VASOACTIVE INTESTINAL POLYPEPTIDE IN THE CHICKEN ANTERIOR PITUITARY GLAND

A thesis submitted in partial fulfilment of the regulations for the Doctor of Philosophy degree of the University of Edinburgh.

by

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PUBLICATIONS ARISING FROM THESIS

Oral communications

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ATP	adenosine tri phosphate
AVP	arginine vasopressin
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
C-terminal	carboxyl terminal
CA	caudal lobe
CE	cephalic lobe
CH_3CN	acetonitrile
CRF	corticotrophin releasing hormone
cDNA	complementary deoxyribonucleic acid
cGnRH-I	chicken gonadotrophin releasing hormone
Ci/mM	curies per milli mole
CNS	central nervous system
cpm	counts per minute
CTP	cytosine tri phosphate
DAB	diaminobenzidine
DAPI	dihydrochloride 4', 6-diamidino-2-phenyl-indole
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminotetra-acetic acid
FSH	follicle stimulating hormone
g	grams
GFAP	glial fibrillary acidic protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GTP	guanine tri-phosphate
GnRH	gonadotrophin releasing hormone
HPLC	high pressure liquid chromatography
ICC	immunocytochemistry
IH	inferior hypothalamic nuclei
IN	infundibular hypothalamic nuclei
LH	luteinising hormone
ME	median eminence
mls	millilitres

mM	milli moles
mRNA	messenger ribonucleic acid
NSB	non specific binding
N-terminal	amino terminal
ng	nano grams
nM	nano moles
ODS	octadecasilane
PACAP	pituitary adenylate cyclase activating polypeptide
PACAP-I-R	PACAP Type I Receptor
PACAP-II-R	PACAP Type II Receptor
PCR	polymerase chain reaction
PHI/M	peptide histidine isoleucine/methionine
PHN	nucleus periventricularis hypothalami
PK	proteinase K
POP	nucleus preopticus periventriculus
PRL	prolactin
PVN	nucleus paraventricularis magnocellularis
PVR	PACAP/VIP receptor
rpm	revolutions per second
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SCN	suprachiasmatic nucleus
SOe	nucleus supraopticus
SOv	nucleus supraopticus, pars ventralis
SSC	standard sodium citrate
TAE	Tris acetate electrophoresis buffer
TBE	Tris borate electrophoresis buffer
TE	Tris EDTA
TEMED	tetramethyl ethylenediamine
TRH	thyrotrophin releasing hormone
TSH	thyroid stimulating hormone
TSM	tractus septomesencephalicus
UV	ultra violet
VIII	3rd ventricle
VIP	vasoactive intestinal polypeptide
VIP-RI	VIP receptor subtype I
VIP-RII	VIP receptor subtype II
µg	micro grams
µl	micro litres
µM	micro moles

ABSTRACT

In birds, prolactin secretion is stimulated by a hypothalamic prolactin releasing hormone, vasoactive intestinal polypeptide (VIP). Active immunization against VIP suppresses both prolactin and LH secretion. Since VIP in physiological doses, does not stimulate LH release, it is suggested that it might act to regulate the function of LH-producing cells in a paracrine manner. The aim of this thesis is to provide biochemical, molecular and anatomical evidence to support this hypothesis.

Vasoactive intestinal polypeptide was shown to be present in the chicken anterior pituitary gland using high performance liquid chromatography (HPLC), radioimmunoassay (RIA) and immunocytochemistry. The VIP antibodies used for both RIA and immunocytochemistry showed no cross reaction with any known VIP-like peptides, including pituitary adenylate cyclase activating polypeptide (PACAP). VIP mRNA was also shown to be present in anterior pituitary gland, using reverse-transcription polymerase chain reaction (RT-PCR) and primers designed from the chicken VIP cDNA sequence, and by solution hybridization RNAse protection assay.

Two VIP-immunoreactive cell (VIP-ir) types were found throughout the cephalic and caudal lobes of the anterior pituitary gland. The morphological features of the VIP-ir cell types were similar to that of the folliculo-stellate cell type (FS-cell). One VIP-cell type contained the Ca 2+ binding protein S-100 protein, a specific marker of FS-cells, while the S-100 protein was not detected in the second VIP-cell type. The VIP-cells were closely associated with gonadotrophs, lactotrophs and unidentified cell types, but VIP-ir was not colocalised with LH or PRL. The VIP-cell characteristically enveloped several adjacent gonadotrophs with cytoplasmic projections, which suggests that intra-pituitary VIP may regulate the gonadotroph in a paracrine manner.

VIP receptors were localised immunocytochemically, in the anterior pituitary gland and hypothalamus, using antibodies raised the peptide sequence of the human subtype-I (VIP-RI) and the subtype-II VIP receptors (VIP-RII). The VIP-RI was not present in PRL-, LH- or GH-cells, but was found exclusively in ACTH-cells. The VIP-RII was diffusely distributed through both lobes of the chicken anterior pituitary gland, but the cell type containing VIP-RII immunoreactivity was not identified.

The effects of VIP and PACAP on pituitary hormone secretion were determined *in vitro* using cultured hemi-pituitaries. VIP and PACAP stimulated PRL secretion in a dose dependent manner. High concentrations of VIP and PACAP stimulated GH and LH secretion but did not affect ACTH secretion.

In conclusion, VIP is produced in the anterior pituitary gland and occurs in a FS-cell type which is closely associated with gonadotrophs. These observations are consistent with the view that intra-pituitary VIP may act in a paracrine manner to regulate the function of gonadotrophs.

Chapter 1: INTRODUCTION

1.1 Vasoactive intestinal polypeptide in the peripheral nervous system

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid polypeptide originally isolated from the porcine duodenum (Said and Mutt, 1970; 1972), and the chicken intestine (Nilsson, 1974). The biological activity of VIP was first determined using a bioassay initially established for secretin, a closely related gastrointestinal peptide extracted from the same source. The assay involved the measurement in the turkey, of the bicarbonate and protein composition and rate of flow of pancreatic secretions (Heatley *et al.,* 1965; Dockray, 1973). The regulation of pancreatic secretion by secretin in birds is essentially the same as that observed in mammals. However, in birds VIP is 30 times more potent than secretin in stimulating flow of pancreatic juices (Dockray, 1973).

1.1.1 Distribution of VIP in the peripheral nervous system

VIP-like immunoreactivity is widely distributed in the peripheral nervous system occurring in the sympathetic ganglia (Hokfelt *et al.,* 1977), in the vagus, splanchnic and sciatic nerves (Lundberg *et al.,* 1978; 1979) and in nerves supplying the gastrointestinal (Fuxe *et al.,* 1977; Polak *et al.,* 1974) and genito-urinary tracts (Alm *et al.,* 1977; Larsson *et al.,* 1977; Hokfelt *et al.,* 1978). It also occurs in the nerves supplying the connective tissue, alveoli, lactiferous ducts in the nipple and parenchyma tissue of the human and rat mammary gland (Erikkson *et al.,* 1996), and within ganglion-like structures around airways, and pulmonary vessels of the mammalian lung (Dey *et al.,* 1981; Eriksson *et al.,* 1996).

VIP-like immunoreactivity (VIP-ir) has been localised in the chicken and quail embryo by immunocytochemistry, where it is distributed throughout the peripheral nervous system. VIP-ir nerve fibres are abundant along the entire digestive tract of the chicken (Fontaine-Perus, 1984), in the submucosal and myenteric plexus and are also prominent in the oesophagus and pancreas (Nilsson, 1974; 1975, Sundler *et al.,* 1979, Vaillant *et al.,* 1980). VIP nerve fibres occur in the pancreas of the chicken in the exocrine portion, the adventia artery and in the connective tissue of the ductal wall. VIP nerve fibres are also found in the intrapulmonary ganglia of the 12-day-old chick embryo, and in the lamina propria of the primary and secondary

bronchi, and in the pulmonary septa of the lung of the chick at hatch (Salvi and Renda, 1992). In the adult chicken, VIP immunoreactive nerve fibres are observed throughout the primary bronchus wall in the lung septa (Salvi and Renda, 1992).VIP immunoreactivity is also present in postganglionic secretory nerves of the glandular parenchyma of the salt gland in the duck (Gertsberger, 1988).

1.1.2 Biological action of VIP

The widespread distribution of VIP in the peripheral nervous system reflects its biological functions. VIP stimulates bronchodilation (Said and Mutt, 1970; 1988) smooth muscle relaxation (Piper *et al.,* 1970) and secretory processes in the gastrointestinal tract (Said, 1982; Barbezat and Grossman, 1971; Makhlouf *et al.,* 1978). Systemic administration of VIP stimulates hypotension by inducing peripheral vasodilation in the guinea-pig and cat, and inhibits vagally induced respiratory insufflation pressure (Lundberg *et al.,* 1984).

In the reproductive system, VIP is a modulator of vascular activity for penile erection (Ottesen *et al.,* 1984; Dixson *et al.,* 1984) and also increases cervical (Allen *et al.,* 1988) and vaginal (Fahrenkrug *et al.,* 1988) blood flow. In the mammary gland VIP released by oxytocin, may stimulate vasodilation in the skin overlying the mammary gland, which is observed in association with milk ejection (Eriksson *et al.,* 1996).

In birds, VIP stimulates the secretion of bicarbonate-rich pancreatic juices in the turkey, possibly in a neuroendocrine manner (Dockray, 1973, Vaillant *et al.,* 1980). VIP also stimulates the secretion of highly concentrated saline from the supraorbital salt gland of the duck acting on both the arteriolar network and the secretory tubules (Gertsberger, 1988).

1.2 Related peptides in the VIP gene family

The structural similarity between VIP and other peptide hormones isolated from the gastrointestinal tract, shows that it is a member of the secretin-glucagon family (Figure 1.1). This family of peptides includes, secretin, glucagon, growth hormone releasing hormone (GHRH), peptide histidine isoleucine/methionine (PHI/M), and the recently isolated pituitary adenylate cyclase activating polypeptide (PACAP; Section 1.8) (Said and Mutt, 1970; 1988; Said 1986; Bloom *et al.,* 1983;

Gozes and Brenneman, 1989). The structural similarity between these peptides, is also reflected in the similarity between the genes encoding for the VIP gene family, and suggests they may have evolved from a common ancestral gene (Gozes and Brenneman, 1989). Distinct regulatory mechanisms controlling the expression of specific peptides in the VIP gene family have also evolved, which may regulate tissue and cell specific expression.

cVIP

HIS SER ASP ALA VAL PHE THR ASP ASN TYR SER ARG PHE ARG LYS GLN MET
ALA VAL LYS LYS TYR LEU ASN SER VAL LEU THR

mVIP

HIS SER ASP ALA VAL PHE THR ASP ASN TYR THR ARG LEU ARG LYS GLN MET
ALA VAL LYS LYS TYR LEU ASN SER ILE LEU ASN

PHI

HIS ALA ASP GLY VAL PHE THR SER ASP PHE SER ARG LEU LEU GLY GLN LEU
SER ALA LYS LYS TYR LEU GLU SER LEU ILE

cPACAP

HIS ILE ASP GLY ILE PHE THR ASP SER TYR SER ARG TYR ARG LYS GLN MET
ALA VAL LYS LYS TYR LEU ALA VAL LEU GLY LYS ARG TYR TYR LYS GLN ARG
VAL LYS ASN LYS

SECRETIN

HIS SER ASP GLY THR PHE THR SER GLU LEU SER ARG LEU ARG ASP SER ALA
ARG LEU GLN ARG LEU LEU GLN GLY LEU VAL

GHRH

TYR ALA ASP ALA ILE PHE THR ASN SER TYR ARG LYS VAL LEU GLY GLN LEU
SER ALA ARG LYS LEU LEU GLN ASP ILE MET SER

GLUCAGON

HIS SER GLN GLY THR PHE THR SER ASP TYR SER LYS TYR LEU ASP SER ARG
ARG ALA GLN ASP PHE VAL GLN TRP LEU MET ASN

Figure 1.1 A comparison of the amino acid sequence of chicken VIP (cVIP) and peptides in the VIP gene family. The differences in amino acids are underlined. Mammalian VIP (mVIP) sequence is found in human, porcine, bovine and rat. peptide histidine isoleucine (PHI) is the porcine sequence; pituitary adenylate cyclase activating polypeptide (PACAP) is chicken. Secretin is the porcine and bovine sequence; growth hormone releasing hormone (GHRH) is the human sequence; glucagon is the porcine and human sequence (Adapted from Said, 1986)

1.2.1 Molecular biology of VIP

Vasoactive intestinal polypeptide cDNAs encoding the prepro VIP were first isolated in man (Itoh *et al.,* 1983), and subsequently rat (Giladi *et al.,* 1990), chicken and turkey. The mammalian and avian VIP genes too are made up of seven exons with each exon encoding a distinct functional domain (Figure 1.2). It can be predicted from the cDNA sequence that transcription of the gene results in the production of a mRNA encoding VIP itself and a second peptide containing 27 amino acids. This second peptide is termed peptide histidine isoleucine (PHI) in rats and the pig, and peptide histidine methionine (PHM) in humans (Itoh *et al.,* 1983; Giladi *et al.,* 1990). VIP and PHI/M are encoded by exon 5 and exon 4 respectively of the VIP gene, and the two peptides are synthesised as part of the same protein.

Mammalian VIP and PHI/M are produced by the fully processed VIP mRNA, and there is evidence that they are not always produced in the same tissue, since a differential distribution has been demonstrated in the brain of the rat (Beinfeld *et al.,* 1984; Christofides *et al.,* 1984). Although, there is no evidence for alternative splicing of the mammalian VIP gene (Linder *et al.,* 1987).

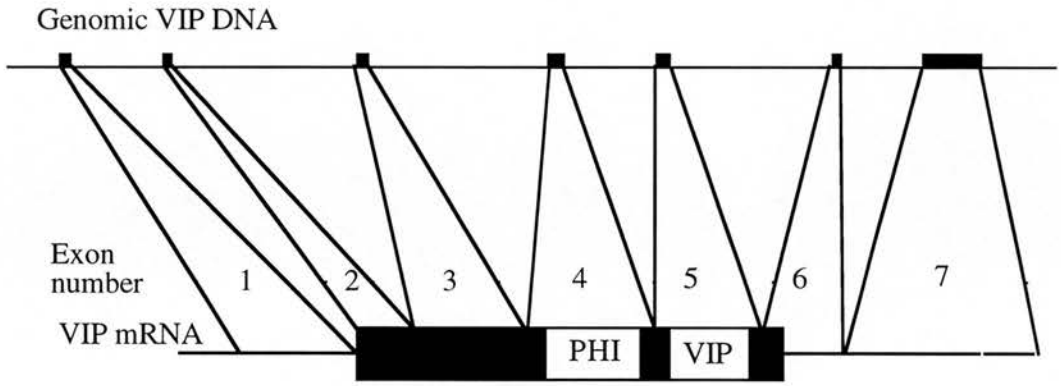


Figure 1.2 Transcription of the mammalian VIP gene.

1.2.2 Comparison between mammalian and avian VIP

The cDNA encoding the avian prepro VIP gene is highly homologous with cDNAs encoding mammalian VIP pre-pro polypeptides (Talbot *et al.,* 1995; McFarlin *et al.,* 1995; You *et al.,* 1995). The chicken genomic DNA sequence was analysed by PCR, and identified exons encoding both VIP and PHI, but analysis of several

clones from a hypothalamic cDNA library failed to confirm the presence of the exon encoding PHI. In fact, the largest VIP clone contained a complete open-reading frame coding for a 165 amino acid pre-pro VIP, which did not contain a sequence encoding PHI (Talbot *et al.,* 1995). It was confirmed by RT-PCR, solution hybridisation, and RNase protection assay that the chicken VIP gene transcript is alternatively spliced (Figure 1.3); and that two forms of the VIP mRNA are expressed in unequal amounts in different tissues, with the form containing PHI being the least abundant (Talbot *et al.,* 1995).

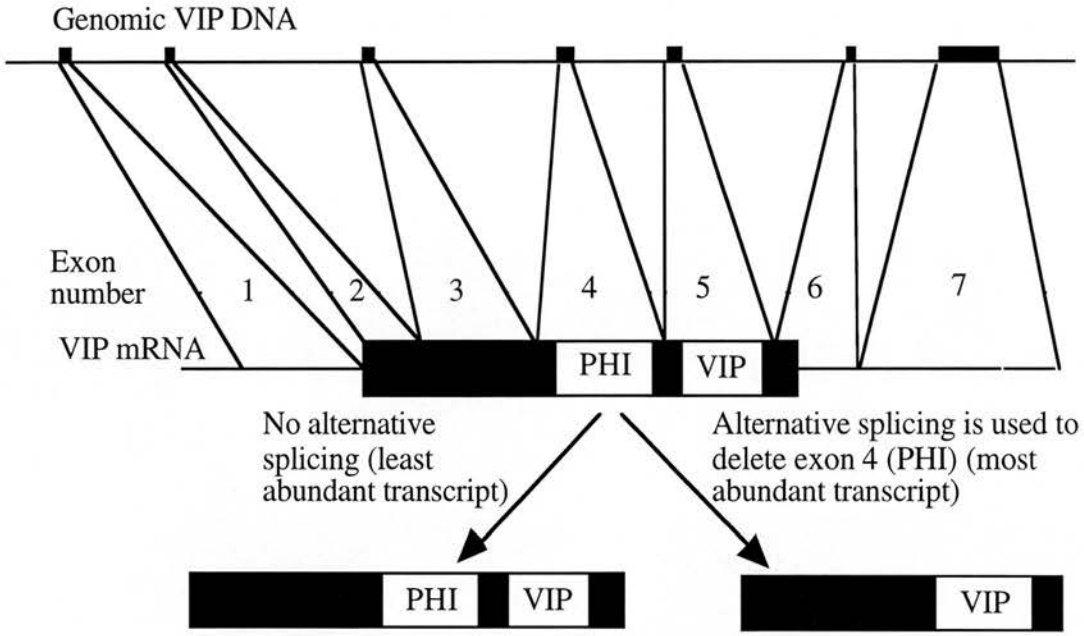


Figure 1.3 Transcription of the avian VIP gene.

1.3 Localisation of VIP in the central nervous system

The chemical characterisation of VIP extracted from various brain tissues showed that it is identical to VIP extracted from the gut (Marley and Emson, 1982). Studies using immunocytochemistry and autoradiography demonstrated that the distribution and biological action of VIP is widespread in the mammalian brain, being present in the cerebral cortex, amygdala, hippocampus, and hypothalamus (Said 1982; 1986; Rostene, 1984; Gozes and Brenneman, 1989).

VIP-positive bipolar neurones occur in all the cortical regions, with dendritic branching in layers I and deep cortical layers, (Morrison and Magistretti, 1983;

Morrison *et al.,* 1984). In the rat, approximately 1% of all cortical neurones are VIP-positive, distributed uniformly in all cortical areas (Morrison *et al.,* 1984). In the forebrain many nuclei including the lateral and basolateral nuclei of the amygdala contain VIP cell bodies (Roberts *et al.,* 1982). VIP is also found in the retina (Loren *et al.,* 1980; Fukuda *et al.,* 1981) caudally in the brainstem and in the dorsal horn of the spinal cord (Loren *et al.,* 1979; Eiden *et al.,* 1982; Honda *et al.,* 1983).

In birds, topographical studies of the distribution of VIP in the CNS have been described for the pigeon (Hof *et al.,* 1991) chicken (Eposito *et al.,* 1994; Kuenzel and Blasher, 1994) and quail (Aste *et al.,* 1995). The VIP-immunoreactivity occurs in several extrahypothalamic regions including the hippocampus, the organum septi laterale (LSO), and the lobus paraolfactorius (LPO) (Eposito *et al.,* 1994; Aste *et al.,* 1995). VIP-ir perikarya are also found in the lateral septum (SL), nucleus striae terninalis (nST), posterior hypothalamus and tuberal hypothalamus (see Section 1.3.2), which are associated with control of reproductive function (Aste *et al.,* 1995). The distribution of VIP in the quail CNS is shown in Fig. 1.4.

