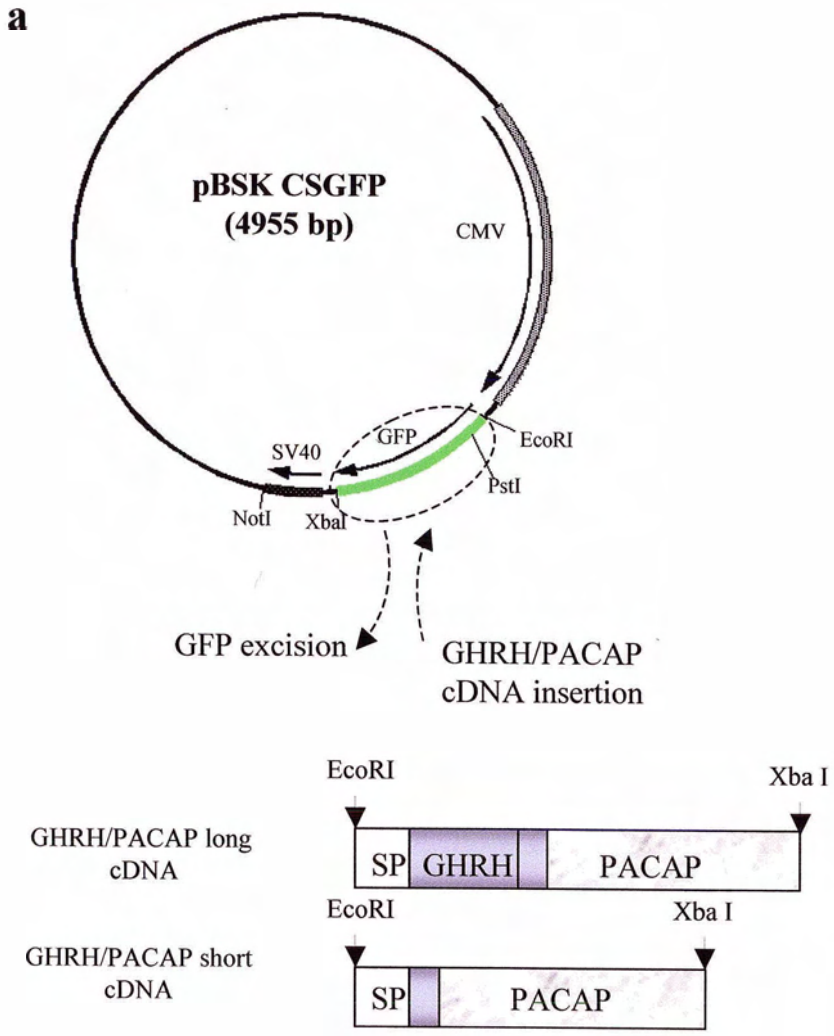
Transgenic frog construct generation:

Two vectors used as the backbone for the final construct design, pBSK CSGFP and xCAR GFP, were kindly provided by Dr. Browder, University of Calgary. The pBSK vector consists of green fluorescent protein (GFP) driven by a constitutive simian cytomegalovirus (CMV) promoter, simian virus (SV) 40 poly adenylation (polyA) sequence and ampicillin resistance. The GFP sequence was excised from pBSK using EcoRI and XbaI restriction enzymes (New England Biolabs Ltd., Pickering, ON) and both forms of GHRH/PACAP cDNA were excised using the same enzymes from the pGEM-T vector system II (Promega). The cut DNA was separated using 1.0% gel electrophoresis and desired fragments including the pBSK backbone and GHRH/PACAP cDNA were cut out of the gel and extracted using the QIAquick gel extraction kit (Qiagen). The two fragments were then ligated using 6 units T4 DNA ligase (Promega)

with a 3:1 ratio of insert to vector (Figure 2.1). The construct with the insert was confirmed by a diagnostic PstI/NotI digest. These first constructs were given the name pBSK-GHRH/PACAP short or pBSK-GHRH/PACAP long depending on the cDNA transcript that was inserted.

The xCAR GFP vector consists of an endogenous *X. laevis* cardiac actin promoter (xCAR), an SV40 polyA site and ampicillin resistance. xCAR is heart and skeletal muscle specific (7) and drives the production of GFP mRNA. To liberate the xCAR-GFP-SV40 module from the xCAR GFP plasmid, NotI restriction enzyme (New England Biolabs) was used. The construct pBSK-GHRH/PACAP was also cut using NotI at a site downstream of the CMV-GHRH/PACAP-SV40 module. After digestion, the pBSK-GHRH/PACAP construct enzyme reaction was heat inactivated at 75^0C for 20 min, allowed to cool to room temperature, and 10 units calf intestinal phosphatase (New England Biolabs) were added followed by 1 h incubation at 37^0C. The reaction was then purified with the QIAquick PCR purification kit (Qiagen). The fragments of DNA were cloned together after gel purification as above in a 4:1 ratio (GFP fragment to GP containing vector) to produce the final GHRH/PACAP construct (Figure 2.2, Figure 2.3). A diagnostic digest using Sca I was done to confirm module orientation since the same restriction site was used on both sides and could result in cloning the insert backwards. Constructs with inserts were screened using a gel cracking method. A master grid was prepared on an agar plate with ampicillin. A desired number of colonies were picked individually with a new sterile toothpick from the original plate. Each colony on a toothpick was touched to the master plate and then the same colony was transferred to an Eppendorf tube with 30µl cracking buffer (50mM NaOH, 0.5% SDS, 5mM EDTA and

Figure 2.1. Vector construct cloning strategy to generate transgenic *Xenopus laevis* frogs that overexpress GHRH and PACAP or truncated GHRH and PACAP. The pBSK CSGFP vector was cut with EcoRI and XbaI enzymes to excise GFP and full length or truncated GHRH/PACAP cDNA was cloned in its place using the same enzymes **(a)**. Two constructs, pBSK-GHRH/PACAP long **(b)** and pBSK-GHRH/PACAP short **(c)** were generated. CMV = cytomegalovirus; GFP = green fluorescent protein; SV40 = simian virus 40 poly adenylation. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.


a
pBSK CSGFP
(4955 bp)
CMV
SV40
GFP
EcoRI
PstI
NotI
XbaI
GFP excision
GHRH/PACAP
cDNA insertion
EcoRI
Xba I
GHRH/PACAP long
cDNA
SP
GHRH
PACAP
EcoRI
Xba I
GHRH/PACAP short
cDNA
SP
PACAP

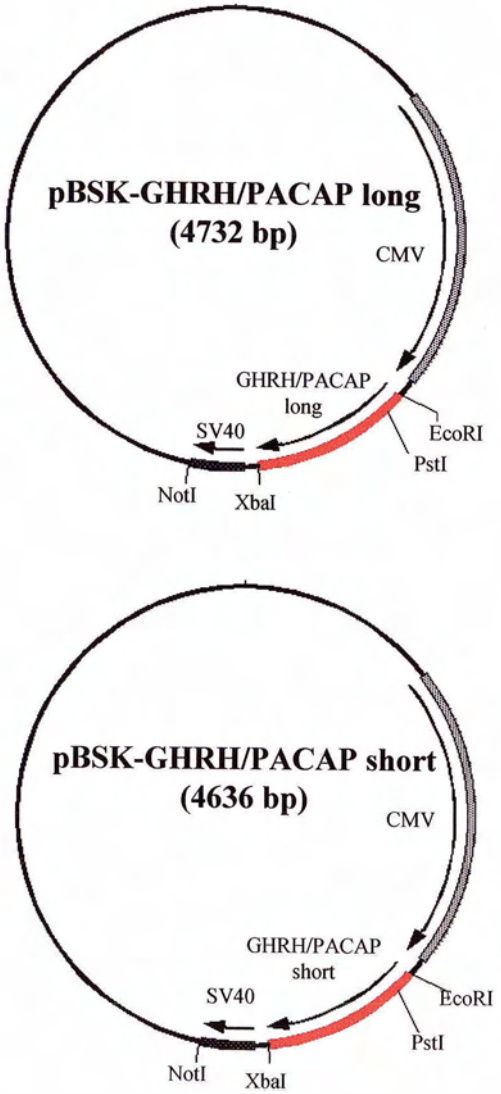

b
pBSK-GHRH/PACAP long
(4732 bp)
CMV
GHRH/PACAP
long
SV40
EcoRI
PstI
NotI
XbaI
c
pBSK-GHRH/PACAP short
(4636 bp)
CMV
GHRH/PACAP
short
SV40
EcoRI
PstI
NotI
XbaI

Figure 2.2. Vector construct cloning strategy to generate transgenic *Xenopus laevis* frogs that overexpress GHRH and PACAP or truncated GHRH and PACAP. Excision and insertion of the xCAR-GFP-SV40 module into the pBSK-GHRH/PACAP construct downstream of GHRH/PACAP is shown using the NotI restriction site. xCAR = cardiac actin promotor; GFP = green fluorescent protein; SV40 = simian virus 40 poly adenylation; CMV = cytomegalovirus; GHRH = growth hormone releasing-hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

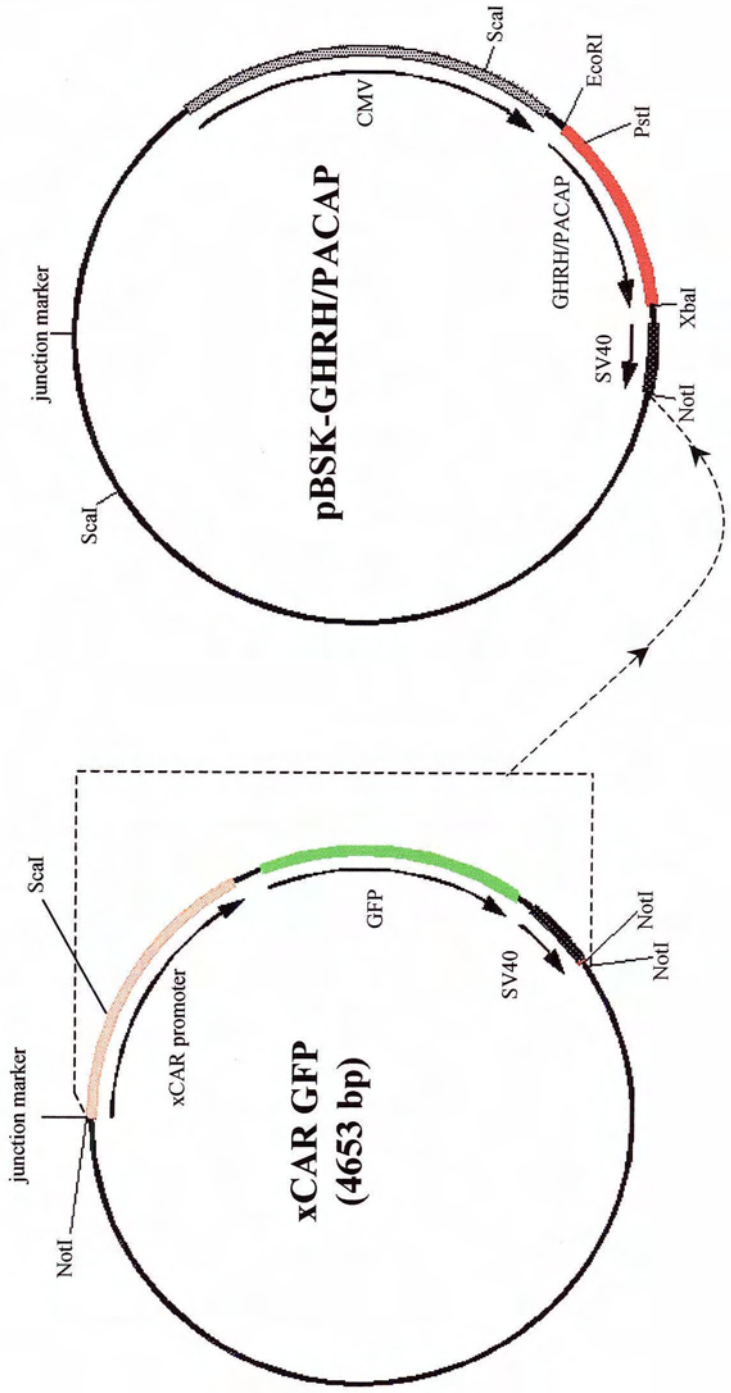
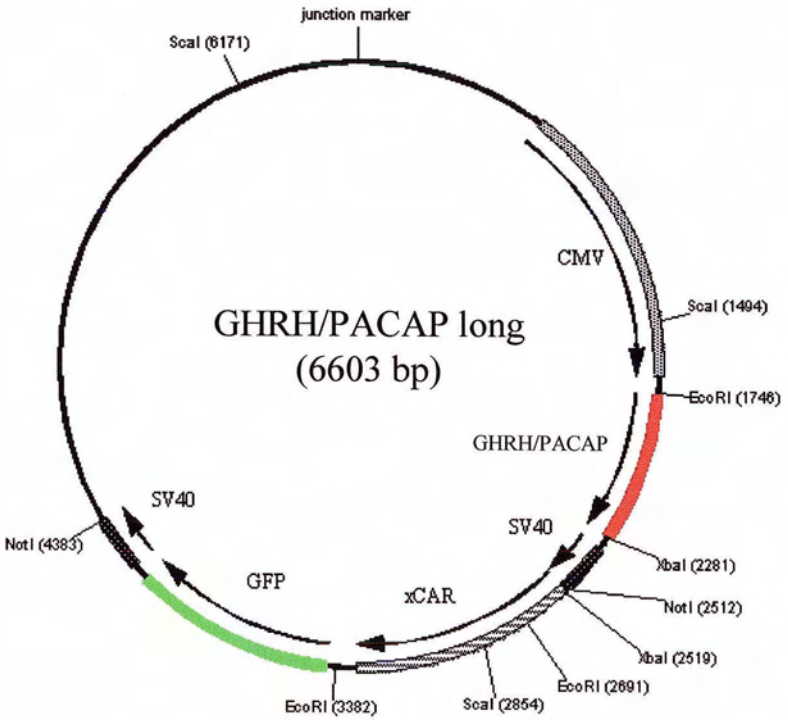

pBSK-GHRH/PACAP
junction marker
ScaI
CMV
ScaI
EcoRI
PstI
GHRH/PACAP
SV40
XbaI
NotI
xCAR GFP
(4653 bp)
junction marker
NotI
ScaI
xCAR promoter
GFP
SV40
NotI
NotI

Figure 2.3. Vector constructs used to generate transgenic *Xenopus laevis* frogs that overexpress GHRH and PACAP **(a)** or truncated GHRH and PACAP **(b)**. xCAR = cardiac actin promotor; GFP = green fluorescent protein; SV40 = simian virus 40 poly adenylation; CMV = cytomegalovirus; GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

a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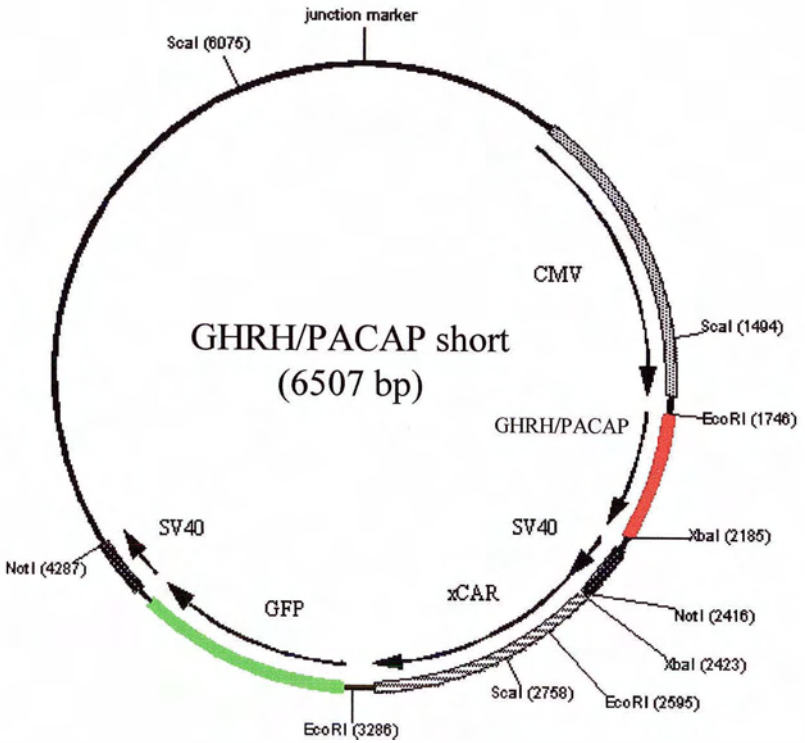
ScaI (6171)
CMV
ScaI (1494)
GHRH/PACAP long
(6603 bp)
EcoRI (1746)
GHRH/PACAP
SV40
SV40
NotI (4383)
XbaI (2281)
GFP
xCAR
NotI (2512)
XbaI (2519)
EcoRI (2691)
EcoRI (3382)
ScaI (2854)

b


junction marker
ScaI (6075)
CMV
ScaI (1494)
GHRH/PACAP short
(6507 bp)
EcoRI (1746)
GHRH/PACAP
SV40
SV40
XbaI (2185)
NotI (4287)
GFP
xCAR
NotI (2416)
XbaI (2423)
ScaI (2758)
EcoRI (2595)
EcoRI (3286)

0.025% bromocresol green dye). The bacterial cells were quickly dispersed in the buffer by rotating the toothpick for a few seconds. Samples were incubated at 68^{0}C for 45 min and then pulsed in the centrifuge at 12 000 rpm for a few seconds. Samples were loaded into dry wells of 0.7% agarose gel; 1 X TAE buffer was added until level with the gel surface. The gel was run for 10 min at 10V/cm, was covered with buffer and was run at 5V/cm until the dye had migrated about ¾ the length of the gel. The gel was stained in EtBr and viewed under UV light.

