The role of pituitary adenylate cyclase-activating polypeptide (PACAP) in cell cycle exit, differentiation and apoptosis during early chick brain development

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We accept this dissertation as conforming to the required standard

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ABSTRACT

Supervisor: Dr. Nancy M. Sherwood

Regulated survival, proliferation and differentiation of cells in the nervous system is crucial for development. Much of regulation is controlled by hormones. There is abundant evidence that a member of the glucagon superfamily, pituitary adenylate cyclase-activating polypeptide (PACAP), is important in this process. PACAP functions have been described in the peripheral and central nervous systems of many species. Although the primary function of PACAP is not known, its high conservation and presence in all species examined to date suggest it is vital to normal development.

My thesis objective was to determine the response of early CNS neuroblasts to PACAP, in conjunction with another glucagon superfamily member, growth hormone-releasing hormone (GHRH). GHRH is best known for causing release of growth hormone from the pituitary, but it also has functions in nervous system development. Because PACAP and GHRH are encoded on the same gene in non-mammalian vertebrates, it is possible that they have similar or coordinated functions. PACAP affects development by altering levels of proliferation and differentiation and decreasing apoptosis. For these reasons, I focused my research in these areas.

Using neuroblast-enriched cultures from embryonic day 3.5 chick, my first goal was to show that PACAP and GHRH affected these cells. Radioimmunoassays for cAMP revealed that all but one form of PACAP, and only one form of GHRH, caused an increase in cAMP relative to controls. As to the former, comparison of differing PACAP structures suggested that conservation at the amino terminus was important in binding the hormone to the receptor. The fact that PACAP, but not GHRH, increased

cAMP, indicated that evolution of PACAP and GHRH had altered their functions. Chick neuroblasts were also shown to produce PACAP and its primary receptor, suggesting an autocrine/paracrine role for PACAP.

My next goal was to examine the nature of the downstream effects of increased cAMP. To study cell cycle, I developed a protocol using proliferating cell nuclear antigen (PCNA) and propidium iodide (PI), in fixed cell populations. PCNA is present in low amounts in non-cycling cells, but rises sharply in actively proliferating cells. The PI helped delineate cell cycle compartments, because in permeabilized cells it binds to and quantifies DNA. Changes in G_0, G_1, S and G_2/M were recorded using flow cytometry. Because the cells were producing PACAP and most were cycling, rather than add more PACAP I chose to block the PACAP receptor. This caused cell cycle exit. I also blocked the cell cycle at two points, and showed that exogenous PACAP could release some cells from the block, and return them to cycling. PACAP affected apoptosis also, but because the protocol was not designed to measure this, I adopted another protocol using flow cytometry. With live cells, and fluorescein diacetate, which is retained and fluoresces in healthy cells, and PI, which enters only cells with damaged membranes, I used the characteristic of apoptotic cells to die with membranes intact to confirm increased apoptosis when the PACAP receptor was blocked.

This left the question of whether PACAP affected differentiation. The cell cycle protocol had shown some cells were still quiescent, not dying, at 24 h, so I hypothesized that they might be differentiating. I used proteomics to test this. With isotope-coded affinity tagged (ICAT) analysis, I measured changes in protein content in cells that had been treated with the receptor blocker, compared to control. This confirmed previous

work and my hypothesis that some cells were differentiating. Because this technique is not commonly used in molecular biology, I also evaluated the effectiveness of the technique. My work showed that endogenous PACAP keeps chick neuroblasts alive and cycling, but will allow some to differentiate rather than die, when the hormone is withdrawn. Obviously, PACAP plays a crucial role in early chick brain development.

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LIST OF ABBREVIATIONS

Apaf: apoptosis protease activating factor

BC: best confidence
BDNF: brain-derived neurotrophic factor
BrdU: bromodeoxyuridine

c (e.g. cPACAP): chicken
cAMP: cyclic adenosine monophosphate
CCT: chaperonin-containing T (complex)
cDNA: complementary deoxyribonucleic acid
CDI: cyclin dependent kinase inhibitor
Cdk: cyclin dependent kinase
CNTF: ciliary neurotrophic factor
cRBC: chick red blood cell

D-MEM: Dulbecco's Modified Eagle's Medium
DMSO: dimethysulfoxide
DTT: dithiothreitol

E: embryonic day
EF-2: elongation factor 2
eIF: eukaryotic translation initiation factor

FBS: fetal bovine serum
EDTA: ethylenediaminetetraaceticacid
FDA: fluorescein diacetate
EF: elongation factor
FGF: fibroblast growth factor
FBP: myc far upstream element-binding protein
FITC: fluorescein isothiocyanate
FL: fluorophore

GFAP: glial fibrillary acidic protein
GHRH: growth hormone-releasing hormone
GIP: glucose-dependent insulinotropic polypeptide
GTP: guanine triphosphatase

h (e.g. hPACAP): human
HLA: human leukocyte antigens
HMGB1 or HMG1: high mobility group box 1
hnRNP: heterogeneous nuclear ribonucleoprotein
HSP: heat shock protein

ICAT: isotope-coded affinity tag
IGF-I/II: insulin-like growth factor I/II

MS: mass spectrometry

NCBI: National Centre for Biotechnology Information
NGF: nerve growth factor
NSE: neuron-specific enolase

P: postnatal day
PAC_1-R: PACAP type 1 (PACAP specific) receptor
PACAP: pituitary adenylate cyclase activating polypeptide
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
PFM: paraformaldehyde
PHAP: putative human HLA class II associated protein
PHI: peptide histidine isoleucine
PHM: peptide histidine methionine
PI: propidium iodide
PI_3: phosphoinositide - 3
PKA: protein kinase A
PKC: protein kinase C
PP1: protein phosphatase I
PP2A: protein phosphatase 2A
PRP: PACAP related peptide

RIA: radioimmunoassay
rt: room temperature

s (e.g. sPACAP): salmon
SDS: sodium dodecylsulfate
SEM: standard error of the mean
snRNP: small nuclear ribonucleoprotein

t (e.g. tPACAP): tunicate
TBST: Tris-buffered saline with Tween

UTR: untranslated regions

VIP: vasoactive intestinal polypeptide

ACKNOWLEDGEMENTS

I will begin by acknowledging my birth family. I have always known that I will never have to be alone in the world; this has done much to keeping me moving forward. I want to acknowledge the day-to-day support of my adopted family in Victoria, especially my partner Doreen, who nurtures me fully and completely as a human being, and helps me stay sane. My thanks also to Dr. Cheryl Malmo and Dr. Stuart Gershman.

I am grateful to Pat Rasmussen for first encouraging me to attempt a university education, then for mental, emotional, and practical support. I am indebted to Nancy Sherwood, for making me an offer when I thought that graduate school was beyond my reach. The ache in my jaw from a smile that stuck to my face for at least two weeks is not something I will ever forget. She has for seven years supplied a generous working environment, allowed a self-defined degree of independence, and all the enthusiasm one could hope to hitch a ride on when one too many little failures loomed too large.

I thank the members of the Sherwood lab, past and present, and will especially remember David Lescheid and Erica Fradinger for their emotional support. I thank Carol Warby for her technical support, and for the contribution of her cheerful dedication to the lab environment. Her presence is a gift. I thank Tom Gore for technical support, and Heather Down for that and more. I thank the members of my committee, who were always as helpful as they could manage. I must single out Dr. Terry Pearson, who tolerated not only my domination of his flow cytometer for two years, but also my immunological ignorance as I was developing my protocols. The members of his lab were not only helpful, but welcoming.

Lastly, I acknowledge myself; I can't believe I've done it.

DEDICATION

I dedicate this work to Dr. Rita Levi-Montalcini. Dr. Levi-Montalcini was awarded the Nobel Prize in Physiology or Medicine in 1986. She was acknowledged for her lifetime of work in neurology. She will always be associated with the discovery of nerve growth factor, the prototypic soluble factor that feeds nerve cells. But it is for her courage and strength that I make this dedication. Levi-Montalcini was born in Italy in 1909. She was discouraged from an education by a father who believed it would interfere with the duties of a wife and mother. But she convinced him to let her complete school, and by 1936 she had graduated from medical school. This was the year that Mussolini issued the "Manifesto per la Difesa della Razza", signed by ten scientists, and this manifesto was followed by laws which barred non-Aryans from academic and professional careers. She and her family chose to stay in Italy, isolated from the community, and Levi-Montalcini built a laboratory in her bedroom to continue her work on chick limb bud development. At one point, heavy bombing by Anglo-American forces drove her and an assistant to a country cottage, where she rebuilt her mini-laboratory. By 1943 even that had become unsafe, as the Germans invaded Italy, and she fled to Florence, where she lived underground. When the Germans retreated, she left her laboratory to work as a nurse and medical doctor until the war ended in 1945. She continued her ground-breaking work in neurology in Rome, St. Louis, and retired in Rome in 1979.

CHAPTER 1

General Introduction

INTRODUCTION

The vertebrate nervous system develops from an infolding of ectodermal tissue, the cells of which then proliferate, migrate throughout the embryo, aggregate into nuclei, structurally and functionally differentiate, and finally establish a network of billions of cells, each connected to thousands of others (Brodal 1992). This process is regulated by soluble and non-soluble factors, and includes phases during which many cells undergo a special kind of cell death, called programmed cell death or apoptosis. It is estimated that as many 85% of nerve cells die during development (Schwartz and Osborne 1995).

Regulation of nervous system development by growth factors and neurohormones

An important category of soluble factors involved in nervous system development is growth factors and hormones. Historically, growth factors have been defined as agents that cause cell division, and it has been commonly accepted that developing nerve cells are regulated in number by diffusion of growth factors from target tissues. However, recent research suggests that growth factors also play a significant role in regulating survival and differentiation of nerve cells, making them similar in function to hormones. As well, they may be transported through the vascular system to act at a distance, as occurs with hormones (Glowinski 1990). Moreover, they may act in an autocrine fashion to regulate the source itself, a capacity again shared by hormones (Ayer-Lelievre et al. 1988). Growth factors and hormones can be large (more than 100 amino acids) whereas hormones can also be relatively small (as few as three amino acids). Growth factors tend to have a protein kinase receptor, whereas hormones

may have a protein kinase receptor or a seven-transmembrane receptor. However, in terms of function, the distinction between the two groups is blurred. One researcher has described the boundary between the two groups as "virtually imperceptible" (Baserga 1981) and another has suggested a shift to the inclusive term "chemical mediators" (Glowinski 1990).

This thesis considers the impact of two of these messengers, specifically pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH), and their roles in stimulating immature nerve cells to continue dividing, differentiate, or die. PACAP and GHRH are both neurohormones, that is they are produced by neurons. This distinguishes them from glandular hormones, which are secreted by glands to circulate in the blood. They are distinguished from neurotransmitters both by their size and by their mode of action. Neurohormones are at least a few amino acids long but usually longer, whereas neurotransmitters consist of at most, a few, sometimes modified amino acids, as well as some purines and gases. Neurohormones generally travel further distances, and elicit a longer-term effect after some delay. Neurotransmitters, on the other hand, travel only short distances, i.e. across a synaptic cleft, to bind to a receptor and initiate a brief but immediate effect (Baulieu 1990). Intermediate types of mediators are neuromodulators, which travel beyond the synaptic cleft to bind to a receptor on an adjacent or neighbouring cell. Neurotransmitters may act as neuromodulators.

Because it is difficult to isolate the impact of soluble factors *in vivo*, cell culture is commonly used for studying their effects. However, lack of consistency in a number of variables makes comparisons between published works difficult. Cellular

composition can alter results; for example in one research study, PACAP decreased proliferation only if fibroblast growth factor was present, otherwise it increased proliferation (Suh et al. 2001). Changes in medium content can have an effect; for instance, addition of serum may enhance or inhibit differentiation to a particular biochemical phenotype (Kentroti and Vernadakis 1990). Serum may also have confounding effects in that it carries immune system components such as antibodies, and proteases such as thrombin and chymotrypsin, which will affect final composition and behaviour of cells (Baserga 1981). Substrate choice can be important. A number of researchers have found that cell-to-cell contact, which is affected by both substrate and plating density, can affect both the behaviour of immature cells (Hartikka and Hefti 1988; Alderson et al. 1990) and the fate of differentiating cells (Mangoura et al. 1990).

The glucagon superfamily of hormones

Both PACAP and GHRH are members of the glucagon superfamily of hormones. The family also includes glucagon, glucagon-like peptides (GLP) I and II, vasoactive intestinal polypeptide (VIP), secretin, glucose-dependent insulinotropic polypeptide (GIP), and peptide histidine isoleucine (PHI), which is known as peptide histidine methionine (PHM) in humans. Glucagon superfamily hormones are produced throughout the body: in the gut, gonads, pancreas, and nervous system. All but GIP have been found in the brain (Sherwood et al. 2000). Functions are also diverse and include regulation of metabolism and the cardiovascular, endocrine, and immune systems.

The glucagon superfamily members are encoded on six genes, which share a similar structure. Although in mammals GHRH and PACAP are on different genes, in other vertebrates they are found on the same gene (Fig. 1.1).

Receptors

All receptors for the glucagon family belong to a subset of the seven transmembrane receptor family (Fig. 1.2). They are all coupled internally to a G protein, and all but one is linked to adenylyl cyclase or phospholipase C. Binding is known to be, to varying degrees, promiscuous. That is, some family members will bind to receptors for other members, usually with lesser affinity (Desbuquois 1990; Sherwood et al. 2000). All the receptors have been isolated and described (Vaudry et al. 2000; Tse et al. 2002). The PACAP receptors are the best characterized. Two major types of PACAP receptor have been described, based on binding affinities (Sherwood et al. 2000; Vaudry et al. 2000). One type ($VPAC_1$-R and $VPAC_2$-R) binds PACAP with equal affinity to VIP, and the other (PAC_1-R), binds PACAP with 100 to 1000 times the affinity of VIP. The predominant PACAP receptor expressed during brain development is the PACAP-specific receptor, PAC_1-R (Sherwood et al. 2000). Alternate splicing of the transcript from a single gene creates nine isoforms of PAC_1-R (Laburthe et al. 2003). Inclusion or exclusion of three cassettes (hip, hop1, hop2) in the third intracellular loop creates six isoforms: short, hip, hop1, hop2, hiphop1 and hiphop2 (Fig. 1.2). A seventh isoform is created by a 21-amino acid deletion in the extracellular domain, and an eighth form is created by a 57-amino acid deletion in the extracellular domain. A ninth form is

Figure 1.1 Comparison of the gene structure for members of the glucagon superfamily of hormones. Boxes represent exons, and lines indicate introns; they are not drawn to scale. SP indicates signal peptides and UTR indicates untranslated regions. White boxes indicate peptides of unknown function. Darkened boxes indicate bioactive peptides. Note that both the mammalian and non-mammalian GHRH and PACAP genes are shown. GHRH: growth-hormone-releasing hormone; GIP: glucose-dependent insulinotropic polypeptide; GLP-I: glucagon-like peptide I; GLP-II: glucagon-like peptide II; PACAP: pituitary adenylate cyclase-activating polypeptide; PHI: peptide histidine isoleucine; PRP: PACAP-related peptide; VIP: vasoactive intestinal polypeptide (Sherwood et al. 2000).

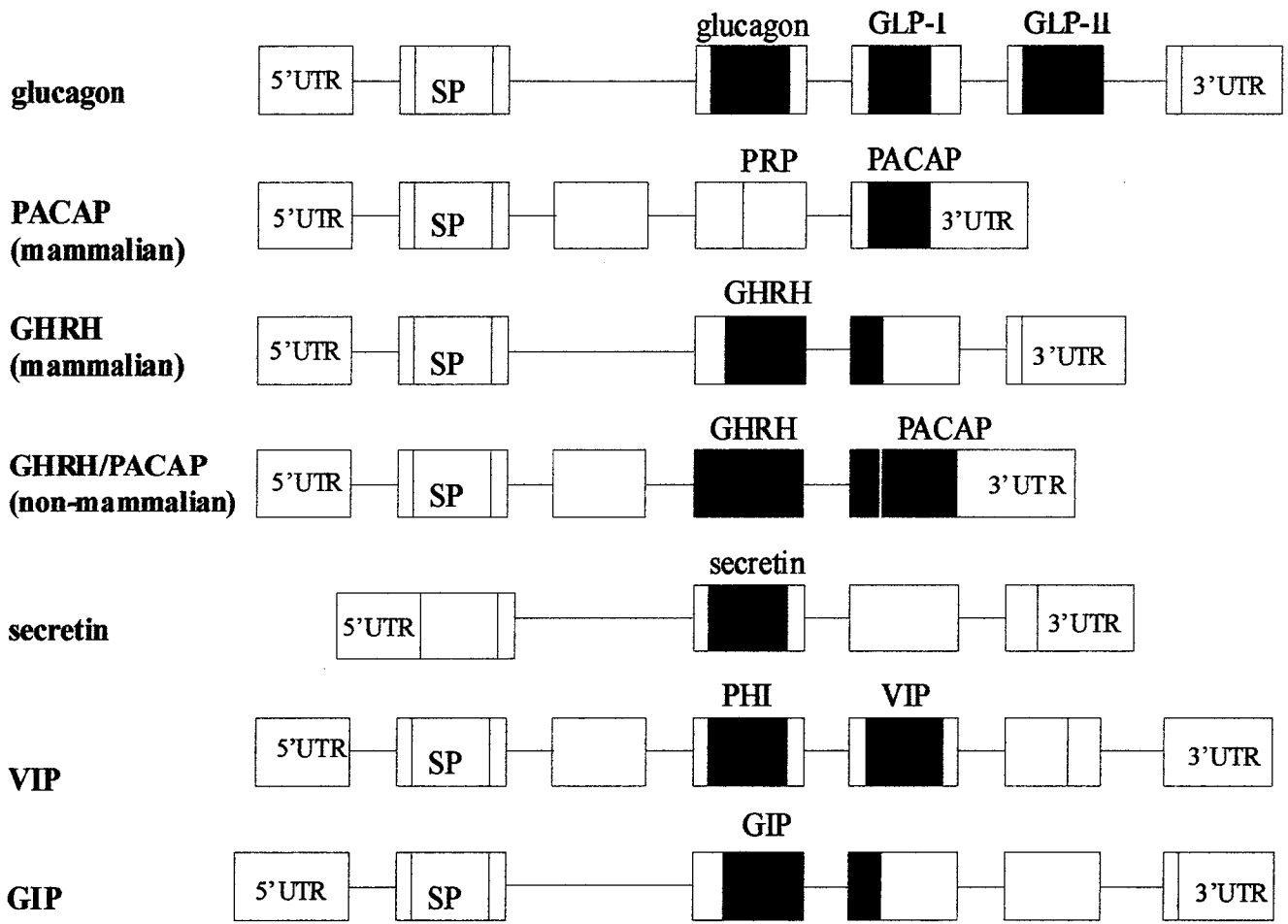
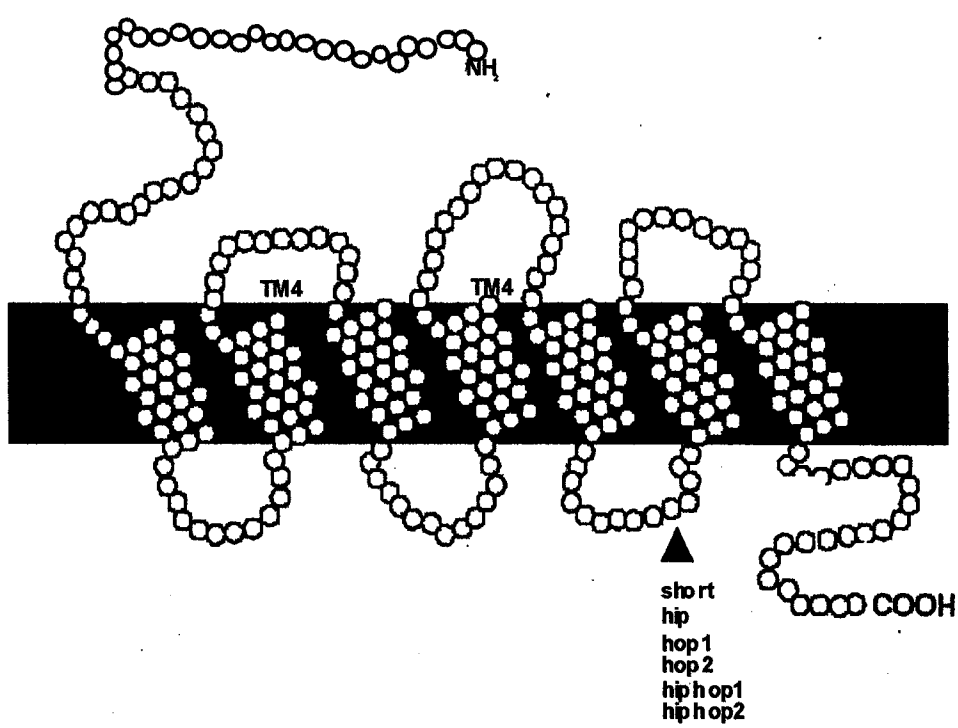

glucagon
5'UTR
SP
glucagon
GLP-I
GLP-II
3'UTR
PACAP
(mammalian)
5'UTR
SP
PRP
PACAP
3'UTR
GHRH
(mammalian)
5'UTR
SP
GHRH
3'UTR
GHRH/PACAP
(non-mammalian)
5'UTR
SP
GHRH
PACAP
3'UTR
secretin
5'UTR
secretin
3'UTR
VIP
5'UTR
SP
PHI
VIP
3'UTR
GIP
5'UTR
SP
GIP
3'UTR

Figure 1.2 Representation of a seven transmembrane receptor. The arrowhead indicates the region in the third intracellular loop where alternate splicing creates six isoforms of the PACAP-specific receptor. Two more isoforms, not indicated, are created by 21 and 57 amino acid deletions in the extracellular domain. A final form, TM4, does not act through adenylyl cyclase or phospholipase C as do the others, but activates instead a calcium channel. The transmembrane (TM) locations that contain the substitutions and deletions which create this isoform are indicated. (Ulloa-Aguirre and Conn 2000).

extracellular



intracellular

TM4, which does not activate adenylyl cyclase or phospholipase C, but acts instead through a calcium channel (Fig. 1.2).

The GHRH receptor has not been as fully characterized as the PACAP receptor, but there are at least two forms, caused by alternative splicing of a single gene transcript. This results in inclusion or exclusion of a cassette in the third intracellular loop (Mayo 1992; Mayo et al. 1996).

Discovery and characterization of GHRH

A human (h)GHRH was first reported in 1982 by two separate groups, following isolation from pancreatic tumour extracts, and based on the peptide's ability to stimulate release of growth hormone from cultured pituitary cells. Two non-amidated forms, 37 and 40 amino acids in length, were characterized from tumour tissue, along with a 44-amino acid form, amidated at the carboxy terminus (Guillemin et al. 1982; Rivier et al. 1982).

Two years after characterization of the pancreatic form of GHRH, a peptide from human hypothalamic-hypophysial tissues was isolated and sequenced, and a comparison showed the 44 amino acid peptide to have the same sequence as the pancreatic form; it was apparent there was only one hGHRH gene (Ling et al. 1984). The amino acid sequence of the peptide placed it in the glucagon superfamily of genes. Only the 27 residues at the amino terminus appeared to be required for *in vitro* potency (Guillemin 1986), although there is a putative cut site after the 29th amino acid (Guillemin et al. 1982; Rivier et al. 1982).

A 43-amino acid rat GHRH was characterized soon after the human form, based on its ability to cause secretion of growth hormone from cultured rat anterior pituitary cells. It has 67% sequence identity with hGHRH, with most differences at the carboxy end (Spiess et al. 1983). At present there are 22 GHRH peptides sequenced from vertebrates, with varying degrees of similarity (39-93%) to the human peptide (Adams et al. 2002). Most of these peptides are amidated, 44-amino acid peptides, although the mouse, rat and fish peptides have 42, 43 and 44 amino acids, respectively, with no amidation.

The hGHRH gene has been found to span 10 kb of genomic DNA (Mayo et al. 1985). It consists of five exons separated by four introns (Fig. 1.1). Exon 1 encodes 5' untranslated (UTR) sequences, and exon 2 encodes some 5' UTR, a signal peptide and part of a small connecting peptide of unknown function. Exon 3 codes for the remainder of the connecting peptide, and all of the biologically active portion of hGHRH, with the remainder on exon 4. Exon 4 also encodes most of a carboxy-terminal peptide, which has no known function. Exon 5 codes for the remainder of the carboxy-terminal peptide, and 3' UTR sequences.

DNA sequencing showed the hGHRH peptide precursor to be either 107 or 108 amino acids in length; alternative RNA processing causes inclusion or exclusion of a serine residue at the beginning of exon 5 (Gubler et al. 1983). A 103-amino acid precursor for mouse GHRH has since been isolated (Suhr et al. 1989) as well as a rat GHRH precursor of 104 amino acids (Campbell and Scanes 1992).

An important distinction between mammalian GHRH and that of other vertebrates and protochordates is that in the non-mammalian group, GHRH is encoded

on the same gene as PACAP. This raises the possibility that they have coordinated functions. A typical non-mammalian gene, deduced from nucleotide sequences from the chicken is included in Figure 1.1.

GHRH is one of the least well conserved members of the superfamily (Hoyle 1998). The chicken form of GHRH is approximately 75% homologous to the teleost forms, but only 43% homologous to the human form.

Discovery and characterization of PACAP

PACAP was discovered as a result of a search for new hypothalamic hormones that would stimulate secretion of hormones from the anterior pituitary, and/or regulate growth, differentiation and general function of both characterized and uncharacterized adenohypophyseal cells (Arimura 1992). Based on the knowledge that some hypothalamic releasing factors stimulated adenylate cyclase and thereby caused an increase in cAMP, and because cAMP is important in intracellular signalling and could be measured easily and accurately, researchers screened ovine hypothalamic tissues for a new substance which would increase levels of cAMP in rat pituitary cells. The assumption was that any peptide that increased adenylate cyclase activity would have a significant biological effect on the cells of the pituitary. A novel 38-residue polypeptide was isolated, amidated at the carboxy-terminus, and this protein was called pituitary adenylate cyclase-activating polypeptide (with 38 residues), or PACAP38 (Miyata et al. 1989). Amino acid analysis of the peptide revealed a glycine residue at position 28, followed by two basic amino acids, which suggested that the peptide could be cleaved to produce another peptide with 27 residues, PACAP27; this peptide was isolated and sequenced one year later (Miyata et al. 1990). The presence of this shorter peptide, also

amidated at the carboxy-terminus, was confirmed in the hypothalamic tissue (Arimura 1992). Analysis revealed a primary structure that placed PACAP in the glucagon superfamily, most homologous to VIP (68%). The area important for retention of biological function is located at the amino-terminus of PACAP38, which corresponds to the amino-terminus of PACAP27 (Vaudry et al. 2000).

The protein structures of human, sheep, rat and mouse PACAP have since been deduced from the cDNA and/or gene, and all appear to be identical (Kimura et al. 1990; Ogi et al. 1990; Hosoya et al. 1992; Okazaki et al. 1995; Cummings et al. 2002). The chicken and turkey forms of PACAP were deduced from the cDNA and differ by only one amino acid from the mammalian form (McRory et al. 1997; Yoo et al. 2000). Analysis of the peptide structure of frog PACAP revealed only one amino acid difference from hPACAP (Chartrel et al. 1991). The primary structures of PACAP have also been deduced in catfish and salmon. The 27 amino acids forms are identical to the human form, but both catfish and salmon PACAP38 have four amino acid changes compared to hPACAP (McRory et al. 1995; Parker et al. 1997). The zebrafish gene was characterized and the structure deduced; two forms of PACAP38 had three or six amino acids changes compared to hPACAP (Fradinger and Sherwood 2000; Adams et al. 2002). The protein structure for both stargazer and stingray were reported, both differing from hPACAP by 4 amino acids (Matsuda et al. 1997; Matsuda et al. 1998). Recently, the sequences from five more fish have been deduced from the cDNA (Adams et al. 2002). PACAP27 from whitefish and grayling are identical to the human forms, but differ by four and three amino acids, respectively, from the hPACAP38 forms. Flounder and halibut share the same sequence, with four amino acid changes compared

to hPACAP38. Sturgeon differs from the human form by three amino acids. A 27-amino-acid form of PACAP was isolated from the invertebrate protochordate *Chelyosoma productum* (sea squirt), and the protein structure deduced; there is only one amino acid difference (McRory and Sherwood 1997).

The human PACAP gene spans approximately 3 kb (Hosoya et al. 1992). The gene is transcribed to create five exons and four introns (Fig. 1.1). Exon 1 encodes a 5' UTR region. Along with some 5' UTR, a signal peptide is encoded on exon 2. A cryptic peptide with no known function spans the end of exon 2, exon 3, and the beginning of exon 4. A peptide called PACAP-related peptide (PRP), with no known function, is located on exon 4 and may continue into the beginning of exon 5. It is either 29 amino acids long if encoded only on exon 4, but 48 amino acids long if it extends into exon 5 (Sherwood et al. 2000). Exon 5 codes for PACAP, and 3' UTR. That PACAP is completely encoded by one exon lends more support to the hypothesis that PACAP27 is produced by post-translational cleavage, rather than alternate splicing of mRNA (Arimura 2003).

Analysis of the human, sheep, rat and mouse PACAP precursor cDNAs revealed an open reading frame of 176 amino acids in human and sheep forms, and 175 amino acids in rat and mouse forms (Kimura et al. 1990; Ogi et al. 1990; Ohkubo et al. 1992; Cummings et al. 2002).

PACAP has the distinction of being the most conserved member of this family of hormones (Hoyle 1998). The human, sheep, rat and mouse 38-amino-acid forms are identical, and there is only one amino acid substitution in the chick (Fig. 1.3) and frog.

Figure 1.3 Comparison of amino acid sequences between human and chicken PACAP and GHRH. Dashes indicate no change in the amino acid. The top set of four sequences show that there is high sequence identity between human and chicken PACAP (97%), but much lower sequence identity between human and chicken GHRH (43%). The second set of four sequences highlights the degree of homology for each peptide within each species. Human GHRH has five amino acids in common with human PACAP, and chicken GHRH has 11 amino acids in common with chicken PACAP. An "a" at the end of a sequence indicates amidation. (Hoyle 1998).

Comparison of human and chicken PACAP, human and chicken GHRH

```
hPACAP  H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K a

cPACAP  - I - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -


hGHRH   Y A D A I F T N S Y R K V L G Q L S A R K L L Q D I M S R Q Q G E S N Q E R G A R A R L a

cGHRH   H - - G - - S K A - - - L - - - - - - - N Y - H S L - A K R V - G A S S G L - D E - E P L S
```

Comparison of human PACAP and GHRH, chicken PACAP and GHRH

```
hPACAP  H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K a
hGHRH   Y A - D A I - T N S Y - R K V L G Q L S A R K L L Q D I M S R Q - G E S - Q E R G A R A R L a

cPACAP  H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K a
cGHRH   - A - - - - S K A - R K L L G - L S A R N - - H S L M A - - V G G A S S G L G D E A E P L S
```

There are 3-4 substitutions in the teleosts compared to mammalian PACAP. It is quite remarkable that there is only one substitution between the human and the invertebrate sea squirt PACAP27 form, given that these two species diverged more than 550 million years ago (Kumar and Hedges, 1998).

Involvement of glucagon superfamily members in nervous system development

Generally, members of this superfamily are best known for regulating metabolism, and the cardiovascular, endocrine, and immune systems. VIP, glucagon, and glucagon-like peptides I and II may have some function during nervous system development. There is little evidence that PHI plays a role as well. No functions have yet been found for secretin and GIP during nervous system development. The three hormones most studied for their effects on development of both the peripheral and central nervous systems are GHRH, VIP and especially PACAP.

VIP and PHI

VIP is a 28-amino acid neuropeptide, isolated in 1970, and identified initially as a potent vasodilator (Desbuquois 1990). It is now known that it has a broad range of activity, and is distributed not only throughout the gut, but also throughout the PNS and CNS. VIP is associated exclusively with neurons, and clear evidence exists for a role during PNS development. In the rat PNS, cultured cells from embryonic day 15.5 (E15.5) to post-natal day 1 (P1) superior cervical ganglia responded to VIP by increased survival, mitosis, and differentiation (Pincus et al. 1990).

VIP function in the developing CNS is not as clear, because evidence from rat

and mouse are contradictory (Muller et al. 1995; Waschek 1995; Hill et al. 1996; Brenneman et al. 1998). VIP receptors have been identified in the rat CNS at E11, but mRNA for the peptide has not been found in the CNS until after birth (Hill et al. 1996). It is likely that VIP is being supplied by embryo-derived placental cells or from the mother, because maternal serum revealed a six to ten-fold increase in VIP at this point and radiolabeled VIP administered to the mother was found in the E10 embryo (Hill et al. 1996). It is possible that VIP is taking the place of another hormone, since PHI, secretin and GHRH will bind with low affinity to VIP receptors (Desbuquois 1990), and PACAP will bind with high affinity (Sherwood et al. 2000).

Studies with mouse, however, revealed expression of the VIP gene as early as E11 in the hindbrain with correct-size mRNA by E14 (Waschek et al. 1996). A receptor gene was also detected at E14 in this study. Culture of E10 whole mouse embryos with VIP resulted in preferential growth of neural over non-neural tissue (Gressens et al. 1993). This result was later confirmed by administration of a VIP antagonist to pregnant dams, and effects were most evident in the brains of the offspring. A decrease in cellular survival and proliferation was evident for these E9-E11 embryos (Gressens et al. 1994). A later study by this group revealed that the increase in development of cultured embyros due to VIP was because the S and G_1 phases of the cell cycle were shortened (Gressens et al. 1998). Another group was unable to increase the growth rate of mouse embryos in culture by incubating with a concentration of VIP that was in the same range as these earlier studies, although conditions were not identical (Sheward et al. 1998).

There is also a possibility that VIP is effective in rescuing developing E12-14

CNS cells that have experienced damage. VIP was able to restore cell numbers in mouse dissociated spinal cord neurons treated with the nerve toxin tetrodotoxin (Brenneman and Foster 1987). (PHI, secretin and GHRH were ineffective in this study.) However, it was likely that VIP was acting through an intermediary cell type, probably glia. As well, blockade of VIP in E17 and E18 mouse neocortex resulted in increased apoptosis and decreased differentiation, but again, the effect was indirect, as VIP was found to be acting through astroglia (Zupan et al. 2000).

VIP may often act through immune cells, glial cells and particularly a potent factor termed activity-dependent neurotrophic factor (Waschek 1995; Brenneman et al. 1998). It is also possible that VIP plays its most important role in brain development after birth, at least in some species. VIP expression has been localized to the rat CNS in high concentrations during the first three post-natal weeks (Graber and Burgunder 1996).

VIP mRNA and protein have been isolated in E10 chick sympathetic ganglia, but this is at a time when the cells are making contact with target cells and beginning to differentiate (Ernsburger et al. 1997). This suggests that production of the hormone is signaling the onset of a maturing phenotype, rather than having an effect on development. Interestingly, these cells could be prompted by ciliary neurotrophic factor (CNTF) to express VIP at E7, but only *in vitro* and not *in vivo*.

PHI is a 27 amino acid peptide, structurally related to, and encoded on the same gene as VIP (Tatemoto and Mutt 1981). The two hormones are co-released, including in the brain, and since PHI will bind to VIP receptors at lowered affinity, it is possible that PHI plays a minor role in brain development. However, a receptor specific for PHI

was recently isolated from goldfish, and does not appear to be expressed in the brain (Tse et al. 2002). Localization of this PHI-specific receptor in the pituitary suggests a primary function that does not involve nervous system development (Tse et al. 2002).

Glucagon, and GLP I and II

Glucagon, GLP I and GLP II are encoded on the same gene (Fig. 1.1). Glucagon is a 29 amino acid peptide, best known for increasing glucose in the blood. GLP I is a 37 amino acid peptide best known for regulation of insulin in response to nutrient ingestion in the gut (Mojsov et al. 1987). GLP II plays a role in gut cell survival (Sherwood et al. 2000).

All three peptides are also produced by nerve cells during development, and have been found in the fetal rat brain (Lui et al. 1990). Glucagon appears to be more abundant earlier in development compared to the other two hormones, which peak after birth (Lui et al. 1990). This could mean that the primary roles of GLP-I and GLP-II involve post-natal development. However, as with VIP, GLP I may rescue embryonic cells from apoptosis; glutamate-induced apoptosis in E18 rat hippocampal neurons was reduced when treated with GLP-I agonists (Perry et al. 2002).

GHRH

GHRH has been identified in rat hypothalamus by E13 (Rodier et al. 1990). Several studies have been undertaken to show that GHRH is involved in determination of fate in differentiating chick nerve cells. Administration of GHRH to E3 neuroblasts both *in ovo* and *in vitro* increased the number of cells expressing a cholinergic

phenotype (Kentroti and Vernadakis 1990). E3 brain cells, treated *in ovo*, also showed an increase in catecholaminergic differentiation when treated with GHRH (Kentroti and Vernadakis 1989) and a decrease in GABAergic expression (Kentroti and Vernadakis 1991). In all three experimental protocols, the researchers were able to show that there is a brief window for these effects: no changes in fate were recorded after E6. Whether these increases in neurotransmitter expression were due to selective survival of subpopulations or to phenotypic induction was not determined.

A response to GHRH was also evident in chick spinal cord cells. Both proliferation and differentiation increased in populations of E10 cells, cultured in serum (Kentroti and Vernadakis 1992). In addition, GHRH caused an increase in cholinergic expression.

PACAP

Most research involving PACAP comes from the study of rat. Expression of PACAP has been recorded throughout the rat PNS by E14 (Lindholm et al. 1993; Nielsen et al. 1998). Expression was also reported at E14 in the CNS in the cerebral cortex (DiCicco-Bloom et al. 1998), cerebellum (Skoglosa et al. 1999), and whole brain (Tatsuno et al. 1994). Binding sites were identified in rat cerebellum at P8 (Basille et al. 1993; Villalba et al. 1997).

Increases in survival, proliferation and differentiation were all reported for cells cultured from E15.5 rat superior cervical ganglion in response to PACAP (DiCicco-Bloom and Deutsch 1992; Takei et al. 1998). Enhanced survival, as well as an increase in morphological and biochemical differentiation, was reported in dorsal root ganglion

cells from E20 to birth (Lioudyno et al. 1998). Mediation by the second messenger cAMP was suggested to be a key part in PACAP's ability to rescue neonatal rat superior cervical ganglion cells from apoptosis (Chang and Korolev 1997). These P1 cells undergo apoptosis when deprived of NGF, and the effect was shown to be correlated with a decrease in cAMP. Administration of PACAP to the cultures restored cAMP levels and rescued the cells.

In the CNS, PACAP has caused increased survival and proliferation in E13 rat olfactory neurons (Hansel et al. 2001). However, the hormone caused E13.5 cerebral cortex cells to exit the cell cycle. In cells that were still receiving a stimulatory message from basic fibroblast growth factor, administration of PACAP caused a decrease in proliferation and an increase in morphological differentiation (Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998). Because these cells expressed both the ligand and receptor, an autocrine/paracrine function was suggested (Lu and DiCicco-Bloom 1997). This appeared to be confirmed by the reduction, to a lesser extent, of proliferation in control cultures. Addition of serum to these cultures allowed for greater survival in both control and treated cultures, suggesting that some undefined trophic needs were supplied by the serum.

PACAP was shown to preferentially support a subset of rat neuroblasts cultured from E14 mesencephalon (DiCicco-Bloom and Deutsch 1992). The peptide increased the number of dopaminergic neurons that survived, without altering overall cell survival. Similar results were obtained when cells from E18 cerebellum were treated in culture: there was no effect on overall numbers, but the number of GABAergic neurons decreased (Skoglosa et al. 1999). This suggests an induction of phenotype from the

population, rather than an effect on proliferation. In addition, treatment of E17 rat hippocampal neurons with PACAP caused a preferential increase in the number of surviving septal cholinergic neurons (Takei et al. 2000).

Several groups have suggested that PACAP is able to prevent apoptosis in populations of cerebellar granule cells within the first post-natal week, as the cells continue to develop. An increase in cAMP, which has been shown to reduce apoptosis induced by deprivation of KCl, was recorded in response to PACAP (Cavallaro et al. 1996; Chang et al. 1996; Campard et al. 1997). Also, reduction in the extent of DNA fragmentation was observed (Villalba et al. 1997; Journot et al. 1998). Increases in survival and morphological differentiation were described (Gonzalez et al. 1997). An increase in cell number was reported, but it was not determined whether this was due to increased proliferation or decreased cell death, or both (Vaudry et al. 1999).

It is obvious from the work with rat neuroblasts that PACAP can enhance proliferation, and play a significant role in rescuing cells that would otherwise undergo apoptosis. Ample evidence supports a role for PACAP in causing cells to exit the cell cycle, with increases in morphological and biochemical differentiation in dorsal root ganglion, cerebral cortex, mesencephalon and cerebellum.

Expression of PACAP and its receptors has been reported at E9.5 in the mouse, in the developing neural tube and rhombencephalon (Sheward et al. 1996; Sheward et al. 1998; Waschek et al. 1998). Addition of PACAP to E10.5 mouse hindbrain cells in culture caused a decrease in DNA synthesis, suggesting a possible decrease in proliferation (Waschek et al. 1998). Prevention of apoptosis in early post-natal mouse cerebellar granule cells was shown to be through the primary PACAP receptor, PAC_1-R

(Tabuchi et al. 2001). PACAP may play an important role in early frog development as well, based on the presence of both the ligand and its receptor mRNAs in the neural tube only 18 h after fertilization (Hu et al. 2001).

In the chick PNS, an increase in cell number in the dorsal root ganglion and the lumbar motor column was recorded in embryos treated *in ovo* between E3.5 and E8.5 with PACAP (Arimura et al. 1994). These increases were due to a decrease in apoptosis, because massive proliferation has ceased in this area by this stage. Although no trophic effect could be measured in cultures of E10 sympathetic neurons in response to the peptide on its own, PACAP was effective in increasing survival in these cultures when they were prompted to undergo apoptosis by withdrawal of NGF (Przywara et al. 1998). The rescuing effect appeared to involve direct targeting of an apoptotic-associated caspase. An increase in survival of these cells in response to PACAP was reported by another group as well (Wakade and Leontiv 1998). It appears that PACAP plays a significant role in survival of cells in the PNS of early chick embryos.

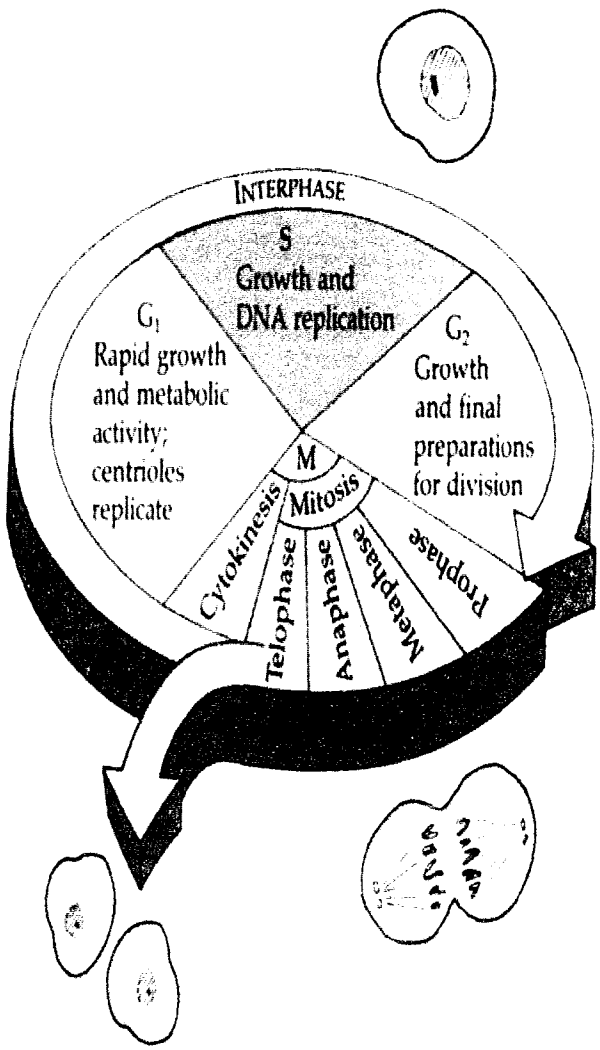
The importance of cell cycle regulation and apoptosis during development

Cell Cycle

The timing and rate of cell proliferation and differentiation is a vital aspect of development. If malfunction does not result in abortion, it can lead to a host of diseases and disorders. The complex process that controls these processes is referred to as the cell cycle (Fig. 1.4). Most people are aware that overproliferation of cells can lead to all forms of cancer, including those in the nervous system, but there are other diseases that result from dysregulation of the balance between proliferation and differentiation. The basis of fetal alcohol syndrome may lie in ethanol stimulation of proliferation which interferes with terminal differentiation (Armant and Saunders 1996). Epidemiological studies suggest that prenatal exposure to nicotine can lead to, among other things, abnormalities in cellular proliferation and differentiation that lead to improper neurodevelopment and a higher risk of psychiatric disorder (Ernst et al. 2001). Non-expression of the protein encoded by the necdin gene is thought to suppress proliferation and probably contributes to the neurobehavioural disorder called Prader-Willi syndrome (Muscatelli et al. 2000; Yoshikawa 2000; Ren et al. 2003).

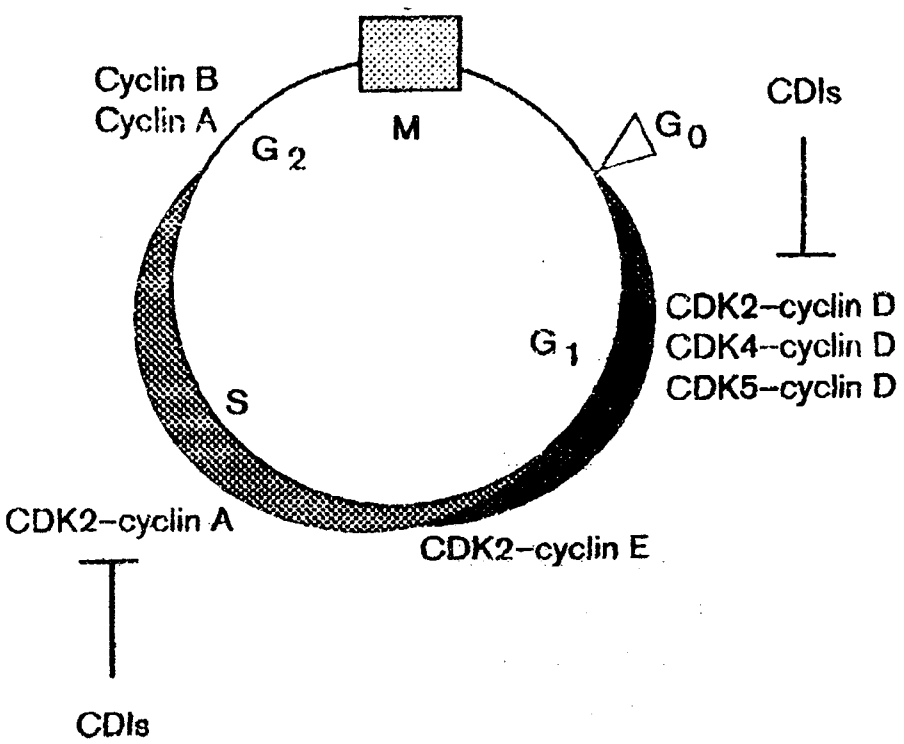
Obviously, understanding the cell cycle is one crucial area in the study of nervous system development. The cell cycle is driven by a complex host of proteins, which activate and deactivate each other (Hung et al. 1996; Crews and Mohan 2000).

Figure 1.4 Phases of cell cycle. Interphase consists of three phases: G_1 is a time when the cell is preparing to duplicate its DNA, synthesis (S) phase involves growth of the cell and DNA replication, and G_2 includes more growth, along with synthesis of final proteins required for division. The mitotic (M) phase consists of both mitosis and cytokinesis. (Campbell 1990).


INTERPHASE
S
Growth and
DNA replication
G1
Rapid growth
and metabolic
activity;
centrioles
replicate
G2
Growth
and final
preparations
for division
M
Mitosis
Cytokinesis
Telophase
Anaphase
Metaphase
Prophase

Much of the regulation takes place by the interaction of cyclins, which fluctuate throughout the cell cycle, and cyclin dependent kinases (CDKs) which do not. A number of cyclins have been identified, as well as the cell cycle compartment in which they are most abundant and active (Fig. 1.5). An active complex in any compartment consists of a phosphorylated CDK bound to an appropriate cyclin. As an example, when cyclin D is upregulated in G_1, and binds to CDK 2, 4, or 5, the cell begins the activities associated with this phase of the cell cycle. Degradation of the cyclins allow progression to the next stage in the cycle. The progression can be halted, however, by CDK inhibitors (CDIs). CDIs provide a method to arrest cells should they become damaged or if conditions are otherwise inadequate for replication, e.g. low levels of nutrients. One of the best known methods involves the major tumour suppressor gene p53, which activates proteins that will activate a CDI when DNA damage is detected (Hung et al. 1996). The quiescent stage of the cell cycle is also indicated on Fig. 1.5 (G_0); it is at this point that growth factors and hormones prompt a non-cycling cell to begin the process of division. Binding of the factor or hormone to the cell stimulates signal transduction cascades that activate genes for production of the necessary proteins, such as the D cyclins (Hung et al. 1996).

Figure 1.5 Cell cycle regulators. Progression of a cycling cell is mediated by a series of cyclins, each of which is more abundant during different phases of the cell cycle. The cyclin dependent kinases (CDKs) do not fluctuate during the cycle. An active complex consists of a cyclin bound to a CDK, which results in production of proteins for that part of the cycle. CDK inhibitors (CDIs) may stop the cell cycle progression. G_0 indicates the quiescent state, and M indicates mitosis. (Crews and Mohan 2000).


Cyclin B
Cyclin A
G 2
M
G 0
CDIs
CDK2–cyclin D
CDK4–cyclin D
CDK5–cyclin D
G 1
S
CDK2–cyclin A
CDK2–cyclin E
CDIs

Apoptosis

Just as properly regulated proliferation and differentiation are requisite to formation of healthy offspring, so too is the survival of an appropriate number of cells. Apoptosis is a naturally-occurring cell death which serves not only to rid the developing embryo of harmful cells, but also shapes the body, including the nervous system. This cell death is thought to be an active process, often involving production of RNA and proteins, rather than a passive degeneration, and as such constitutes an intrinsic cellular suicide program. It is characterized by condensation of chromatin and degradation of nuclear DNA, followed by pinching off of plasma membrane-bound vacuoles of cytoplasm and nucleus called apoptotic bodies, and finally phagocytosis of these vacuoles. These morphological characteristics of naturally-occurring cell death were first described in 1972 in two papers. The first was a comprehensive review that compared cell death that occurs during development and maintenance of homeostasis, to that which occurs during pathological cell death or necrosis (Wyllie et al. 1972). The second paper by the same group considered the implications of these findings, and named the process apoptosis (Kerr et al. 1972). Table 1.1 compares the major differences between apoptosis and necrosis.

Many biochemical features and pathways of apoptosis have since been identified, fueled by researchers who were investigating the nematode *Caenorhabditis elegans* in the 1980s and 1990s. They discovered several genes that appeared to be dedicated to an intrinsic cellular suicide program, and then identified homologous genes in mammals (Horvitz et al. 1982; Ellis and Horvitz 1986; Yuan et al. 1993; Hengartner and Horvitz 1994). This suggested the process existed purely to initiate physiological

Table 1.1 Comparison of some of the major features of apoptosis versus necrosis.

	Apoptosis	**Necrosis**
cells affected	individual	contiguous
energy	required	not required
membranes	remain intact	lose integrity
cytoplasm	shrinks	swells
chromatin	condenses, moves to periphery	aggregates loosely, disappears
DNA	cleaved into equal fragments	degraded
organelles	undamaged, compacted	destroyed
apoptotic bodies	yes	no
lysis	no	yes
inflammation	not normally	always
phagocytosis	tissue macrophages, surrounding cells	circulating macrophages

death, and was evolutionarily conserved. In fact, the process has been described in a unicellular protozoan parasite (Amiesen et al. 1995; Wellburn et al. 1996). It is now accepted that apoptosis is a constitutively expressed, but normally suppressed program, existing in virtually all cells with active nuclei (Jacobson et al. 1997). The program becomes activated by default when circumstances dictate (Raff et al. 1993; Wood et al. 1993; Jacobson et al. 1997).

Apoptosis may be set in motion by the binding of a ligand to a death receptor, by lack of binding of a hormone or growth factor to a receptor, or by an intracellular event (Oppenheim 1996). The complexity of the transduction system is demonstrated by the fact that binding of some steroid receptors can keep some cells alive but cause the death of others (Vaux and Korsmeyer 1999). Apoptosis often involves production of RNA and proteins (Vaux and Korsmeyer 1999). An important family of genes that regulate apoptosis is the Bcl-2 family. The genes in this family share a high degree of homology, yet some act to set apoptosis in motion, and others act to inhibit it (Table 1.2). The various members can dimerize with one another, and either enhance or antagonize the function of the other. In this way, it may be the proportion of pro-apoptotic to anti-apoptotic members that eventually determines the final fate of the cell (Jacobson et al. 1997; George 2002). Phosphorylation of these proteins may also play a role (Jacobson et al. 1997). A key set of proteins are the ICE family of proteases, also called caspases. These are *c*ysteine-specific *asp*arate prote*ase*s that exist as inactive zymogens in the cell. When apoptosis is triggered and adaptor proteins activate the caspases, they set in motion a proteolytic cascade that breaks down substrates necessary for structure and function, as well as DNA replication (O'Connor et al. 2000).

Table 1.2 Pro-apoptotic and anti-apoptotic members of the Bcl-2 gene family.

Anti-Apoptotic	Pro-apoptotic
A1	Bad
Bcl-2	Bak
Bcl-w	Bax
Mcl-1	Bid
Bfl-1	Bok
BHRF-1	Bik
Boo	Bim
Bcl-X_L	Bcl-X_S
	Bod
	DP5
	Hrk
	Mtd
	Nip3
	Noxa
	PUMA

Not every morphological or biochemical feature is found in every case described as programmed cell death or apoptosis (Clarke 1990; Schwartzman and Cidlowski 1993; Ueda and Shah 1994). In fact, some researchers have suggested that the terms "programmed cell death" and "apoptosis" may not actually describe the same phenomenon, and that naturally-occurring cell death actually includes several distinct forms of cell death (Majno and Joris 1995; Bursch 2001). In support, although some elements of naturally-occurring cell death have been preserved, various initiators, effectors and pathways can be utilized depending on the cell type and conditions (Deshmukh and Johnson 1997). For this thesis, the only distinction I will make is between naturally-occurring cell death, which I will call apoptosis, and that which results from pathological damage, which I will call necrosis.

Like overproliferation of cells, underexpression of apoptosis during development can also lead to cancers of the nervous system. Decreased apoptosis during the earliest stages of nervous system development is responsible for the devastating childhood cancer, neuroblastoma (Catchpoole and Lock 2001). Mutations to the gene that encodes the receptor for nerve growth factor (NGF) probably leads to increased apoptosis and a syndrome that includes insensitivity to pain and mental retardation (Indo 2002). Antiepileptic drugs can cause birth defects, and the cause appears to be excess apoptotic neurodegeneration in the fetus (Bittigau et al. 2002). Interference with the actions of growth factors can cause unnatural apoptosis during development in response to alcohol ingestion by the mother (Goodlett and Horn 2001). Paralytic poliomyelitis results from apoptotic death of cells in the CNS (Couderc et al. 2002).