Abbreviations for **Figure 1.4**. (*next page*) AA, archistratium anterius; AM, nucleus anterior medialis hypothalami; Ap, archistratium posterius; AVT, area ventralis (Tsai); CP, commissura posterior; DMN, nucleus dorsomedialis hypothalamii; DSV, decussatio supraoptica ventralis; EW, nucleus of Edinger-Westphal; GCt, substantia grisea centralis; Hb, habenula, ICo, nucleus intercollicularis, IP, nucleus interpeduncularis; LA, nucleus lateralis anterior thalami; LFS, lamina fontalis superior; LH, lamina hyperstriatica; LHy, regio lateralis hypothalami; LMD, lamina medullaris dorsalis; LPO, lobus paraolfactorius; LSO, organum septi laterale; MLd. nucleus mesnephalicus lateralis, pars dorsalis; NC, neostriatum caudale; nST, nucleus striae terminalis; OM, nucleus nervi oculomotori; PA, paleostriatum augmentatum; POM, nucleus preopticus medialis; PVN, nucleus paraventricularis magnocellularis, PVT, paleostriatum ventrale; QF, Tratus quintofrontalis, SGFS, stratum griseum et fibrosum superficiale; SGP, srtatum griseum periventriculare; SL, nucleus septalis lateralis, TO, tuberculum olfactorium, Tn, nucleus taeniae, Va, vallecula telecephali.

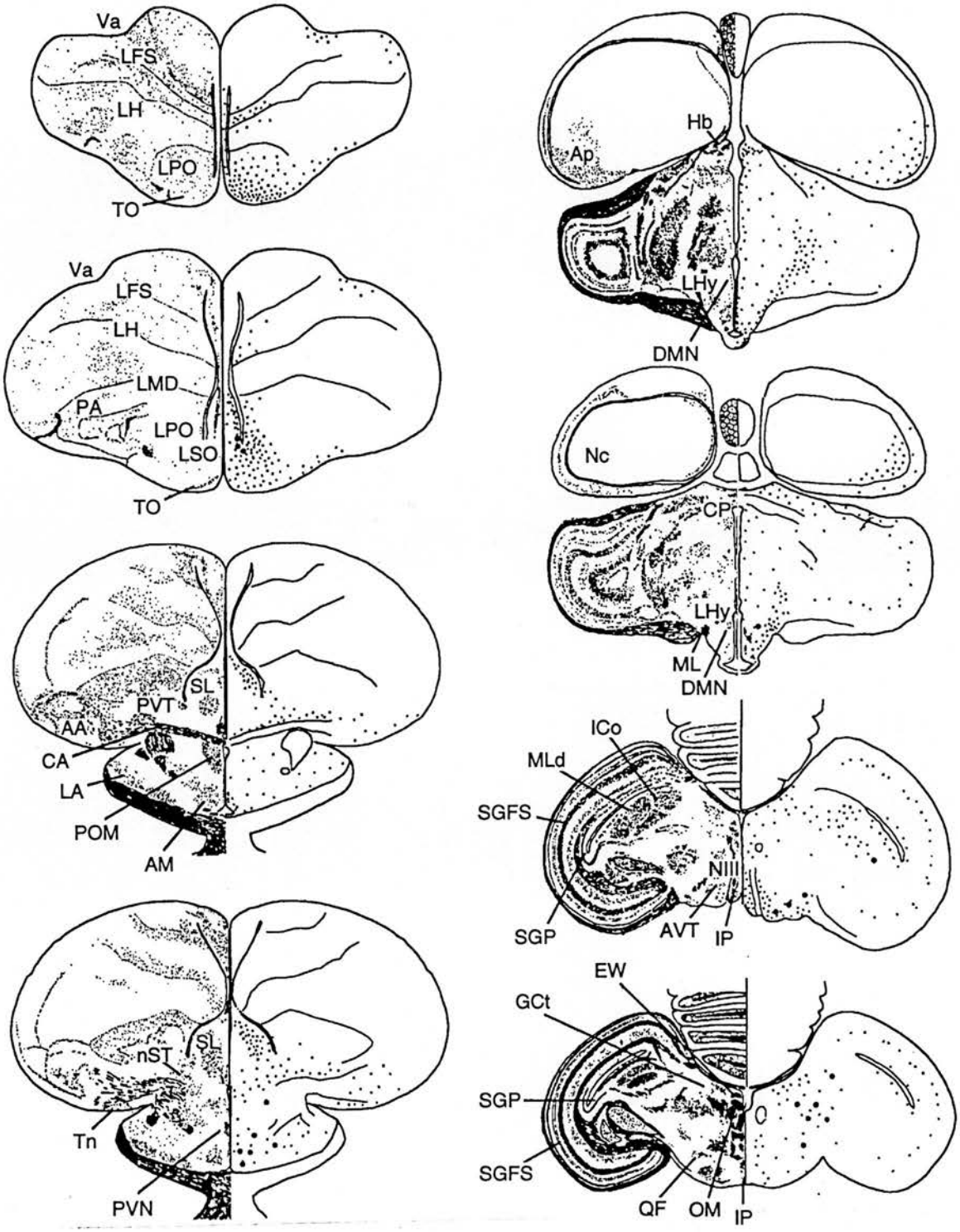


Figure 1.4 Schematic drawings of frontal sections of quail brain illustrating the distribution of VIP-immunoreactivity. Immunopositive cell bodies and fibres are indicated by large dots and small dots respectively. (Aste *et al.*, 1995). For a list of abbreviations see previous page.

1.3.1 VIP in the mammalian hypothalamus

Vasoactive intestinal polypeptide containing cell bodies and nerve terminals are found in the rat anterior hypothalamus, are concentrated in the suprachiasmatic nucleus (SCN) (Besson *et al.,* 1979a, b; Loren *et al.,* 1979; Card *et al.,* 1988). Other more diffusely distributed VIP cell bodies are found elsewhere in the hypothalamus. In the rat, VIP occurs in the hypophyseal portal vasculature at a concentration 10 times greater than that in the peripheral plasma (Said and Porter, 1979; Shimatsu *et al.,* 1981). This suggests that VIP is released from the median eminence, and does not originate from peripheral regions, such as the gut. This view is supported by the observation that VIP concentrations in the portal vasculature are not affected by the removal of the gut (Brar *et al.,* 1985).

Since lesions centred on the VIP cell bodies in the SCN do not depress concentrations of VIP in the portal vasculature, it seems that VIP cell bodies other than those in the SCN, project to the median eminence (Rostene *et al.,* 1982). Retrograde tracing and immunocytochemistry studies in the rat, show that VIP cell bodies in the parvocellular and periventricular parts of the paraventricular nucleus (PVN) project to the median eminence (Dalcik and Phelps, 1993).

1.3.2 VIP in the avian hypothalamus

Vasoactive intestinal polypeptide-immunoreactivity occurs in the septal and preoptic regions of the quail and chicken (Yamada *et al.,* 1982; Macnamee *et al.,* 1986). This corresponds to the distribution of luteinising hormone releasing hormone (LHRH) cell bodies and fibres (Sterling and Sharp, 1982; Panzica *et al.,* 1992), and suggests that VIP may influence LHRH secretion in birds. VIP-like immunoreactivity also occurs in cell bodies and terminals throughout the hypothalamus of the Japanese quail (Yamada *et al.,* 1982), ring dove (Silver *et al.,* 1988) turkey (Mauro *et al.,* 1989) and bantam hen (Macname *et al.,* 1986).

A subset of VIP-ir neurons occur adjacent to the ventral lateral ventricles, which are proposed to be encephalic photoreceptors because they also contain opsin-like immunoreactivity (Silver *et al.,* 1988). Cell bodies containing VIP-like material in the mediobasal hypothalamus (MBH) project to the external layer of the median eminence (ME) (Silver *et al.,* 1988; Sharp *et al.,* 1989). The distribution of VIP in the medial basal hypothalamus and median eminence is consistent with the view that VIP is an avian hypothalamic releasing hormone (Section 1.3.1).

1.4 Central action and neuroendocrine role of VIP in mammals

1.4.1 Biological effects of VIP in the CNS

Vasoactive intestinal polypeptide acts as a neurotransmitter and neuromodulator in the rat CNS, and stimulates adenylate cyclase activity in the retina, olfactory bulb, hippocampus, cerebral cortex, thalamus and hypothalamus, in the presence of calcium (Quik *et al.,* 1978) (see Section 1.8.1). The application of VIP to corticospinal cells induces an excitatory response (Phillis *et al.,* 1978; Phillis and Kirkpatrick, 1980), while depolarisation is induced after the administration of VIP to motor and inter-neurones in the dorsal horn of the toad spinal cord (Phillis and Kirkpatrick, 1980) and pyramidal neurones of the CA1 subfield of the rat hippocampus (Dodd *et al.,* 1979; Dingeldine *et al.,* 1980).

The immunocytochemical demonstration of VIP in nerves innervating blood vessels in the CNS, suggests that VIP may regulate cerebral blood flow (Larsen *et al,.* 1981). The anterior arteries receive a greater VIP-ergic innervation than the caudal arteries (Edvinsson *et al.,* 1980), and a role for VIP in the regulation of central blood flow is established in several species (Heistad *et al.,* 1980; Larsen *et al.,* 1981; Wei *et al.,* 1980). Although it is not certain whether this effect is a direct or an indirect effect through the regulation of neurotransmitters, particularly acetylcholine or norepinephrine.

Vasoactive intestinal polypeptide increases cAMP levels and tyrosine hydroxylase (TH) gene expression (or stability), in cultured avian sympathetic neurons (Zurn *et al.,* 1993). This observation suggests that VIP may play a long term neuromodulatory role in the nervous system via the regulation of TH gene expression, which is an enzyme involved in the biosynthesis of catecholamines (Zurn *et al.,* 1993).

1.4.2 Neurotrophic action of VIP

The amount of VIP in several brain areas changes markedly during embryogenesis, development and growth of the nervous system (Said, 1982; 1984; Gozes and Brenneman, 1989; 1993; Waschek, 1995) and these changes have been shown to reflect a neurotrophic role for VIP in neural tissue. A 30-fold increase in VIP mRNA occurs in the rat cerebral cortex between birth and 30 day of age, after

which VIP mRNA levels decrease to about 60% of the peak levels. This decrease is associated with an increase in the SCN (Gozes *et al.,* 1987; Gozes, 1988), which demonstrates that different factors may operate at different stages of development, to regulate VIP gene expression in specific regions of the brain.

1.4.2.1 Electrical activity and the regulation of VIP

Changes in electrical activity may influence the neurotrophic action of VIP, by regulating VIP gene expression in specific regions of the CNS. This view is supported by the observation that the addition of tetrodotoxin to spinal cord cultures, which blocks synaptic and electrical activity, also accelerates cell death (Brenneman *et al.,* 1985a), and inhibits the secretion and synthesis of endogenous VIP (Brenneman *et al.,* 1985a; Foster *et al.,* 1989; Agoston *et al.,* 1991). Furthermore, the addition of VIP to spinal-cord dorsal root ganglion neurons incubated with tetrodotoxin, prevents neuronal cell death (Kasper and Lipton, 1990). VIP-like immunoreactivity is also found in spinal cord cultures, and incubation with VIP antisera results in significant neuronal cell death (Brenneman and Eiden, 1986; Brenneman and Foster, 1987). These observations suggest that the electrical activity of neurons may modulate cell survival by regulating the expression and secretion of VIP.

1.4.2.2 Trophic action of VIP in the CNS

Many neurotrophic neuropeptides which affect development and or differentiation act directly on the target cell, but the neurotrophic actions of VIP may involve indirect cellular mechanisms (Gozes and Brenneman, 1989; Gozes and Brenneman, 1993). VIP released by neurons may diffuse to adjoining cells and promote neuronal survival, by stimulating high affinity receptors on astroglia (Banker, 1980; Brenneman *et al.,* 1987; 1990; Gozes and Brenneman, 1989; 1993). Glia are thought to have a supportive role for developing neurons by providing directional cues (Wessells *et al.,* 1980; Silver *et al.,* 1982; Noble *et al.,* 1984) and growth promoting substances (Banker, 1980; Muller *et al.,* 1984; Eagleson *et al.,* 1985). The neuronal survival factors released by glial cells in response to VIP are not fully characterised, but one candidate is interleukin-1-like substance (Brenneman *et al.,* 1992), which stimulates glial proliferation (Giulian *et al.,* 1988).

1.4.2.3 Effects of VIP on cell proliferation and differentiation

At micromolar concentrations VIP acts as a mitogenic factor and stimulates cAMP formation and DNA synthesis in embryonic neurons in the superior cervical ganglion (Pincus *et al.,* 1990; 1991). Low concentrations of VIP significantly increase the rate of mitosis in neural and non-neural tissue over a 4 hour period in whole embryos (mouse) (Gressens *et al.,* 1993). VIP may also act as an autocrine regulator of neurogenesis (Wollman *et al.,* 1993). For example, Northern analysis demonstrates that VIP mRNA occurs in a neuroblastoma cell line (Wollman *et al.,* 1993), and VIP is reported to stimulate cell differentiation (O'Dorisio *et al.,* 1992; Pence and Shorter, 1993) and mitosis in some neuroblastoma cell lines (Wollman *et al.,* 1993).

The importance of VIP in the development of the brain is demonstrated in the following studies. The administration of a VIP antagonist, during the development of the CNS in neonatal rats, results in the impairment of neuronal development, acquisition of reflexes, and learning and memory mechanisms, but these effects are reversed by coadministration of VIP (Gozes *et al.,* 1990; Panlilo et al 1990; Hill *et al.,* 1991). It appears that changes in VIP gene expression and VIP secretion from neurons, coupled with changes in the production of neurotrophic factors by glial cells, may mediate the development and growth of the central nervous system.

1.4.3 Effect of VIP on energy metabolism in the CNS

VIP may locally regulate glycogenolysis in several regions of the cortex, an effect which may be mediated by cAMP production (Magistretti *et al.,* 1981). A quantitative autoradiographic ^{14}C-deoxyglucose study demonstrated increased glucose utilisation in caudate nucleus, cingulate cortex and several other regions, in response to VIP administration (McCulloch *et al.,* 1983; McCulloch and Kelly, 1983). In addition, VIP stimulates an increase in glycogenolysis and changes in morphology in astroglia cells acting through specific VIP receptors (see Section 1.5.5) (Magistretti, 1988).

1.4.4 Effect of VIP on pituitary hormone production

1.4.4.1 Prolactin releasing factor in mammals

The release of PRL in mammals is controlled primarily by the inhibitory action of hypothalamic dopamine (Ben-Jonathan *et al.,* 1989; Gala, 1990), although releasing factors do influence PRL secretion to a lesser extent. VIP for example, stimulates PRL secretion in man and rats (Said and Mutt, 1970; Ruberg *et al.,* 1978; Malarkey *et al.,* 1981; Nagy *et al.,* 1988) and passive immunization against VIP in ovariectomized rats, reduces peripheral PRL levels (Lasaga *et al.,* 1989; Murai *et al.,* 1989) and PRL secretion *in vitro* (Hagen *et al.,* 1986; Nagy *et al.,* 1988). VIP stimulates PRL release at plasma concentrations similar to those found in the hypophyseal portal vasculature of lactating rats (Gozes and Shani, 1986; Gozes and Brenneman, 1989; Lam, 1991). This suggests that hypothalamic VIP plays a major role in the release of prolactin during lactation. In support of this view, VIP concentration in the rostral anterior hypothalamus (rAHN) and paraventricular nuclei (PVN) of lactating rats, is higher than in virgin diestrous rats (Chiocchio *et al.,* 1991). Quantitative autoradiography using [I^{125}]-VIP demonstrates that the number of VIP binding sites increase in the striatum, thalamus, anterior portion of the anterior paraventricular hypothalamus and anterior pituitary gland of the female rat during lactation, when PRL levels are significantly elevated (Viau *et al.,* 1992). This observation is consistent with the view that VIP produced by the PVN plays a key role in the suckling-induced release of PRL in rats (Kiss *et al.,* 1986).

1.4.4.2 Growth hormone

VIP is reported to stimulate GH release in mammals (Rotsztejn *et al.,* 1980a; Westendorf *et al.,* 1983) by a direct action on the anterior pituitary gland (Dorflinger and Schonbrunn, 1983). GH release is stimulated by VIP from GH tumor cells *in vitro* (Matsushita *et al.,* 1981) and from superfused rat anterior pituitary cells *in vitro*, provided the cells are treated with dexamethasone (Denef *et al.,* 1985). Intracerebroventricular infusion of VIP in the rat induces GH secretion (Vijayan *et al.,* 1979), and VIP antagonises the action of the GH-inhibiting factor somatostatin *in vitro* (Enjalbert *et al.,* 1982).

1.4.4.3 Adrenocorticotrophic hormone

VIP stimulates ACTH release from primary cultures of rat anterior pituitary cells, but only at high concentrations (1-3μM) which may not be physiologically significant (Westendorf *et al.,* 1983). VIP also induces the release of adrenocorticotrophic hormone *in vitro* (ACTH) from a mouse pituitary corticotroph-like AtT20 cell line (Reisine *et al.,* 1982) and from a human ACTH-secreting pituitary adenoma (Oliva *et al.,* 1982), by stimulating cAMP production. The ability of VIP to stimulate ACTH release from human corticotropinoma cells is additive with arginine vasopressin (AVP), and is modulated by hydrocortisone (White *et al.,* 1982). It seems that VIP may regulate ACTH secretion in certain physiological states by a direct action on the pituitary (Westendorf *et al.,* 1983).

Vasoactive intestinal polypeptide infused into the aorta or cerebral ventricle of the rat stimulates the release of oxytocin, AVP (Bardrum *et al.,* 1987; 1988) and ACTH (Itoh *et al.,* 1982) into the systemic circulation. Similarly infusion of VIP, into the PVN the site of AVP and CRF cell bodies, also increases plasma ACTH and corticosterone (CORT) levels in the rat (Alexander and Sander, 1994). This stimulatory action of VIP is reduced when CRF or AVP antagonists are co-administered (Alexander and Sander, 1995).

Feeding has been shown to influence the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Ishizuka *et al.,* 1983; Al-Dumluji *et al.,* 1987). Central infusion of a VIP antagonist into the PVN, reduces the increase in plasma ACTH and CORT levels associated with feeding (Alexander *et al.,* 1995). The imunocytochemical observation that synaptic connections occur between VIP-containing nerve terminals and AVP neuronal perikarya, supports the view that hypothalamic VIP may play a key role in the release of ACTH releasing factors and ACTH secretion (Ibata *et al.,* 1993). In conclusion, hypothalamic VIP may stimulate ACTH release directly from the corticotroph, but it is more likely to stimulate the release of corticotrophin releasing factors such as CRF or AVP from the PVN (Alexander and Sander, 1995; Alexander *et al.,* 1995).

1.4.4.4 Luteinising hormone

In rats and man VIP may stimulate or inhibit LH secretion acting at the level of the hypothalamus. Vijayan *et al.* (1979) found that the infusion of VIP into the third cerebral ventricle (ICV) of ovariectomised (OVX) rats, increases luteinising

hormone release, presumably by inducing GnRH release. Similarly, studies *in vitro* show that VIP stimulates gonadotrophin hormone releasing hormone (GnRH) from the rat medio-basal hypothalamus (Ohtsuka *et al.,* 1988) and median eminence synaptosomes *in vitro* (Samson *et al.,* 1981).

There is also evidence that VIP inhibits LH secretion at the level of the hypothalamus. Akema *et al.* (1988) found that infusion of VIP into the rat preoptic hypothalamus or third cerebral ventricle in OVX rats significantly reduces LH secretion. This finding has been confirmed in OVX rats treated with oestrogen and progesterone (Alexander *et al.,* 1985; Weick and Stobie, 1992).

The inhibitory action of VIP on GnRH release from the hypothalamus may be mediated through the suprachiasmatic nucleus (SCN) or the PVN. The destruction of the SCN in the rat, by electrolytic lesion abolishes LH pulses, and renders the rat hyper-responsive to the inhibitory action of VIP, on LH release (Stobie and Weick, 1990). However, lesions in the PVN block the inhibitory influence of VIP on LH secretion (Stobie and Weick, 1990). In conclusion, it seems that VIP originating from the SCN, acts directly on the VIP receptors in the PVN (Shaffer and Moody, 1986) to inhibit LH secretion, via a non-VIPergic connection between the PVN and the GnRH pulse generator (Figure 1.5) (Stobie and Weick, 1990; Weick *et al.,* 1992; Weick and Stobie, 1995).