Sperm extraction from male adult frogs and nuclei preparation:

Jill Johnston in Dr. Browder's laboratory at University of Calgary prepared this portion of materials and methods. Jill made the sperm extract and nuclear preparation, but the method is included here for completion.

Adult *Xenopus laevis* (Nasco, Fort Atkinson, WI) frogs were kept in Z-Mod tanks (Marine Biotech Inc., Beverly, Mass) at 24^{0}C on a 12h/12h/ light/dark cycle and fed daily with Nasco frog pellets (1 pellet/frog/day). Adult males were euthanized in 0.05% benzocaine for approximately 20 min. The frog was dissected, testes were removed and placed in a petri dish. Testes were rinsed once in cold 1 X Marc's modified Ringers (MMR) solution (pH 7.5) followed by two rinses in 1 X nuclear preparation buffer (NPB) (Appendix). The liquid was blotted with KimWipes and excess fat and debris were removed from testes under a dissecting scope. Testes were moved to a clean dry petri dish and were macerated with scissors thoroughly until clumps of tissue were not visible. A cut pipet tip (1mm) was used to mix the macerated tissue with 1ml 1 X NPB. The suspension was filtered through an 80μm nylon mesh into a 14ml plastic Falcon test tube.

The petri dish and scissors were rinsed off with 1 X NPB, 1ml at a time, through the nylon mesh into the Falcon tube to a final volume of 10ml. The sample was centrifuged at 678 x g (Sorvall HB-4) for 10 min at 4^0C. The supernatant was immediately poured off and the sperm pellet was resuspended in 8ml 1 X NPB. Centrifugation was repeated and the supernatant was poured off before the pellet was resuspended with 1ml of 1 X NPB. Digitonin (20 µl of 10mg/ml) was added. The solution was mixed gently and was incubated at room temperature for 5 min. To stop the reaction, 10 ml of 1 X NPB with 3% bovine serum albumin (BSA) was added to the suspension. The sample was centrifuged as previously. After the supernatant was poured off, the pellet was resuspended gently with a cut tip in 5ml 1 X NPB with 3% BSA and was centrifuged as previously.

To count sperm, the supernatant was poured off completely without drying out the pellet. The pellet was gently resuspended in 250µl – 500µl sperm storage buffer (SSB) and suspended sperm nuclei were transferred to a 1.5ml Eppendorf tube (Appendix). A 1:100 dilution was made using 10µl of the suspension and nuclei were counted on a hemocytometer at 400X magnification. The nuclei were diluted to $1x10^5$ nuclei/µl using SSB. The nuclei were stored at 4^0C for 48 hours, were aliquoted into volumes sufficient for a single day's set of injections and were snap frozen in liquid N_2. The aliquots were stored at -80^0C.

Transgenic frog generation via in vitro microinjection:

The GHRH/PACAP construct was linearized in the noncoding region using Sac I restriction enzyme (New England Biolabs). The concentration of the linearized plasmid

was brought to 72 ng/μl. Two *X. laevis* females were primed with 800IU human chorionic gonadotropin (Intervet, Whitby, ON) and placed in a 17^0C degree incubator for 12-16 hours. Eggs were squeezed from females and degelled in 2% cysteine in 1 x MMR (pH 7.8 – 8.0) while shaking at 200rpm for no longer than 4 min. The eggs were washed well (at least four times) in 1 X MMR. Eggs were loaded into 2% agarose base 60mm injection petri dishes with 0.4 X MMR and 6% ficoll and were allowed to sit for 5 minutes to adhere to the agarose. Sperm nuclear DNA was taken from -80^0C and put on ice. Linearized plasmid (2.5μl) and 2.5 x 10^5 nuclei (2.5 μl) were incubated together at room temperature for 10 min. Sperm dilution buffer was added to bring the volume to 500 μl. Cut pipette tips were used at all times when dealing with sperm nuclear DNA to avoid shearing. Microinjection needles were made from glass capillary Drummond Microcaps (Fisher Scientific LTD, Nepean, ON) with a P-87 needle puller (Sutter Instruments, Novato, CA). This was done using a 3mm box filament at a settings of: heat = 950, pull = 70, velocity = 20, time = 1 and pressure = 330. Glass needles were clipped to about 60μm in diameter using fine forceps. The needles were backloaded with the diluted reaction mixture for microinjection. Eggs were injected with a Harvard PHD2000 infusion pump with 2.5ml Hamilton gas-tight syringes attached to Tygon tubing filled with mineral oil at 0.9μl/min so that one sperm nucleus entered per egg. After injection, eggs were incubated at 16^0C for three hours to the 4-cell stage. Transgenic frogs were generated in batches over several weeks.

The method of generating transgenic *Xenopus laevis* frogs has been developed by Kroll and Amaya (7) and was partially modified by Dr. Browder, University of Calgary. For buffer solutions see Appendix 1.

Embryo and juvenile frog care:

Using a clipped, fire-polished Pasteur pipet, normally cleaving embryos were transferred to 100mm petri dishes (40 per dish) containing 0.1 X MMR + 6% Ficoll 400 + 50 μg/ml gentamycin and were cultured at 16^0C. The next day, normal embryos at stage 10-11 were transferred to 0.1 X MMR and gentamycin. Injected embryos were cultured at 16^0C and the next day (stage 15) abnormal and dead embryos were removed. The tadpoles were changed into the same fresh medium and were incubated at room temperature. At about stage 45, tadpoles were visualized for fluorescence in cardiac muscle and somites to identify true transgenesis. Starting at stage 45, tadpoles were transferred into 0.1 X MMR which was changed weekly and from this point were fed every 2 days until metamorphosis with powedered food for fry (Sera Micron, Heinsberg, Germany) suspended in water. Juvenile frogs were fed with frog brittle (Nasco) and were transferred into glass aquaria. Tadpole developmental stages were identified according to Nieuwkoop and Faber (16).

Fluorescent embryo visualization and photography:

Tadpoles were visualized under a fluorescent stereomicroscope with camera (Zeiss SV11) at 500 nm wavelength. Pictures were taken as black and white images and were then modified to color using Openlab and Adobe Photoshop software. Later stage tadpoles that were too motile for photography were immobilized by anaesthesia using 0.015% benzocaine in 0.1X MMR, for a few seconds.

In vitro fertilization to generate wild type frogs:

Adult *X. laevis* females were primed and eggs were squeezed out as previously described into a 35mm petri dish containing 0.4 X MMR. Testes were dissected from an adult euthanized *X. laevis* male and were stored in 1 X MMR with 10% fetal bovine serum at 4^0C up to one week. When needed, half a testis was washed in 1 X MMR and the tissue was macerated in a couple drops of 1 X MMR. This suspension was pipetted into the dish of eggs and was shaken vigorously for 4 min at approximately 200rpm. The eggs and sperm were allowed to sit for 20 min. If fertilization was successful, the animal poles of the eggs rotated to face upward. At this point the embryos were degelled in 2% cysteine as above except that 0.1 X MMR was used instead of 1 X MMR. Embryos were cultured in 0.1 X MMR in 100mm petri dishes in batches of about 40/dish at room temperature.

PCR to confirm construct integration in juvenile transgenic frogs:

A piece of muscle tissue was cut from euthanized frogs and was placed in a mixture of 5% Chelex® 100 Resin (BIO-RAD Laboratories Ltd., Mississauga, ON), 0.1% Tween 20, and 0.2mg/ml proteinase K to extract genomic DNA. The mixture was incubated in the Perkin Elmer Cetus DNA Thermal Cycler at 50^0C for 50 min and then proteinase K was inactivated at 94^0C for 15 min. Primers were designed to GFP on the construct used to generate transgenic frogs and the product was amplified using the same PCR reaction mixture as previously. The primers produced a 220 bp product and were as follows: C) 5' ATCTTCTTCAAGGACGACGGCAACT 3' and D) 5' TGTTGTGGC

GGGTCTTGAAGTT 3'. The first cycle had a denaturation step of 4.5 min at 94^{0}C. The next 31 cycles consisted of denaturation at 94^{0}C for 1 min, annealing at 59^{0}C for 1 min, and extension at 72^{0}C for 1 min. The final cycle had an extension of 8 min.

Detection of ectopic GHRH/PACAP expression in transgenic frog liver:

Liver was collected from juvenile euthanized *X. laevis* wild type and transgenic frogs by dissection. Tissue was quick frozen in a tube in liquid nitrogen and stored at –80^{0}C. The tissue was ground to a fine powder using a chilled mortar and pestle. Approximately 40mg tissue was used to extract total RNA with Trizol® LS reagent (Invitrogen) as previously described. Total RNA (approximately 2.5μg) was treated with 2 units Turbo^{TM} DNAse in 1XDNase buffer (Ambion) and the reaction was incubated at 37^{0}C for 30 min. The reaction was inactivated using 1:1 phenol to chloroform extraction. Approximately 1μg was reverse transcribed to cDNA using Superscript II reverse transcriptase (Gibco, BRL). The cDNA reaction was diluted (1:20) and one μl cDNA template was added to a 50 μl PCR reaction and the mixture was the same as previously. Primers to detect GHRH/PACAP expression were the same as before used on frog brain cDNA (A and B). Primer design for the ribosomal protein L8 was kindly provided by Nick Veldhoen from Dr. Helbing's lab, University of Victoria, and the primers were as follows: E) 5' CAGGGGACAGAGA AAAGGTG 3' and F) 5' TGAGCTTTCTTGC CACAG 3' to yield an approximately 270 bp product. The PCR consisted of a denaturation at 94^{0}C for 30 s; annealing at 57^{0}C for 30 s; extension at 72^{0}C for 45 s for 35 cycles and 7 min extension for the last cycle. PCR products generated with primers A and B were cloned into pGEM-T vector system II (Promega) as previously and were

dideoxy sequenced to confirm that they correspond to the expected GHRH/PACAP transcript. To ensure that the total RNA was not contaminated by genomic DNA even after DNAse treatment, primers specific to CMV were used in a PCR reaction. The primers used were as follows: G) 5' TGCCAACTGGGGAGGGGTCTA 3' and H) 5' GGGGAGTGGCTAT GGGCGGTA 3' and yielded a 318bp product. The PCR mixture was the same as before and the conditions consisted of a denaturation at 94^0C for 30s; annealing at 62^0C for 30 s; extension at 72^0C for 45 s for 35 cycles and 7 min extension for the last cycle.

Frog growth and survival rates:

Frog length was measured from tip of head to cloaca region, 148 days post-fertilization with a ruler. Frog death was recorded for a period of four months starting at one week after microinjection or *in vitro* fertilization. Embryos with a defect in the first week post-fertilization were not included in counts. Embryos with a defect more than one week post-fertilization were counted as dead as they had to be euthanized.

Tadpole histology:

Stage 50 control and transgenic tadpoles with either a long or short GHRH/PACAP construct were euthanized in 0.015% benzocaine and fixed in 4% paraformaldehyde in 1 X phosphate buffered saline (PBS). The fixed tadpoles were rinsed twice with distilled H_2O and were dehydrated for 20 min in 1ml volume of ethanol of the following concentrations: 30%, 50%, 70% twice, 95% and 100% three times. The tissues were embedded in hydroxyethyl methacrylate embedding medium (Technovit,

Germany) and transverse sections were cut serially at 7 μm with glass knives on a JB-4 microtome. Sections were stained for a few minutes with filtered and diluted (3 drops in 35ml distilled water) Richardson's stain (equal ratio of 1% azure II to 1% methylene blue in 1% borax) rinsed with distilled H_2O, air dried and mounted in Entellen medium (BDH, Germany). Sections were photographed with a Nikon digital E990 camera (Nikon Corp., Japan) using the Universal microscope (Zeiss, Germany) with a blue filter. Picture brightness and contrast were adjusted using Adobe Photoshop software.

RESULTS

GHRH/PACAP mRNA amplification

Primers designed to *X. laevis* GHRH/PACAP yielded three PCR products of approximately 570 bp, 549 bp and 453 bp (Figure 2.4). After performing a BLAST search on the remaining two sequences, the 549 bp product matched with full-length *X. laevis* GHRH/PACAP transcript (longGP). The 453 bp product was identical to the longer transcript except for a truncation of the first 32 amino acids within the GHRH sequence (shortGP). The splicing removed exon four. The largest product of 570 bp did not correspond to any known sequence as stated previously.

Transgenesis

Xenopus laevis frog eggs that were injected with sperm nuclear DNA and a construct bearing [xCAR-GFP; CMV-GHRH/PACAP], developed into tadpole embryos that expressed GFP in muscle tissue uniformly (transgenic) (Figure 2.5), expressed GFP in muscle tissue mosaically, or did not express GFP at all (non-transgenic). Embryos that had integrated the construct into genomic DNA, expressed GFP past one month of age. Genomic transgene integration was confirmed by PCR of genomic DNA in juvenile transgenic frog muscle tissue specific for GFP (Figure 2.6).

Ectopic and/or elevated expression of PACAP in transgenic frogs

Transgenic tadpoles expressed GHRH/PACAP mRNA in liver, whereas wild type tadpoles did not (Figure 2.7). A transgenic frog generated using longGP cDNA

Figure 2.4. RT-PCR on *Xenopus laevis* brain tissue using primers specific for the GHRH/PACAP (GP) gene. ld = 100 bp ladder; -ve = negative control. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

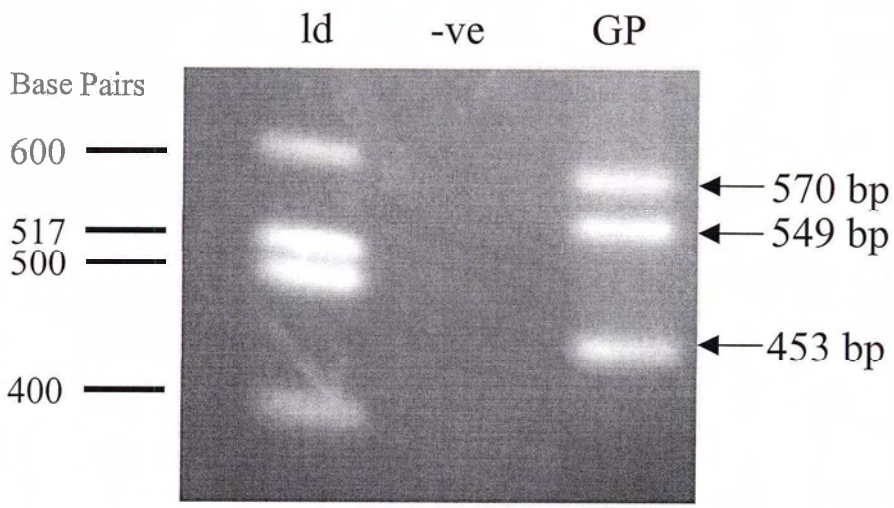
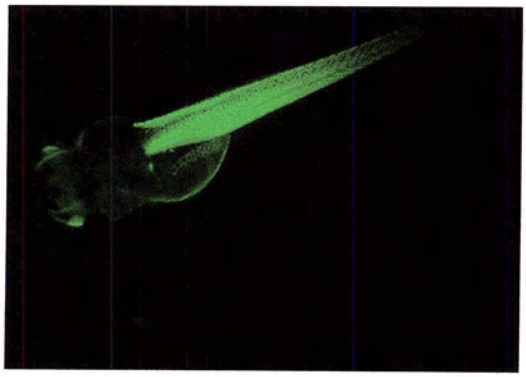

ld
-ve
GP
Base Pairs
600
517
500
400
570 bp
549 bp
453 bp

Figure 2.5. Transgenic *Xenopus laevis* tadpole stage 49 injected with a construct consisting of green fluorescent protein and either a transcript with full length GHRH/PACAP or a transcript with truncated GHRH and full length PACAP. Fluorescent image of whole tadpole where green fluorescent protein is the most intense in tail muscle tissue **(a)**. Merged white light and fluorescent image of tadpole **(b)**. Top view of tadpole where jaw muscle is indicated by white arrow **(c)**. Ventral view of tadpole where jaw muscles are indicated by a black arrow **(d)**. Side view of tadpole tail where individual somites are indicated by white arrows **(e)**. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

a



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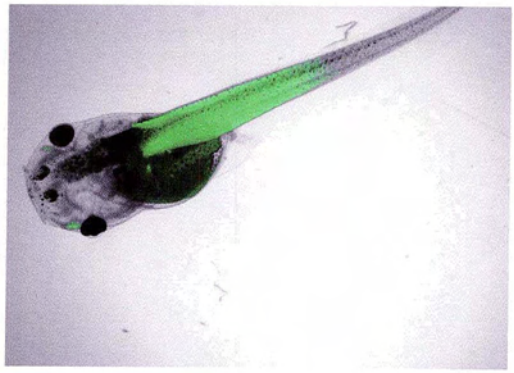