However, apoptosis in the nervous system is probably most often associated with what has become known as the neurotrophic theory. This theory states that neurons are produced in excess and only those that receive neurotrophic support from the appropriate target tissue will survive (Raff et al. 1993). The neurotrophic theory is associated with the time during development when neurons are extending their axons to their target tissues, i.e. around the time of differentiation. What this process ensures is that neurons that do not reach the proper tissue, that are not healthy, or are in excess will be eliminated, and allows an orderly system wherein the final number of neurons matches the final number of target cells (Raff et al. 1993).

As well, proliferating cells that have made the commitment to differentiate, but are not forming synaptic connections, may undergo apoptosis (Blaschke et al. 1998). The neurotrophic theory would be completely ruled out in this case, so obviously other factors are involved, and could include limitation of neurotrophic factors or malfunctioning receptors, or other as yet unknown forms of differentiation-associated selection mechanisms (Blaschke et al. 1998). Control of neuronal survival relies on other factors as well, such as support from neighbouring cells of the same or a different type, and interactions with the substrate (Williams and Smith 1993; Jacobson et al. 1997). Even post-mitotic cells are now known to undergo apoptosis, possibly due to conflicting signals that cause an abortive attempt to re-enter the cell cycle (Freeman et al. 1994).

Objectives

The high conservation of PACAP across species and through time suggests an important role for this peptide. Although the major function is yet to be elucidated, PACAP appears to play an important role in nervous system development. It enhances cellular survival, regulates cellular proliferation and differentiation, and inhibits apoptosis. Recent discovery of a novel zinc-finger protein, Zac-1, that regulates cell cycle and apoptosis by separate mechanisms, also induced expression of the gene encoding PAC_1-R (Spengler et al. 1997). This elevates the likelihood that PACAP plays a vital role during development, as Zac-1 is only the second gene identified, the first being the major tumour suppressor p53, that can both induce cell cycle arrest and apoptosis.

PACAP, VIP and GHRH are the three hormones in the glucagon superfamily known to be most involved in nervous system development. Although VIP has high sequence identity, shares some receptors with PACAP and would seem a good second candidate for study, it is also likely that its effects on nervous system development are indirect, and possibly more important after birth. However, PACAP and GHRH are encoded on the same gene in non-mammalian vertebrates, and could therefore have a coordinated function. Also, GHRH has been shown to have an effect on chick nervous system development. Therefore, even though sequence identity between the two is low (Fig. 1.3), I chose to study GHRH in conjunction with PACAP.

I chose the chick as a study model because it is easy and inexpensive to obtain sterile embryos at any stage. Brain tissue can be collected before glial cells begin to develop, which allows development of cell cultures that are highly neuroblast-enriched.

My first objective was to develop serum-free cultures of neuroblasts at an age when cells would be both proliferating and differentiating, and hence, suitable for studying the effects of hormones on both those processes, as well as on apoptosis.

My second objective was to determine if the hormones had some effect on the cultured cells. To test this, I exposed the cells to various concentrations of the hormones, and measured changes in the intracellular messenger cAMP. When this experiment indicated that only PACAP had an effect on the cells, I discontinued my study of GHRH.

My third objective was to test the effects of PACAP on cell cycle and apoptosis. I tested several simple methods, but the complex nature of the cultures required more complex protocols. Because flow cytometry is a sensitive and versatile method for measuring even small changes in cell populations, I developed protocols based on its use. To understand the natural cycle of these cells, I first characterized movement of untreated cells through the cell cycle using proliferating cell nuclear antigen and propidium iodide. A modified version of this protocol measured changes in apoptosis, which was followed up by a protocol designed specifically to measure changes in apoptosis.

My fourth objective was to test the effects of PACAP on the cells directly, and indirectly by blocking the PACAP-specific receptor. I also blocked the cell cycle in both G_1, and G_2/M, and tested the ability of PACAP to release the cells from the block and return them to cycling.

My fifth and final goal was to discover if PACAP could increase differentiation in these cells. The method I chose also allowed confirmation of previous results. Using

isotope-coded affinity tag (ICAT) analysis, I compared the changes in protein production in untreated cell populations, and populations that had been treated with the PACAP-specific blocker, for both 5 and 24 h.

REFERENCES

Adams, BA, DW Lescheid, ED Vickers, LA Crim and NM Sherwood (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.

Alderson, RF, AL Alterman, Y-A Barde and RM Lindsay (1990). "Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture." Neuron **5**: 297-306.

Amiesen, JC, T Idziorek, O Billaut-Mulot, M Loyens, J-P Tissier, A Pontentier and A Ouaissi (1995). "Apoptosis in a unicellular eukaryote (*Trypanosoma cruzi*): implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival." Death and Differentiation **2**: 285-300.

Arimura, A (1992). "Pituitary adenylate cyclase-activating polypeptide (PACAP): discovery and current status of research." Regulatory Peptides **37**: 287-303.

Arimura, A (2003). PACAP: discovery, genes, receptors. Pituitary Adenylate Cyclase-Activating Polypeptide. H Vaudry and A Arimura. Norwell, MASS, Kluwer Academic Publishers**:** 1-24.

Arimura, A, A Somogyvari-Vigh, C Weill, RC Fiore, I Tatsuno, V Bay and DE Brenneman (1994). "PACAP functions as a neurotrophic factor." Annals of the New York Academy of Science, USA **739**: 228-243.

Armant, DR and DE Saunders (1996). "Exposure of embryonic cells to alcohol: contrasting effects during preimplantation and postimplantation development." Seminars in Perinatology **20**: 127-139.

Ayer-Lelievre, C, L Olson, T Ebendal, A Seiger and H Persson (1988). "Expression of the brain-derived neurotrophic factor gene in hippocampal neurons." Science **240**: 1339-1441.

Baserga, R, Ed. (1981). Tissue Growth Factors. Springer-Verlag.New York

Basille, M, BJ Gonzalez, P Leroux, L Jeandel, A Fournier and H Vaudry (1993). "Localization and characterization of PACAP receptors in the rat cerebellum during development: evidence for a stimulatory effect of PACAP on immature cerebellar granule cells." Neuroscience **57**: 329-338.

Baulieu, E-E (1990). Hormones: a complex communication network. Hormones. E-E Baulieu and PA Kelly. New York, Chapman and Hall**:** 3-169.

Bittigau, P, M Sifringer, K Genz, E Reith, D Pospischil, S Govindarajalu, M Dziet, S Pesditschek, I Mai, K Dikrainian, JW Olney and C Ikonomidou (2002). "Antiepileptic drugs and apoptotic neurodegeneration in the developing brain." Proceedings of the National Academy of Science USA **99**: 15089-15094.

Blaschke, AJ, JA Weiner and J Chun (1998). "Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system." Journal of Comparative Neurology **396**: 39-50.

Brenneman, DE and GA Foster (1987). "Structural specificity of peptides influencing neuronal survival during development." Peptides **8**: 687-694.

Brenneman, DE, GW Glazner, JM Hall, J Hauser, A Davidson and I Gozes (1998). "VIP neurotrophism in the central nervous system: multiple effectors and identification of a femtomolar-acting neuroprotective peptide." Annals of the New York Academy of Science, USA **865**: 207-212.

Brodal, P (1992). The Central Nervous System: Structure and Function. New York, Oxford University Press.

Bursch, W (2001). "The autophagosomal-lysosomal compartment in programmed cell death." Cell Death and Differentiation **8**: 569-581.

Campard, KP, C Crochemore, F Rene, D Monnier, B Koch and JP Loeffler (1997). "PACAP Type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA pathway." DNA and Cell Biology **16**: 323-333.

Campbell, NA (1990). Biology. Redwood City, CA, Benjamin/Cummings Publishing Company.

Campbell, RM and CG Scanes (1992). "Evolution of the growth hormone-releasing factor (GRF) family of peptides." Growth Regulation **2**: 175-191.

Catchpoole, DR and RB Lock (2001). "The potential tumour suppressor role for caspase-9 (CASP9) in the childhood malignancy, neuroblastoma." European Journal of Cancer **37**: 2217-2221.

Cavallaro, S, A Copani, V D'Agata, S Musco, S Petralia, C Ventra, F Stivala, S Travali and PL Canonico (1996). "Pituitary adenylate cyclase-activating polypeptide prevents apoptosis in cultured cerebellar granule neurons." Molecular Pharmacology **50**: 60-66.

Chang, JY and VV Korolev (1997). "Cyclic AMP and sympathetic neuronal programmed cell death." Neurochemistry International **31**: 161-167.

Chang, JY, VV Korolev and J-Z Wang (1996). "Cyclic AMP and pituitary adenylate cyclase-activating polypeptide (PACAP) prevent programmed cell death of cultured rat cerebellar granule cells." Neuroscience Letters **206**: 181-184.

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

Clarke, PGH (1990). "Developmental cell death: morphological diversity and multiple mechanisms." Anatomy and Embryology (Berlin) **181**: 195-213.

Couderc, T, F Guivel-Benhassine, V Calaora, AS Gosselin and B Blondel (2002). "An *ex vivo* murine model to study poliovirus-induced apoptosis in nerve cells." Journal of General Virology **83**: 1925-1930.

Crews, CM and R Mohan (2000). "Small-molecule inhibitors of the cell cycle." Current Opinion in Chemical Biology **4**: 47-53.

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

Desbuquois, B (1990). Gastrointestinal hormones. Hormones. E-E Baulieu and PA Kelly. New York, Chapman and Hall**:** 541-590.

Deshmukh, M and EMJ Johnson (1997). "Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons." Molecular Pharmacology **51**: 987-906.

DiCicco-Bloom, E and PJ Deutsch (1992). "Pituitary adenylate cyclase activating polypeptide (PACAP) potently stimulates mitosis, neuritogenesis and survival in cultured rat sympathetic neuroblasts." Regulatory Peptides **37**: 319.

DiCicco-Bloom, E, N Lu, JE Pintar and J Zhang (1998). "The PACAP ligand/receptor system regulates cerebral cortical neurogenesis." Annals of the New York Academy of Science, USA **865**: 274-289.

Ellis, HM and HR Horvitz (1986). "Genetic control of programmed cell death in the nematode *C. elegans*." Cell Death and Differentiation **44**: 817-829.

Ernsburger, U, H Patzke and H Pohrer (1997). "The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation." Mechanisms of Development **68**: 115-126.

Ernst, M, ET Moolchan and ML Robinson (2001). "Behavioural and neural consequences of prenatal exposure to nicotine." Journal of the American Academy of Child and Adolescent Psychiatry **40**: 630-641.

Fradinger, EA and NM Sherwood (2000). "Characterization of the gene encoding both growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP) in the zebrafish." Molecular and Cellular Endocrinology **165**: 211-219.

Freeman, RS, S Estus and EM John (1994). "Analysis of cell-cycle-related gene expression in post-mitotic neurons: selective induction of cyclin D1 during programmed cell death." Neuron **12**: 343-355.

George, DA (2002). The mitochondria and apoptosis. Innovations in Cellular Dynamics, BD Biosciences.

Glowinski, J (1990). Nerve cells and their chemical messages. Hormones. E-E Baulieu and PA Kelly. New York, Chapman and Hall**:** 181-186.

Gonzalez, BJ, M Basille, D Vaudry, A Fournier and H Vaudry (1997). "Pituitary adenylate cyclase-activating polypeptide promotes cell survival and neurite outgrowth in rat cerebellar neuroblasts." Neuroscience **78**: 419-430.

Goodlett, CR and KH Horn (2001). "Mechanisms of alcohol-induced damage to the developing nervous system." Alcohol Research and Health **25**: 175-184.

Graber, M and JM Burgunder (1996). "Ontogeny of vasoactive intestinal peptide gene expression in rat brain." Anatomy and Embryology (Berlin) **194**: 595-605.

Gressens, P, JM Hill, I Gozes, M Fridkin and DE Brenneman (1993). "Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos." Nature **362**: 155-157.

Gressens, P, JM Hill, B Paindaveine, I Gozes, M Fridkin and DE Brenneman (1994). "Severe microcephaly induced by blockade of vasoactive intestinal peptide function in the primitive neuroepithelium of the mouse." Journal of Clinical Investigation **94**: 2020-2027.

Gressens, P, B Paindaveine, JM Hill, P Evrard and DE Brenneman (1998). "Vasoactive intestinal peptide shortens both G_1 and S phases of neural cell cycle in whole postimplantation cultured mouse embryos." European Journal of Neuroscience **10**: 1734-1742.

Gubler, U, JJ Monhan, PT Lomedico, RS Bhatt, KJ Collier, BJ Hoffman, P Böhlen, F Esch, N Ling, F Zeytin, P Brazeau, MS Poonian and LP Gage (1983). "Cloning and sequence analysis of cDNA for the precursor of human growth hormone-releasing factor, somatocrinin." Proceedings of the National Academy of Science, USA **80**: 4311-4314.

Guillemin, R (1986). Hypothalamic control of pituitary functions. The Growth Hormone Releasing Factor. Liverpool, U.K., Liverpool University Press. **The Sherrington Lectures XVII:** 73.

Guillemin, R, P Brazeau, P Bohlen, F Esch, N Ling and WB Wehrenberg (1982). "Growth hormone-releasing factor from a human pancreatic tumor that caused acomegaly." Science **218**: 585-587.

Hansel, D, V May, BA Eipper and GV Ronnett (2001). "Pituitary adenylate cyclase-activating polypeptides and alpha-amidation in olfactory neurogenesis and neuronal survival *in vivo*." Journal of Neuroscience **21**: 4625-4636.

Hartikka, J and F Hefti (1988). "Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons *in vitro*." Journal of Neuroscience Research **21**: 352-364.

Hengartner, MO and H Horvitz, R. (1994). "*C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2." Cell Death and Differentiation **76**: 665-676.

Hill, JM, SK McCune, RJ Alvero, GW Glazner and DE Brenneman (1996). "VIP regulation of embryonic growth." Annals of the New York Academy of Science USA **805**: 268-269.

Horvitz, H, H Ellis and P Sternberg (1982). "Programmed cell death in nematode development." Neuroscience Commentaries **1**: 56-65.

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

Hoyle, CHV (1998). "Neuropeptide families: evolutionary perspectives." Regulatory Peptides **73**: 1-33.

Hu, Z, V Lelievre, WI Rodriguez, J Tam, JW Cheng, S Cohen-Corey and JA Wascheck (2001). "Embryonic expression of pituitary adenylyl cyclase-activating polypeptide and its selective type I receptor gene in the frog *Xenopus laevis* neural tube." The Journal of Comparative Neurology **441**: 226-275.

Hung, DT, TF Jamison and SL Schreiber (1996). "Understanding and controlling the cell cycle with natural products." Chemistry and Biology **3**: 623-639.

Indo, Y (2002). "Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV. Clinical, biological and molecular aspects of mutations in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor." Clinical Autonomic Research **12 (Suppl)**: I 20-32.

Jacobson, MD, M Weil and MC Raff (1997). "Programmed cell death in animal development." Cell Death and Differentiation **88**: 347-354.

Journot, L, M Villalba and J Bockaert (1998). "PACAP-38 protects cerebellar granule cells from apoptosis." Annals of the New York Academy of Science, USA **865**: 100-110.

Kentroti, S and A Vernadakis (1989). "Growth hormone-releasing hormone influences neuronal expression in developing chick brain. I. Catecholaminergic neurons." Developmental Brain Research **49**: 275-280.

Kentroti, S and A Vernadakis (1990). "Growth hormone-releasing hormone and somatostatin influence neuronal expression in developing chick brain. II. Cholinergic neurons." Brain Research **512**: 297-303.

Kentroti, S and A Vernadakis (1991). "Growth hormone-releasing hormone and somatostatin influence neuronal expression in developing chick brain. III. GABA-ergic neurons." Brain Research **562**: 34-38.

Kentroti, S and A Vernadakis (1992). "Neuron-enriched cultures derived from spinal cord of 10-day-old chick embryos: influence of neuropeptides on neuronal survival, proliferation and cholinergic expression." International Journal of Developmental Neuroscience **10**: 535-544.

Kerr, JFR, AH Wyllie and AR Currie (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." British Journal of Cancer **26**: 239-257.

Kimura, C, S Ohkubo, K Ogi, M Hosoya, Y Itoh, H Onda, A Miyata, L Jiang, RR Dahl, HH Stibbs, A Arimura and M Fujino (1990). "A novel peptide which stimulates adenylate cyclase: molecular cloning and characterization of the ovine and human cDNAs." Biochemical and Biophysical Research Communications **166**: 81-89.

Kumar, S and SB Hedges (1998). "A molecular timescale for vertebrate evolution." Nature **392**:917-920.

Laburthe, M, A Couvineau and P Nicole (2003). Molecular pharmacology and structure-function analysis of PACAP/VIP receptors. Pituitary Adenylate Cyclase-Activating Polypeptide. H Vaudry and A Arimura. Dordrecht, Kluwer Academic Publishers Group: 69-94.

Lindholm, D, G Dechant, C-P Heisenberg and H Thoenen (1993). "Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity." European Journal of Neuroscience **5**: 1455-1464.

Ling, N, F Esch, P Böhlen, P Brazeau, WB Wehrenberg and R Guillemin (1984). "Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor." Proceedings of the National Academy of Science, USA **81**: 4302-4306.

Lioudyno, M, Y Skoglosa, N Takei and D Lindholm (1998). "Pituitary adenylate cyclase-activating polypeptide (PACAP) protects dorsal root ganglion neurons from death and induces calcitonin gene-related peptide (CGRP) immunoreactivity in vitro." Journal of Neuroscience Research **51**: 243-356.

Lu, N and E DiCicco-Bloom (1997). "Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells." Proceedings of the National Academy of Science, USA **94**: 3357-3362.

Lui, EY, SL Asa, DJ Drucker, YC Lee and PL Brubaker (1990). "Glucagon and related peptides in fetal rat hypothalamus *in vivo* and *in vitro*." Endocrinology **126**: 110-117.

Majno, G and I Joris (1995). "Apoptosis, oncosis, and necrosis." American Journal of Pathology **146**: 315.

Mangoura, D, N Sakellaridis and A Vernadakis (1990). "Evidence for plasticity in neurotransmitter expression in neuronal cultures derived from 3-day-old chick embryo." Developmental Brain Research **51**: 93-101.

Matsuda, K, T Oshida, Y Nagano, K Kashimoto, T Yatohgo, H Shimomura, D Shioda, A Arimura and M Uchiyama (1998). "Purification and primary structure of pituitary adenylate cyclase activating polypeptide (PACAP) from the brain of an elasmobranch, stingray, *Dasyatis akajei*." Peptides **19**: 1489-1495.

Matsuda, K, Y Takei, J Katoh, S Shioda, A Arimura and M Uchiyama (1997). "Isolation and structural characterization of pituitary adenylate cyclase-activating polypeptide (PACAP)-like peptide from the brain of a teleost, stargazer, *Uranoscopus japonicus*." Peptides **18**: 723-727.

Mayo, KE (1992). "Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone." Molecular Endocrinology **6**: 1734-1744.

Mayo, KE, GM Cerelli, RV Lebo, BD Bruce, MG Rosenfeld and RM Evans (1985). "Gene encoding human growth hormone-releasing factor precursor: structure, sequence, and chromosomal assignment." Proceedings of the National Academy of Science, USA **82**: 63-67.

Mayo, KE, TL Miller, V DeAlmeida, J Zheng and PA Godfrey (1996). "The growth-hormone-releasing hormone receptor: signal transduction, gene expression, and physiological function in growth regulation." Annals of the New York Academy of Science **805**: 184-203.

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

McRory, JE, DB Parker, S Ngamvongchon and NM Sherwood (1995). "Sequence and expression of cDNA for pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish." Molecular and Cellular Endocrinology **108**: 169-177.

McRory, JE, RL Parker and NM Sherwood (1997). "Expression and alternative processing of a chicken gene encoding both growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP)." DNA and Cell Biology **16**: 95-102.

Mitchell, LG, JA Mutchmor and WD Dolphin (1988). Zoology. Don Mills, Ontario, Benjamin/Cummings: 689.

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

Miyata, A, L Jiang, R Dahl, C Kitada, K Kubo, M Fujino, N Minamino and A Arimura (1990). "Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38)." Biochemical and Biophysical Research Communications **170**: 643-648.

Mojsov, S, G Weir and J Habener (1987). "Insulinotropin: glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas." Journal of Clinical Investigation **79**: 616-619.

Muller, J-M, V Lelievre, L Becq-Giraudon and A-C Meunier (1995). "VIP as a cell-growth and differentiation neuromodulator role in neurodevelopment." Molecular Neurobiology **10**: 115-134.

Muscatelli, F, DN Arbrous, A Massacrier, I Boccaccio, M Le Moal, P Cau and H Cremer (2000). "Disruption of the mouse Necdin gene results in hypothalamic and behavioural alterations reminiscent of the human Prader-Willi syndrome." Human Molecular Genetics **9**: 3101-3110.

Nielsen, HS, J Hannibal and J Fahrenkrug (1998). "Embryonic expression of pituitary adenylate cyclase-activating polypeptide in sensory and autonomic ganglia and in spinal cord of the rat." Journal of Comparative Neurology **394**: 403-415.

O'Connor, L, DCS Huang, LA O'Reilly and A Strasser (2000). "Apoptosis and cell division." Current Opinion in Cell Biology **12**: 257-263.

Ogi, K, C Kimura, H Onda, A Arimura and M Fujino (1990). "Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP)." Biochemical and Biophysical Research Communications **173**: 1271-1279.

Ohkubo, S, C Kimura, K Ogi, K Okazaki, M Hosoya, H Onda, A Miyata, A Arimura and M Fujino (1992). "Primary structure and characterization of the precursor to human pituitary adenylate cyclase activating polypeptide." DNA and Cell Biology **11**: 21-30.

Okazaki, K, Y Itoh, K Ogi, S Ohkubo and H Onda (1995). "Characterization of the murine PACAP mRNA." Peptides **16**: 1295-1299.

Oppenheim, RW (1996). "Neurotrophic survival molecules for motoneurons: an embarrassment of riches." Neuron **17**: 195-197.

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

Perry, T, NJ Haughey, MP Mattson, JM Egan and NH Greig (2002). "Protection and reversal of excitotoxic neuronal damage by glucagon-like peptide I and exendin-4." Journal of Pharmacology and Experimental Therapeutics **302**: 881-888.

Pincus, DW, EM DiCicco-Bloom and IB Black (1990). "Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts." Nature **343**: 564-575.

Przywara, DA, JS Kulkarni, TD Wakade, DV Leontiv and AR Wakade (1998). "Pituitary adenylyl cyclase-activating polypeptide and nerve growth factor use the proteasome to rescue nerve growth factor-deprived sympathetic neurons cultured from chick embryos." Journal of Neurochemistry **71**: 1889-1897.

Raff, MC, BA Barres, JF Burne, HS Coles, Y Ishizaki and MD Jacobson (1993). "Programmed cell death and the control of cell survival: lessons from the nervous system." Science **262**: 695-700.

Ren, J, S Lee, S Pagliardini, M Gerard, CL Stewart, JJ Greer and R Wevrick (2003). "Absence of Ndn, encoding the Prader-Willi syndrome-deleted gene necdin, results in congenital deficiency of central respiratory drive in neonatal mice." Journal of Neuroscience **23**: 1569-1573.

Rivier, J, J Speiss, M Thorner and W Vale (1982). "Characterization of a growth hormone-releasing factor from a pancreatic islet tumour." Nature **300**: 276-278.

Rodier, PM, B Kates, WA White and CJ Phelps (1990). "Birthdates of the growth hormone releasing factor cells of the rat hypothalamus: an autoradiographic study of immmunocytochemically identified neurons." Journal of Comparative Neurology **291**: 363-372.

Schwartz, LM and BA Osborne (1995). Preface. Methods in Cell Biology. Toronto, Academic Press. **46**.

Schwartzman, RA and JA Cidlowski (1993). "Apoptosis: the biochemistry and molecular biology of programmed cell death." Endocrine Reviews **14**: 133-151.

Sherwood, NM, SL Krueckl and JR McRory (2000). "The origin and function of the PACAP/glucagon superfamily." Endocrine Reviews **21**: 619-670.

Sheward, JW, EM Lutz, AJ Copp and AJ Harmar (1998). "Expression of PACAP, and PACAP type 1 (PAC1) receptor mRNA during development of the mouse embryo." Developmental Brain Research **109**: 245-253.

Sheward, WJ, EM Lutz and AJ Harmar (1996). "Expression of pituitary adenylate cyclase-activating polypeptide receptors in the early mouse embryo as assessed by reverse transcription polymerase chain reaction and *in situ* hybridization." Neuroscience Letters **216**: 45-48.

Skoglosa, Y, C Patrone and D Lindholm (1999). "Pituitary adenylate cyclase activating polypeptide is expressed by developing rat Purkinje cells and decreases the number of cerebellar gamma-amino butyric acid positive neurons in culture." Neuroscience Letters **265**: 207-210.

Spengler, D, M Villalba, A Hoffman, C Pantaloni, S Houssami, J Bockaert and L Journot (1997). "Regulation of apoptosis and cell cycle arrest by Zac-1, a novel zinc finger protein expressed in the pituitary gland and the brain." EMBO Journal **16**: 2814-2825.

Spiess, J, J Rivier and W Vale (1983). "Characterization of rat hypothalamic growth hormone-releasing factor." Nature **303**: 532-535.

Suh, J, N Lu, A Nicot, I Tatsuno and E DiCicco-Bloom (2001). "PACAP is an anti-mitogenic signal in developing cerebral cortex." Nature **4**: 1-2.

Suhr, ST, JO Rahal and KE Mayo (1989). "Mouse growth hormone-releasing hormone: precursor structure and expression in brain and placenta." Molecular Endocrinology **3**: 1693-1700.

Tabuchi, A, M Koizumi, J Nakatsubo, T Yaguchi and M Tsuda (2001). "Involvement of endogenous PACAP expression in the activity-dependent survival of mouse cerebellar granule cells." Neuroscience Research **39**: 85-93.

Takei, N, Y Skoglosa and D Lindholm (1998). "Neurotrophic and neuroprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on mesencephalic dopaminergic neurons." Journal of Neuroscience Research **54**: 698-706.

Takei, N, E Torres, A Yuhara, H Jongsma, C Otto, L Korhonen, Y Abiru, Y Skoglosa, G Schutz, H Hatanaka, MV Sofroniew and D Lindholm (2000). "Pituitary adenylate cyclase-activating polypeptide promotes the survival of basal forebrain cholinergic neurons *in vitro* and *in vivo*: comparison with effects of nerve growth factor." European Journal of Neuroscience **12**: 2273-2280.

Tatemoto, K and V Mutt (1981). "Isolation and characterization of the intestinal peptide porcine PHI (PHI-27), a new member of the glucagon-secretin family." Proceedings of the National Academy of Science, USA **78**: 6603-6607.

Tatsuno, I, A Somogyvari-Vigh and A Arimura (1994). "Developmental changes of pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor in the rat brain." Peptides **15**: 55-60.

Tse, DL-Y, RT-K Pang, AO-L Wong, S-M Chan, H Vaudry and BK-C Chow (2002). "Identification of a potential receptor for both peptide histidine isoleucine and peptide histidine valine." Endocrinology **143**: 1327-1336.

Ueda, N and SV Shah (1994). "Apoptosis." Journal of Laboratory and Clinical Medicine **124**: 169-177.

Ulloa-Aguirre, A and PM Conn (2000). G protein coupled receptors and G proteins. Principles of Molecular Regulation. PM Conn and AR Means. Totowa, New Jersey, Humana Press: 3-25.

Vaudry, D, BJ Gonzalez, M Basille, A Fournier and H Vaudry (1999). "Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development." Proceedings of the National Academy of Science, USA **96**: 9415-9420.

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

Vaux, DL and SJ Korsmeyer (1999). "Cell death in development." Cell Death and Differentiation **96**: 245-254.

Villalba, M, J Bockaert and L Journot (1997). "Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway." Journal of Neuroscience **17**: 83-90.

Wakade, AR and D Leontiv (1998). "Sympathetic neurons of the chick embryo are rescued by PACAP from apoptotic death." Annals of the New York Academy of Science, USA **865**: 176-180.

Waschek, JA (1995). "Vasoactive intestinal peptide: an important trophic factor and developmental regulator?" Developmental Neuroscience **17**: 1-7.

Waschek, JA, RA Casillas, TB Nguyen, EM DiCicco-Bloom, EM Carpenter and WI Rodriguez (1998). "Neural tube expression of pituitary adenylate cyclase-activating polypeptide (PACAP) and receptor: potential role in patterning and neurogenesis." Proceedings of the National Academy of Science, USA **95**: 9602-9607.

Waschek, JA, J Ellison, DT Bravo and V Handley (1996). "Embryonic expression of vasoactive intestinal peptide (VIP) and VIP receptor genes." Journal of Neurochemistry **66**: 1762-1765.

Wellburn, SC, C Dale, D Ellis, RP Beecroft and TW Pearson (1996). "Apoptosis in procyclic *Trypanosoma brucei rhodesiense in vitro*." Death and Differentiation **3**: 229-236.

Williams, GT and CA Smith (1993). "Molecular regulation of apoptosis: genetic controls on cell death." Cell Death and Differentiation **74**: 777-779.

Wood, KA, B Dipasquale and RJ Youle (1993). "*In situ* labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum." Neuron **11**: 621-632.

Wyllie, AH, JFR Kerr and AR Currie (1972). "Cell death: the significance of apoptosis." Inernational Review of Cytology **68**: 251-306.

Yoo, SJ, S You, H Kim, SC Kim, YJ Choi, M El Halawani, J Farris and DN Foster (2000). "Molecular cloning and characterization of alternatively spliced transcripts of the turkey pituitary adenylate cyclase-activating polypeptide." General and Comparative Endocrinology **120**: 326-335.

Yoshikawa, K (2000). "Molecular mechanisms of differentiation and death of human neurons: With special reference to necdin and APP." Japanese Journal of Neuropsychopharmacology **20**: 155-159. (Abstract only).

Yuan, J, S Shaham, S Ledoux, HM Ellis and HR Horvitz (1993). "The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme." Cell Death and Differentiation **75**: 641-652.

Zupan, V, A Nehlig, P Evrard and P Gressens (2000). "Prenatal blockade of vasoactive intestinal peptide alters cell death and synaptic equipment in the murine neocortex." Pediatric Research **47**: 53-63.

CHAPTER 2

PACAP but not GHRH has an autocrine/paracrine role in neuroblast-enriched brain cell cultures

A version of this chapter has been published as:

Nola M. Erhardt, Erica A. Fradinger, Laura A. Cervini, Jean E. River, and Nancy M. Sherwood (2001) Early Expression of Pituitary Adenylate Cyclase-Activating Polypeptide and Activation of its Receptor in Chick Neuroblasts. **Endocrinology 142: 1616-1625**

INTRODUCTION

PACAP and GHRH are both produced in the hypothalamus of the central nervous system, as well as in the peripheral nervous system, and in non-neural tissues. Both have a range of functions, inside and outside the nervous system.

GHRH is best known for release of growth hormone from the pituitary, and is also involved in paracrine and endocrine secretions, fetal growth, and in the functions of the immune, reproductive and digestive systems (Sherwood et al. 2000). GHRH affects the cell cycle outside the nervous system, by enhancing proliferation and differentiation of pituitary cells (Mayo et al. 1988, 1996). GHRH also affects the cell cycle within the central nervous system of chick, by increasing survival and proliferation of E10 neuroblasts (Kentroti and Vernadakis 1992). As well, GHRH can influence the differential fate of cells when applied early in development (Kentroti and Vernadakis 1989, 1990, 1991).

PACAP is known to have many functions. It is active in smooth and cardiac muscle function, bone metabolism, immune system function, and paracrine, endocrine and exocrine secretions (Sherwood et al. 2000). PACAP also regulates the cell cycle outside the nervous system, by enhancing survival, inhibiting apoptosis, and through changes in proliferation and differentiation. The particular effect depends on concentration, interaction with other factors, and utilization of receptor variants (Sherwood et al. 2000). In rodents, a response to physiological doses of PACAP has been recorded for primordial germ cells, splenocytes, thymocytes, astrocytes and follicle cells (Tatsuno et al. 1991; Delgado et al. 1996; Pesce et al. 1996; Moroo et al. 1998; Lee et al. 1999; Vallejo and Vallejo 2002).

Within the nervous system, regulation of development has been well documented for PACAP during the past 14 years. Most of what is known comes from work with rodents. PACAP increases cell numbers by enhancing cell survival and inhibiting apoptosis (DiCicco-Bloom and Deutsch 1992; Canonico et al. 1996; Cavallaro et al. 1996; Chang et al. 1996; Campard et al. 1997; Gonzalez et al. 1997; Villalba et al. 1997; Journot et al. 1998; Lindholm et al. 1998; Lioudyno et al. 1998; Takei et al. 1998; Vaudry et al. 1999; Vaudry et al. 2000; Hansel et al. 2001; Tabuchi et al. 2001), and regulating cell cycle by enhancing or inhibiting proliferation and differentiation (DiCicco-Bloom and Deutsch 1992; Gonzalez et al. 1997; Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998; Waschek et al. 1998; Suh et al. 2001; Lelievre et al. 2002).

There are few reports on the involvement of PACAP during chick brain development. An increase in cell numbers between E3.5 and E9 in the dorsal root ganglion and lumbar motor column, probably due to a decrease in apoptosis, has been reported (Arimura et al. 1994).

Generally, little is known about the role of hormones and growth factors during the early stages of chick brain development. There is evidence to suggest that fibroblast growth factor is required *in utero* for acquisition of neural cell fate (Wilson et al. 2000), and plays a role in organization of the midbrain by E2 (Crossley et al. 1996). Two soluble factors secreted by specialized cells of the developing neural tube, bone morphogenetic protein and sonic hedgehog, are active by E3.5, inducing differentiation of neural cell types in the brain (Dale et al. 1997; Lee and Jessell 1999).

There are reports of early involvement by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor I (IGF-I). NGF

mRNA transcripts have been located in the chick brain at E3.5 (Ebendal and Persson 1988), and receptors are present by at least E5 (Heuer et al. 1990). Increases in survival have been recorded in E3.5 cell populations in response to NGF (Rahman et al. 1994). BDNF was expressed in chick embryos at E4 (Hallbook et al. 1993). Receptors that bind these neurotrophins are present at this stage (Nagtegaal et al. 1998). A peak in the production of IGF-I peptide and receptors was reported between E3 and E6 in chick brain (Bassas et al. 1989; Hernandez-Sanchez et al. 1995).

The hypothesis for my thesis was that PACAP, perhaps in conjunction with GHRH, would affect early brain development by regulating cell cycle and apoptosis. My first objective was to develop neuroblast-enriched cell cultures from early chick brain tissue, which would consist of both proliferating and differentiating cells. Because I did not know how long it would take for changes in the system to become detectable in response to hormones, I wanted to develop cultures which did not require chemical dissociation methods, since at least one of these chemicals (trypsin) has been shown to disrupt receptors for up to 48 hours after treatment (Banker and Goslin 1991). I also wanted to develop serum-free cultures, because serum can promote glial cell proliferation (Mattson 1995), and can confound the effects of hormones, for example, by increasing proliferation due to mitogenic factors in the serum (Baserga 1981). As well, different lots of serum vary in content, and may cause poor consistency of results (Freshney 1988). I also wanted to positively identify the cultured cells as neuroblasts.

The final part of this goal was to show that GHRH and PACAP had some effect on the cells by measuring changes in the intracellular messenger cAMP in response to the hormones. As well, in conjunction with a colleague, Erica A. Fradinger, we tested

for the presence of PACAP in the cells using immunocytochemistry (N.M.E.) and Western blot (E.A.F.). Erica Fradinger also contributed by determining whether mRNA for both peptides and their receptors were present in the cells.

MATERIALS AND METHODS

Cell culture

Preliminary work established a dissecting, dissociating and culturing protocol suitable for testing the effect of PACAP and GHRH on chick neuroblasts. An embryonic age at which to dissect, a chemical-free method of dissociating brain tissue into a single cell suspension, and a defined medium that would support the cells for at least several days had to be established. Unless otherwise specified, chemicals and reagents were purchased from Sigma (Oakville, ON).

Embryonic Age

Initial experiments involved dissection of embryonic tissue between E1.5 and E4, equivalent to stages 10-24 (Hamburger and Hamilton 1992). My goal was to find a developmental stage at which cultures could develop, without contamination by glia and others types of cells, such as blood cells. At the same time, the cultures needed to consist of cells that would not just proliferate, but differentiate as well. Dissections were restricted to embryos no younger than E1.5 because smaller embryos yielded small amounts of tissue and seemed less likely to produce both proliferating and differentiating cells, and to embryos no older than E4 because gliogenesis begins at E8 in the chick (Bellairs and Osmond 1998). Dissecting methods were taken from Freshney

(1988), with some modifications. Eggs were cleaned with 70% ethanol, and the shell and chorioallantoic membrane peeled away with sterile forceps. The embryos were quickly removed to a dry plastic petri dish where the heads were at once removed and placed in approximately 10 ml PBS (Invitrogen, Burlington, ON) containing 500 μg/ml streptomycin and 500 U/ml penicillin, on ice.

To simplify the search for an appropriate embryonic age, traditional chemical dissociation methods were used, which also followed Freshney (1988), with some modifications. All were based on the use of trypsin. A warm trypsin method was briefly assessed, but abandoned in favour of a cold trypsin method, which allowed for a longer period of time during which the enzyme could penetrate the tissue with lowered enzymatic activity, as this produced a higher yield of healthy cells. The original concentration of trypsin was 0.25% (2.5 g/L) in PBS, which was eventually decreased to 0.025% (0.25 g/L), again to lessen damage to cells.

Typically, tissue minced into small pieces was rinsed twice in the same PBS/antibiotic solution used during dissection, chopped into 1-2 mm segments, and transferred to the trypsin solution at a volume of about 2 ml per 100 mg of tissue. The cells were incubated overnight at 4 C, and the bulk of the trypsin removed using a Pasteur pipette. Tissue fragments were incubated in the residual trypsin for 20 min at 37 C, then trypsin inhibitor was added to the tube, and the contents triturated with a Pasteur pipette for 10 min. The solution was centrifuged at 5000 X g for 5 min at 10 C, and the supernatant was removed. A concentration of 25 mg/ml DNase in PBS was added, at a volume of approximately 2 ml per 100 mg of tissue. Cells were dispersed and centrifuged as above. The supernatant was removed, and cells resuspended in plating

medium. Addition of DNase was eventually removed from the protocol, because it did not appear to lower the amount of debris in the final plating solution. This allowed the cells to be resuspended in plating medium directly following neutralization of the trypsin. As well, centrifugation was decreased to 3000 X g, for 5 min. Using these chemical methods, I was able to obtain single cell suspensions which appeared to be healthy and virtually free from contamination. At this point, cells were plated in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen #10316), supplemented with 1.5 mM glutamine, 8 μg/ml insulin, 500 μg/ml streptomycin and 500 U/ml penicillin, and 5 to 10% fetal bovine serum (FBS), purchased from Cansera (Etobicoke, ON).

Of the cultures produced from E1.5 to E4 embryos, I chose to continue with cells from the E3.5 tissue, because there appeared to be a high number of round, phase-bright cells with no processes, which I judged to be proliferating cells. As well, small neuritic processes could be seen extending from some cells which had attached to the wells, within hours of plating.

Serum-free Medium

My next goal was to develop a serum-free system which allowed the cells to flourish at least as well as they did in the serum-added medium. Although I had done some experimentation with simply reducing the amount of FBS to as low as 1%, this did not produce cultures that were as healthy or that had the same longevity. Serum-free media had been developed for cell lines and older embryonic cell cultures, and a search of the literature and the products available led me to choose a commercially available product that was a modified version of D-MEM. Neurobasal medium (Invitrogen

#21103; Appendix 1) had been adapted by lowering the osmolality, and reducing the concentrations of two excitatory neurotransmitters (glutamate and asparate), as well as optimizing a number of other ingredients such as amino acids and sodium bicarbonate (Brewer and Cotman 1989). It had been designed to optimize growth of rat embryonic hippocampal neurons at low density. A recommended supplement to the Neurobasal medium was B27 (Invitrogen #17504; Appendix 2), a controlled mixture of 20 hormones, vitamins, fatty acids and anti-oxidants. Addition of glutamic acid and glutamine, which are not included in the formula, was also recommended (Brewer et al. 1993). As well as improving the survival of rat hippocampal neurons, this combination had improved survival in cultures of neurons from other areas of rat embryonic CNS, such as the cortex, septum, substantia nigra, striatum, cerebellum and dentate gyrus. It had the added promise of keeping any glial cell growth to a minimum (Brewer et al. 1993).

Plating the E3.5 chick brain cells in Neurobasal medium allowed me to discontinue use of all supplements except B27, glutamic acid and glutamine. Visual inspection of the cultures suggested no loss of quality, and a possible increase in proliferation.

Chemical-free Dissociation

My final goal prior to testing the impact of hormones on the cells was to develop a chemical-free method for dissociating the cells. Initially, minced cells were simply filtered through screens of various diameters. This approach was abandoned due to the difficulty in obtaining high numbers of cells, and concerns regarding maintaining

sterility of the screens. Following this, I returned to Freshney (1988) and attempted trituration through fire-polished Pasteur pipettes to break up the minced pieces. The larger pieces were allowed to settle for about 20 min, on ice, and the supernatant was collected for counting using a Neubauer hemacytometer (VWR International, West Chester, PA). This approach was better, but was also abandoned due to the difficulty of reducing the diameter of the pipettes to the same size with each dissection, and again, because cell counts were still too low.

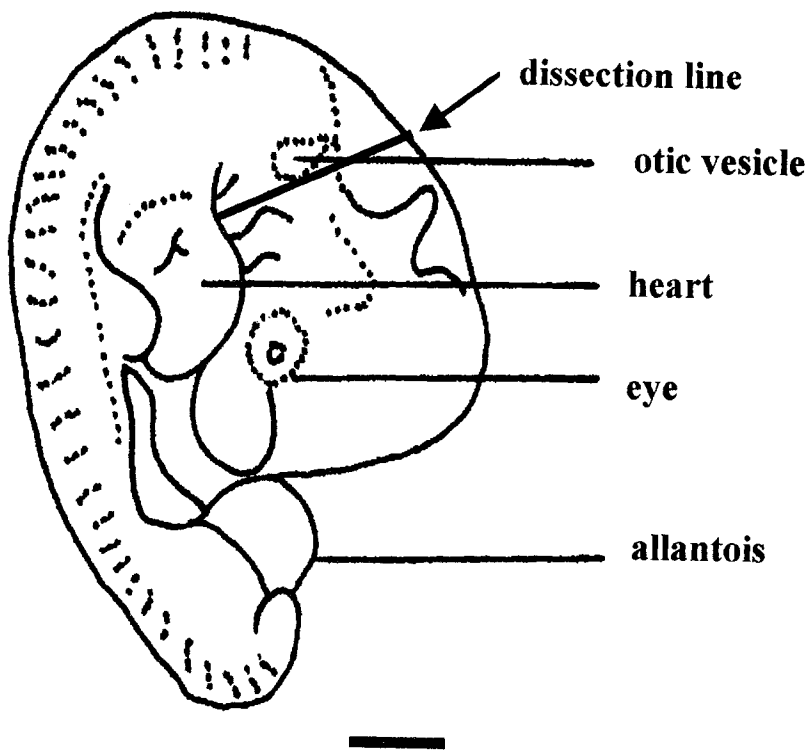
The method I chose to solve both the problem of sterility and standardization of the diameter of the trituration apparatus was to gently triturate the cell suspension, in a small amount of Neurobasal medium, through decreasing diameters of individually-packaged sterile needles. I tested a range of needle gauges between 30 and 18, and experimented with trituration between 20 to 75 times. Again, larger fragments were allowed to settle out, and the single cells from the supernatant were counted and plated. The system allowed me the added advantage of being able to plate the cells within about one hour of dissection. However, I also dissected and dissociated tissue the previous day using the trypsin method I had been using, and plated cultures side by side until I was satisfied that the mechanically dissociated cultures contained at least the same approximate number of healthy cells. This experimentation suggested that trituration through a 20 gauge needle, 30 times over about 20 min, while keeping the cells on ice, produced the highest yield of healthy cells. Although it was not possible to confirm due to the tendency of the cells to form aggregates, visual observation suggested an increase in proliferating cells using this method, which would be reasonable if the trypsin had

been inactivating surface receptors. It could also be that the method was simply less harsh. Cultures remained healthy for up to two weeks using this protocol.

Final Cell Culture Protocol

In summary, the final cell culture protocol developed for testing the effects of GHRH and PACAP on chick neuroblasts was as follows. White leghorn chicken eggs were incubated in a humidified, forced air incubator that automatically rocked the eggs. Eggs were removed from the incubator when embryos were at Stage 21-22 (Hamburger and Hamilton 1992) and brains (Fig. 2.1) removed to sterile PBS containing 500 µg/ml streptomycin and 500 U/ml penicillin. The tissue was washed twice in the same solution, then briefly minced with a sterile razor blade. Fragments were placed in culture medium, and slowly triturated 30 times through a sterile 20 gauge needle. The mixture was allowed to settle and individual cells in the supernatant were used for experiments. Cells were counted using a Neubauer hemacytometer and diluted to 0.9 - 1.1 X 10^6 cells/ml, then plated 0.5 ml/well in 24-well, flat-bottom, tissue culture-coated plates (Corning, Acton, MA). Cells were kept on ice until plated. Cells were cultured in Neurobasal medium (Invitrogen), with manufacturer's recommended supplements, in a humidified atmosphere of 5% CO_2 at 37 C.

Figure 2.1 Chick embryo after 3.5 days of 21-day gestation period. Development corresponds to Hamburger and Hamilton's (1992) stage 21-22. Heads (below dissection line) were used for experiments. Figure is adapted from Belairs and Osmond (1998). Scale bar = 1 mm.


dissection line
otic vesicle
heart
eye
allantois

Immunocytochemistry

Before assays involving the hormones could be undertaken, it was necessary to confirm that the cultures were neuroblast-enriched. My goal at this point was to identify cells using antibodies against neuron-specific enolase (NSE), which recognizes neurons only, and glial fibrillary acidic protein (GFAP), which recognizes astrocytes, the most common form of glial cell. I began experimentation with antibodies against NSE first, followed by antibodies against GFAP, then combined the antibodies for a dual labeling protocol.

Preliminary Labeling

Initial attempts to identify the cells were based on labeling cells from suspension, immediately after dissection. Briefly, 10 μl of the cell suspension were air dried on glass slides, fixed in either 100% cold acetone, or 2% paraformaldehyde (PFM) in PBS for 5-20 min, washed, exposed to antiserum against NSE raised in a rabbit (Incstar, Stillwater, MN) for 30 min at room temperature (rt), washed and exposed to goat anti-rabbit IgG bound to fluorescein isothiocyanate (FITC) for another 30 min at rt. Cells were washed again, mounted, and then viewed using an epifluorescence microscope. Cell concentrations varied from 10^5 to 10^7 cells/ml. Anti-NSE concentration varied from 1:100 in PBS to undiluted, and the secondary antibody, FITC, from 1:120 to 1:30 in PBS. However, this method produced no labeling, and because I did not know the length of time it would take for a majority of cells in the culture to begin to produce NSE, I began culturing cells prior to labeling.

In a similar protocol, cells were plated in 8-chamber culture slides (Becton Dickinson, Franklin Lakes, NJ). Cells were grown for as briefly as 4.5 h, and for as long as nine days, before fixation. Because the cells tended to detach during washes, and acetone fixation had retained cells on the slides during suspension labeling slightly better than PFM, 100% ice-cold acetone was retained as the fixative. The same concentrations of cells and antibodies were tested, although the multi-chamber slide allowed more efficiency in testing various fixation times and antibody concentrations. Also, to allow more time for anti-NSE to infiltrate the cells, I began exposing the cultures to the antiserum overnight, then for 48 hours, at 4 C, before exposure to the secondary antibody for as long as two hours at rt. A non-specific binding step was added before anti-NSE exposure, when longer culturing began to show labeling but was accompanied by a high level of background colour. Labeling was detected at nine days in culture, but since more cells were lost during washing of older cultures, days in culture were reduced until labeling disappeared at 4 days of culture. I chose to label at 6 days of culture, to ensure the cells were labeled well. After the anti-NSE labeling protocol had been established, several experiments were performed to determine the proper concentration of anti-GFAP bound to the fluorophore Cy3, to use in the cultures.

In an attempt to improve attachment during the procedure, culture slides were treated with several extracellular matrix components. These included 0.34 $\mu g/cm^2$ and 1.35 $\mu g/cm^2$ poly-l-lysine, 2 $\mu g/cm^2$ fibronectin, 1.35 $\mu g/cm^2$ laminin, and 2.5, 5 and 10 $\mu g/cm^2$ collagen. All substances were dissolved according to manufacturer's instructions, and rinsed after air-drying if necessary. The poly-l-lysine produced a very

uneven coating, and although the fibronectin and laminin tended to keep cells from aggregating, they did not inhibit cell loss. The collagen appeared to have no effect.

Final NSE and GFAP Labeling Protocol

In summary, to detect neurons and glia, an aliquot of 250 µl of a 4.5 X 10^5 cells/ml suspension was plated per chamber in 8-chamber, tissue culture-treated culture slides (Bectin Dickinson, Franklin Lakes, NJ). Cells were cultured for 6 days, to allow for attachment and differentiation. Medium was gently aspirated, and chambers allowed to air-dry 1.5 h in a sterile environment. Cells were fixed by addition of ice-cold 100% acetone for 1 min, air dried, and rehydrated in ice-cold PBS. Non-specific binding was blocked by addition of 5% sheep serum in PBS for 40 min at rt. To detect neurons, undiluted rabbit antiserum raised against NSE was added. To detect glia at the same time, a 1:100 dilution of monoclonal mouse anti-GFAP conjugated to Cy3 diluted in PBS was added. Both primary antisera contained 5% sheep serum. A control lacking both primary antisera was included. Each primary antibody with its conjugate was also tested separately on the cells. The cells were incubated in a humidity chamber for 48 h at 4 C, then washed 3 times for 5 min in cold PBS. Incubation for 2 h at rt followed, in goat antiserum raised against rabbit IgG and conjugated to FITC, diluted 1:60 in PBS. The cells were washed again, then mounted using a SlowFade Light Antifade kit (Molecular Probes, Eugene, OR). Slides were examined and photographed under a Leitz Aristoplan epifluorescence microscope.

cAMP assays

To determine whether GHRH and PACAP had any effect on early chick neuroblasts, several forms of both hormones were obtained for testing. Six species-specific forms of PACAP were chosen, including human(h) / salmon(s) PACAP27 (they are the same), chicken(c) PACAP27, tunicate(t) PACAP27, hPACAP38, cPACAP38 and sPACAP38. Seven forms of GHRH were chosen, including hGHRH29, cGHRH29, carp GHRH28 (used in place of sGHRH28; there's only one amino acid change), tGHRH27-like peptide, hGHRH44, cGHRH46 and sGHRH45. Human PACAP27 and PACAP38 were purchased from Peninsula Laboratories (Belmont, CA). All other peptides were the kind gift of Jean E. Rivier and Laura A. Cervini (Salk Institute for Biological Studies, La Jolla, CA).

Preliminary work involved testing some of these peptides using cAMP enzyme-linked immunosorbent assay (ELISA) kits, produced by Amersham (Oakville, ON) and Cayman Chemicals (Ann Arbor, MI). The effects of several hormones were tested at periods between 20 and 60 min, at concentrations between 1 and 400 nM. Although poor reproducibility caused abandonment of this project, it did suggest that cAMP peaked at some point after one hour, and that the range of concentrations was acceptable.

The next step involved measurement of cAMP using radioimmunoassay (RIA) kits. Peptides were dissolved in PBS and added to the cultures immediately upon plating. Because of the presence of methionine residues in the peptides, ascorbic acid (0.5 mM) was added with the peptides to prevent oxidation, following tests to confirm that this concentration had no effect on basal cAMP production. 1-Isobutyl-3-

methylxanthine (0.5 mM) was added at the same time to preserve the cAMP, following similar tests. Cells were lysed 0.5, 1, 2 and 24 h after plating, and cAMP was assayed from the combined cells and medium. Cells were lysed by addition of ice-cold 100% ethanol to the medium, to a final concentration of 65% ethanol. The mixture was allowed to settle and the supernatant collected. The settled material was washed with 200 µl of ice-cold 100% ethanol, and the wash combined with the supernatant prior to centrifugation at 5000 rpm for 15 min at 4 C. The supernatant was collected and the ethanol was evaporated by vacuum centrifugation at 4 C. Samples were covered and stored at 4 C until assayed. RIA (^{125}I) kits were supplied by PerSeptive Biosystems (Framingham, MA) for all assays except those which measured a response to tPACAP and tGHRH-like peptide. When the PerSeptive kit was no longer available, a kit supplied by NEN Life Sciences was used, and for comparison purposes, h/sPACAP27 was re-assayed, along with tPACAP27, using this kit.

Statistics

A minimum of 3 independent values, obtained in 2 separate experiments, were averaged to obtain each data point. The data were analyzed by ANOVA, followed by Dunnett's test. Dunnett's test was chosen because it is considered a stringent test for comparing treatment means with a control mean, and allows unequal sample sizes. Scheffé's method was used to compare two treatment means (100 nM and 1000 nM hPACAP38) when it appeared that the lower concentration might be generating a greater response and thus altering a generally observed dose-dependent response curve.

Detection of PACAP in cells

To detect PACAP using immunocytochemistry, an aliquot of 250 µl of a 1 X 10^6 cells/ml suspension was plated in 8-chamber culture slides (Nalge Nunc, Naperville, IL), cultured for two days, and processed in the same manner as for anti-NSE, with the following changes: cells were fixed in 4% PFM in PBS, and incubated in primary antiserum for 24 h. The primary antiserum was undiluted HB7, a rabbit antiserum raised in our laboratory against human PACAP38 conjugated to bovine thyroglobulin.

To detect PACAP using Western blot, a colleague, Erica. A. Fradinger, extracted protein from E3.5 chick brain using 400 µl lysis buffer (4% wt/vol SDS, 5% vol/vol 2-mercaptoethanol, 5% wt/vol sucrose). The protein extract (6 µl) was separated on a 16% Tris-Tricine gel (BioRad, Hercules, CA) at 100 V until the dye front reached the bottom of the gel. The protein was transferred to a polyvinylidene fluoride membrane (NEN Life Sciences, Boston MA) at 100 V for 30 min in transfer buffer (12.5 mM Tris-HCl pH 8.2, 200 mM glycine, 10% methanol). Immunolocalization was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. The membrane was blocked with 10 ml of Tris-buffered saline with 0.05% Tween-20 (TBST) containing three drops of goat serum, for 1 h at rt. Rabbit antisera against human PACAP27 and human PACAP38 (Peninsula Laboratories), were diluted 1:2000 in 10 ml of TBST with one drop of goat serum, and added to the membrane for overnight incubation at 4 C. The membrane was washed with TBST three times for 5 min, then incubated with one drop of goat antiserum raised against rabbit IgG, diluted in 10 ml of TBST with three drops of goat serum, for 45 min at rt. The membrane was washed again, incubated in ABC reagent for 30 min at rt, and

washed again. The DAB substrate with NiCl was added and the color allowed to develop for 3-5 min. The reaction was stopped by rinsing the membrane two times for 5 min in distilled water, and the membrane was allowed to air dry.

mRNA isolation and cDNA synthesis

Work also performed by Erica. A. Fradinger was to isolate the mRNA for GHRH, PACAP and the PAC_1 receptor, and to prepare, amplify and sequence the cDNA. Freshly dissected E3.5 chick brain cells were harvested and flash frozen on dry ice. The cells were ground to a fine powder using a micropestle (Diamed, Missisauga, ON) in 1.5 ml tubes chilled with liquid nitrogen. Duplicate samples of mRNA were isolated using the Poly (A) Pure Kit (Ambion, Austin, TX), as outlined by the manufacturer. Single-stranded cDNA was synthesized using 2 mM of oligo (dT) 20 in 1x First Strand Buffer, 2 mM dNTPs, 10 mM DTT, 5 U Ribonuclease Inhibitor (Gibco) and 200 U Superscript II (Gibco) to a final volume of 50 µl. The mRNA was combined with the oligo (dT) primer and the mixture was heated to 70 C for 7 min, then placed on ice. The remaining reagents were added, the reaction was incubated at 42 C for 90 min, then Superscript II was denatured at 95 C for 10 min. The quality of the cDNA was verified by PCR amplification of tubulin using the primers T10 (5′-CAGGTGTCCACGGCTGTGGTG-3′) and T11 (3′-AGGGCTCCATCGAAACGCAG-5′).