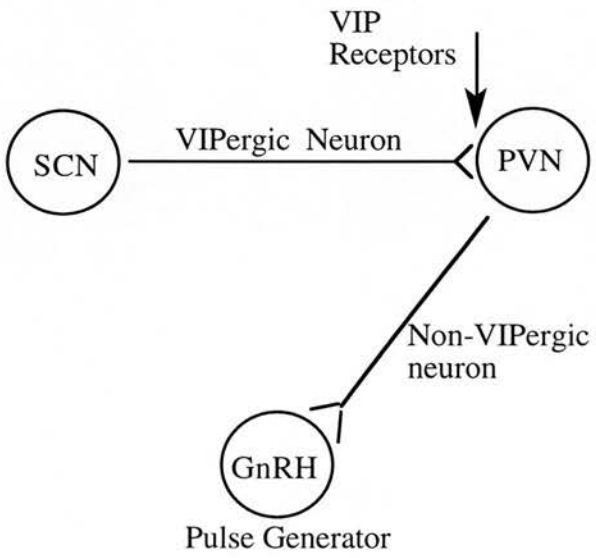


Figure 1.5 A hypothetical model of the SCN-PVN VIPergic pathway that mediates the inhibitory effect of exogenous VIP on pulsatile LH secretion in the rat (Weick and Stobie, 1995).

VIP may also act at the level of the anterior pituitary gland to modulate the action of GnRH. In man, an infusion of VIP into the peripheral circulation, followed by an infusion of GnRH 30 min later, significantly augments the release of LH but not FSH (Hammond *et al.,* 1993). This demonstrates that VIP may act directly at the level of the anterior pituitary to potentiate GnRH-stimulated LH release. However, the LH releasing activity of GnRH in rat anterior pituitary cultures (Culler and Paschall, 1991) or *in vivo* (Alexander *et al.,* 1985; Stobie and Weick, 1989) is not potentiated by pre-treatment with VIP. These observations suggest, that the ability of VIP to augment the LH releasing activity of GnRH is species dependent, and occurs in man, but not the rat.

1.5 The neuroendocrine role of VIP in control of incubation behaviour in birds

Incubation behaviour is characterised by continuous sitting on eggs, aggressive nest protection and warning vocalisations. Riddle *et al.* (1935) was first to demonstrate that PRL plays a major role in broodiness, and this was confirmed by the observation that PRL increases in the pituitary gland and plasma at the onset of incubation behaviour (Sharp *et al.,* 1979; Lea *et al.,* 1981). The main endocrine features associated with incubation are an increase in the concentration of plasma prolactin (PRL), (Burke and Dennison, 1980; Proudman and Opel, 1981; Etches and Cheng, 1982), and a decline in plasma luteinising hormone (LH) and ovarian steroids (Cogger *et al.,* 1979; Sharp *et al.,* 1979). VIP is the avian prolactin releasing hormone and plays a major role in the neuroendocrine control of incubation behaviour in birds (Sharp, 1989, El Halawani *et al.,* 1990) (Section 1.5.3).

1.5.1 Ovarian steroids and the induction of broodiness

In laying hens, rapidly growing ovarian follicles secrete oestrogen which stimulates sexual responsiveness, and induces in concert with progesterone, nesting behaviour (Sharp, 1980). The pre-ovulatory ovarian follicles release large amounts of progesterone during the final stages of maturation (Sharp, 1980). Nesting behaviour associated with oviposition of the resulting ovulated ovum, occurs 24-32 hours after the preovulatory release of progesterone. Bantam and turkey hens lay large clutches of eggs and nesting behaviour is associated with the laying of each egg. After several eggs have been laid, the bird starts to sit on the nest at night,

thereby transforming nesting behaviour gradually into full incubation behaviour (Lea *et al.,* 1981).

1.5.2 Prolactin and the induction of broodiness.

Increased plasma PRL is associated with the expression of incubation behaviour in poultry (Sharp *et al.,* 1979; El Halawani *et al.,* 1986; Sharp *et al.,* 1988) and doves (Goldsmith *et al.,* 1981). The administration of PRL to oestrogen-progesterone primed ovariectomised turkeys, but not to ovariectomised turkeys increases the persistency of nesting behaviour, and transforms it to incubation behaviour. The transformation of oestrogen-progesterone primed dependent nesting behaviour into full incubation behaviour in poultry therefore depends on increased prolactin secretion (El Halawani *et al.,* 1986; Sharp *et al.,* 1988).

Plasma PRL concentration falls when the nest and eggs are removed from the incubating turkey or bantam, but readiness to incubate appears to persist for 2-3 days (El Halawani *et al.,* 1980; Sharp *et al.,* 1988). Active immunization against PRL in the bantam hen blocks the action of PRL and inhibits the development of incubation behaviour (March *et al.,* 1994). This illustrates the importance of circulating prolactin in the induction of incubation in the turkey and chicken. Prolactin may induce incubation behaviour by acting directly on the brain, because intracranial infusion of PRL induces full incubation behaviour in the laying turkey (Youngren *et al.,* 1991).

In the dove, PRL appears to play a less important role in the maintenance of incubation behaviour, than in the turkey or bantam. Peripheral administration of PRL to doves deprived of their nest, maintains their readiness to incubate (Janik and Buntin, 1985), and prolongs the time doves will normally incubate by 5 days (Lea et al., 1986). However, nest deprivation and return studies have also shown that there is no concomitant increase in prolactin secretion in the dove (Lea and Sharp, 1989). Recent studies also suggest that peripheral PRL is not required for the maintenance of incubation behaviour in doves (Lea *et al.,* 1991). These authors showed that passive immunisation against VIP, the major PRL releasing factor in birds, prevents the development of the prolactin-dependent crop sac in incubating doves but does not inhibit the expression of incubation behaviour (Lea *et al.,* 1991). These studies demonstrate that there is difference in the physiological requirement for peripheral prolactin in the dove compared to that of the bantam and turkey.

1.5.3 VIP as the avian prolactin releasing factor

Increased plasma PRL levels associated with the development of incubation behaviour are related to increased amounts of VIP, VIP-cell number, area and immunoreactive density in the mediobasal hypothalamus in bantams, turkeys and doves (Sharp *et al.,* 1989; Mauro *et al.,* 1988; 1989; Cloues *et al.,* 1990; You *et al.,* 1995). Hypothalamic extracts containing VIP stimulate PRL secretion from turkey anterior pituitary cells in culture, while VIP immunoneutralization depresses PRL secretion (El Halawani *et al.,* 1990). The disruption of incubation behaviour by nest deprivation results in a parallel decline in plasma PRL levels and number of VIP neurons in the medio-basal hypothalamus (Mauro *et al.,* 1989). However, VIP neurones found elsewhere in the hypothalamus do not show morphological changes associated with the development of incubation behaviour in the dove (Cloues *et al.,* 1990).

The importance of VIP in the regulation of PRL is demonstrated by the finding that active immunization against VIP in the turkey (El Halawani *et al.,* 1995), or passive immunization against VIP in the chicken (Sharp *et al.,* 1989), suppresses incubation behaviour, and depresses plasma PRL secretion. The release of a prolactin releasing factor and PRL secretion by electrical stimulation of the hypothalamus, is blocked in turkeys which are actively immunized against VIP (Youngren *et al.,* 1994).

VIP increases PRL mRNA in the anterior pituitary gland when injected into laying bantam hens, and PRL mRNA in the pituitary is reduced by passive immunization against VIP in the incubating chicken (Talbot *et al.,* 1991) and turkey (Youngren *et al.,* 1994). The direct action of VIP is mediated in the turkey pituitary gland by specific VIP binding sites (see Section 1.7.3). The number of VIP binding sites in the anterior pituitary gland increase in incubating hens, which correlates with an increase in plasma PRL concentration (Rozenboim and El Halawani, 1993). In conclusion, radioimmunoassay, immunocytochemical, and passive and active immunization studies support the hypothesis that hypothalamic VIP is the main PRL releasing factor in birds.

1.5.4 Active immunization against VIP in the chicken

Active immunization against prolactin in the bantam chicken blocks the biological actions of prolactin, preventing broodiness without disrupting egg production (March *et al.,* 1994). It was predicted that active immunization against VIP would have a similar effect. However, plasma PRL and plasma LH were suppressed in immunized birds, after they were transferred from short to long days (photostimulated) (Fig. 1.6) (Sharp *et al.,* 1993). Photostimulation (week 10) of the control birds stimulated a resumption of egg laying in association with an increase in plasma LH and PRL (Fig. 1.6). The immunized birds began to come back into lay, after all the control birds were laying and plasma PRL was suppressed throughout the experiment. In the immunised birds the plasma LH concentration increased transiently compared to that of the controls, and remained moderately low for a 8-10 week period after photostimulation. The results of this study suggest that in addition to controlling PRL secretion, VIP may also facilitate the function of gonadotrophs and modulate LH secretion.

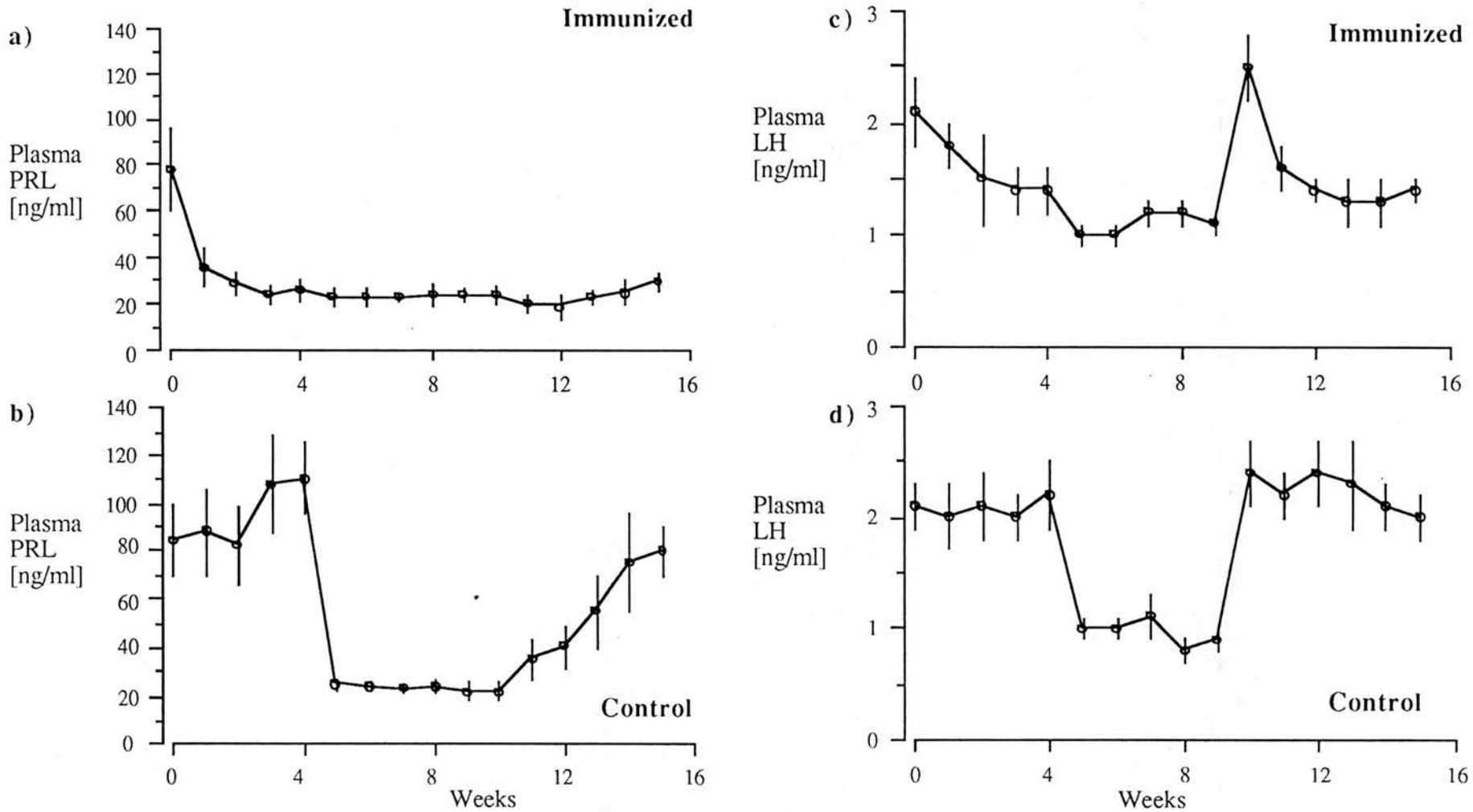


Figure 1.6 The effect of active immunization against VIP on plasma (a, b) prolactin (PRL) and (c, d) luteinising hormone (LH) in the laying bantam hen (mean±SEM, n=12). The immunized and control birds were kept on long days for 4 weeks, and then transferred to short days for 6 weeks to induce ovarian regression, before being transferred back to long days.(a) Plasma prolactin, and after a transient increase, plasma LH, were suppressed in the immunized birds after they were transferred from short to long days. Photostimulation of control birds stimulated a rapid increase in plasma LH, and a gradual increase in plasma PRL (Sharp et al., 1993).

1.6 Discovery of VIP in the anterior pituitary gland

The observation that basal PRL release from rat dispersed anterior pituitary cells is suppressed in the presence of VIP antibodies, suggests that PRL secretion may be regulated by VIP which is synthesised by the anterior pituitary (Hagen *et al.,* 1986). This was confirmed by the observation that anterior pituitary fragments incorporated [^{3}H]-labelled leucine into immunoreactive intra-pituitary VIP *in vitro* (Arnaout *et al.,* 1986), and immunoreactive VIP was immunoprecipitated by a specific anti-VIP antiserum (Lam *et al.,* 1989).

Early immunocytochemical studies demonstrated that a VIP-cell type is not easily detected in the pituitary gland of normal rats, but is observed following thyroidectomy or chronic administration of oestrogen (Lam *et al.,* 1989; Steel *et al.,* 1989; Koves *et al.,* 1990a). Immunocytochemical studies showed that VIP is present in cells in the anterior pituitary gland, but there are still doubts concerning the cell type synthesising VIP. While some authors have reported that intra-pituitary VIP in the rat is synthesised and colocalised in lactotrophs (Morel *et al.,* 1982; Nagy *et al.,* 1988), others have evidence that this is not the case (Lam *et al.,* 1989; Segerson *et al.,* 1989; Carrillo and Phelps, 1992). The latter authors reported that the pituitary VIP immunoreactive cell type is distinct from lactotrophs and thyrotrophs (Lam *et al.,* 1989; Segerson *et al.,* 1989; Carrillo and Phelps, 1992). Therefore it appears that VIP synthesis occurs in the rat anterior pituitary gland, but there is doubt whether the lactotroph or a different cell type cell, contains VIP immunoreactivity.

1.6.1 The influence of steroids on intra-pituitary VIP

Intra-pituitary VIP content is manipulated by a several endocrine factors. VIP gene expression is reduced after ovariectomy in rats (O'Halloran *et al.,* 1990), and increases after treatment with oestrogen (Lam, 1991; Kasper *et al.,* 1992). Oestrogen administration in the female rat also increases the number of VIP-ir cells (Steel *et al.,* 1989). However, the effect of oestrogen on VIP pituitary content and gene expression, may only be of pathological significance. The high doses of oestrogen (70μg) (Carrillo *et al.,* 1991) required to induce changes in VIP content, induced an abnormal hyperprolactinemia (Maletti *et al.,* 1982; Montagne *et al.,* 1995). Low doses of oestrogen (7μg) stimulate a significant increase in pituitary PRL content, without changing plasma PRL levels or VIP content (Carrillo *et al.,* 1991). The increases in pituitary VIP and PRL content following oestrogen

treatment support the view that VIP may play a role in the development of oestrogen-induced hyperprolactinaemia in the rat (Carrillo *et al.,* 1991; Montagne *et al.,* 1995).

1.6.2 Effect of thyroidectomy on intra-pituitary VIP

Thyroidectomy results in an increase in VIP content and mRNA in the pituitary, which is reversed following thyroxine (T4) replacement, but these effects are not observed in the hypothalamus (Lam *et al.,* 1989; Lam and Srivastava, 1990; Jones *et al.,* 1994). This evidence suggests that the response of the anterior pituitary to hypothyroidism is tissue specific, but the factor or factors which influence VIP expression in the anterior pituitary are not known.

1.6.3 Effect of adrenalectomy on intra-pituitary VIP

There is evidence that VIP levels in the brain are controlled by corticosteroids. Adrenalectomy reduces the concentration of VIP in the hippocampus (Rotsztejn *et al.,* 1980b) and of VIP mRNA in the SCN and anterior pituitary gland. The depressive effect of adrenalectomy on VIP mRNA in the juvenile rat anterior pituitary but not in the hypothalamus, is reversed by treatment with dexamethasone (Lam *et al.,* 1992). In contrast, adrenalectomy in the adult rat reduces VIP mRNA in the SCN of the hypothalamus, but this effect is not reversed by corticosterone replacement (Gozes *et al.,* 1994). These observations suggest that adrenal steroids regulate VIP in the pituitary gland, but indirect mechanisms influenced by the adrenal gland regulate VIP in the SCN.

1.6.4 The influence of the hypothalamic factors on intra-pituitary VIP

Anterior pituitary VIP content is significantly reduced in the rat, after removing the hypothalamic input to the anterior pituitary. This was achieved by transplanting the anterior pituitary under the kidney capsule, (Prysor-Jones *et al.,* 1987) or by colchicine administration which blocks the release of hypothalamic factors, which act on the anterior pituitary gland (Carretero *et al.,* 1992). These studies support the view that hypothalamic factor(s) may modulate intra-pituitary VIP.

Lesions of hypothalamic nuclei, also block the release of hypothalamic factors which may influence intra-pituitary VIP content. For example, the ablation of the hypothalamic arcuate-median eminence, results in a reduction of concentration of VIP in the anterior pituitary gland (Carrillo and Dluzen, 1993). Furthermore, the increase in pituitary VIP concentration, which is induced by hypothyroidism, is

significantly reduced by anterolateral deafferentation of the hypothalamus (Mickalkiewicz and Suzuki, 1994). This observation shows that the biosynthesis of VIP in the pituitary is not due solely to the withdrawal of thyroid hormones (see Section 1.6.2), but is mediated by a factor released by the hypothalamus in response to hypothyroidism.

In rats it has been suggested that serotonin may be released from the hypothalamus to stimulate PRL secretion (Ben-Jonathan, *et al.,* 1989), and by inference VIP secretion. Serotonin may stimulate intra-pituitary VIP mRNA, which in turn, stimulates PRL release. This view is supported by the observation that the tryptophan hydroxylase inhibitor, para-chlorophenylalanine methyl ester (pCPA) an inhibitor of serotonin biosynthesis, depletes the stores of serotonin in the medial basal hypothalamus, and reduces VIP mRNA in the rat anterior pituitary gland (Signs *et al.,* 1993). The administration of 5-hydroxytryptophan a precursor for serotonin, supplements the loss of serotonin in pCPA treated rats, and partially restores intra-pituitary VIP mRNA (Signs *et al.,* 1993). The use of specific 5-HT receptor antagonists also significantly reduces VIP mRNA levels in the anterior pituitary, and supports the hypothesis that hypothalamic serotonin may play a major role in the expression of VIP mRNA in the rat anterior pituitary gland (Signs *et al.,* 1993; 1994).

1.6.5 A paracrine or autocrine role for VIP in the regulation of lactotroph function

Pituitary VIP may play an autocrine role in the regulation of PRL secretion. Nagy *et al.,* (1988) used a haemolytic plaque assay to study PRL release from individual lactotrophs and demonstrated that VIP antiserum suppressed PRL secretion. Therefore, it appears that VIP may stimulate PRL secretion from the lactotroph in an autocrine manner. This view is supported by the observation that VIP was localised in a small population of lactotrophs (Morel *et al.,* 1982; Steel *et al.,* 1989; Koves *et al.,* 1990a), although these immunocytochemical observations have not been confirmed by other authors (see Section 1.6).

The influence of VIP on PRL secretion in rats has been studied by implanting the anterior pituitary gland under the kidney capsule, and to induce hyperprolactinaemia or by implanting GH_3 (PRL cell line) tumors. Under these conditions hyperprolactinaemia suppresses intrapituitary VIP content, an effect which is reversed by oestrogen treatment (Prysor-Jones *et al.,* 1988; Montagne *et al.,* 1995). Therefore, it seems likely that intra-pituitary VIP mediates the action of

oestrogen in the rat anterior pituitary and possibly induces hyperprolactinaemia in certain physiological situations (Montagne *et al.,* 1995), but it is still not clear whether this is in an autocrine/paracrine manner.