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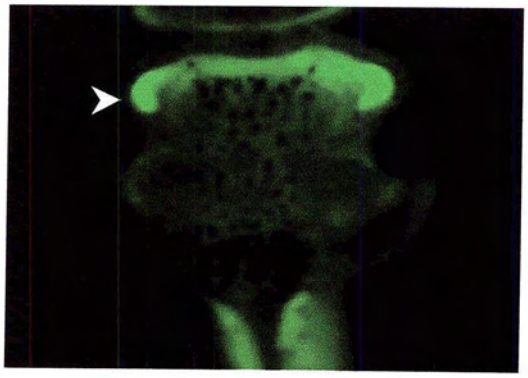

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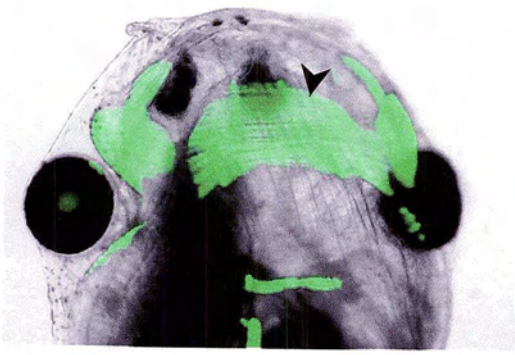


e

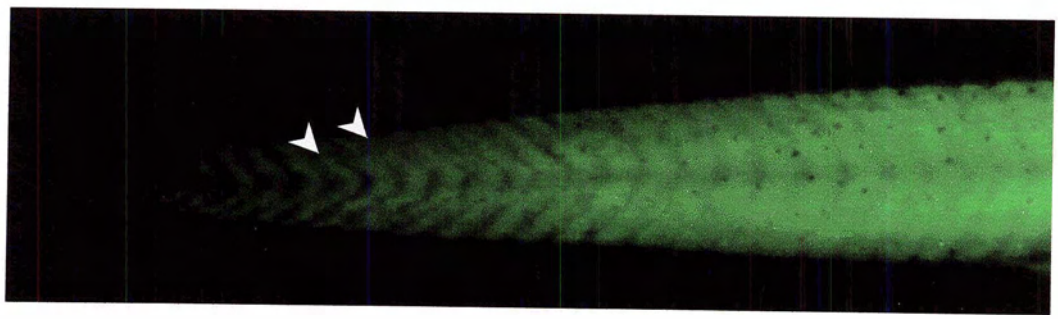


Figure 2.6. Confirmation of green fluorescent protein (220 bp) genomic integration in transgenic (tg) *Xenopus laevis* frog muscle tissue compared to wild type frogs (wt) using PCR. ld = 100 bp ladder; -ve = negative control.

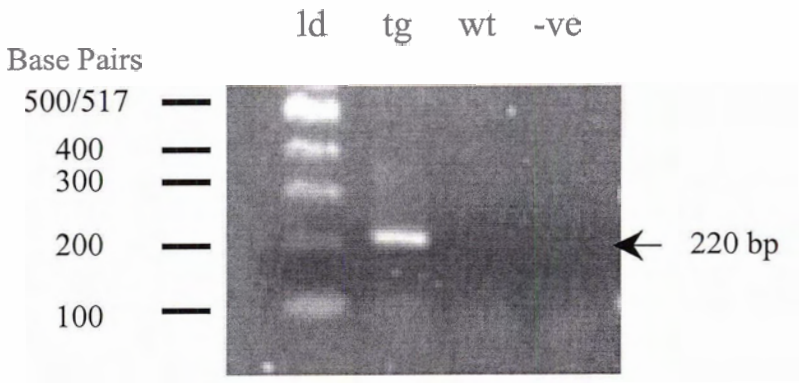

ld
tg
wt
-ve
Base Pairs
500/517
400
300
200
100
220 bp

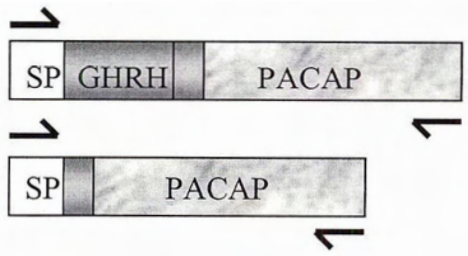
Figure 2.7. A diagram of methods used to confirm ectopic expression of GHRH/PACAP transcripts in transgenic *Xenopus laevis* liver **(a)**. RT-PCR of L8 housekeeping gene and GHRH/PACAP (GP) in frog liver. GP expression was present in transgenic frogs that express the longGP (L) or truncated shortGP (S) transcript but not in wild type (W) frogs **(b)**. Ld = 100bp ladder; -ve = negative control. ⟶ = primer; SP = signal peptide; GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

a

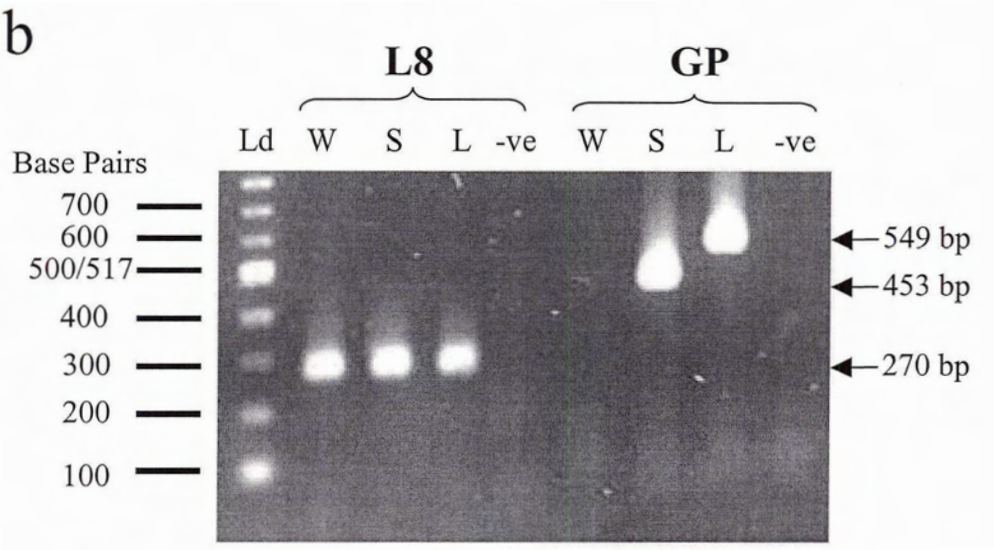
1. Dissect liver from transgenic frog

2. Extract mRNA from liver

3. PCR on cDNA



b



expressed a transcript of the appropriate size in liver. Similarly, a transgenic frog that was generated using shortGP cDNA, expressed a shorter transcript of expected size in the liver. Sequencing confirmed that both transcripts corresponded to *X. laevis* GHRH/PACAP mRNA. To ensure that mRNA was expressed from the transgene and that the primers were not amplifying genomic DNA (transgene) instead, primers designed to CMV promoter located on the transgene were used in a PCR reaction. A PCR product would indicate that genomic DNA containing the integrated transgenic vector is present. A band of 318bp indicating presence of CMV was visible only for RNA extracted from liver of transgenic frogs overexpressing shortGP (Figure 2.8). No bands were present for wild type or transgenic frog overexpressing longGP.

Frog external morphology and histology

Transgenic tadpoles, at stage 44, had external morphology that appeared normal when compared to wild type tadpoles (Figure 2.9). Some frogs had deformities and abnormal growth, including anteriorized phenotype but this was apparent in both control and transgenic embryos. Embryo defects were especially frequent in early developmental stages again in both control and transgenic animals. Frogs were monitored until they reached a juvenile age and no visible difference in external appearance was noted between wild types and transgenic frogs. When frogs were sacrificed, autopsy did not reveal any organ abnormalities.

Stage 50 control (wild type) and transgenic tadpoles either overexpressing longGP or shortGP were examined for histology. Tissues that were analyzed included eye, heart, brain, glottis, kidney, liver and intestines (Figure 2.10). Two differences were noted in

Figure 2.8. RT-PCR on *Xenopus laevis* liver mRNA (cDNA) using primers designed to cytomegalovirus (CMV) promoter located on the transgene to ensure cDNA is not contaminated by genomic DNA. Genomic contamination was present in transgenic frogs that express truncated GHRH/PACAP (S) transcript but not in frogs expressing full length GHRH/PACAP (L) transcript. Wild type (W) frogs do not contain CMV promoter. Ld = 100bp ladder; -ve = negative control. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

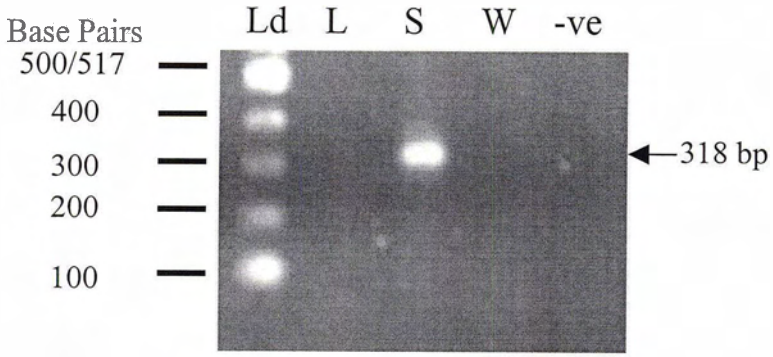

Base Pairs
500/517
400
300
200
100
Ld
L
S
W
-ve
318 bp

Figure 2.9. Stage 44 *Xenopus laevis* wild type control (C) and transgenic (TG) tadpole that overexpress GHRH and PACAP. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

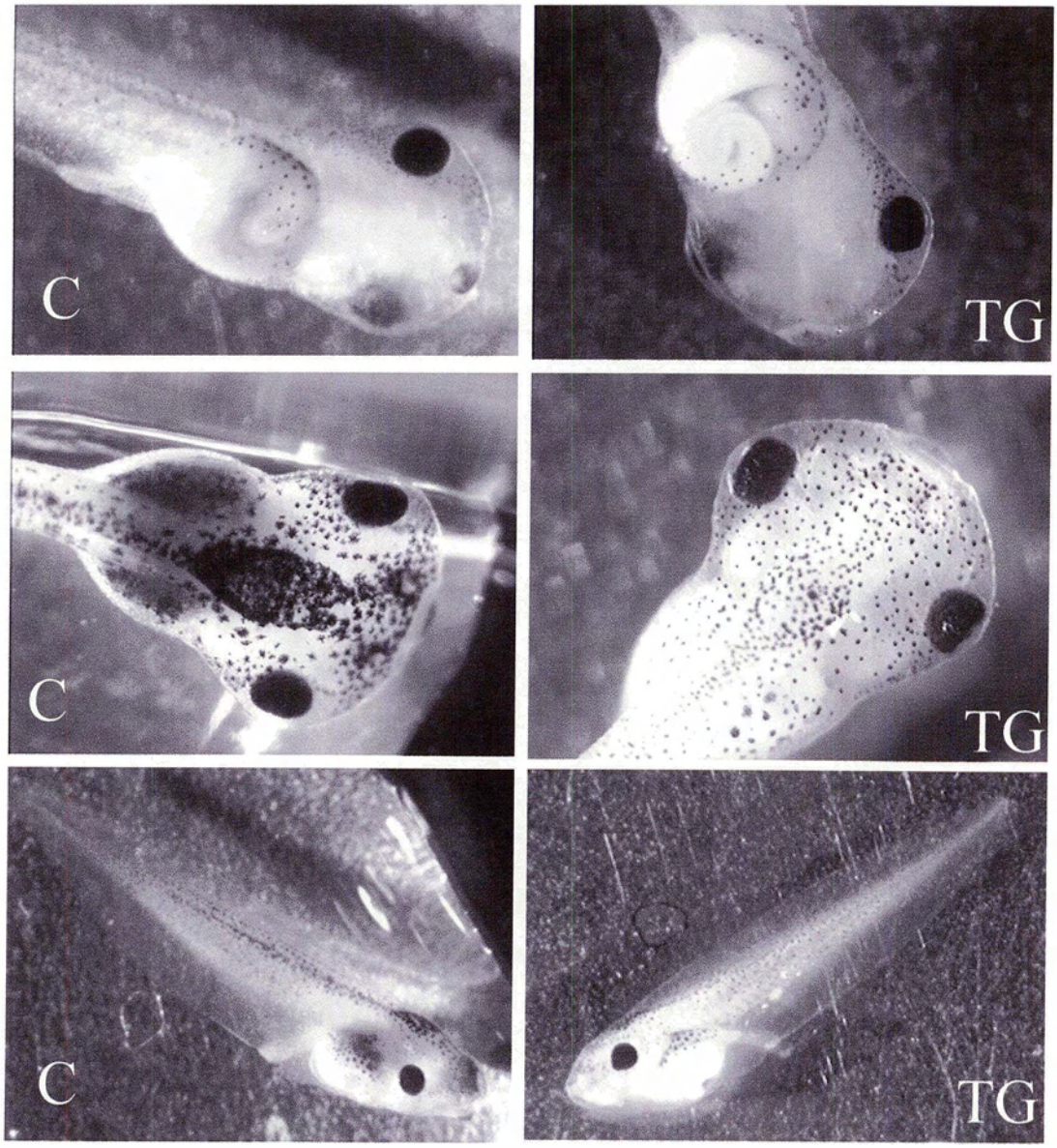
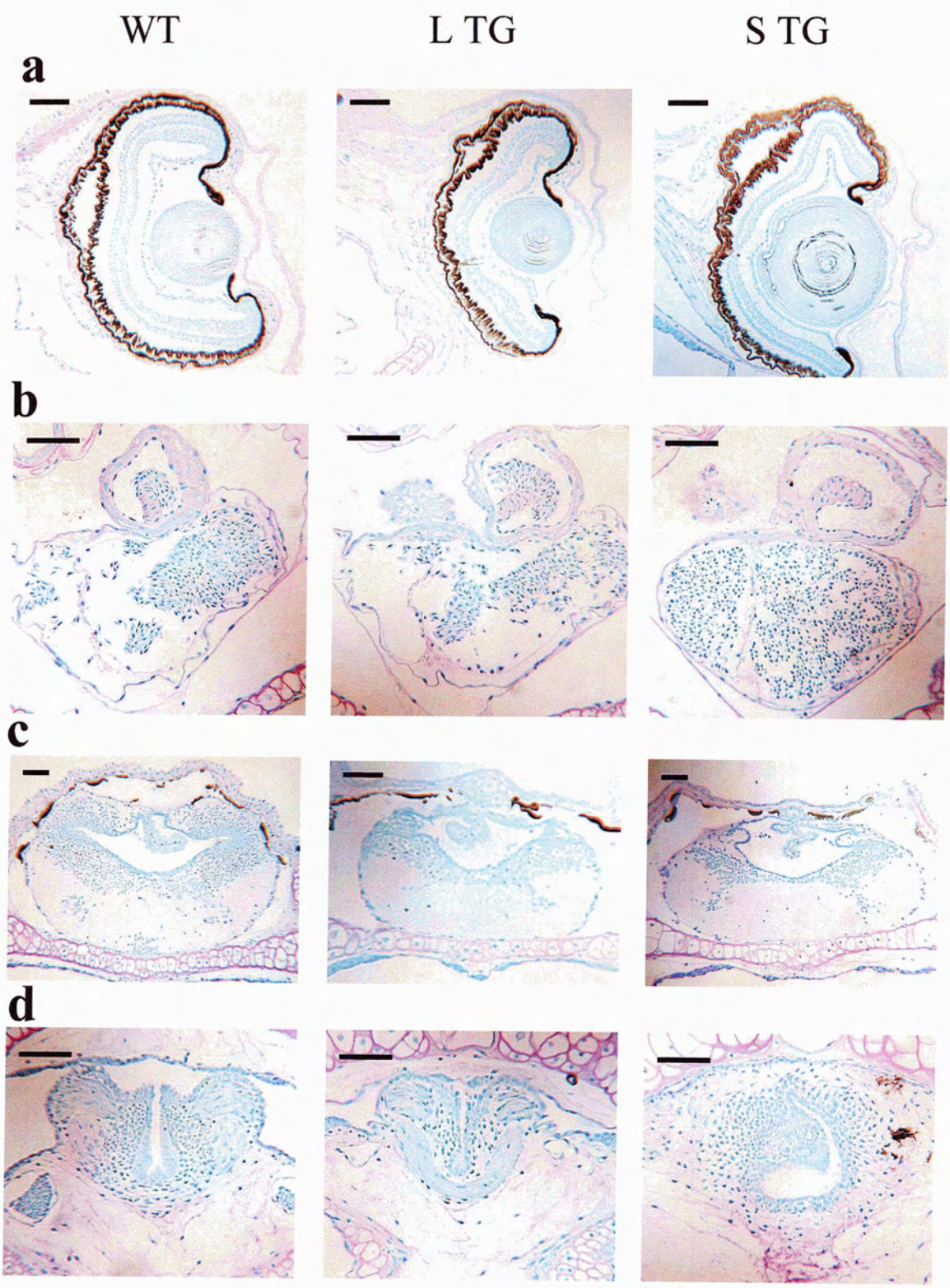
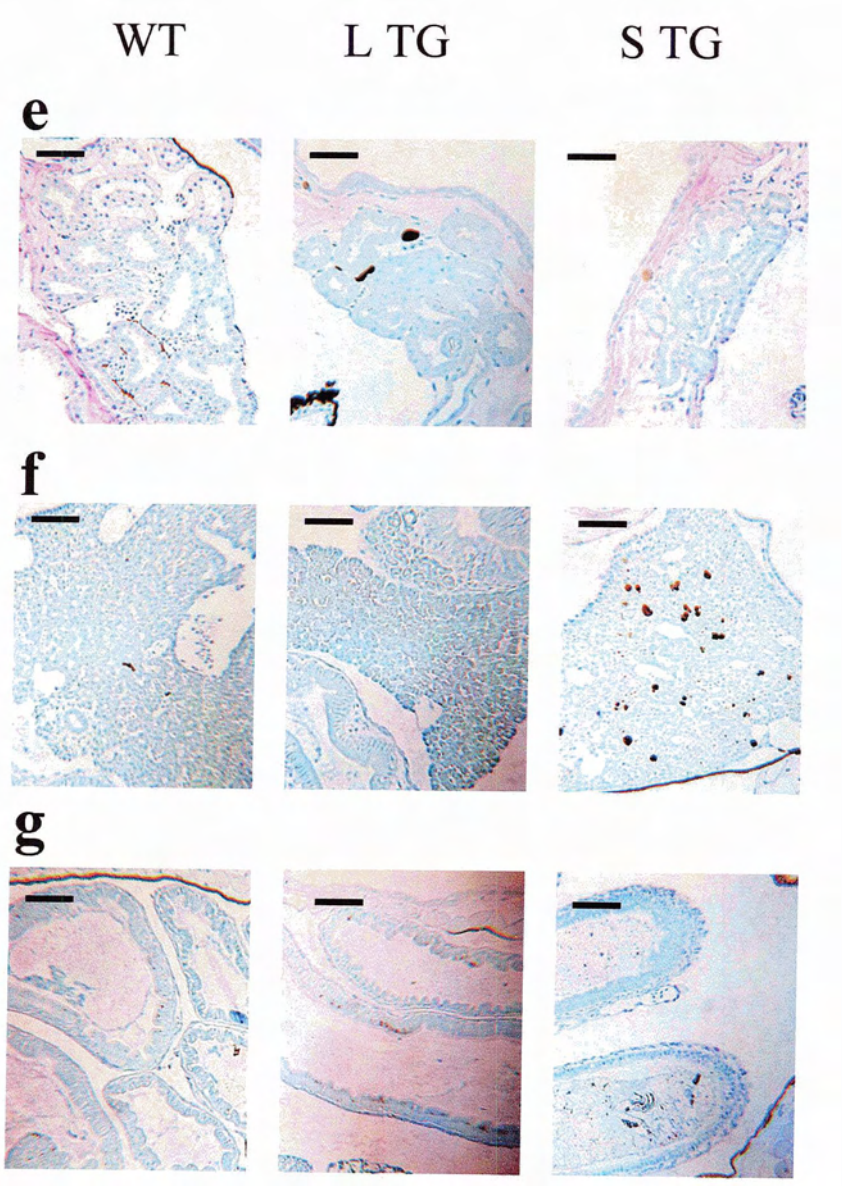