PCR amplification of the PACAP receptor was performed using primers 5′-GCGTTGTACACAGTTGGATA-3′ and 5′-TTGAATTGGGACTGGGATCT-3′ designed against transmembrane regions 1 and 7 of the chicken PAC_1-R (Peeters et al. 1999). Amplification of ligands was performed using the primers 5′ -

CAAAGCCTACAGGAAACTCCTGGGCC-3′ and 5′ - CGCTATTTGTAGGA TGAGCAACCGCC-3′ designed against the 5′ region of GHRH and the 3′ UTR of the chicken PACAP gene (McRory et al. 1997). A 2 µl volume of cDNA was added to a 50 µl volume containing 200 µM dNTPs, 2 mM $MgCl_2$, 0.4 µM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen). The reaction was heated to 94 C for 2 min, then cycled 30 times at 94 C for 30 sec, 52 C for 45 sec, and 72 C for 1 min. A 10 µl aliquot of PCR product was separated on a 1.5% agarose gel. The PCR product was ligated into pGEM-T vector as specified by the manufacturer (Promega, Madison, WI) and cloned. Two recombinant plasmids from each of the duplicate samples were sequenced using an ABI Prism 377 DNA Sequencer.

RESULTS

Methods generate healthy cultures

Near the time of plating, primary cultures from E3.5 chick brain contained proliferating cells, based on their round, smooth, phase-bright appearance, as well as lack of dendritic or axonal projections and the fact that they were not attached to the culture plates (Fig.2.2). Some of these cells elongated, and occasionally appeared to undergo cytokinesis. By 12 hours, small aggregates had begun to form, and short processes could be seen extending from some of the cells (Fig. 2.3). Over the next several days, cultures developed into large aggregates of cells connected by fasciculated axons (Fig. 2.4). Some cells surrounding the aggregates had elongated and formed tracts or sheets of flattened cells (Figs. 2.4, 2.5). Few blood cells, identifiable by their oblong shape and darker centres, were visible.

Neuroblasts but not glia are identified at E3.5

The cultures consisted primarily of neuroblasts, based on labeling with antibodies against neurons and glial cells. Although evidence was difficult to obtain because of the tendency of the cells to detach during the labeling procedures, photographs of a typical culture reveal an abundance of nerve cells (Fig. 2.6, top) and no glial cells (Fig. 2.6, bottom). Cultures labeled with single antibodies confirmed the results of the double labeling procedure. Antiserum against NSE labeled cells in 6-day cultures, but did not label cells prior to 4 days in culture (data not shown). This suggested that the E3.5 cells were undifferentiated, because NSE is found only in differentiated cells.

Figure 2.2 Embryonic day 3.5 neuroblasts shortly after plating. The cells were proliferating, based on their round, smooth, phase-bright appearance and their motility. Cells increased in size, and occasionally one could be seen to undergo cytokinesis. Scale bar = 7 μm.

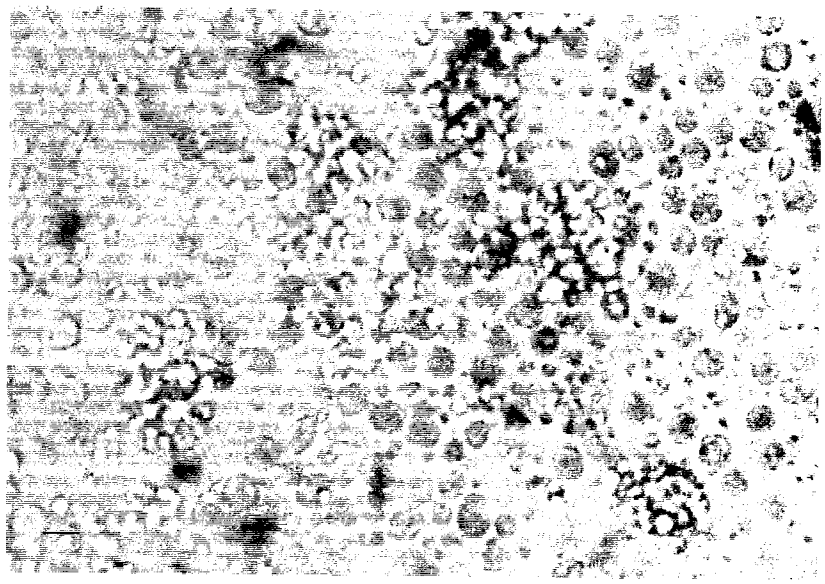


Figure 2.3. Embryonic day 3.5 neuroblasts about 12 hours after plating. Cells had attached and had begun to form small aggregates. Some were extending neuritic processes (arrows). Scale bar = 7 μm.

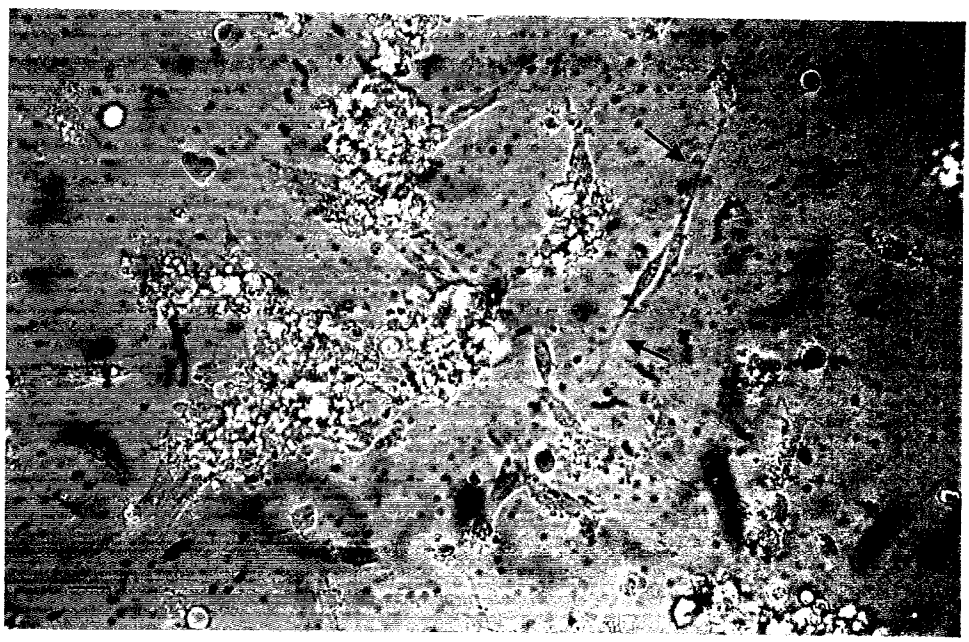


Figure 2.4 Embryonic day 3.5 neuroblasts after several days in culture. Cells formed large aggregates, connected by fasciculated axons. Proliferating cells were still visible. Scale bar = 7 μm.

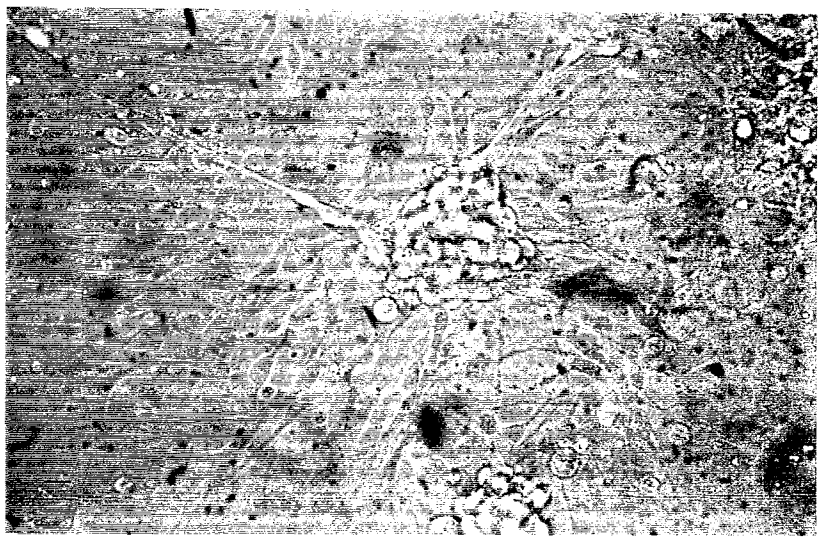


Figure 2.5 Embryonic day 3.5 neuroblasts after several days in culture. Flattened tracts or sheets of cells surrounded some of the aggregated cells. Scale bar = 7 μm.

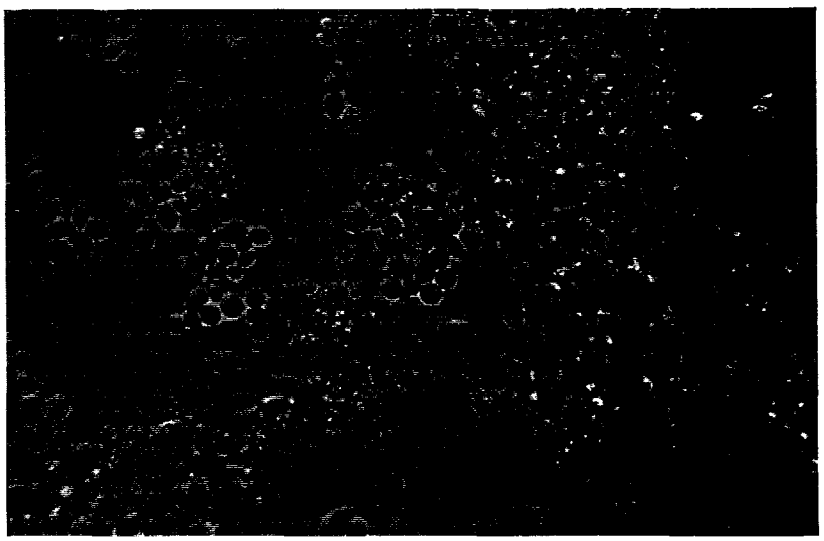
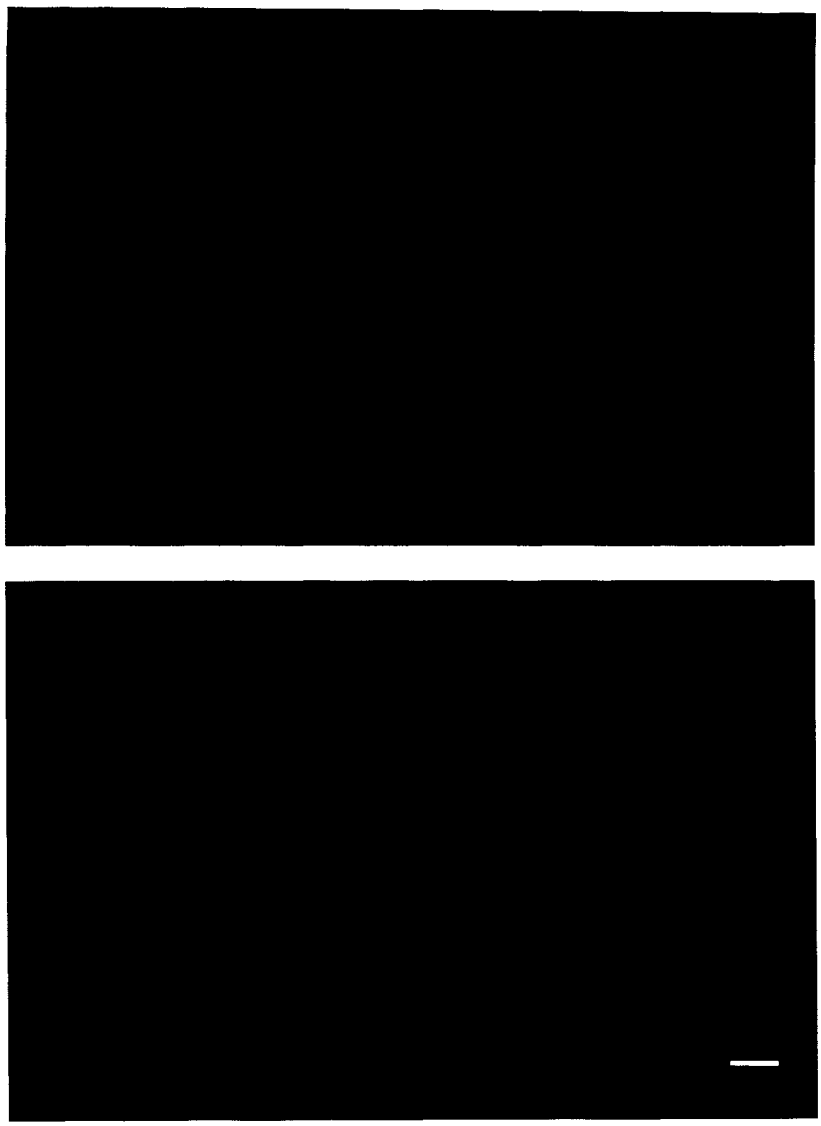


Figure 2.6 Embryonic day 3.5 neuroblasts after 6 days in culture, labeled with an antibody against neuron specific enolase conjugated to the green fluorophore FITC (top) and an antibody against glial fibrillary acidic protein conjugated to the red fluorophore Cy3 (bottom). The same frame is shown. Immunostaining reveals an abundance of neural cells. A large aggregate shows up in the upper left corner, with a few individual cells beneath. Glial cells were absent. Scale bar = 10 μm.



cAMP pathway is activated by five PACAP peptides

A general dose-dependent increase in cAMP production in the neuroblast-enriched cultures was observed in response to physiological concentrations of h/sPACAP27 (Fig. 2.7), cPACAP27 (Fig. 2.8), hPACAP38 (Fig. 2.9) and cPACAP38 (Fig. 2.10). In some cases, a statistically significant response compared to control was evident for 1 nM. Administration of 10 nM enhanced production further, and 100 nM concentrations resulted in peak production levels. Statistically, values did not rise further with administration of 1000 nM hormone, indicating that a plateau had been reached. An apparent drop in production above 100 nM in response to hPACAP38 was not borne out statistically. Responses to h/sPACAP27 obtained with the NEN Life Sciences kit (Fig. 2.11, bottom) were lower than with the PerSeptive Biosystems kit (Fig. 2.7), with values reaching only 50-75%. However, the typical trend in cAMP production is evident in response to tPACAP27 when measured with the NEN kit (Fig. 2.11, top), and the magnitude of the response is similar to that elicited by h/sPACAP27 (Fig. 2.11, bottom) using the NEN kit. Statistical increases were not recorded in response to sPACAP38 for any concentration tested, at any point in time up to 24 h (Fig. 2.12).

cAMP pathway is activated by only one GHRH peptide

Although a trend was not evident, a statistical increase in cAMP production over control was measured in response to the tGHRH-like peptide. Increases were in response to 1 nM PACAP 0.5 h after plating, and 10 nM PACAP 1 h after plating (Fig. 2.13). The response to the tGHRH-like peptide was measured using

Figure 2.7 Response by embryonic day 3.5 neuroblasts to nM concentrations of human/salmon (they are the same) PACAP27. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, and dark asterisks indicate significance at $p<0.01$, as determined by ANOVA followed by Dunnett's test.

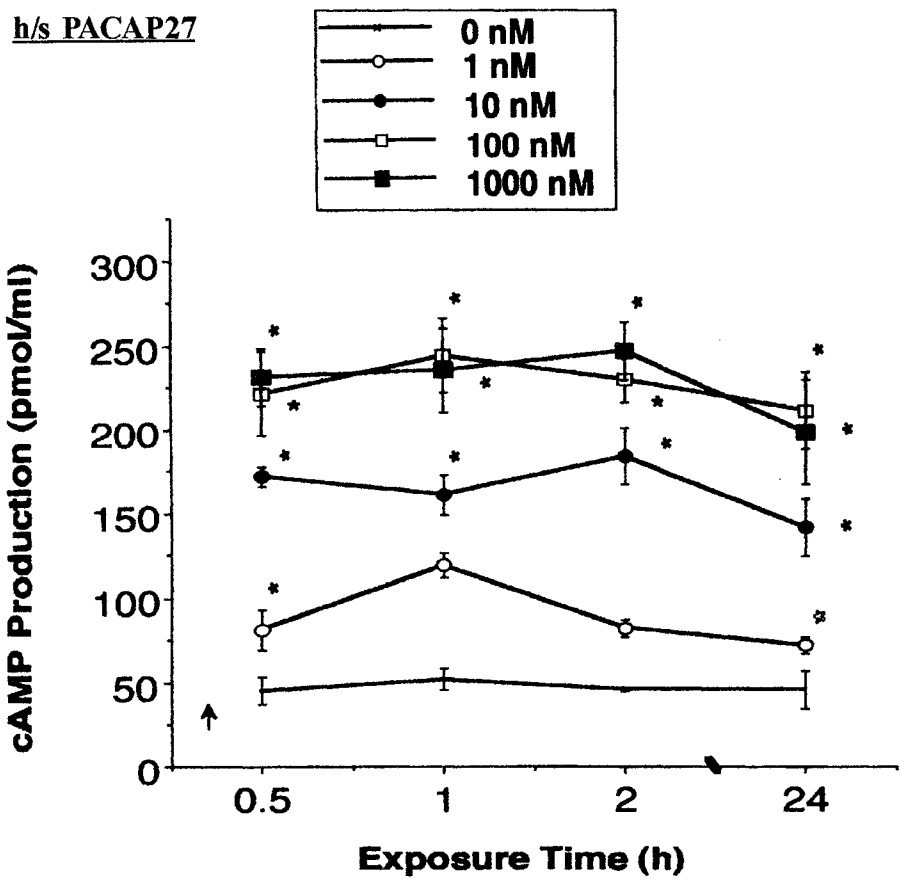

h/s PACAP27
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
300
250
200
150
100
50
0
0.5
1
2
24
Exposure Time (h)

Figure 2.8 Response by embryonic day 3.5 neuroblasts to nM concentrations of chicken PACAP27. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, and dark asterisks indicate significance at $p<0.01$, as determined by ANOVA followed by Dunnett's test.

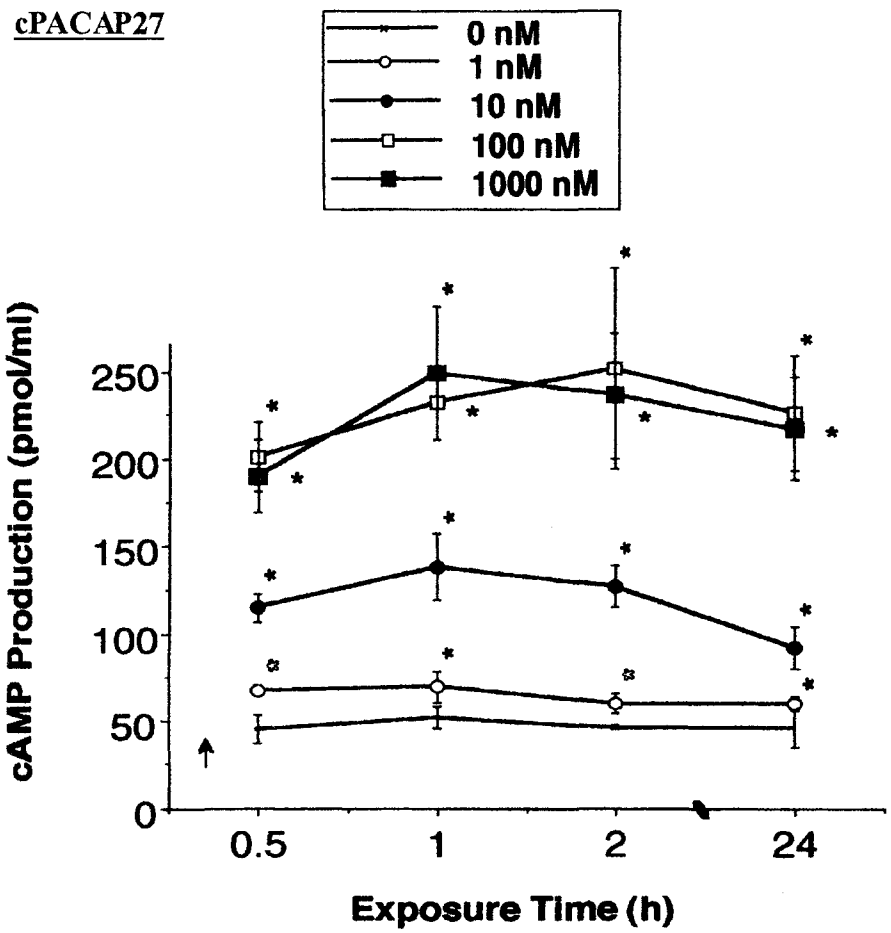

cPACAP27
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
250
200
150
100
50
0
0.5
1
2
24
Exposure Time (h)

Figure 2.9 Response by embryonic day 3.5 neuroblasts to nM concentrations of human PACAP38. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, and dark asterisks indicate significance at $p<0.01$, as determined by ANOVA followed by Dunnett's test.

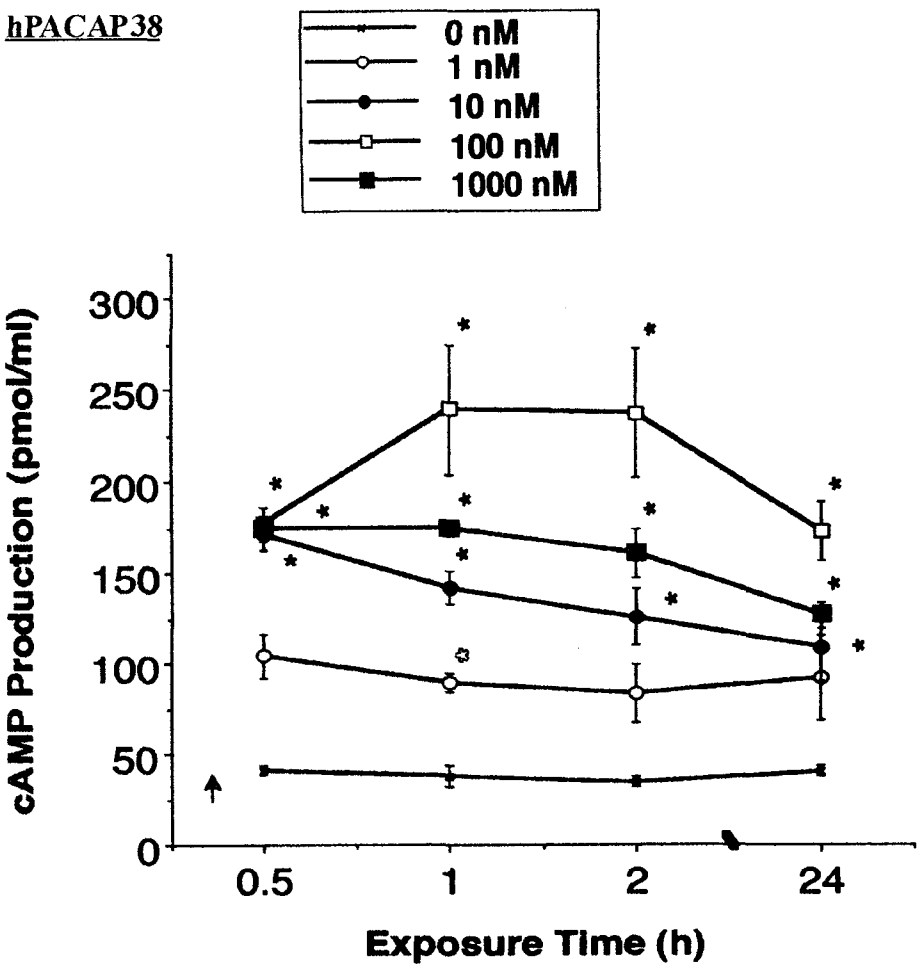

hPACAP38
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
300
250
200
150
100
50
0
0.5
1
2
24
Exposure Time (h)

Figure 2.10 Response by embryonic day 3.5 neuroblasts to nM concentrations of chicken PACAP38. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, and dark asterisks indicate significance at $p<0.01$, as determined by ANOVA followed by Dunnett's test.

cPACAP38

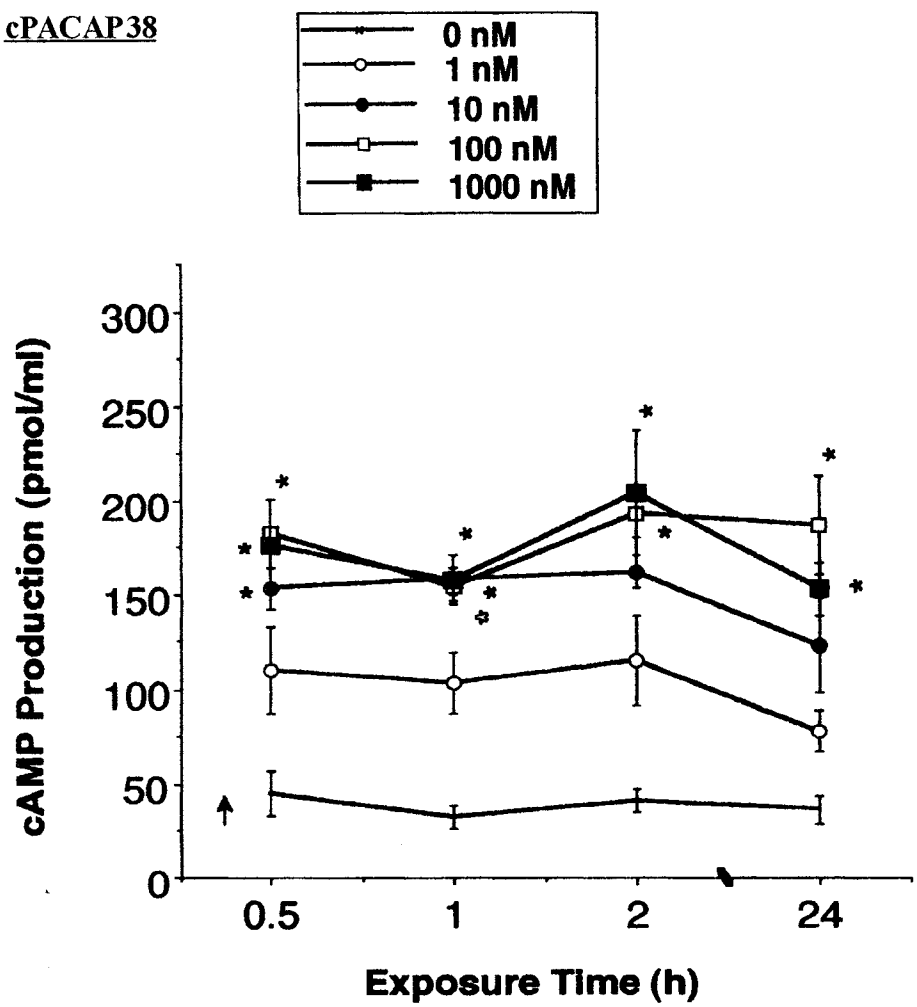

0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
300
250
200
150
100
50
0
0.5
1
2
24
Exposure Time (h)

Figure 2.11 Response by embryonic day 3.5 neuroblasts to nM concentrations of tunicate PACAP27 (upper graph), using a different manufacturer's kit (NEN) than was used to collect the data in Figures 2.7 to 2.10. For comparison, human/salmon PACAP27 was re-assayed with the same (NEN) kit (lower graph). Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, and dark asterisks indicate significance at $p<0.01$, as determined by ANOVA followed by Dunnett's test.

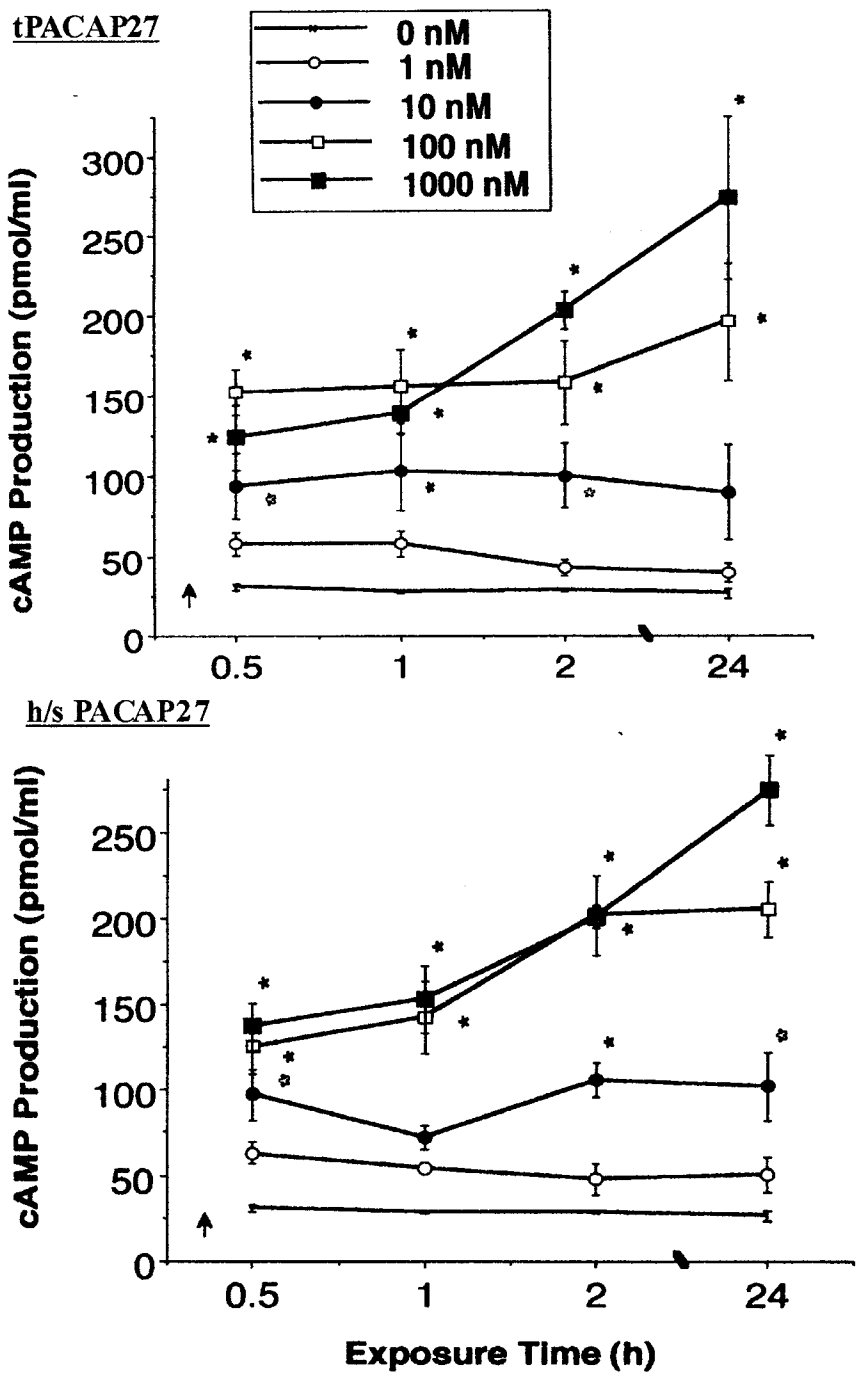

tPACAP27
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
300
250
200
150
100
50
0
0.5
1
2
24
h/s PACAP27
cAMP Production (pmol/ml)
250
200
150
100
50
0
0.5
1
2
24
Exposure Time (h)

Figure 2.12 Response by embryonic day 3.5 neuroblasts to nM concentrations of salmon PACAP38. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. None of the data points were significant, as determined by ANOVA.

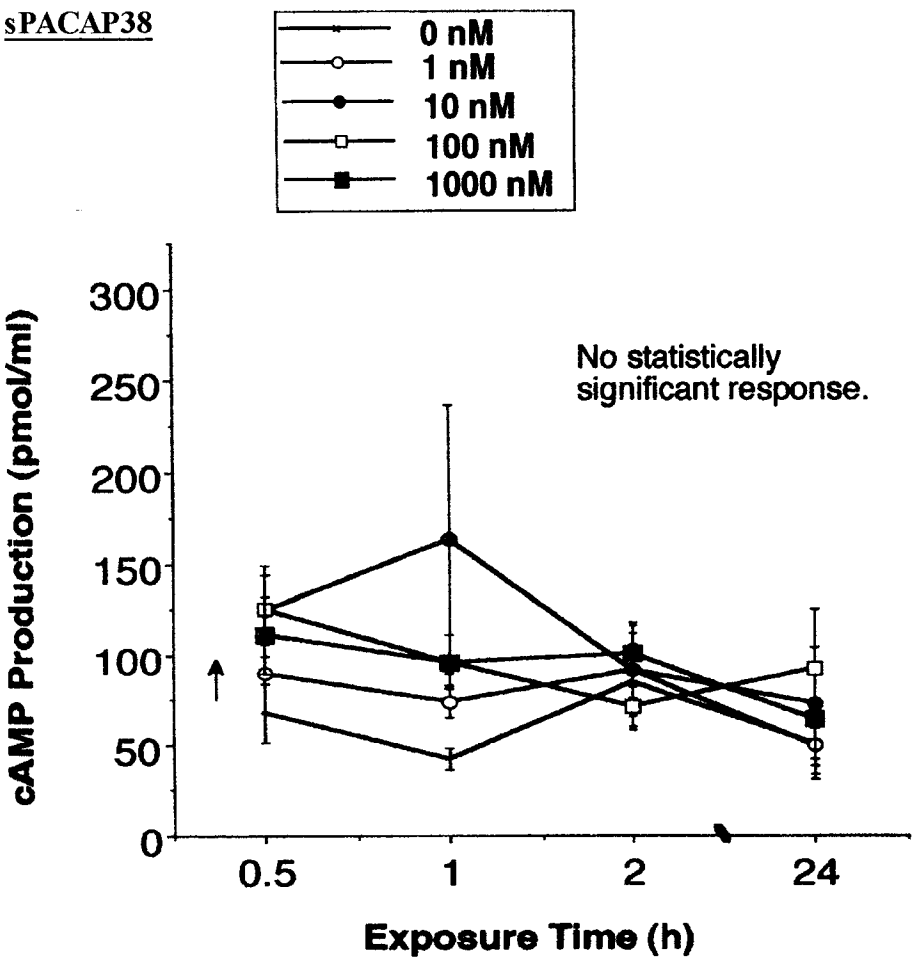
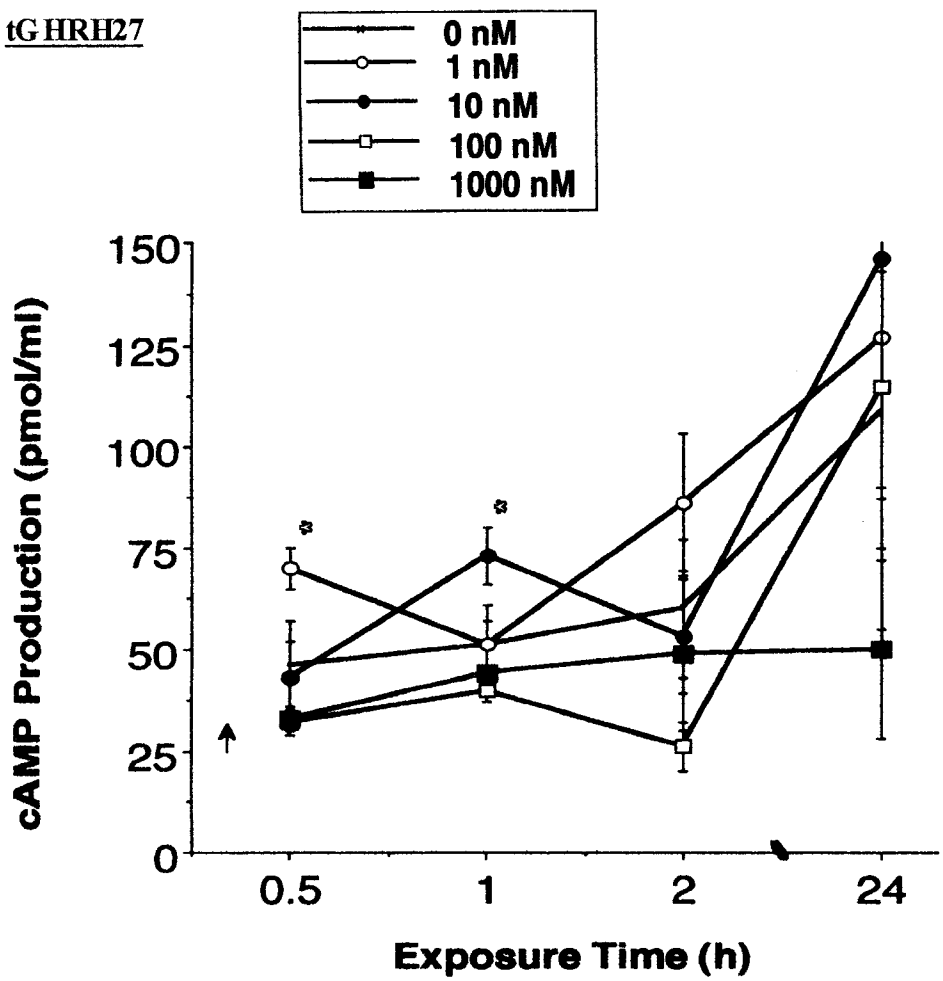

sPACAP38
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
300
250
200
150
100
50
0
No statistically
significant response.
0.5
1
2
24
Exposure Time (h)

Figure 2.13 Response by embryonic day 3.5 neuroblasts to nM concentrations of tunicate GHRH27-like peptide. Each data point is the mean of at least three independent determinations. The arrow indicates cAMP production at the time the hormone was administered. Clear asterisks indicate a significant difference from control at $p<0.05$, as determined by ANOVA followed by Dunnett's test.


tG HRH27
0 nM
1 nM
10 nM
100 nM
1000 nM
cAMP Production (pmol/ml)
150
125
100
75
50
25
0
0.5
1
2
24
Exposure Time (h)

the RIA kit that produced lower values overall, but it is still evident that the magnitude of increase is not as great as the increases recorded in response to other hormones using this kit (Figs. 2.11, 2.13). No statistically significant increases were recorded in response to hGHRH29, cGHRH29, carpGHRH28, hGHRH44, cGHRH46, or sGHRH45 (data not shown). *P* values were between 0.08 and 0.96.

PACAP and its specific receptor are expressed in neuroblasts

Photographs of E3.5 chick brain cells cultured for two days and labeled to reveal PACAP38 showed that most or all of the cells contained the protein (Fig. 2.14). Western blot analysis of protein from freshly-dissected cells revealed a band of approximately 4.5 kDa. This corresponded to the PACAP38 standard, and confirmed the presence of PACAP38, but not PACAP27, in the cells (Fig. 2.15). An additional band larger than 4.5 kDa may represent an unprocessed precursor.

mRNA isolated from the cells and converted to cDNA was amplified using RT-PCR with exact match primers for PACAP receptor and its ligand. The reactions yielded 708 bp (Fig. 2.16) and 276 bp bands (Fig. 2.17). Sequence analysis confirmed that the 708 bp band was the PAC_1-R (short, i.e. lacking any insert) transcript (Fig. 2.18). Our sequence has one nucleotide change at position 555 compared to the previously isolated cDNA (Peeters et al. 1999). This change does not cause an amino acid change. The transcript did not contain the TM-4 variation.

The 276 bp band was confirmed to be the chicken GHRH-PACAP transcript (Fig. 2.19). Our sequence has 3 nucleotide changes at positions 43, 46 and 124 compared to

Figure 2.14 Embryonic day 3.5 neuroblasts after two days in culture, labeled to show the presence of human PACAP38 (upper picture). The primary antiserum was conjugated with the green fluorophore FITC. The control, in which the primary antiserum was omitted, is shown for comparison (lower picture). Scale bar = 8 μm.

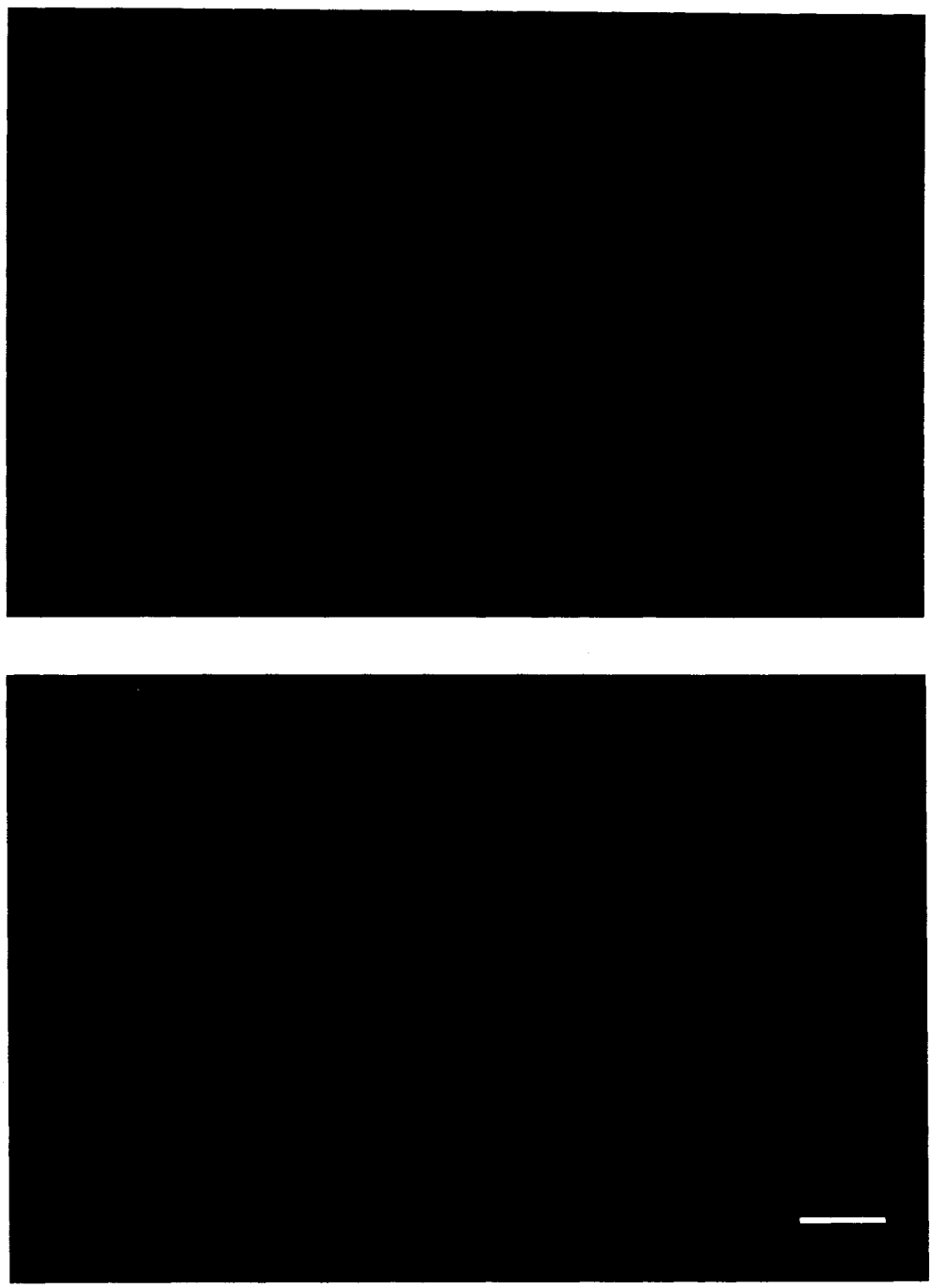


Figure 2.15 Western blot analysis of protein isolated from embryonic day 3.5 chick brain cells, using antisera against human PACAP38 and human PACAP27. Because initial staining for the standards was very heavy (lanes 4 and 5), standards were repeated on a separate gel to enhance band clarity (lanes 6 and 7). The molecular masses of the PACAP38 peptide (4.5 kDa) and the PACAP27 peptide (3.1 kDa) are indicated. A heavy band in the first and third lanes, above the 4.5 kDa bands, may represent a precursor.

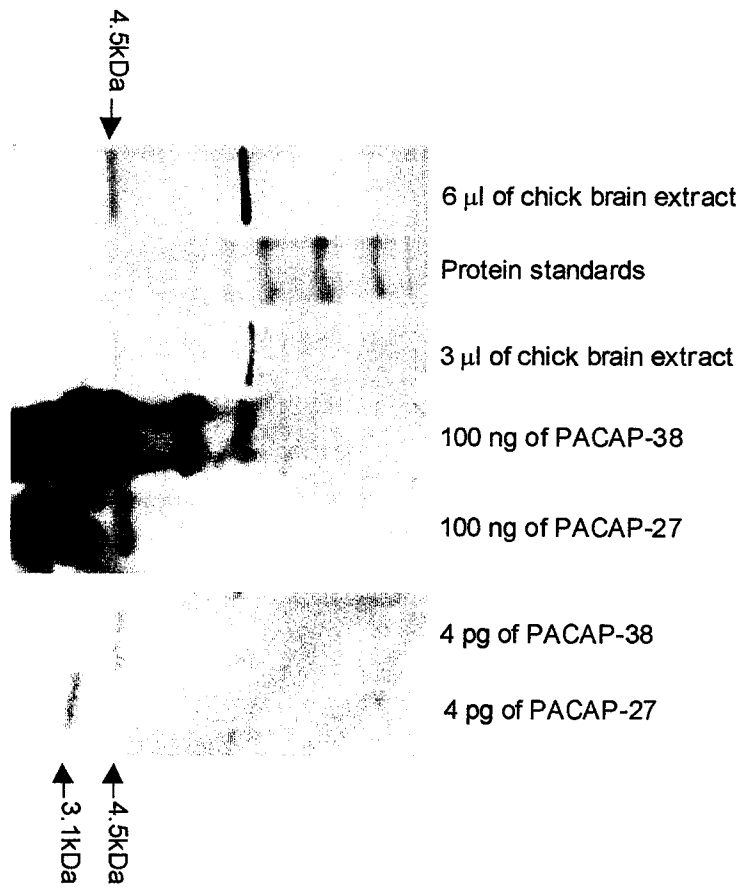

4.5kDa
6 μl of chick brain extract
Protein standards
3 μl of chick brain extract
100 ng of PACAP-38
100 ng of PACAP-27
4 pg of PACAP-38
4 pg of PACAP-27
3.1kDa
4.5kDa

Figure 2.16 RT-PCR amplification of mRNA for the PACAP receptor isolated from embryonic day 3.5 chick brain cells.

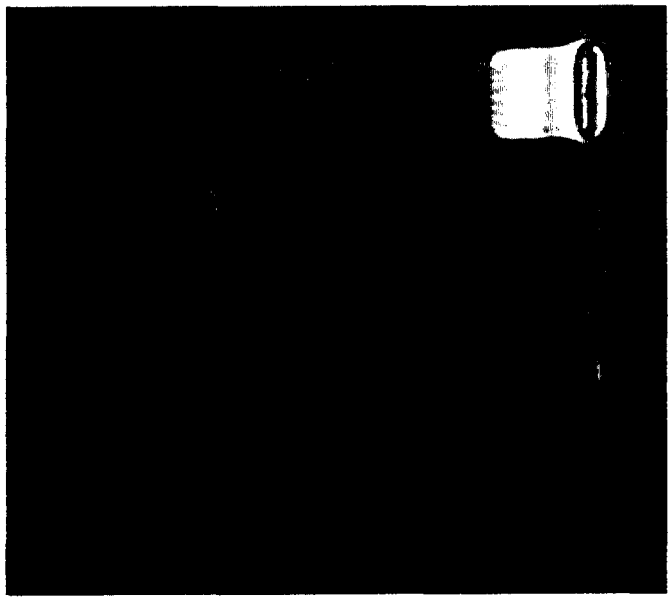
123 bp ladder

PAC_1 receptor

+ve tubulin control

-ve control

Figure 2.17 RT-PCR amplification of mRNA for the GHRH-PACAP ligand isolated from embryonic day 3.5 chick brain cells.



123 bp ladder

PACAP-GHRH

+ve tubulin control

-ve control

Figure 2.18 Partial nucleotide sequence from cDNA isolated from embryonic day 3.5 chick brain cells encoding the PAC_1-receptor, (short, i.e. lacking any insert, isoform). Transmembrane domains are underlined and primer sequences are in italics (first 20 bp, last 21 bp). The arrow at nucleotide 555 indicates the site of a nucleotide change from a previously isolated sequence (Peeters et al. 1999).

GCG TTG TAC ACA GTT GGA TAC AGC ACG TCT CTC GTT 36
TMD-1
TCG CTC ACC ACC GCC ATG GTG ATC CTG TGT CGT TTC 72

AGG AAG CTT CAC TGC ACT CGG AAC TTT ATC CAC ATG 108

AAC CTC TTT GTT TCA TTC ATC CTA CGT GCA ATA TCA 144
TMD-2
GTA TTC ATT AAA GAT GGG GTT CTT TAT GCT GAG CAA 180

GAT GGC AAC CAC TGT TTT ATC TCC ACT GTG GAA TGC 216

AAA GCA GTG ATG GTG TTT TTT CAC TAC TGT GTC ATG 252
TMD-3
TCC AAC TAC TTC TGG CTG TTC ATT GAG GGA TTG TAC 288

CTT TTC ACG TTA TTG GTA GAA ACC TTT TTC CCA GAG 324

AGG AGG TAT TTC TAC TGG TAC ACC ATT ATT GGC TGG 360

GGC ACC CCG ACA ATT TGT GTC ACT GTC TGG GCA GTG 396
TMD-4
CTG AGG CTT CAC TTT GAT GAT ACT GGC TGC TGG GAC 432

ATG AAT GAC AAC ACT GCC TTG TGG TGG GTG ATC AAG 468

GGT CCT GTG GTT GGG TCA ATC ATG ATA AAC TTT GTG 504
TMD-5
CTT TTT ATT GGC ATC ATT GTG ATA CTT GTG CAG AAA 540

CTC CAG TCA CCT GAT↓ ATT GGA GGC AAT GAA TCC AGC 576

ATT TAT TTG AGA TTG GCT CGC TCT ACA CTA CTG CTT 612

ATC CCC TTG TTT GGA ATT CAC TAC ACG GTG TTT GCT 648
TMD-6
TTT TCT CCG GAA AAT GTC AGT AAG CGG GAG AGA CTT 684

GTG *TTT GAA TTG GGA CTG GGA TCT* 708
TMD-7

Figure 2.19 Partial nucleotide sequence from cDNA isolated from embryonic day 3.5 chick brain cells encoding GHRH and PACAP. The sequence corresponding to the GHRH peptide is double underlined and the sequence corresponding to the PACAP peptide is single underlined. Primer sequences are indicated in italics (first 26 bp, last 26 bp) and arrows indicate sites of nucleotide changes from previously isolated sequences (McRory et al. 1997).

[GRF- *C AAA GCC TAC AGG AAA CTC CTG GGC CAG CTG* 31

TCC GCA AGG↓ AAA↓ TAC CTG CAC TCC CTG ATG GCC AAG 67

CGG GTC GGC GGT GCC AGC AGC GGC CTG GGG GAC GAG 103

GCG GAA CCG CTC AGC_{46}] AAG CGG↓ **[PACAP** CAC ATA 130

GAC GGC ATC TTC ACG GAC AGC TAC AGC CGC TAC CGG 166

AAA CAA ATG GCT GTC AAG AAA TAC TTA GCG GCC GTC 202

CTG_{27}GGG AAA AGG TAT AAA CAA AGA GTT AAA AAC AAA 238

$_{38}$] GGA CGC CGA GTA *GCG TAT TTG* tag_{stop} *gatgagcaa* 271

ccgcc 276

the previously isolated gene (McRory et al. 1997). The change at position 46 causes an amino acid change from Asp to Lys.

DISCUSSION

PACAP and GHRH affect early chick nervous system development

It is clear that E3.5 chick brain cells produce mRNA for PACAP and that this transcript is translated to yield the mature PACAP38 peptide. Although the PACAP27 peptide was not detected, it is possible that it is present at levels not detectable by Western blot. In adult rat, PACAP27 accounted for less than 10% of total PACAP in the brain (Arimura et al. 1991). E3.5 chick brain cells also produce mRNA for a PACAP-specific receptor, and physiological doses of PACAP strongly activated the cAMP pathway. This suggests that an autocrine/paracrine system may be present. GHRH, which is encoded on the same gene as PACAP in chickens, was also shown to be present as mRNA at this stage. However, only one of seven forms of GHRH tested produced a statistical increase in cAMP production. Only the tunicate GHRH-like hormone initiated a response, and then only in response to a 1 nM concentration at 0.5 h after plating, and a 10 nM concentration at 1 h after plating. A critical question is whether the GHRH receptor is expressed in E3.5 chick brain cells. To date, cDNA for the chicken GHRH receptor has not been reported, although the receptor has been cloned in mammals and goldfish (Sherwood et al. 2000). It is also possible that GHRH has an effect at this early stage, but that the effect is not mediated through the cAMP pathway.

The cell cultures in these experiments were virtually all neuroblasts based on three lines of evidence. Firstly, the cells were cultured in medium designed to virtually eliminate glial cell growth (Brewer et al. 1993), and this effect was confirmed by immunocytochemical results. Secondly, gliogenesis in chick brain does not begin until E8 *in vivo*. Thirdly, concentrations of antiserum against NSE that identified neurons in

6-day cultures did not recognize cells in 4-day cultures. Because NSE does not recognize undifferentiated nerve cells, this supports the conclusion that the cells in these experiments, cultured only up to 24 h, were primarily neuronal precursors or neuroblasts.

There are few reports on the involvement of PACAP in chick brain development. An increase in cell numbers between E3.5 and E9 in the dorsal root ganglion and lumbar motor column (Arimura et al. 1994), and an increase in cAMP in post-natal chicken hypothalamus and cerebral cortex have been reported (Nowak et al. 1999). Therefore, the present study is the first one to show an activation of early chick brain cells by PACAP.

PACAP receptor is activated in chick neuroblast-enriched cultures

Our results suggest that the physiological actions of PACAP in neuroblast-enriched cultures from E3.5 chick brain are mediated through one of the PACAP-specific receptors, PAC_1-R, short isoform. The PAC_1-R short isoform and the PAC_1-R hop1 isoforms were expressed in the brain of the adult chicken (Peeters et al. 1999). However, we found only the PAC_1-R short isoform in the developing brain of E3.5 chick. This could indicate that the PAC_1-R short isoform is the only form, or the predominant form of this receptor in neuronal precursors. The PAC_1-R short isoform is strongly coupled to the cAMP pathway (Sherwood et al. 2000), indicating that the observed increase in cAMP in these cultures in response to hormone addition is likely achieved by activation of this receptor.