1.7 The VIP receptor

1.7.1 Characterisation of the VIP binding site

The VIP-binding protein has been characterised using a covalent cross-linking technique, in several tissues (Laburthe *et al.,* 1984: Laburthe and Couvineau, 1988; Luis *et al.,* 1988). The apparent molecular weights of the VIP-binding protein, vary according to species and tissue, ranging from 45kDa to 73kDa. Structural differences in the protein core or post-translational glycosylation of the VIP binding site, may account for the differences in molecular weight. The cloning of the rat VIP receptor has confirmed the presence of glycosylation sites (see Section 1.8.3) (Ishihara *et al.,* 1992), and differences in the composition of the oligosaccharide chains attached to the VIP receptor may be responsible for its heterogeneity (Fabre *et al.,* 1993).

1.7.2 Signal transduction pathways linked to the VIP receptor

The VIP receptor is coupled to guanosine tri-phosphate (GTP)-binding proteins, (G-protein) which are heterotrimers, consisting of α, ß and γ subunits (see Figure 1.6). The G-proteins bind to guanine nucleotides, providing a bridge between the receptor and the signal transduction pathway (Fig. 1.7). When guanosine diphosphate (GDP) is bound (α subunit) to the heterotrimer, the receptor complex is inactivated. However, when VIP interacts with the membrane receptor protein, the ß and γ subunits bind to the receptor-ligand complex and dissociate from the α subunit which has GTPase activity, and exchanges GDP for GTP. The α subunit undergoes a transformation and interacts with adenylate cyclase, which has the ability to catalyse the dephosphorylation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The cAMP molecule acts as a second messenger, responsible for the induction of downstream events, including the stimulation of protein kinase A (PKA), and Ca^{2+} influx, which may also lead to a changes in gene transcription.

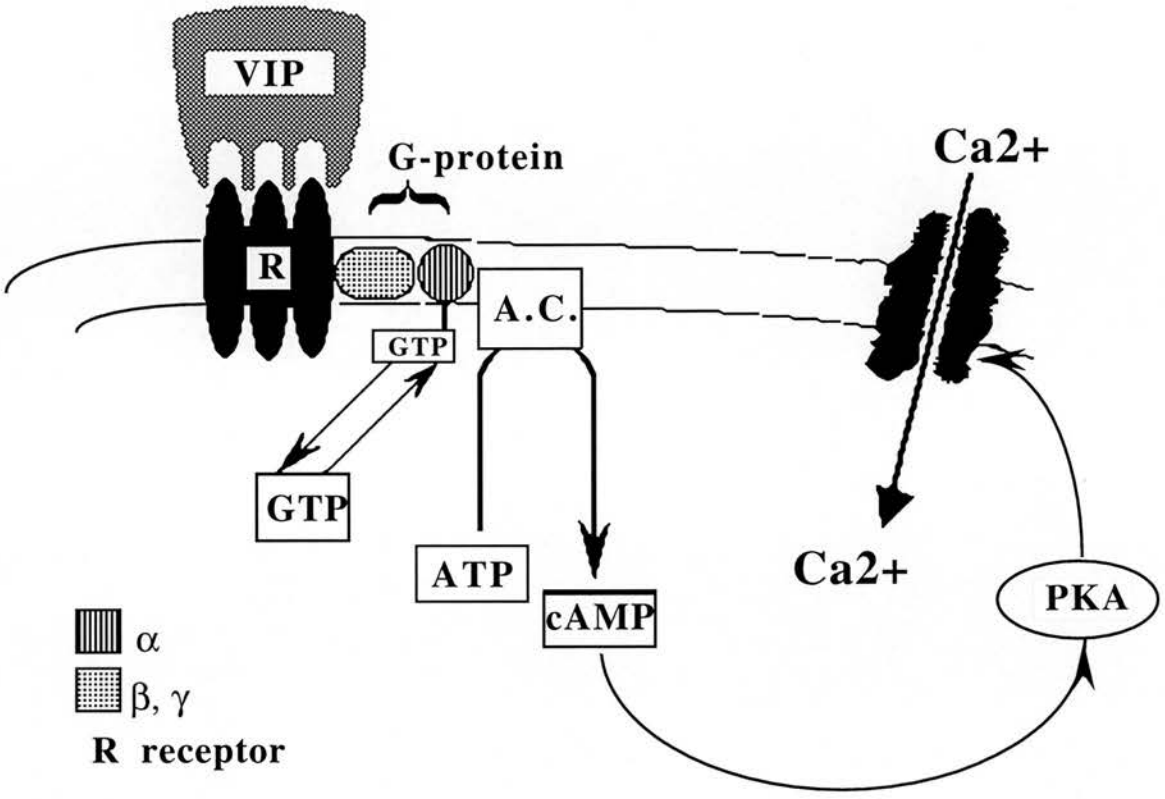


Figure 1.7 Schematic representation of the proposed second messenger pathway for VIP. *Abbreviations*: *AC* adenylate cyclase; *ATP* adenosine triphosphate; Ca2+ calcium; *cAMP* cyclic adenosine monophosphate; *GDP* guanosine diphosphate: *GTP* guanosine triphosphate: *PKA* protein kinase A (Adapted from Bennett and Whitehead, 1983).

1.7.3 The avian VIP receptor

The avian VIP receptor has not been cloned but has been characterised by covalent cross linking radiolabelled VIP to chicken pineal gland membranes (Meunier *et al.*, 1991). Two high affinity binding sites were observed with apparent molecular weights of 57kDa and 70kDa (Meunier *et al.*, 1991). The distribution of VIP binding sites has been mapped in the brain of a pigeon, using autoradiography (Hof *et al.*, 1994; Kuenzel *et al.*, 1996). Intense labelling occurs in the periventricular area and moderate to low binding occurs in the nucleus posteromedialis hypothalami and lateral hypothalami (Hof *et al.*, 1994). These areas contain VIP immunoreactive fibres as reported in the quail (Yamada *et al.*, 1982) and the chicken (Macnamee *et al.*, 1986; Aste *et al.*, 1995).

The turkey anterior pituitary contains high- and low-affinity binding sites for VIP (Rozenboim and El Halawani, 1993). This is consistent with observations in the rat anterior pituitary (Wanke and Rorstad, 1990b) uterus (Huang and Rorstad, 1990), pineal (Kaku *et al.,* 1983) and blood vessels (Huang and Rorstad, 1987). The number of VIP-binding sites in the anterior pituitary increases in the incubating turkey hen (Rozenboim and El Halawani, 1993), in association with the increase in concentration of pituitary PRL content and plasma PRL (Mauro *et al.,* 1989; Sharp *et al.,* 1989).

A VIP receptor has been characterised in the chicken anterior pituitary gland, hypothalamus, and median eminence, and shown to be represented by a single class high affinity binding site (Gonzales *et al.,* 1994b). The specific binding of the VIP receptor in the cephalic lobe of the anterior pituitary gland is greater than in the caudal lobe of the anterior pituitary, but increases in both lobes after progesterone treatment (Gonzales *et al.,* 1994a). The specific binding of the VIP receptor is also greater in the cephalic lobe of the incubating hen compared to the cephalic lobe of the laying hen (Gonzales *et al.,* 1994c). This evidence supports the view that avian VIP, as in the rat, stimulates PRL secretion directly from the lactotroph, or a cell type which occurs predominantly in the cephalic lobe.

1.7.4 Molecular biology of the mammalian subtype-I VIP receptor

The subtype-I VIP receptor has been cloned from man (Sreedharan *et al.,* 1993) and rat (Ishihara *et al.,* 1992), and was shown to be structurally related to the secretin (Ishihara *et al.,* 1991), calcitonin (Lin *et al.,* 1991) and parathyroid hormone (Juppner *et al.,* 1991) receptors. These receptors all have seven transmembrane domains, are linked to G-protein and are part of a signal transduction pathway involving adenylate cyclase (see Section 1.8.2).

The human VIP receptor (hVIP-RI) cDNA was isolated from Nalm 6 leukaemia pre-B cells and HT-29 human colon adrenocarcinoma cells (Sreedharan *et al.,* 1993), which are two cell lines which possess specific receptors for VIP (O'Dorisio *et al.,* 1989; Sambrook *et al.,* 1989). The potential membrane topography of the seven hydrophobic segments is shown in Fig. 1. 8

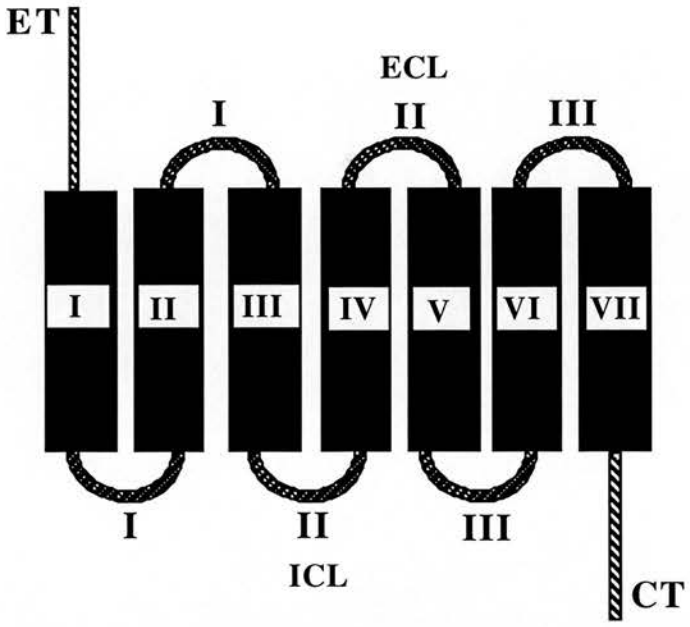


Figure 1.8 A diagram illustrating the potential membrane topography of the human subtype-I VIP receptor. The seven hydrophobic transmembrane segments are shaded black. The extracellular amino terminus is designated ET, and three extracellular loops are designated ECL-I, -II, and -III, respectively. The intracellular carboxyl terminus is labelled CT, and the three intracellular loops are labelled ICL-I, -II, and-III respectively (Sreedharan *et al.,* 1993).

The rat VIP-R (rVIP-RI) cDNA was isolated by generating a cDNA library from the human colon adrenocarcinoma WiDr cell line (derivative of human HT-29), and screened using a rat secretin receptor cDNA (Ishihara *et al.,* 1991) as a probe (Ishihara *et al.,* 1992). An intact cDNA clone was isolated after Northern analysis of rat lung mRNA, using the human WiDr cell line as a probe (Ishihara *et al.,* 1992). The specificity of the VIP-RI cDNA isolated from both man and the rat, was confirmed by transfection into a cell line (human- COS-7; rat-COP), which does not express the VIP receptor. When the clones were expressed in these cell lines, VIP stimulated adenylate cyclase and cAMP production (Sreedharan *et al.,* 1993; Ishihara *et al.,* 1992). The VIP-RI mRNAs are detected in tissues which contain specific VIP-binding sites, including the liver, lung, small intestine and brain (Sreedharan *et al.,* 1993; Ishihara *et al.,* 1992).

Following the discovery of the subtype-II VIP receptor (see Section 1.7.5) the original rat and human VIP-RI described in this section, were designated the subtype-I VIP-receptor (VIP-RI).

1.7.5 Molecular biology of the subtype-II VIP-receptor

VIP-binding proteins occur in the brain and periphery with a range of molecular weights (see Section 1.8) and it was originally suggested that there are several VIP receptors (Laburthe *et al.,* 1989; Robberecht *et al.,* 1990). Similarly, studies using peptide analogues have demonstrated the existence of more than one pharmacologically distinct class of VIP receptor (Robberecht *et al.,* 1986; Gozes *et al.,* 1991a, b; Blum *et al.,* 1992). Two subtypes of the VIP receptor are known which differ in their sensitivity to GTP analogues (Hill *et al.,* 1992). These two receptor subtypes, in the mouse embryo, can be differentially regulated by the treatment with a VIP antagonist (Gressens *et al.,* 1993).

Although, mRNA for the hVIP-RI is abundant in the lung and small intestine, there are several tissues which have specific VIP binding sites, such as the spleen, pancreas and adrenal, which do not express subtype-I VIP-R mRNA (Sreedharan *et al.,* 1993). A second VIP receptor cDNA clone was isolated by PCR, from a rat pituitary cDNA library. Degenerate oligonucleotide primers corresponding to the third and seventh transmembrane domains of the G-protein linked receptor family were used to isolate a full-length cDNA clone from a rat olfactory bulb cDNA library (Lutz *et al.,* 1993). When the clone is expressed in COS-7 cells, VIP stimulates cAMP production. The distribution of this VIP receptor mRNA is also consistent with that of VIP-binding sites (Lutz *et al.,* 1993), but differs from that of the human and rat subtype-I VIPR mRNA (Sreedharan *et al.,* 1993; Ishihara *et al.,* 1992). This second VIP-R has a different sequence and distribution from the first human and rat VIP receptors. The second VIP-R is designated the subtype-II VIP-receptor (VIP-RII) by Lutz *et al.* (1993), and the first VIP receptor cloned by Sreedharan et al (1993) and Ishihara et al (1992), has been designated the subtype-I VIP-receptor (VIP-RI).

1.7.6 Differential distribution of the type-I and type-II VIP receptor

The distribution of the VIP-RI and VIP-RII, has been described in the central and peripheral nervous systems of the rat, using PCR, Northern analysis and *in situ* hybridization (Usdin *et al.,* 1994). The study revealed that the two receptors are pharmacologically similar, but are differentially expressed in most tissues (Table 1.1) (Usdin *et al.,* 1994).

TABLE 1.1 The distribution of mRNA for the VIP-RI and VIP-RII in several regions of the rat brain. *Abreviations*: anteroventral nucleus (*AVN*); suprachiasmatic nucleus (*SCN*), organum vasculosum lamina terminalis (*OVLT*); paraventricular nucleus (*PVN*); supraoptic nucleus (*SON*); accessory magnocellular nucleus (*AMN*); dorsal medial nucleus (*DMN*) (Usdin *et al.,* 1994).

Location	***VIP-RI***	***VIP-RII***
Cerebral cortex:	Layers III and V	Layers VI
Thalamus:	Anteroventral nucleus (AVN)	Throughout the thalamic nuclei except the AVN
Hippocampus:	Uniform expression	Also expressed
Hypothalamus:	No expression	Medial & lateral preoptic nuclei; OVLT; periventricular portion & ventral subdivision of the SCN; Magnocellular cells of the PVN, SON & AMN; DMN, posterior hypothalamic nucleus & mammillary nuclei.
Anterior pituitary:	Little or no expression	Highly expressed in many cells

In conclusion, the VIP-RI mRNA is found in peripheral tissues where VIP acts through specific VIP-binding sites, but not in the testes and stomach. In these tissues the action of VIP may be mediated by a second VIP receptor subtype, and this is supported by the observation that VIP-RII mRNA, and not VIP-RI is expressed in the testes and stomach (Usdin *et al.,* 1994). In addition, the VIP-RII mRNA is also found in a number of regions in the central nervous system associated with neuroendocrine function, including several hypothalamic nuclei and the pituitary gland (Usdin *et al.,* 1994).

1.7.7 VIP binding sites in the anterior pituitary gland

VIP-binding sites have been demonstrated in anterior pituitary tissue, in a corticotroph-like (AtT20) cell line, and in two prolactin secreting GH cell lines. In the anterior pituitary, the high affinity VIP binding sites are the same as those that are found in other VIP responsive tissues (Rosselin, 1986; Wanke and Rorstad, 1990b). A specific VIP-binding site is also found on lactotrophs (Wanke and Rorstad, 1990a).

A second low affinity VIP binding site also occurs in the pituitary gland (Wanke and Rorstad, 1990b), but its significance is not known. It is possible that VIP may also bind to VIP-like receptors present in the anterior pituitary which have a lower affinity for VIP, than the specific VIP receptor (Magistretti et al. 1986). Several peptide receptors in the peripheral and central nervous system share a structural homology with the VIP receptor (secretin-glucagon receptor family), and these VIP-like receptors can bind VIP and other VIP-like peptides to a varying degree. For example, VIP may bind to the subtype-I PACAP receptor (PACAP-RI) which is highly expressed in the anterior pituitary gland. The PACAP-RI has a high affinity for PACAP, but a lower affinity for VIP (see Section 1.8.4). Low affinity VIP binding sites also occur in a GH_3 cell line (Wood *et al.,* 1985), whereas low and high affinity binding sites occur in the GH_4C_1 cell line (Bjoro et al. 1987).

Steroids affect VIP-binding sites in the pituitary gland. In male castrated rats the number of VIP binding sites increase in the rat anterior pituitary gland, an effect which is reversed by a prior injection of testosterone. In contrast, VIP-binding sites in the liver, prostate or brain are not affected by steroids (Wanke *et al.,* 1990). This suggests that testosterone may play a role in the regulation of VIP receptor function in the male anterior pituitary gland.

1.7.8 VIP receptor mRNA in the anterior pituitary gland

The mRNA for the VIP-RII occurs in the rat anterior pituitary gland (Lutz *et al.,* 1993; Rawlings *et al.,* 1995) and VIR-RII mRNA is greater in the pituitary of a pregnant rat compared to that of a nonpregnant rat (Usdin *et al.,* 1994). The VIP-RII also occurs in the somatotroph-like GH_4C_1 cell line (Rawlings *et al.,* 1995), consistent with reports of VIP-binding sites in normal somatotrophs and clonal GH_3 cells (Rawlings *et al.,* 1993; Coleman and Bancroft, 1993; Deutsch and Sun, 1992). VIP-RII mRNA is also found in a gonadotroph-like αT3-1 cell line, and

analysis of PRL and GH enriched cell populations with PCR primers, suggests that type-II VIP receptor mRNA is the principal VIP-R in these cell types (Vertongen *et al.,* 1995a).

Little or no mRNA for the VIP-RI is found in the pituitary (Usdin *et al.,* 1994; Rawlings *et al.,* 1995). VIP does stimulate differentiation in the corticotroph-like AtT20 cell line (Braas *et al.,* 1994) and the mRNA encoding the VIP-RI and VIP-RII is present in this cell line (Journot and Rawlings, unpublished data). The VIP-RI mRNA has not been demonstrated in normal corticotroph cells, but its presence would explain the low level of VIP-RI mRNA detected in the rat anterior pituitary gland (Usdin *et al.,* 1994; Rawlings *et al.,* 1995).

1.8 Pituitary adenylate cyclase activating polypeptide

A hypothalamic peptide similar to VIP originally isolated from the ovine hypothalamus, stimulates adenylate cyclase, and cAMP production in anterior pituitary gland cells. It is known as pituitary adenylate cyclase activating polypeptide (PACAP) (Miyata *et al.,* 1989) and occurs as a 38 amino acid, C-terminally amidated peptide (PACAP38) and a 27 amino acid form (PACAP27) (Miyata *et al.,* 1989: 1990).

PACAP cDNA has been isolated from several species (Kimura *et al.,* 1990; Ogi *et al.,* 1990; 1993), and the deduced amino acid sequences are identical. The chicken form of PACAP38 is identical to mammalian PACAP, except at position 2 where Ile replaces Ser (Yasuhara *et al.,* 1993) (see Section 1.2.2). The first 28 amino acids of mammalian PACAP38 share a 68% homology with porcine VIP (Miyata *et al.,* 1989), and is also a member of the VIP/Secretin gene family. It is possible that many biological effects of VIP could be mediated by PACAP or vice versa, since there is a high degree of structural similarity between these neuropeptides.

1.8.1 Distribution of PACAP

Like VIP, PACAP is widely distributed in the mammalian peripheral and central nervous systems (see reviews Arimura, 1992; Christophe, 1993), but there are a number of cases where the distribution of PACAP is different from that of VIP. PACAP-immunoreactivity occurs in nerve fibres in the gut wall of the rat, human, sheep and chicken (Sundler *et al.,* 1992). PACAP-ir also occurs in endocrine cells

of the proventriculus, and is colocalised with VIP in nerve cell bodies and nerve fibres in the chicken gut (Sundler *et al.,* 1992). This observation suggests that PACAP like VIP, is involved in the regulation of motor and secretory activities of the gastrointestinal tract.