C
TG
C
TG
C
TG

Figure 2.10. Histology of stage 50 wild type (WT) and transgenic (TG) *Xenopus laevis* tadpoles. Tissues or organs that were compared included eye **(a)**, heart **(b)**, brain **(c)**, glottis **(d)**, kidney **(e)**, liver **(f)**, and intestine **(g)**. Sections were stained with Richardson's stain. Scale bar represents 0.2mm. S = frog overexpressing truncated GHRH/PACAP transcript; L = frog overexpressing full length GHRH/PACAP transcript. GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.


WT
L TG
S TG
a
b
c
d


WT
L TG
S TG
e
f
g

the transgenic frog overexpressing shortGP when compared to the control frog. First, a structure that most likely resembles the glottis was deformed in the transgenic frog because cells were not arranged symmetrically when compared to control (Figure 2.10d). Second, the transgenic liver was full of black or dark brown granules (Figure 2.10f). No striking histological differences were noted between the transgenic frog ovexpressing longGP when compared to the control frog.

Frog survival rates and size

Juvenile frog survival rates were examined at the end of a four-month period starting at one week post-fertilization (Figure 2.11). Wild type *in vitro* fertilized frogs (n=50) had the highest survival rate of 52%. All other types of tadpoles generated by egg injection with sperm nuclear DNA combined with or without a DNA construct had lower survival rates. Non-transgenic frogs (n=12) that did not express GFP had a survival rate of 38.8%. Frogs that were injected with sperm nuclear DNA only (sperm-injected) (n=11) had a survival rate of 27.2%. Transgenic frogs had the lowest survival rates of 16.5% and 12.5% corresponding to frogs overexpressing longGP (n=23) and shortGP (n=24), respectively.

Tadpole survival rates were also analyzed over a period of 45 days, starting at one week after fertilization (Figure 2.12). Both groups of transgenic tadpoles overexpressing either longGP (n=23) or shortGP (n=24) had a sharp drop in numbers starting after the first week post fertilization. The sharp decrease in surviving frogs gradually leveled off but continued until day 45. Non-transgenic frogs (n=12) also exhibited a sharp drop in numbers in the first week recorded and then once again at 34 days. Wild type frogs

2.11. Survival rates of *Xenopus laevis* frogs at the age of four months, starting at one week after fertilization. The graph shows the difference in survival rate of frogs produced by two types of fertilization methods and also compares transgenic versus non-transgenic frog survival rates. Embryos with a defect in the first week post-fertilization were not included in counts. Embryos with a defect more than one week post-fertilization were counted as dead as they had to be euthanized. STG = transgenic frogs produced by injection of sperm nuclear DNA and a DNA construct that overexpressed truncated GHRH and full length PACAP; LTG = transgenic frogs produced by injection of sperm nuclear DNA and a DNA construct overexpressing both GHRH and PACAP; SPE = control frogs produced by injection of sperm nuclear DNA without a DNA construct; NTG = frogs that did not appear transgenic (no green fluorescent protein visible) but were produced by injection with sperm nuclear DNA and DNA construct; WT = wild type frogs produced by *in vitro* fertilization; GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.

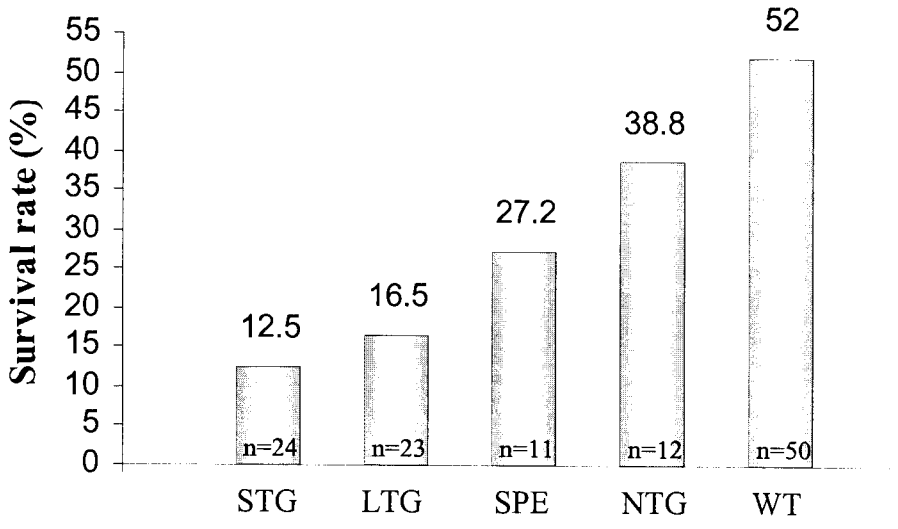
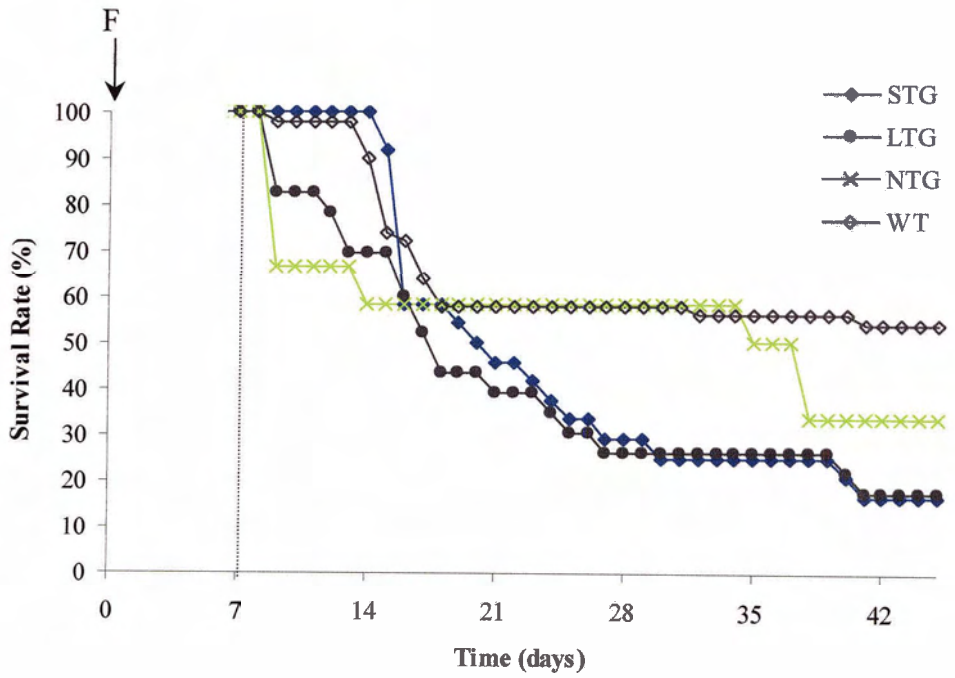

Survival rate (%)
55
50
45
40
35
30
25
20
15
10
5
0
12.5
16.5
27.2
38.8
52
n=24
n=23
n=11
n=12
n=50
STG
LTG
SPE
NTG
WT

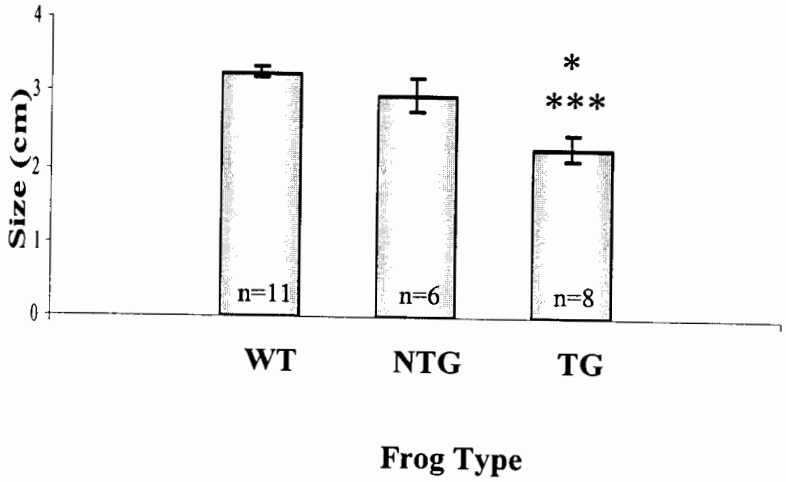
Figure 2.12. Survival rates of *Xenopus laevis* frogs over a 45 day period starting at one week after fertilization. The graph shows the difference in survival rates of frogs produced by two types of fertilization methods (sperm nuclear DNA injection and *in vitro* fertilization) and also compares transgenic versus wild type frog survival rates. Embryos with a defect in the first week post-fertilization were not included in counts. Embryos with a defect more than one week post-fertilization were counted as dead as they had to be euthanized. F = fertilization; vertical line = beginning of death rate recording; STG = short form transgenic frogs produced by injection of sperm nuclear DNA and a DNA construct overexpressing truncated GHRH and full length PACAP; LTG = long form transgenic frogs produced by injection of sperm nuclear DNA and a DNA construct overexpressing GHRH and PACAP; NTG = frogs that did not appear transgenic (no green fluorescent protein was visible) but were produced by injection with sperm nuclear DNA and DNA construct; WT = wild type frogs produced by *in vitro* fertilization; GHRH = growth hormone-releasing hormone; PACAP = pituitary adenylate cyclase-activating polypeptide.


F
STG
LTG
NTG
WT
Survival Rate (%)
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
42
Time (days)

(n=50) exhibited a sharp drop in numbers after the first week, but when compared to other groups of frogs, mortality was minimal starting at day 17 until day 45.

The size of juvenile frogs was compared at 148 days post-fertilization (Figure 2.13). On average, wild type frogs (n=11) were the largest, followed by non-transgenic frogs (n=6), and transgenic frogs (n=8) were the smallest in size. Since numbers of transgenic frogs were low at this point, both types expressing either longGP or shortGP transcripts, were grouped together for analysis. There was a significant size difference between wild type and transgenic frogs ($P<0.001$) and between non-transgenic and transgenic frogs ($P<0.05$). There was no significant difference in size between wild type and non-transgenic frogs. Significance ($P<0.05$) was determined using the Tukey-Kramer multiple comparison test.

Figure 2.13. Measurement of size for 148-day-old wild type *in vitro* fertilized (WT), injected non-transgenic (NTG) and injected transgenic (TG) *Xenopus laevis*. Error bars represent SEM; n represents sample size; *** indicates a significant difference ($P<0.001$) between the values obtained from TG frogs and WT frogs; * indicates a significant difference ($P<0.05$) between the values obtained from TG frogs and NTG frogs (Tukey-Kramer Multiple Comparison Test).


Size (cm)
4
3
2
1
0
n=11
n=6
n=8
*
***
WT
NTG
TG
Frog Type

DISCUSSION

The goal of this study was to generate transgenic frogs overexpressing a pleiotropic neuropeptide, PACAP, and to examine the growth, development, physiology, and metamorphosis of these modified amphibians. This is the first study that applies transgenesis to study PACAP *in vivo* in amphibians. The DNA construct that was created and used in this experiment served a dual purpose; to identify true transgenic frogs by homogeneous GFP expression in muscle tissue and at the same time to overexpress PACAP constitutively in most frog tissues by the use of the ubiquitous CMV promoter. Because PACAP is encoded on the same gene as GHRH in non-mammalian species resulting in one transcript for both peptides, primers were designed to amplify the complete GHRH/PACAP transcript from *X. laevis* brain tissue. After amplification by PCR, three products were present and sequencing revealed that two products could be used in the present study. A 549bp product exactly matched the full length GHRH/PACAP mRNA (longGP) that has been previously isolated from *X. laevis* brain tissue and sequenced by Hu *et al.*(13). The shorter product of 453bp was identical to the full-length transcript except for truncation of the first 32 amino acids within the GHRH sequence (shortGP) and has not been previously reported in *X. laevis*. Similarly, two forms of GHRH/PACAP have been isolated from brain tissue of *R. ridibunda* (15). Alexandre *et al.* proposed that the two types of PACAP encoding mRNAs in frog could be derived from a single gene by alternative splicing (15). Earlier, Parker *et al.* showed that amino acids 1-32 in the salmon GHRH/PACAP gene are encoded on a separate exon which can also be spliced out to generate two PACAP precursor variants (17, 18). Additionally, a truncated form of GHRH/PACAP lacking amino acids 1-32 has also been

identified in *X. laevis* brain. However, the transcript has 11 amino acid differences within the coding region when compared to the full-length transcript or the truncated transcript identified in the present project. The researchers hypothesized the mRNA may be transcribed from another PACAP gene present in *Xenopus* (13). Both transcript types isolated in the present study were used to generate frogs that either overexpress both GHRH and PACAP or PACAP alone.