Downstream effects of increased cAMP production are unknown

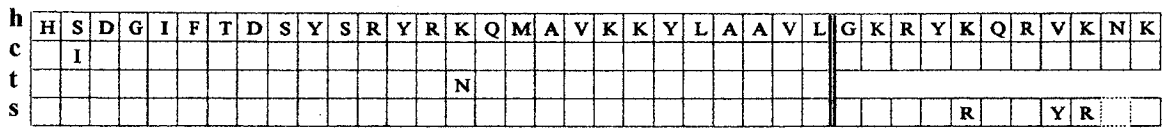
This work did not elucidate the nature of downstream effects resulting from increases in cAMP production. Although the protocol was not designed to rule out simple trophic effects, it can be expected that this alone would not account for the magnitude of the cAMP response generated during the first 2 h of culture, a time when the cells should be healthy. It is possible that the increase in second messenger leads to enhancement of proliferation, or differentiation, or both. Both proliferating and differentiating cells were evident during the first 24 h, and increases in cAMP can enhance both these processes. Proof that these cells had differentiated was shown by the presence of NSE after six days in culture. Studies on chick embryogenesis show that by E3.5 the neural tube has closed, and cranial nerves, sensory organs, Rathke's pouch and the infundibulum (which will become the pituitary), are forming (Bellairs and Osmond 1998). Generally, cells are still in a proliferative state, but some are differentiating in the hindbrain, olfactory epithelium and optic tectum (Mulrenin et al. 1999). It is also possible that PACAP plays a role in keeping the cells alive by inhibiting apoptosis at this stage.

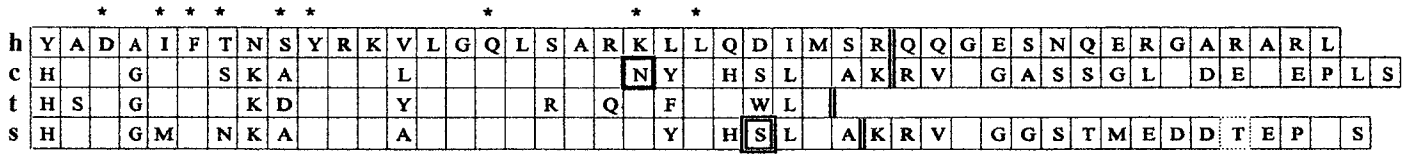
Evolution of PACAP and GHRH alters function in chick neuroblasts

This study suggests that the single amino acid differences between the human/salmon, chicken and tunicate forms of PACAP27 (Table 2.1) do not cause an ascertainable difference in production of cAMP in neuroblast-enriched chick brain cell cultures. However, substitutions in the carboxy-terminal region of PACAP38 appear to

Table 2.1 PACAP and GHRH amino acid sequences.*

PACAP





GHRH

*Comparisons are to the human form of each peptide. Double line indicates end of short forms. Amino acids common to human PACAP and human GHRH are indicated by an asterisk. The amino acids outlined by dotted lines in the carboxy-terminal region of salmon PACAP38 and salmon GHRH45 were synthesized according to the first reported sequences (Parker et al, 1993), but later sequenced as S in both cases (Parker et al, 1997). The amino acid outlined in bold in the middle region of chicken GHRH was synthesized according to a previously reported sequence (McRory et al, 1997) but sequenced by us as K. The amino acid outlined by a double border in the carboxy-terminal region of salmon GHRH is replaced by T in carp (used in place of salmon for this study).

have a marked effect on the function of the peptide. There are four amino acid substitutions in the carboxy- terminal region (amino acids 28-38) of sPACAP38, compared to hPACAP38 and cPACAP38 (Table 2.1). It is possible that the carboxy-terminal changes lessen the effectiveness of the peptide in other species, probably by altering its binding affinity to receptors. In support of this, it has been reported that a VIP analogue in which hPACAP29-38 is added to VIP29 resulted in enhanced binding to PAC_1-R, suggesting a stabilizing effect of the carboxy-terminus of PACAP38 on optimal peptide conformation (Schafer et al. 1999).

The only form of GHRH that prompted a statistical increase in cAMP was the tunicate GHRH27-like peptide. Examining the structure of this peptide compared to the other GHRH hormones tested in this experiment provides a possible explanation. Within the first 27 amino acids, tGHRH-like peptide is more similar in sequence to hPACAP27 than are hGHRH, cGHRH, sGHRH or carp GHRH. The latter four peptides have nine amino acids in common with hPACAP27, but the tGHRH-like peptide has 12 that match the human peptide (Table 2.1). As well, within the glucagon superfamily of hormones, four amino acids at the amino-terminus of the ligands have been identified as crucial for binding to the PAC_1 receptor, and tGHRH-like peptide is the only GHRH tested here that includes all four of these amino acids. These crucial amino acids include histidine in position 1, serine in position 2, phenylalanine in position 6 and threonine in position 7 (Schafer et al. 1999). One or two of these amino acids are changed in hGHRH, cGHRH, sGHRH and carp GHRH (Table 2.1). Because it is not known whether cells at this stage express the GHRH receptor, and because the GHRH

and PACAP receptors are related, it is possible that the tGHRH-like peptide is binding to the PACAP receptor.

Summary

In summary, in this portion of my thesis I established cell cultures from E3.5 chick brain, positively identified the cells as neuroblasts, and measured dose-dependent increases in cAMP up to five-fold of control values in response to physiological concentrations of h/sPACAP27, cPACAP27 and tPACAP27. Responses to PACAP38 were more variable, ranging from five-fold for hPACAP38, to four-fold for cPACAP38, to no significant response for sPACAP38, compared to control values. The responses to PACAP38 may reflect a greater difference in peptide structure among species. Increases in cAMP generated by hGHRH, cGHRH and s/carpGHRH were not statistically significant, whereas increases in response to lower-range doses of tGHRH27-like peptide were significant, but small. We also used immunocytochemistry and Western blot to show the presence of the PACAP38 peptide. RT-PCR was used to demonstrate that mRNAs for PACAP and GHRH, and a PACAP-specific receptor were present in the cells. This was a first report suggesting an autocrine/paracrine system for PACAP in early chick brain development, based on the presence of the ligand, mRNA for the ligand and receptor, and activation of the receptor in neuroblast-enriched cultures.

REFERENCES

Arimura, A, A Somogyvari-Vigh, A Miyata, K Mizuno, DH Coy and C Kitada (1991). "Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes." Endocrinology **129**: 2787-2789.

Arimura, A, A Somogyvari-Vigh, C Weill, RC Fiore, I Tatsuno, V Bay and DE Brenneman (1994). "PACAP functions as a neurotrophic factor." Annals of the New York Academy of Science, USA **739**: 228-243.

Banker, G and K Goslin, Eds. (1991). Culturing Nerve Cells. Cambridge, Mass, MIT Press.

Baserga, R, Ed. (1981). Tissue Growth Factors. New York, Springer-Verlag.

Bassas, L, M Girbay, MA Lesniak, J Roth and F de Pablo (1989). "Development of receptors for insulin and insulin-like growth factor I in head and brain of chick embryos: autoradiographic localization." Endocrinology **125**: 2320-2327.

Bellairs, R and M Osmond (1998). The Atlas of Chick Development. Toronto, Academic Press.

Brewer, GJ and C Cotman (1989). "Survival and growth of hippocampal neurons in a defined medium at low density: advantages of a sandwich culture or low oxygen." Brain Research **494**: 65-74.

Brewer, GJ, JR Torricelli, EK Evege and PJ Price (1993). "Optimized survival of hippocampal neurons in B27-supplemented Neurobasal™, a new serum-free medium combination." Journal of Neuroscience Research **35**: 567-576.

Campard, KP, C Crochemore, F Rene, D Monnier, B Koch and JP Loeffler (1997). "PACAP Type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA pathway." DNA and Cell Biology **16**: 323-333.

Canonico, PL, A Copani, V D'Agata, S Musco, S Petralia, S Travali, F Stivala and S Cavallaro (1996). "Activation of pituitary adenylate cyclase-activating polypeptide receptors prevents apoptotic cell death in cultured cerebellar granule cells." Annals of the New York Academy of Science, USA **805**: 470-472.

Cavallaro, S, A Copani, V D'Agata, S Musco, S Petralia, C Ventra, F Stivala, S Travali and PL Canonico (1996). "Pituitary adenylate cyclase-activating polypeptide prevents apoptosis in cultured cerebellar granule neurons." Molecular Pharmacology **50**: 60-66.

Chang, JY, VV Korolev and J-Z Wang (1996). "Cyclic AMP and pituitary adenylate cyclase-activating polypeptide (PACAP) prevent programmed cell death of cultured rat cerebellar granule cells." Neuroscience Letters **206**: 181-184.

Crossley, PH, S Martinez and GR Martin (1996). "Midbrain development induced by FGF8 in the chick embryo." Nature **380**: 66-68.

Dale, JK, C Vesque, TJ Lints, K Sampath, A Furley, J Dodd and M Paczek (1997). "Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm." Cell **90**: 257-269.

Delgado, M, E Garrido, C Martinez, J Leceta and RP Gomariz (1996). "Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptides (PACAP27 and PACAP38) protect CD4+CD8+ thymocytes from glucocorticoid-induced apoptosis." Blood **87**: 5152-5161.

DiCicco-Bloom, E and PJ Deutsch (1992). "Pituitary adenylate cyclase activating polypeptide (PACAP) potently stimulates mitosis, neuritogenesis and survival in cultured rat sympathetic neuroblasts." Regulatory Peptides **37**: 319.

DiCicco-Bloom, E, N Lu, JE Pintar and J Zhang (1998). "The PACAP ligand/receptor system regulates cerebral cortical neurogenesis." Annals of the New York Academy of Science, USA **865**: 274-289.

Ebendal, T and H Persson (1988). "Detection of nerve growth factor mRNA in the developing chicken embryo." Development **102**: 101-106.

Freshney, RI (1988). Culture of Animal Cells: a Manual of Basic Technique. New York, Alan R. Liss, Inc.

Gonzalez, BJ, M Basille, D Vaudry, A Fournier and H Vaudry (1997). "Pituitary adenylate cyclase-activating polypeptide promotes cell survival and neurite outgrowth in rat cerebellar neuroblasts." Neuroscience **78**: 419-430.

Hallbook, F, CF Ibanez, T Ebendal and H Persson (1993). "Cellular localization of brain derived neurotrophic factor and neurotrophin-3 mRNA expression in the early chicken embryo." European Journal of Neuroscience **5**: 1-4.

Hamburger, V and HL Hamilton (1992). "A Series of Normal Stages in the Development of the Chick Embryo." Developmental Dynamics **195**: 231-272.

Hansel, D, V May, BA Eipper and GV Ronnett (2001). "Pituitary adenylate cyclase-activating polypeptides and alpha-amidation in olfactory neurogenesis and neuronal survival *in vivo*." Journal of Neuroscience **21**: 4625-4636.

Hernandez-Sanchez, C, A Lopez-Carranza, C Alarcon, E de la Rosa and F de Vablo (1995). "Autocrine/paracrine role of insulin related growth factors in neurogenesis: local expression and effects in cell proliferation and differentiation in retina." Proceedings of the National Academy of Science, USA **92**: 9834-9838.

Heuer, JG, CS von Bartheld, Y Kinoshita, PC Evers and M Bothwell (1990). "Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system." Neuron **5**: 283-296.

Journot, L, M Villalba and J Bockaert (1998). "PACAP-38 protects cerebellar granule cells from apoptosis." Annals of the New York Academy of Science, USA **865**: 100-110.

Kentroti, S and A Vernadakis (1989). "Growth hormone-releasing hormone influences neuronal expression in developing chick brain. I. Catecholaminergic neurons." Developmental Brain Research **49**: 275-280.

Kentroti, S and A Vernadakis (1990). "Growth hormone-releasing hormone and somatostatin influence neuronal expression in developing chick brain. II. Cholinergic neurons." Brain Research **512**: 297-303.

Kentroti, S and A Vernadakis (1991). "Growth hormone-releasing hormone and somatostatin influence neuronal expression in developing chick brain. III. GABA-ergic neurons." Brain Research **562**: 34-38.

Kentroti, S and A Vernadakis (1992). "Neuron-enriched cultures derived from spinal cord of 10-day-old chick embryos: influence of neuropeptides on neuronal survival, proliferation and cholinergic expression." International Journal of Developmental Neuroscience **10**: 535-544.

Lee, J, H-J Park, H-S Choi, H-B Kwon, A Arimura, B-J Lee, W-S Choi and S-Y Chun (1999). "Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (PACAP) messenger ribonucleic acid in the rat ovary and the role of PACAP as a follicle survival factor." Endocrinology **140**: 818-826.

Lee, KJ and TM Jessell (1999). "The specification of dorsal cell fates in the vertebrate central nervous system." Annual Review of Neuroscience **22**: 261-294.

Lelievre, V, Z Hu, JY Byun, Y Ioffe and J Waschek (2002). "Fibroblast growth factor-2 converts PACAP growth action on embryonic hindbrain precursors from stimulation to inhibition." Journal of Neuroscience Research **67**: 566-573.

Lindholm, D, Y Skoglosa and N Takei (1998). "Developmental regulation of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor 1 in rat brain: function of PACAP as a neurotrophic factor." Annals of the New York Academy of Science, USA **805**: 189-196.

Lioudyno, M, Y Skoglosa, N Takei and D Lindholm (1998). "Pituitary adenylate cyclase-activating polypeptide (PACAP) protects dorsal root ganglion neurons from death and induces calcitonin gene-related peptide (CGRP) immunoreactivity in vitro." Journal of Neuroscience Research **51**: 243-356.

Lu, N and E DiCicco-Bloom (1997). "Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells." Proceedings of the National Academy of Science, USA **94**: 3357-3362.

Mattson, MP (1995). Neuronal Death in Primary Cell Cultures. Methods in Cell Biology. LM Schwartz and BA Osborne. San Diego, CA, Academic Press, Inc. **46: Cell Death**.

Mayo, KE, RE Hammer, LW Swanson, RL Brinster, MG Rosenfeld and RM Evans (1988). "Dramatic pituitary hyperplasia in transgenic mice expressing a human growth hormone-releasing factor gene." Molecular Endocrinology **2**: 606-612.

Mayo, KE, TL Miller, V DeAlmeida, J Zheng and PA Godfrey (1996). "The growth-hormone-releasing hormone receptor: signal transduction, gene expression, and physiological function in growth regulation." Annals of the New York Academy of Science, USA **805**: 184-203.

McRory, JE, RL Parker and NM Sherwood (1997). "Expression and alternative processing of a chicken gene encoding both growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP)." DNA and Cell Biology **16**: 95-102.

Moroo, I, I Tatsuno, D Uchida, T Tanaka, J Saito, Y Saito and A Hirai (1998). "Pituitary adenylate cyclase-activating polypeptide stimulates mitogen-activated protein kinase (MAPK) in cultured rat astrocytes." Brain Research **795**: 191-196.

Mulrenin, EM, JW Witkin and A-J Silverman (1999). "Embryonic development of the gonadotropin-releasing hormone (GnRH) system in the chick: a spatio-temporal analysis of GnRH neuronal generation, site of origin, and migration." Endocrinology **140**: 422-433.

Nagtegaal, ID, EAJF Lakke and E Marani (1998). "Trophic and tropic factors in the development of the central nervous system." Archives of Physiology and Biochemistry **106**: 161-202.

Nowak, JZ, K Kuba and JB Zawilska (1999). "Stimulatory effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on cyclic AMP formation in the hypothalamus and cerebral cortex of four avians and rat." Polish Journal of Pharmacology **51**: 87-91.

Parker DB, Coe IR, Dixon GH, and NM Sherwood (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

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

Peeters, K, HHJ Gerets, K Princen and F Vandesande (1999). "Molecular cloning and expression of a chicken pituitary adenylate cyclase-activating polypeptide receptor." Molecular Brain Research **71**: 244-255.

Pesce, M, R Canipari, GL Ferri, G Siracusa and M De Felici (1996). "Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates adenylate cyclase and promotes proliferation of mouse primordial germ cells." Development **122**: 215-221.

Rahman, H, S Kentroti and A Vernadakis (1994). "Neuroblast cell death *in ovo* and in culture: interaction of ethanol and neurotrophic factors." Neurochemical Research **19**: 1495-1502.

Schafer, H, J Zheng, C Morys-Wortmann, UR Fölsch and WE Schmidt (1999). "Structural motifs of pituitary adenylate cyclase-activating polypeptide (PACAP) defining PAC-$_1$ receptor selectivity." Regulatory Peptides **79**: 83-92.

Sherwood, NM, SL Krueckl and JR McRory (2000). "The origin and function of the PACAP/glucagon superfamily." Endocrine Reviews **21**: 619-670.

Suh, J, N Lu, A Nicot, I Tatsuno and E DiCicco-Bloom (2001). "PACAP is an anti-mitogenic signal in developing cerebral cortex." Nature **4**: 1-2.

Tabuchi, A, M Koizumi, J Nakatsubo, T Yaguchi and M Tsuda (2001). "Involvement of endogenous PACAP expression in the activity-dependent survival of mouse cerebellar granule cells." Neuroscience Research **39**: 85-93.

Takei, N, Y Skoglosa and D Lindholm (1998). "Neurotrophic and neuroprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on mesencephalic dopaminergic neurons." Journal of Neuroscience Research **54**: 698-706.

Tatsuno, I, PE Gottschall and A Arimura (1991). "Inhibition of mitogen-stimulated proliferation of murine splenocytes by a novel neuropeptide, pituitary adenylate cyclase activating polypeptide: a comparative study with vasoactive intestinal peptide." Endocrinology **128**: 728-734.

Vallejo, I and M Vallejo (2002). "Pituitary adenylate cyclase-activating polypeptide induces astrocyte differentiation of precursor cells from developing cerebral cortex." Molecular and Cellular Neuroscience **21**: 671-683.

Vaudry, D, BJ Gonzalez, M Basille, A Fournier and H Vaudry (1999). "Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development." Proceedings of the National Academy of Science, USA **96**: 9415-9420.

Vaudry, D, BJ Gonzalez, M Basille, TF Pamantung, A Fournier and H Vaudry (2000). "PACAP acts as a neurotrophic factor during histogenesis of the rat cerebellar granule cortex." Annals of the New York Academy of Science, USA **921**: 293-299.

Villalba, M, J Bockaert and L Journot (1997). "Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway." Journal of Neuroscience **17**: 83-90.

Waschek, JA, RA Casillas, TB Nguyen, EM DiCicco-Bloom, EM Carpenter and WI Rodriguez (1998). "Neural tube expression of pituitary adenylate cyclase-activating polypeptide (PACAP) and receptor: potential role in patterning and neurogenesis." Proceedings of the National Academy of Science, USA **95**: 9602-9607.

Wilson, SI, E Graziano, R Harland, TM Jessell and T Edlund (2000). "An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo." Current Biology **10**: 421-429.

Appendix 1. Composition of Neurobasal medium.

Inorganic salts
$CaCl_2$ (anhydrous)
$Fe(NO_3)_3 \cdot 9H20$
KCl
$MgCl_2$ (anhydrous)
NaCl
$NaHCO_3$
$NaH_2PO_4.H_2O$

Amino Acids
L-alanine
L-arginine·HCl
L-asparagine·H20
L-cysteine
glycine
L-histidine·HCl·H_2O
L-isoleucine
L-leucine
L-lysine·HCl
L-methionine
L-phenylalanine
L-proline
L-serine
L-threonine
L-tryptophan
L-tyrosine
L-valine

Vitamins
D-Ca pantothenate
choline chloride
folic acid
i-inositol
niacinamide
pyridoxal·HCl
roboflavin
thiamine·HCl
vitamin B12

Other
D-glucose
phenol red
HEPES
sodium pyruvate

Appendix 2. Composition of B27 supplement.

biotin
L-carnitine
cortisone
ethanolamine
D(+)-galactose
glutathione (reduced)
linoleic acid
linolenic acid
progesterone
putrescine
retinyl acetate
selenium
T3 (triodo-1-thyronine)
DL-α-tocopherol (vitamin E)
DL-α-tocopherol acetate
Proteins
albumin, bovine
catalase
insulin
superoxide dismutase
transferrin

CHAPTER 3

Flow cytometry studies show that PACAP decreases apoptosis and increases proliferation in chick neuroblasts

INTRODUCTION

PACAP, a highly conserved member of the glucagon superfamily of hormones, was first characterized by its ability to stimulate adenylate cyclase, and thereby increase cAMP in pituitary cells (Miyata et al. 1989; Hosoya et al. 1992). PACAP exerts its effect either through two receptors it shares with VIP, or through a receptor with much higher affinity for PACAP, PAC_1-R. The latter is the most dominant form in the brain during development (Sherwood et al. 2000), and this was the form we identified in these neuroblast-enriched cultures (Chapter 2). Alternate splicing of a single gene encoding PAC_1-R creates nine isoforms that act through adenylate cyclase and a G protein, probably G_s (Sherwood et al. 2000; Laburthe et al. 2003). It is the variation in these receptors that can lead to a variety of downstream effects. For example, the PAC_1-R-hop isoform was identified in sympathetic neuroblasts, which responded to PACAP by activating both the cAMP and phospholipase C pathways (Lu et al. 1998). In contrast, the PAC_1-R-short was the predominant isoform expressed in cerebral cortical precursors, and these cells responded to PACAP by activating only the cAMP pathway (Lu et al. 1998). In the sympathetic neuroblasts, proliferation was increased, and in the cerebral cortical precursors, proliferation was decreased.

PACAP was shown to cause a dose-dependent increase in cAMP in neuroblast-enriched primary cell cultures derived from embryonic day 3.5 chick brain (Chapter 2). It seemed unlikely that the increase in cAMP was due simply to a neurotrophic effect, because it occurred at time when the cultures should not yet be expected to be degenerating. Several downstream effects were possible, including regulation of final cell numbers through control of proliferation and apoptosis. In rat and mouse nervous

system, PACAP has been reported to affect proliferation by acting through cAMP (Lu and DiCicco-Bloom 1997; Waschek et al. 1998), and has also been shown to inhibit apoptosis through the cAMP pathway (Campard et al. 1997; Chang and Korolev 1997; Journot et al. 1998).

My goal was to assess the impact of the peptide on proliferation and apoptosis. Several methods were briefly tested before the final decision was made to use an antibody against proliferating cell nuclear antigen (PCNA), with propidium iodide (PI), in conjunction with flow cytometry. PCNA is essential for DNA synthesis, and is present throughout the cell cycle (McCormick and Hall 1992; Jónsson and Hübscher 1997). Quiescent cells show a small amount of immunolabeling (Garrido et al. 2000), but intensity increases five to ten-fold in cycling cells (Belyavskyi et al. 1995). To measure DNA content, cells were fixed to allow entry and binding of PI to DNA. Cells were tested by adding PACAP to the cultures, by blocking a PACAP-specific receptor, and by blocking the cell cycle to test whether PACAP could overcome the block.

MATERIALS AND METHODS

Cell culture

Cell culture was as previously described (Chapter 2) with the exception of cell concentration and addition of peptides. Briefly, brain tissue from E3.5 chicks was dissociated mechanically and single cell suspensions were plated in Neurobasal medium (Invitrogen, Burlington, ON), with the manufacturer's recommended supplements. Cells were plated at various concentrations for preliminary work, and were used at 3 X 10^6 cells/ml for flow cytometry. Peptides were added as described below.

Peptides and reagents

Chicken PACAP38 was a kind gift from Dr. Jean Rivier at the Salk Institute for Biological Studies (La Jolla, CA). Ascorbic acid (0.5 mM) was added to prevent degradation of the peptide. Human PACAP6-38 was purchased from American Peptide Company (Sunnyvale, CA) and AnaSpec Inc (San Jose, CA), and was used to block the PAC_1 receptor. Culture slides were purchased from Bectin Dickinson (Mississauga, ON). Chick red blood cell nuclei were also purchased from Bectin Dickinson, as part of a kit (349523). The 1{ß-[3-(4-Methoxyphenyl)-propoxy]-4-methoxyphenylethyl (SK&F 96365), used to block capacitative calcium entry, and the bis-benzimide dye (Hoechst 33258), used to stain apoptotic nuclei, were purchased from Cedarlane Laboratories (Hornby, ON). A BrdU Incorporation kit was purchased from CalBiochem (San Diego, CA). The Alexa Fluor 488®, a Cyquant Proliferation Assay kit, and an Ezchek Caspase 3 Assay kit were purchased from Molecular Probes (Eugene, OR). Staurosporine, used to induce apoptosis, was purchased from Sigma (Oakville, ON) and diluted in DMSO,

with the final concentration not exceeding 1%. All other reagents, including the anti-mouse monoclonal antibody against PCNA (clone PC10), were purchased from Sigma.

Preliminary work

Since a valid and easy method of testing whether cell numbers are increasing or decreasing in culture is to simply count them, I began by plating cells at various densities, treating them with hormone concentrations known to increase cAMP, and observing them. My goal was to find a plating density at which the cells remained healthy for at least 1-2 days, and did not aggregate to the point that counting individual cells would be unreliable. From the cAMP studies I judged that frequent observation over the first 24 hours could give me at least preliminary results. This approach was unsuccessful, because cells began to aggregate within 2-3 hours, and cultures did not remain healthy at low densities for even 24 hours.

My next approach was to fix aliquots of cells, in a manner similar to the protocols used to label for NSE and PACAP (Chapter 2), but label for PCNA. Briefly, I plated cells cultured for 24 h at concentrations varying from 3 X 10^6 cell/ml (about 750,000 per chamber) to 0.25 X 10^6 cells/ml (about 30,000 per chamber) in 8-chamber culture slides. I carefully removed the supernatant, and fixed the cells with ice-cold methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for 0.5-5 min at –20 C. Cells were air dried, rehydrated, and blocked with 5% sheep serum in PBS for 15 min at rt. Dilutions between 1:1000 and 1:10 of anti-PCNA were tested, for 24 h at 4 C. Cells were washed in PBS and dilutions between 1:100 and 1:40 anti-mouse IgG bound

to Alexa Fluor 488® were applied for 1-1.5 h at room temperature (rt), which was about 20 C. After additional washes, cells were mounted and viewed.

Although it was possible to use concentrations of cells closer to normal in this protocol, difficulties still arose with the appearance of clumped cells, and additionally, with the degree of subjectivity required to determine not only what constituted a cell, versus a cell fragment, but also what constituted positive labeling. Also, since the procedure again required washing, cell loss was a problem and a particular concern was that the loss was selective, since proliferating cells were less likely to attach to chamber slides as tightly as did the more differentiated cells.

A manual counting protocol using the bis-benzimide dye (Hoechst 33258) to identify apoptotic cells encountered similar problems. Briefly, cells were plated at 100,000 cells per chamber in 4-chamber culture slides for 2.5 h to 3 days, carefully drained, and fixed in 3:1 methanol: acetic acid for 30 min at rt. Cells were washed, and 10-50 μg/ml bis-benzimide was added and cells incubated for 15-30 min at rt. Cells were washed, mounted, and viewed. Again, it was difficult to distinguish individual cells, and identification of apoptotic nuclei required subjective assessment.

Furthermore, a kit based on incorporation of BrdU, and a kit based on binding of a fluorophore to nucleic acids (Cyquant assay), were tested for assessment of proliferation. A kit based on cleavage of a caspase 3 substrate was tested for assessment of apoptosis. All gave variable results, perhaps due to some of the problems described above.

It was my opinion at this point that what was needed was a method that would allow me to plate at normal densities, minimize manipulation of the cells, especially the

number of washes, and be able to measure small changes in large populations, with great sensitivity and reliability. This led me to the flow cytometer. Initial studies using propidium iodide as a relative measure of DNA content in fixed cells, and a software program designed to analyze transformed human blood cells (ModFit) was unsuccessful, at least partially because primary cell cultures do not consist of discrete cell populations of different cell sizes and densities. Developing a protocol using a more generalized software program (Cellquest) involved a great degree of trouble-shooting, partially again because primary cell cultures produce gradients of cell sizes and densities, and because fixation resulted in a large amount of debris. However, I was able to develop a working protocol to examine the effects of PACAP on cell cycle, and apoptosis, using anti-PCNA and PI in fixed cell populations.

Final protocols using flow cytometry

Defining the Cell Cycle Compartments

Untreated cells were cultured for 5, 12, 24, 48 and 72 h, then processed for flow cytometry in the following manner. Cell culture supernatants were removed and kept on ice. Attached cells were detached from plates using gentle agitation in 0.025% trypsin in PBS for 10 min at rt. Enzyme action was stopped by addition of trypsin inhibitor. Also, any remaining cells were scraped from all wells using a soft rubber spatula before being combined with their respective supernatants.

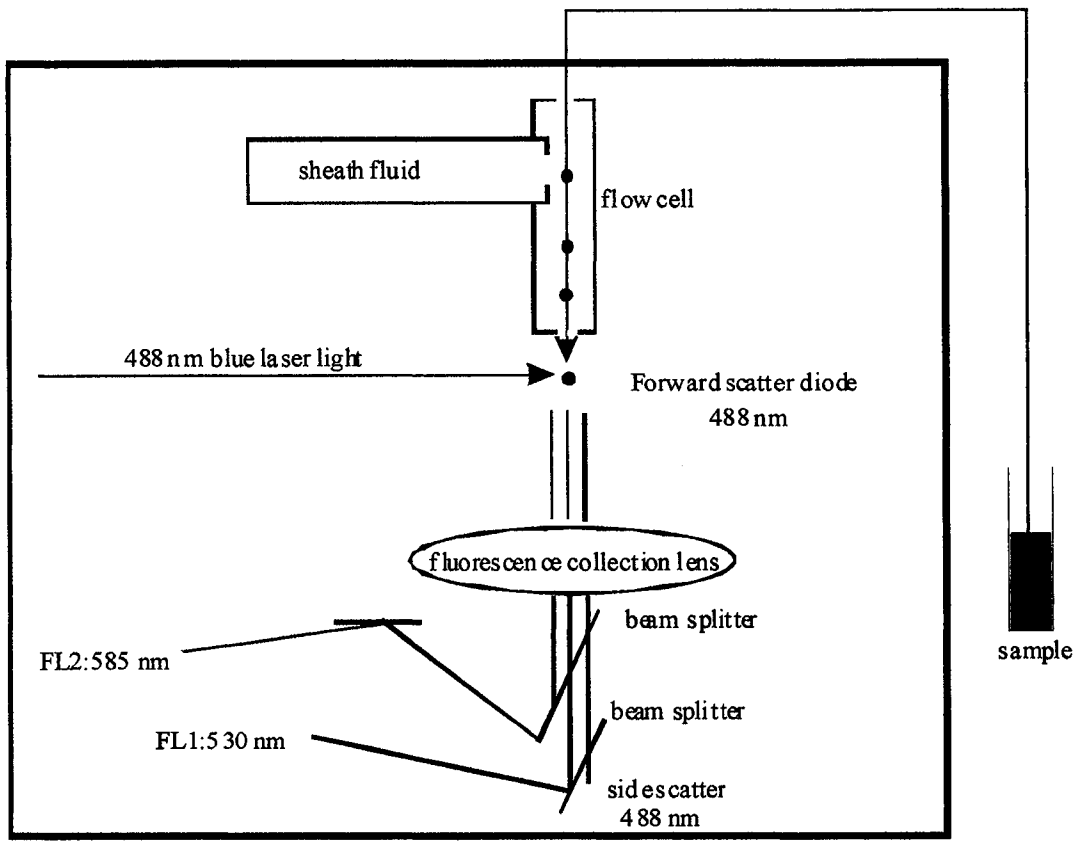
Cells were centrifuged at 1000X g for 5 min at rt, and washed in 0.5 ml cold PBS. Cells were resuspended by gentle vortexing in 0.25 ml cold PBS, then fixed by addition of 0.75 ml ice-cold 100% ethanol, added drop by drop while vortexing to minimize clumping. Alcohol fixation was chosen because it not only fixes but also

permeabilizes cells, and allows highly reproducible detection of PCNA (Casasco et al. 1993; Garrido et al. 2000). Fixation proceeded for 30 min at –20 C.

Cells were centrifuged as above to remove the fixative, then washed with 0.5 ml cold PBS. Vortexing to resuspend cells was quite vigorous to minimize cell clumping. The wash procedure left approximately 30 µl PBS with the cells in each tube, and to each tube was added 80 µl primary antibody solution, consisting of a 1:80 dilution of anti-PCNA and 10% sheep serum in PBS. Cells were incubated for 60 min at rt, then diluted in 0.5 ml rt PBS before centrifugation as above. The secondary antibody solution was added, consisting of a 1:100 dilution of goat anti-mouse IgG conjugated to FITC with 10% sheep serum in PBS. Cells were again incubated for 60 min at rt, then diluted and centrifuged as above. Cells were finally resuspended in 0.5 ml rt PI solution consisting of 100 µg/ml RNase and 10 µg/ml PI in PBS, then incubated for 45 min at rt. Cells were moved to ice and analyzed immediately. Controls for these assays consisted of one tube with no reagents (unstained control), one tube with only the secondary antibody FITC (non-specific staining control), one tube with only the anti-PCNA conjugated to FITC (PCNA positive control), and a fourth with PI only (PI positive control). All PBS used in the protocol was Mg^{++} and Ca^{++} free.

To analyze the cell cycle, two-parameter flow cytometric analysis was performed using a Bectin Dickinson FACSCalibur™ equipped with an argon ion laser operating at 488 nm, and analysis was performed using Cellquest software (Fig. 3.1). Threshold was set at 52 on forward scatter. The forward scatter diode was set at E00 (no multiplication of signal), with linear amplification. The secondary antibody, FITC, bound to PCNA

Figure 3.1 Drawing outlining main parts of the flow cytometer. Treated cells from the sample tube are drawn into the machine, combined with sheath fluid, and sprayed individually past a laser light beam which records forward scatter (a measure of relative size), side scatter (a measure of relative granularity), and the intensity of as many as four fluorophores, for each cell. For simplicity, the diagram shows collection of only the two fluorescence intensities used in these experiments. Data is recorded by a connected computer for immediate or later analysis. FL=fluorophore


sheath fluid
flow cell
488 nm blue laser light
Forward scatter diode
488 nm
fluorescence collection lens
beam splitter
FL2:585 nm
beam splitter
FL1:530 nm
side scatter
488 nm
sample

emits fluorescence at near 530 nm and was recorded on the channel for fluorophore 1 (FL1), and the PI emits at near 585 nm and was recorded on FL2. Voltage was adjusted to 250 for side scatter, 450 for PCNA-FITC and 620 for PI fluorescence. The side scatter and PCNA-FITC fluorescence were measured with logarithmic amplification, and the PI fluorescence was measured using linear amplification. Based on control tubes, compensation for overlapping fluorescence was set to remove 10% PCNA fluorescence from the channel recording PI fluorescence.

Both forward and side angle light scatter were recorded for the total cell population so that each sample could be monitored for changes in the range of normal cell size and cell granularity. A gate was generated from these data by plotting PI width against PI height to exclude debris and apoptotic cells (lower than normal width and height), and aggregated cells (higher than normal width and height). The flow cytometer was set to collect events only from inside this gate, which contained primarily single and healthy cells, and stop after 10,000 events had been collected. Samples were assayed in duplicate, and each assay performed twice, unless otherwise noted. Data were converted to DNA content (based on PI fluorescence) versus cycling activity (based on PCNA content) on dot plots. Preliminary cell cycle compartment gates were drawn on these plots based on the results of control tubes and DNA histograms.

Although apoptosis was of interest in these studies, DNA width versus height plots do not allow clear distinction between debris and apoptotic cells. Therefore, apoptotic cells were excluded from the cell cycle analyses to eliminate as much debris as possible. This was necessary because large amounts of debris could obscure small changes in the cell cycle. Apoptosis quantification is explained below.

The tentative parameters of all cell cycle compartments were tested. Chick red blood cells (cRBCs) were used to confirm the DNA content of the G_1 region, since both are 2N. Because the cRBCs used here were from a kit for calibrating the flow cytometer, the cells formed equidistant scatter patterns of single cells, doublets, triplets, etc. The far left heavier scatter was used as the 2N, single cell standard. Because cRBCs are also non-cycling and therefore express minimal PCNA, they were also used to test the parameters of the G_0 region. The cRBCs were removed from the manufacturer's ethanol fixative at the same time and in the same way as neuroblasts cultured for 24 h and fixed in ethanol, and processed identically after that point. The parameters of the G_2/M region were tested by treating neuroblasts with SK&F 96365, which blocks capacitative calcium entry. SK&F 96365 has been shown to inhibit DNA synthesis in E3 chick retinal cells (Sugioka et al. 1999), but data have also suggested that this chemical will cause a block at the G_2/M stage (Nordstrom et al. 1992; Barbiero et al. 1995). At plating, and at 12 h incubation, SK&F 96365 solution was added to the cultures to achieve a final concentration of 3 μM, and the cells were harvested at 24 h.

Characterizing and Blocking the Cell Cycle

Untreated cells cultured for 5, 12, 24, 48 and 72 h were used to assess the proportion of cells in each cell cycle compartment (G_0, G_1, S, G_2/M). Culture medium was not changed in any of these assays, but cultures were examined under a light microscope prior to processing to confirm normal appearance of growth. Data were collected from at least six independent samples from at least two separate assays.

To test whether PACAP could release cells from cell cycle blocks, SK&F 96365 was again used, at a concentration below that had been used to block proliferation in chick retinal cells without increasing mortality (Sugioka et al. 1999). The cells were treated as described above. Whereas SK&F inhibits the actions of calcium by blocking entry, the calcium chelator EGTA blocks calcium effects extracellularly. Therefore, EGTA was tested to determine if it would also block cells during the cell cycle, possibly at a different stage. Cells were treated with a final concentration of 1 mM EGTA. The EGTA was added at plating, and again at 12 h. Cells were harvested at 24 h.

Defining and Characterizing the Natural Apoptotic Populations

The distinction between debris and apoptotic cells was based on DNA versus PCNA dot plots generated from collection of *ungated* populations, because apoptotic cells appear as a discrete population with low DNA (small cleaved fragments leak out of the cell) and low PCNA (because it is down-regulated), whereas debris appears as a denser region virtually devoid of DNA and PCNA. (Aggregated cells appear as a dense region with very high amounts of DNA and PCNA). Therefore, a tentative apoptotic gate was drawn on these plots, and tested by treatment with staurosporine. Staurosporine is an indolocarbazole alkaloid which inhibits several kinases, but particularly protein kinase C. This compound has been used to induce apoptosis in various cell types. Staurosporine has been used by two groups to induce apoptosis in cells derived from E7 chick brain (Ahlemeyer et al. 2000; Dawson 2000). Cultures were treated with 1 μM staurosporine for 24 h before processing. Six independent samples were collected from two experiments.

Naturally-occurring apoptosis was assessed in the same manner as for cell cycle compartments, with the exception that the flow cytometer was set to collect all events, including those in the apoptotic, debris and aggregate regions, and to stop after 10,000 events had been recorded from inside the gate that delineated healthy, single cells. Because debris, apoptosis and aggregation were generally about 40-50% of the cultures, this meant that total cell counts that determined the proportion of apoptotic cells in each assay were generally between 20,000 and 30,000 events.

Assessing the PACAP Receptor Blockade

Although the ability of PACAP6-38 to block the PAC_1 receptor in chick has not yet been tested, the chicken PAC_1-R shares 81-83% homology with mammalian PAC_1-R (Peeters et al. 1999). PACAP6-38 is commonly used to block mammalian PAC_1-R (Shoge et al. 1999; Jamen et al. 2002; Silveira et al. 2002; Vaudry et al. 2003). Cultures treated with PACAP6-38 were harvested at 5 and 24 h only. A final concentration of 10 μM was tested in 5 h cultures, and a final concentration of 5 μM was tested in 24 h cultures. For 24 h cultures, the block was added at plating and replenished at 12 h, so that the total amount added was the same as for the 5 h cultures. Again, cell cycle analysis was based on gated populations, and changes in apoptosis were based on ungated populations.

Assessing the Effects of PACAP on Cell Cyle, Cycle Blockage and Natural Apoptosis

For assessment of PACAP effects on cell cycle, data collected from previous experiments (Chapter 2) were used to determine which concentrations and what form of

PACAP to use. Because both 10^{-7} M and 10^{-6} M PACAP caused peak increases in cAMP in those assays, both concentrations were used in these experiments. Because the human and chicken forms of PACAP had produced similar results, the chicken form was used here. Also, the short form of PAC_1-R was identified in the neuroblasts in those studies, and this receptor isoform has been shown to bind PACAP38 with greater affinity than PACAP27 (Sherwood et al., 2000). Therefore, the 38 amino acid form of chicken PACAP was used here.

Cells were cultured for 5, 12, 24, 48 and 72 h. Peptides were diluted in PBS and added at plating. Peptides were added again at 12, 24, 36, 48, and 60 h for longer assays. Ascorbic acid was only replenished at 24 and 48 h as required, since more frequent additions caused a buildup of crystals in the medium (data not shown). Data on peptide effects were collected from at least six independent samples taken from at least two separate assays.

To determine the effects of PACAP on blockage of the cell cycle, 10^{-7}M PACAP was added at plating to cultures treated with EGTA and SK&F 96365. Cells were harvested at 24 h.

To assess the effects of PACAP on natural apoptosis, cells were treated with both 10^{-7} M and 10^{-6} M concentrations of PACAP, for 5, 12, 24, 48 and 72 h. Peptides and ascorbic acid were replenished as above. Again, the flow cytometer was set to collect *all* events, and stop after 10,000 events had been recorded inside the gate that delineated healthy, single cells.

Data analysis

Unless otherwise stated, data were collected from at least four independent samples from at least two separate assays. Tukey's test was used to identify statistical changes in adjacent harvesting times (e.g. 5 h versus 12 hours, 12 hours versus 24 hours, etc.) for the three-day cultures. The values obtained during cell cycle arrest experiments were also analyzed using Tukey's test. ANOVA was used to analyze cultures in which cells had been blocked by PACAP6-38, and in cultures treated with staurosporine. All other statistical analysis was done using ANOVA followed by Dunnett's test. Standard error bars are included on all graphs. One asterisk is used to indicate significance at $p<0.05$, two asterisks are used to indicate significance at $p<0.01$, and three asterisks are used to indicate statistical significance at $p<0.001$ or less.

RESULTS

Analysis reveals massive proliferation

Light scatter for the cell population showed a broad range of forward scatter indicating a wide range of cell sizes, which is expected for a primary culture of both proliferating and differentiating neuroblasts (Fig. 3.2, upper panel). Tentative cell cycle compartments, and an area of mixed apoptosis and debris, as well as an area of aggregates, could be identified by DNA width versus height contour and dot plots considered jointly (Fig. 3.2, lower panels). The gate drawn to exclude all but healthy single cells was used to plot the DNA versus PCNA content of the cells. This revealed several distinct regions which could be tentatively identified as G_0, G_1, S and G_2/M (Fig. 3.3). In all assays, at least 90% of the cells within the gate drawn in Fig. 3.2 were included in the four compartment gates in Fig. 3.3, with the exception of 72 h cultures, where the percentage dropped to 80% or more (data not shown). The gates for G_0/G_1 and G_2/M were based on the presence of two heavy scatter regions, with the second region showing double the DNA content as the first region (see inset histograms, Fig. 3.3). The region of positive PCNA labeling was based on control tubes, where most PCNA-FITC scatter was above 10^2 on the y axis, and most scatter for unstained and non-specific labeling was consistently below this mark (Fig. 3.4). In all assays, almost all of the cells were above 10^2 on the y axis, which showed that they were in a proliferative state.

The parameters of the G_1 gate were tested by comparing neuroblast and cRBC DNA content on DNA versus PCNA dot plots (Fig. 3.5). A gate drawn around the heaviest scatter for the cRBCs (lower panel) and copied to the same vertical location

Figure 3.2 Typical flow cytometer plots used to analyze cell populations in cultured E3.5 chick neuroblasts. Cells were analyzed initially only for DNA content. Forward scatter (an indication of relative cell size), and side scatter (an indication of relative cell granularity) were recorded for total cell populations (upper panel). This scatter was then converted to contour and dot plots to compare the intensity of propidium iodide (PI) fluorescence height to width (lower panels). Consideration of these scatter patterns allowed creation of a gate to exclude debris, apoptotic cells and aggregated cells.

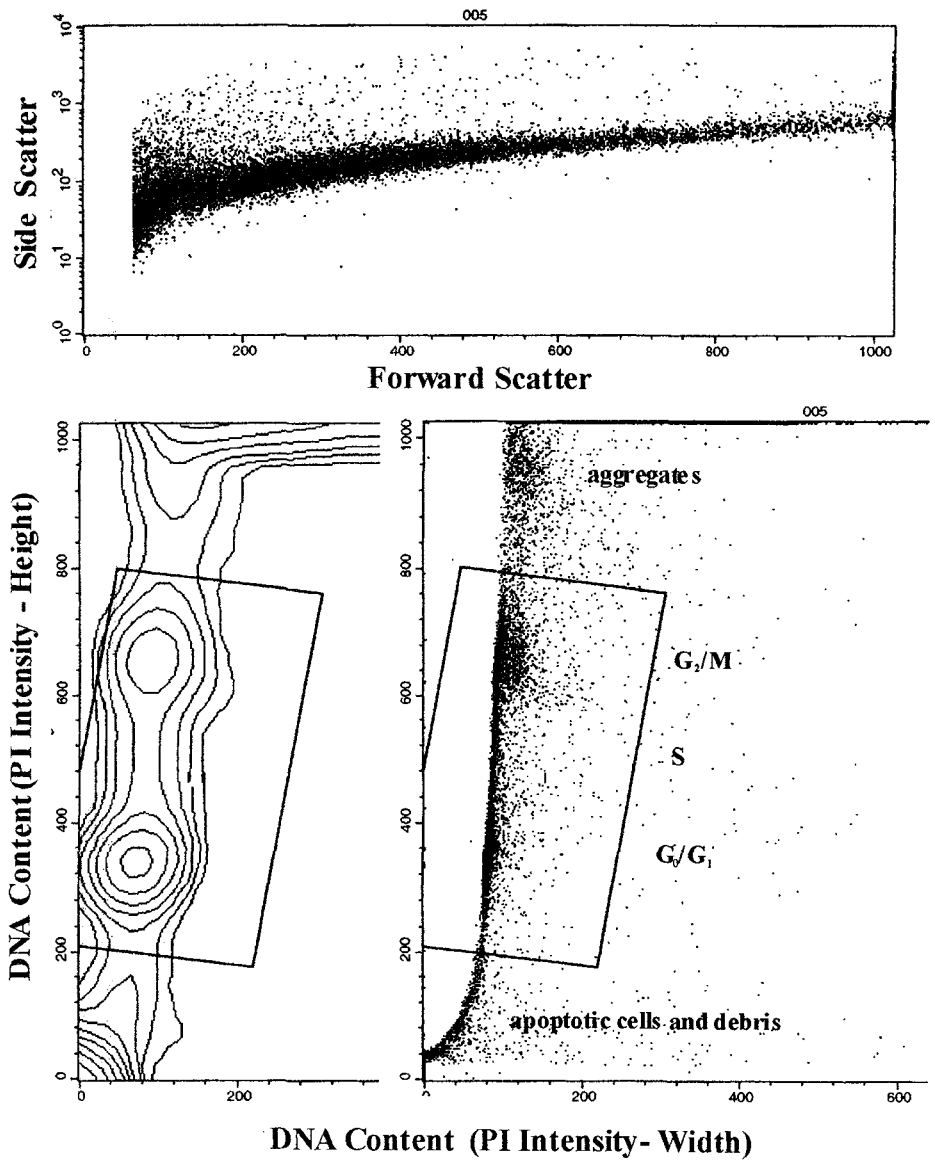

005
Side Scatter
Forward Scatter
005
aggregates
G2/M
S
G0/G1
apoptotic cells and debris
DNA Content (PI Intensity - Height)
DNA Content (PI Intensity- Width)

Figure 3.3 Typical scatter of cultured E3.5 chick neuroblasts from gate drawn in Fig. 3.2, plotted as DNA content against PCNA content. Preliminary cell cycle compartment gates were drawn as shown, then tested as described in the following text and figures. Green=quiescent cells; blue=G_1; orange=S; red=G_2/M. Black scatter was outside the range of healthy individual cells and was not counted.

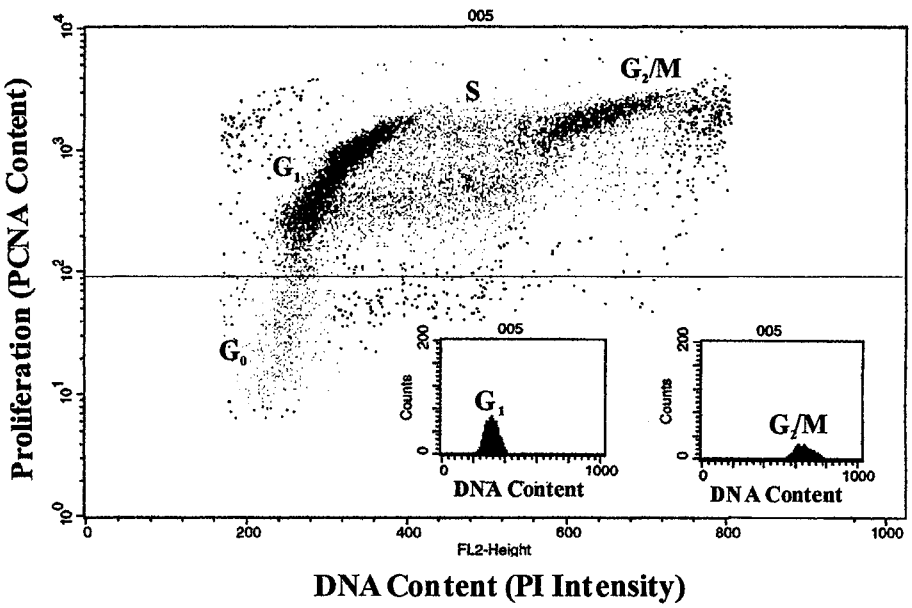

005
Proliferation (PCNA Content)
S
G2/M
G1
G0
005
Counts
G1
DNA Content
005
Counts
G2/M
DNA Content
FL2-Height
DNA Content (PI Intensity)

Figure 3.4 Control scatter used to tentatively set the region of G_0. The unstained cells were primarily below 10^2 on the y axis. The non-specifically labeled cells were in the same general area, although some non-specific labeling did bring the scatter a little higher. The PCNA-FITC control tube, which could be expected to contain primarily labeled cells, with some unlabeled cells representing debris and apoptotic cells, was for the most part above 10^2 on the y axis. The parameters of the G_0 region were further tested using chick red blood cells; see text for more details.

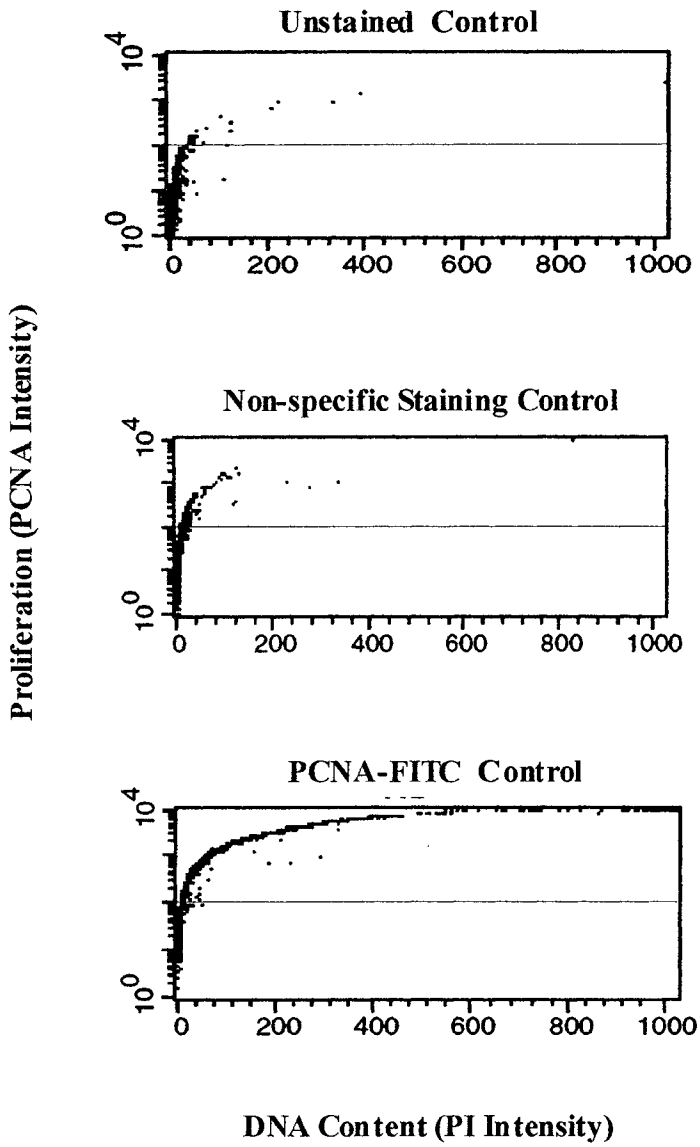
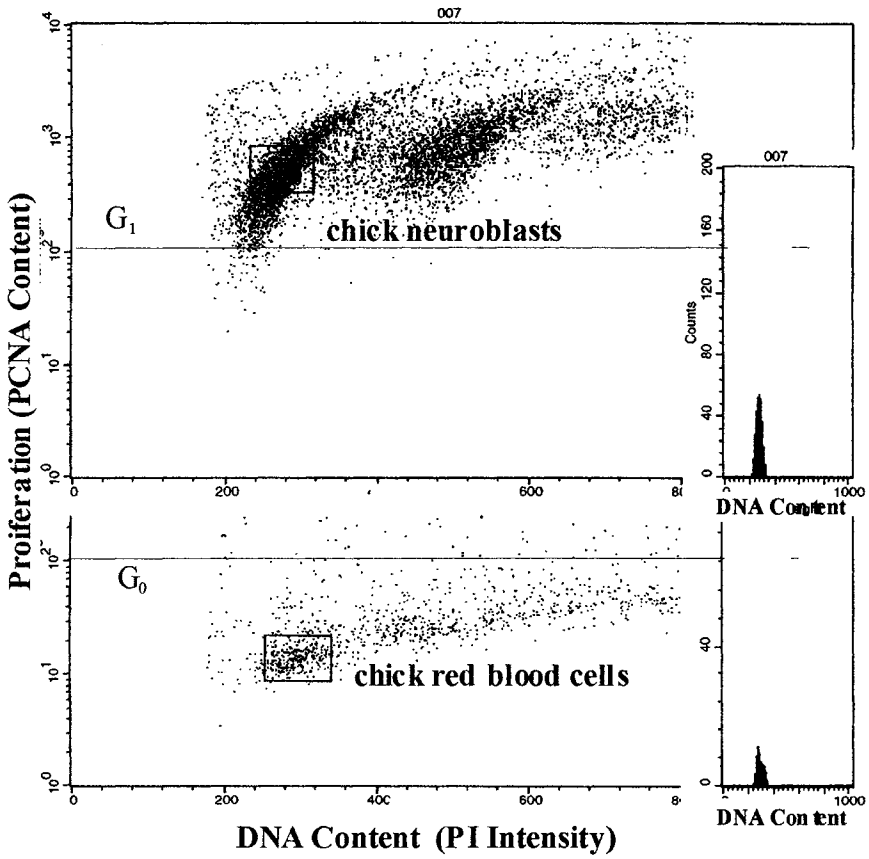

Unstained Control
Non-specific Staining Control
PCNA-FITC Control
Proliferation (PCNA Intensity)
DNA Content (PI Intensity)
10^4
10^0
0
200
400
600
800
1000

Figure 3.5 Effects of tests of G_1 and G_0 parameters. Chick red blood cells (lower panel) were processed with 24 h neuroblasts (upper panel). Histograms (inset) revealed 2N DNA in a gate placed in the same vertical location in both panels. (As noted in the text, scatter from the cRBCs spread horizontally across the screen, because the cRBCs formed doublets and triplets etc.; therefore the main scatter, representing single cells, was used as the standard.) Scatter from the blood cells also suggested that non-cycling, or G_0 cells, could be identified by their position below 10^2 on the y axis.