The distribution of PACAP immunoreactive neurons has been studied in the brain of the rat, human and sheep (Koves *et al.,* 1990b: 1991; Vigh *et al.,* 1991). PACAP cell bodies are present in the supraoptic and paraventricular nuclei, of the rat and ovine hypothalamus, and immunopositive fibres are also found in the external and internal zones of the median eminence (Koves *et al.,* 1990b: 1991; Vigh *et al.,* 1991). These observations support the view that PACAP like VIP, may also act as a hypothalamic releasing factor to regulate anterior pituitary function or secretion. PACAP immunoreactivity also occurs in the chicken brain (Yasuhara *et al.,* 1992), but there are no published reports describing its distribution. Based on the observations in mammalian species, it is likely that PACAP would have a similar distribution and role in the chicken.

1.8.2 Multiple receptors for PACAP and VIP

Binding studies show that PACAP and VIP bind to two major groups of receptor. PACAP is 1000 times more potent than VIP, in stimulating cAMP production in the rat anterior pituitary gland (Miyata *et al.,* 1989), and specific receptors have been identified in the pituitary (Vigh *et al.,* 1993) which have a greater affinity for PACAP than VIP (Gottschall *et al.,* 1990). The PACAP receptor which binds PACAP38 and PACAP27 with a greater affinity than VIP is referred to as the PACAP Type I receptor (PACAP-I-R or PVR1), and its encoding gene has been cloned by several groups (Rawlings, 1994; Harmar and Lutz, 1994; Arimura and Shioda, 1995). The PACAP-I-R gene is expressed in several regions of the rat CNS, and pituitary, adrenal, and testis, but there is little or no expression in other peripheral tissues (Spengler *et al.,* 1993; Hashimoto *et al.,* 1993; Ogi *et al.,* 1993).

The second receptor subtype which binds PACAP38, PACAP27 and VIP with approximately the same affinity, is the classic VIP receptor (VIP-RI/II) (Section 1.7.4 and 1.7.5), which is also known as the PACAP Type II receptor (PACAP-II-R or PVR2/PVR3). Both type I and type II PACAP receptors are linked to G-proteins and are part of the VIP/secretin family of receptors (Christophe, 1993; Rawlings, 1994).

1.8.3 Effect of PACAP on pituitary hormone cells

PACAP stimulates ACTH, GH, LH and PRL secretion from superfused rat pituitary cells, but has no effect on static cultures despite an increase in cAMP production (Miyata *et al.,* 1989). PACAP also stimulates a change in intracellular Ca2+ ([Ca2+i]) in several pituitary cell types, notably somatotrophs, gonadotrophs and follicular stellate cells (Canny *et al.,* 1992; Yada *et al.,* 1993). In gonadotrophs PACAP stimulates a 'Ca2+ spike' response which is independent of extracellular Ca2+, and is believed to involve the release of Ca2+ from an intracellular store (Canny *et al.,* 1992) through a 1,4,5-inositol triphosphate [Ins(1,4,5)P3]-dependent mechanism (Fig. 1.9) (Rawlings *et al.,* 1993; 1994).

In addition, PACAP stimulates an extracellular Ca2+ dependent 'Ca2+ plateau' response in gonadotrophs and somatotrophs (Canny *et al.,* 1992) , which is likely to result from the stimulation of adenylate cyclase and cAMP production through the PACAP-II-R (VIP-R). There are cases where it appears that the PACAP-I-R is coupled to both the adenylate cyclase and inositol phosphate turnover (PI) signalling pathways, through different G-proteins (Christophe, 1993). This view is supported by the observation in the T3-1 (LH-like) cell line, that PACAP stimulates a Ca2+ store-dependent Ca2+ spike, through a inositol phosphate pathway and Ca2+ influx. In contrast, VIP only stimulates Ca2+ influx, and with a lower potency than that of PACAP. (Rawlings *et al.,* 1995). These observations suggest that PACAP-I-R is more abundant than the VIP-RII in the αT3-1 cell line. Alternatively, the PACAP-I-R may be more efficiently coupled to adenylate cyclase than the VIP-RII, or may compete more effectively for available G-proteins (Rawlings *et al.,* 1995).

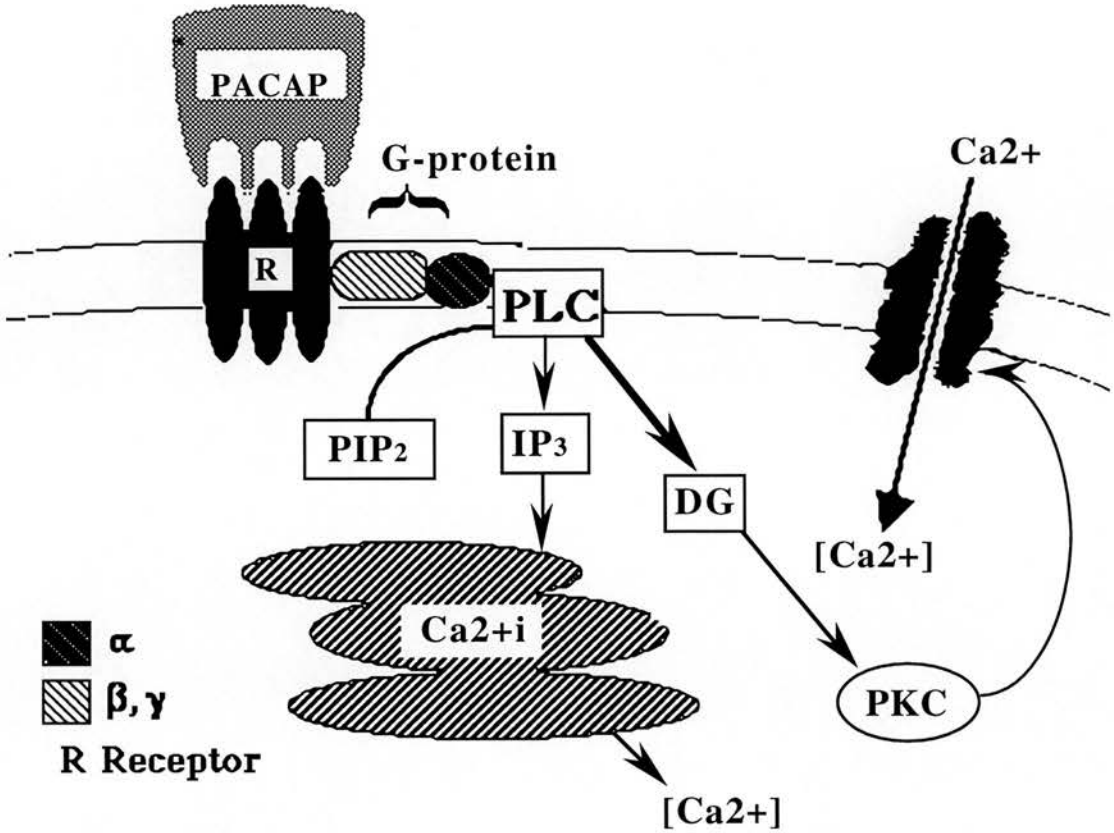


Figure 1.9 Schematic representation of the inositol phosphate (PI) signal transduction pathway triggered by the Type-I PACAP receptor in gonadotrophs. *Abbreviations*: *Ca2+* Calcium; *Ca2+i* intracellular Calcium; *DG* diacylglycerol; *IP3* inositol 1,4,5-triphosphate, *R* VIP receptor; *PIP2* phosphatidylinositol 4,5,-biphosphate; *PKC* Protein kinase C; *PLC* Phospholipase C.

1.8.4 VIP-PACAP receptors in the anterior pituitary

Observations on VIP-PACAP receptors in the anterior pituitary gland and pituitary derived cell lines suggest that differential expression might result in a complex regulation of pituitary cell function by VIP and PACAP. This point is illustrated in Table 1.2 which shows the relationship between VIP/PACAP receptors and their signal transduction pathways in various cell types.

TABLE 1.2 . Expression of VIP-PACAP receptors in pituitary derived cell lines and pituitary tissue, and the signal transduction mechanisms linked to each receptor subtype. *Abbreviations*: *A.C.* adenylate cyclase; *αT3-1* gonadotroph-like cell line; *AtT-20* corticotroph-like cell line; *GH4C1* somatotroph/lactotroph-like cell line; *PKA* Protein kinase A; *PKC protein* kinase C; *PLC* phospholipase C; *PVR1* PACAP receptor subtype I; *PVR2* PACAP receptor subtype II & VIP receptor subtype I; *PVR3* PACAP receptor subtype III & VIP receptor subtype II; (+) expression; (-) no expression (Rawlings *et al.,* 1995).

	RECEPTOR TYPE		
	PVR1	***PVR2***	***PVR3***
Cell type			
αT3-1	+	-	+
GH_4C_1	-	-	+
AtT-20	-	+	-
Pituitary	+	+	+
Signal transduction pathway			
A.C. & PKA	+	+	+
PLC & PKC	+	-	-

1.9 Paracrine control of anterior pituitary function

In an endocrine tissue such as the anterior pituitary gland, hormones are released from cells into the peripheral circulation to reach distant specific target cells (Denef, 1986). Hormones can also be released locally to influence neighbouring cells to act in a paracrine manner. When the secretory cell regulates its own function, through receptors on its surface for its own secretory product, the cell is regulated in an autocrine manner (Le Roith *et al.,* 1983, Denef, 1986; Schwartz and Cherny, 1992).

A number of features of the anterior pituitary cells are consistent with a role for paracrine control mechanisms within the gland. These include the topographical arrangement of different pituitary cells, the presence of neuropeptides, growth factors and biogenic amines which effect pituitary hormone release *in vitro* and the local action of pituitary hormones within the gland (Denef, 1986).

1.9.1 Folliculo-stellate cell

The folliculo-stellate cell (FS-cell) is of particular interest to researchers studying paracrine and autocrine mechanisms within the anterior pituitary gland. The FS-cells were first described in the rat and human pituitary gland by Farquhar (1957), and are distinct from normal hormone secreting pituitary cell types (Vila-Porcile, 1972; Perryman, 1983). The FS-cell is also present in the avian anterior pituitary gland (Harrisson *et al.,* 1982a, b; Fernandez *et al.,* 1986). Morphological features used to identify the FS-cell include: 1) stellate appearance, 2) a lack of secretory granules, 3) junctional complexes linking similar cells, 4) cilia and microvilli projecting into the follicular lumen and 5) a limited number of organelles and ribosomes (Harrison et al 1982b) (Figure 1.10)

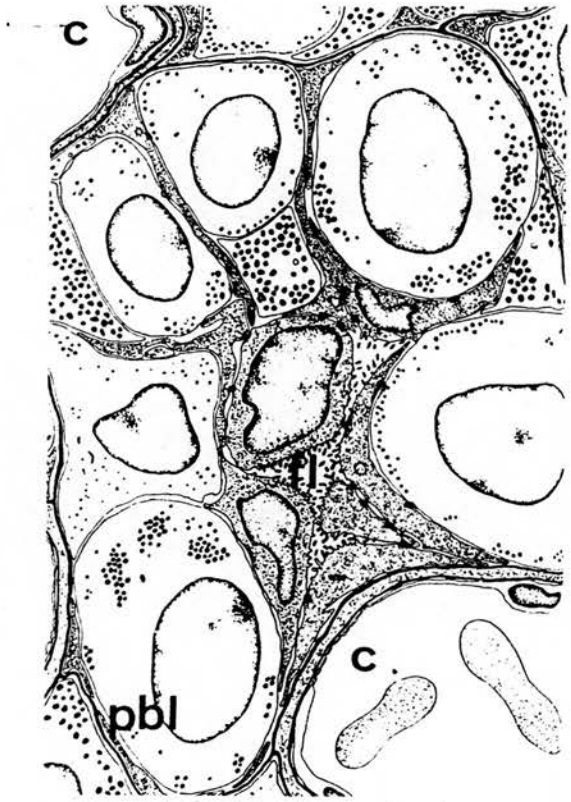


Figure 1.10 A diagrammatic representation of the FS-cell in the anterior pituitary of the rat. The FS-cell (dark background) surround various follicular cavities (fl). Their processes extend between granulated cells (GC) (clear background), and abut onto the parenchymal basal lamina (pbl). Some fenestrated capillaries (c) are also shown. (Allaerts *et al.,* 1990).

1.9.2 Localisation of S-100 protein in FS-cells

Folliculo-stellate cells are characterised by the presence of a soluble membrane bound protein called S-100 (Cocchia and Miani, 1980; Nakajima *et al.,* 1980; Shirasawa *et al.,* 1983). S-100 protein was first isolated from the central nervous system, where it has unique Ca2+ binding properties (Moore, 1965; Calissano *et al.,* 1969). In mammals, S-100 protein is found in glial cells in the CNS, and in several cell types (Boyes *et al.,* 1986; Donato, 1986; Vanstapel *et al.,* 1986; Haimoto *et al.,* 1987). Although the precise function of S-100 protein remains uncertain, S-100 immunocytochemistry (Kahn *et al.,* 1983; Takahashi *et al.,* 1984) has provided a useful tool in identifying FS-cells in the mammalian (Cocchia and Miani, 1980; Nakajima *et al.,* 1980; Shirasawa *et al.,* 1983) and avian (Atoji *et al.,* 1990) anterior pituitary gland.

1.9.3 Origin of FS-cells

Folliculo-stellate cells may be related to glial cells, and therefore be of neuroectodermal origin, although this is not consistent with the classical view that the anterior pituitary is ectodermal in origin (Cocchia and Miani, 1980). The glial cell and FS-cell share common features, such as cell membrane ATPase activity (Semoff and Hadley, 1978), similar structural features (Barberini and Correr, 1984; Stokeef *et al.,* 1986) and a common supportive role (Dingemans and Feltkamp, 1972).

The cytoskeletal intermediate filament proteins vimentin and glial fibrillary acidic protein (GFAP) also occur in glial cells (Gown and Gabbiani, 1984). The distribution of GFAP-immunoreactive glial cells has also been mapped out in the central nervous system of several species including the rat (Hajos and Gallatz, 1987) and quail (Cameron-Curry *et al.,* 1991). The use of antisera to specific glial cell markers such as, S-100 protein (Cocchia and Miani, 1980), GFAP (Calvo *et al.,* 1988) and vimentin (Marin *et al.,* 1989) demonstrates that FS-cells in anterior pituitary are glial-like, supporting the view that FS-cells share a common origin with glial cells.

The FS-cells represent about 5-10% of the anterior pituitary cell population and are dispersed throughout the gland (Allaerts *et al.,* 1990). The FS-cells may associate in groups and form follicles, or be closely associated with hormone secreting cells, with long cytoplasmic extensions between neighbouring cells (Allaerts *et al.,* 1990).

1.9.4 Function of FS-cells in the anterior pituitary

The function of the FS-cell in the mammalian pituitary gland is suggested by its morphological structure and its association with the granulated hormone-secreting pituitary cells. The FS-cells may play a role in the transfer of waste material from degenerating cells into the lumen of follicular cavities (Fernandez, et al 1986), and the phagocytosis of cells during endocrine dysfunction (Perryman *et al.,* 1980; Vila-Porcile, 1972). In female rats primed with oestrogen for several weeks, lysosomes and autophagic vacuoles occur in the FS-cells, after the withdrawal of the oestrogen treatment (Stokeef *et al.,* 1986). The FS-cells may also provide mechanical support in the pituitary (Blanco *et al.,* 1978), in addition to providing trophic support for the hormone secreting cells (Vila-Porcile, 1972).

1.9.5 Growth factors, cytokines and FS-cells

Growth factors and cytokines produced and localised within FS-cells together with their effects on pituitary hormone secretion are listed in Table 1.3

Table 1.3 The effect of substances known to be expressed by FS-cells, on pituitary hormone secretion. *Abbreviations*: (+) stimulatory action (-) inhibitory action *ACTH* adrenocorticotroph ; *bFGF* basic fibroblast growth factor; *GH* somatotroph; *IGF-I* Insulin-like growth factor-I; *IL-6* interleukin-6; *LH* gonadotroph; ***ne*** indicates no effect.; *PRL* prolactin; *S-100* S-100 protein; *TSH* Thyrotroph; (Allaerts *et al.,* 1990, Houben and Denef, 1994).

	Pituitary cell type				
	PRL	**GH**	**TSH**	**LH**	**ACTH**
Growth Factor					
S-100	+				
bFGF	**+/-/ne**	**-**	**+**		
IL-6	**+/ne**	**+/ne**		**+/ne**	**+/ne**
IGF-1	**-**	**+/-**		**+/ne**	

The development of a method to culture FS-cells *in vitro* has facilitated a direct experimental approach to the study of the functional properties of FS-cells, and the identification of paracrine factors they produce (Ferrara *et al.,* 1986; Allaerts and Denef, 1989). FS-cells contain and release S-100 protein, which stimulates PRL secretion from cultured clonal PRL cells (Ishikawa *et al.,* 1983), and is implicated in cell division and growth (Fan, 1982; Kligman and Hilt, 1988).

Basic fibroblast growth factor (bFGF) is secreted by FS-cells, which enhances the responsiveness of lactotrophs and thyrotrophs to TRH (Baird *et al.,* 1985). A novel vascular endothelial growth factor (VEGF) also occurs in FS-cells (Ferrara and Henzel, 1989; Gospodarowicz and Lau, 1989) and may regulate the microvasculature of the anterior pituitary gland. The cytokine interleukin-6 (IL-6) occurs in S-100(+) FS-cells (Vankelecom *et al.,* 1993), which stimulates PRL, GH (Spangelo *et al.,* 1989) LH (Yamaguchi *et al.,* 1990) secretion *in vitro* and ACTH secretion *in vivo* (Bateman *et al.,* 1989) and *in vitro* (Woloski *et al.,* 1985).

A number of studies have demonstrated that the FS-cell attenuates the secretory activity of most pituitary cell types by reducing their response to stimulatory (Baes *et al.,* 1987) as well as inhibitory hypothalamic releasing factors (Allaerts and Denef, 1989). Follistatin inhibits follicle stimulating hormone (FSH) secretion, and is released by FS-cells in monolayer cultures, and therefore may act directly on the gonadotroph and regulate FSH secretion in the pituitary (Gospodarowicz and Lau, 1989). This observation is consistent with a paracrine role of the FS-cell in modulating pituitary hormone secretion. It is therefore interesting to note, that after gonadectomy in the rat, FS-cells closely surround gonadotrophs but not thyrotrophs (Shirasawa *et al.,* 1983), which further supports the view that FS-cells may modulate the function of pituitary hormone cells.

In conclusion, FS-cells in the anterior pituitary gland are involved in a number of physiological responses to changes in the endocrine environment. The production and secretion of paracrine factors by FS-cells, and their relationship with pituitary hormone secreting cells, suggests that this cell type plays an important role in cell-to-cell communication and regulation of pituitary function.

1.10 Research objectives

The preceding literature shows that one of the functions of VIP in the central nervous system is to regulate the secretion of hormones from the anterior pituitary gland. In birds, VIP is the major hypothalamic prolactin releasing factor, with no apparent LH-releasing activity. However, a recent study showed that active immunization against VIP in the bantam hen results in the expected depression in PRL secretion, and an unexpected depression of LH secretion (Sharp *et al.,* 1993). On the basis of this observation it was suggested that VIP may also facilitate the function of gonadotrophs.

Vasoactive intestinal polypeptide immunoreactivity (Lam *et al.,* 1989; Steel *et al.,* 1989; Carrillo and Phelps, 1992) and VIP mRNA (Lam *et al.,* 1989; O'Halloran *et al.,* 1989) occurs in the rat anterior pituitary gland. A VIP-cell type demonstrated immunocytochemically appears to be distinct from that of the lactotroph (Lam *et al.,* 1989; Sergeson *et al.,* 1989; Carrillo and Phelps, 1992), and may secrete VIP to affect pituitary hormone function. It is suggested that VIP might also be produced in the chicken anterior pituitary gland, and act in a similar fashion. The depression of plasma LH secretion in the chicken, after actively immunizing against VIP, may be due to the suppression of intra-pituitary VIP, which has a trophic action on the gonadotroph and thereby modulates LH secretion.

The objectives of this thesis were:-

- To determine whether VIP-immunoreactivity occurs in the chicken anterior pituitary gland.
- To localise cells containing VIP in the anterior pituitary and determine their relationship with other cell types.
- To establish whether the VIP mRNA occurs in the anterior pituitary, and if so which cell type expresses the VIP gene.
- To identify VIP binding sites on pituitary cell types by immunocytochemistry.
- To establish whether VIP modulates pituitary hormone release *in vitro*, and in particular LH secretion.