Three types of embryos developed after injecting eggs with sperm nuclear DNA and construct DNA. Non-transgenic embryos that did not express GFP and did not integrate the linearized construct into genomic DNA; the construct then became degraded. Embryos that expressed GFP mosaically in a few muscle fibers were not considered as true transgenic frogs because the construct integration occurred in some cells after embryonic differentiation already took place. Finally, embryos that expressed GFP homogeneously in muscle tissues were considered as true transgenic animals that had integrated construct DNA into the genome before embryogenesis began. Non-transgenic embryos were used as positive controls for the injection. Other controls used were embryos injected with sperm nuclear DNA only or wild type embryos created by *in vitro* fertilization. Juvenile frogs that were identified initially as true transgenic animals by fluorescence had integrated the construct DNA into the genome as shown by PCR on muscle tissue. This indicates that genomic transgene integration was successful in the process of generating transgenic frogs.

To assess whether transgenic juvenile frogs had GHRH/PACAP transcript expression originating from the integrated construct in addition to endogenous gene expression, mRNA was extracted from liver and PCR was used to detect ectopic

GHRH/PACAP expression. Because wild type *X. laevis* does not express GHRH/PACAP in liver tissue (13), the presence of a product from a transgenic liver would indicate that the source of the transcript was the integrated transgene. A product of expected size and sequence for both transgenic types of frogs was present. However, as GHRH/PACAP cDNA was used in the vector construct and was integrated into the transgenic frog genome, genomic contamination of RNA extracted from liver would also result in the exact same product and sequence. To rule out this possibility, primers designed to the CMV promotor were used to check for presence of genomic DNA in the sample as transcribed mRNA would not contain the CMV promotor. Indeed, contamination was present in the liver sample from a transgenic frog overexpressing shortGP. It is likely that this sample contained more genomic DNA than the sample used from a transgenic frog overexpressing longGP and the DNase concentration was not sufficient. Also, only a few contaminating molecules are necessary to amplify a PCR product. Even though only one sample shows that GHRH/PACAP is expressed from the integrated transgene ectopically, it can be used as sufficient evidence that generation of transgenic frogs was successful because the transgene was transcribed.

Because GHRH/PACAP expression is driven by CMV, a constitutive and ubiquitous promoter, the GHRH/PACAP transcript was expected to be present and elevated in most tissues of transgenic frogs. Therefore, quantitative PCR could be used to examine whether GHRH/PACAP mRNA levels were elevated in brain tissue of transgenic frogs compared to wild types.

The expression and immunoreactivity of both PACAP and its receptors is predominant in the CNS in frogs implicating PACAP's role as a neurotransmitter,

neuromodulator and/or neurotrophic factor in amphibians as in mammals. For example, *in vivo* administration of PACAP to the cerebellar cortex of postnatal rats resulted in a transient enlargement of the cerebellum due to increased proliferation of granule cells (19). In *Xenopus*, PACAP is also thought to be involved in brain and neural tube development because PACAP and PAC_1-R gene expression patterns resemble those of mammals (14). However, PACAP's role in amphibian development has to be clarified further. Otto *et al.* examined PACAP's actions during early *Xenopus* development by injecting either PACAP-27 or PACAP-38 peptide into the early embryo. Both PACAP-38 and GHRH mRNA were shown to induce neuralization when injected into embryonic blastocoel but only PACAP-38 injection resulted in dorso-anteriorized embryos, clearly impeding embryonic development (12). Based on these findings, tadpole morphology and histology was examined in the present study to determine whether PACAP overexpression had an effect on *Xenopus* embryogenesis. Even though some anteriorized transgenic embryos were identified, such phenotype was also noted among non-transgenic embryos that were generated by injection. This phenotype was more frequent initially when the first few batches of embryos were generated and can be explained mainly by poor sperm quality (personal experience) or by poor egg quality used for injection (7). Also, developmental defects were more frequent in injected embryos independent of whether frogs were transgenic or not when compared to *in vitro* fertilized embryos. The defects are thought to arise from egg damage during the injection process such as leakage of egg yolk (personal experience). Once frogs survived past metamorphosis, which was normal in transgenic and wild type frogs, no defects in external body structure were noted. Furthermore, autopsy did not reveal any difference

in organ appearance between transgenic and wild type frogs. In summary, these results indicate that excessive PACAP does not seem to affect metamorphosis or organ development in frogs. However, because frogs were made in batches and quality of eggs and/or sperm vary between groups, it is hard to maintain a homogenous population of frogs. In addition, the number of transgene copies that integrate into the genome vary between embryos (7) resulting in a potential gradient of a particular phenotype which could not be distinguishable in the first generation of transgenic frogs. Ideally, the first generation transgenic frogs should be grown to adulthood, mated, and second generation transgenic offspring should be examined for phenotype because the population of offspring would be homogenous (9). This was not attainable during the present study as *X. laevis* take between one to two years to sexually mature. Also, in the present study, transgenic frogs had a high mortality rate and all died before reaching sexual maturity.

Histology did not reveal any major differences in brain shape or cell organization when comparing transgenic or wild type frogs indicating that excess PACAP does not affect neural development in an anatomical sense. Other organs or tissues that were examined also appeared normal in transgenic tadpoles. The only differences noted in the transgenic tadpole overexpressing shortGP was an abnormal structure most closely resembling the glottis and a liver that contained brown granules. PACAP has been implicated in mammalian respiration (20, 21). Therefore, it is possible that PACAP could affect the development of the glottis, a structure used for respiration in amphibians (22). However, the other type of transgenic frog did not have an abnormal glottis, so this is most likely a defect in the individual tadpole. To gain further insight into PACAP's effect on respiration in amphibians, more than one tadpole would have to be sectioned

and analyzed for histology. Brown granules identified in transgenic frog liver are most likely pigmented melanomacrophage centers since pigmentation in lower vertebrates is commonly found in other tissues than the skin (23). Because only one of the transgenic frogs had this pigmentation, it does not seem to be related to hormone overexpression. It is possible that the tadpoles with no pigments in their liver originated from albino frogs since both albino and pigmented parents were used to generate the tadpoles examined in this study.

Even though transgenic frogs did not appear different after external examination when compared to wild type frogs, it was of interest to examine their body size in order to determine whether excess GHRH or PACAP affected growth. Both GHRH and PACAP belong to the glucagon superfamily of hormones and have originated from the same precursor gene existing in all vertebrates except for mammals where the peptides are located on separate genes. Both hormones exhibit hypophysiotropic properties and mediate biological effects through 7-transmembrane domain receptors (1). In mammals, GHRH stimulates the secretion of growth hormone (GH). However, controversial results exist with regards to PACAP and its effect on GH secretion. Findings indicate that in amphibians both GHRH and PACAP are involved in the neuroendocrine control of somatotropes because they are stimulatory factors of GH. Frog PACAP has been shown to stimulate GH release in two frog species, *R. ridibunda* and *Rana catesbeiana* and human GHRH has been shown to stimulate GH release from bullfrog pituitary cells (24). The function of growth hormone in mammals is mainly responsible for body growth and metabolic processes. GH function in amphibians has been examined using transgenic frogs overexpressing the hormone. Transgenic tadpoles took the same amount of time to

reach metamorphosis but were larger than control tadpoles. Also, transgenic frogs developed skeletal defects resembling acromegaly and died sooner than controls. Because phenotypic features of elevated GH in frog resemble those of mammals, the investigators have concluded that the function of GH in amphibians is to regulate growth (10). Since PACAP and GHRH are upstream positive effectors of GH, it seems plausible that transgenic frogs in the present study would be larger than wild type frogs. In contrast, transgenic frogs were significantly smaller when compared to non-transgenic or wild type frogs. Even though the method of generating wild type frogs and transgenic frogs differed (*in vitro* fertilization versus egg injection), this did not seem to have any effect on frog growth and can be ruled out. This assumption is based on the fact that non-transgenic frogs were also generated by injection and there was no significant difference between wild type frogs and non-transgenic frogs. Findings from the present study suggest that excess PACAP and/or GHRH resulted in decreased frog growth. It is possible that high hormone levels induced PACAP or GHRH receptor down-regulation resulting in a different phenotype than expected. It is also likely that the construct could have integrated into a susceptible area of the genome involved in growth as discussed below. Due to low sample sizes, this study should be repeated and second generation transgenic frogs should be used.

Mouse knockout studies have shown that PACAP is critical for survival in mammals since null pups die at an early postnatal age (2). Therefore, I expected that excess PACAP would not be lethal to frogs. The present study indicates that the method used to generate frogs has an effect on survival rate. Wild type frogs that were generated through *in vitro* fertilization had the highest survival rate and all embryos generated by

injection independent of whether they were transgenic or not, had lower survival rates. However, both types of transgenic frogs either overexpressing PACAP alone or PACAP and GHRH, had the lowest survival rates when compared to either wild type, sperm injected or non-transgenic frogs. Clearly, excess PACAP and GHRH resulted in very low survival rates by the end of a four-month period. To determine when the mortality of frogs was the highest, frog deaths were also examined over a period of 45 days starting at one week after fertilization. It seems that the majority of frog types died in these first 45 days of development. Both types of transgenic frogs exhibited the sharpest drop in numbers when compared to non-transgenic or wild type frogs. Once frogs made it past metamorphosis, the death rate was not as dramatic. Once again, it is possible that excess PACAP in transgenic frogs resulted in receptor down-regulation or that a gene function was lost as a result of random construct integration as discussed below.

In summary, based on previous studies, I hypothesized that transgenic frogs overexpressing PACAP and/or GHRH would develop abnormally, would grow larger in size and would have normal survival rates and metamorphosis. The findings from this study imply that PACAP and/or GHRH at higher than normal physiological levels may be involved in developmental processes to some extent. Results from the present experiment indicate that transgenic frogs that survive seem to develop normally, undergo metamorphosis as usual, and have normal organs as examined externally and using histology. Results have also revealed that transgenic frogs were smaller in size when compared to wild type frogs and had lower survival rates. This was unexpected since both GHRH and PACAP are positive upstream regulators of growth promoting GH. This phenomenon can be explained in several ways.

First, when levels of hormones are too elevated for extended periods of time, receptors can become down-regulated. To maintain homeostasis, hormone receptors are down-regulated by desensitization to the hormone or by concentration decrease as a result of internalization. Such down-regulation may result in different physiological effects than what is normally expected. In addition, in wild type animals short-term pulsatile secretion of both peripheral and pituitary hormones exists. The pulses of hormone can range from a few minutes to a few hours. It is thought that discontinuous hormone secretion allows for sufficient time for receptor synthesis (25). In the present study, transgenic frogs are expected to constantly express both PACAP and GHRH as opposed to oscillating release. Also, feedback on the GHRH/PACAP construct is not possible as only a CMV promoter is present. The endogenous frog PACAP promoter has not been used in the construct due to difficulty of its isolation and size limitation of the construct. As a result, it is very likely that elevated hormone levels have promoted a dramatic reduction in receptor number or sensitivity.

Second, the reduced growth and especially increased mortality could have resulted from random construct integration and disruption of vital genes even though it would be expected that such an event would cause death at very early stages of development (most likely by the first week). It has been well established that the construct integration process is random and can affect expression of nearby genes endogenous to the animal or cause a complete loss of function of a gene (26, 27). Because *X. Laevis* is tetraploid and has extra copies of many genes, a DNA disruption event might be less harmful to the frog when compared to a diploid organism (28). To exclude this possibility, an attempt was made to generate transgenic frogs that

overexpress GFP only. However, due to time limitation, such frogs were not generated. This would be a control for random insertional mutation of genes within the chromosome and for potential toxicity of GFP.

Third, PACAP causes a wide variety of effects by increasing intracellular cAMP or Ca^{2+} levels. At a very early stage of development, an organism is sensitive to abnormal concentrations of ubiquitous molecules including secondary messengers. Excessive exposure to cAMP or Ca^{2+} during embryogenesis may have resulted in death or abnormal development and in the long run in retarded growth.

Xenopus laevis offers several advantages for studying development and cellular biology and is a potential animal model to study hormone function. The present study has revealed some interesting insights into PACAP's function in amphibians *in vivo*. These findings can become more valuable by improving some aspects of experimental design and avoiding problems that were encountered. In future studies, second generation transgenic offspring should be used for analysis if first generation transgenic frogs survive until sexual maturity to establish a homogeneous population of the same phenotypic characteristics and to increase the sample size (9). In addition, a loss-of-function experiment would complement the overexpression study to enhance the understanding of PACAP's function. This experiment could be performed by overexpressing antisense cDNA to PACAP mRNA in transgenic frogs to eliminate translation of PACAP mRNA. Finally, it might be more meaningful to use the diploid *X. tropicalis* in place of tetraploid *X. laevis* since increased complexity of the genome makes interpretation of gene function studies difficult (29).

REFERENCES

1. **Sherwood NM, Krueckl SL, McRory JE** 2000 The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocrine Reviews 21:619-670

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

3. **Gray SL, Yamaguchi N, Vencova P, Sherwood NM** 2002 Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 143:3946-3954

4. **Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, Eiden LE** 2002 Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. Proceedings of the National Academy of Sciences of the United States of America 99:461-466

5. **Nutt SL, Bronchain OJ, Hartley KO, Amaya E** 2001 Comparison of morpholino based translational inhibition during the development of Xenopus laevis and Xenopus tropicalis. Genesis 30:110-113

6. **Zhou Y, Ching YP, Kok KH, Kung HF, Jin DY** 2002 Post-transcriptional suppression of gene expression in Xenopus embryos by small interfering RNA. Nucleic Acids Research 30:1664-1669

7. **Kroll KL, Amaya E** 1996 Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173-3183

8. **Fu L, Buchholz D, Shi YB** 2002 Novel double promoter approach for identification of transgenic animals: A tool for in vivo analysis of gene function and development of gene-based therapies. Molecular Reproduction and Development 62:470-476

9. **Marsh-Armstrong N, Huang H, Berry DL, Brown DD** 1999 Germ-line transmission of transgenes in Xenopus laevis. Proceedings of the National Academy of Sciences of the United States of America 96:14389-14393

10. **Huang H, Brown DD** 2000 Overexpression of Xenopus laevis growth hormone stimulates growth of tadpoles and frogs. Proceedings of the National Academy of Sciences of the United States of America 97:190-194

11. **Sherwood NM, Gray SL, Cummings KJ** 2003 Consequences of PACAP Gene Knockout. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 347-360

12. **Otto C, Schutz G, Niehrs C, Glinka A** 2000 Dissecting GHRH- and pituitary adenylate cyclase activating polypeptide-mediated signalling in Xenopus. Mechanisms of Development 94:111-116

13. **Hu Z, Lelievre V, Tam J, Cheng JW, Fuenzalida G, Zhou X, Waschek JA** 2000 Molecular cloning of growth hormone-releasing hormone/pituitary adenylyl cyclase-activating polypeptide in the frog Xenopus laevis: brain distribution and regulation after castration. Endocrinology 141:3366-3376

14. **Hu Z, Lelievre V, Rodriguez WI, Tam J, Cheng JW, Cohen-Cory S, Waschek JA** 2001 Embryonic expression of pituitary adenylyl cyclase-activating polypeptide and its selective type I receptor gene in the frog Xenopus laevis neural tube. Journal of Comparative Neurology 441:266-275

15. **Alexandre D, Vaudry H, Jegou S, Anouar Y** 2000 Structure and distribution of the mRNAs encoding pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in the frog, Rana ridibunda. Journal of Comparative Neurology 421:234-246

16. **Nieuwkoop PDaF, J.** 1994 Normal Table of Xenopus laevis (Daudin). © Garland, New York, Garland.