007
Proiferation (PCNA Content)
G1
chick neuroblasts
G0
chick red blood cells
DNA Content (PI Intensity)
007
Counts
DNA Content
DNA Content

on the scatter plot for the neuroblasts (upper panel) appeared to verify the location of 2N neuroblasts. This was supported by the DNA histograms (inset). In addition, scatter from the blood cells supported data from control tubes showing that non-cycling, or G_0 cells, could be identified by their position below 10^2 on the y axis.

SK&F 96365 treatment did appear to arrest cells in G_2/M, because there was an increase in the region of 4N cells in response to treatment (Fig. 3.6). DNA versus PCNA dot plots show typical scatter for both control (upper panel) and SK&F 96365-treated cultures (lower panel), but a denser region in the area that had been tentatively identified as G_2/M is easily noted in the cultures treated with the calcium blocker (lower panel). The DNA histograms (inset) also show a clear buildup in the G_2/M area in response to SK&F 96365.

Proliferation continues over time

Typical areas representing G_0, G_1, S and G_2/M throughout three days of continuous cell culture are visualized in Fig. 3.7. It appeared that the population did not shift markedly until about 24 h, when a lower portion of G_2/M began to build and a higher area of G_2/M scatter decreased in size. Fig. 3.8, which compares the mean proportion of cells within each compartment over the three days, would not reveal this change, since G_2 and mitotic cells are combined within the G_2/M region. However, the distinctness of the population and its location, after 24 h, suggests that the

Figure 3.6 Effects of SK&F treatment. Cultured chick neuroblasts were treated with SK&F 96365, which blocked calcium entry and arrested cells in G_2/M. Scatter and histograms (inset) revealed a clear block in treated cells (lower panel), and this matched the location of 4N cell scatter in the neuroblast cultures (upper panel). The arrow indicates a putative mitotic region, where PCNA has begun to decrease but the cells are not yet separated into 2N cells.

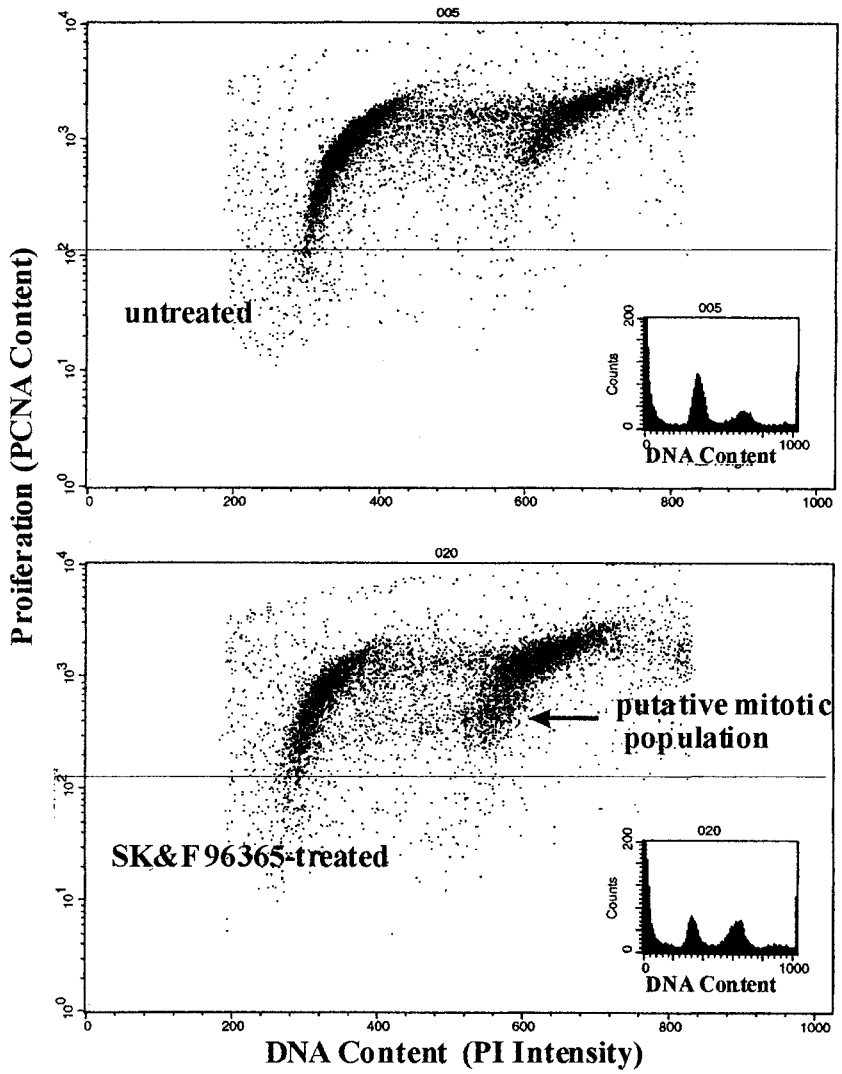

005
untreated
005
Counts
DNA Content
020
putative mitotic
population
SK&F 96365-treated
020
Counts
DNA Content
Proiferation (PCNA Content)
DNA Content (PI Intensity)

Figure 3.7 Typical scatter plots showing changes in cell cycle compartments during 72 h of continuous neuroblast culture. Cells were dissected from E3.5 chicks, grown in serum-free medium, then fixed and analyzed for DNA and PCNA content. Dot and contour plots for (a) 5 h, (b) 12 h, (c) 24 h, (d) 48 h and (e) 72 h suggested little change for the first 24 h, at which point a build-up of cells below and on the left of the main population of the G_2/M region probably represented a mitotic cell population. This suggested a cell cycle of approximately 24 h, which was supported by an increase in the G_1 region concurrent with a decrease in the G_2/M region by 48 h. Changes in cycling patterns were confirmed by analysis of cell numbers (see Figure 3.8).

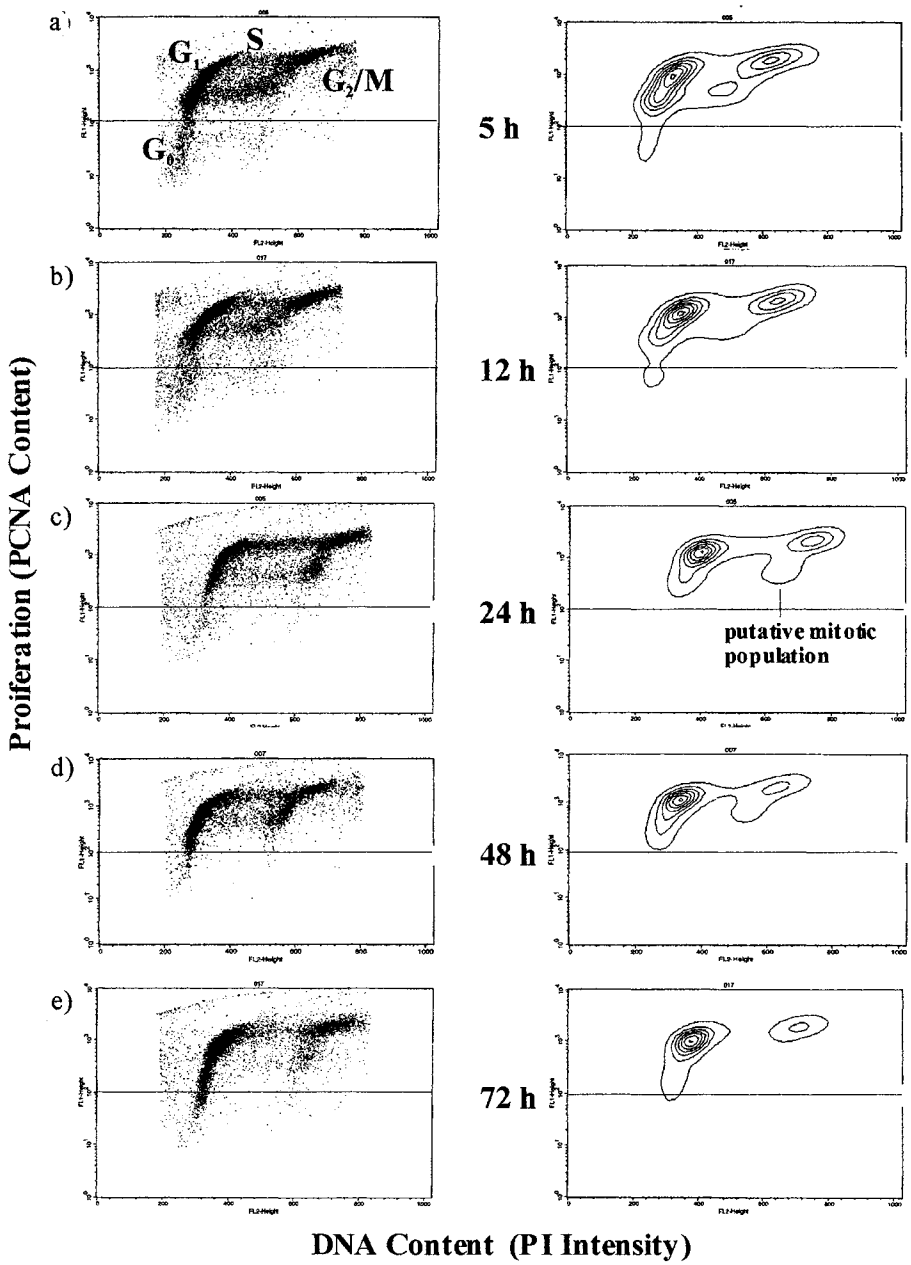
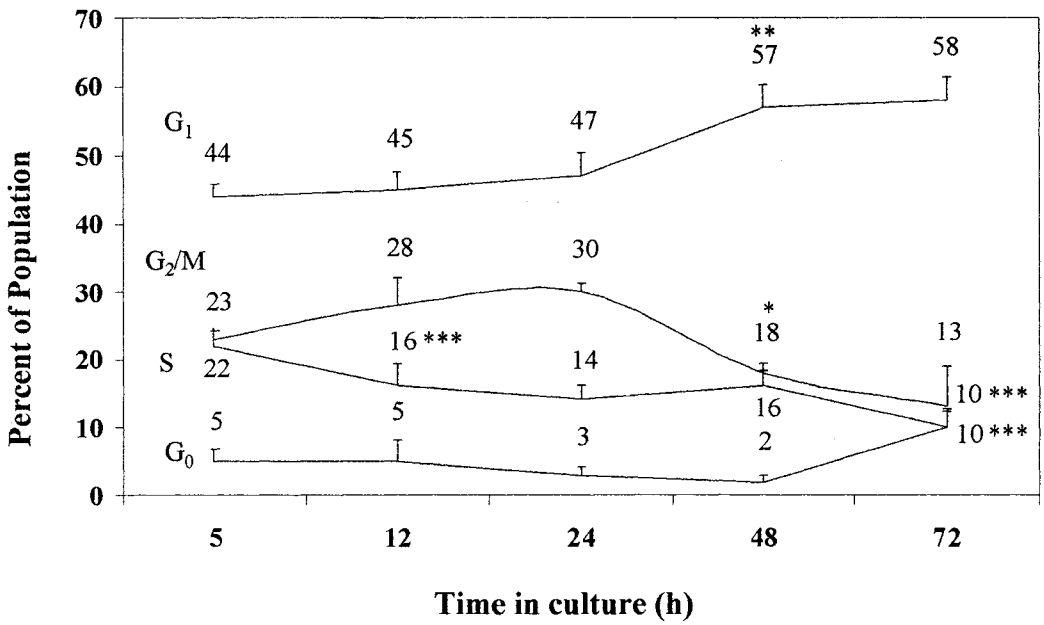

a)
b)
c)
d)
e)
G1
S
G2/M
G0
5 h
12 h
24 h
48 h
72 h
putative mitotic population
Proliferation (PCNA Content)
DNA Content (PI Intensity)

Figure 3.8 Changes in the proportions of cells in each cell cycle compartment over three days of neuroblast cell culture. A decrease in synthesis occurred between 5 and 12 h, then again between 48 and 72 h. A decrease in G_2/M was concurrent with an increase in G_1 at 48 h. An increase in G_0 was recorded at 72 h.

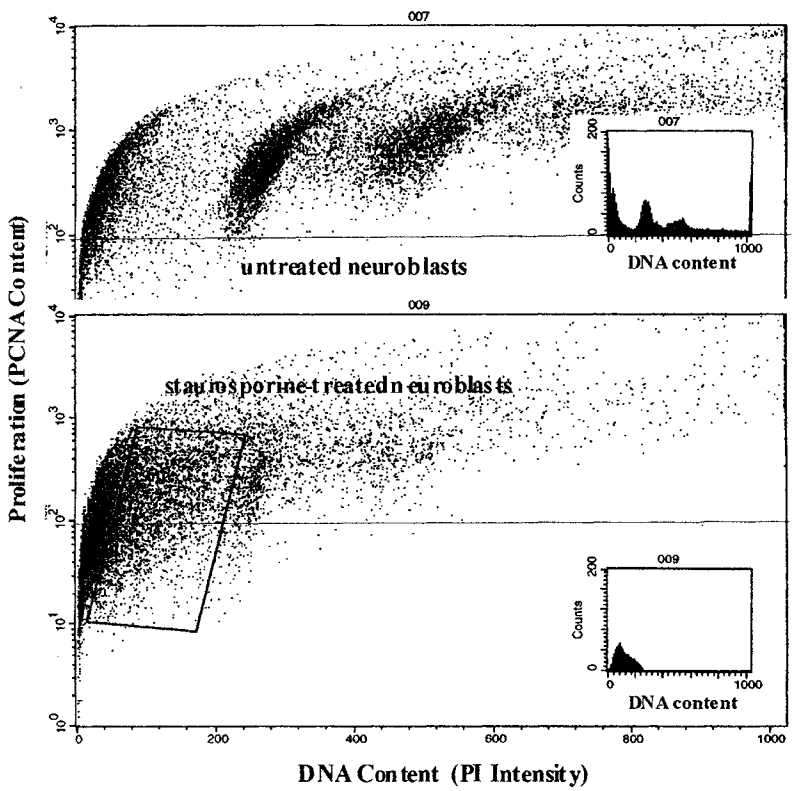

70
60
50
40
30
20
10
0
Percent of Population
G1
44
45
47
**
57
58
G2/M
23
28
30
*
18
13
S
22
16 ***
14
16
10 ***
G0
5
5
3
2
10 ***
5
12
24
48
72
Time in culture (h)

cells were a mitotic population. This population was in the same location as the putative mitotic population identified in Fig. 3.6. By 48 h, the number of cells in G_1 appeared to be building and the number of cells in G_2/M decreasing (Fig. 3.7). This was confirmed statistically: G_1 cells increased from 47% to 57% of the population and G_2/M cells decreased, from 30% to 18% of the population (Fig. 3.8). This increase in G_1 was sustained at 72 h, and G_2/M continued non-statistically to decrease, from 18% to 13% of the population (Fig. 3.8). Although there was an initial decrease in synthesis between 5 and 12 h, from 22% to 16% of the population (Fig. 3.8), Tukey's test revealed no other statistical significance for sequential changes in this region, i.e. there was no trend. There was a statistical decrease in the synthesis region between 48 and 72 h, from 16% to 10% of the population (Fig. 3.8). G_0 was consistently below 10% of the population, until a statistical rise from 2% to 10% of the population between 48 and 72 h (Fig. 3.8).

Staurosporine helps to delineate apoptotic region

An increase in cells containing low DNA and low PCNA in response to staurosporine helped delineate the area containing apoptotic cells (Fig. 3.9). The DNA histogram inset into the upper panel (untreated cells) shows DNA content for all cells in the scatter plot, with typical peaks for debris, G_0/G_1, and G_2/M. However, the DNA histogram inset into the lower panel (staurosporine-treated cells) reveals only the DNA inside the gated area. This DNA peak, situated between the G_0/G_1 and the debris peaks, appears to be an apoptotic peak. The increase in the number of cells in this region was a mean of 26% ($p<0.001$) based on four independent values from two separate experiments.

Figure 3.9 Effects of 24 h of treatment with 1 μM staurosporine. Untreated neuroblasts (upper panel) showed typical scatter, and the inset histogram shows typical DNA scatter for the population. However, most treated cells (lower panel) had lesser amounts of both DNA and PCNA, indicative of apoptotic cells (gated region), or secondarily necrotic cells and debris (left of gate). The inset histogram on the lower panel shows DNA from the gate only, and reveals what appears to be a typical sub-G_1 apoptotic peak.


007
untreated neuroblasts
DNA content
Counts
009
staurosporine-treated neuroblasts
DNA content
Counts
Proliferation (PCNA Content)
DNA Content (PI Intensity)

Blocking PACAP receptor arrests cell cycle, then increases apoptosis

Five-hour blockade of PAC_1-R is visualized in Fig. 3.10, where a clear increase in quiescent cells occurs in treated cells (lower panel) compared to control (upper panel). Although increased apoptosis would also move cells toward the lower left of the screen, the increased scatter shows cells with no PCNA and 2N DNA, rather than low PCNA and low DNA, suggesting quiesence, not apoptosis. The picture is confirmed by numbers in Table 3.1. The block caused a statistical decrease in G_2/M and G_1, in favour of G_0, of about 12% of the population. Synthesis and apoptosis were unchanged.

Twenty-four-hour blockade of PAC_1-R is presented in Fig. 3.11. The dot plot shows that PACAP6-38 has moved cells from G_0 into apoptosis. Table 3.2 shows that there is still some cell cycle exit. Synthesis is again unchanged. The notable result is that apoptosis is higher: a mean of about 11% of the population. The decreases in G_1 and G_2/M could be as a result of increased exit or increased apoptosis.

PACAP has little effect on proliferating neuroblasts

Only one significant value was obtained in assays which measured the impact of PACAP on cells from 5 to 72 h in culture. At 12 h, a mean increase of 5% in the G_1 population compared to control was recorded for 10^{-6} M PACAP (Fig. 3.12).

PACAP does release some cells from cell cycle arrest

Analysis revealed that the calcium chelator EGTA was able to rescue cells from natural apoptosis, and block them in G_1 (Table 3.3). After 24 h, two regions showed a statistical change: the G_1 region increased and the apoptotic region decreased,

Figure 3.10 Effects of blocking the primary PACAP receptor with 10 μM PACAP6-38 for 5 h. An increase in chick neuroblasts exiting the cell cycle (bottom panel) was apparent when cells were blocked with PAC_1-R. The population below 10^2 on the PCNA axis defines the cells as G_0. Untreated cells are shown for comparison (upper panel).

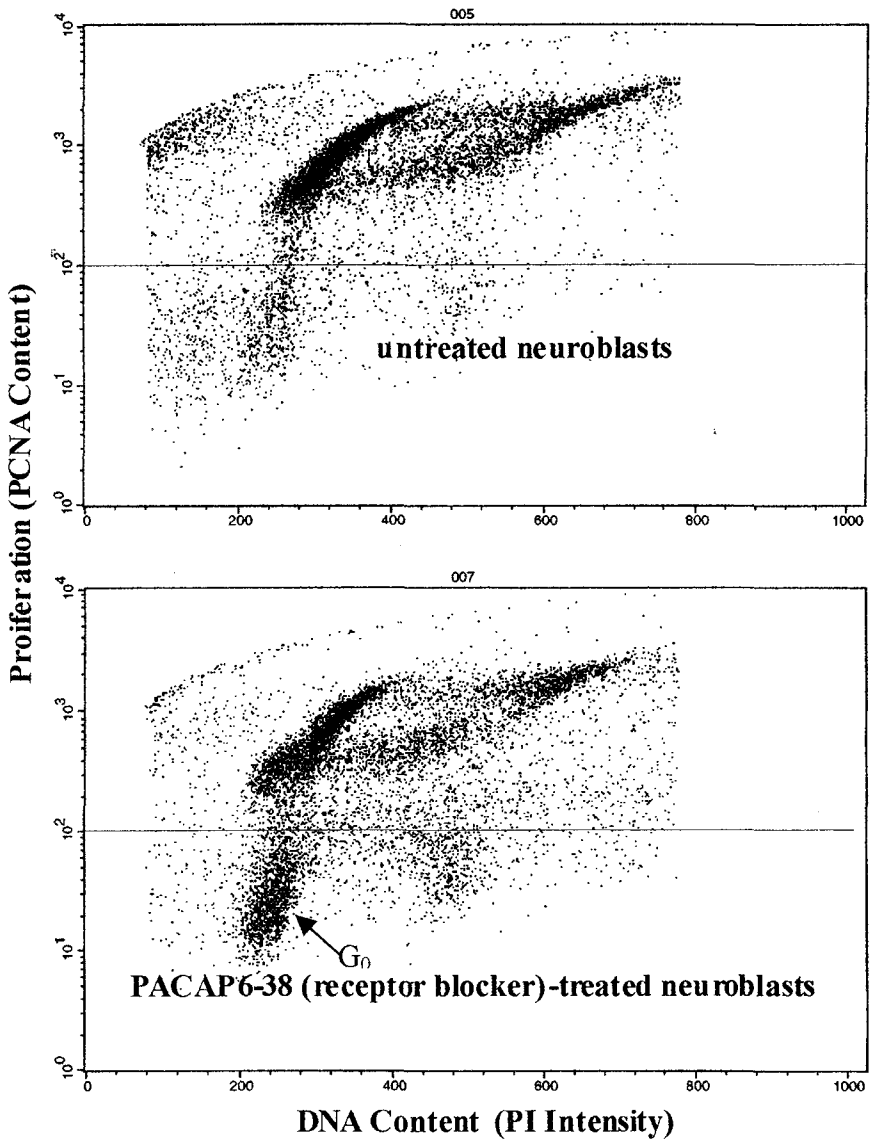

005
untreated neuroblasts
007
G0
PACAP6-38 (receptor blocker)-treated neuroblasts
Proliferation (PCNA Content)
DNA Content (PI Intensity)

Table 3.1 Exit from cell cycle after 5 h of treatment with 10 μM final concentration of the PACAP receptor blocker, PACAP6-38.*

	No Treatment		PACAP6-38		
	% Population	SEM	% Population	SEM	*p* value
G_0	9.5	1.6	21.8	1.9	<0.05
G_1	43.8	1.0	37.0	1.1	<0.05
S	22.8	1.9	21.2	1.9	
G_2/M	20.0	0.4	14.0	0.9	<0.001
Apoptosis	3.0	0.4	3.3	1.0	

*Cell cycle compartments were assessed based on populations that excluded mixed debris/apoptotic regions, whereas apoptosis was assessed based on ungated, or entire populations.

Figure 3.11 Effects of 24 h of treatment with 5 μM of the PACAP receptor blocker, PACAP6-38. Cells appear to move from G_0 into apoptosis after 24 h of treatment (bottom panel). Untreated cells are shown for comparison (upper panel).

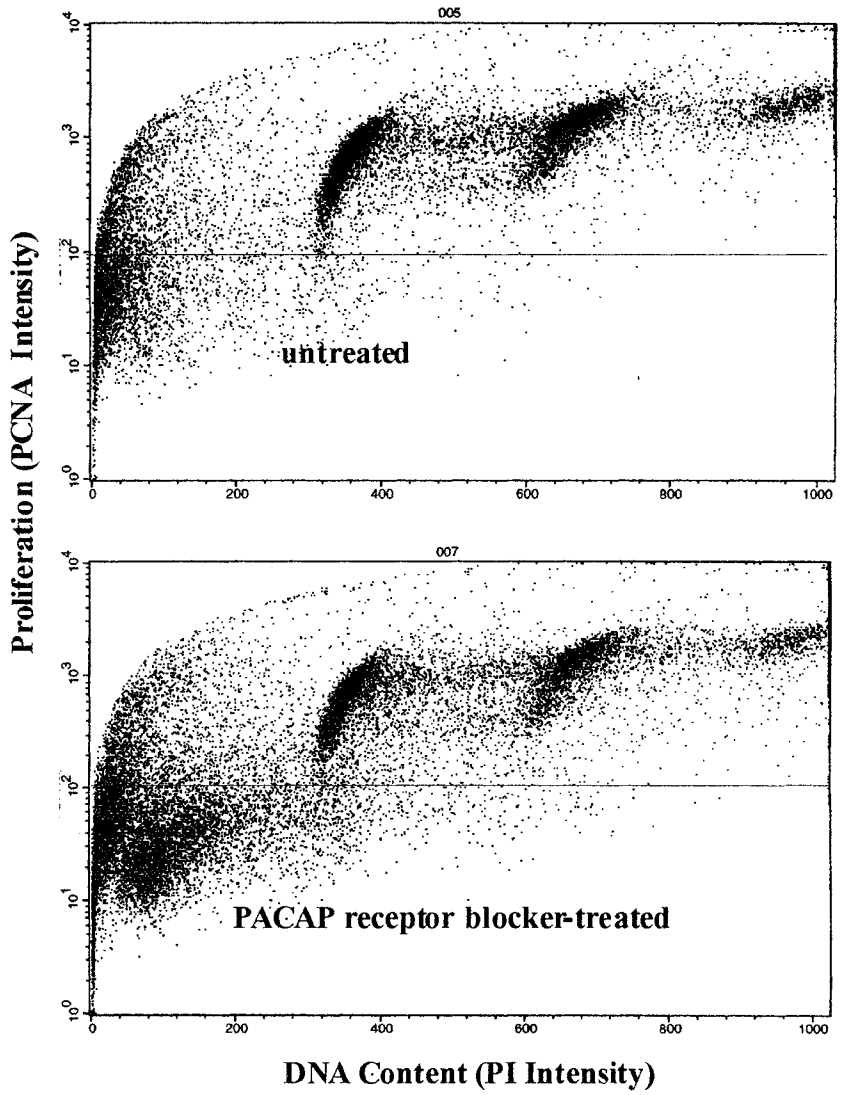

005
untreated
007
PACAP receptor blocker-treated
Proliferation (PCNA Intensity)
DNA Content (PI Intensity)
0
200
400
600
800
1000

Table 3.2 Cell cycle exit and an increase in apoptosis after 24 h of treatment with 5 μM final concentration of the PACAP receptor blocker, PACAP6-38.*

	No Treatment		PACAP6-38		
	% Population	SEM	% Population	SEM	*p* value
G_0	3.5	0.7	8.0	0.0	<0.001
G_1	46.0	1.2	40.0	0.4	<0.01
S	14.8	0.5	15.0	0.6	
G_2/M	30.5	0.3	29.0	0.5	<0.05
Apoptosis	17.3	2.2	28.0	1.2	<0.05

*Cell cycle compartments were assessed based on populations that excluded mixed debris/apoptotic regions, whereas apoptosis was assessed based on ungated, or entire populations.

Figure 3.12 The effect of 10^{-7} M and 10^{-6} M chicken PACAP38 on cell cycle during three days of neuroblast culture. The proportion of cells in each of the cell cycle compartments G_0, G_1, S and G_2/M was calculated. (The proportion of cells entering apoptosis was analyzed separately.) Only one statistically significant point was found, in the G_1 compartment at 5 h, in response to 10^{-6} M chicken PACAP38 (arrow).

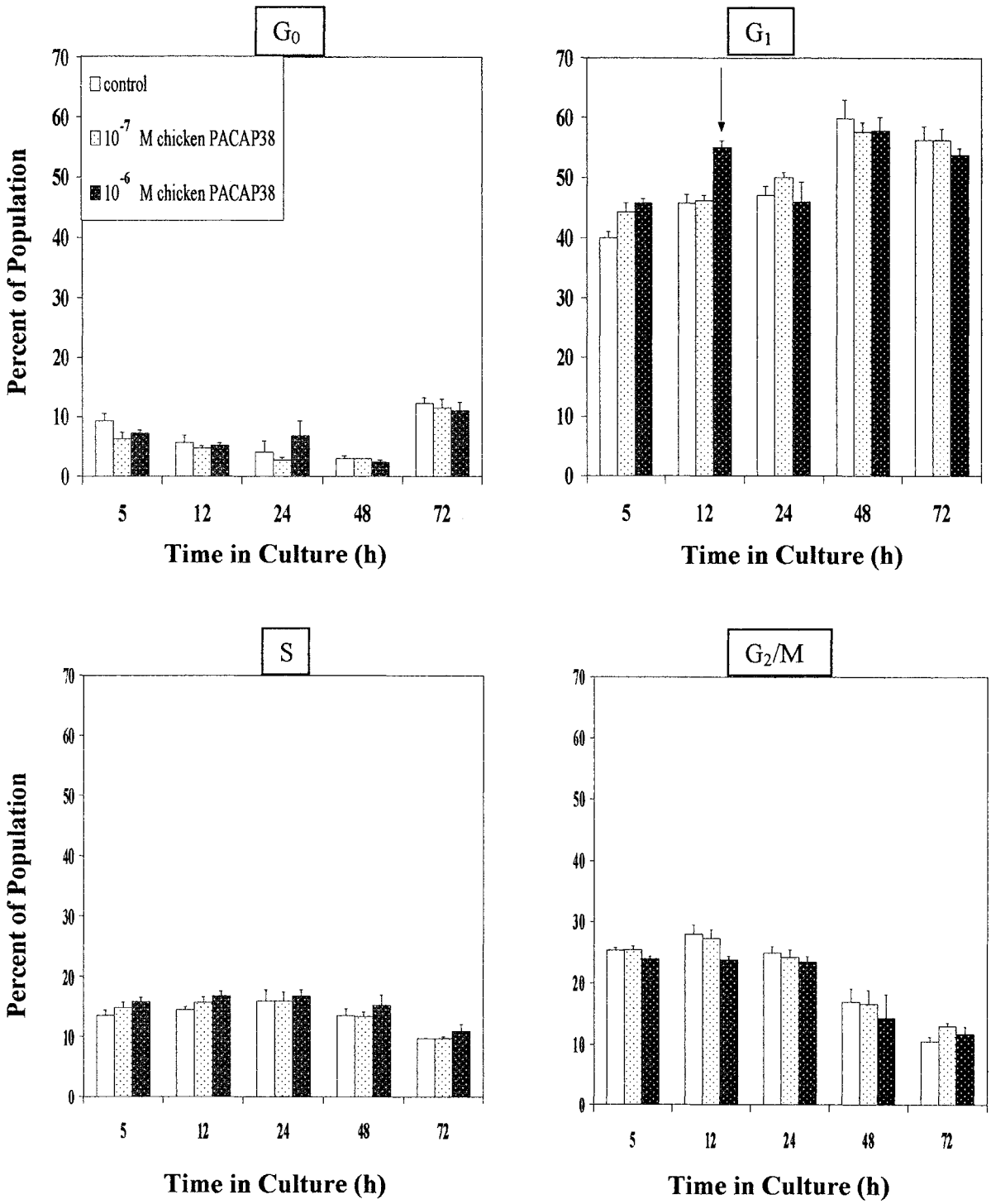

G0
G1
S
G2/M
control
10-7 M chicken PACAP38
10-6 M chicken PACAP38
Percent of Population
Time in Culture (h)
0
10
20
30
40
50
60
70
5
12
24
48
72

Table 3.3 Arrest in G_1 by EGTA, and rescue from apoptosis by EGTA or EGTA with 10^{-7} M PACAP, after 24 h of treatment. *p* values are in comparison to untreated cells; no statistical difference was present between EGTA and EGTA + PACAP treated cells.

	No Treatment		EGTA			EGTA + 10^{-7} M PACAP		
	% Population	SEM	% Population	SEM	*p* value	% Population	SEM	*p* value
G_0	3.8	0.6	2.8	0.5		3.3	1.1	
G_1	46.5	2.2	57.8	2.5	<0.05	53.8	2.8	
S	13.3	1.4	9.3	1.6		11.8	2.5	
G_2/M	29.8	0.6	28.0	0.7		25.8	1.7	
Apoptosis	20.8	1.9	11.5	1.6	<0.05	11.8	1.9	<0.05

*Cell cycle compartments were assessed based on populations that excluded mixed debris/apoptotic regions, whereas apoptosis was assessed based on ungated, or entire populations

by a mean of about 10% of the population in each case. Adding PACAP to EGTA did not change the outcome in the either population.

Cells were successfully blocked in the G_2/M region by SK&F 96365 (Table 3.4). The values for G_1 dropped by about 13%, whereas the values for G_2/M climbed about 15%. However, in this case, adding PACAP to SK&F did change the outcome. PACAP reduced the block in the expected region (G_2/M), to the point where there was no statistical difference compared to control. This was confirmed by a statistical difference between the two treated populations. In other words, PACAP released some cells from the block created by SK&F. As well, although the numbers are small, a statistical increase was recorded in the synthesis region for PACAP compared both to control, and to the SK&F 96365-treated region (Table 3.4). Cells did not leave the cell cycle or undergo apoptosis.

PACAP does not decrease natural apoptosis

Excluding one time frame, PACAP did not appear to have any effect on natural apoptosis, as calculated from ungated populations. Table 3.5 shows that the only statistically significant values occur at 48 h, and these actually show an increase in apoptosis of several percent due to hormone addition.

Table 3.4 Blockage of cells in G_2/M after 24 h of treatment with the calcium entry blocker SK&F 96365, and SK&F 96365 with 10^{-7}M PACAP.*

	1. No Treatment		2. SK&F		*p* value	3. SK&F + 10^{-7} M PACAP		*p* value	
	% Population	SEM	% Population	SEM	vs 1	% Population	SEM	vs 1	vs 2
G_0	2.5	0.1	2.5	0.3		4.3	1.6		
G_1	52.8	2.8	39.8	2.8	<0.05	31.5	2.3	<0.001	
S	10.5	0.5	10.0	0		12.3	0.3	<0.05	<0.01
G_2/M	29.3	1.8	44.5	1.8	<0.01	35.5	3.1		<0.05
Apoptosis	16.8	2.8	15.8	1.3		23.0	4.6		

*Cell cycle compartments were assessed based on populations that excluded mixed debris/apoptotic regions, whereas apoptosis was assessed basedon ungated, or entire populations.

Table 3.5 Changes in natural apoptosis after treatment with chicken PACAP38.*

	No Treatment		10^{-7} M PACAP			10^{-6} M PACAP		
	% Population	SEM	% Population	SEM	*p* value	% Population	SEM	*p* value
5 h	3.4	1.5	3.0	1.4		3.3	1.7	
12 h	6.0	0.7	5.8	0.7		7.8	0.8	
24 h	11.2	1.0	10.8	0.9		16.5	3.5	
48 h	14.2	0.5	18.0	0.6	<0.001	19.0	2.0	<0.05
72 h	14.3	2.3	19.0	2.3		18.5	1.7	

*Cell cycle compartments were assessed based on populations that excluded mixed debris/apoptotic regions, whereas apoptosis was assessed based on ungated, or entire populations.

DISCUSSION

Flow cytometry reveals cell cycle patterns

This chapter shows that flow cytometry is a reliable method with which to study primary cell cultures, whether undisturbed or after one or more treatments. Survival, apoptosis, proliferation and differentiation can all be evaluated. We know that primary cultures from E3.5 chick brain are a highly enriched population of neuroblasts, cultured at a time when glia are not yet present (Chapter 2). In this chapter, I show that the cells are both cycling and non-cycling, and can be identified in every compartment of the cell cycle. A high level of neuroblast survival continued for three days of culture, and survival could possibly be extended (with medium changes) up to E8 in chick, when glial cells begin to develop (Bellairs and Osmond 1998).

Only 10% compensation for overlapping fluorescence was required, so there was little loss in the data due to a need to separate fluorescence emissions. Identification of gates was convincing. Of some concern is that access to an internal antigen (PCNA) required fixation and resulted in a large loss of cells to debris and aggregation. However, of the remaining cells identified in the initial gate (Fig. 3.2), more than 90% were intact single cells available for analysis, in all but 72 h cultures.

Data was collected over three days of culture, and proportions analyzed. The data suggested a cycle of about 24 h, because this was when a putative mitotic population became prominent. An increase in G_1 concurrent with a decrease in G_2/M evident by 48 h provided support for this observation. Although a build-up of toxins and depletion of medium components may have affected cells after 24 h, the amount of synthesis did not decrease statistically between 12 and 48 hours, and there was no

indication that populations were seriously declining until 72 h, when the percent of single cells captured from the initial gate dropped from 90% or more to 80% or more.

Blocking PACAP receptor stops cells cycling

The focus in this study was on proliferation and apoptosis. Immediate results showed that most of the cells in these cultures were in an actively cycling state. In fact, the data suggested that proliferative cells were of such a high proportion (more than 90% of the cells were in G_1, S and G_2/M compared to G_0 as shown in Figs. 3.7 and 3.8), that only slight increases could occur in response to the addition of more PACAP. An alternative was to block the PACAP receptor.

Blocking the PAC_1 receptor for 5 h did show that the cells exited the cycle, suggesting that the hormone was needed to keep the cells proliferating. The results from cell cycle arrest in G_2/M supported this theory as well. Adding PACAP to SK&F 96365 lowered the number of cells trapped in G_2/M by SK&F 96365, to the point that there was no longer statistical difference from control. To put it another way, adding PACAP to SK&F 96365 cut in half the number of neuroblasts that were in cell cycle arrest. PACAP also caused a small increase in synthesis.

These results seem at first to contradict a body of research in which PACAP decreased proliferation and increased differentiation in rat and mouse CNS (Gonzalez et al. 1997; Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998; Suh et al. 2001). The present study suggests that PACAP keeps neuroblasts proliferating, rather than releasing them from the cell cycle. This could be due to the age of the cells in these cultures. The research on rodent cells involved studies of older animals, generally mid-

gestation until after birth. It could be that the signal from PACAP would change to one that causes differentiation at an older age when other developmental signals have changed.

The response of the cells to blocks in G_1 and G_2/M may reveal something about the pathways through which PACAP operates at this stage of development. Only the short form of the PAC_1 receptor was isolated from these chick neuroblasts (Chapter 2). The TM4 variation and the five isoforms created by insertion of cassettes into the third intracellular loop could only have been present in small amounts if at all, because the sequencing protocol included those portions of the receptor. However, the presence or absence of isoforms created by deletions in the extracellular domain could not have been verified, because the changes that identify them are outside the area that was sequenced. In addition to increasing cAMP, these PAC_1 receptor isoforms can also increase production of intracellular calcium by acting through the phospholipase C transduction pathway. Therefore, it is possible that the effects observed here could be due to activation of either the cAMP or the phospholipase C pathways. However, PACAP had no effect on cell cycle arrest or apoptosis when calcium was kept from entering the cell for 24 h by addition of the calcium chelator EGTA. This suggests that it did not activate phospholipase C, or did not activate it strongly enough to produce sufficient IP3 to restore homeostasis through release of calcium from intracellular stores. With lack of calcium, the cells stopped cycling, and could not undergo apoptosis. PACAP was not able to affect this decline. However, PACAP was able to restore at least some cells to cycling when blockage of capacitative calcium entry with SK&F 96365 arrested cells in G_2/M. With only store-operated channels blocked, and calcium available in the medium,

PACAP could have opened voltage-operated or non-selective cation channels activated by cAMP to allow calcium entry in an attempt to restore homeostasis. The PAC_1-R (short) isoform, identified in these cells, was also the predominant isoform expressed in cerebral cortical precursors, which responded to PACAP by activating only the cAMP pathway (Lu et al. 1998). The question of which pathways are most active in these cells could be confirmed by use of analogues, antagonists and agonists at various locations in the pathways.

Blocking the PACAP receptor also increases apoptosis

It is clear that PACAP maintains survival of chick CNS cells early in development by blocking apoptosis. Blocking the PACAP receptor for 24 h revealed the same pattern as blocking it for 5 h, except that apoptosis was increased. The effect of PACAP on apoptosis matches that found by other researchers, who reported that the hormone was necessary to prevent older chick neuroblasts from dying in the peripheral and autonomic nervous systems. Apoptosis was decreased by PACAP in E8 ciliary ganglion neurons (Pugh and Margiotta 2000), and E10 sympathetic ganglion cells (Przywara et al. 1998; Wakade and Leontiv 1998). This present study is a first report of PACAP's ability to do so at such an early age in the chick, and in the brain. This widens the scope of the function of PACAP in brain development, as we now know that it plays a significant role in blocking apoptosis not only in rat and mouse brain, but also in chick brain, and is active at a very early age, at least in the chick.

Also, cell cycle exit was still statistically above control after 24 h of treatment with a PACAP receptor blocker, although the proportion of cells leaving the cycle was

less than at 5 h. Because differentiation was not measured in this study, it is possible that some of these cells, which had so recently left the cell cycle, were actually committed to differentiate rather than to die. Cell cycle arrest of proliferating cells can result in either apoptosis or differentiation (Howard et al. 1993).

PACAP's effects on natural apoptosis inconclusive

Based on my results, it was obvious that the effect of PACAP on natural apoptosis required further study. In my experiments, natural apoptosis slowly increased from 5 h to 48 h, then levelled off at 72 h, but was still less than 15% even after three days in culture (Table 3.5). A method specifically designed to measure apoptosis, using live cells to avoid loss to debris and aggregation, was required.

Summary

In conclusion, in this chapter of my thesis I show that flow cytometry is a versatile system for studying the actions of hormones and growth factors on normal neuroblasts during very early brain development, and could be utilized as well to test disturbances to development. The populations that are in G_0, G_1, S, G_2/M and apoptosis can be quantified, first based on age and time in culture, and then based on treatment.

Because most E3.5 chick neuroblasts were proliferative in these cultures, it was clear that the most effective way to study the action of PACAP on cell cycle and apoptosis was to block its action on the PACAP-specific receptor. This work was supplemented by arresting cycling cells and testing the ability of PACAP to remove the block. This system shows that PACAP plays an important role in the regulation of cell

cycle and apoptosis in early chick brain development. The results show that chick neuroblasts require PACAP and its receptor for survival and full proliferation at this stage of chick brain development.

REFERENCES

Ahlemeyer, B, D Fischer, T Kissel and J Krieglstein (2000). "Staurosporine-induced apoptosis in cultured chick embryonic neurons is reduced by polyethylenimine of low molecular weight used as a coating substrate." Neuroscience Research **37**: 245-253.

Barbiero, G, L Munaron, S Antoniotti, FM Baccino, G Bonelli and D Lovisolo (1995). "Role of mitogen-induced calcium influx in the control of the cell cycle in Balb-c 3T3 fibroblasts." Cell Calcium **18**: 542-556.

Bellairs, R and M Osmond (1998). The Atlas of Chick Development. Toronto, Academic Press.

Belyavskyi, M, J Miller, E Belyavskaya and V Wilson (1995). "BPV E1 protein alters the kinetics of cell cycle entry of serum starved mouse fibroblasts." Cytometry **21**: 257-264.

Campard, KP, C Crochemore, F Rene, D Monnier, B Koch and JP Loeffler (1997). "PACAP Type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA pathway." DNA and Cell Biology **16**: 323-333.

Casasco, A, M Giordana, M Danova, M Csasco, AI Cornaglia and A Calligaro (1993). "PC10 monoclonal antibody to proliferating cell nuclear antigen as probe for cycling cell detection in developing tissues." Histochemistry **99**: 191-199.

Chang, JY and VV Korolev (1997). "Cyclic AMP and sympathetic neuronal programmed cell death." Neurochemistry International **31**: 161-167.

Dawson, G (2000). "Mechanisms of apoptosis in embryonic cortical neurons (E6 and E7) in culture involved lipid signalling, protein phosphorylation and caspase activation." International Journal of Developmental Neuroscience **18**: 247-257.

DiCicco-Bloom, E, N Lu, JE Pintar and J Zhang (1998). "The PACAP ligand/receptor system regulates cerebral cortical neurogenesis." Annals of the New York Academy of Science, USA **865**: 274-289.

Garrido, SM, C Willman, FR Appelbaum and DE Banker (2000). "Three-color versus four-color multiparameter cell cycle analyses of primary acute myeloid leukemia samples." Cytometry **42**: 83-94.

Gonzalez, BJ, M Basille, D Vaudry, A Fournier and H Vaudry (1997). "Pituitary adenylate cyclase-activating polypeptide promotes cell survival and neurite outgrowth in rat cerebellar neuroblasts." Neuroscience **78**: 419-430.

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

Howard, KM, LC Burke, C Mailhos, A Pizzey, CS Gilbert, DW Lawson, MKL Collins, NSB Thomas and PS Latchman (1993). "Cell cycle arrest of proliferating neuronal cells by serum deprivation can result in either apoptosis or differentiation." Journal of Neurochemistry **60**: 1783-1791.

Jamen, F, R Puech, J Bockaert, P Brabet and G Bertrand (2002). "Pituitary adenylate cyclase-activating polypeptide receptors mediating insulin secretion in rodent pancreatic islets are coupled to adenylate cyclase but not to PLC." Endocrinology **143**: 1253-1259.

Jónsson, ZO and U Hübscher (1997). "Proliferating cell nuclear antigen: more than a clamp for DNA polymerases." BioEssays **19**: 967-975.

Journot, L, M Villalba and J Bockaert (1998). "PACAP-38 protects cerebellar granule cells from apoptosis." Annals of the New York Academy of Science, USA **865**: 100-110.

Laburthe, M, A Couvineau and P Nicole (2003). Molecular pharmacology and structure-function analysis of PACAP/VIP receptors. Pituitary Adenylate Cyclase-Activating Polypeptide. H Vaudry and A Arimura. Dordrecht, Kluwer Academic Publishers Group**:** 69-94.

Lu, N and E DiCicco-Bloom (1997). "Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells." Proceedings of the National Academy of Science, USA **94**: 3357-3362.

Lu, N, R Zhou and E DiCicco-Bloom (1998). "Opposing mitogenic regulation by PACAP in sympathetic and cerebral cortical precursors correlates with differential expression of PACAP receptor (PAC_1-R) isoforms." Journal of Neuroscience Research **53**: 651-662.

McCormick, D and PA Hall (1992). "The complexities of proliferating cell nuclear antigen." Histopathology **21**: 591-594.

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

Nordstrom, T, HA Nevanlinna and LC Anderson (1992). "Mitosis-arresting affect of the calcium channel inhibitor SK&F 96365 on human leukemia cells." Experimental Cell Research **202**: 487-494.

Peeters, K, HHJ Gerets, K Princen and F Vandesande (1999). "Molecular cloning and expression of a chicken pituitary adenylate cyclase-activating polypeptide receptor." Molecular Brain Research **71**: 244-255.

Przywara, DA, JS Kulkarni, TD Wakade, DV Leontiv and AR Wakade (1998). "Pituitary adenylyl cyclase-activating polypeptide and nerve growth factor use the proteasome to rescue nerve growth factor-deprived sympathetic neurons cultured from chick embryos." Journal of Neurochemistry **71**: 1889-1897.

Pugh, PC and JF Margiotta (2000). "Nicotinic acetylcholine receptor agonists promote survival and reduce apoptosis of chick ciliary ganglion neurons." Molecular and Cellular Neuroscience **15**: 113-122.

Sherwood, NM, SL Krueckl and JR McRory (2000). "The origin and function of the PACAP/glucagon superfamily." Endocrine Reviews **21**: 619-670.

Shoge, K, HK Mishima, T Saitoh, K Ishihara, Y Tamura, H Shiomi and M Sasa (1999). "Attenuation by PACAP of glutamate-induced neurotoxicity in cultured retinal neurons." Brain Research **839**: 66-73.

Silveira, MS, MR Costa, M Bozza and L R (2002). "Pituitary adenylate cyclase-activating polypeptide prevents induced cell death in retinal tissue through activation of cyclic AMP-dependent protein kinase." Journal of Biological Chemistry **277**: 16075-16080.

Sugioka, M, W-L Zhou, H-D Hofmann and M Yamashita (1999). "Ca2+ mobilization and capacitative Ca2+ entry regulate DNA synthesis in cultured chick retinal neuroepithelial cells." International Journal of Developmental Neuroscience **17**: 163-172.

Suh, J, N Lu, A Nicot, I Tatsuno and E DiCicco-Bloom (2001). "PACAP is an anti-mitogenic signal in developing cerebral cortex." Nature **4**: 1-2.

Vaudry, D, A Falluel-Morel, M Basille, TF Pamantung, M Fontaine, A Fournier, H Vaudry and BJ Gonzalez (2003). "Pituitary adenylate cyclase-activating polypeptide prevents C2-ceramide-induced apoptosis of cerebellar granule cells." Journal of Neuroscience Research **72**: 303-316.

Wakade, AR and D Leontiv (1998). "Sympathetic neurons of the chick embryo are rescued by PACAP from apoptotic death." Annals of the New York Academy of Science, USA **865**: 176-180.

Waschek, JA, RA Casillas, TB Nguyen, EM DiCicco-Bloom, EM Carpenter and WI Rodriguez (1998). "Neural tube expression of pituitary adenylate cyclase-activating polypeptide (PACAP) and receptor: potential role in patterning and neurogenesis." Proceedings of the National Academy of Science, USA **95**: 9602-9607.

CHAPTER 4

PACAP is confirmed to block apoptosis in early chick neuroblasts, but does not rescue cells from induced apoptosis

INTRODUCTION

It is clear that one important function of PACAP during brain development in several species is to regulate proliferation through control of cell cycle. PACAP increases or decreases proliferation depending on animal, age, and tissue. In rodent cerebellar granule cells, PACAP decreased proliferation (Gonzalez et al. 1997; Lu and DiCicco-Bloom 1997; DiCicco-Bloom et al. 1998; Suh et al. 2001). We now know that in younger cells from chick brain, PACAP increases proliferation (Chapter 3).

However, the function of PACAP with regard to neurotrophism and apoptosis is much more straightforward. PACAP maintains or increases cell numbers by enhancing survival and/or inhibiting apoptosis. In the rodent embryonic nervous system this effect has been reported in cells in the dorsal root ganglion, lumbar motor column, superior cervical ganglion, cerebellar granule layer, cerebellum, mesencephalon, cortex, hippocampus and septal nuclei (DiCicco-Bloom and Deutsch 1992; Arimura et al. 1994; Canonico et al. 1996; Cavallaro et al. 1996; Chang et al. 1996; Campard et al. 1997; Gonzalez et al. 1997; Villalba et al. 1997; Journot et al. 1998; Lindholm et al. 1998; Lioudyno et al. 1998; Takei et al. 1998; Waschek et al. 1998; Skoglosa et al. 1999; Vaudry et al. 1999; DiCicco-Bloom et al. 2000; Vaudry et al. 2000; Tabuchi et al. 2003). In E10 chick, this effect has been reported in the sympathetic nervous system (Przywara et al. 1998; Wakade and Leontiv 1998). We have now discovered that PACAP inhibits apoptosis in very early chick brain as well.

Also, there is evidence that PACAP is able to rescue cells that have been induced to undergo apoptosis due to damage or toxicity. PACAP has rescued cells from apoptotic death due to ethanol exposure, oxidative stress, ischemia, and glutamate-

induced neurotoxicity (Morio et al. 1996; Uchida et al. 1996; Ito et al. 1999; Vaudry et al. 2002a; Vaudry et al. 2002b).

Based on the results from Chapter 3, it was obvious that the effect of PACAP on natural apoptosis in chick cells required further study. Although fixation had complicated the protocol by creating high amounts of debris and aggregation, the suggestion was that natural apoptosis remained low for at least 48 h. For this reason, I searched for a protocol that was sensitive and specific for detecting early changes in apoptosis, and which would allow treatment of live cells so that the entire population could be evaluated.

Two protocols for measuring both natural and induced apoptosis were evaluated. Both made use of live cells, and both used flow cytometry. The first was based on movement of phosphatidylserine residues from the inner leaflets to the outer leaflets of cell membranes, and binding of an anticoagulant, annexin V, to these residues. The change in location of phophatidylserine is a very early marker of apoptosis, preceding nuclear breakdown, DNA fragmentation, and the appearance of many apoptosis-associated molecules (Rimon et al. 1997; Van-Den-Eijnde et al. 1997; Kravtsov et al. 1999). It constitutes a signal to neighbouring cells of the impending event (Fadok et al. 1992). Annexin V is bound to a fluorescent molecule to allow visualization, and an increase in fluorescence indicates an increase in apoptosis. The cells are counterstained with propidium iodide (PI), which is a measure of necrosis, because PI can only enter cells in an unfixed cell population if the membranes have been compromised. Populations can be identified as apoptotic (annexin V positive, PI negative), necrotic (PI positive, annexin V negative), and vital (no staining).

The second method employed fluorescein diacetate (FDA) and PI, and was based on the different responses of live cells to these two stains. FDA passively crosses cell membranes, is converted to fluorescein in healthy cells, and is retained as long as the cell membrane remains intact. Again, PI penetrates only cells with damaged membranes. Populations can therefore be defined as vital (fluorescein positive, PI negative), necrotic (fluorescein negative, PI positive), and apoptotic (mid-range fluorescein, because the cells are increasingly unable to convert FDA, and mid-range PI, because the cells slowly lose membrane integrity).

My goal was to confirm that the PACAP receptor blocker did cause apoptosis in these cells, then to retest the effect of PACAP on natural apoptosis. I also wanted to test the ability of PACAP to rescue cells induced to undergo apoptosis, using several compounds. Staurosporine is a general protein kinase inhibitor known to induce apoptosis in E7 chick neuroblasts (Weisner and Dawson 1996; Ahlemeyer et al. 2000; Dawson 2000). Ceramide is a second messenger in the lipid metabolism pathway, and has been reported to cause apoptosis in both E3.5 (Frago et al. 1998) and E7 chick neuroblasts (Weisner and Dawson 1996; Ahlemeyer et al. 2000). Ethanol is a neuronal toxin which kills cells chick and rodent neuroblasts by necrosis or apoptosis (Kentroti and Vernadakis 1990; Rahman et al. 1994; Vaudry et al. 2002a). Induction of apoptosis was confirmed by blocking protein synthesis with cycloheximide, and by visualization of cells with a DNA dye to show characteristic apoptotic morphology.

MATERIALS AND METHODS

Cell culture

Cell culture was as previously described (Chapter 2) with the exception of cell concentration and addition of peptides. Briefly, brain tissue from E3.5 chicks was dissociated mechanically and single cell suspensions were plated in Neurobasal medium (Invitrogen, Burlington, ON), with the manufacturer's recommended supplements. Cells were plated at 2 X 10^6 cells/ml. Peptides were added as described below.

Peptides and reagents

Chicken PACAP38 was a gift from Dr. Jean Rivier (Salk Institute for Biological Studies, La Jolla, CA). Ascorbic acid (0.5 mM) was added to prevent degradation of the peptide. The PACAP receptor antagonist, human PACAP6-38, was purchased from American Peptide Company (Sunnyvale, CA). The Annexin V-Biotin Apoptotic Detection kits were purchased from Oncogene Research Products (San Diego, CA). The bis-benzimide dye (Hoechst 33258) used to visualize apoptotic nuclei, and the FDA were purchased from Cedarlane Laboratories (Hornby, ON). All other chemicals, including the C6 form of ceramide, were purchased from Sigma (Oakville, ON), and diluted according to the manufacturer's instructions.