Chapter 2: MATERIALS AND METHODS

2.1 General materials

2.1.1 Standard reagents

All reagents were obtained from Fisons (Loughborough, Leicestershire, U.K.), BDH (Poole, Dorset, U.K.) or Sigma (Poole, Dorset, U.K.), unless indicated. The water was purified by pre-filtration and reverse osmosis purification by Milli-RO and Milli-Q systems (Millipore Waters, Watford, U.K.).

Peptides were obtained from the following sources: chicken vasoactive intestinal polypeptide (cVIP) (Penninsula Laboratories plc.), ovine pituitary adenylate cyclase activating polypeptide (oPACAP) (Penninsula Laboratories plc.), chicken pituitary adenylate cyclase activating polypeptide (cPACAP) (A gift from Prof. A. Arimura, Tulane University Herbert Centre, Los Angeles, U.S.A.), and chicken gonadotrophin releasing hormone 1 (cGnRH-1) (Penninsula Laboratories plc.).

2.1.2 Standard solutions

Blocking buffer: 2 x SSC, 0.05% Triton X-100.

Buffer 1(x10): 1M Tris NaCl (pH 8.0).

Denhardts solution: 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA.

Equilibrated phenol: Re-distilled phenol was repeatedly equilibrated with equal volumes of 0.5M Tris until the pH was greater than 7.0. This was then stored with an equal volume of TE and 1.0% hydroxyquinoline at -20°C.

Gel loading dye: 50% glycerol, 50% 2 x TAE, 2.5 mg bromophenol blue, 2.5 mg xylene cyanol.

Phosphate buffer (PB): 0.1 M Na H_2 PO_4, 0.1M Na_2 HPO_4 (pH 7.4).

PBS: 0.1 M PB, mM NaCl (pH 7.4).

RNase buffer: 0.01 M Tris (pH 8.0), 0.5 M NaCl, 1 mM EDTA

SSC: 15mM Sodium citrate, 150 mM NaCl.

TE Tris EDTA: 10mM Tris-HCL (pH 8.0), 1 mM EDTA.

TAE Tris acetate electrophoresis buffer: 40 mM Tris-acetate, 1 mM EDTA.

TAE acetate : 100mM TAE ammonium acetate pH 2.5.

TAE formate : 100mM TAE formic acid pH 3.5.

TBE Tris borate electrophoresis buffer: 89mM Tris-borate, 2.5 mM EDTA.

2.1.3 Animals

The animals used in these experiments were adult female bantam hens obtained from the Roslin Institute flock (Roslin, Midlothian, U.K.). They were individually caged, fed layers pellets *ad libitum*, and water was freely available through drinking nipples. The lighting schedule was 16-hours light and 8-hours dark (lights on at 00-00 GMT). Broodiness was induced by transferring groups of five birds to floor pens (4m x 2m) containing 5 nest boxes, and maintaining the same light pattern. The birds were inspected daily for 3-5 weeks for evidence of broodiness, which was recognised by persistent nesting, and characteristic clucking. Furthermore ovarian regression in incubating hens, was confirmed by surgical examination at autopsy.

2.1.4 Blood samples

Blood samples were taken from the brachial wing vein with heparinised (Pump-hep, Leo Laboratories, Princes Risborough, UK) syringes, and plasma obtained by centrifugation at 250 x g for 15 min. Samples were then stored at -20°C before radioimmunoassay for hormones (see Section 2.9).

2.2 Immunocytochemistry

2.2.1. Reagents

All the solutions and the washes used were made with 0.1M PBS pH 7.4, and all incubations were carried out in a humid incubation chamber at 4°C, unless otherwise indicated. The following antibodies used in this study will be described in Chapters 3, 5 & 6: anti-chicken vasoactive intestinal polypeptide (anti-cVIP) (code/6DL/31/4); anti-chicken prolactin (anti cPRL) (code/31/1); anti-turkey luteinising hormone ß-subunit (anti-LH; 783) anti-chicken growth hormone (anti cGH); anti- S-100 and anti-glial fibrillary acidic protein (anti-GFAP) were

purchased from DAKO (High Wycombe. Bucks., U.K.). The antibodies to type-I and type II VIP-Receptor were kindly donated by Dr. E.J. Goetzl (Dept. of Medicine and Microbiology-Immunology, University of California, San Francisco, CA, U.S.A.), mammalian pituitary adenylate cyclase activating polypeptide (anti-mPACAP) was donated by Prof. A. Arimura (Tulane University Medical Centre, U.S..-Japan Biomedical Research Laboratories, Los Angeles, U.S.A.) and the anti-chicken adrenocorticotrophin hormone (anti-ACTH; code/5. 81) was supplied by Prof. P. Lowry (University of Reading). All sera (NDS, NSS, NRS, DARS) were obtained from the Scottish Antibody Production Unit (SAPU, Law Hospital, Carluke, U.K.).

2.2.2 Tissue collection

The anterior pituitary glands were obtained from laying and broody bantam hens. The hens were killed by cervical dislocation (Scientific Procedures Act, 1986) and decapitated. The lower mandible was removed from the head and trimmed by removing the tissue and bone rostral to the orbital recess and overlying the base of the brain. The anterior pituitary was detached from the median eminence and then separated from the neural lobe. The basal hypothalamus was then dissected with a scalpel by making vertical cuts posterior and anterior to the optic chiasma. The tissue fragments were then placed in fixative or liquid nitrogen according to the experimental protocol.

2.2.3 Perfusion

The hens were deeply anaesthetised with sodium pentobarbitone (60mg/kg) (Sagatal, Rhone Merieux), injected into the brachial wing vein. Then the brain was perfused via the carotid artery with a cannulae (Portex Ltd, Kent, U.K.) directed towards the head, with physiological saline 250-300ml (0.9% NaCl w/v) containing heparin (10U/ml), After severing the jugular vein, the brain was perfused with 400-500 ml of Zamboni's fixative (4% paraformaldehyde, 15% aqueous saturated picric acid, 0.1 M PBS, pH 7.4)

2.2.4 Microwave Fixation

The tissue was fixed in Bouin's (0.1M copper acetate; 0.015% aqueous saturated picric acid; 4% formaldehyde) for 2-3 hours and processed further by microwave irradiation (H2500 Microwave processor, Bio-Rad, Hemel Hempstead). The tissue was irradiated in a series of steps: 4 min at 55°C in fixative; 60 min at 67°C

in 100% ethanol; 45 min at 74% in isopropanol and for 15 min in xylene with a 250 ml water load (Boon and Kok, 1988). The tissue was embedded in polystyrene (100,000 Mr) (100g polystyrene, 400ml xylene, 25ml benzyl alcohol, 5ml dibutyl phthalate) (Fragioni and Borgioli, 1979), in a tissue embedding mould (Peel-A-Way, Polysciences Inc., Warrington, PA, U.S.A.) and baked in an oven for 24-36 hours at 60°C (Fragioni and Borgioli, 1979). When the polystyrene had hardened the plastic mould was removed and the block trimmed (5 x 5 x 5 mm) and attached to a perspex chuck (Roslin Institute, Dept. of Engineering Services) with adhesive. The embedded tissue was then stored at room temperature or sectioned immediately on a microtome.

2.2.5 Microtome sectioning

A motorised microtome (Microtome, LKB Instruments, Surrey, U.K.) equipped with a glass blade, was used to section the polystyrene embedded tissue at a thickness of 2-5μm. Two hundred to three hundred microlitres of paraldehyde solution (10%v/v), was dispensed onto a subbed-slide, and the sections were removed from the glass blade with a pair of forceps and placed onto the fluid. The slide was then transferred to a hot plate (80°C) for 3-5 seconds, until the solution became milky and the sections flattened. The slide was then removed, allowed to cool at room temperature for 1 min; the excess paraldehyde solution aspirated off the slide, and dried on a warm plate (40°C) to ensure tissue adhesion to the slide. The sections were then stored (1 month) in a suitable container or used immediately for immunocytochemistry.

2.2.6 Vibratome sectioning

A Vibratome (shaking microtome) (Bio-Rad, Hemel Hempstead, U.K.) was used because the morphological preservation of the sectioned material is better than that of frozen sections and is capable of sectioning larger blocks of material (Priestley, 1987). Therefore this was the method of choice when sectioning large areas of the brain (Chapters 3 and 5). The perfused tissue was attached to a horizontally orientated chuck with adhesive and then embedded in 2% agar to provide structural support. The chuck was placed in a small tank on the shaking microtome with buffer (PBS). The vibrating razor blade was then advanced horizontally through the tissue, and the sections (70μm) floated off into the buffer for collection, and transfered to suitable receptacles for immunocytochemical staining.

2.2.7 Immunostaining on mounted slides

All the washes were carried out in 0.1M PBS, pH 7.4 unless otherwise indicated. The immunocytochemical procedure described below was used for all the experiments, but variations were made, and described in later chapters. The slides were cleared with xylene and rehydrated (5 min) with a graded series of alcohols; 100%; 95%; 90%; 70%; 50% and then washed 3 times in PBS.

The most commonly used enzyme label was horseradish peroxidase, and sections stained by this method were incubated in methanol containing 0.3% H_2O_2 (30 min) to block endogenous peroxidase activity in the tissue. All sections were washed in: PBS for 5 min; Triton X-100 (0.4%) for 5 min and PBS for 5 min. The area around the tissue sections was dried with a strip of fibre-free blotting paper (Agar, UK), and encircled with a layer of synthetic wax (DAKO-PEN, DAKO, High Wycombe. Bucks., U.K.). A suitable blocking (5%; NDS or NGS) solution was then applied to each tissue section (50µl) and stored in a horizontal slide holder and incubated for 30 min. The antibody was prepared to the desired concentration (1: 100-20 000 see Table 2.1), in 0.1% blocking solution, and 50µl applied to each section, after the excess block solution had been removed, and incubated overnight.

Table 2.1 Antibodies used for immunocytochemical studies and the dilution used in the pituitary and hypothalamus. Abbreviations: *A.P.* anterior pituitary; *Bov* bovine; *Gp* guinea pig; *Hu* human; *Hyp.* hypothalamus; *Rb* rabbit; *Sh* sheep; and *Tur* turkey. The antibodies were described in Section 2.2.1.

Antibody	**Specificity**	**Working**	**Dilution**
(code)		**A.P.**	**Hyp.**
ACTH (5.81)	Rb anti-Ch.	1:500	/
GH	Rb anti-Ch	1:500	/
LH (783)	Rb anti-Tur.	1:1000	/
LH α subunit	Sh anti-Ch.	1:1000	/
PRL (31/1)	Rb anti-Ch	1:20 000	/
PRL	Gp anti-Ch.	1:1000	/
S-100 (Dako)	Rb anti-Bov.	1:500	1:500
Vasotocin	Rb anti-Ch	/	1:10 000
VIP (6DL/34/1)	Sh anti-Ch	1:2000	1:10 000
VIP-RI (A, B)	Rb anti-Hu.	1:100	1:100
VIP-RII (4, 5, 6, 22 &23)	Rb anti-Hu.	1:100	1:100

The method used to identify a specific primary antibody is described in more detail in the Results, however the Figure (2.1) below shows two staining methods used in this study.

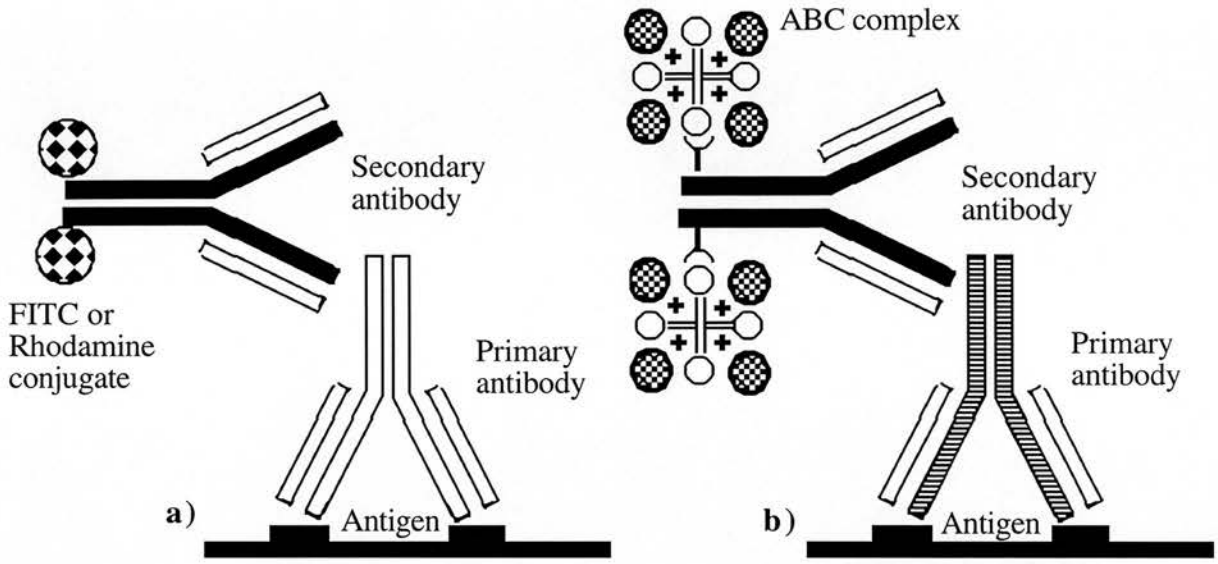


Figure 2.1 A schematic representation of the two immunocytochemical staining methods used in this study. The (a, b) primary antibody recognises a specific antigenic site and binds to it. A second antibody is then used to bind to the primary antibody. The secondary antibody may be (a) conjugated directly to a marker, such as FITC or rhodamine, or (b) be biotinylated (*open circle*). The biotinylated second antibody acts as a bridge linking the avidin-peroxidase enzyme complex to the primary antibody. The avidin-biotin complex (ABC) is visualised when DAB is added (*filled circle*), and is oxidised by peroxidase (+) to a brown product (Adapted from Sternberger, 1979).

The sections stained with the fluorochromes fluorescein isothiocyanate (FITC; SAPU) or Lissamine rhodamine isothiocyanate (LSRC; Jackson Laboratories, Stratech Scientific, Beds., UK), were mounted in an aqueous mounting medium (90% v/v glycerol/PBS), containing the anti-fading agent diazo-bicyclo-2,2,2,-octane (DABCO). The addition of dihydrochloride, 4', 6-diamidino-2-phenyl-indole (DAPI), facilitated the visualisation of nuclei. The sections were then examined on a fluorescent microscope (Nikon Optiphot) or a scanning laser confocal microscope (Bio-Rad MRC-, Herts., U.K.).

The sections stained with a peroxidase conjugate were incubated with the chromagen 3,3'-diaminobenzidine (DAB) in 50 mM Tris pH 7.4, 0.3% hydrogen

peroxide, until the desired staining was obtained. DAB acts as an electron donor for peroxidase, and when oxidised, forms an insoluble precipitate at the reaction site. The sections were washed in PBS and several times (x3) in distilled H_2O, and dried in an oven for 2-3 hours at 37°C. The slides were then washed twice in KEMSOL-30 (Kem Serv, Ltd, U.K.) for 10 min and mounted in DPX mountant (Agar Scientific, Stansted, U.K.) and viewed by light microscopy (Olympus, Loughborough, Leics., U.K.).

2.2.8 Immunostaining on free floating sections

The method used to stain free floating sections is principally the same used to stain sections on mounted slides (Section 2.2.7). The sections were stored in a receptacle (2-3 sections) which allowed the sections to be transferred to different solutions, without handling or damaging the sections. The immunostaining was performed by transferring the receptacle holding the sections to the required development solution, such as DAB for the desired amount of time (1-5 min).

2.3 RNA Isolation

2.3.1 Reagents

All materials and reagents used for RNA isolation were purchased free of ribonuclease (RNAse) activity, and any solutions which were not, were treated with diethlypyrocarbonate (DEPC) 0.1% v/v for 24 hours and then autoclaved.

2.3.2 Tissue collection

The anterior pituitary glands were obtained from laying and broody bantam hens in the same manner as described in 2.2.1. The tissue was placed in sterile tubes, weighed, snap frozen in liquid nitrogen and stored frozen at -70°C.

2.3.3 Isolation of total RNA

Total RNA was extracted from tissue using a modified version of the acidified phenol-chloroform method of Chomczynshi and Sacchi (1987). Approximately five to ten anterior pituitary glands or hypothalamic tissue (50-100mg) was homogenised in 0.9 mls of RNAzol B solution (Witney, Oxon, U.K.) and left in ice for 5 minutes. A further 0.9 mls of RNAzol B solution was added to each tube and vortexed thoroughly, and placed on ice for another 5 minutes. Chloroform

(0.2 mls) was added to each tube which was vortexed and placed on ice for 15 minutes, before spinning at 12 000 rpm for 15min at 4°C.

The clear aqueous phase, containing the total RNA, was carefully removed and precipitated with an equal volume of pre-chilled (-20°C) isopropanol and stored at -20°C for 1 hour. The RNA was precipitated into a pellet by centrifugation at 12 000 rpm for 30 minutes at 4°C. The pellet was washed with 75% ethanol and then 70% ethanol, before being vacuum dried. The pellet was re-dissolved in water, and the yield and purity of the total RNA was estimated by measuring the optical density of an aliquot (2µl) by UV spectrophotometry at 260nm and 280nm. The sample was then diluted with water to a pre-determined RNA concentration and stored frozen at -70°C.

2.4 Polymerase chain reaction

Genomic DNA can be amplified using the polymerase chain reaction (PCR) (Saiki et al. 1990), which comprises three steps, which when combined, make 1 PCR cycle. Firstly, the double stranded DNA is heat-denatured, and then the single stranded DNA is cooled to allow two oligonucleotide primers to anneal to complementary regions on the target DNA. Finally the thermo-stable DNA polymerase *Taq* amplifies the oligonucleotide targeted DNA.

2.4.1 Reverse transcription-PCR

The PCR does not amplify RNA because the Taq enzyme used in the reaction is DNA dependent, therefore the RNA is reverse transcribed (RT) to generate complementary DNA (cDNA). Reverse transcription polymerase chain reaction (RT-PCR) uses similar principals and protocols as standard PCR, but the template or target is derived from reverse transcribed RNA or complementary DNA (cDNA) (Frogman et al. 1988).

The total RNA was prepared by the method described in Section 2.3.3 and the optical density measured to determine the concentration required for the RT reaction. RNA was reverse transcribed to cDNA using a first strand synthesis kit (Pharmacia Ltd.) containing the following reaction components: 5µg RNA in 8µl DEPC-H_2O: 2µl of mix (3.5µl DDT, 3.5µl Oligo. DT primer); 5µl first strand synthesis kit. The RT reaction was allowed to proceed at 37°C for 1 hour and stopped by increasing the temperature to 95°C for 10mins.

The PCR was also set up using reagents supplied as a kit (Promega). The reaction mixture consists of (Table 2.2): RT cDNA; oligo primer mix; reaction buffer; NTPs (Promega); DEPC-H2O; *Taq* enzyme (Boehringer) and mineral oil. The volumes of reagents are shown in Table 2.2. The samples were dispensed into microcentrifuge tubes and placed in the hot block of a PCR machine (Hybaid, Teddington, Middlesex, U.K.).

Table 2.2 Reagents used in the polymerase chain reaction, and the format used for PCR.

Sample	**H_2O**	**MIX**	**cDNA/RNA**
H_2O	25µl	25µl	/
Tissue cDNA	15µl	25µl	10µl
Tissue RNA	15µl	25µl	10µl

Cycle 1: 94°C for 4 min; 65°C for 45 sec and 74°C for 45 sec.
Cycle 2: 94°C for 45 sec; 65°C for 45 sec and 74°C for 45 sec.

The cDNA templates were amplified by oligonucleotide primers, which were selected from the chicken VIP cDNA sequence (Fig. 2.2). The primers were designated 215L and 275Z (Oswell DNA Service, Edinburgh, UK) (Table 2.3).

Table 2.3 The oligonucleotide primers derived from the chicken VIP cDNA sequence used for PCR. The base numbers relate to the position of primers in the VIP 19 cDNA.