17. **Parker DB, Coe IR, Dixon GH, Sherwood NM** 1993 Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. European Journal of Biochemistry 215:439-448

18. **Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM** 1997 Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology 138:414-423

19. **Vaudry D, Gonzalez BJ, Basille M, Fournier A, Vaudry H** 1999 Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development. Proceedings of the National Academy of Sciences of the United States of America 96:9415-9420

20. **Cummings KJ, Pendlebury JD, Sherwood NM, Wilson RJ** 2004 Sudden neonatal death in PACAP-deficient mice is associated with reduced respiratory chemoresponse and susceptibility to apnoea. The Journal of Physiology 555:15-26

21. **Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H** 2000 Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacological Reviews 52:269-324

22. **Jorgensen CB** 2000 Amphibian respiration and olfaction and their relationships: from Robert Townson (1794) to the present. Biological Reviews of the Cambridge Philosophical Society 75:297-345

23. **Zuasti A, Jimenez-Cervantes C, Garcia-Borron JC, Ferrer C** 1998 The melanogenic system of Xenopus laevis. Archives of Histology and Cytology 61:305-316

24. **Montero M, Yon L, Kikuyama S, Dufour S, Vaudry H** 2000 Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. Journal of Molecular Endocrinology 25:157-168

25. **Beaulieu E-E, Kelly AK** 1990 Hormones from Molecules to Disease. Hermann publishers, New York p.38 and pp 85-87

26. **Si-Hoe SL, Wells S, Murphy D** 2001 Production of transgenic rodents by the microinjection of cloned DNA into fertilized one-cell eggs. Molecular Biotechnology 17:151-182

27. **Hofker MH, Breuer M** 1998 Generation of transgenic mice. Methods in Molecular Biology 110:63-78

28. **Yon L, Alexandre D, Montero M, Chartrel N, Jeandel L, Vallarino M, Conlon JM, Kikuyama S, Fournier A, Gracia-Navarro F, et al.** 2001 Pituitary adenylate cyclase-activating polypeptide and its receptors in amphibians. Microscopy Research and Technique 54:137-157

29. **Hirsch N, Zimmerman LB, Grainger RM** 2002 Xenopus, the next generation: X. Tropicalis genetics and genomics. Developmental Dynamics 225:422-433

APPENDIX 1

10 X Marc's Modified Ringers (pH to 7.5)

1 M NaCl
20 mM KCl
10 mM $MgCl_2$
20 mM $CaCl_2$
50 mM HEPES

Sterilised by autoclaving and stored at room temperature.

2 X Nuclear Preparation Buffer (pH to 7.7)

1.5 M Sucrose
1 M HEPES
500 mM EDTA
100 mM DTT
4 mM spermine/ 10 mM spermidine mix

Stored at 4^0C.

Sperm Storage Buffer

1 X Nuclear Preparation Buffer
30% glycerol
0.3% BSA

Stored at 4^0C.

CHAPTER 3

Carbohydrate metabolism in response to PACAP infusion in mice (*Mus musculus*)

A version of this chapter will be submitted following the completion of the experiments. Petra Venc(ova) Drncova, Greg Gillespie, Kaaren Gibb, Emma Isaac and Nancy Sherwood. Carbohydrate metabolism in mice given chronic PACAP by micro-osmotic pump.

INTRODUCTION

Several lines of evidence point to PACAP as a regulator of the carbohydrate metabolism. First, PACAP and its receptors have been located in the pancreas (1). Second, PACAP has been shown to release insulin and glucagon (2, 3) from the pancreas and catecholamines from the adrenals (4). Third, transgenic mice lacking the PACAP specific receptor or PACAP itself exhibit carbohydrate dysfunction. Gray *et al.* have demonstrated that fasted PACAP null pups exhibit hyperinsulinemia and hypoglycemia at 5 days after birth (5). In addition, Jamen *et al.* found that PAC_1 receptor knockout mice have an impaired insulinotropic response to an oral glucose challenge (6). Because PACAP and its receptors are ubiquitously distributed in the central nervous system and peripheral organs, the phenotype of null mice may be too complex to interpret.

Another approach to study PACAP's actions *in vivo* is the use of receptor antagonism. PAC_1-R antagonists dramatically reduce insulin release from pancreas indicating that PACAP enhances the insulin response during feeding (7). An opposite effect was studied by PACAP administration at concentrations exceeding physiological levels. PACAP administration has been performed either by injection at one time point (8), at multiple time points (9) or by infusion over a longer period lasting up to 75 min (3). These studies provide insight into PACAP's effects on cAMP, insulin and glucagon concentration. Finally, one study has generated a transgenic mouse line that overexpresses PACAP in pancreatic β-cells allowing a more prolonged local exposure of mice to the peptide. Although PACAP was not detected in the plasma of transgenic mice, elevated plasma insulin levels after glucose loading were observed when compared

to controls (10). Clearly, elevated PACAP in the pancreas seems to perturb carbohydrate metabolism in mammals.

In the present study, the role of PACAP has been examined *in vivo* by the administration of the hormone through a micro-osmotic pump. Osmotic pumps are advantageous because they continuously deliver the peptide over long periods of time at a controlled rate, reducing the amount of animal handling and eliminating problems with frequent injections. The goal of this study was to mimic hormone overexpression and to gain insight on PACAP's role in carbohydrate metabolism when normal mice are treated with a higher than normal neuropeptide concentration over a 12 day period.

METHODS AND MATERIALS

Pump surgery

Weaned wild type C57/Bl6 mice (Charles River) of at least 10g were used for pump surgery. Mice were kept at 24^0C - 25^0C on a 12 hour light/dark cycle. Single mice were fed rodent Lab Diet® 5001 (PMI® Nutrition International Inc., Brentwood, MO) and breeding pairs were fed high fat rodent Lab Diet® 5015. Alzet micro-osmotic pumps, model 1002 (Cupertino, CA), were filled with 100µl synthetic human/rat/mouse PACAP-27 obtained from Jean Rivier (Salk Institute, La Jolla, CA) in sterile Dulbecco's phosphate buffered saline (PBS) (Invitrogen Canada Inc., Burlington, ON) at a concentration of 1µg peptide/µl. Mice received a dose of 70 pmol of PACAP per hour over a twelve day period because the pump model used delivered 0.22µl/hour for 14 days at a constant rate. Control mice received plastic sham pumps of approximately the same dimensions and mass as the real micro-osmotic pumps. PACAP-27 solution was stored at -80^0C until the day of the surgery. Pump surgeries and other procedures in this experiment were approved by the Animal Care Committee, University of Victoria. Mice were anesthetized by isoflurane gas and a small incision was made between the scapulae. Skin was loosened with a hemostat, the pump was implanted subcutaneously in the interscapular region and the skin opening was sealed with Vetbond™ glue (3M Animal Care Products, St. Paul, MN). Mice were allowed to recover under a heat lamp beside a 50ml falcon tube filled with warm tap water.

Glucose measurement and body mass

Blood glucose was measured prior to surgery on day 0 and after surgery on days 6 and 12 prior to anaesthesia. Glucose data was collected for mice fed *ad libidum* and for mice that were fasted for approximately 12 hours. Mice were restrained and the saphenous vein was punctured with a needle to obtain a drop of blood. Glucose levels were measured using the Elite® Glucometer with blood glucose test strips (Bayer Inc., ON). Body mass was measured on day 0 prior to surgery and on day 12 after the surgery.

Serum and plasma collection

Mice were euthanized on day 12 after pump surgery by isoflurane anaesthesia followed by blood collection by cardiac puncture and cervical dislocation. Blood was collected using a chilled syringe and was treated appropriately to obtain serum or plasma. To collect serum, blood was allowed to stand at room temperature for half an hour and was spun at 1,600 x g using an IEC Centra-R7 refrigerated centrifuge (Damon) for 15 min at 0^0C. To obtain plasma, 1mg/ml EDTA and 500 KIU/ml Aprotinin were added to blood that was kept on ice until it was spun at 1,600 x g for 15 min at 0^0C. Serum and plasma were collected, quick frozen on dry ice and stored at -80^0C until further use.

PACAP and insulin radioimmunoassays

Plasma PACAP-27 levels were collected 12 days following surgery, then measured with a competitive binding peptide radioimmunoassay (Peninsula Laboratories Inc., San Carlos, CA). The PACAP (1-27) amide (Human, Ovine, Rat) assay has a typical sensitivity of 6pg PACAP/tube; the standards ranged from 1pg to 128pg

PACAP/tube. The trace was ^{125}I-labeled PACAP27 and was detected by rabbit antiserum specific for the peptide. Samples of 100μl in duplicate were set up for control and PACAP treated mice. Serum insulin levels were measured in fasted and fed mice on day 12 following surgery using a radioimmunoassay (Sensitive Rat Insulin RIA Kit, Linco Research Inc., St. Charles, MO). The Sensitive Rat Insulin assay utilized ^{125}I-labeled insulin and rat insulin antiserum to determine insulin levels in plasma. The ability of detection ranged from 0.02ng insulin/ml to 1.0ng insulin/ml. In this experiment samples of 50μl in duplicate were used for control and PACAP treated mice for both fed and fasted states.

Histology

Heart, liver and skeletal muscle were dissected from PACAP treated and control mice on day 12 after pump surgery. Tissue was fixed in 4% paraformaldehyde in 1 X phosphate buffered saline. Samples were rinsed twice with distilled H_2O and were dehydrated for 20 min in 1ml volume of ethanol of the following concentrations: 30%, 50%, 70% twice, 95% and 100% three times. The tissues, cut to approximately 2mm^3, were embedded in Technovit hydroxyethyl methacrylate embedding medium (Heraeus Kulzer, Germany) and were sectioned serially at 7 μm with glass knives on a Sorvall JB-4 microtome. Sections were dried under a heat lamp for 10 min and were stained with Gill's No. 3 hematoxylin (Sigma Diagnostic, St. Louis, MO) and Eosin Y. An immersion in hematoxylin of 30 min to 1 hour depending on tissue was followed by a wash in tap water for 10 min. Sections were immersed in Scott's tap water for 2 min and were rinsed in distilled water. Eosin Y was used as a counterstain for 2-10 min depending on tissue

type. Sections were dehydrated with an ethanol series of 70%, 95%, and 100% for 5 min each. Tissue was immersed in 100% xylene for 5 min followed by mounting in Entellen medium (BDH, Darmstadt, Germany). The tissues were photographed with a Nikon digital E990 camera (Nikon Corp., Japan) with a Universal microscope (Zeiss, Germany) at a magnification of 16X or 40X.

Interperitoneal PACAP challenge to test receptor sensitivity

Mice that were 27 days old were surgically operated on to insert either a control pump or a PACAP-27 filled pump as previously. At 12 days post surgery, these mice were injected intraperitoneally with 100pmol PACAP-27 in Dulbecco's PBS. In addition, mice of the same age (39 days) with no pumps were injected with 100µl sterile Dulbecco's PBS. Blood was collected via cardiac puncture before the injection or at 5, 10, 20, 30 or 50 minutes after the injection. Each mouse was used for only one time point as the total volume of blood collected was only sufficient for one insulin assay in duplicate. Glucose concentration was measured from collected blood, whereas serum was collected and stored for the insulin radioimmunoassay.

RESULTS

Survival rate and body mass

Both control mice (n=34) and mice treated with PACAP-27 (n=35) for a period of twelve days had 100% survival rate for the duration of the study. Body mass of control and PACAP treated mice was recorded on the day of the surgery (day 0) and 12 days after the surgery (day 12). No significant difference in mass was observed for the period of PACAP-27 infusion when comparing control and PACAP treated mice (Figure 3.1). Significance ($P<0.05$) was determined using the unpaired t test for both days 0 and 12. Welch correction was also performed on day 12.

Plasma PACAP concentration

Blood plasma PACAP-27 concentration was measured in control (n=11) and PACAP treated (n=11) mice using a radioimmunoassay. PACAP-27 concentration for all samples except one did not fall within the standard curve and was lower than 1.0pg/100μl of plasma. Only one sample corresponding to a PACAP treated mouse had a plasma reading of 12.9pg/100μl of PACAP.

Histology

Liver, muscle, and heart tissue samples from control and PACAP treated mice were analyzed for histology using Hematoxylin and Eosin staining. No observable difference was noted in cell morphology, size or organization in these tissues (Figure 3.2). Oil Red O staining (WaxIt, Vancouver, BC) for lipids of liver tissue from control and PACAP treated mice did not reveal any difference.

Figure 3.1 Body mass of control (Con) and PACAP treated (Pac) mice at the day of pump surgery (day 0) and 12 days after the surgery (day 12). PACAP treated mice received 70 pmol/hr PACAP-27 over a 12 day period. Error bars represent SEM; n represents sample size. Day 0, $P = 0.9529$ and day 12, $P = 0.2027$, indicating no significant difference ($P<0.05$) (unpaired t test).

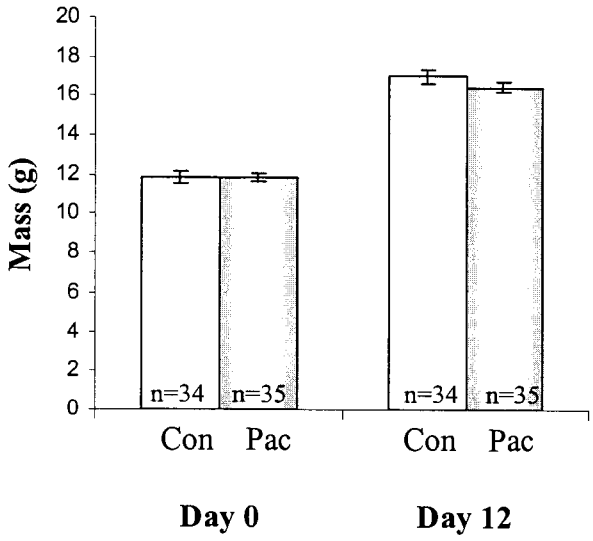
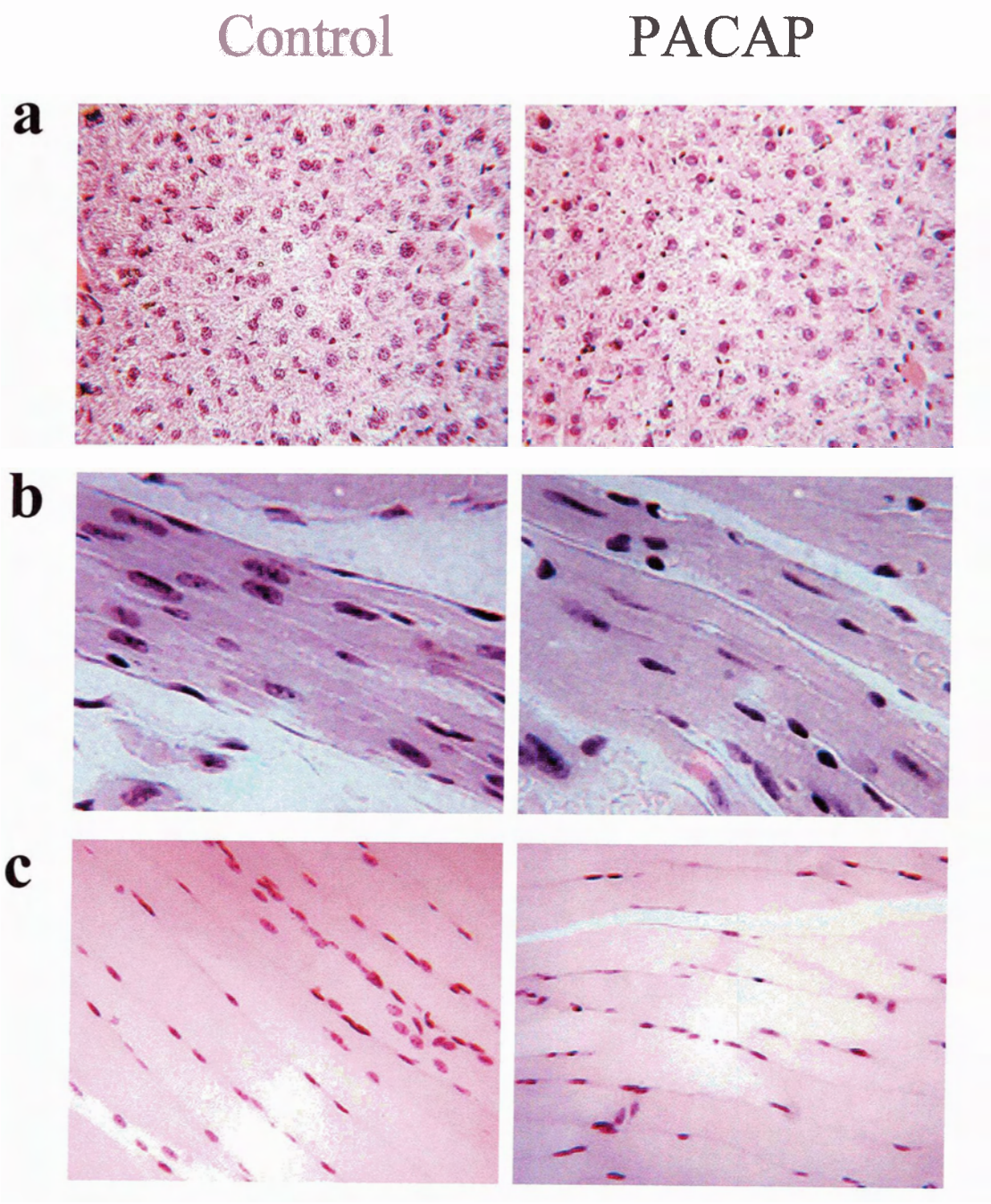

20
18
16
14
12
10
8
6
4
2
0
Mass (g)
n=34
n=35
n=34
n=35
Con
Pac
Con
Pac
Day 0
Day 12

Figure 3.2. Liver (a), heart (b), and skeletal muscle (c) tissue histology of control and PACAP treated mice. PACAP treated mice received 70 pmol/hr PACAP-27 over a 12 day period. Tissues were stained with Hematoxylin and Eosin.