Annexin V protocol

Plated neuroblasts were treated according to the kit manufacturer's instructions, with minor modifications. The protocol was adjusted for processing lower volumes of cells, trypsin was inactivated using trypsin inhibitor rather than medium, and one extra

wash was added to ensure removal of all trypsin. Briefly, supernatant from cultured cells was removed and kept on ice. Attached cells were washed with 0.5 ml rt PBS, then detached from plates using gentle agitation in 0.025% trypsin in PBS for 10 min at rt. Enzyme action was stopped by addition of trypsin inhibitor. In some experiments, cells were scraped from wells instead of removed enzymatically. Supernatants and attached cells were combined, and washed by centrifuging at 1000X g for 5 min at rt, then resuspending in 0.5 ml ice-cold binding buffer. An aliquot of 1.25 µl annexin V-biotin solution was added to the tubes, and the cells were incubated for 15 min at rt in the dark. Cells were washed and resuspended in 0.5 ml ice-cold binding buffer, and a 15 µl aliquot of a 15 µg/ml solution of streptavidin-FITC in binding buffer was added. A 10 µl aliquot of a 30 µg/ml PI solution was added, and the cells were put on ice, in the dark, for immediate analysis. Each assay included control tubes containing no annexin V-FITC or PI (unstained control), annexin V-FITC only (annexin V-FITC positive control), streptavidin-FITC only (non-specific labeling control) and PI only (PI positive control). Cells were tested between 2.5 h and 7 h after plating, because in this protocol, apoptosis was visible within only a few hours.

Cytometric analysis was as described in Chapter 3, with the following changes. The annexin V-FITC fluorescence was recorded on FL1, and all parameters except forward scatter were recorded using logarithmic amplification. Voltages for side scatter, annexin V-FITC and PI fluorescence were set at 250, 625 and 575, respectively. No compensation was required. Vital, apoptotic and necrotic gates were set using control tubes. Because apoptotic cells with very low amounts of annexin V-FITC would be present in the unstained control, and those with slightly higher amounts of annexin V-

FITC would be present in the annexin V-FITC-positive control, the demarcation between the vital and apoptotic regions was set visually based on control tubes. The necrotic cell region was easily identifiable using the control tube. The regions or gates set using control tubes were not changed during the collection of data within any assay. A total of 10,000 events was collected for each sample, with samples assayed in duplicate.

A modified Annexin V protocol was also tested, which had been designed specifically for adherent transformed cells (Van-Engeland et al. 1996). This protocol differed in one important way: the annexin V-biotin was incubated with the cells prior to any manipulation. The supernatant was again removed, and washed free of excess reagent. The attached cells were washed free of excess reagent as well, detached as above, and combined with supernatant. At this point the streptavidin-FITC conjugate and the PI was added, and the cells immediately analyzed.

These methods were repeated numerous times, using only untreated cells, and included several experiments in which staurosporine, in a range of concentrations, was added to the cultures in an attempt to increase the amount of apoptosis.

FDA and PI protocol

Plated cells were removed from culture plates using trypsin as described above. Cells were washed with 0.5 ml rt PBS, and gently resuspended in 0.5 ml 0.05 μg/ml FDA in rt PBS. After 30 min incubation at 37 C, cells were washed with rt PBS, and resuspended in 0.5 ml 0.4 μg/ml PI in rt PBS. Following incubation for 30 min at rt in the dark, cells were resuspended in 0.5 ml cold PBS, and put on ice, in the dark. Each assay included control tubes containing no FDA or PI (unstained control), FDA only (FDA positive control), and PI only (PI positive control). Because the level of necrosis in the cultures was low, another control was added consisting of cells that had been heat-killed for approximately 60 min in a 60-70 C water bath, then stained with PI only (heat-killed PI positive control).

Cytometric analysis was as described above, with the following differences. The FDA-FITC fluorescence was recorded on FL1. Voltages for side scatter, FDA-FITC and PI fluorescence were set at 280, 575 and 725, respectively. Compensation was set to remove 10% FDA fluorescence from the PI detector. Vital and apoptotic cell gates were set as described above. In addition, because cells in the heat-killed PI positive control contained some apoptotic cells, and cells in the PI-only positive control contained some necrotic cells, the demarcation between the apoptotic region and the necrotic region was also set visually based on control tubes. Final gates were adjusted based on cells stained with both FDA and PI. These gates were not changed during the collection of data within any assay. A total of 10,000 events was collected for each sample, with samples assayed in duplicate, and each assay performed twice, unless otherwise noted.

Confirmation of apoptotic region

Before conducting any experiments involving hormones using the FDA and PI protocol, cells were treated with staurosporine to confirm the region of apoptosis. A final concentration of 1 μM staurosporine was added at plating, and cells were harvested after 5 h.

To prove that the increased scatter identified as apoptotic cells did consist of apoptotic and not necrotic cells, cycloheximide was added to the staurosporine-treated cultures. Cycloheximide is a protein synthesis inhibitor that has been shown to inhibit apoptosis by blocking synthesis of death proteins (Schwartzman and Cidlowski 1993). Concentrations of 1.25 mg/ml and 5 mg/ml were added to the cultures at plating, and staurosporine was added 30 min later. Cells were harvested at 5 h.

To further confirm that the increased scatter in the apoptotic region consisted of apoptotic cells, some of the untreated and the staurosporine-treated cultures were stained to reveal morphology typical of apoptotic cells. The DNA in an apoptotic cell condenses, causing brighter staining with DNA-binding dyes, and in later stages the cell breaks up into smaller bodies, some of which still contain DNA. Cells were removed from culture plates after 5 h and 24 h, using trypsin as described above, but left in suspension. Aliquots of 10 μl were dispensed onto glass slides and allowed to air dry. Fixation followed in 3:1 ethanol:acetic acid for 15 min at rt. Slides were again allowed to air dry, and 30 μl bis-benzimide dye (Hoechst 33258) in PBS was added. After 15 min at rt, slides were washed three times in PBS, and mounted in 1:1 PBS:glycerol with 1.5% propyl gallate. Photographs were taken using a Leitz Aristoplan epifluorescence microscope.

Changes in vital, apoptotic, and necrotic populations

To characterize the cell cultures using the FDA and PI system, untreated cells were cultured for 5, 12, 24, 48 and 72 h and the proportion of cells that were vital, apoptotic and necrotic were quantified. As in cell cycle and proliferation assays, medium was not changed at any point, but cultures were examined under a light microscope prior to processing to confirm normal appearance of growth.

Receptor blockade

To confirm the impact of PAC_1-R blockade, PACAP6-38 was added to cultures at plating to achieve a final concentration of 10 μM, and cultures were harvested at 5 h. In a separate set of experiments, PACAP6-38 was added at plating to achieve a final concentration of 5 μM, replenished at 12 h, and the cells were harvested at 24 h. (The concentrations and culture times were exactly as those used in Chapter 3 to assess receptor blockade.)

Assessment of PACAP on natural apoptosis

To examine the response of these neuroblasts to PACAP over time, cells were cultured for 5, 12, 24, 48 and 72 h before staining with FDA and PI. Concentrations of 10^{-7} M and 10^{-6} M PACAP were added to the cultures at plating, and replenished every 12 h as necessary for longer protocols. (Again, the concentrations and culture times were the same as those used in Chapter 3 to assess this effect.)

Assessment of PACAP on induced apoptosis

For all assays testing the effects of PACAP on induced apoptosis, PACAP was added at plating, and chemicals were added after 30 min. Staurosporine was added to achieve a final concentration of 1 μM. Ceramide was tested in a range between 50 and 500 mM final concentration. Ethanol was tested in a range between 5 and 10% final volume. In all experiments, cells were harvested after 5 h.

Data analysis

Unless otherwise stated, data were collected from at least four independent samples from at least two separate assays. Tukey's test was used to identify statistical changes in adjacent harvesting times (e.g. 5 h versus 12 hours, 12 hours versus 24 hours, etc.) for the three-day cultures. ANOVA was used in all other cases, followed by Dunnett's test if statistical differences were present. Three asterisks indicates statistical significance at $p<0.001$, two asterisks indicate significance at $p<0.01$ and one asterisk indicates significance at $p<0.05$.

RESULTS

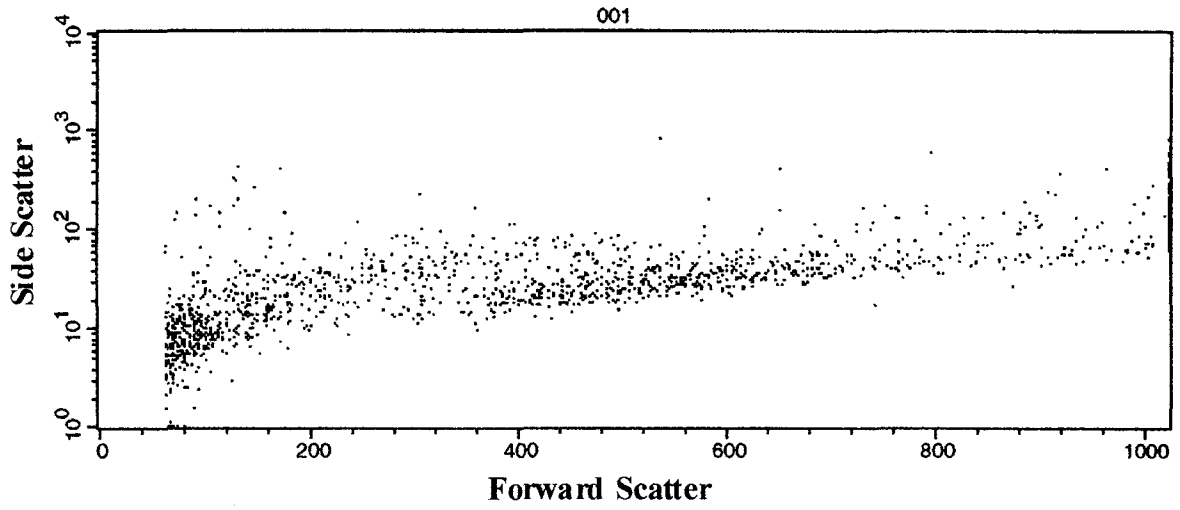
Annexin V protocols give questionable results

Experiments using annexin V-FITC and PI revealed a light scatter pattern that indicated fewer cells in the mid-size range (as recorded by forward scatter) and mid-granularity range (as recorded by side scatter), compared to the cell cycle and proliferation assays (Fig. 4.1, top panel, compared to Fig. 3.2, top panel). Fluorescence analysis of untreated cells exposed to both stains showed a normal amount of necrosis, about 15%. However, the percentage of apoptotic cells, 30-40% (Fig. 4.1, lower panel), was much higher than expected, given the results from the cell cycle and proliferation assays. This occurred using both variations of the protocol, and was the case whether the cells were detached using trypsin or were scraped free from the wells. Addition of increasing amounts of the staurosporine did not raise the levels of apoptosis any further (data not shown). These protocols were abandoned.

FDA and PI protocol identifies vital, apoptotic and necrotic populations

In the FDA and PI protocol, forward and side scatter for the cell population were the same as in the cell cycle and proliferation protocols (data not shown). Three discrete regions were identifiable on control scatter plots as vital cells, apoptotic cells, and necrotic cells, based on staining with FDA and PI (Fig. 4.2, top panel). A line of vital (FDA positive) cells, which ranged above unstained control scatter (near 10^2 on the x axis) was clear. Apoptotic cells (low FDA and low PI) could be identified as an oblong scatter nearer mid-plot. Necrotic cells (no FDA and high PI) were obvious in the upper left corner. The gates drawn for analysis are shown on the bottom panel of the figure.

Figure 4.1 Scatter plots from Annexin V-based apoptosis assays. Upper panel shows light scatter which differed from that observed in cell cycle and proliferation assays; fewer events were recorded in the mid-size and mid-granularity region. The lower panel shows that whereas necrosis was typically light (about 15%), the apoptotic region was much higher than expected (30-40%). The gates were drawn based on scatter from control tubes (as described under the heading "Annexin V protocol" in the Materials and Methods section).


001
Side Scatter
Forward Scatter
0
200
400
600
800
1000

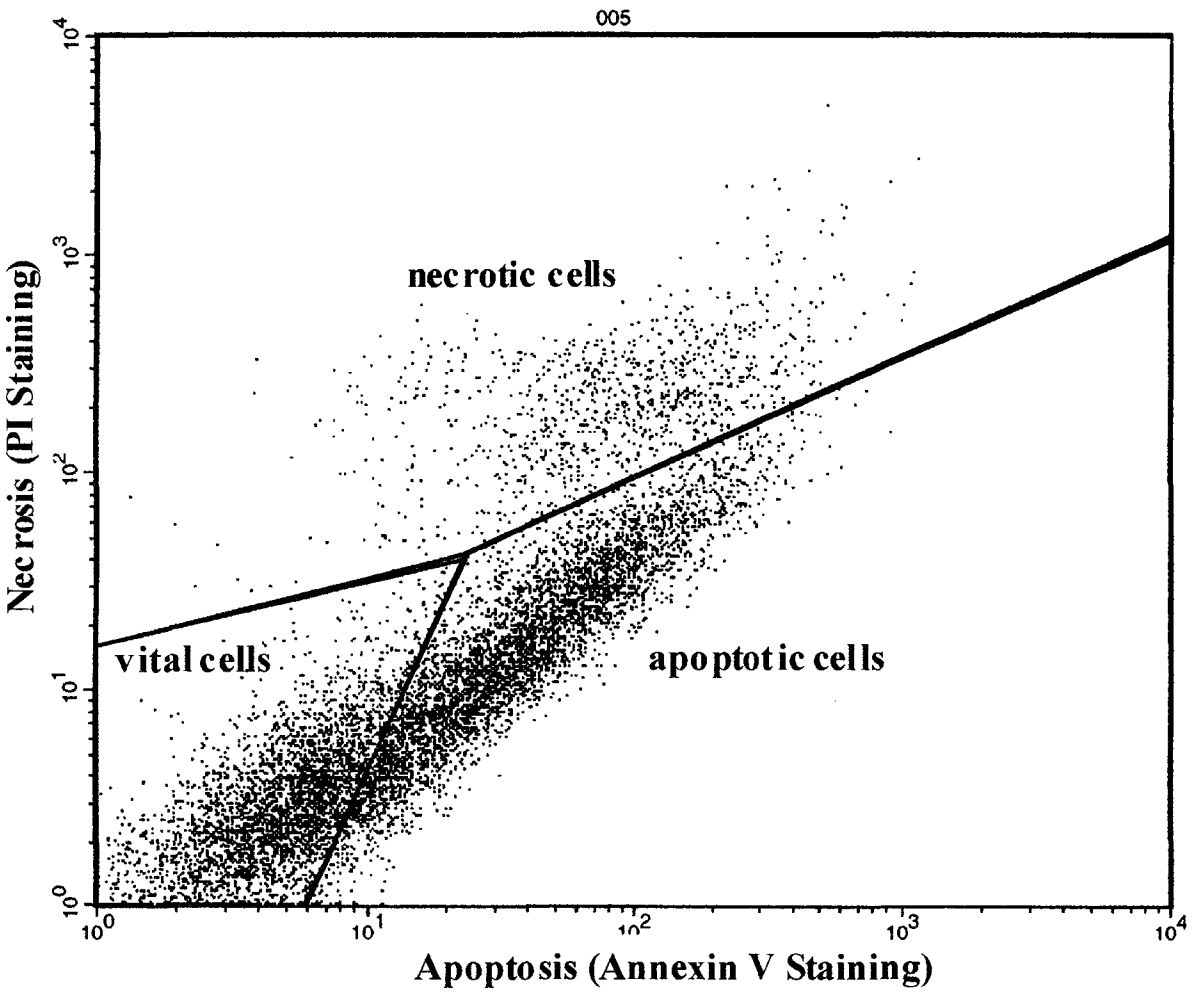
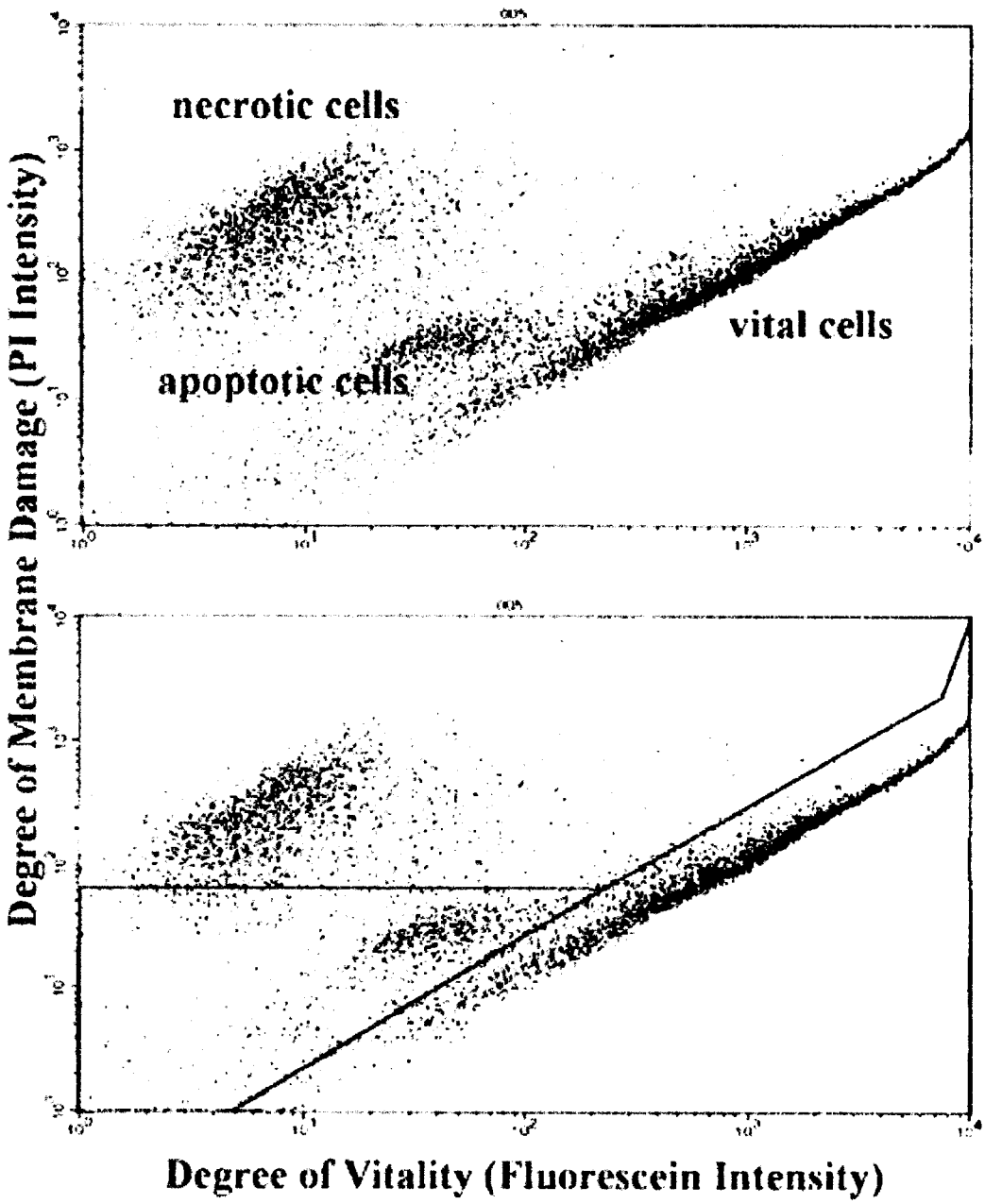

005
Necrosis (PI Staining)
Apoptosis (Annexin V Staining)
necrotic cells
vital cells
apoptotic cells

Figure 4.2 Typical scatter of cultured chick neuroblasts after staining live cell populations with fluorescein diacetate and propidium iodide. Typical analysis gates are indicated on the lower panel.


necrotic cells
vital cells
apoptotic cells
Degree of Membrane Damage (PI Intensity)
Degree of Vitality (Fluorescein Intensity)

The apoptotic region was confirmed by treatment with staurosporine (Fig. 4.3). Significant changes were created in vital, apoptotic and necrotic populations. Staurosporine decreased vital cells, and increased apoptotic cells, as well as necrotic cells. Some of the latter may have been secondarily necrotic cells, that is, late apoptotic cells that cannot be engulfed by surrounding cells *in vitro* and disintegrate.

Affirmation that the decline in health is due to apoptosis was provided by the rescuing effects of cycloheximide (Fig. 4.4). Fluorescence photographs confirmed an increase in cells with typical apoptotic morphology in cell populations induced to undergo apoptosis, as compared to controls (Fig. 4.5).

Populations change slowly

Similar to the results obtained with fixed cells in the cell cycle and proliferation protocol, Fig. 4.6 shows that unfixed cells also remained healthy for at least 24 h, and were still reasonably viable after 72 hours, even without medium changes. There were statistically significant changes in the necrotic population from 12 h onwards, although the number never climbed over 10% of the total population. The only other significant change was an increase in apoptosis at 48 h, which was still high at 72 h.

Blocking PACAP receptor increases apoptosis and necrosis

The increase in apoptosis recorded in the cell cycle and proliferation protocol in response to blocking PAC_1-R was clearly confirmed by the apoptosis protocol. Significant increases in apoptosis and necrosis were recorded after 5 h of treatment with 10 μM PACAP6-38 (Fig. 4.7A). After 24 h of treatment, there was still a significant

Figure 4.3 Treatment with the protein kinase inhibitor staurosporine caused an increase in apoptosis and necrosis in chick neuroblasts. Some of the necrotic cells may be late apoptotic, or secondarily necrotic cells. The treatment confirmed the region of apoptosis.

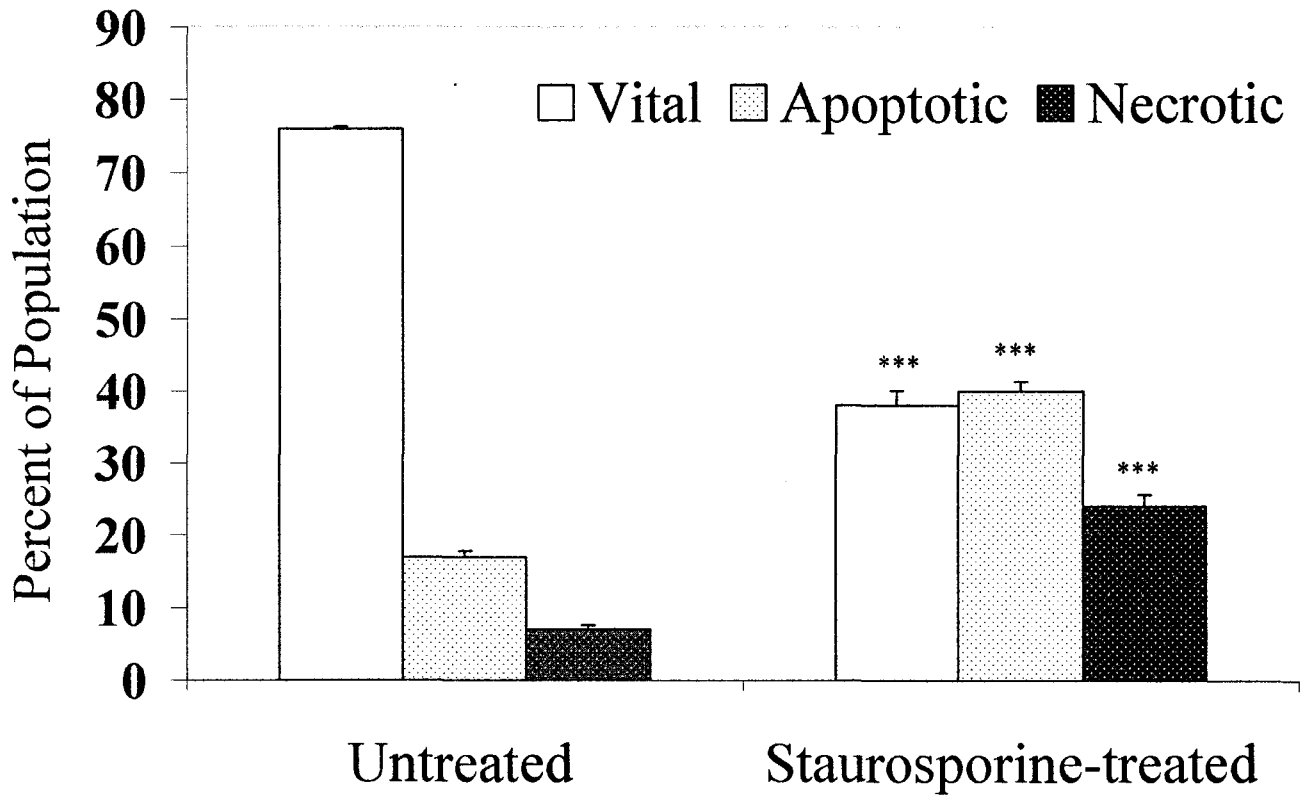

90
80
70
60
50
40
30
20
10
0
Percent of Population
Vital
Apoptotic
Necrotic
***
***
***
Untreated
Staurosporine-treated

Figure 4.4 Inhibition of staurosporine by cycloheximide. Because apoptosis often requires synthesis of proteins, death may be inhibited by protein synthesis inhibitors such as cycloheximide. The cell population identified as apoptotic was confirmed by the rescue of staurosporine-treated cells (the "untreated" controls in this experiment) in a dose-dependent manner by cycloheximide.

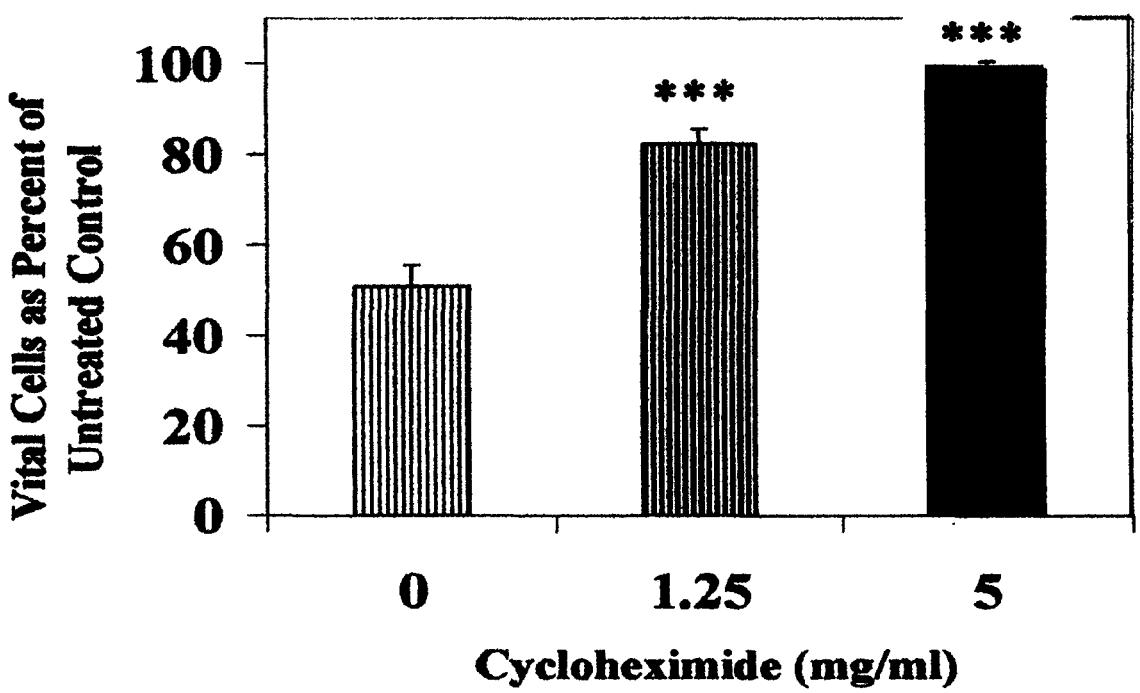

Vital Cells as Percent of Untreated Control
100
80
60
40
20
0
***
***
0
1.25
5
Cycloheximide (mg/ml)

Figure 4.5 Cells treated with staurosporine (right) were stained with the DNA dye Hoechst 33258 to highlight the condensed nuclei and apoptotic bodies that form during apoptosis. Solid arrows point to condensing nuclei, whereas dotted arrows highlight cells breaking into apoptotic bodies. The higher number of smaller cells with darker staining indicate condensed DNA, and demonstrate the efficiency of the apoptosis-inducer when compared to the controls on the left. Scale bar represents 10 μm.

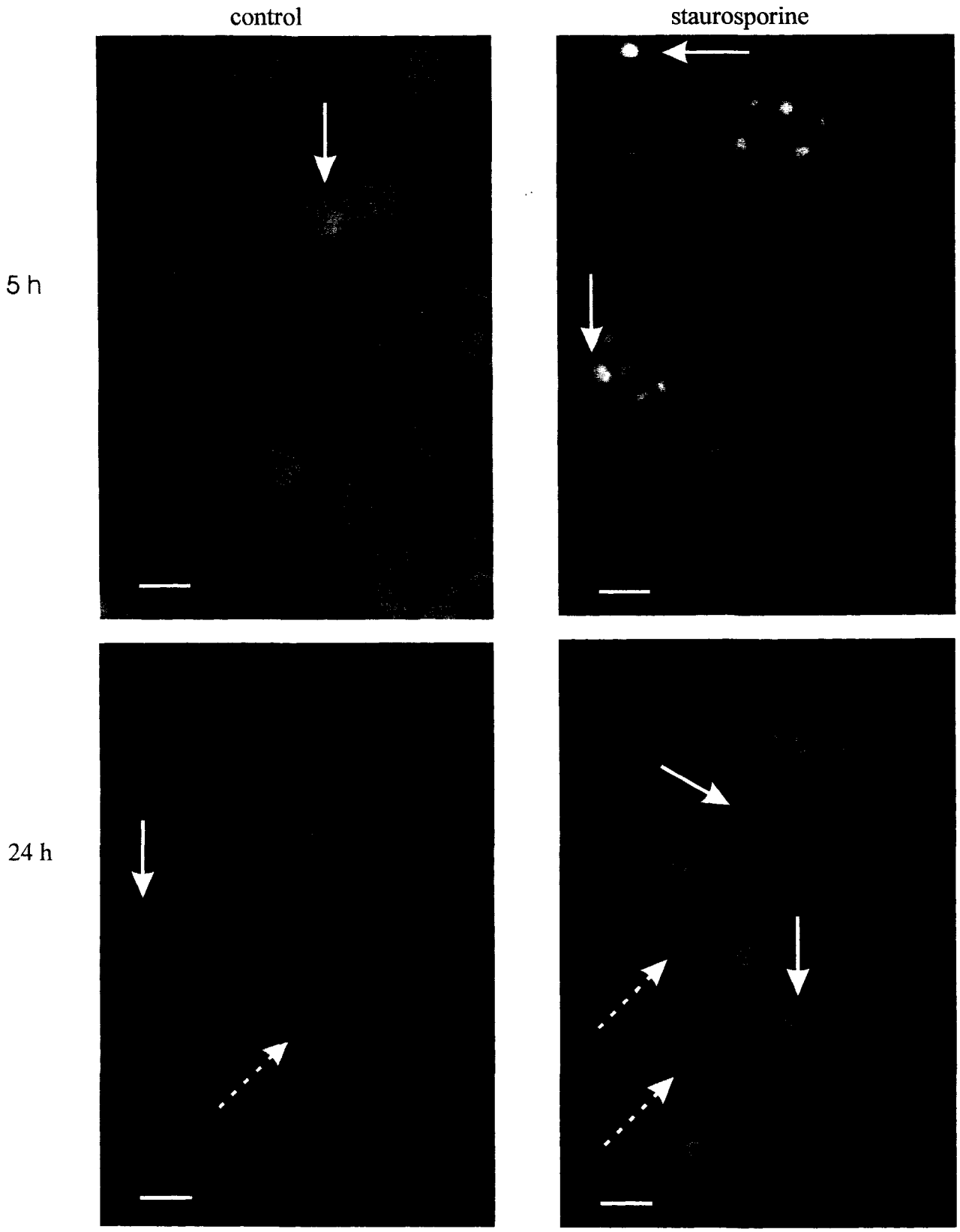

control
staurosporine
5 h
24 h

Figure 4.6 Congruent with results from cell cycle and proliferation experiments, FDA and PI staining showed that cultured E3.5 chick neuroblasts remained very healthy for about 24 h, and were still viable cultures even after three days with no medium change. Cultures up to 12 h generally yielded a vital cell population of more than 80%, and the mean stayed greater than 70% until at least 48 h. The low levels of natural apoptosis throughout made assessment of PACAP's ability to rescue cells from apoptosis unconvincing without induction.

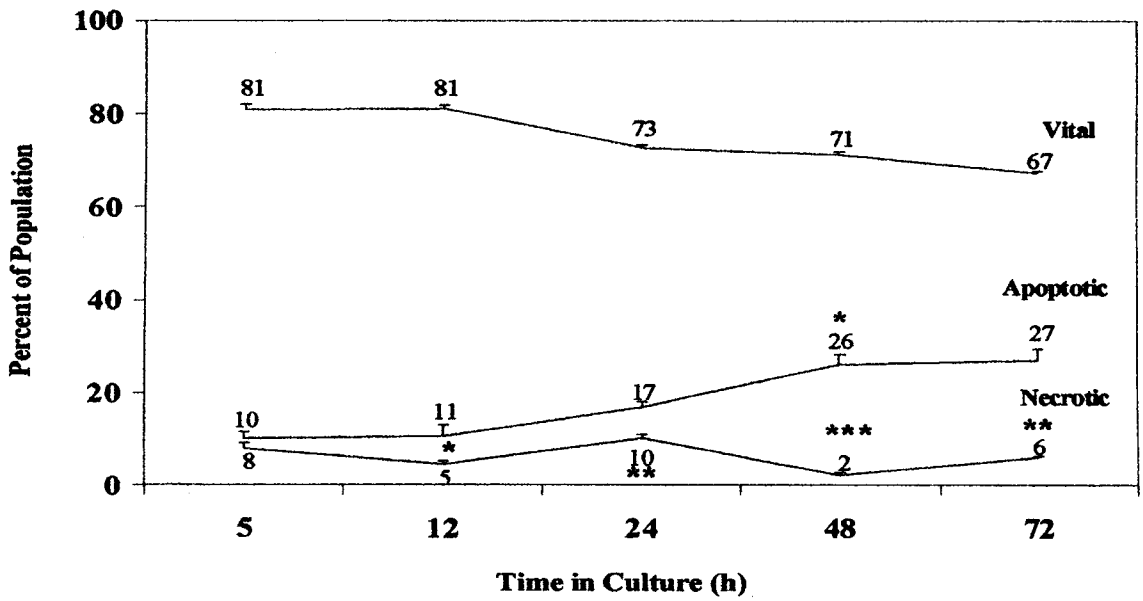
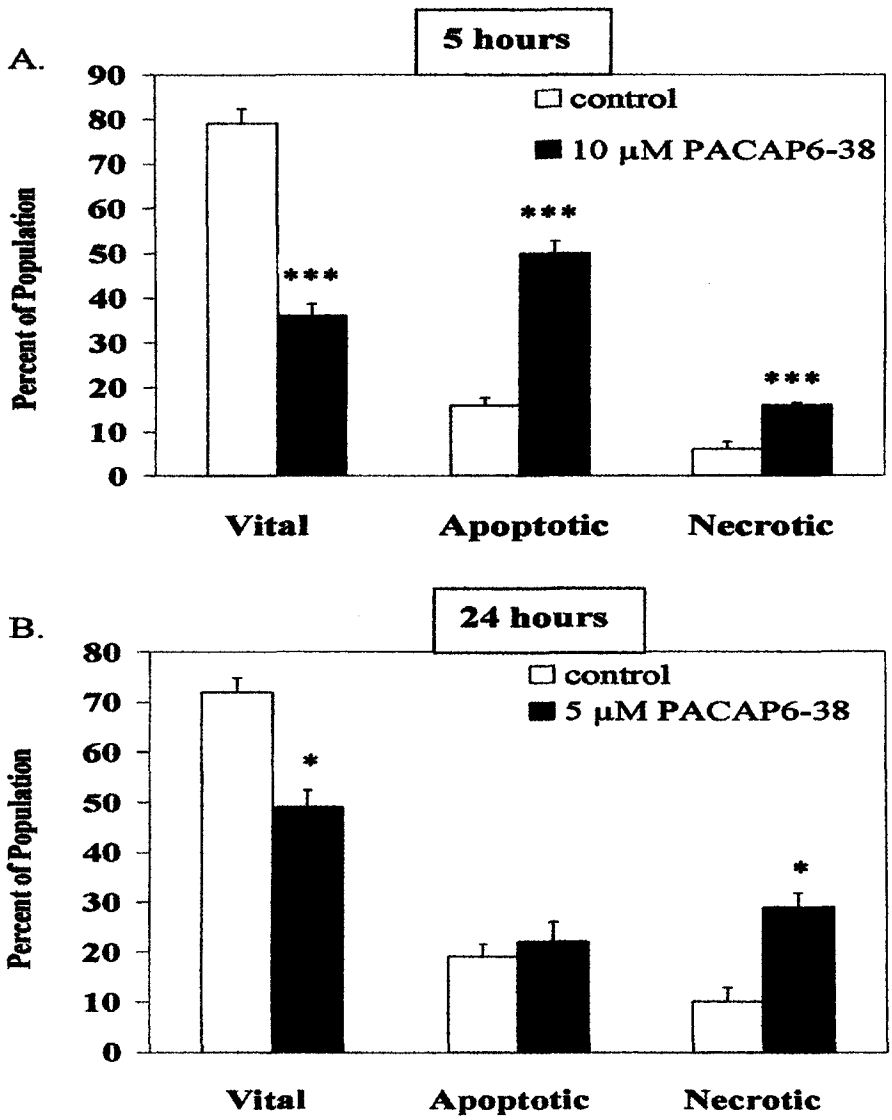

100
80
60
40
20
0
Percent of Population
81
81
73
71
67
Vital
Apoptotic
*
26
27
17
11
10
Necrotic
***
**
6
*
8
5
10
**
2
5
12
24
48
72
Time in Culture (h)

Figure 4.7 The ability of the PACAP receptor blocker, PACAP6-38, to cause cell cycle exit and apoptosis, discovered in the cell cycle and proliferation experiments, was confirmed using a protocol designed more specifically to study apoptosis. A. After 5 h, 10 μM of the block caused highly significant decreases in the vital population, and increases in both the apoptotic and necrotic regions. B. After 24 h, PACAP6-38 still caused a significant decrease in vital cells, and a significant increase in necrotic cells (which could include late apoptotic or secondarily necrotic cells).


A.
5 hours
control
10 μM PACAP6-38
Percent of Population
90
80
70
60
50
40
30
20
10
0
***
***
***
Vital
Apoptotic
Necrotic
B.
24 hours
control
5 μM PACAP6-38
Percent of Population
80
70
60
50
40
30
20
10
0
*
*
Vital
Apoptotic
Necrotic

decrease to the vital population, with an increase in the necrotic (and probably secondarily necrotic) population (Fig. 4.7B).

PACAP increases natural apoptosis, has little effect on induced apoptosis

Although cell cycle and proliferation results showed little change in natural apoptosis in response to PACAP, in this protocol specific for measurement of apoptosis, 10^{-6} M PACAP significantly increased apoptotic cells at 5 h, and 10^{-7} M PACAP significantly decreased the number of vital cells at 24 h (Fig. 4.8). Both concentrations decreased the number of vital cells while increasing the number of apoptotic cells at 48 h (Fig. 4.8). Treatment with 10^{-7} M PACAP increased necrosis at 72 hours.

PACAP was generally unable to rescue cells from staurosporine-induced apoptosis, although both concentrations of hormone caused a statistical decrease in necrosis after 5 h (Fig. 4.9). PACAP was also unable to rescue cells treated with ceramide and ethanol (data not shown).

Figure 4.8 Chick neuroblasts treated with both 10^{-7} M and 10^{-6} M chicken PACAP38 were assayed to assess impact on survival and apoptosis. There was a statistical increase in apoptosis for the higher concentration of hormone at 5h, and a decrease in vital cells for the lower concentration at 24 h. At 48 h, both concentrations caused a decrease in vital cells and an increase in apoptosis. At 72 h, the higher concentration caused an increase in the number of necrotic cells.

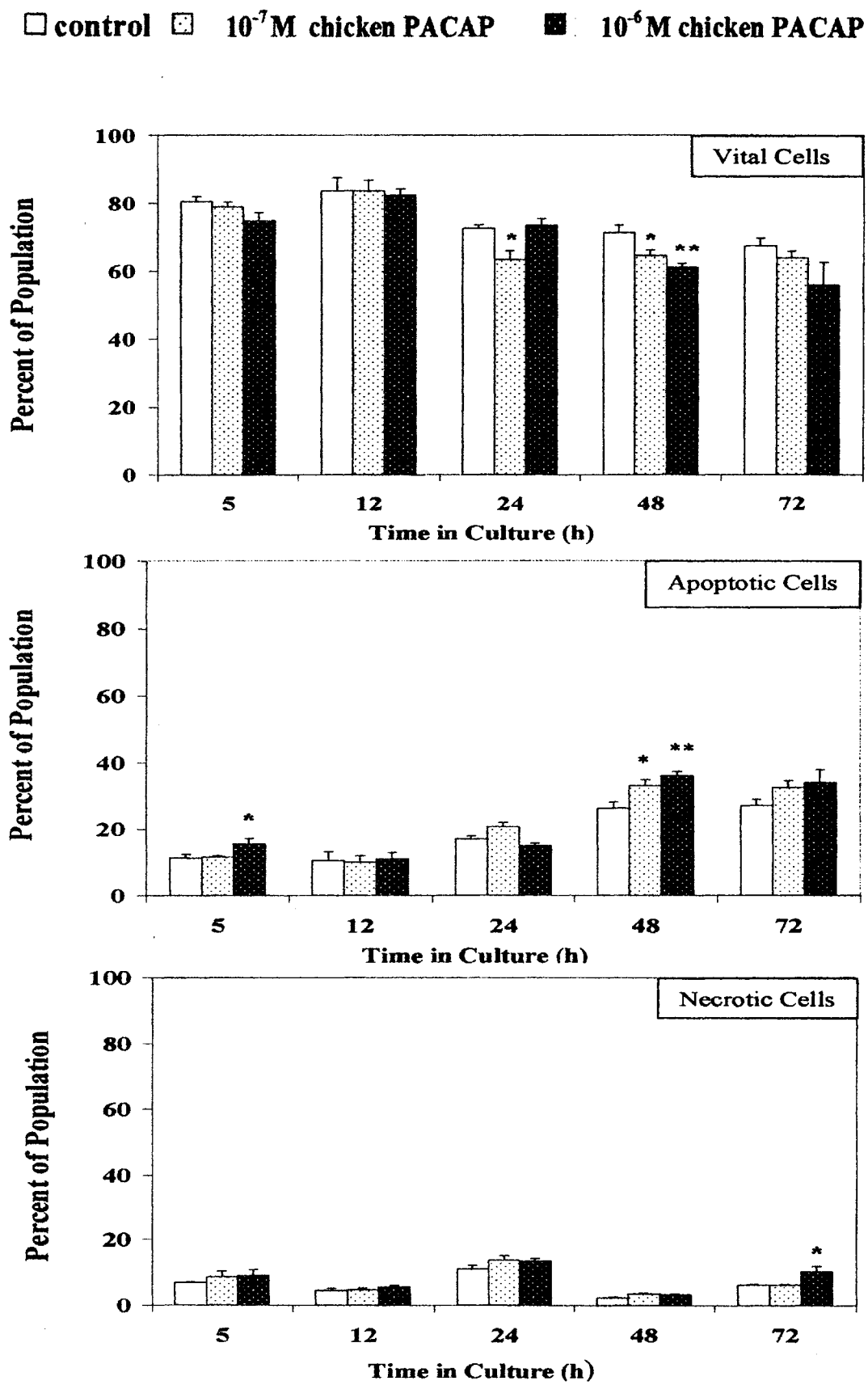
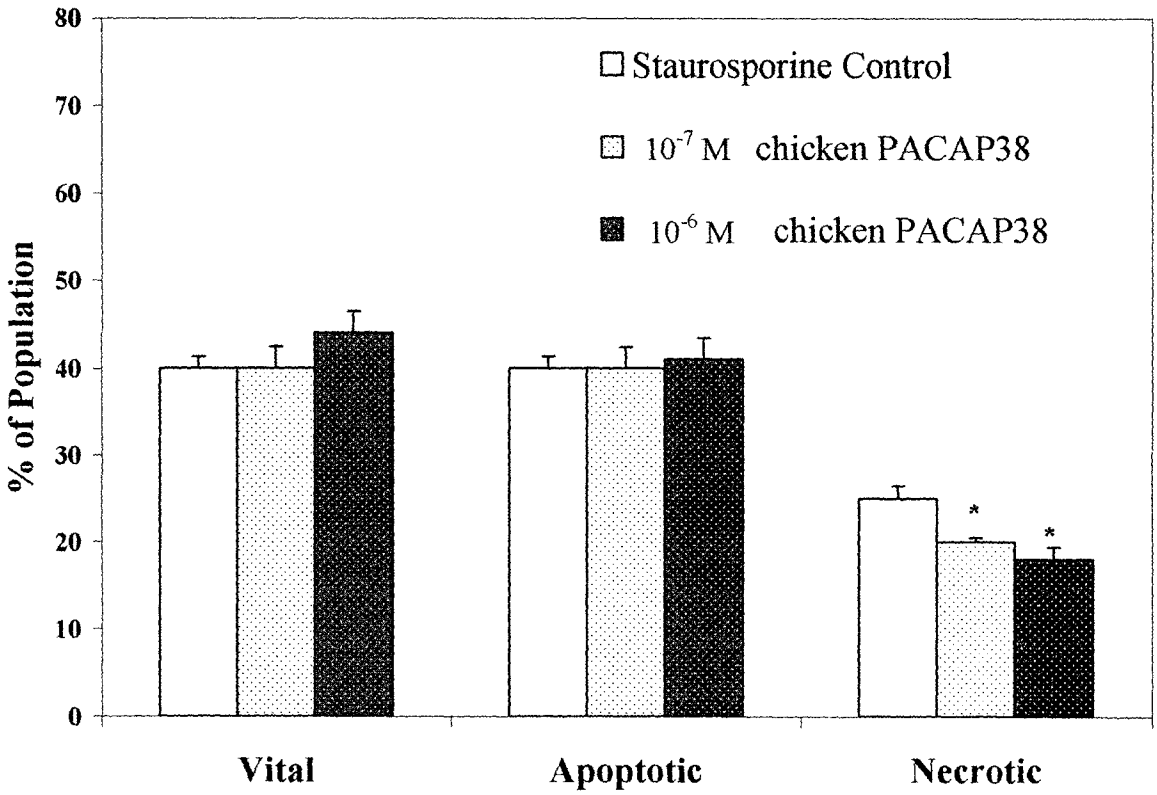

control
10-7M chicken PACAP
10-6M chicken PACAP
Vital Cells
Percent of Population
100
80
60
40
20
0
5
12
24
48
72
Time in Culture (h)
Apoptotic Cells
Percent of Population
Time in Culture (h)
Necrotic Cells
Percent of Population
Time in Culture (h)

Figure 4.9 Cells induced to undergo apoptosis with staurosporine did not appear to be rescued after 5 h incubation with PACAP. However, there were statistical decreases in necrosis (which could include late apoptotic or secondarily necrotic cells) for both concentrations of PACAP.


80
70
60
50
40
30
20
10
0
% of Population
Staurosporine Control
10-7 M chicken PACAP38
10-6 M chicken PACAP38
*
*
Vital
Apoptotic
Necrotic

DISCUSSION

Annexin V is an ineffective protocol

Both protocols based on use of annexin V appeared to produce inflated levels of apoptosis in this study. Addition of a known apoptosis-inducer for these cells could not raise the levels any further. This suggested that manipulation of the cells during processing altered the cell membranes to the degree that, even without treatment of any kind, the maximum number of phosphatidylserine residues were exposed for binding to annexin V. The inflated levels that appeared using the modified protocol are more difficult to explain. In this protocol, washes should have removed any excess annexin V prior to manipulation of cells, so there should have been no increase in binding after that point. The lack of success with this modified protocol may relate to differences in the properties of adherence between the transformed cells used to develop that protocol (Van-Engeland et al. 1996) and cells from primary cell culture. It could also be that there is a cell surface component of chick neuroblasts that non-specifically binds annexin V.

FDA and PI protocol identified three populations

However, the FDA and PI protocol is a simple and reliable method for examining changes in live, apoptotic and necrotic populations. As with the cell cycle and proliferation protocol, only 10% compensation for overlapping fluorescence was required, so there was little loss in data due to a need to separate fluorescence emissions. There was also very little debris and no aggregation. Although a build-up of toxins and depletion of medium components may have affected cells after about 24 h, the apoptotic

populations did not really begin to climb until about 48 h, and the necrotic population stayed low throughout the three-day culture.

An apoptotic region was easily delineated, and confirmed by induction of apoptosis using staurosporine. The apoptotic program in these cells obviously requires production of proteins, as evidenced by the ability of cycloheximide to dose dependently rescue staurosporine-treated cells. This matches the complete rescue of E8 chick ciliary ganglion cells by addition of cycloheximide, when the cells were induced to undergo apoptosis by withdrawal of trophic support (Villa et al. 1994). It also matches the rescue of E8 chick retinal ganglion cells by cycloheximide, when the cells were induced to undergo apoptosis by addition of fibroblast growth factor (Yokoyama et al. 1997). The apoptotic cells showed typical morphology.

Effects of PAC_1-R confirmed

The increase in apoptosis in response to blockade of PAC_1-R, reported in Chapter 3, was clearly confirmed. The FDA and PI protocol revealed higher amounts of apoptosis after 5 h incubation than after 24 h incubation, contrary to the cell cycle and proliferation protocol. This can be explained. The cell cycle and proliferation protocol was not designed to measure apoptosis, and would not have measured it as accurately as the FDA/PI protocol, for two reasons. In Chapter 3, apoptotic cells were defined by an absence of PCNA, because they do not cycle, and by a decrease in DNA, because in a population that has been permeabilized and fixed, small fragments of DNA are able to leak out of the cells. Therefore, the apoptotic gate was drawn below the region of positive PCNA staining, and to the left of the G_0 region. The parameters of the gate

were also based on an increase in heavy cell scatter in this area when the cells were treated with the apoptosis-inducer staurosporine. However, PCNA has a half-life of 20 to 30 hours, so it is likely that some apoptotic cells were left out of the gate, above it. As well, cells can undergo apoptosis from any compartment of the cycle, and would therefore register varying amounts of DNA depending on the compartment from which they had exited. Therefore, some apoptotic cells that had left the cycle from S or G_2/M would have been left out of the gate, to the right of G_0, and also not been included in the total of apoptotic cells. Both these factors would have caused an underestimation of the true apoptotic proportions.

However, the FDA and PI protocol was designed to measure apoptosis, based on intracellular enzyme activity and membrane integrity, in a live cell population. Therefore there was no delay comparable to the PCNA degradation delay, nor was there varying amounts of DNA leakage since the apoptotic cell membranes remained intact. Because they were live cells, there was also less debris. Although there was some overlap between the vital and apoptotic populations, it was relatively easy using control tubes to gate three quite distinct populations: live, apoptotic and necrotic.

As well, because apoptotic cells in culture are not phagocytosed, they undergo secondary necrosis. The sensitivity of this protocol could explain why there was an increase in the region defined as necrotic at 24 h. For these reasons, the picture of a rapid increase in apoptosis at 5 h with a build-up of necrosis and secondary necrosis at 24 h, suggested by the FDA and PI protocol, is reasonable.

PACAP does not rescue cells

It is interesting that PACAP did not rescue cells from staurosporine induced apoptosis, but increased the number of cells that were undergoing apoptosis and necrosis. Earlier experiments showed these concentrations of PACAP to maximally increase cAMP in the cells (Chapter 2), but it could be that the doses are not in the physiological range and cultured cells are prone to apoptosis at these concentrations.

The possibility that PACAP has the ability to rescue cells induced to undergo apoptosis by chemical or environmental insults is interesting. The impact of PACAP on staurosporine-induced apoptosis was only tested at 5 h in this study, but there was a statistical decrease in necrotic cells. cAMP has been reported to rescue cells from similar concentrations of staurosporine, although the cells were older (E7), the treatment was longer (24 h), and the rescue was only partial (Dawson 2000). Of concern with staurosporine studies is that it is best known (and even marketed) as a PKC inhibitor, but it will inhibit a variety of protein kinases, including PKA, through which cAMP acts (Herbert et al. 1990). However, PACAP was also not able to rescue cells from ceramide and ethanol-induced apoptosis. There is a possibility that the production of PACAP in these cells serves simply to keep them alive and proliferating, and does not serve any other function.

Summary

In this chapter, I confirm that PACAP is necessary to prevent apoptosis at this stage of chick brain development, but find no convincing evidence that it plays a role in rescuing cells either from natural or induced apoptosis.

REFERENCES

Ahlemeyer, B, D Fischer, T Kissel and J Krieglstein (2000). "Staurosporine-induced apoptosis in cultured chick embryonic neurons is reduced by polyethylenimine of low molecular weight used as a coating substrate." Neuroscience Research **37**: 245-253.

Arimura, A, A Somogyvari-Vigh, C Weill, RC Fiore, I Tatsuno, V Bay and DE Brenneman (1994). "PACAP functions as a neurotrophic factor." Annals of the New York Academy of Science, USA **739**: 228-243.

Campard, KP, C Crochemore, F Rene, D Monnier, B Koch and JP Loeffler (1997). "PACAP Type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA pathway." DNA and Cell Biology **16**: 323-333.

Canonico, PL, A Copani, V D'Agata, S Musco, S Petralia, S Travali, F Stivala and S Cavallaro (1996). "Activation of pituitary adenylate cyclase-activating polypeptide receptors prevents apoptotic cell death in cultured cerebellar granule cells." Annals of the New York Academy of Science, USA **805**: 470-472.

Cavallaro, S, A Copani, V D'Agata, S Musco, S Petralia, C Ventra, F Stivala, S Travali and PL Canonico (1996). "Pituitary adenylate cyclase-activating polypeptide prevents apoptosis in cultured cerebellar granule neurons." Molecular Pharmacology **50**: 60-66.

Chang, JY, VV Korolev and J-Z Wang (1996). "Cyclic AMP and pituitary adenylate cyclase-activating polypeptide (PACAP) prevent programmed cell death of cultured rat cerebellar granule cells." Neuroscience Letters **206**: 181-184.

Dawson, G (2000). "Mechanisms of apoptosis in embryonic cortical neurons (E6 and E7) in culture involved lipid signalling, protein phosphorylation and caspase activation." International Journal of Developmental Neuroscience **18**: 247-257.

DiCicco-Bloom, E and PJ Deutsch (1992). "Pituitary adenylate cyclase activating polypeptide (PACAP) potently stimulates mitosis, neuritogenesis and survival in cultured rat sympathetic neuroblasts." Regulatory Peptides **37**: 319.

DiCicco-Bloom, E, PJ Deutsch, J Maltzman, J Zhang, JE Pintar, J Zheng, WF Friedman, X Zhou and T Zaremba (2000). "Autocrine expression and ontogenetic functions of the PACAP ligand/receptor system during sympathetic development." Developmental Biology **219**: 197-213.