215L Bases 259-279 (5' exon 3) 5'-GATGCAGCCAGTGAATCTGAC-3'

275Z Bases 514-534 (3' exon 6) 5'-GGTCGGTTTGAAGCTCCACTT-3'

The PCR machine was pre-programmed with the number and duration of cycles, in addition to the desired melting, annealing and extending temperatures. When the reaction had completed, the PCR products were visualised on a 3% GTG-NuSieve agarose gel (Section 2.5.1).

```
1                   20                  40                  60
CCGTCATCGCACCCCGACAGCCGTTCTTTCGGCAGCAGCGCAGGCGACAGGGCGCCGTCCCCATAGCGCA

         80                  100                 120
GGCAGCGCCCTCCCGAGCAGCCCCACCGACGGACTCGCGGCTCCGTGGCGGTCATGGAGCACCGCGGC
                                                   MetGluHisArgGlu

 140                 160                 180
GCCTCCCCGCTCCTCCTCGCCCTCGCCCTCCTCAGCGCCCTCTGCTGGCGGGCGCGGGCG
AlaSerProLeuLeuLeuAlaLeuAlaLeuLeuSerAlaLeuCysTrpArgAlaArgAla

 200                 220                 240
CTGCCCCCGCGGGGCGCCGCCTTCCCTGCTGTGCCGCGACTGGGAAACAGACTGCCCTTT
LeuProProArgGlyAlaAlaPheProAlaValProArgLeuGlyAsnArgLeuProPhe

 260                 280                 300
GATGCAGCCAGTGAATCTGACCGCGCCCATGGGTCCTTAAAGTCTGAATCAGACATTTTG
AspAlaAlaSerGluSerAspArgAlaHisGlySerLeuLysSerGluSerAspIleLeu

 320                 340                 360
CAGAACACACTACCTGAAAATGAGAAATTCTATTTCGATCTGTCCAGAATTATTGATAGC
GlnAsnThrLeuProGluAsnGluLysPheTyrPheAspLeuSerArgIleIleAspSer

 380                 400                 420
TCCCAGGACAGTCCTGTCAAACGCCACTCTGATGCTGTCTTCACTGACAACTACAGCCGC
SerGlnAspSerProValLysArgHisSerAspAlaValPheThrAspAsnTyrSerArg

 440                 460                 480
TTTCGAAAGCAAATGGCTGTGAAGAAATACTTAAACTCAGTTTTAACTGGAAAACGAAGC
PheArgLysGlnMetAlaValLysLysTyrLeuAsnSerValLeuThrGlyLysArgSer

 500                 520                 540
CAGGAAGAGTTAAATCCAGCCAAACTTCGAGGTGAAGCAGAAATTCTTGAACCTTCCTTT
GlnGluGluLeuAsnProAlaLysLeuArgGlyGluAlaGluIleLeuGluProSerPhe

 560                 580                 600
TCAGAAAACTATGATGATGTTTCTGTAGATGAACTGCTGAGCCACCTCCCATTGGACCTC
SerGluAsnTyrAspAspValSerValAspGluLeuLeuSerHisLeuProLeuAspLeu

 620                 640                 660                 680
TGAAGGACACCTAGCAAACTCTTCAACAAGAACAAGTTATTTTTGAGTTCCACATAGTATTTCAAAGAGA
end

          700                 720                 740
TGACTTTAGTCATCAAACCAGAACAAATATGTTGTGAAGTGAAAGTTGTGATATATTTGTTTCTTATGTAA

 760                 780                 800                 820
TAAAAGTTGATATTTACATTGTAAATACTATTCTAGAGTTCTCTACTGAAAGCTGTACATATGGATGCCAG

          840                 860                 880                 900
TTAAACAAATGAGAAGTCTGTAAGTCCATATGCTGTAAATCCTTTACTTCAATAAATTCATTTGAAAATGAA


AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 2.2 The sequence of chicken VIP cDNA (VIP19) and the corresponding predicted amino acid sequence as described by Talbot *et al.,* 1995. The amino acid sequence of mature VIP is in bold. The underlined sequences are those of oligonucleotide primers used for PCR, 5' (215L, *) and 3' (275Z, **).

2.4.2 Restriction enzymes

The restriction enzymes were purchased from Boehringer Manneheim (BCL, Lewes, Sussex, U.K.) or Pharmacia (Milton Keynes, Buckinghamshire, U.K.). The use of restriction enzymes was according to manufacturers recommended instructions.

2.5 Agarose gel electrophoresis

2.5.1. Double stranded DNA

The PCR products were analysed by agarose gel electrophoresis using a submarine electrophoresis tank (BRL, Paisley, U.K.) and 1 x Tris acetate electrophoresis buffer (TAE; 40mM Tris acetate, 1mM EDTA). The most frequently used agarose was 3 or 4% NuSieve gels (Flowgen Inst., Ltd.), because it was designed to improve the separation of small DNA fragments and has a low melting point. The agarose was added to TAE buffer and dissolved by gently heating the mixture in a microwave. The mixture was allowed to cool until 'hand hot' and 1-2µl ethidium bromide (5mg/ml) was added. The gel was cast into a plastic mould with a casting comb. When the gel had set the casting comb was removed and the gel placed in the electrophoresis tank and covered with TAE buffer. The PCR product samples were mixed with (1:1) 2-3µl of blue gel marker (bromophenol blue 0.25%w/v, xylene cyanol 0.25% w/v & glycerol 30%w/v in DEPC-H_2O). The samples and DNA size markers (Boehringer Mannheim, Lewes, U.K.) were dispensed into appropriate wells, and the gel run for 2-3 hours at 70mV. After electrophoresis the gel was removed and observed with an UV transilluminator and photographed. The ethidium bromide has an affinity for nucleic acids and was used for the visualisation of DNA by UV transillumination (UV products).

2.5.2 RNA

The analysis of RNA was performed on 3% NuSieve agarose (3:1) containing 2.2M formaldehyde and 1 x gel running buffer (MOPS) (1M morpholinopropanesulphonic acid, pH 7.0, 50mM sodium acetate, 5mM EDTA pH 8.0). The agarose was dissolved in the H_2O, 25 x gel running buffer and 12.3M formaldehyde. The samples 1-2µl (10-20µg) were treated with 2µl 12.3M formaldehyde and 0.7µl formamide and heated at 56°C for 15 minutes. The

samples were then mixed with gel loading dye and run with a RNA size ladder (Gibco) submerged in 1 x gel running buffer for 2-3 hours at 55mV.

2.5.3 Northern analysis

The formaldehyde agarose gel was washed 4 times in H_2O for 5 min and then soaked for 45 min in 50 mM NaOH and 10 mM NaCl. The gel was neutralised in 0.1M Tris HCL pH 7.5 for 45 min, and 20 x SSC for 1 hour. The gel was placed on a glass plate and a piece of Hybond-N (Amersham International) was placed on top of the gel, followed by 2-3 cm of blotting paper and paper towels. A weight (0.5kg) was then applied and the blot left overnight. After transfer the blot was washed briefly in 2 X SSC, and then exposed to UV radiation in a UV Cross linker (Stratagene).

2.6 In situ Hybridization

In situ hybridization allows the localisation of mRNA to be visualised at the cellular level. This method involves the hybridization of a specific nucleotide probe with a specific target nucleic acid within a tissue section. The use of cloning techniques has increased the development of *in situ* hybridization techniques, which has allowed the production of probes for the detection of specific mRNA sequences.

2.6.1 Probe Production

RNA probes for *in situ* hybridization were produced as described by Cox *et al.* (1984). The single-stranded RNA probe (riboprobe) was generated by a RNA polymerase (T7 or T3) transcribing a cDNA sequence cloned downstream of the appropriate polymerase initiation site. The probe sequences used were cloned into the pBluescript sk (+) plasmid and flanked by two RNA polymerase promoter sites. The linearization of the plasmid by different restriction enzymes enabled the generation of specific sense or antisense probe sequences (Fig. 2.3).

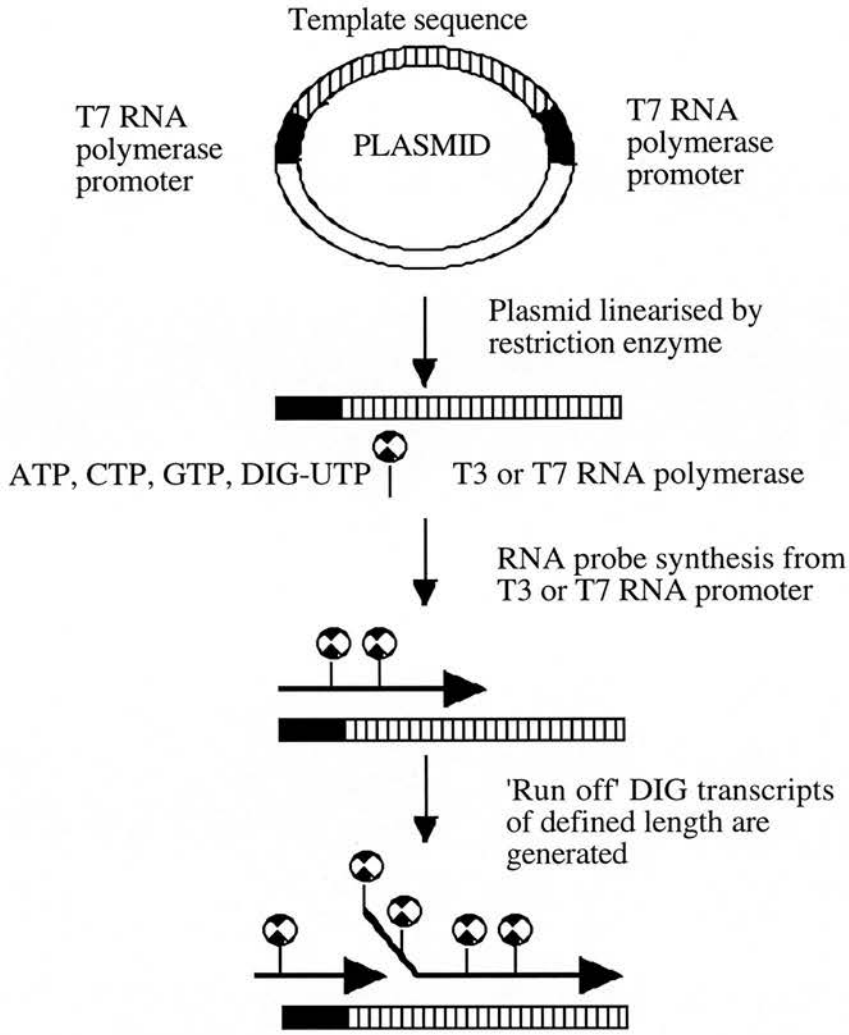


Figure 2.3 A schematic representation of method used to generate RNA probes from a nucleotide sequence, which has been cloned into the multiple cloning site of a plasmid (VF 48). The plasmid was linearised by a restriction enzyme, and labelled probes are generated by incorporating labelled nucleotides (DIG-UTP) in the RNA transcription reaction.

The RNA probe was transcribed from the linearised template, by an DNA-polymerase RNA polymerase enzyme (T3 or T7), which recognises the promoter sequence and generates 'run off' transcripts. The labelled probe was generated by incorporating labelled nucleotides, such as digoxigenin labelled uridine triphosphate (DIG-UTP) with unmodified nucleotides in the reaction mix. Approximately 1µg of DNA template generates 10µg of full length DIG-labelled RNA transcript, of which every 20-25th nucleotide incorporated was DIG-UTP (Fig. 2.4).

Table 2.4 The components of the RNA probe transcription reaction mix

1µg-2µg Linearised DNA template
2µl DIG-labelling kit (x10, Boehringer Mannheim)
1µl RNase block
4µl Transcription buffer (x5, Boehringer Mannheim)
40U T7 or T3 RNA polymerase (Stratagene)
Incubation volume 20µl

The transcription mix was incubated for 1-2 hours at 37°C, and the template was removed by incubating 2µl RNAse-free DNase for a further 15mins at 37°C. Precipitation of the probe was achieved by adding 2µl 2.0M EDTA, 1.25µl 8M LiCl, 75µl pre-chilled ethanol and incubating for 2 hours or overnight at -20°C. The pellet was obtained by centrifugation and washed with 70% ethanol, prior to vacuum drying. The probe was then resuspended in 98µl DEPC-H_2O, 1µl RNase block, 1µl tRNA (10mg/ml), incubated for 30 min at 37°C, and stored at -20°C.

The probe was analysed to ensure that the transcription reaction had produced long transcripts of the correct size. A 1µl sample of probe was subjected to Northern transfer (see Section 2.5.3) and the Hybond-N washed in Buffer 1 (100 mM Tris HCL pH 7.5, 150 mM NaCl) for 5 min. Then washed in buffer 2 (buffer 1, 1% marvel) for 30 min. The probe was stained with a suitable primary antibody (Boehringer) conjugated to either alkaline phosphatase or peroxidase and developed according to the manufacturers instructions.

2.6.2 Tissue collection

The brain was rapidly removed and lowered into a beaker (100ml) containing isopentane, which was pre-chilled (-25-30°C) by immersing the base of the beaker in liquid nitrogen for about 1 minute. An alternative procedure was to freeze the brain by placing it on a layer of aluminium foil placed on dry ice. This method was considered to be a less abrasive method for freezing the brain, and was less likely to produce large cracks or fractures in the tissue. The brain was then stored at -80°C prior to hybridisation. Frozen sections 10-15µm were cut on a cryostat (Shandon) and thaw mounted on gelatin coated slides (Supafrost/Plus, CellPath). The sections were dried out on the slides at room temperature for 1 minute to allow attachment, and then the slides were stored at -80°C until required (2-3 weeks).

2.6.3. Tissue processing

Several steps were performed prior to hybridization to enhance tissue preservation and the probability of hybrid formation. A number of precautions were also taken to prevent RNAse contamination: gloves worn at all times, all glassware was heat baked (180°C); all materials were RNAse-free and solutions made with DEPC-treated H_2O. The slides were removed from cold storage (-80°C) and dried quickly with a hair dryer to remove condensation and moisture from the sections, and placed into the chosen fixative. The Table (2.5) below lists the processing steps used to prepare the tissue sections for hybridization with the RNA probe.

Table 2.5 An *in situ* hybridization protocol used to process the tissue sections up to the hybridization stage. The slides were placed in a slide rack and passed through the following RNase-free solutions.

Treatment	**Time**
0.1M PBS pH 7.4, 4% Paraformaldehyde	5 min x 1.
0.1M PBS pH 7.4	5 min x 2.
DEPC-H_2O	5 seconds.
TEA	5 seconds.
TEA, Acetic anhydride (1% v/v)	10 min x 1.
2 x SSC	3 min x 1.
Ethanol: 70%; 95% & 100%	3 min each
Chloroform	5 min x 1.

2.6.4 Hybridization

The protocol for the localization of VIP mRNA by *in situ* hybridization developed in this study, is discussed in the results chapters. Briefly, the previous steps were designed to provide the optimum conditions for the hybridization reaction. There were several variables which can enhance the hybridization signal including, probe length, probe concentration, stringency of hybridization conditions and preservation of tissue morphology. The hybridization buffer used routinely in this study is shown in the Table (2.6) below.

Table 2.6 A list of components in the standard hybridization buffer.

Component	Volume
De-ionised Formamide	12.5 ml
Dextran Sulphate 50%	5 ml
NaCl 5M	1.5 ml
Tris 1M, pH 8.0	0.2 ml
EDTA 0.5M	0.04 ml
Denhardts Solution x 50	0.5 ml
DEPC-H_2O	0.06 ml

The final hybridization buffer also included probe and tRNA in the following composition: 75% hybridization buffer; 20% TE containing probe and 5% tRNA. For hybridization, the tissue sections were covered with 70-100µl of hybridization buffer, then covered with a parafilm coverslip (Sigma) ensuring no air bubbles were trapped, and sealed with rubber cement (Sanford Corporation, Newell UK.). The slides were then placed in a humid incubation chamber and incubated overnight in an oven set to the desired temperature.

2.6.5 Post-hybridization washes

The reason for post-hybridization washes was to reduce the non specific binding (background) of the RNA probe to tissue, and non-homologous RNA sequences. The most effective method of reducing the background was RNase treatment (John *et al.,* 1969; Lynn *et al.,* 1983). In addition, a series of washes of increasing stringency was used to reduce background without compromising the level of specific hybridization signal. This was achieved by varying the salt concentration (2 x SSC- 0.1 SSC) and temperature (37°C-55°C) of the post-hybridization washes.

2.6.6 Immunolabelling

After the hybridization and post-hybridization steps the tissue sections were immunostained in a similar manner to mounted sections described previously (Section 2.2.7). Briefly, the slides were placed in a blocking buffer (Section 2.1.2) containing 2% normal sheep serum, for 1 hour at room temperature, then

washed twice in Buffer 1 (0.01M Tris pH 8.0, 0.05% Triton X-100) for 10 min. Then 100µl of anti-digoxigenin antibody (anti-DIG<POD>; 1: 100) solution (Buffer (1), containing 0.3% Triton X-100 (Boehringer Mannheim) was applied to each slide, covered with a parafilm coverslip and sealed with rubber cement. The slides were placed in a humid incubation chamber and incubated overnight at 4°C.

2.6.7 Visualisation

The slides were re-racked in buffer (1), and washed twice in buffer (1) for 15min. Then 400-500µl of DAB solution (see Section 2.2.7) was applied to each slide. The slides were placed in a humid incubation chamber, which was wrapped in foil to protect the slides from the light. In addition 2-3 slides were placed in a petri dish wrapped in foil, these were checked periodically for staining development. The DAB solution was reapplied if the staining period was prolonged. The length of staining was approximately 1-3 hours and varied according to the type of tissue. The reaction was stopped by placing the slides in a PBS wash for 10 min (x2).

A cresyl violet stain was used to identify the nuclei of neuronal cell bodies and pituitary cells, and confirm that specific staining by the DIG-labelled antisense probes. Sections were dehydrated through a series of graded ethanol washes: 50% 3 min ; 70% 3 min; 95% 3 min and 100% 3 min (x2). The slides were then placed in xylene for 5 min (x2) and mounted in DPX (Agar Scientific, Stansted, U.K.). The staining was visualised by light microscopy.

2.7 Solution hybridisation and RNase protection assay

The most sensitive and accurate technique used to show specific mRNA expression in a tissue is the solution hybridisation/RNase protection assay (Jakubowski and Roberts, 1991). A high-specific-activity radiolabelled RNA probe is used to hybridise to a complementary target mRNA sequence in a liquid phase. The resulting RNA-RNA hybrid is protected from RNase digestion, which digests all the remaining single stranded mRNA and unhybridized RNA probe in the liquid phase.

2.7.1 Probe Production

The antisense RNA probe was transcribed with an RNA transcription kit (Stratagene) from pVP39G2 (Talbot *et al.,* 1995) digested with XbaI. The sense strand targets complementary to the radiolabelled probe, were transcribed from

either VIP 19 or pVP39G2 digested with *Xba* I or *Eco* RI respectively. The nucleotide rCTP was replaced by [^{32}P]CTP (Amersham International plc) in the reaction mix. Table (2.7) below shows the components of the RNA transcription reaction used to generate the probe.

Table 2.7 A list of components required for RNA transcription reaction, used to generate a radiolabelled [^{32}P] RNA probe.

Component	**Volume**
5 x Buffer	2µl
rGTP [10mM]	0.5µl
rATP	0.5µl
rUTP	0.5µl
rCTP [0.2mM]	0.5µl
DDT	0.5µl
RNA guard (10U)	0.5µl
DNA template (VIP19 or pVP39G2)	0.2µl
[^{32}P]CTP	2µl
Enzyme (T7 or T3) (Promega)	1µl
DEPC H_2O	1.8µl

The reaction mixture was incubated for 30 min at 37°C, and then 1µl DNase was added to each tube and incubated for a further 15 min at 37°C. The probe was diluted with 90µl of DEPC-H_2O, and 1µl was placed onto two pieces of DE-81 paper discs (Whatman, Maidstone, Kent, U.K.) to measure the incorporation of radioactivity in the probe. When the drops of probe mix had dried onto the paper discs, one disc was placed into a scintillation tube and filled with scintillant (2ml) (Pharmacia), and the remaining disc was washed three times (5 min) in 0.5M sodium di-hydrogen orthophosphate (NaH_2 PO_4). This disc was also placed into another scintillation tube and filled with scintillant (2 ml), and both tubes were placed on a ß-counter (Pharmacia, Wallac 1410, Liquid scintillator Counter). Purification of the RNA probe was achieved using G-50 microcentrifuge spin columns (TE midi select-D, 5'Prime-3'Prime, U.K.), and either stored at -80°C or used immediately.