Control
PACAP
a
b
c

Glucose and insulin serum levels

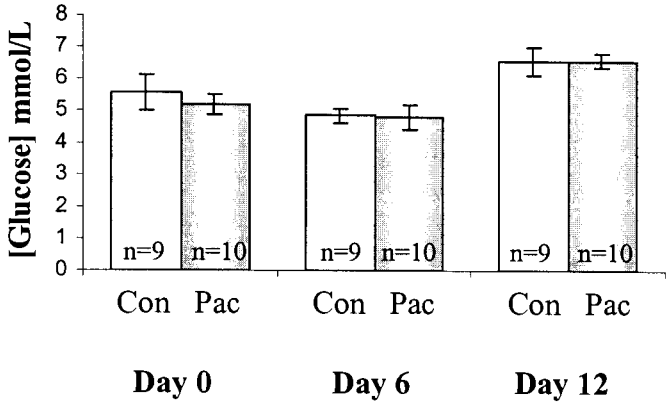
Blood glucose concentration was measured in control and PACAP treated mice at days 0, 6, and 12 after surgery. There was no significant difference in glucose readings when comparing control (n=9) and PACAP treated mice (n=10) that were fasted for 12 hours (Figure 3.3a). Similarly, when glucose readings were compared between control (n=10) and PACAP treated mice (n=10) that were fed *ad libidum*, no significant difference was observed (Figure 3.3b). Insulin serum levels were compared in control (n=9) and PACAP treated mice (n=10) that were fasted for 12 hours using radioimmunoassay; no significant difference was observed (Figure 3.4). In addition, after comparing insulin serum levels in control (n=10) and PACAP treated mice (n=10) that were fed *ad libidum*, no significant difference was found (Figure 3.4). Significance ($P<0.05$) was determined using the unpaired t test for glucose readings at days 0, 6, and 12 after surgery during fed or fasted states. Unpaired t test with a Welch correction was performed for serum insulin concentrations at day 12 after surgery during a fed or fasted state.

Interperitoneal PACAP challenge

Mice that did not have surgery were injected with PBS and glucose readings were recorded for separate groups of mice at times 0 min (n=11), 5 min (n=10), 10 min (n=10), 20 min (n=5), 30 min (n=6), and 50 min (n=5). Blood glucose concentration peaked at 5 min with a mean of 12.44 mmol/L and dropped back down to baseline by 30 min (Figure 3.5a). Mice with control pumps were injected with PACAP and glucose readings were recorded in separate groups of mice at times 0 min (n=11), 5 min (n=12), 10 min (n=12),

Figure 3.3. Blood glucose levels of control (Con) and PACAP treated (Pac) mice at the day of pump surgery, day 0, and 6 or 12 days after the surgery, day 12. PACAP treated mice received 70 pmol/hr PACAP-27 over a 12 day period and were either fasted for 12 hours **(a)** or fed *ad libidum* **(b)**. Error bars represent SEM; n represents sample size. Fasted mice **(a)** day 0, $P = 0.2797$; day 6 $P = 0.7106$; day 12, $P = 0.6174$, indicating no significant difference (unpaired t test). Fed mice **(b)** day 0, $P = 0.5850$; day 6, $P = 0.8979$; day 12, $P = 0.9783$, indicating no significant difference ($P<0.05$) (unpaired t test).

a Fasted



b Fed

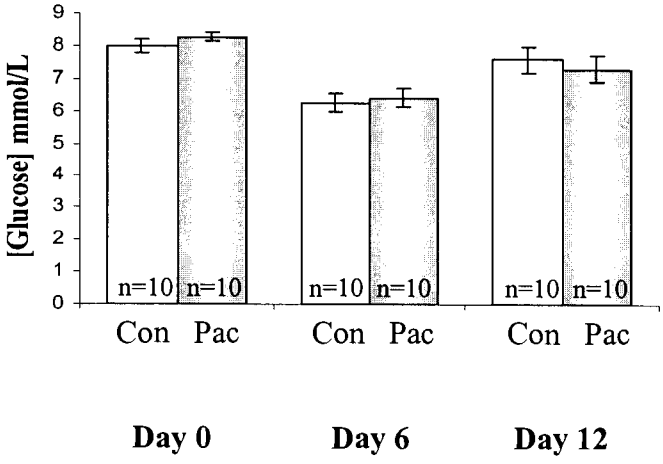


Figure 3.4. Serum insulin levels of control (Con) and PACAP treated (Pac) mice 12 days after pump surgery. PACAP treated mice received 70pmol/hr PACAP-27 over a 12 day period and were either fasted for 12 hours or fed *ad libidum*. Error bars represent SEM; n represents sample size. Fed mice $P = 0.1322$, indicating no significant difference. Fasted mice $P = 0.1879$, indicating no significant difference ($P<0.05$) (unpaired t test).

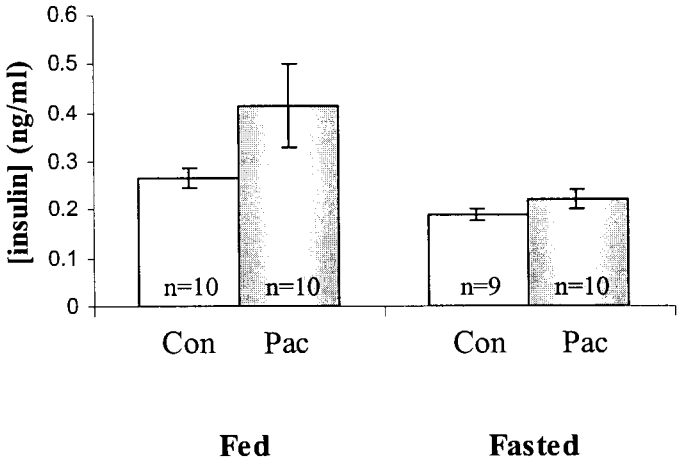
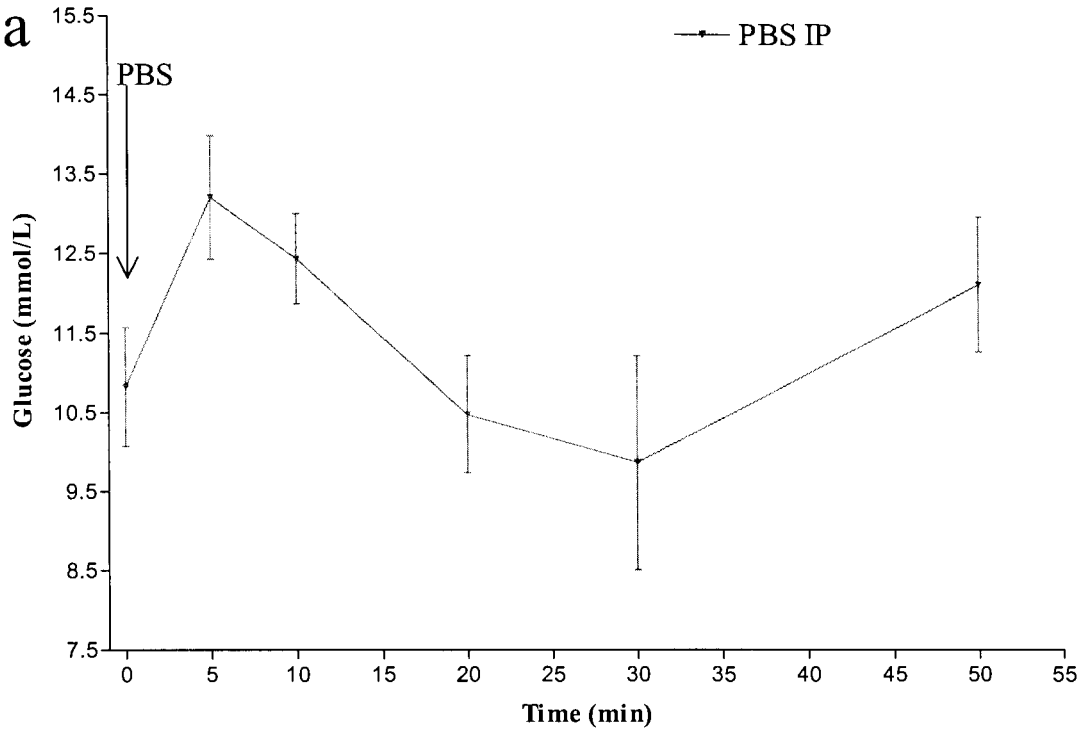
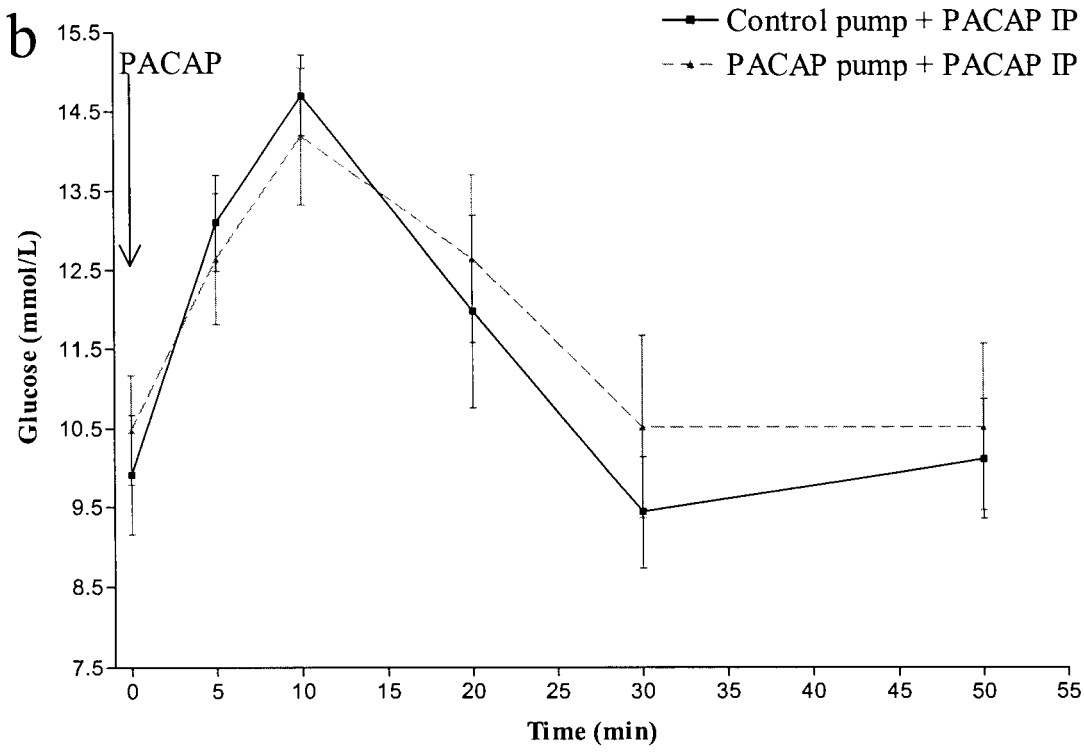

[insulin] (ng/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
n=10
n=10
n=9
n=10
Con
Pac
Con
Pac
Fed
Fasted

Figure 3.5. Blood glucose concentration of mice that received an intraperitoneal injection of saline (PBS IP) without surgery **(a)** or mice that received a control pump for a period of 12 days and an intraperitoneal PACAP-27 injection (PACAP IP) on day 12 and mice that received a PACAP filled pump (PACAP pump) for a period of 12 days and an intraperitoneal PACAP-27 injection on day 12 **(b)**. Glucose levels were measured in separate groups of mice at 0 min (pre-injection) or at 5, 10, 20, 30, and 50 min following the injection or at 0 min where no injection was given. Means ± SEM are shown.


a
PBS IP
PBS
Glucose (mmol/L)
15.5
14.5
13.5
12.5
11.5
10.5
9.5
8.5
7.5
0
5
10
15
20
25
30
35
40
45
50
55
Time (min)

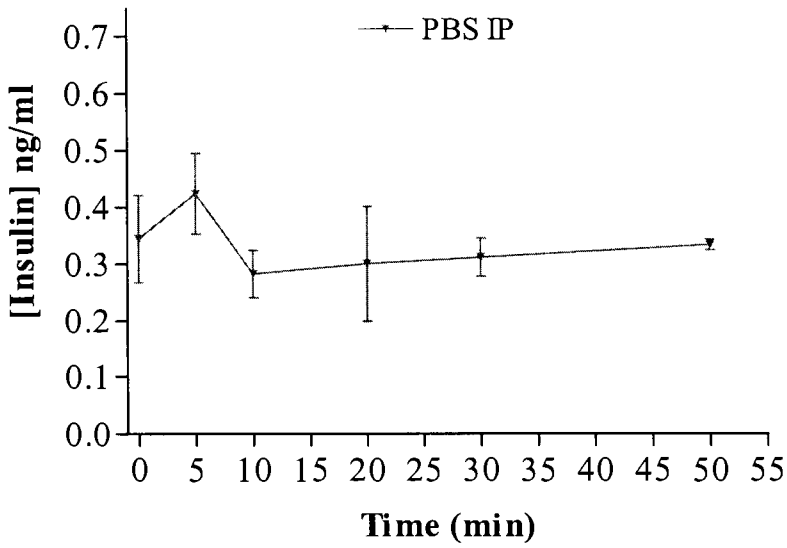

b
Control pump + PACAP IP
PACAP pump + PACAP IP
PACAP
Glucose (mmol/L)
15.5
14.5
13.5
12.5
11.5
10.5
9.5
8.5
7.5
0
5
10
15
20
25
30
35
40
45
50
55
Time (min)

20 min (n=12), 30 min (n=11), and 50 min (n=12). Blood glucose concentration peaked at 10 min with a mean of 14.71 mmol/L and returned to normal by 30 min (Figure 3.5b). Mice treated with PACAP filled micro-osmotic pumps for 12 days were injected with PACAP and glucose readings were recorded in separate groups of mice at times 0 min (n=9), 5 min (n=10), 10 min (n=11), 20 min (n=11), 30 min (n=9), and 50 min (n=10). Blood glucose concentration peaked at 10 min with a mean of 14.19 mmol/L and returned to basal level by 30 min (Figure 3.5b). Both groups of mice that had surgery and were injected with PACAP had a very similar transient glucose response to the injection. There was no overall significant difference in glucose concentration when comparing treatment and time interaction in mice that received pumps and PACAP injection, $P=0.9295$. Significance ($P<0.05$) was determined using two-way ANOVA.

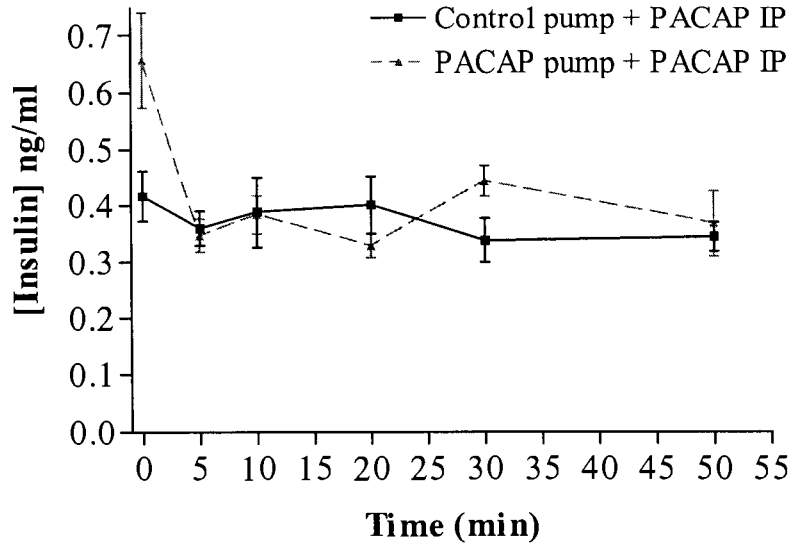
Mice were injected with PBS followed by measurement of insulin concentration in separate groups of mice at times 0 min (n=10), 5 min (n=10), 10 min (n=10), 20 min (n=5), 30 min (n=6), and 50 min (n=5) (Figure 3.6a). Mice with control pumps were injected with PACAP, then blood was collected for insulin concentration in separate groups of mice at times 0 min (n=11), 5 min (n=15), 10 min (n=12), 20 min (n=11), 30 min (n=12), and 50 min (n=12) (Figure 3.6b). Mice treated with PACAP for 12 days were injected with PACAP and blood was collected for insulin concentration in separate groups of mice at times 0 min (n=9), 5 min (n=13), 10 min (n=11), 20 min (n=11), 30 min (n=9), and 50 min (n=10) (Figure 3.6b). Values for duplicates that differed by more than 10% coefficient of variance were not used in data analysis. There was an overall significance in treatment and time interaction in mice that received pumps and PACAP

Figure 3.6. Serum insulin concentration of mice that received an intraperitoneal injection of saline (PBS IP) without surgery **(a)** or mice that received a control pump for a period of 12 days and an intraperitoneal PACAP-27 injection (PACAP IP) on day 12 and mice that received a PACAP filled pump (PACAP pump) for a period of 12 days and an intraperitoneal PACAP-27 injection on day 12 **(b)**. Insulin levels were measured in separate groups of mice at 0 min (pre-injection) or at 5, 10, 20, 30, and 50 min following the injection or at 0 min where no injection was given. Means ± SEM are shown.

a


PBS IP
[Insulin] ng/ml
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0 5 10 15 20 25 30 35 40 45 50 55
Time (min)

b


Control pump + PACAP IP
PACAP pump + PACAP IP
[Insulin] ng/ml
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0 5 10 15 20 25 30 35 40 45 50 55
Time (min)

injection $P<0.05$ when including all data. However, when the zero time point values were not included in the analysis and only values after the injection were compared, time and treatment interaction was not significantly different $P=0.2890$. Significance ($P<0.05$) was determined using two-way ANOVA.