DiCicco-Bloom, E, N Lu, JE Pintar and J Zhang (1998). "The PACAP ligand/receptor system regulates cerebral cortical neurogenesis." Annals of the New York Academy of Science, USA **865**: 274-289.

Fadok, VA, DR Voelker, PA Campbell, JJ Cohen, DL Bratton and PM Henson (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." Journal of Immunology **148**: 2207-2216.

Frago, LM, Y León, EJ de la Rosa, A Gómez-Muñoz and I Varela-Nieto (1998). "Nerve growth factor and ceramides modulate cell death in the early developing inner ear." Journal of Cell Science **111**: 549-556.

Gonzalez, BJ, M Basille, D Vaudry, A Fournier and H Vaudry (1997). "Pituitary adenylate cyclase-activating polypeptide promotes cell survival and neurite outgrowth in rat cerebellar neuroblasts." Neuroscience **78**: 419-430.

Herbert, JM, E Seban and JP Maffrand (1990). "Characterization of specific binding sites for [3H]-staurosporine on various protein kinases." Biochemical and Biophysical Research Communications **171**: 189-195.

Ito, Y, M Arakawa, K Ishige and H Fukuda (1999). "Comparative study of survival signal withdrawal- and 4-hydroxynonenal induced cell death in cerebellar granule cells." Neuroscience Research **35**: 321-327.

Journot, L, M Villalba and J Bockaert (1998). "PACAP-38 protects cerebellar granule cells from apoptosis." Annals of the New York Academy of Science, USA **865**: 100-110.

Kentroti, S and A Vernadakis (1990). "Neuronal plasticity in the developing chick brain; interaction of ethanol and neuropeptides." Developmental Brain Research **56**: 205-210.

Kravtsov, VD, TO Daniel and MJ Koury (1999). "Comparative analysis of different methodological approaches to the *in vitro* study of drug-induced apoptosis." American Journal of Pathology **155**: 1327-1339.

Lindholm, D, Y Skoglosa and N Takei (1998). "Developmental regulation of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor 1 in rat brain: function of PACAP as a neurotrophic factor." Annals of the New York Academy of Science, USA **805**: 189-196.

Lioudyno, M, Y Skoglosa, N Takei and D Lindholm (1998). "Pituitary adenylate cyclase-activating polypeptide (PACAP) protects dorsal root ganglion neurons from death and induces calcitonin gene-related peptide (CGRP) immunoreactivity in vitro." Journal of Neuroscience Research **51**: 243-356.

Lu, N and E DiCicco-Bloom (1997). "Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells." Proceedings of the National Academy of Science, USA **94**: 3357-3362.

Morio, H, I Tatsuno, A Hirai, Y Tamura and Y Saito (1996). "Pituitary adenylate cyclase-activating polypeptide protects rat cultured cortical neurons from glutamate-induced toxicity." Brain Research **741**: 82-88.

Przywara, DA, JS Kulkarni, TD Wakade, DV Leontiv and AR Wakade (1998). "Pituitary adenylyl cyclase-activating polypeptide and nerve growth factor use the proteasome to rescue nerve growth factor-deprived sympathetic neurons cultured from chick embryos." Journal of Neurochemistry **71**: 1889-1897.

Rahman, H, S Kentroti and A Vernadakis (1994). "Neuroblast cell death *in ovo* and in culture: interaction of ethanol and neurotrophic factors." Neurochemical Research **19**: 1495-1502.

Rimon, G, CE Bazenet, KL Philpott and LL Rubin (1997). "Increased surface phosphatidylserine is an early marker of neuronal apoptosis." Journal of Neuroscience Research **48**: 563-570.

Schwartzman, RA and JA Cidlowski (1993). "Apoptosis: the biochemistry and molecular biology of programmed cell death." Endocrine Reviews **14**: 133-151.

Skoglosa, Y, C Patrone and D Lindholm (1999). "Pituitary adenylate cyclase activating polypeptide is expressed by developing rat Purkinje cells and decreases the number of cerebellar gamma-amino butyric acid positive neurons in culture." Neuroscience Letters **265**: 207-210.

Suh, J, N Lu, A Nicot, I Tatsuno and E DiCicco-Bloom (2001). "PACAP is an anti-mitogenic signal in developing cerebral cortex." Nature **4**: 1-2.

Tabuchi, A, K Funaji, J Nakatsubo, M Fukuchi, T Tsuchiya and M Tsuda (2003). "Inactivation of aconitase during the apoptosis of mouse cerebellar granule neurons induced by a deprivation of membrane depolarization." Journal of Neuroscience Research **71**: 504-515.

Takei, N, Y Skoglosa and D Lindholm (1998). "Neurotrophic and neuroprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on mesencephalic dopaminergic neurons." Journal of Neuroscience Research **54**: 698-706.

Uchida, D, A Arimura, A Somogyvari-Vigh, S Shioda and WA Banks (1996). "Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide (PACAP)." Brain Research **736**: 280-286.

Van-Den-Eijnde, SM, AJM Luijsterburg, L Boshart, CI De-Zeeuw, JH Van-Dierendonck, CPM Reutelingsperger and KC Vermeij (1997). "*In situ* detection of apoptosis during embryogenesis with annexin V: from whole mount to ultrastructure." Cytometry **29**: 313-320.

Van-Engeland, M, CS Ramaekers-Frans, B Schutte and CPM Reutingsperger (1996). "A novel assay to measure loss of asymmetry during apoptosis on adherent cells in culture." Cytometry **24**: 131-139.

Vaudry, D, BJ Gonzalez, M Basille, A Fournier and H Vaudry (1999). "Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development." Proceedings of the National Academy of Science, USA **96**: 9415-9420.

Vaudry, D, BJ Gonzalez, M Basille, TF Pamantung, A Fournier and H Vaudry (2000). "PACAP acts as a neurotrophic factor during histogenesis of the rat cerebellar granule cortex." Annals of the New York Academy of Science, USA **921**: 293-299.

Vaudry, D, TF Pamantung, M Basille, C Rouselle, A Fournier, H Vaudry, JC Beauvillain and BJ Gonzalez (2002a). "Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death." Proceedings of the National Academy of Science, USA **99**: 6398-6403.

Vaudry, D, C Rouselle, M Basille, MA Falluel, TF Pamantung, M Fontaine, A Fournier, H Vaudry and BJ Gonzalez (2002b). "PACAP protects cerebellar granule neurons against oxidative stress-induced apoptosis." European Journal of Neuroscience **15**: 1451-1460.

Villa, P, M Miehe, M Sensenbrenner and B Pettman (1994). "Synthesis of specific proteins in trophic factor-deprived neurons undergoing apoptosis." Journal of Neurochemistry **62**: 1468-1475.

Villalba, M, J Bockaert and L Journot (1997). "Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway." Journal of Neuroscience **17**: 83-90.

Wakade, AR and D Leontiv (1998). "Sympathetic neurons of the chick embryo are rescued by PACAP from apoptotic death." Annals of the New York Academy of Science, USA **865**: 176-180.

Waschek, JA, RA Casillas, TB Nguyen, EM DiCicco-Bloom, EM Carpenter and WI Rodriguez (1998). "Neural tube expression of pituitary adenylate cyclase-activating polypeptide (PACAP) and receptor: potential role in patterning and neurogenesis." Proceedings of the National Academy of Science, USA **95**: 9602-9607.

Weisner, DA and G Dawson (1996). "Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway." Journal of Neurochemistry **66**: 1418-1425.

Yokoyama, Y, S Ozawa, Y Seyama, H Maniki, Y Hayashi, K Kaji, K Shirama, M Shioda and K Kano (1997). "Enhancement of apoptosis in developing chick neural retina cells by basic fibroblast growth factor." Journal of Neurochemistry **68**: 2212-2215.

CHAPTER 5

Proteomics reveals that blocking the PACAP receptor leads to differentiation, in addition to cell cycle exit and apoptosis in chick neuroblasts

INTRODUCTION

In Chapter 3, I found that blocking the PACAP receptor during cell cycle and proliferation assays led to cell cycle exit at 5 h, with cells progressing to apoptosis by 24 h. The results of a more sensitive assay for apoptosis in Chapter 4 suggested there was a more rapid increase in apoptosis, followed by some decline. But the cell cycle and proliferation assays also suggested that at 24 h, some cells were still quiescent, not yet undergoing apoptosis. It was possible that some of those cells that had so recently left the cell cycle were actually committed to differentiate rather than to die. Cell cycle arrest of proliferating cells can lead to either apoptosis or differentiation (Howard et al. 1993). Therefore, the final goal of my thesis was to examine whether some of the cells blocked from the cycling effects of PACAP were differentiating. Although it would have been simplest to examine growth of neurites, aggregation of cultured cells made this impossible. It would have been possible to continue using flow cytometry methods, for example, by labeling cells for an early differentiation marker like microtubule-associated-protein 5, I felt the necessity to permeabilize the cells by fixing them would compromise the accuracy of the numerical data. So I investigated other possibilities.

It is increasingly common in molecular biology to determine differentially expressed cDNA or mRNA using microarrays (Galaviz-Hernandez et al. 2003; Handwerger and Aronow 2003; Kinoshita et al. 2003; Shankar et al. 2003; Tabakoff et al. 2003; Udtha et al. 2003). There are many benefits to what some have termed an hypothesis-free, holistic approach, but limitations as well. Microarrays allow rapid identification of gene activity on a massive scale: evaluation of 20,000 genes or expressed sequence tags is not uncommon (Burton et al. 2002). Even if only a few

percent of these genes exhibit what is considered to be an important level of change (typically 2 to 5 fold) there can still be several hundred genes to investigate. For example, researchers using an adipocyte cell line, using a cut-off of at least a 5-fold increase or decrease, found altered regulation of 285 genes following stimulation to differentiate (Burton et al. 2002). Organizing such large data sets so that relevant biological data emerges can be daunting, but several methods have been developed (Eisen et al. 1998; Tamayo et al. 1999; Tavazoie et al. 1999). These methods are used to group genes in various ways so that inter-relationships can be investigated. A major benefit to microarray data is that some of the upregulated or downregulated genes may not yet have been associated with the biological process being studied. Researchers investigating the effects of thyroid hormones on the liver discovered 7 upregulated and 38 downregulated genes that were not previously known to be been associated with thyroxine (Feng et al. 2000).

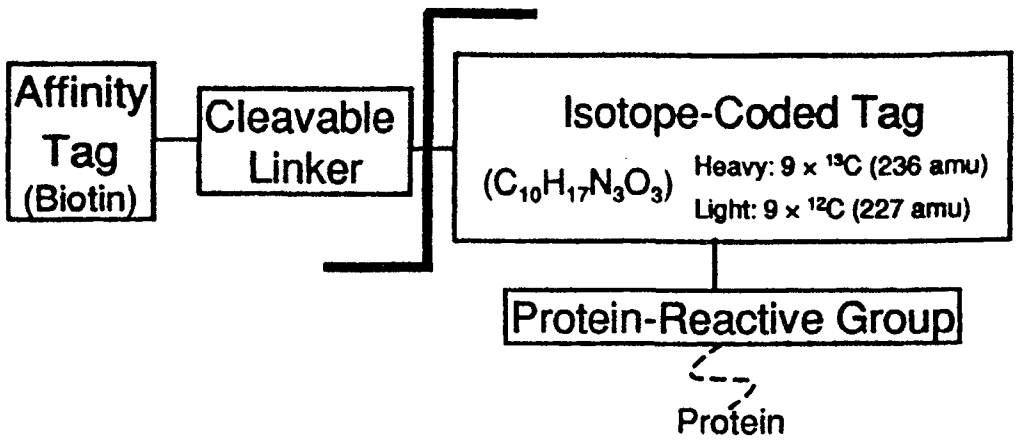
One of the most obvious limitations with microarrays is that an increase in mRNA will not necessarily produce a protein product, or production may be delayed, so it is impossible to confidently correlate stimuli with actual changes in cell dynamics. The production of mRNA does not necessarily reflect protein levels (Futcher et al. 1999; Gygi et al. 1999a). In addition, activation of a gene may result in the generation of a variety of proteins that have been post-transcriptionally and post-translationally modified, and these changes cannot be distinguished in microarrays. It is estimated that the approximate 30,000 genes in the human genome code for about 100,000 proteins (Clynen et al. 2003). Traditional methods are generally still required to confirm microarray data and draw conclusions.

Specific to this thesis, there were further disadvantages in using microarray. Isolation of mRNA from brain tends to produce very low yields (Ambion 2003), and preliminary work proved this to be true. Generation of useful data also relies on the availability of a microarray designed for the species under investigation. There are only a few microarrays available for analysis of chicken genes and these arrays are relatively small. For these reasons, I decided to examine the possibility of differentiation in these cell populations by evaluating the direct effectors of cell changes, the proteins.

Isotope-coded affinity tagged (ICAT) analysis is a recently introduced technique based on labeling samples with isotopes of different masses (Gygi et al. 2002). The control sample is generally labeled with a molecule that incorporates a light isotope, and the treated sample with an identical molecule that incorporates a heavy isotope. The samples are then mixed, digested into fragments, and the fragments are sequenced and quantified using mass spectrometry.

The ICAT label binds through a reactive group to proteins at the thiol group of a cysteine residue. The label is also attached to an affinity tag, which allows separation of labeled from unlabeled fragments by avidin affinity chromatography (Fig. 5.1). Labeled fragments can be separated into as many as 30 fractions prior to analysis by mass spectrometry (MS). Because a mass spectrometer cannot identify fragments above a certain size, cleavage of the affinity tag prior to analysis greatly increases the number of labeled fragments that can be processed. The mass spectrometer is able to identify the amino acid composition of each fragment and compare the amount of heavy to light isotope by alternating between identification and quantification modes.

Figure 5.1 Representation of an isotope-coded affinity tag. The coded tag may contain a light isotope, or a heavy isotope (both are shown here). A control sample is generally labeled with the light isotope, and a treated sample with the heavy isotope. The tag is bound at one end to a reactive group that will bind a protein at the thiol group of a cysteine residue. At the other end, it is bound to a cleavable affinity tag. This affinity tag allows separation of labeled fragments by avidin affinity chromatography prior to mass spectrometry. Figure taken from Applied Biosystems (2003).


Affinity Tag (Biotin)
Cleavable Linker
Isotope-Coded Tag
$(C_{10}H_{17}N_3O_3)$
Heavy: 9 x ^{13}C (236 amu)
Light: 9 x ^{12}C (227 amu)
Protein-Reactive Group
Protein

A sequence of as few as five contiguous amino acids is sufficient to identify a protein. Parent proteins are identified using databases, and, as with microarrays, tools have been developed to allow clustering of data so that inter-relationships can be established and novel proteins to the process under investigation can be identified (Koonin and Galperin 2003).

There is another advantage that ICAT analysis has compared to microarrays. Most proteins have a cysteine residue, for example, only 8% of yeast proteins do not (Gygi et al. 1999b). Therefore, most proteins will be labeled. However, only the cleaved fragments that contain the cysteine groups will be collected on the affinity column, reducing the number of samples that will be processed by the mass spectrometer. Furthermore, although most proteins have a cysteine residue, they are relatively rare compared to other amino acids within proteins, which lowers further the number of fragments collected. Together, this reduces the number of samples that will be processed by the mass spectrometer to about one-tenth that which would otherwise be present, greatly simplifying analysis (Patton 2002). The method has been proven to be accurate and reproducible, and allows identification of low abundance proteins, which can be important when a biological system changes (Gygi et al. 2002).

Specific to this thesis, ICAT analysis was superior because it was easy to obtain large amounts of protein. Although the chick proteome has not yet been as well characterized as those of other species, it is possible to consider the general conservation of a protein in order to decide whether an ICAT analysis identification based on another species is likely to be valid.

Of course, one obvious weakness of the ICAT method is that any protein lacking a cysteine residue will not be labeled or identified. Another disadvantage, shared by microarrays, is an inability to identify whether proteins have been post-translationally modified, for example, by proteolytic processing or phosphorylation. One author suggests that there may be as many as several hundred thousand to millions of different proteins in the human body (Clynen et al. 2003). But the mass spectrometer identifies only a few hundred in an ICAT scan.

As well, the data collected does not tell the researcher anything about the rate at which proteins are being synthesized or degraded, or moved in and out of complexes or compartments of the cell, all of which are relevant to a picture of the cellular response (Han et al. 2001). For example, an increase in heat shock protein activity has been shown in response to hydrogen peroxide treatment, but there was no change in mRNA or protein accumulation (Bruce et al. 1993)

There are still other, more mundane, disadvantages. Contamination in the sample will result in fewer relevant results. Consider, for instance, that bovine serum albumin, commonly used in cell cultures, contains 35 cysteine residues according to the National Institutes of Health National Centre for Biotechnology Information (NCBI). Therefore, even small amounts left in the protein mixture will sequester large amounts of ICAT reagent and greatly decrease the number of other proteins that can be labeled and identified.

The main reason for undertaking the ICAT analyses was to detect protein markers that would prove or disprove some degree of differentiation when the cells were blocked by the PACAP receptor. There were several other reasons why I chose the

method. I considered ICAT analysis a good opportunity to corroborate the data collected by flow cytometry. Also, I was interested in evaluating the ICAT technique because it can be utilized to complement other protocols used in this thesis, as well as more traditional molecular biology techniques. Lastly, I wanted to know if the data would suggest good candidate markers for differentiation, proliferation, exit, and apoptosis.

MATERIALS AND METHODS

Cell culture and preparation

Cells were dissected and dissociated as in previous chapters, and plated at 2 X 10^6 cells/ml (5 h ICAT analysis) or 3 X 10^6 cells/ml (24 h ICAT analysis). PACAP6-38 (AnaSpec Inc; San Jose, CA) was added at plating. As with all other assays, the receptor blocker was added for a final concentration of 10 μM for the 5 h experiment, and 5 μM given twice for the 24 h experiment. That is, the blocker was replenished at 12 h for the 24 h culture.

To harvest, cells were scraped free from the wells with a soft rubber spatula, centrifuged at 1000X g for 5 min at rt. Then the supernatant was removed, leaving approximately 60 μl of fluid with the cells in each tube. Aliquots of 60 μl of Mg^{+2} and Ca^{+2}-free PBS were added, and the cells resuspended and centrifuged again. Supernatants were removed and the cells resuspended in PBS as above. Cells were then pooled into one tube of untreated cells and one tube of treated cells. Following a final centrifugation, cells were drained of all liquid and frozen at –80 C until analysis.

Cells cultured for 5 h were used in one ICAT. Cells cultured for 24 h were used in two ICATs, with the first portioned into 10 fractions, and the second into 30 fractions. In this way, a technical replicate was provided for the 24 h protocol. It also allowed a comparison of the amount of data collected dependent on the number of fractions.

ICAT Analysis

ICAT analysis was performed by members of the UVic-Genome BC Proteomics Centre (University of Victoria, Victoria, B.C.). Cells were solubilized by adding 100 μl

of 0.2% SDS, boiled for 15 min, vortexed and cooled, then 100 µl freshly-made saturated urea was added. The solution was centrifuged and the supernatant was used in a modified Bradford assay to confirm there was sufficient protein in the sample. Using the protocols from an Applied Biosystems kit (Foster City, CA), proteins were denatured and reduced, and treated with an ICAT reagent that used $9C^{12}$ as the light isotope and $9C^{13}$ as the heavy isotope. Samples were digested with trypsin at 37 C overnight, then cleaned of chemical background such as SDS, urea and excess ICAT label by using strong cation exchange chromatography. The labeled fragments were isolated by biotin affinity chromatography. The biotin tag was cleaved, and the labeled peptides were separated into fractions by two dimensional chromatography using strong cation exchange and reversed-phase high performance liquid chromatography. Fragments were identified and quantified by nanoflow liquid chromatography combined with tandem mass spectrometry. Sequences were identified based on collision-induced dissociation, and correlation of the resulting spectra to amino acids by searching a database using ProICAT (Applied Biosystems) computer algorithm. Quantification was based on comparison of relative signal intensities for identical pairs of fragments bearing either light or heavy label. Parent proteins were identified using the NCBI database.

Data Analysis

Data was normalized using at least 20 fragments of bovine serum albumin from each ICAT, with the assumption that no alteration in this protein had occurred due to treatment with the PACAP receptor blocker. This seemed reasonable, as the resulting number was close to the general mean of all peptides. This resulted in untreated to

treated ratios of 1.25 for the 5 h assay, and 0.59 and 0.60 for the 24 assays of 10 and 30 fractions, respectively. Data was then analyzed by accepting a *mean* change of 1.6 fold as significant. Although this amount of change might be considered low for some experiments, our data came from a population of asynchronous cells, and only a small proportion of the population had been shown by other methods to leave the cell cycle when the PACAP receptor was blocked. Proteins for both 5 and 24 h assays were considered to have changed expression if the machine identified at least two separate fragments, both with a best confidence (BC) number (likelihood of a correct identification), of 75 or greater. In addition, proteins were accepted for the 5 h assay if only one fragment was identified, but the BC was at least 90, and for the 24 h assay if the same fragment appeared in both assays, each time with a BC of at least 75.

Although complex data analysis tools are available, because we knew in advance our areas of interest (differentiation, cell cycle changes, and apoptosis), and because our data set was relatively small, we used the simplest approach possible. We explored the known functions of proteins that had changed in response to the receptor blocker, and grouped them into the following categories: differentiation markers, proteins that showed a decrease in proliferation or an increase in exit, and apoptosis-associated proteins. There was overlap, because changes in some proteins may have led eventually to exit, quiescence, differentiation, or apoptosis. Not all proteins that fit the criteria were discussed, if to do so would have added little to the overall picture. Two proteins that did not fit into any of the categories were discussed.

RESULTS

More than 50 proteins change at 5 h, and more than 20 change at 24 h

Analysis of cells cultured for 5 h resulted in a data set with more fragments, and identified the most parent proteins, compared with those cultured for 24 h and fractioned either 10 or 30 times (Table 5.1). About 2% to 6% of the proteins were changed, depending on the assay and whether 1.6 or 2 fold was accepted as significant. The individual proteins with details of the assays are summarized in Table 5.2 (Parts I-III).

Table 5.1 Comparison of numbers of fragments and proteins from different assays.

Description of Assay	Fragments Sequenced	*Proteins Identified	*Proteins with >2 Fold Change	*Proteins with >1.6 fold Change
5 h culture, 10 fractions	963	864	24	55
24 h culture, 10 fractions	538	459	13	15
24 h culture, 30 fractions	762	637	21	22

* numbers include only proteins that fit the analysis criteria

Table 5.2, Part I. Change in proteins levels after treatment with a PACAP-specific receptor blocker.

(h:hours; n:unique fragments; f:fractions; D:differentiation; E:exit; A:apoptosis; +:increase; no symbol:decrease; italics indicate not considered significant)

NCBI#	Name of Protein	5h change (n)	24h/10f change (n)	24 h/30f change (n)	D	E	A
A decrease in a ribosomal and a myc-associated protein suggest differentiation							
16117787	ribosomal protein L34	*1.5 (1)*	2.4 (1)	1.9 (1)	√		
1082624	myc far upstream element-binding protein	1.8 (1)	2.3 (1)	2.2 (1)	√		
20467247	myc-like regulatory element	1.9 (1)	-	-	?	?	
A decrease is observed in proteins that create the working cell							
10835049	RhoA	1.6 (3)	0 (3)	0 (2)		√	√
1173054	ribosomal protein L5	-	1.9 (1)	2.2 (2)		√	
1362935	ribosomal protein S5	*1.4 (1)*	1.9 (2)	2.2 (1)		√	
119168	elongation factor 2	2.2 (5)	1.6 (6)	1.6 (1)		√	
1350717	ribosomal protein L30	1.7 (2)	*1.5 (2)*	*1.3 (2)*		√	
133883	ribosomal protein S20/S22	2.3 (1)	-	-		√	√
7159674	ribosomal protein L3	-	1.9 (2)	1.9 (1)	√	√	
28529052	ribosomal unit S12	+1.9 (2)	-	-		?	
7242148	eukaryotic translation initiation factor 2	1.8 (2)	-	-		√	
25014072	eukaryotic translation initiation factor 1A	1.9 (1)	-	-		√	
1708419	eukaryotic translation initiation factor 5	-	2.4 (1)	2.3 (1)		√	
17999537	U5 snRNP-specific protein (220 kD)	1.7 (1)	-	-		?	?
14141166	hnRNP E2	2.2 (3)	-	-		?	?
14141152	hnRNP M4	2.5 (3)	-	-		√	?
17221616	nuclear protein matrin 3	1.9 (2)	-	-		?	?
2500530	ATP-dependent RNA helicase p47/bat1	1.6 (1)	1.7 (2)	2.0 (4)		√	?
113825	ATP-dependent RNA helicase An3	2.5 (3)	-	-		?	?
10863927	cyclophilin A	2.2 (1)	-	-	?	?	?
13929124	cyclophilin A	2.7 (1)	-	-	?	?	?
20838011	cyclophilin A	2.2 (1)	-	-	?	?	?
4454307	cyclophilin A	2.2 (1)	-	-	?	?	?

Table 5.2, Part II. Change in proteins levels after treatment with a PACAP-specific receptor blocker.

(h:hours; n:unique fragments; f:fractions; D:differentiation; E:exit; A:apoptosis; +:increase; no symbol:decrease; italics indicate not considered significant)

NCBI#	Name of Protein	5h change (n)	24h/10f change (n)	24 h/30f change (n)	D	E	A
A decrease is observed in proteins that keep the cell working							
1083177	DNA topoisomerase I	3.9 (1)	-	-		√	
122070	Histone H3		2.4 (1)	1.7 (1)		√	
8670805	chaperonin-containing T-complex 1 delta	*1.4 (3)*	2.3 (2)	-		√	
21105454	chaperonin-containing T-complex 1 beta	-	1.9 (2)	1.8 (2)		√	
123678	heat shock protein 90 alpha	2.4 (1)	-	2.4 (2)	√	√	
11968062	protein phosphatase 1, catalytic subunit	2.3 (2)	-	-		?	
129336	protein phosphatase 2A	1.9 (2)	-	-		?	?
6434958	26S proteasome complex subunit p42D	1.6 (1)	-	-		?	?
11095437	valosin-containing protein	1.7 (3)	-	-		?	?
28279681	high mobility group box 1 protein	1.7 (2)		*1.2 (2)*		?	
6855513	syndesmos	1.9 (1)	-	-		?	
1703102	actin	2.0 (1)	-	-		?	
113271	actin	2.1 (1)	-	-		?	
28396657	actin	2.0 (1)	-	-		?	
118463	destrin	2.2 (3)	-	-		?	
20140786	thymosin beta	1.7 (1)	0 (1)	0 (1)		?	
1729834	tubulin alpha chain	1.9 (1)	-	-		?	
125956	lamin B2	1.7 (2)	-	-		?	
1125065	laminin-binding protein	1.7 (1)	-	-		?	
Apoptosis is confirmed directly and indirectly							
5453880	putative HLA class II associated protein 1	-	+2.1 (1)	+2.1 (1)			√
112696	14-3-3 delta/zeta protein	-	1.8 (1)	1.6 (1)			?
11610628	proliferating cell nuclear antigen	*1.4 (1)*	*0 (1)*	*+1.7?*			?
104472	lactate dehydrogenase	1.9 (2)	2.1 (2)	1.8 (1)		?	?
126897	malate dehydrogenase	1.8 (1)	-	-		?	?

Table 5.2, Part III. Changes in proteins levels after treatment with a PACAP-specific receptor blocker.

(h:hours; n:unique fragments; f:fractions; D:differentiation; E:exit; A:apoptosis; +:increase; no symbol:decrease; italics indicate not considered significant)

NCBI#	Name of Protein	5h change (n)	24h/10f change (n)	24 h/30f change (n)	D	E	A
Apoptosis in confirmed (continued)							
112981	aspartate aminotransferase	-	1.8 (1)	1.8 (1)		?	?
113159	aconitate hydratase	1.8 (1)	2.3 (1)	2.2 (1)		?	?
22775582	ATP/ATP-ADP antiporter	1.8 (3)	2.7 (3)	2.4 (3)		?	?
The anomalous ones							
11275220	insulin/EGF single chain fusion protein	-	+4.9 (1)	+5.2 (1)	X		
15826759	chain C human insulin-Hla-Dq8 complex	-	+4.2 (1)	-	X		
1480467	mutant cysteine-rich FGF receptor	+3.6 (1)	-	*2.0?*	?	?	?
Some not discussed							
12229876	polyadenylate-binding protein 2	1.7 (2)	-	-			
123647	heat shock protein 71	1.8 (2)	-	-			
1055224	cellular nucleic acid binding protein	2.4 (2)	-	-			
1199816	CCT gamma protein	1.6 (1)	-	-			
20872723	similar to retinoic acid binding protein II	2.4 (1)	-	-			
1079438	ribonucleoprotein	1.8 (1)	-	-			
1362609	glial cells missing (gcm) protein	2.4 (1)	-	-			
24710922	orphan nuclear receptor Dax 1	2.0 (1)	-	-			
2134412	superoxide dismutase Cu-Zn	-	-	2.0 (2)			
21105454	T-complex protein 1- beta	-	1.9 (2)	(1.8)			

DISCUSSION

Determining appropriate statistical methods by which to evaluate ICAT analysis is somewhat problematic. It could be said that the value of this data is compromised because there was no biological replication, which relies on independence of sample generation (Yang and Speed 2002). However, using two different fragments from one biological sample to confirm parent identity has been accepted as valid by at least one group (Han et al. 2001). Another group included proteins that had been identified by only one fragment in their tables of parent proteins (Shiio et al. 2002). It is reasonable for this study to accept the validity of the data, because it confirms previous flow cytometry results (with the exception of differentiation proteins) and because it generally reveals trends. The data confirm that proteins active in maintaining cell cycling were decreased, and those associated with apoptosis were increased. A degree of differentiation was also revealed.

Because proteins play different roles within a cell, and inter-relationships are complex, what follows is an attempt to describe a changing cell population. This is why some proteins that changed less than 2-fold are included, and why some that changed more than two-fold, but did not contribute to the picture, were not discussed. Some of the discussion is necessarily speculative. Published scientific literature was the primary source for determining functions, and was used wherever there was a claim for evidence of differentiation. However, for some proteins, a description provided by the NCBI database was accepted.

A decrease in a ribosomal protein and a myc-associated protein suggest differentiation

Ribosomal Protein L34

The increasing downregulation of the cytoplasmic ribosomal protein L34 suggested an increasing amount of differentiation over 24 h of receptor blockade. At 5 h, the decrease in L34 was only 1.5 fold (insignificant). At 24 h, the decrease was more than 2.1 fold (Table 5.2, Part I). L34 was shown by several methods to inhibit a neuron-specific protein, p35, which is the only known activator of, and specific for, Cdk5 (Moorthamer and Bhabatosh 1999). This decrease in L34 suggests less inhibition of p35, i.e. more p35 activity, and hence more activation of Cdk5. Cdk5 binds to cyclin D in the G_1 phase of the cell cycle (Fig. 1.5). And although this would at first glance suggest enhancement of cell cycle progression, the catalytic subunit of Cdk5 has been found to be predominantly expressed in post-mitotic cells in the mammalian nervous system (Lew and Wang 1995; Tang and Wang 1996). Therefore it appears that the primary function of activated Cdk5 is to remove cells from the cycle at G_1, by enhancing differentiation. This role is supported by evidence that blocking Cdk5 inhibits neuritic outgrowth and association of Cdk5 to growth cones (Nikolic et al. 1996). As well, Cdk5 knockout mice have an underdeveloped cortex and decreased cerebellar foliation (Ohshima et al. 1996; Tang and Wang 1996). L34 was found to inhibit the activity of Cdk4/cyclin D1, located in the G_1 phase of the cycle, which would stop progression to S phase (Fig. 1.5). (Neither Cdk5, Cdk4, or any cyclin, was detected in these assays.) Ribosomal protein L34 has not been sequenced in chicken, but one of the fragments identified here from chick is identical to that fragment in human, mouse and pig, and is

89% identical to the same fragment in zebrafish and catfish. The protein fragment is well-conserved.

Myc Far Upstream Element-Binding Protein (FBP)

The transcription factor c-myc is well-known for its role in cell cycle control and oncogenesis. C-myc is present in actively dividing cells, and plays a key role in growth. It disappears following terminal differentiation, as discussed by Davis-Smyth et al (1996). Further evidence that the cells were differentiating in response to blockade of PAC_1-R was a decrease in an activator of myc, myc far upstream element-binding protein (FBP). FBP has been found to contribute directly to transcription of c-myc by binding to a single strand site in the 5' flanking region (Duncan et al. 1996). At 5 h, FBP had decreased 1.8 fold, and by 24 h it was down by more than 2.2 fold (Table 5.2, Part I). FBP is an immediate early response gene, and a member of a highly conserved family of transcription factors (Bazar et al. 1995; Davis-Smyth et al. 1996). According to the NCBI database, it has only been sequenced in humans.

Both FBP and c-myc mRNA and protein were down-regulated when HL60 cells were stimulated to differentiate, and no evidence of FBP could be found in quiescent cells (Bazar et al. 1995). Data suggest that FBP destabilizes GAP-43 mRNA, which produces a protein necessary for differentiation and the development of neural connections (Irwin et al. 1997). Thus, a decrease in FBP reduced c-myc and proliferation, and lessened the destabilizing influence on GAP-43, allowing the cells to differentiate, when the PACAP-specific receptor was blocked. Of note here, is that the half-life of the FBP protein is 1.5 h (Bazar et al. 1995).

The only other direct link to myc in these assays was a 1.9 fold decrease at 5 h in what was identified as a myc-like regulatory protein sequenced from the lotus plant. This identification was based on an unpublished submission to NCBI, and the fragment itself was poorly conserved even amongst plants.

A general loss of cellular activity can signal cell cycle exit

Much of the activity of the myc gene involves ribosome biogenesis and protein synthesis, and it has been hypothesized that a major role of c-myc is to promote growth of cells, particularly by increasing cell mass that is requisite to division. A review is provided by Boon et al (1997), and a list of corroborating references can be found in Shiio et al (2001). The embryonic and neuroendocrine form of c-myc is n-myc, and like c-myc, it regulates proliferation. Upregulation of n-myc is correlated with neuroblastoma progression, and tumours with the highest activity of n-myc follow the most aggressive course (Seeger et al. 1985).

Several proteins involved in ribosome formation and protein synthesis, as well as chaperone functions, were found to be upregulated by n-myc in a neuroblastoma cell line, but downregulated in the current study. These included heat shock protein 90 alpha, chaperonin-containing T-complex (CCT), ribosomal proteins L5 and S5, and elongation factor 2 (Boon et al. 2001). However, decreases in some structural and functional cellular components may also be a result of apoptosis. Proteome analysis of Fas-induced apoptosis revealed that many of the proteins that contained motifs related to RNA binding and processing were decreased (Thiede et al. 2001).

Interestingly, ICAT analysis has been performed on myc-expressing cells and myc-null cells from a rat fibroblast cell line (Shiio et al. 2002). Again, in the proliferative, myc-positive cells, there was a general reduction in proteases, induction of protein synthesis pathways, and an increase in processes that led to cell growth. A total of 528 proteins were identified and quantified, although the paper does not report the number of fractions analyzed. Of these, 177 showed more than a 2-fold change. It is notable that this ICAT analysis confirmed results of earlier microarray data, but also detected changes in proteins that were not revealed by microarray. Three of the proteins increased in myc-positive cells were decreased in the present study: ribosomal protein S5, the CCT complex delta subunit, and the RNA helicase p47 (Shiio et al. 2002). One protein, RhoA, was decreased in myc-positive cells (Shiio et al. 2002), and was also decreased in the present assay.

RhoA

Rho genes are a subfamily of the ras oncogene family, and like the ras family function as guanine triphosphatases (GTPases). The Rho family was originally thought to be primarily involved in modification of cell morphology, but it is now known that it plays a role in membrane trafficking, regulation of growth and development, and apoptosis (Lacal 1997; Van Aelst and D'Souza-Schorey 1997; Zohn et al. 1998). In their role as growth regulators, the family induces the activation of a host of transcription factors (Lacal 1997; Zohn et al. 1998). Inhibition of RhoA activity by various means causes cell cycle blocks and decreased DNA synthesis; overexpression leads to transformation (Zohn et al. 1998; Seasholtz et al. 1999b).

At 5 h, the decrease in RhoA protein was at the low end of the scale, at 1.6 fold (Table 5.2, Part I). The protein was identified but unchanged at 24 h. This suggests that the cells exited the cycle quickly after the receptor was blocked, and backs up data from Chapter 3, in which exit was more pronounced at 5 h. Although the amount of change was low, as has been discussed, post-translational modifications are not visualized by ICAT analysis. RhoA deactivation takes place when phosphorylation increases its affinity for guanine nucleotide dissociation inhibitors, which move it away from the cellular membrane and sequester it in the cytosol (Van Aelst and D'Souza-Schorey 1997). It is conceivable that the low amount of downregulation here is reflective of a greater degree of deactivation. Rho family members can be components of G-coupled protein receptor pathways (Lang et al. 1996; Zohn et al. 1998; Seasholtz et al. 1999a), including the G protein-cAMP-PKA pathway blocked in this study. The decrease in RhoA in the myc-positive rat cells, which were highly proliferative, seems contradictory. It could be explained by recalling the long list of RhoA functions, or by considering the very different nature of the cells.

Rho GTPases have also been implicated in the onset of apoptosis, due either to inactivation or overexpression (Aznar and Lacal 2001; Li et al. 2002). It is possible then that the decrease in RhoA was a result of the onset of apoptosis. This matches Chapter 4 results, which showed that apoptosis was higher at 5 h compared to 24 h.

A decrease is observed in proteins that create the working cell

If a decline in ribosome biogenesis and protein synthesis are indications that cells are leaving the cycle, then these ICAT analyses were very successful in proving that was

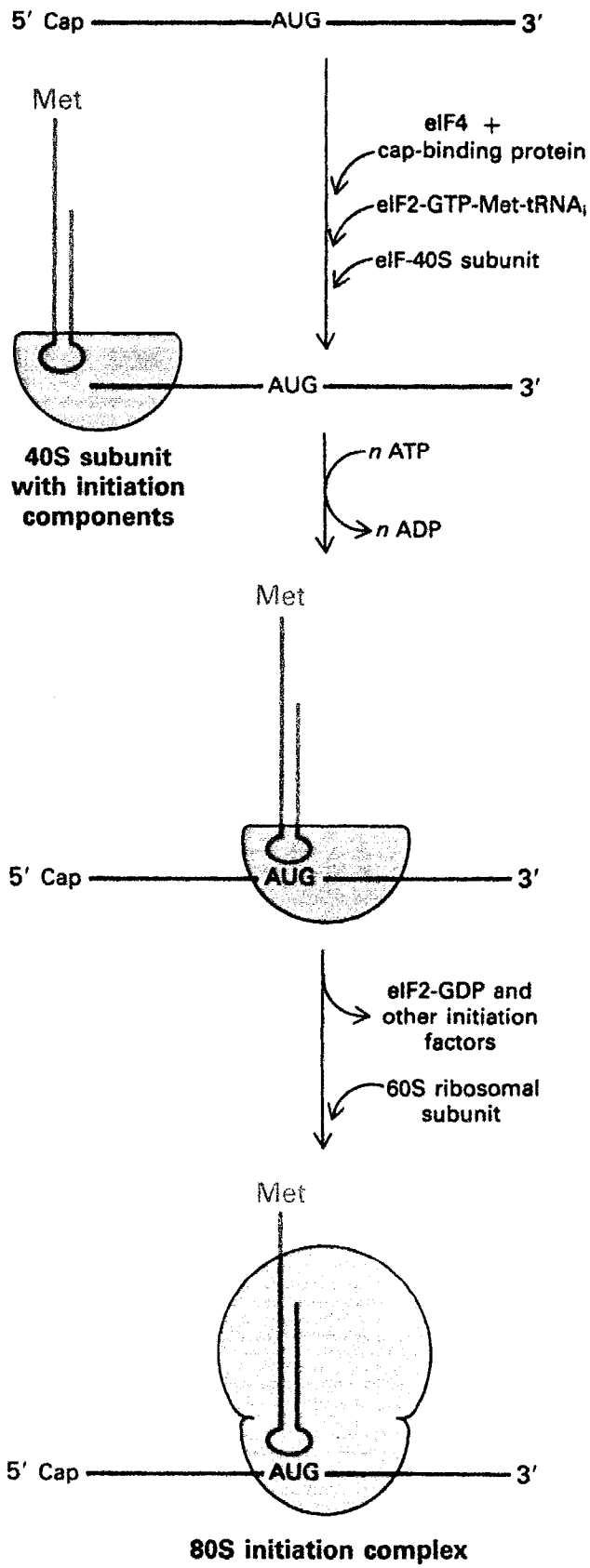
the case, because a high proportion of proteins that were decreased were in these two areas.

Ribosome Activity

As mentioned, ribosomal proteins L5 and S5, as well as elongation factor 2 (EF-2), were upregulated by n-myc in a neuroblastoma line (Boon et al. 2001). In the present ICAT study, along with several other ribosomal subunits, they were decreased. They are summarized in Table 5.2, Part I. Like RhoA, there were generally greater decreases at 5 h, and in at least two cases there was evidence of recovery by 24 h. An exception was S5, which had decreased by 1.4 fold at 5 h (insignificant), but was more than 2-fold below normal at 24 h. L5 had decreased more than 2 fold at 24 h. EF-2 decreased 2.2 fold by 5 h, and 1.9 fold by 24 h. At 5 h, L30 was down 1.7 fold, but had recovered to only a 1.3 fold decrease at 24 h. S20 (also known as S22) was down 2.3 fold at 5 h, and L3 was decreased by almost 2 fold at 24 h. Only one ribosomal unit, S12, had increased, by 1.9 fold at 5 h.

All these proteins come together to form a functional initiation complex so that protein translation can proceed (Fig. 5.2). S20 and S5 are part of the 40S subunit. L5 and L30 are part of the 60S subunit. S5 binds to L5. L5 comprises part of the EF-2 binding site. EF-2 transfers growing polypeptide chains along the ribosome. Ribosomal protein L3 participates in the formation of the peptidyltransferase centre of the functioning complex, and is essential for its catalytic activity. S12 is a key regulatory protein regulating translation fidelity, and its increase suggests that even though production is declining, the system is not simply being degraded. As well, a decrease in

Figure 5.2 Assembly of the ribosomal complex. The 40s ribosomal subunit, accompanied by initiator transfer RNA and eukaryotic initiation factors (eIFs), scans and locates a start site (usually AUG) on a messenger RNA. At this point the initiation factors disperse, and the 60S ribosomal unit is able to bind, forming the 80S initiation complex, and production of proteins from the complete ribosome can begin. Met = methionine, the first amino acid in a polypeptide chain, which will bind to the AUG codon. Figure from (Stryer 1988).



80S initiation complex

L3 could have been an indication of differentiation. L3 was reported to be down-regulated when a human cell line was induced to differentiate (Bevort and Leffers 2000). There is also a possibility that the decline in S20 was related to the onset of apoptosis, as it was in a human leukaemic cell line (Goldstone and Lavin 1993).

Generally, conservation amongst ribosomal proteins is high. The S20 fragment was matched exactly to human and frog, and was still 83% similar to that fragment in corn. One of the S5 fragments, matched to human, was 100% identical to the same fragment in *Caenorhabditis elegans*. An L5 fragment, matched to human, also matched fragments in several other animals and plants. The L3 fragment, matched to cow, was 93% similar to that fragment in human, rat and mouse. The S12 fragment was matched to human, mouse, and pig and was 81% similar to that fragment in yeast. L30 and the EF-2 fragments were matched to chick proteins.

Eukaryotic Translation Initiation Factors

Eukaryotic translation initiation factors (eIFs), like the ribosomal units discussed above, assist in creating a functional ribosome complex for protein translation (Merrick 1992). At 5 h, eIF-2 and eIF-1A were decreased almost 2 fold, and eIF-5 was down by more than 2 fold at 24 h (Table 5.2, Part I). eIF-2 and eIF-1A (also known as eIF-4C), are involved in the first steps of forming the 40S subunit, and the availability of eIF-2 is one of the major regulating steps controlling translation (Merrick 1992). eIF-5 causes release of initiation factors from the 40S subunit, so that the 60S subunit can bind (Fig. 5.2). eIF-5 is the most catalytically active of the translation factors (Merrick 1992). All of these proteins were identified as human, but fragment conservation is high at least for

eIF-2 (95% similar to yeast), and eIF-5 (68% similar to fruit fly). The eIF-1A fragment conservation varies more, but is still 88% similar to mouse and 81% to rabbit. Again, because ribosome biogenesis and protein synthesis have been associated with cycling, the decrease in eIFs suggests a movement out of the cycle.

U5 snRNP-specific protein (220 kD)

Introns are excised from pre-mRNAs by formation of a spliceosome, created by integration of small nuclear ribonucleoproteins (snRNPs). U5 snRNP is the initial protein that begins formation of this complex, providing a substrate for other particles, and the 220 kD protein is a component of it (Anchsel et al. 1998). U5 snRNP is also responsible for aligning the spliceosome so that ligation can occur (Anchsel et al. 1998). This protein was decreased 1.7 fold by 5 h (Table 5.2, Part I), and the fragment sequence was matched to human. Conservation is high; human snRNPs have extensive homology with those from yeast. This decrease could be as a result of decreased cellular activity, that is, cell cycle exit, but because it involves RNA processing, it could relate (as well) to apoptosis.

hnRNPs E2 and M4

The ICAT analysis recognized two ubiquitously-expressed heterogeneous nuclear ribonucleoproteins (hnRNPs), both downregulated more that 2 fold by 5 h (Table 5.2, Part I). hnRNP E2 plays a role in RNA stabilization and binding. The hnRNP M isoform, or M4 protein, is also involved in RNA binding, and pre-mRNA processing, as well as other aspects of mRNA metabolism and transport. Interestingly, it has also been

found to bind to a carcinoembryonic antigen in rat liver macrophages (Bajenova et al. 2001). This suggests a decrease in M4 may also relate to exit. Both fragments were linked to a human protein, and a search of the NCBI database suggested that conservation is not extremely high, so this data must be included tentatively. Again, since RNA binding and processing is involved, this decrease could be related to increased apoptosis.

Nuclear Protein Matrin 3

ICAT analysis identified a chick inner nuclear structural protein, matrin 3, which had been decreased 1.9 fold at 5 h (Table 5.2, Part I). This protein participates in anchoring improperly-edited RNAs to the nuclear matrix, preventing export to the cytoplasm (Zhang and Carmichael 2001). This likely reflects lowered RNA activity, which could lead to exit or apoptosis.

Putative ATP-dependent RNA Helicase p47/Bat1, and An3

The proteins p47 and Bat 1 are both members of a DEAD box family of helicases, that unwind double-stranded RNA so that it can be altered and modified. The 5 h ICAT recognized a DEAD box helicase, most closely related to p47, that decreased 1.6 fold (Table 5.2). The fragment matched human, rat, mouse and pig fragments exactly. The 24 h ICAT recognized a fragment identified as rat Bat 1, that decreased almost 2 fold (Table 5.2, Part I). Neither p47 nor Bat 1 have been sequenced in chicken. However, human Bat 1 shares 99.4% protein sequence identity to rat p47 (Allcock et al. 1999). It could be then that these fragments represent the same protein in chick, either

p47, or Bat 1, or another closely related DEAD box member. Rat p47 was another protein upregulated by n-myc (Boon et al. 2001), so this does suggest one more piece of evidence that the cells were leaving the cell cycle.

The protein identified as putative ATP-dependent RNA helicase An3 was decreased 2.5 fold at 5 h (Table 5.2, Part I). An3 is also a member of the DEAD box family. An3 belongs to the DDX subfamily, and shuttles RNA between the nucleus and the cytoplasm (Askjaer et al. 1999). This helicase was originally identified in frog, and is active throughout the embryo during development (Gururajan et al. 1991). However, an homologous protein in mouse, ERH, becomes more restricted to brain and kidney after about E9 (Sowden et al. 1995). Conservation of the fragment appears to be fairly high, identical in human and mouse, and 67% conserved in yeast. Because these proteins are involved in RNA processing, decreases may reflect the increase in apoptosis at 5 h.

Cyclophilin A

Four forms of cyclophilin A, matched to four different species, were identified in the 5 h ICAT analysis. Because none have been sequenced in chick, but the family is highly conserved, it is possible they represent the same protein, or at least highly-related proteins, and I will consider them as a group. All were decreased as much as 2.2 fold, and one as much as 2.7 fold (Table 5.2, Part I).

Cyclophilins are expressed in many tissues, and function as enzymes that convert proteins into another isomeric form. They are also known as peptidyl prolyl cis/trans isomerases, or PPIases. However, there is controversy regarding their true biological

function. For a discussion, see Yurchenko et al (2002). It may be that they are simple enzymes involved in accelerating RNA splicing and folding, or they may act more like molecular chaperones, with a capacity to transport and manipulate folded proteins. It could be that their functional roles are as important as cell cycle regulation and inhibition of growth factors like epidermal growth factor. The decrease in these cell cultures is notable, and it could be important, but there is no way to elucidate a function.

A decrease is observed in proteins that keep the cell working

DNA Topoisomerase I

DNA topoisomerase I unravels supercoiled DNA prior to replication. Because it is decreased by almost 4 fold (Table 5.2, Part II) when treated with a PACAP receptor blocker strongly suggests that DNA replication is decreasing, that is, that the cells are leaving the cycle. The fragment matched 100% to those in human, mouse and frog.

Histone H3

Inactive DNA is packed in chromatin, and complexed to histones to form nucleosomes. The nucleosome consists of an octamer of four pairs each of the replication-dependent histones 2a, 2b, 3 and 4. Histone H3 mRNA levels fluctuate by as much as 50 times during the cell cycle, due to a combination of changes in the rate of gene transcription, and mRNA biosynthesis and degradation (Marzluff and Pandey 1988; Osley 1991). Gene transcription increases 3 to 5 fold as the cells progress from G_0 and G_1 into S phase, and the half-life of the mRNA drops from about 45 minutes to 10 minutes by the end of S phase (Marzluff and Pandey 1988). Histone H3 mRNAs are

commonly used as markers of proliferation, especially by cancer researchers, and could rival classical markers such as Ki67 and PCNA (Stenger et al. 1992; Carr et al. 1995; Nagao et al. 1996; Arakura et al. 2001; Gomez et al. 2001; Alpini et al. 2002; Bettuzzi et al. 2002; Delva et al. 2002; Schumm et al. 2002). If histone H3 protein levels follow mRNA levels, then the approximate 2 fold decrease at 24 h (Table 5.2, Part II) would suggest decreased synthesis, and decreased cycling. Because this is not a synchronized cell population, it would be unreasonable to expect to see changes of 50 fold, although a 2 fold decrease does seem somewhat small considering the activity of histone H3 mRNA. The histone H3 fragments were well conserved; one fragment that matched to human was 100% identical to that in corn.

CCT protein 1, delta and beta subunits

The chaperonin-containing T-complex (CCT) protein 1 was also upregulated by n-myc in the neuroblastoma cell line (Boon et al. 2001), and downregulated in the present assays (Table 5.2, Part II). CCT is a cytosolic molecular chaperone that folds newly synthesized and denatured proteins, such as actins and tubulins, during cellular proliferation. The complex consists of sixteen subunits, but not all these subunits play an equal role in regulation of the complex (Shin-ichi et al. 2001). The levels of alpha and delta subunits in an asynchronous population were lower than those in a population arrested in S phase, but higher than those arrested in M phase. This suggests that the activity of both the subunits and CCT is highest during S phase and lowest during M phase (Shin-ichi et al. 2001). As well, the delta subunit was shown to be degraded more rapidly than any other subunit, and probably represents the rate-limiting step for

chaperone activity (Shin-ichi et al. 2001). This type of control of a large subunit constitutes an efficient and energy-saving form of regulation. Maturation of cyclin E, which is involved in the G_1 to S transition (Fig. 1.5) is also mediated by the CCT complex (Won et al. 1998).

The delta subunit of CCT, matched to a chicken protein, was decreased only 1.4 fold (insignificant) in the 5 h ICAT, but by 24 h it had dropped 2.3 fold (Table 5.2, Part II). Because degradation occurs at the end of S phase (Shin-ichi et al. 2001), and because less than 25% percent of the cells in these cultures were ever recorded in S-phase, it is not surprising that it took time for enough of the cells to traverse the cycle through S-phase and downregulate production of delta subunit and the CCT complex.

HSP 90

Heat shock protein (HSP) 90 is a member of a group of highly conserved proteins, whose general role is to cushion the cell from any type of shock, such as heat, ischemia or energy depletion. It also was upregulated by n-myc in the neuroblastoma cell line (Boon et al. 2001), and downregulated in the present assays (Table 5.2, Part II). HSPs are believed to perform diverse functions such as protein transport, folding, and degradation, as well as dissolution of protein complexes, prevention of protein aggregation, and control of regulatory proteins (Garrido et al. 2001). They do their work in the cytosol, the mitochondria, and the endoplasmic reticulum (Garrido et al. 2001). HSP 90 alpha was down a steady amount of 2.4 fold at both 5 h and 24 h.

HSP 90 showed a decrease during early post-natal development of the rat cerebellum, a time when extensive neuronal differentiation is occurring (D'Souza and

Brown 1998). This decrease in HSP 90 is an indicator that PACAP receptor blockade causes cessation of cycling, and may also promote some degree of differentiation.

However, HSPs have also been implicated in apoptosis. In line with their chaperone activities in the cell, they have a strong cytoprotective role. It is postulated that HSPs are involved in clean-up and repair (Bruce et al. 1993). It is not surprising then that they also act as pro-apoptotic or anti-apoptotic factors. So far, HSP 27, HSP 70 and HSP 90 have been shown to be anti-apoptotic, and HSP 60 pro-apoptotic (Bruce et al. 1993). However, the amount of decline in HSP 90 did not change over the 24 h. In Chapter 4, using a sensitive assay designed to measure apoptosis, the picture that emerged was one in which apoptosis built up rapidly, then dropped by 24 h (Fig. 4.7). Thus, it is more likely that the decline in HSP is due to decreased proliferation, than to increased apoptosis.

Protein Phosphatases 1 and 2A

The gamma subunit of protein phosphatase I (PP1) was decreased 2.3 fold in the treated sample at 5 h (Table 5.2. Part II), and the fragment was found to be conserved from human to yeast. This form of the enzyme has been found to be the dominant form in rat brain (Shima et al. 1993). The gamma subunit is also the catalytic subunit, and can bind with more than 50 regulatory subunits, to form a variety of different complexes, which will interact with a wealth of substrates (Cohen 2002). In its many forms, PP1 is thought to be essential for cell division, participate in the regulation of glycogen metabolism, and regulate protein synthesis. A particular function cannot be assigned here for chick neuroblasts, but this is another example of decreased cellular activity.

Protein phosphatase 2A was also decreased, slightly less at 1.9 fold at 5 h (Table 5.2, Part II). The PP2A fragment was matched to fruit fly, which is 84% similar to its human counterpart. Both these proteins are regulated by putative HLA class II associated protein, which is discussed below, and the significance of the decreases in these enzymes will be addressed in more detail later.

26S Proteasome Regulatory Complex Subunit p42D, and Valosin-Containing Protein

Degradation of most proteins occurs at the proteasome, a large molecular complex of 30 subunits. The proteasome is shaped like a barrel with proteolytic processing occurring in the centre, controlled by regulatory subunits, such as p42D, at one or both ends. For more details, see Holzl et al (2000). The p42D regulatory subunit was decreased 1.6 fold at 5 h (Table 5.2, Part II), and the fragment was matched to fruit fly. The fragment is well-conserved. Valosin-containing protein is important in vesicular trafficking, and one of its functions is to use ATP to bind ubiquinated substrates and chaperone them to the proteasome for degradation (Song et al. 2003). It was decreased a similar amount at 5 h (Table 5.2, Part II). The valosin-containing fragment is also well-conserved. It seems likely this decrease in protein degradation is reflective of the decrease in protein synthesis. If so, then the decreases in the proteasome regulatory subunit and valosin would reflect exit or apoptosis.

HMGB 1 Protein/Amphoterin

The number of functions associated with high mobility group box I (HMGB1), also known as amphoterin, makes it possible to assign only a tentative explanation to the

decrease of 1.7 fold chicken HMGB1 at 5 h, with a return to normal by 24 h (Table 5.2, Part II). HMGB1 has been associated with both intracellular and extracellular functions, and with increases in proliferation, differentiation, neurite outgrowth, and migration (Cabart et al. 1991; Passalacqua et al. 1997; Yamamura et al. 1999; Fages et al. 2000; Chou et al. 2001; Muller et al. 2001; Huttunen et al. 2002; Kuniyasu et al. 2003). It is unlikely that the function here involves a secretory role, because cells that respond to extracellular HMGB1 appear to contain little of it themselves (Muller et al. 2001). The cell cultures here contain virtually all neuroblasts; there are no other cells to respond to a secretory signal.

Therefore, the effect is probably intracellular, and two possibilities are likely. HMGB1 assists site-specific DNA binding proteins to the proper chromatin locations (Muller et al. 2001). If this is the function that is being inhibited, then the decrease here simply reflects a general decrease in cellular activity as the cells leave the cycle. However, a more exciting possibility exists. This protein has been shown to function as a regulator of transcription, by enhancing the binding affinity of transcription factors (Muller et al. 2001). Transformed cells, and a variety of cell types including neuroblasts, have shown increased expression of HMGB1 mRNA and protein during the proliferative, undifferentiated stage (Seyedin et al. 1981; Johnson et al. 1990; Cabart et al. 1991; Yamamura et al. 1999; Kuniyasu et al. 2003). In one study, no HMGB1 protein could be measured in quiescent cells compared to their proliferating counterparts (Cabart et al. 1991). In another study, simple blocking of mitosis in neuroblastoma cells did not affect HMGB1 levels, but cessation of cell cycling with a commitment to differentiate did decrease the levels (Seyedin et al. 1981). The evidence suggests that

HMGB1 enhances transcription of genes that cause cycling, and the downregulation here is indicative not only of downregulation of proliferation, but differentiation as well. If so, again there is a suggestion that exit occurred largely at 5 h, then declined.

However, there are some reports of *increases* in differentiation in response to HMGB1 (Passalacqua et al. 1997; Huttunen et al. 2002). In one study, the neuroblastoma cells that differentiated were clearly being "fed" by neighbouring astrocytes (Passalacqua et al. 1997). In the other study, both neuroblastoma cells and embryonic stem cells were induced to differentiate, as measured by an increase in differentiation markers, when treated with amphoterin (Huttunen et al. 2002). The differentiation proceeded through the cAMP response element-binding protein, but it was shown that cAMP was not involved. This suggests that different pathways can lead to different effects. As well, decreases in amphoterin have been shown to inhibit cellular migration (Fages et al. 2000). Given the age of these cultures, and their highly proliferative state, inhibition of migration is probably not occurring.

Although it is a multi-faceted protein, the best explanation does seem to be that the decrease in HMGB1 was due to cessation of cycling, and possibly an increase in differentiation.

Cytoskeletal Components

A number of cytoskeletal proteins were decreased in the treated cell population at 5 h; details are summarized in Table 5.2, Part II. Syndesmos is a cytoplasmic protein involved in cellular adhesion and related to actin cytoskeletal organization (Baciu et al. 2000). It was decreased 1.9 fold. Several forms of actin were decreased, and

investigation of the specific fragments showed all to be forms of cytoplasmic, non-muscle, beta actin. Decreases were at least 2 fold for each. Destrin, an actin-depolymerizing factor, was decreased by 2.2 fold at 5 h, and 1.5 fold at 24 h (insignificant). Thymosin, an actin sequestering protein, was down 1.7 fold at 5 h and registered no change at 24 h (data not shown). As well, a tubulin alpha chain was decreased 1.9 fold at 5 h. Lamin B2, a protein that constitutes part of the nuclear envelope, was down 1.7 fold, as was a laminin-binding protein.

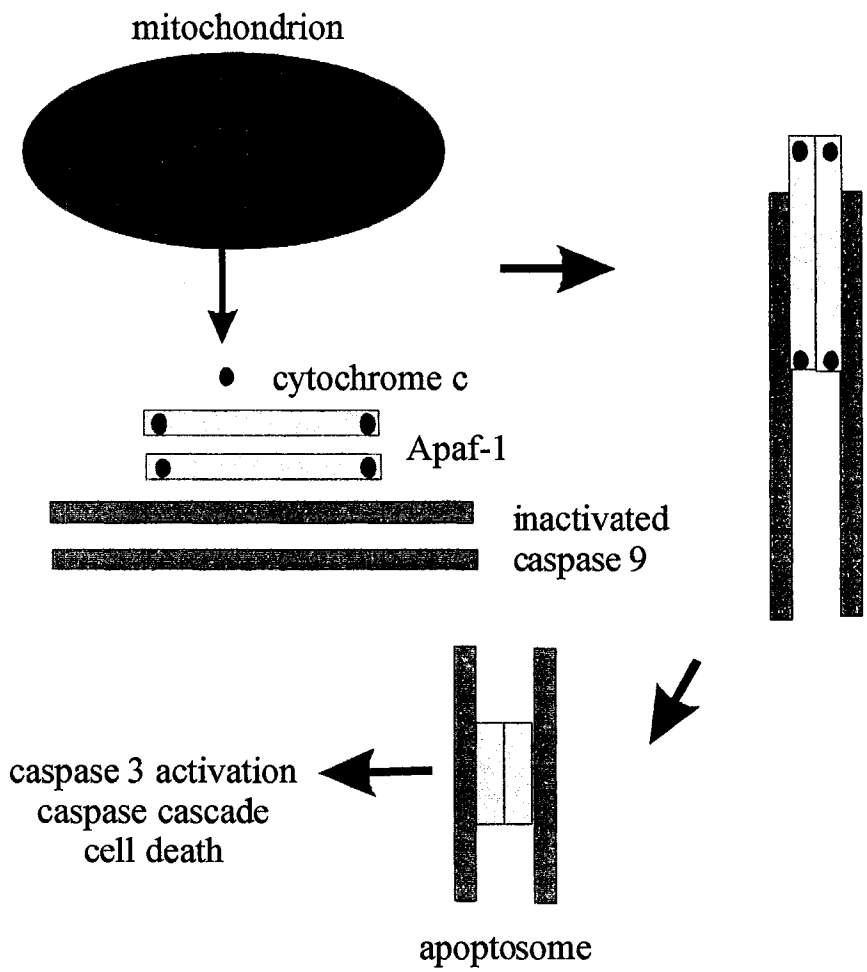
The data here suggest a trend toward recovering cytoskeletal function by 24 h. This follows the trend described for RhoA, several of the ribosomal subunits, and HMGB1. It suggests that this transient decrease in cytoskeletal elements may also relate to exit at 5 h, which declined by 24 h. Again, this supports data from Chapter 3 that 5 h was when cell cycle exit was highest. Combined with data suggesting that the differentiation markers ribosomal protein L34 and FBP were increasing over the 24 h, this paints a picture of exit that led to differentiation.

Apoptosis is confirmed directly and indirectly

Putative HLA class II Associated Protein I (PHAP 1)

An important early event in apoptosis which involves the mitochondrion is the release of cytochrome c, normally held in place by Bcl-2 proteins (George 2002). This disrupts the electron transport chain and energy production, but also allows cytochrome c to bind to apoptotic protease activating factor (Apaf-1), which is the beginning of the formation of the apoptosome, the complex that directs cell death (Fig. 5.3). One of the

Figure 5.3 Apoptosome activation. Cell death begins with release of cytochrome c from the mitochondrion, allowing it to activate apoptotic protease activating factor (Apaf-1), which binds with procaspase 9 to form a heterodimer. Cleavage of procaspase 9 activates the apoptosome, and caspase activation and cell death follow.


mitochondrion
cytochrome c
Apaf-1
inactivated
caspase 9
caspase 3 activation
caspase cascade
cell death
apoptosome

proteins that associate with the apoptosome is PHAP 1. This protein, also known as PP32, and LANP, is a well-conserved tumour suppressor; it has the ability to inhibit tumour formation when confronted with a variety of oncogenes (Chen et al. 1996). PHAP I has recently been shown to exert its tumour-fighting effects by accelerating activation of caspase 9, which increases the activity of apoptosomes (Jiang et al. 2003). PHAP I was one of only three proteins that increased in the 24 h ICAT analysis, and probably the only one convincingly (Table 5.2, Part II). The fragment sequenced, identified as human, is 92% identical to rat and mouse. PHAP I was not identified by the mass spectrometer at 5 h, so it is not possible to compare levels at 5 and 24 h to the patterns found in the other protocols.

However, PHAP I is a potent inhibitor of protein phosphatase 2A (Li et al. 1996). As noted, this enzyme decreased 1.9 fold at 5 h (Table 5.2, Part II). The decrease is in the typical range for 5 h, and suggests that apoptosis was a significant factor at that point. This confirms data reported in Chapter 4, which showed that PACAP receptor blockade results in a rapid rise in apoptosis. PHAP I also regulates protein phosphatase 1, but has an opposite effect, and acts instead to stimulate it (Cohen 2002). Therefore the decrease recorded for PP1 was not due to apoptosis, but likely to a general decrease in cellular activity.

14-3-3- delta/zeta

The 14-3-3 family of proteins is a highly conserved and ubiquitously expressed multi-gene family known for association with signalling molecules, cell cycle proteins, and cell death regulators (Yaffe 2002). In mammals, these proteins are most abundant in

the nervous system (Skoulakis and David 1998). Mutations in fruit fly14-3-3 genes disrupt normal neuronal development and function of the nervous system in a variety of ways, generally during the time of differentiation (Skoulakis and David 1998).

Generally, 14-3-3 proteins bind a variety of ligands to change their level of activity or location. It has been speculated that 14-3-3 proteins only bind to ligands after phosphorylation, and hence represent phosphodependent chaperones (Yaffe 2002). This could allow conformational changes in the ligands that would allow modification or interaction with other molecules. More than 100 proteins have been found to interact with 14-3-3 proteins, including various protein kinases, receptors, enzymes, structural components, cytoskeletal elements, small G proteins and their regulators, scaffolding molecules, proteins involved in transcriptional control of gene expression, and proteins involved in cell cycle control and apoptosis (Yaffe 2002).

A decrease of about 1.7 fold in 14-3-3 delta/ zeta was recorded, in the 24 h assay (Table 5.2, Part II). Protein zeta differs from protein delta only in that it is not phosphorylated. Distinct roles for the various isotypes have yet to be elucidated. There is evidence that, during interphase and when DNA damage occurs, 14-3-3 proteins sequester and inactivate cell cycle components that initiate mitosis, so that division cannot occur (Peng et al. 1997). This can involve moving an attached complex out of the nucleus and into the cytoplasm (Lopez-Girona et al. 1999). This type of change would be impossible to detect using ICAT analysis. In adult rats poisoned with kainic acid, 14-3-3 zeta was localized, with p53, in apoptotic cells (van der Brug et al. 2002). The authors concluded that zeta may act as a mediator in the apoptotic process. But if this

were to be occurring in these cells, one would expect an increase, not a decrease in protein.

However, it could still be that a 14-3-3 protein is involved in recognition of cellular damage and apoptosis in these cells. A model has been postulated that in a normal cell phosphorylated pro-apoptotic BAD is sequestered in the cytosol by a 14-3-3 protein (Zha et al. 1996). When damage occurs, BAD is dephosphorylated and 14-3-3 is released. BAD is free to bind to a anti-apoptotic cousin such as Bcl-X_L, disabling it. If enough BAD is dephosphorylated, the balance shifts, and the cell undergoes apoptosis (Fig. 5.4). Evidence was presented that in the presence of a survival factor BAD was phosphorylated and subsequently sequestered by a 14-3-3 protein (Zha et al. 1996). Furthermore, if BAD phosphorylation sites were blocked by amino acid substitutions, apoptosis increased (Zha et al. 1996). And whereas it was the tau form of 14-3-3 used in these experiments, the authors showed that BAD will bind to multiple forms of 14-3-3.

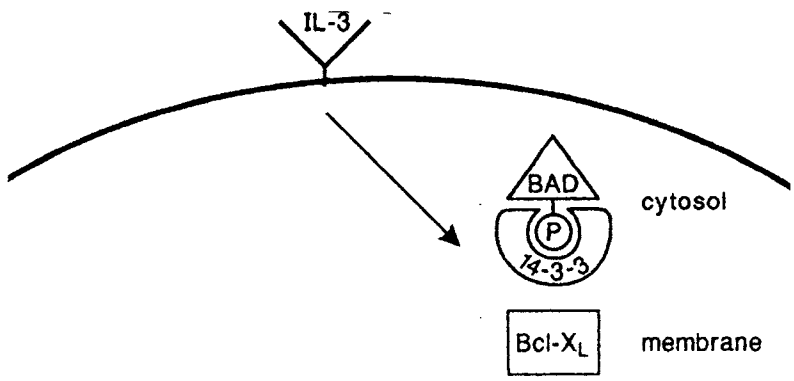
The cells in these ICAT experiments were deprived of an important survival factor. This could cause dephosphorylation of BAD, and an increase in apoptosis. A decrease in 14-3-3 would not be unreasonable, possibly as an impetus to allow dephosphorylation of BAD. But it is probably impossible using this technique to define a role for 14-3-3 proteins when the PACAP receptor is blocked.

The Special Case of PCNA

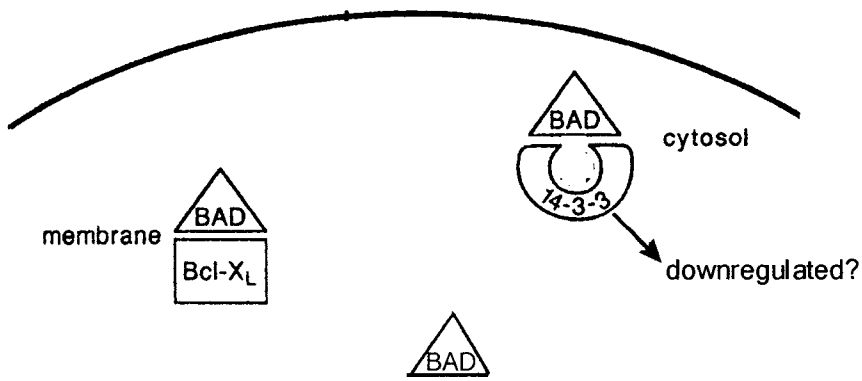
The changes in proliferating cell nuclear antigen (PCNA) were below the level of any criteria, and they were confusing. However, largely because I had used PCNA as a measure of proliferation in Chapter 3, I decided to include it in this discussion. To

Figure 5.4 Representation of one possible way that apoptosis could be inhibited by a 14-3-3 protein. Binding of a growth factor such as interleukin 3 (IL-3) causes the phosphorylation of pro-apoptotic BAD, which attracts binding by a 14-3-3 protein, which sequesters BAD in the cytosol. When damage occurs, BAD is dephosphorylated and released, and binds to an anti-apoptotic Bcl family member. If the balance shifts towards more free pro-apoptotic members than anti-apoptotic members, then the death process begins. Figure adapted from (Zha et al. 1996).

Survival



Apoptosis



reiterate, PCNA is a highly conserved molecule, essential for DNA synthesis. It is present throughout the cell cycle, but immunonegative in quiescent cells (McCormick and Hall 1992; Belyavskyi et al. 1995; Jónsson and Hübscher 1997; Garrido et al. 2000). Everything about the cells tested in this assay suggest a decrease in cycling, and levels of PCNA did fall 1.4 fold at 5 h (insignificant). However, the 24 h ICAT analysis showed no change in the 10 fraction assay, and a mean 1.7 fold increase in the 30 fraction assay (Table 5.2, Part II). The range for this increase was large: a poor BC value suggested a 1.2 fold increase, and a good BC value suggested a 2.3 fold increase. Where does the this messy data come from? The answer may lie in the nature of early responses of cells to the apoptotic signal. Several lines of evidence suggest that cells about to undergo apoptosis actually start to cycle, then abort the attempt. For a review, see O'Connor et al (2000). This seems to occur only with quiescent or post-mitotic cells, and may result from the receipt of conflicting signals. For instance, cells driven to cycle by c-myc when growth factors are limiting, will undergo apoptosis (Evan and Littlewood 1998). Among the cell cycle components which may increase are Cdks and cyclins and their complexes (Frade 2000; O'Connor et al. 2000). One study of thymocytes showed there was actually a requirement for increase of Cdks during apoptosis (Hakem et al. 1999). Specifically, PCNA has been upregulated during the apoptotic death of density arrested Swiss 3T3 cells (Pandey and Wang 1995). Another group studying the dopamine-triggered apoptosis of post-mitotic cells, discovered not only upregulation of PCNA, but also CCT complex delta 1 protein (Shirvan et al. 1997a; Shirvan et al. 1997b; Zilkha-Falb et al. 2000). Oscillations in increased PCNA were observed, one before the commitment to die, and one afterwards.

If there was an increase in PCNA here as a result of an aborted attempt to re-enter the cycle, then it would apply only to the small population of quiescent cells present in these cultures. This would be interesting, because it would mean that chick neuroblasts that have left the cycle still require PACAP for survival. But the picture of the changes in PCNA is too unclear, and is complicated by at least one other factor. PCNA is involved in cell repair (McCormick and Hall 1992). Since the cells were obviously dying in response to treatment with PAC_1-R blocker, PCNA may have been increased in an attempt to repair damage. Even a tentative conclusion is impossible.

Mitochondrial Proteins

Several proteins involved in the production of energy were decreased in these assays. Glycolysis takes place in the cytosol, and is the first step in the metabolism of glucose, to start the production of energy from the electron transport chain in the mitochondria. Lactate dehydrogenase converts lactate to pyruvate in the final step of this process. It was identified in all three assays as reduced about 2-fold (Table 5.2, Part II). The pyruvate is fed into the citric acid cycle in the mitochondria. Malate dehydrogenase interconverts malate to oxaloacetate in the citric acid cycle. It was reduced 1.8 fold at 5 h (Table 5.2, Part II). Aspartate aminotransferase plays a similar role, by interconverting oxaloacetate and aspartate. It was downregulated in the 24 h assay, by about 1.8 fold (Table 5.2, Part III). Aconitate hydratase or aconitase converts citrate into cis-aconitate and iso-citrate during the citric acid cycle (Darnell et al. 1990). It was decreased 2 fold by 5 h, and just over that at 24 h (Table 5.2, Part III). Inactivation of aconitase, measured by its reducing activity, accompanied an increase in

apoptosis when mouse cerebellar granule cells were induced to undergo apoptosis by deprivation of membrane depolarization (Tabuchi et al. 2003). Notably, mRNA levels did not change. Because oxidative stress was increased in the Tabuchi study, it was suggested that aconitase is a key mitochondrial enzyme influencing the viability of neurons under oxidative stress (Tabuchi et al. 2003). One of the hormones that was able to rescue the cells was PACAP38. Because the type and age of the cells, and the conditions inducing apoptosis, were different between that study and the present one, it is not possible to assess the degree of importance of the decrease in aconitase here.

Decreases in these mitochondrial enzymes lower the level of transfer of electrons from the cytosol to the electron transport chain in the mitochondria, and a resulting loss of ATP is to be expected. ATP was decreased about 1.8 fold at 5 h, and more than 2.5 fold at 24 h (Table 5.2, Part III). The decreased activity of this system probably relates to the lesser amounts of protein synthesis and biogenesis, although it could also be as a result of secondary necrosis.

The anomalous ones

Insulin

Appendix 1 lists the components of the B27 supplement and the medium in which these neuroblasts were cultured. Retention of insulin from the medium in the treated sample was revealed by identification of what appeared to be two distinct proteins, each with several fragments identified in the 24 h assays. Examination of these proteins revealed that one was a fusion protein containing insulin, and the other was an insulin complex. All the fragments matched human insulin; none matched chicken

insulin. This insulin increased 4 to 5 fold in treated cells, which was the largest reliable increase recorded. The explanation could involve a change in the property of the PACAP-deprived cells that allowed retention of insulin, perhaps by upregulation of insulin receptors or a binding protein. No satisfactory conclusion can de drawn at this time.

Mutant Cysteine-Rich Fibroblast Growth Factor (FGF) Receptor

The mass spectrometer identified two fragments in the 5 h assay, and four fragments in the 30 fraction assay as a mutant chicken FGF receptor (Table 5.2, Part III). The identification is not surprising, since it is a "cysteine-rich" protein. However, whether the protein increased or decreased was impossible to determine. One fragment from the 5 h assay was not quantified, the other suggested a 3.6 fold increase. For the 24 h assay, the machine also did not record a treated/untreated ratio for one fragment. Two other fragments with unacceptable BC levels suggested approximate 2-fold and 3-fold increases. The only fragment with an acceptable BC at 24 h suggested a 2-fold decrease. This receptor is concentrated in the Golgi apparatus, and regulates acidic and basic FGF by reducing intracellular accumulation of these growth factors in Chinese hamster ovary cells (Zuber et al. 1997). Therefore, it may be involved in intracellular FGF trafficking. Since this is the only growth factor or receptor found to be altered in response to blocking PACAP at the receptor, it would be interesting to investigate it further.

Summary

Flow Cytometry Data are Confirmed, as is Differentiation Hypothesis

ICAT analysis provided an overview of changing proteins in cells treated with a PACAP-specific receptor blocker, and these changes confirmed data generated by flow cytometry. Cessation of cell cycling or exit was suggested by decreases in a variety of proteins regulating ribogenesis and protein synthesis, as well as cellular housekeeping proteins and at least one protein directly involved in cell cycling (histone H3). Apoptosis was confirmed by increases in putative HLA class II associated protein I, with support from several other proteins. There was also enough evidence to accept the hypothesis that some cells were differentiating. Decreases in ribosomal protein L34 and myc far upstream binding protein (FBP) were convincing, and decreases in several other proteins lent tentative support. Using ICAT analysis to study the effects of blocking the PACAP-specific receptor allowed a picture to emerge: some cells rapidly (within 5 h) exited the cycle as others began to undergo apoptosis, then both processes slowed as the level of differentiation (around 24 h) began to increase. This confirms a general conclusion: PACAP is produced in early chick brain to keep cells cycling, and when deprived of this hormone some cells will either undergo apoptosis or differentiation.

As well, new data were generated suggesting some of the pathways that might be involved when the PACAP-specific receptor is blocked. There is good reason to believe that blocking the PACAP receptor leads to apoptotic death by increasing the level of PHAP I, which stimulates apoptosome activity through recruitment of caspase 9. A decrease in PHAP may lead to a decrease in PP2A, although the results of this can only

be speculative. There is a possibility that the apoptotic pathway involves BAD and 14-3-3 proteins.

The anomalous effects of insulin and the presence of a mutant FGF receptor deserve more investigation.

Potential Protein Markers for Changing Cell Cycle and Apoptosis are Identified

In addition to gaining a lot of information on the proteins that change and a small amount of data regarding possible pathways involved when the PACAP receptor is blocked, these ICAT analyses generated information on some proteins that might be good markers for proliferation, cell cycle exit, differentiation, and apoptosis. This could be valuable for continued research on the effects of PACAP, or perhaps the effects of other hormones, on chick brain development.

It is obvious that both FBP and ribosomal protein L34 are good candidates for differentiation markers. So too could be p35, the neuron-specific protein inhibited by L34. Of the three, FBP is the most attractive because of the suggestion that quiescent cells contain so little FBP that researchers could not measure any amount. As well, it has a short half-life (1.5 h), which would allow time-course studies. If cell cultures were used, there would be the added advantage that cells would not have to be in culture long.

Although researchers are already using histone H3 mRNA as a proliferation marker, it would be worthwhile to determine if histone H3 protein levels change as significantly as do mRNA levels. If so, it could be a very clear proliferation marker.

This exercise could be quite useful, as the long-life of PCNA probably resulted in underestimation of proliferation-related changes.

DNA topoisomerase seems an ideal candidate as an exit marker, although it might prove difficult to isolate. The delta subunit of the CCT complex is also a potential marker. It is a conserved molecule that changes rapidly with the cell cycle, and baseline amounts for untreated cells in primary culture could be determined by arresting the cells at various stages.

As an apoptosis marker, PHAP 1 is a strong candidate. Because it responds to a number of oncogenes, it probably acts through several pathways. It could be used as a marker for apoptosis, and for any research involving tumorigenesis.

ICAT is a Valuable Tool

Based on these results, I would conclude that ICAT analysis lends itself well to cell culture-based experiments, especially when the cells consist of only one type. An abundance of protein was easily prepared, and little contamination was present. Certainly, more biological replication, and a fuller analysis of how proteins change over time once the receptor has been blocked could have added clarification and would have added validation. Costs can be reduced by limiting analysis to 10 fractions, and my observations here suggest that 10 fractions are sufficient. When used in conjunction with other techniques, ICAT appears to be a powerful tool easily accessible to scientists without extensive knowledge of proteomics. New advances which promise to more than double the sensitivity of the ICAT method lend even greater sway to the argument (Zhou et al. 2002). Proteomics coupled with the expected publication of the chicken genome in

March, 2004 (Burt and Pourquie 2003) will add considerably to our understanding of brain development using the chicken model.

REFERENCES

Allcock, RJN, PJ Price, S Gaudieri, C Leelayuwat, CS Witt and RL Dawkins (1999). "Characterization of the human central MHC gene, Bat 1: genomic structure and function." Experimental and Clinical Immunogenetics **16**: 98-106.

Alpini, G, L Baiocchi, S Glaser, Y Ueno, M Marzioni, H Francis, JL Phinizy, M Angelico and G LeSage (2002). "Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha." Hepatology **35**: 1041-1052.

Ambion (2003). RNAqueous™: Small scale phenol-free total RNA isolation kit. Austin, TX, Ambion. **Catalog #1912:** 24.

Anchsel, T, Ahrens, K Kaji, H Brahms, S Teigelkamp and R Luhrmann (1998). "The human U5-220kD protein (Prp8) forms a stable RNA-free complex with several U5-specific proteins, including an unwindase, a homologue of ribosomal elongation factor EF-2, and a novel WD-40 protein." Molecular and Cellular Biology **18**: 6756-6766.

Applied Biosystems (2003). Cleavable ICAT™ Reagent Kit for Protein Labeling (manual). Foster City, CA**:** 2.

Arakura, N, M Hayama, T Hondo, K Matsuzawa, T Akamatsu and H Ota (2001). "Histone H3 mRNA *in situ* hybridization for identifying proliferating cells in human pancreas, with special reference to the ductal system." Histochemical Journal **33**: 183-191.

Askjaer, P, A Bachi, M Wilm, FR Bischoff, DL Weeks, V Ogniewski, M Ohno, C Niehrs, J Kjems and IW Mattaj (1999). "RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase." Molecular and Cellular Biology **19**: 6276-6285.

Aznar, S and JC Lacal (2001). "Rho signals to cell growth and apoptosis." Cancer Letters **165**: 1-10.

Baciu, PC, S Saoncella, SH Lee, F Denhez, D Leuthardt and PF Goetinck (2000). "Syndesmos, a protein that interacts with the cytoplasmic domain of syndecan-4, mediates cell spreading and actin cytoskeletal arrangement." Journal of Cell Science **113**: 315-324.

Bajenova, OV, R Zimmer, E Stolper, J Salisbury-Rowswell, A Nanji and P Thomas (2001). "Heterogeneous RNA-binding protein M4 is a receptor for carcinoembryonic antigen in Kupffer cells." Journal of Biological Chemistry **276**: 1067-1073.

Bazar, L, V Harris, I Sunitha, D Hartmann and M Avigan (1995). "A transactivator of c-myc is coordinately regulated with the proto-oncogene during cellular growth." Oncogene **10**(2229-2238).

Belyavskyi, M, J Miller, E Belyavskaya and V Wilson (1995). "BPV E1 protein alters the kinetics of cell cycle entry of serum starved mouse fibroblasts." Cytometry **21**: 257-264.

Bettuzzi, S, F Scorcioni, S Astancolle, P Davilla, M Scaltriti and A Corti (2002). "Clusterin (SGP-2) transient overexpression decreases proliferation rate of SV40-immortalized human prostate epithelial cells by slowing down cell cycle progression." Oncogene **21**(4328-4334).

Bevort, M and H Leffers (2000). "Down regulation of ribosomal protein mRNAs during neuronal differentiation of human NTERA2 cells." Differentiation **66**: 81-92.

Boon, K, HN Caron, R van Asperen, L Valenttijn, M-C Hermus, P van Sluis, I Roobeek, I Weis, PA Voute, M Schwab and R Versteeg (2001). "N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis." EMBO Journal **20**: 1383-1393.

Bruce, JL, BD Price, N Coleman and SK Calderwood (1993). "Oxidant injury rapidly activates the heat shock transciption factor but fails to increase levels of heat-shock proteins." Cancer Research **53**: 12-15.

Burt, D and O Pourquie (2003). "Chicken genome - science nuggets to come soon." Science **300**: 1669.

Burton, GR, Y Guan, N Radhakrishnan and RE McGehee Jr. (2002). "Microarray analysis of gene expression during early adipocyte differentiation." Gene **293**: 21-31.

Cabart, P, I Kalousek and D Jandova (1991). "Expresssion of chromosomal protein HMG-1 in transformed and normal human cells." Haematologia **24**: 71-75.

Carr, MC, RN Schlussel, CR Peters, T Uchida, J Mandell and MR Freeman (1995). "Expression of cell growth regulated genes in the fetal kidney: relevance to *in utero* obstruction." Journal of Urology **154**(242-246).

Chen, TH, JR Brody, FE Romantsev, JG Yu, AE Kayler, E Voneiff, FP Kuhajda and GR Pasternak (1996). "Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci." Molecular Biology of the Cell **7**: 2045-2056.

Chou, DKH, JE Evans and FB Jungalwala (2001). "Identity of nuclear high-mobility group protein, HMG-1, and sulfoglucuronyl carbohydrate-binding protein, SBP-1, in brain." Journal of Neurochemistry **77**: 120-131.

Clynen, E, A De Loof and L Schoofs (2003). "The use of peptidomics in endocrine research." General and Comparative Endocrinology **132**: 1-9.

Cohen, PTW (2002). "Protein phosphatase 1 - targeted in many directions." Journal of Cell Science **115**: 241-256.

Darnell, J, H Lodish and D Baltimore (1990). Molecular Cell Biology. Chapter 15: Energy conversion: the formation of ATP in mitochondria and bacteria. New York, Scientific American Books.

Davis-Smyth, T, RC Duncan, T Zheng, G Michelotti and D Levens (1996). "The far upstream element-binding proteins comprise an ancient family of single-strand DNA-binding transactivators." Journal of Biological Chemistry **271**: 31679-31687.

Delva, P, A Lechi, C Pastori, M Degan, I Sheiban, G Montesi, M Pea, A Meneguzzi and M Menegazzi (2002). "Collagen I and III mRNA gene expression and cell growth potential of skin fibroblasts in patients with essential hypertension." Journal of Hypertension **20**: 1393-1399.

D'Souza, SM and IR Brown (1998). "Constitutive expression of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp 60 in neural and non-neural tissues of the rat during post-natal development." Cell Stress and Chaperones **3**: 188-199.

Duncan, R, I Collins, T Tomonaga, T Zhang and D Levens (1996). "A unique transactivation sequence motif is found in the carboxyl-terminal domain of the single-strand-binding protein FBP." Molecular and Cellular Biology **16**: 2274-2282.

Eisen, MB, PT Spellman, PO Brown and D Botstein (1998). "Cluster analysis and display of genome-wide expression patterns." Proceedings of the National Academy of Science, USA **95**: 14863-14868.

Evan, G and T Littlewood (1998). "A matter of life and cell death." Science **281**: 1317-1321.

Fages, C, R Nolo, HJ Huttunen, E-L Eskelinen and H Rauvala (2000). "Regulation of cell migration by amphoterin." Journal of Cell Science **113**: 611-620.

Feng, X, Y Jiang, P Meltzer and PM Yen (2000). "Thyroid hormone regulation of hepatic genes *in vivo* detected by complementary DNA microarray." Molecular Endocrinology **14**: 947-955.

Frade, JM (2000). "Unscheduled re-rentry into the cell cycle induced by NGF precedes cell death in nascent retinal cultures." Journal of Cell Science **113**: 1139-1148.

Futcher, B, GI Latter, P Monardo, CS McLaughlin and JI Garrels (1999). "A sampling of the yeast proteome." Molecular and Cellular Biology **19**: 7357-7768.

Galaviz-Hernandez, C, C Stagg, G de Ridder, TS Tanaka, MS Ko, D Schlessinger and R Nagaraja (2003). "Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis." Gene **309**(89-89).

Garrido, C, G Sandeep, L Ravagnan and K Guido (2001). "Heat shock proteins: endogenous modulators of apoptotic cell death." Biochemical and Biophysical Research Communications **286**: 433-442.

Garrido, SM, C Willman, FR Appelbaum and DE Banker (2000). "Three-color versus four-color multiparameter cell cycle analyses of primary acute myeloid leukemia samples." Cytometry **42**: 83-94.

George, DA (2002). The mitochondria and apoptosis. Innovations in Cellular Dynamics, BD Biosciences.

Goldstone, SD and MF Lavin (1993). "Isolation of a cDNA clone, encoding the ribosomal protein S20, downregulated during the onset of apoptosis in a human leukaemic cell line." Biochemical and Biophysical Research Communications **196**: 619-623.

Gomez, G, HM Lee, Q He, EW Englander, T Uchida and GHJ Greeley (2001). "Acute pancreatitis signals activation of apoptosis-associated and survival genes in mice." Experimental Biology and Medicine **226**: 692-700.

Gururajan, R, H Perry-O'Keefe, DA Melton and DL Weeks (1991). "The Xenopus localized messenger RNA An3 may encode an ATP-dependent RNA helicase." Nature **349**: 717-719.

Gygi, SP, B Rist, TJ Giffin, J Eng and R Aebersold (2002). "Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags." Journal of Proteome Research **1**: 47-54.

Gygi, SP, Y Rochon, BR Franza and R Aebersold (1999a). "Correlation between protein and mRNA abundance in yeast." Molecular and Cellular Biology **19**: 1720-1730.

Gygi, SP, B Rist, SA Gerber, F Turecek, MH Gelb and R Aebersold (1999b). "Quantitative analysis of complex protein mixtures using isotope-coded affinity tags." Nature. Biotechnology **17**: 994-999.

Hakem, A, T Sasai, I Kozieradzki and JM Penninger (1999). "The cyclin-dependent kinase Cdk2 regulates thymocyte apoptosis." European Journal of Cell Biology **69**: 143-150.

Han, DK, J Eng, H Zhou and R Aebersold (2001). "Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry." Nature. Biotechnology **19**: 946-951.

Handwerger, S and B Aronow (2003). "Dynamic changes in gene expression during human trophoblast differentiation." Recent Progress in Hormone Research **58**: 263-281.

Howard, KM, LC Burke, C Mailhos, A Pizzey, CS Gilbert, DW Lawson, MKL Collins, NSB Thomas and PS Latchman (1993). "Cell cycle arrest of proliferating neuronal cells by serum deprivation can result in either apoptosis or differentiation." Journal of Neurochemistry **60**: 1783-1791.

Huttunen, HJ, J Kuja-Panula and H Rauvala (2002). "Receptor for advanced glycation end products (RAGE) signaling induces CREB-dependent chromogranin expression during neuronal differentiation." Journal of Biological Chemistry **277**: 38635-39646.

Irwin, N, V Baekelandt, L Goritchenko and L Benowitz (1997). "Identification of two proteins that bind to a pyrimidine-rich sequence in the 3'-untranslated region of GAP-43 mRNA." Nucleic Acids Research **25**: 1281-1288.

Jiang, X, H-E Kim, H Shu, Y Zhao, H Zhang, J Kofron, J Donnelly, D Burns, S Ng, S Rosenberg and X Wang (2003). "Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway." Science **299**: 223-226.

Johnson, KR, JE Disney, CR Wyatt and R Reeves (1990). "Expression of messenger RNA encoding mammalian chromosomal proteins HMG-1 and HMG-Y during cellular proliferation." Experimental Cell Research **187**: 69-76.

Jónsson, ZO and U Hübscher (1997). "Proliferating cell nuclear antigen: more than a clamp for DNA polymerases." BioEssays **19**: 967-975.

Kinoshita, A, H Wanibuchi, K Morimura, M Wei, J Shen, S Imaoka, Y Funae and S Fukushima (2003). "Phenobarbitol at low dose exerts hormesis in rat hepatocarcinogenesis by reducing oxidative DNA damage, altering cell proliferation, apoptosis and gene expression." Carcinogenesis **Jun 5; E pub ahead of print**.

Koonin, EV and MY Galperin (2003). Informational Sources for Genomics. Sequence-Evolution-Function: Computational Approaches in Comparative Genomics. Boston, Kluwer Academic Publishers: Chapter 3.

Kuniyasu, H, Y Chihara and T Takahashi (2003). "Co-expression of receptor for advanced glycation end products and the ligand amphoterin associates closely with metastasis of colorectal cancer." Oncology Reports **10**:445-448.

Lacal, JC (1997). "Regulation of proliferation and apoptosis by Ras and Rho GTPases through specific phospholipid-dependent signaling." FEBS Letters **410**: 73-77.

Lang, P, F Gesbert, CM Delaspine, R Stancou, M Pouchelet and A Bertoglio (1996). "Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes." EMBO Journal **15**: 510-519.

Lew, J and JH Wang (1995). "Neuronal cdc2-like kinase." Trends in Biochemical Sciences **20**: 33-37.

Li, M, A Makkinje and Z Damuni (1996). "Molecular identification of I_1^{PP2A}, a novel potent heat-stable inhibitor protein of protein phosphatase 2A." Biochemistry **35**: 6998-7002.

Li, X, L Liu, JC Tupper, DD Bannerman, RK Winn, SM Suebti, AD Hamilton and JM Harlan (2002). "Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells." Journal of Biological Chemistry **277**: 15309-15316.

Lopez-Girona, A, B Furnari, O Mondesert and P Russell (1999). "Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein." Nature **397**: 172-175.

Marzluff, WF and NB Pandey (1988). "Multiple regulatory steps control histone concentrations." Trends in Biological Science **13**: 49-52.

McCormick, D and PA Hall (1992). "The complexities of proliferating cell nuclear antigen." Histopathology **21**: 591-594.

Merrick, WC (1992). "Mechanism and regulation of eukaryotic protein synthesis." Micobiological Reviews **56**: 291-315.

Moorthamer, M and C Bhabatosh (1999). "Identification of ribosomal protein L34 as a novel Cdk5 inhibitor." Biochemical and Biophysical Research Communications **255**: 631-638.

Muller, S, P Scaffidi, B Degryse, T Bonaldi, L Ronfani, A Agresti, M Betrame and ME Bianchi (2001). "The double life of HMGB1 chromatin protein: architectural factor and extracellular signal." EMBO Journal **20**: 4337-4340.

Nagao, T, Y Ishida and Y Kondo (1996). "Determination of S-phase cells by *in situ* hybridization for histone H3 mRNA in hepatocellular carcinoma: correlation with histologic grade and other cell proliferation markers." Modern Pathology **9**: 99-104.

Nikolic, M, H Dudek, YT Kwon, YFM Ramos and L-H Tsai (1996). "The cdk5 kinase is essential for neurite outgrowth during neuronal differentiation." Genes and Development **10**: 816-825.

O'Connor, L, DCS Huang, LA O'Reilly and A Strasser (2000). "Apoptosis and cell division." Current Opinion in Cell Biology **12**: 257-263.

Ohshima, T, JM Ward, CG Huh, G Longnecker, P Veeranna, HC Pant, RO Brady, LJ Martin and AB Kulkarni (1996). "Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death." Proceedings of the National Academy of Science, USA **93**: 11173-11178.

Osley, MA (1991). "The regulation of histone synthesis in the cell cycle." Annual Review of Biochemistry **60**: 827-861.

Pandey, S and E Wang (1995). "Cells en route to apoptosis are characterized by the upregulation of c-fos, c-myc, c-jun, cdc2, and RB phosphorylation, resembling events of early cell cycle traverse." Journal of Cellular Biochemistry **58**: 135-150.

Passalacqua, M, M Patrone, GB Picotti, M Del Rio, B Sparatore, E Melloni and S Pontremoli (1997). "Stimulated astrocytes release high-mobility group 1 protein, and inducer of LAN-5 neuroblastoma cell differentiation." Neuroscience **82**: 1021-1028.

Patton, WF (2002). "Detection technologies in proteome analysis." Journal of Chromatography B **771**: 3-31.

Peng, C-Y, PR Graves, RS Thoma, Z Wu, AS Shaw and H Piwnica-Worms (1997). "Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216." Science **277**: 1501-1505.

Schumm, MA, DA Castellanos, BR Frydel and J Sagen (2002). "Enhanced viability and neuronal differentiation of neural progenitors by chromaffin cell co-culture." Developmental Brain Research **137**: 115-125.

Seasholtz, TM, M Majumdar and JH Brown (1999a). "Rho as a mediator of G protein-coupled receptor signaling." Molecular Pharmacology **55**: 949-956.

Seasholtz, TM, M Majumdar, DD Kaplan and JH Brown (1999b). "Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration." Circulation Research **84**: 1186-1193.

Seeger, RC, GM Brodeur, H Sather, A Dalton, SE Siegel, KY Wong and D Hammond (1985). "Association of multiple copies of the n-myc oncogene with rapid progression of neuroblastomas." New England Journal of Medicine **313**: 1111-1116.

Seyedin, SM, JR Pehrson and RD Cole (1981). "Loss of chromosomal high mobility group proteins HMG1 and HMG2 when mouse neuroblastoma and Friend erythroleukemia cells become committed to differentiation." Biochemistry **78**: 5988-5992.

Shankar, SL, K O'Guin, M Cammer, FA McMorris, TN Stitt, RS Basch, K Varnum and B Shafit-Zagardo (2003). "The growth arrest-specific gene product Gas6 promotes the survival of human oligodendrocytes via a phosphatidylinositol 3-kinase-dependent pathway." Journal of Neuroscience **23**(4208-4218).

Shiio, Y, S Donohoe, C-Y Eugene, DR Goodlett, R Aebersold and RN Eisenman (2002). "Quantitative proteomic analysis of Myc oncoprotein function." EMBO Journal **21**: 5088-5096.

Shima, H, Y Hatano, YS Chun, T Sugimura, Z Zhang, EYC Lee and M Nagao (1993). "Identification of PP1 catalytic subunit isotypes PP1-gamma-1, PP1-alpha in various rat tissues." Biochemical and Biophysical Research Communications **192**: 1289-1296.

Shin-ichi, Y, Y Hideki, Y Takashi and H Kubota (2001). "Cytosolic chaperonin-containing t-complex polypeptide 1 changes the content of a particular subunit species concomitmant with substrate binding and folding activities during the cycle." European Journal of Biochemistry **268**: 4664-4673.

Shirvan, A, I Ziv, A Barzilai, R Djaldeti, R Zilkha-Falb, T Michlin and E Melamed (1997a). "Induction of mitosis-related genes during dopamine-triggered apoptosis in sympathetic neurons." Journal of Neural Transmission (Supplement) **50**: 67-78.

Shirvan, A, I Ziv, T Machlin, R Zilkha-Falb, E Melamed and A Barzilai (1997b). "Two waves of cyclin B and proliferating cell nuclear antigen expression during dopamine-triggered neuronal apoptosis." Journal of Neurochemistry **69**: 539-549.

Skoulakis, EM and RL David (1998). "14-3-3 proteins in neuronal development and function." Molecular Neurobiology **16**: 269-284.

Song, C, Q Wang and CCH Li (2003). "ATPase activity of p97 valosin-containing protein (VCP). D2 mediates the major enzyme activity, and D1 contributes to the heat-induced activity." Journal of Biological Chemistry **278**(3648-3655).

Sowden, J, W Putt, K Morrison, R Bedington and Y Edwards (1995). "The embryonic RNA helicase gene (ERH): A new member of the DEAD box family of RNA helicases." Biochemical Journal **308**: 839-846.

Stenger, AM, ML Garre, L Andreussi, A Cama, F Callea, M Brisigotti, G Fabretti, D Di Martino and GP Tonini (1992). "Expression of histone H3 cell cycle-related gene vimentin and myc genes in pediatric brain tumors. A preliminary analysis showing the different malignant cell growth potential." Molecular Brain Research **13**: 273-275.

Stryer, L (1988). Chapter 30. Protein Synthesis. Biochemistry. New York, W.H. Freeman and Company.

Tabakoff, B, SV Bhave and PL Hoffman (2003). "Selective breeding, quantitative trait locus analysis, and gene arrays identify candidate genes for complex drug-related behaviors." Journal of Neuroscience **23**: 4491-4498.

Tabuchi, A, K Funaji, J Nakatsubo, M Fukuchi, T Tsuchiya and M Tsuda (2003). "Inactivation of aconitase during the apoptosis of mouse cerebellar granule neurons induced by a deprivation of membrane depolarization." Journal of Neuroscience Research **71**: 504-515.

Tamayo, P, D Slonim, J Mesirov, Q Zhu, S Kitareewan, E Dmitrovsky, ES Lander and TR Golub (1999). "Interpreting patterns of gene expresssion with self-organizing maps: Methods and application to hematopoietic differentiation." Proceedings of the National Academy of Science, USA **96**: 2907-2912.

Tang, D and JH Wang (1996). "Cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activators." Progress in Cell Cycle Research **2**: 205-216.

Tavazoie, S, JD Hughes, MJ Campbell, RJ Cho and GM Church (1999). "Microarray analysis of gene expression during early adipocyte differentiation." Nature. Genetics **22**: 281-285.

Thiede, B, C Dimmler, Siejak and T Rudel (2001). "Predominant identification of RNA-binding proteins in Fas-induced apoptosis by proteome analysis." Journal of Biochemistry **276**: 26044-26050.

Udtha, M, SJ Lee, R Alam, K Coombes and V Huff (2003). "Upregulation of c-MYC in WT-1-mutant tumors: assessment of WT1 putative transcriptional targets using cDNA microarray expression profiling of genetically defined Wilms' tumors." Oncogene **22**: 3821-3826.

Van Aelst, L and C D'Souza-Schorey (1997). "Rho GTPases and signaling networks." Genes and Development **11**: 2295-2322.

van der Brug, MP, S Goodenough and P Wilce (2002). "Kainic acid induces 14-3-3 zeta expression in distinct regions of rat brain." Brain Research **956**: 110-115.

Won, K-A, RJ Schumaker, GW Farr, AL Horwich and SI Reed (1998). "Maturation of human cyclin E requires the function of the eukaryotic chaperonin CCT." Molecular and Cellular Biology **18**: 7584-7589.

Yaffe, MB (2002). "How do 14-3-3 proteins work? Gatekeeper phophorylation and the molecular anvil hypothesis." FEBS Letters **513**: 53-57.

Yamamura, J-I, Y Takada, M Goto, M Kumegawa and S Aoe (1999). "High mobility group-like protein in bovine milk stimulates the proliferation of osteoblastic MC3T3-E1 cells." Biochemical and Biophysical Research Communications **261**: 113-117.

Yang, Y-H and T Speed (2002). "Design issues for cDNA microarray experiments." Nature Reviews **3**: 579-588.

Zha, J, H Harada, E Yang, J Jockel and J Korsmeyer (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not Bcl-X_L." Cell and Tissue Research **87**: 619-628.

Zhang, Z and GG Carmichael (2001). "The fate of dsRNA in the nucleus: a $p54^{nrb}$-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs." Cell **106**: 465-475.

Zhou, H, JA Ranish, JD Watts and R Aebersold (2002). "Quantitative proteome analysis by solid-phase isotope tagging amd mass spectrometry." Nature. Biotechnology **19**: 512-515.

Zilkha-Falb, R, A Barzilai, R Djaldeti, I Ziv, E Melamed and A Shirvan (2000). "Involvement of T-complex protein 1 delta in dopamine triggered apoptosis in chick embryo sympathetic neurons." Journal of Biological Chemistry **275**: 36380-36387.

Zohn, IM, SL Campbell, R Khosravi-Far, KL Rossman and CJ Der (1998). "Rho family proteins and Ras transformation: the RHOad less traveled gets congested." Oncogene **17**: 1415-1438.

Zuber, ME, Z Zhou, LW Burrus and BB Olwin (1997). "Cysteine-rich FGF receptor regulates intracellular FGF-1 and FGF-2 levels." Journal of Cellular Physiology **170**: 217-227.

CHAPTER 6

Summary

and

Suggestions for Future Research

On PACAP and chick brain development

Functions of the glucagon superfamily of hormones are diverse, and include regulation of metabolism and the cardiovascular, endocrine, and immune systems. PACAP is the most highly conserved hormone in this family, sharing sequence similarity of 97% with the tunicate (McRory and Sherwood 1997), from which it diverged more than 650 million years ago. The PACAP gene is found in a large and diverse group of animals, and it is always highly conserved. Although it is active in both the embryo and adult of many species, and in various tissues, a large body of evidence suggests that it plays an important role as a neurotrophin and cell cycle regulator during development.

I showed in this thesis that PACAP is an autocrine/paracrine regulator important in the development of early chick brain. Blocking the PACAP-specific receptor caused cells to stop proliferating, and either undergo differentiation or apoptosis. The work could easily be extended to more completely examine development of chick brain. The cell culture and flow cytometry system described here could be used to characterize the activity of chick neuroblast monocultures up to embryonic day 8. Development could be further characterized after day 8, in the presence of glial cells. Although this might complicate results, it would provide a model to study neuron-glial interaction. This system could be used to test the effects not only of PACAP, but of other growth factors and hormones, and their interactions, as well.

On PACAP and disease

Disturbances to cell cycle and apoptosis pathways in the nervous system create a multitude of human diseases, including cancers, fetal alcohol syndrome, forms of mental retardation, psychiatric disorder, and later in life, a host of neurodegenerative and autoimmune disorders (see Chapter 1). The greatest devastation occurs when the cell cycle and apoptotic pathways are conjointly dysfunctional: too little apoptosis and too much proliferation leads to cancers that are virtually impossible to counteract. The range of human suffering associated with these two processes is immense.

PACAP was shown to affect cell cycle and apoptosis through the PACAP-specific receptor, PAC_1-R, in these neuroblasts. It has also been shown, elsewhere, that two major tumour suppressors which regulate both cell cycle and apoptosis, Zac1 and p53, also upregulate PAC_1-R (Spengler et al. 1997; Hoffman et al. 1998). For this reason, PACAP deserves continued study in the realm of its effects on cell cycle and apoptosis, especially in the nervous system, but perhaps in other tissues as well. Zac1 and p53 upregulated PAC_1-R in two nervous system cell lines: neuroblastomas and pheochromocytomas (Vertongen et al. 1996; Hoffman et al. 1998). As well, PAC_1-R was the predominant type of PACAP/VIP receptor found in glial tumours, pituitary adenomas, paragangliomas and endometrial carcinomas, and Zac1 was able to upregulate PAC_1-R in a cell line that did not normally express PACAP (Spengler et al. 1997; Reubi 2000).

On scientific methodology

This thesis tested a variety of methods, and examined changes at the level of mRNA to signaling molecules to proteins to whole cells. This integrated approach not only elucidated particular functions of a particular hormone in one species during a small range in time, it also accentuated the benefit of versatility and variety in scientific exploration. From this work, I was able to use proteomics to identify several good candidate proteins that could act as markers for proliferation, cell cycle exit, differentiation, and apoptosis during early brain development. These proteins are not necessarily new to the scientific field, but this thesis provides a novel view of their functioning which could add to their value as experimental, and perhaps therapeutic markers, in the study and treatment of development and disease.

On the use of the chick as a study model (Or, are bird brains really worth studying?)

That this thesis showed PACAP affected both proliferation and apoptosis in the chick embryo is not surprising, although the very early age at which this occurred was exciting. It was surprising to learn that the proliferative effect was opposite to that which occurs in mammalian brain later in development. It has been well documented in these pages that PACAP causes cell cycle exit in rodent cerebellar granule cells during the later stages of embryogenesis and early post-natal development. Thus, PACAP appears to either function differently in different species, or to change functions during development. Further investigation into these possibilities could enhance our understanding of the primary function of this hormone.

It was recently said during a guest lecture at the University of Victoria that one does not really earn respect in this field until one works with the furry creatures. From my personal observations and experience during production of this thesis, I do see a trend toward more work with rats and mice, and less with animals like frogs and chickens. This thesis was more challenging due to the relative lack of information on chick at the molecular biology level, and the difficulty in finding tools such as antibodies that are characterized for use in chicken. This resulted in a higher degree of risk and more trial and error work than would be necessary working with rat or mouse. Yet I find no reason that studies in this area should be narrow. While it may be that the mouse shares 90% of the genes associated with human disease, and this does indeed make it a good model for study, there is another approach to elucidating basic functioning of molecules like hormones and growth factors. That approach is a comparative and evolutionary one, which takes into account difference as well as sameness, and relates changing structure to changing function.

Furthermore, on a practical, economic, and for some, perhaps even on an ethical level, the chick embryo simply cannot be surpassed in its suitability as a model organism for developmental studies. Chicken eggs are inexpensively obtained and stored, and do not require maternal sacrifice. The embryo exists in sterile conditions, and the stages of development have been described in meticulous detail (Hamburger and Hamilton 1992). It is not too surprising that the woman to whom I have dedicated this thesis, who later shared a Nobel Prize for her work in neurogenesis, chose chick rather than mouse to study in her bedroom laboratory. I do not in any way mean to downplay the importance of mouse and rat as models, or contradict my earlier suggestion that a

variety of integrated approaches will be most useful. But I do believe that bird brains can teach us a lot. Perhaps Rita Levi-Montalcini will live to see the important work she began in 1936 regain a degree of respect. The recent news that assembly of a draft sequence of the chicken genome could be available as early as March 2004 is a hopeful sign (Burt and Pourquie 2003).

REFERENCES

Burt, D and O Pourquie (2003). "Chicken genome - science nuggets to come soon." Science **300**: 1669.

Hamburger, V and HL Hamilton (1992). "A Series of Normal Stages in the Development of the Chick Embryo." Developmental Dynamics **195**: 231-272.

Hoffman, A, E Ciani, S Houssami, P Brabet, L Journot and D Spengler (1998). "Induction of type 1 PACAP receptor expression by the new zinc finger protein Zac1 and p53." Annals of the New York Academy of Science, USA **865**: 49-58.

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

Reubi, JC (2000). "*In vitro* evaluation of VIP/PACAP receptors in healthy and diseased human tissues." Annals of the New York Academy of Science, USA **921**: 1-25.

Spengler, D, M Villalba, A Hoffman, C Pantaloni, S Houssami, J Bockaert and L Journot (1997). "Regulation of apoptosis and cell cycle arrest by Zac-1, a novel zinc finger protein expressed in the pituitary gland and the brain." EMBO Journal **16**: 2814-2825.

Vertongen, P, C Devalck, E Sariban, M-H De Laet, H Martelli, F Paraf, P Helardot and P Robberecht (1996). "Pituitary adenylate cyclase activating peptide and its receptors are expressed in human neuroblastomas." Journal of Cellular Physiology **167**: 36-46.