DISCUSSION

In this study, long-term effects of mammalian PACAP-27 infusion at an elevated concentration in weaned mice were examined. The 12 day infusion period of PACAP in this experiment is the longest reported to date with respect to carbohydrate metabolism. In the present study we used the mammalian isoform, PACAP-27, to treat mice. Even though PACAP-38 is the predominant isoform in mammalian tissues, both isoforms are equipotent in glucose and insulin regulation (1). The concentration of the peptide to be infused at 70pmol/hr and injected at 100pmol was based on previous literature (3, 9, 11-14). Based on earlier findings, the endpoints of interest in the present study included histology (liver, heart, and skeletal muscle), body mass, blood glucose levels and serum insulin concentration.

PACAP and GHRH both belong to the glucagon superfamily of hormones and originate from one precursor in all vertebrates except mammals. This suggests a possibility that PACAP may be involved in growth. In addition, PACAP is known to release GH from the pituitary, further supporting this notion (15). As a result, I hypothesized that mice undergoing treatment would have a greater body mass than controls. In contrast, there was no significant difference in body mass of treated and normal mice indicating that PACAP does not seem to have an effect on growth in mice under these experimental conditions.

Gray *et al.* have reported that PACAP knockout mice have lipid deposits in liver, cardiac muscle and skeletal muscle as a result of carbohydrate and/or lipid metabolism dysfunction (5). Therefore, it was of interest to examine tissue histology of mice exposed to PACAP. As expected, histology of PACAP treated mice did not reveal any difference

in cell distribution and morphology or lipid content in these tissues when compared to normal mice. It seems unlikely that PACAP excess would result in the same phenotype as was seen in the knockout mouse. On the contrary, I would not expect to find lipid accumulation in these tissues as normal mice of the same age lack lipid accumulation.

Circulating PACAP-38 in systemic blood exists at very low concentrations of 24fmol/mL in rats (16). If the same concentration applies to mice then 24 fmol/ml or 109pg/mL should be present in the total blood of a mouse (assuming that total blood volume in mice used was 1mL). This corresponds to an estimated value of 38pg/tube in the radioimmunoassay we used, and should have been detected. Given that PACAP-38 is the more abundant isoform, it is possible that PACAP-27 is found at lower concentrations in rodents as in humans at a circulating level of <10fmol/mL (3). This concentration would be near or below the detection limit of the assay that was used in this experiment and could explain why PACAP was not detected in control mice. However, a constant infusion of PACAP at 70pmol/hr/mouse (317ng/hr/mL) was not detected either even though it was expected to fall within the assay limits. This finding could be explained in several ways. First, PACAP has a very short half-life in circulating blood of less than 1 min, resulting in fast degradation of the peptide (1). Second, PACAP may have bound to receptors in target tissues and was no longer present in the blood. Target tissues would include pancreas and the brain as PACAP is known to cross the blood brain barrier (17). Third, PACAP may not be stable in the pump at mouse body temperature for a period of 2 weeks in PBS. Fourth, the commercial PACAP assay has a design flaw due to labeling of up to three iodines per one molecule of PACAP. A combination of any of these

factors could have contributed to a lower than expected PACAP concentration in circulating blood of treated mice.

Because PACAP is a potent releaser of insulin, it would be expected that glucose disposal should be altered with exogenous PACAP administration. However, studies have shown that PACAP can potentiate insulin secretion *in vivo* in mice and humans without modifying blood glucose (3, 18). Furthermore, mice that overexpress PACAP in pancreas show no difference in basal glucose when compared to wild type littermates (10). This effect can be attributed to PACAP's ability to act as a co-transmitter in releasing adrenalin which counteracts the action of insulin (19). Ahren *et al.* have speculated that the combined release of insulin and adrenalin would result in no net change in basal glucose. This hypothesis was supported by experimental evidence where adrenalectomy in mice resulted in augmented glucose elimination rate (20). It was recently found that PACAP administration by repeated intraperitoneal injection to mice over 5 days seems to have a different effect on glucose disposal than in short term since repeated intraperitoneal PACAP administration in both control and high fat fed mice resulted in significantly reduced basal glucose levels (13). Based on these findings I would expect to see either the same or reduced glucose levels in PACAP chronically treated mice when compared to controls. In support of this, blood glucose of mice that were exposed to PACAP either for 6 or 12 days did not differ significantly from controls.

PACAP is an important regulator with respect to carbohydrate metabolism because it has been shown to initiate insulin release from pancreatic islets both *in vitro* and *in vivo* in several species including mice. Furthermore, PACAP seems to contribute to the insulin response after food intake in a glucose-dependent manner as shown by

gastric glucose gavage (21). Mice overexpressing PACAP in the pancreas had undetectable levels of the PACAP38 isoform in plasma but responded with more enhanced insulin levels to a glucose tolerance test when compared to wild types, indicating that PACAP can act at very low levels (10). As an extremely potent releaser of insulin, PACAP has been shown to have insulinotropic properties at a low concentration of 10fM *in vitro* (22). This suggests that mice in the present study may have received a sufficient dose of PACAP to evoke a significant response in the pancreas even though the assay used was not sensitive enough to detect the neuropeptide in plasma. Based on insulinotropic properties of PACAP mentioned above, insulin levels were expected to be higher 12 days after PACAP infusion treatment especially during the fed state. In contrast, no significant difference was seen in serum insulin concentration between control and PACAP treated mice. In support of this, a recent study showed that transgenic mice overexpressing PACAP in pancreas had the same basal plasma insulin levels as controls in both *ad libitum* fed and fasted states. However, following a glucose tolerance test, insulin levels were significantly higher in transgenic mice when compared to wild types (10). The next step in the current study is to perform a glucose tolerance test on mice treated with pumps but initially we wanted to test PACAP receptor sensitivity to the hormone. If PACAP did not degrade in the pump or in the blood and successfully bound to receptors in target tissues including the pancreas but caused no increase in insulin, it is likely that the PACAP receptors became down-regulated in response to a prolonged exposure to elevated concentrations of the hormone.

To test the hypothesis of reduced receptor sensitivity, an interperitoneal PACAP challenge was performed on mice pre-exposed to the hormone for a period of 12 days.

The purpose of the injection was to measure glucose levels and insulin release to determine if pancreatic and other cells of treated mice still responded as well to PACAP as control mice. This study found that glucose levels rose following the saline injection, most likely caused by stress from the injection itself (23). In addition, glucose levels also peaked in both control and experimental animals with pumps that received a PACAP injection. The pattern of glucose levels over time was very similar between the two groups and the interaction between treatment and time was not statistically significant. Because both groups that received a PACAP injection also underwent pump surgery, it is clear that a sham pump group with PBS injection is a necessary control as post-operative stress may be a factor (23). To validate the PACAP challenge, mice that do not receive a pump of the same age as other treatment groups (39 days old) should be injected with PACAP only. To obtain more valuable results, the sample size for each treatment should be increased to reduce the standard error of the mean. In summary, glucose data from this experiment to date suggest that glucose levels following a PACAP injection were not affected by a 12 day pre-exposure to PACAP.

Measuring insulin in response to PACAP is an indirect way to determine whether pancreatic islet cells respond to the hormone. There was a pronounced difference in insulin levels at time zero (at the end of pump treatment) between treated and control mice prior to the PACAP injection, making the overall difference in insulin significant between the treatment groups. However, no overall difference was found in serum insulin concentration when comparing time and treatment interaction after the injection when the zero time point values were excluded. Mice injected with saline solution had somewhat elevated insulin at 5 min perhaps due to peaking glucose levels at the same

time. As already mentioned, to make the information more valuable mice without surgery need to be injected with PACAP and compared with saline injected mice. Mice used for this experiment were fed *ad libidum* resulting in random time and quantity of food intake for each mouse which might cause variability in blood glucose levels and corresponding serum insulin concentrations. Data from this study reveal there were no significant differences in glucose or insulin after a chronic exposure to PACAP followed by a PACAP challenge. This finding suggests that continuous PACAP may result in PACAP resistance but additional work needs to be done to make this conclusive. It would be interesting to repeat the experiment with a more stringent control of feeding, for example, by fasting the mice for an hour prior to the injection and giving them a glucose gavage or injection along with PACAP administration. Because insulin release is much more exaggerated in the presence of both glucose and PACAP, it would be easier to test the proposed hypothesis.

For this part of the study, I hypothesized that weaned mice would not die, would not have altered histology, may grow larger and would have differences in metabolites/hormones involved in the carbohydrate metabolism when exposed to PACAP in excess for several days. In conclusion, augmented PACAP in mature mice proved not to be lethal in contrast to frogs that are exposed to the hormone since fertilization. Also, there was no difference in histology, body mass, glucose or insulin between treated and control mice. It must be mentioned that this experimental design poses a few problems that need to be addressed in future studies. Long-term, constant delivery of PACAP can be obtained only if the hormone is stable at body temperature of the mouse for the

duration of infusion. Therefore, PACAP should be tested for stability at 37^0C to ensure the peptide did not degrade during the treatment. The sample size for this study should be increased because low numbers of mice per treatment result in higher variability and smaller probability of identifying a significant difference. The glucometer is an accepted measuring device for glucose levels in research animals (24). However, we may need to do repeated measurements of each sample to reduce variability. Factors that could affect glucose readings are blood oxygenation and the feeding state of the animal. In our experiment blood was collected from the saphenous vein at days 6 and 12 and the heart ventricle for the remaining glucose readings. It is difficult to collect blood from the same ventricle each time to control for oxygen content. However, mice can be fasted to control for glucose obtained from the diet. Stress is another factor that definitely affects glucose levels. Studies show that frequent handling of mice and circumvention of stress by anaesthesia improves the accuracy and reproducibility of data when measuring endogenous glucose levels in mice (18, 23). Since experimental and control groups in this study were handled equally, stress levels would be expected to be the same.

To complete this study, it would be necessary to test for the PACAP challenge effect by also injecting groups of mice that had no pumps implanted with PACAP at the same time points as the other groups. Also, it would be interesting to do an intravenous or a gastric glucose tolerance test on PACAP treated mice since PACAP acts in a glucose-dependent manner to release insulin and causes a several fold increase in insulin concentration in the presence of glucose. Finally, the generation of transgenic mice overexpressing PACAP in specific tissues may yield some very interesting results. So far this has been done in pancreas, but since PACAP is predominantly located in the brain

and peripheral nerves, the use of a neuron specific promoter may provide different results.

REFERENCES

1. **Filipsson K, Kvist-Reimer M, Ahren B** 2001 The neuropeptide pituitary adenylate cyclase-activating polypeptide and islet function. Diabetes 50:1959-1969

2. **Bertrand G, Puech R, Maisonnasse Y, Bockaert J, Loubatieres-Mariani MM** 1996 Comparative effects of PACAP and VIP on pancreatic endocrine secretions and vascular resistance in rat. British Journal of Pharmacology 117:764-770

3. **Filipsson K, Tornoe K, Holst J, Ahren B** 1997 Pituitary adenylate cyclase-activating polypeptide stimulates insulin and glucagon secretion in humans. The Journal of Clinical Endocrinology and Metabolism 82:3093-3098

4. **Arimura A** 1998 Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Japanese Journal of Physiology 48:301-331

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

6. **Jamen F, Persson K, Bertrand G, Rodriguez-Henche N, Puech R, Bockaert J, Ahren B, Brabet P** 2000 PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. The Journal of Clinical Investigation 105:1307-1315

7. **Ahren B** 2003 PACAP in the Endocrine Pancreas. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 185-206

8. **Ahren B, Filipsson K** 1998 Pituitary adenylate cyclase activating polypeptide stimulates insulin secretion in a glucose-dependent manner in vivo. Annals of the New York Academy of Sciences 865:466-470

9. **Absood A, Chen D, Hakanson R** 1992 Neuropeptides of the vasoactive intestinal peptide/helodermin/pituitary adenylate cyclase activating peptide family elevate plasma cAMP in mice: comparison with a range of other regulatory peptides. Regulatory Peptides 40:311-322

10. **Yamamoto K, Hashimoto H, Tomimoto S, Shintani N, Miyazaki J, Tashiro F, Aihara H, Nammo T, Li M, Yamagata K, et al.** 2003 Overexpression of PACAP in transgenic mouse pancreatic beta-cells enhances insulin secretion and ameliorates streptozotocin-induced diabetes. Diabetes 52:1155-1162

11. **Onaga T, Uchida M, Kimura M, Miyazaki M, Mineo H, Kato S, Zabielski R** 1996 Effect of pituitary adenylate cyclase-activating polypeptide on exocrine and endocrine secretion in the ovine pancreas. Comparative biochemistry and physiology. Comparative Pharmacology and Toxicology 115:185-193

12. **Radcliff RP, Lookingland KJ, Chapin LT, Tucker HA** 2001 Pituitary adenylate cyclase-activating polypeptide induces secretion of growth hormone in cattle. Domestic Animal Endocrinology 21:187-196

13. **Yada T, Sakurada M, Filipsson K, Kikuchi M, Ahren B** 2000 Intraperitoneal PACAP administration decreases blood glucose in GK rats, and in normal and high fat diet mice. Annals of the New York Academy of Sciences 921:259-263

14. **Fridolf T, Sundler F, Ahren B** 1992 Pituitary adenylate cyclase-activating polypeptide (PACAP): occurrence in rodent pancreas and effects on insulin and glucagon secretion in the mouse. Cell and Tissue Research 269:275-279

15. **Montero M, Yon L, Kikuyama S, Dufour S, Vaudry H** 2000 Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. Journal of Molecular Endocrinology 25:157-168

16. **Rawlings SR, Hezareh M** 1996 Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. Endocrine Reviews 17:4-29

17. **Banks WA, Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S** 1996 Transport of pituitary adenylate cyclase-activating polypeptide across the blood-brain barrier and the prevention of ischemia-induced death of hippocampal neurons. Annals of the New York Academy of Sciences 805:270-279

18. **Filipsson K, Pacini G, Scheurink AJ, Ahren B** 1998 PACAP stimulates insulin secretion but inhibits insulin sensitivity in mice. American Journal of Physiology 274:E834-842

19. **Hamelink C, E. W, Eiden LE** 2003 PACAP: An 'Emergency Response' Co-Transmitter in the Adrenal Medulla. **In:** Pituitary Adenylate Cyclase-Activating Polypeptide. Vaudry H and Arimura A., eds. Kluwer Academic Publishers, Norvell, Massachusetts p. 227-250

20. **Ahren B, Filipsson K** 2000 The effects of PACAP on insulin secretion and glucose disposal are altered by adrenalectomy in mice. Annals of the New York Academy of Sciences 921:251-258

21. **Filipsson K, Holst JJ, Ahren B** 2000 PACAP contributes to insulin secretion after gastric glucose gavage in mice. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 279:R424-432

22. **Yada T, Sakurada M, Ihida K, Nakata M, Murata F, Arimura A, Kikuchi M** 1994 Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet beta-cells. The Journal of Biolical Chemistry 269:1290-1293

23. **Tabata H, Kitamura T, Nagamatsu N** 1998 Comparison of effects of restraint, cage transportation, anaesthesia and repeated bleeding on plasma glucose levels between mice and rats. Lab Animal 32:143-148

24. **Rheney CC, Kirk JK** 2000 Performance of three blood glucose meters. The Annals of Pharmacotherapy 34:317-321