2.7.2 Hybridisation

The total RNA used for the solution hybridisation reaction was isolated using the RNAzol B method described in Section 2.3.3. The concentration of RNA was determined by optical density measurement and the required amount of RNA was incubated with radiolabelled probe in 20µl 4M guanidine isothiocyanate (GuSCN) at 32°C (see Table 2.8).

Table 2.8 The components used to incubate with sample RNA for solution hybridization.

RNA	**Label**	**4M GuSCN**
Radiolabelled probe	0.5µl	0
tRNA	2µl	23µl
Sense target	2µl	23µl
Sample RNA	5µl	25µl

2.7.3 RNase protection

A stock RNase solution was made containing 400µl 1M Tris pH 7.5, 300µl 4M NaCl; 16µl RNase A and 8µl RNase T (Boehringer Mannheim) 300µl was added to each tube except the 'probe only tube', and incubated for 1 hour at 32°C. Then 20µl SDS (0.1% w/v) and 5µl Proteinase K (10µg/ml) was added to each tube, heated for 2 min at 68°C, and finally at 48°C for 30min. The RNA-RNA hybrids protected from RNase digestion were then separated and purified by phenol-chloroform extraction described in Section 2.3.3, and precipitated with 100% ethanol for 1 hour at -20°C. The sample was dried down in a vacuum desiccator and re-dissolved in stop solution before separation on a polyacrylamide gel (PAGE). The Figure (2.4) below summarises the steps used in the solution hybridization RNase protection assay.

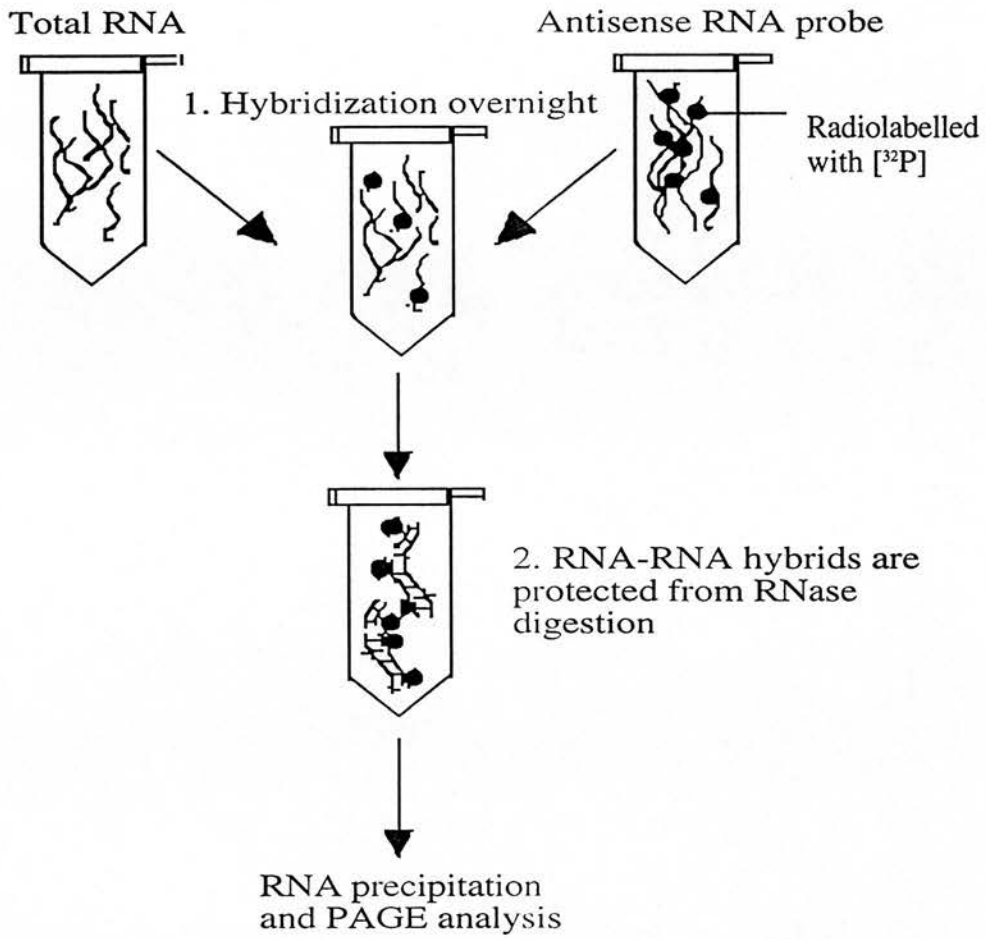


Figure 2.4. A simplified version of the solution hybridization RNase protection assay.

2.7.4 Polyacrylamide gel electrophoresis

All the gels were 14 x 14 x 1.5 mm and run in a vertical slab gel unit model SE400 (The Sturdier, Hoefer Scientific Instr., California, U.S.A.). The gel mix comprised, 6% 19:1 acrylamide:bisacrylamide, 6M urea, 1 x TBE, and polymerisation was initiated with 0.03% ammonium persulphate and 1.28 µl/ml of TEMED. The gels were typically run at 400V in 0.5 x TBE for approximately 1.5 hours or until the xylene blue dye had run off the bottom of the gel. After electrophoresis was complete the gel was transferred to 3M filter paper (Whatman, Maidstone, Kent, U.K.), and the gel surface covered in Saran wrap (Dow, Genetic Research instr., Essex, U.K.) and dried on a gel drier (Zabona AG Basel, Switzerland). A phosphor imager (Molecular Dynamics, U.K.) was then used to quantify the amount of radioactivity in each protected band.

2.8 Tissue concentration of VIP

2.8.1 Acetic acid extraction

The tissue was obtained as described in Section 2.2.2, weighed, placed in microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until required. The cVIP was extracted by boiling the tissue in 1ml of 2M acetic acid for 10 min. The tissue was sonicated (Heat Systems-Ultrasonics Inc.) and centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was removed, stored on ice, and the pellet resuspended in 1M acetic acid sonicated and boiled for a further 5 min. The centrifugation step was repeated and the second supernatant fraction removed and stored in a second tube. All the supernatant fractions were then dried down with a vacuum concentrator (Hetovac) and stored at -70°C.

2.8.2 RP-HPLC purification

Reverse-phase HPLC (RP-HPLC) incorporates a hydrocarbon-bonded (octadecyl, C18) stationary support, with an aqueous eluent containing a proportion of organic solvent (methanol or acetonitrile) (Knox, 1978). The more polar solutes have greater affinity for the eluent and so elute in reversed order of polarity i.e. most polar first. The advantages of chemically bonded phases is that polar and ionic molecules such as cVIP can be chromatographed more efficiently, and an elution gradient can be used without stripping the stationary phase. The addition of trifluoroacetic acid (TFA) can improve the retention of packing material, by (ion suppression) reducing the pH of the mobile phase and suppressing the secondary ionisation of the phosphate groups.

2.8.3 Column characterisation

This investigation was designed to characterise the elution profile of synthetic vasoactive intestinal polypeptide (Penninsula), and compare with the elution profile of peptides, including VIP, extracted from brain tissues. The preliminary studies used a C-18 Apex ODS column (Jones Chromatography, Mid Glamorgan, UK). The samples were pumped onto the column by a HPLC pump with a flow rate 1.5ml/min, (Millipore Waters, Watford, UK) and the column eluents were monitored for absorbance at 280nm (Millipore Waters, Watford, UK). Four successive step-wise elutions were performed with acetonitrile (ACN) 0.1% TFA,

100mM TEA phosphate pH 2.5, TEA acetate pH 2.5, TEA formate pH 3.5 with a linear gradient for 40 min.

However, the characteristics of the Apex ODS column were not suitable for the elution of cVIP, regardless of the solvent system used. Therefore, a Vydac column (218TP5415) was used, which produced the desired elution profile for synthetic cVIP. This type of column was also used for the purification of cVIP from brain tissue extracts and iodinated VIP ([^{125}I]-VIP). The samples were subjected to RP-HPLC using a linear gradient (Table 2.9) from (A) to (B) for 40 min, with an ACN buffer system (H_2O: ACN: 10%TFA), where (A)=90%:10:1 and (B)=10:90:1. The results are described in Chapter 3 (Section 3.4.1).

Table 2.9 The linear gradient used to elute synthetic VIP from a RP-HPLC column.

Time	**Flow rate**	**% Concentration**	
(min)	**(ml/min)**	**A**	**B**
0	1.5	100	0
20	1.5	65	35
25	1.5	50	50
30	1.5	100	0

2.9 Hormone measurements

The pituitary hormones LH, PRL, GH, ACTH and VIP were measured by the appropriate assay, and are described in the following sections.

2.9.1 Luteinising hormone radioimmunoassay

The chicken LH radioimmunoassay used in these studies was a modification of the double antibody method developed by Sharp *et al.* (1987), and an LH assay diluent : 160 ml 0.5M sodium phosphate buffer; 17.5g NaCl; 5.84g EDTA, 2g sodium azide and 40 ml horse serum (Gibco).

2.9.1.1 Iodination of LH traces

All reagents used were made in 50 mM Na phosphate (NaP) buffer, pH 7.4, unless indicated elsewhere. A 1.25µg aliquot of chicken LH (PRC-AE1-s-1) was

mixed with 25µl of NaP in a 1.5 ml microcentrifuge tube, by a magnetic stirrer. A 37 MBq unit (10µl) of [^{125}I]-Na (IMS-30, Amersham International plc) was added to the tube, and the iodination reaction started with 10µl of 3.55 mM chloromine T, which proceeded for 1 min at room temperature. The reaction mix was stopped by adding 10µl of 5.26 mM sodium metabisulphite, and diluted with 100µl of 0.6M KI.

2.9.1.2 Purification of iodinated LH

A PD-10 Sephadex column (LKB-Pharmacia was pre-equilibrated, with column buffer (NaP containing 0.2% gelatine). The reaction mix was added to the PD-10 column and column buffer (200µl) was used to rinse the microcentrifuge tube, and added to the column. Column buffer was added to the column when required, and 25 fractions of 10 drops were collected into LP4 plastic tubes (Denley-Luckham Ltd.). The iodinated LH was typically eluted in fractions 4-7, and was diluted with RIA diluent (Section 2.9.1) to a concentration of approximately 500 000 counts per minute (cpm) per 10µl and stored at 4°C for up to 4 weeks.

2.9.1.3 Preparation of standards

A chicken LH standard (RI-LH-1) was stored lyophilised at -20°C containing 192µg. This standard was reconstituted with 1 ml of NaP, and split into aliquots of 26µl. The dilution of this aliquot with 974µl of LH diluent produced a working standard of 5µg/ml. A series of 16 dilutions produced a series of 14 standards designated (Std14-Std1) 5.0, 4.0, 2.5, 2.0, 1.0; 0.5, .0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.007, 0.003 and 0.002 ng/ml, which were used in the LH assay.

2.9.1.4 LH assay procedure

The LH assay procedure was also used as a standard assay protocol for the PRL and VIP radioimmunoassay, and any divergence from this method is discussed in the appropriate section. Briefly, the samples and standards (200µl of each) were dispensed into plastic LP2 (Denley-Luckham) tubes with an automated dispenser (Hamilton MicroLab-M, Howe & Co. Ltd); and mixed with the anti-chicken LH primary antiserum (LH 3/3), used at a final dilution of 1: 19 000, and stored at 4°C for 24 hours (See Table 2.10). Radiolabelled [^{125}I]-LH was added to all tubes (12 000 cpm per 50µl) and incubated for another 24 hours. On day 3 of the LH assay, 50µl of normal rabbit serum (NRS) diluted to 1: 200, and 50µl of donkey anti-

rabbit serum (DARS) diluted to 1: 20, were added to each tube and incubated for 24 hours. All the tubes, except the TC were then centrifuged (Sorvall RC-3B) at 1500 x g for 30 min at 4°C. Then 50µl of a 6% starch solution was added to each tube to maintain the integrity of the pellet and centrifuged for another 15 min. The supernatant fraction was then aspirated to waste and the pellets and TC were counted for 60 seconds on a gamma counter (1277 GammaMaster, LKB-Pharmacia), and the data analysed with AssayZap™ (AssayZap Universal Assay calculator, Biosoft).

Table 2.10 The protocol for the radioimmunoassay of chicken LH. *Abbreviations*: *TC* Total counts; *NSB* Non specific binding; *TB* Total binding; *STD* LH standards; *SAM* Samples; *NRS* Normal sheep serum; *DARS* Donkey anti-rabbit serum; *Nil.* No addition made; *Dil* LH diluent.

Solution	**TC**	**NSB**	**TB**	**STD**	**SAM**
DAY 1					
Dil.	Nil.	250µl	200µl	Nil	Nil.
SAMPLE	Nil.	Nil.	Nil.	200µl	200µl
ANTIBODY	Nil.	Nil.	50µl	50µl	50µl
DAY 2					
LH LABEL	50µl	50µl	50µl	50µl	50µl
DAY 3					
NRS (1: 200)	Nil.	50µl	50µl	50µl	50µl
DARS (1: 20)	Nil.	50µl	50µl	50µl	50µl
DAY 4					
		CENTRIFUGATION AT 1500 x g at 4oC for 30 min.			
STARCH	Nil.	50µl	50µl	50µl	50µl
		CENTRIFUGATION AT 1500 x g at 4oC for 15 min.			

2.9.2 Prolactin radioimmunoassay

The PRL radioimmunoassay used in this study was a modification of the method developed by Talbot and Sharp (1994).

2.9.2.1 Preparation of PRL standards

A 5µg aliquot of prolactin stored in microcentrifuge tubes, was diluted to a working concentration of 500ng/ml. The top standard was produced by diluting

the stock 1: 5 with diluent. (Std10-Std1, 125, 62.5, 31.25. 15.63, 7.81, 3.91, 1.95, 0.98, 0.49 and 0.24ng/ml).

2.9.2.2 Iodination of PRL traces

The reaction mixture was composed of 8µl prolactin (5µg), 22µl NaP buffer, 10µl 125I (1MCi) (Amersham International, Amersham UK) and 10µl 3.55mM chloromine T, and incubated for 1min. The reaction was stopped with 200µl 20mM Tris/HCL, pH 7.5, containing 0.1% Tween 20. The reaction mixture was added to a PD10 column and topped up with 0.1M Tris. The eluent was collected (12 drops) in 18 tubes, and the peak identified by counting the tubes with a monitor. The peak of bound activity was further chromatographed, on a Sephacryl HR100 column, equilibrated with 0.1M Tris/HCL, pH 8.5, containing 0.1% Tween 20, and eluted with a flow rate of 1ml/min. The four fractions with the highest activity were pooled and diluted 1:2 with assay diluent and stored at -70°C until used in the assay.

2.9.2.3 PRL assay procedure

The PRL assay procedure was essentially the same as that of the LH-RIA described in Section (2.9.1.4), except that all sample and standard volumes were 100µl, and the NSB and TB were 150µl and 100µl respectively.

2.9.3 VIP radioimmunoassay

Chicken VIP was labelled using a modification of the method described by Sharp et al. (1989). The ^{125}-I labelled VIP was separated from the reaction mixture by the reverse-phase HPLC method described in Section 2.8. .

2.9.3.1 Preparation of VIP standards

The chicken VIP standard (5µg) (Peninsula Laboratories plc.) was diluted with assay diluent (LH diluent) to a volume of 1.2 ml of diluent (4µg/ml). A series of 17 dilutions produced a series of 13 standards designated Std13-Std1: 4000, 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.9, 1.9, 0.98 and 0.49 pg/ml.

2.9.3.2 VIP assay procedure

The VIP assay protocol was the same format as that of the LH radioimmunoassay (Section 2.9.1.4), but ^{125}-I VIP (15 000 cpm/ tube in 50µl) was added to each tube on day 3. The bound cVIP was precipitated on day 4 with anti-sheep precipitating serum (DASS) and non-immune sheep serum (NSS) at final concentrations of 1:20 and 1:200 respectively.

2.9.3.3 Validation of VIP antibodies for VIP radioimmunoassay

Three sheep anti-chicken VIP antibodies 33/8, 33/4 and 31/4, were analysed for their suitability in a radioimmunoassay. Dilution curves were used to establish the binding characteristics of each antibody over a range of dilutions 1:100-1:128 000. Standard curves were set up for each antibody, with a predetermined antibody dilution which was extrapolated from the dilution curves, to give a specific binding of 25%. The cVIP standard displaced [^{125}I]-VIP from the antibody 33/4 more effectively than the other two antibodies, and was used as the standard VIP antibody in the VIP radioimmunoassays .

2.9.4 Growth hormone

GH was measured by an enzyme linked immunoabsorbant assay (ELISA) modified by Houston *et al.* (1991).

2.9.4.1 Preparation of the pre-coated plates

The antibody code 6F5 was diluted with 50 mM $NaHCO_3$ pH 9.6, to give a working concentration of 1µg/ml 6F5 / 100µl; which was added to each well (100µl) of a number of 96 well plates and incubated for 24 hours at 4°C. The plates were washed 3 times (Wash buffer, PBS containing 0.25%(v/v) Tween 20) with a plate washer and then incubated (200µl per well) with a block solution of 2% RIA grade BSA, for 1 hour at 37°C. The plates were washed another 3 times (wash buffer) and then each well was incubated with 200µl of a 30% sucrose solution, for 2 hours at room temperature. The sucrose solution was aspirated to waste and the plates allowed to dry at room temperature, and stored at 4°C until required.

2.9.4.2 Preparation of biotinylated antibody

The purified GH (B4E4) antibody (1.5-2.0 mg) was dialysed overnight at 4°C in 4 litres 0.1 M $NaHCO_3$ pH 8.0, and the protein concentration measured by absorbance at 280 nm. Five mg of biotin succinimide ester was dissolved in 2 ml of DMSO and then added to the antibody at a concentration of 50µl per ml antibody, and incubated at room temperature for 2 hours. The anti- chicken GH biotinylated antibody was dialysed for 24 hours at 4°C in PBS pH 7.4, and the concentration measured by absorbance at 280 nm.

The samples and GH standards (800, 400, 200, 100, 50, 25, 12.5, 6.25pg/100µl) were made in PBS pH 7.4 containing 2% BSA, and 100µl were dispensed to each well and incubated for 2 hours at room temperature. The wells were washed 6 times (wash buffer) and then incubated with (100µl per well) biotinylated B4E4 at a concentration of 0.5µg/100µl, for 1 hour at room temperature. The plates were washed 6 times again, and a streptavidin-horseradish peroxidase solution (1:1000 in blocking buffer), was (100µl) added to each well. The plates were washed (6 times) for a final time, and then 100µl of the developing solution (26 mM NaHPO4, pH 5, 24 mM citric acid, 0.5mg/ml phenylenediamine) was added to each well and incubated for 3 min, followed by 50µl of stop solution (2M H_2SO_4). The optical density of each sample was read at 490nm in a MR700 plate reader (Dynatec Laboratories, Burgess Hill, UK), against a blank (PBS only).

2.9.5 Adrenocorticotrophic hormone

The measurement of ACTH in tissue culture media was kindly performed by Dr. F. Antoni using a double antibody precipitation ACTH-RIA described by Werner *et al.* (1989). The antibody no. 6 (G.B. Mahara, Budapest, Hungary), and the assay standard human $ACTH_{1-39}$ (CIBA Geigt, Basel, Switzerland), were diluted with an assay buffer (0.5mM NaP buffer, pH 7.4), containing 6% polyethylene glycol (PEG, MW 6000), 1% BSA, 0.1% Tritron X-100, 2.5mM EDTA, 100 kallikrein U/ml of aprotinin. A 50µl sample was incubated with 50µl antiserum, and 10µl tracer (10 000 cpm/10µl) for 24hrs. Then precipitating serum, anti-rabbit IgG and non-immune rabbit serum were added and incubated for 3hrs at room temperature. Finally, 1ml of 3% PEG was added to each tube, spun for 30min, the supernatant decanted and the pellet counted on a gamma counter.

2.10 Cell Division

2.10.1 Bromodeoxyuridine incorporation

DNA replication in a cell is principally confined to the S phase of the cell cycle, and it has been shown that cells in the S phase can incorporate modified DNA precursors. The use of radiolabelled DNA precursors such as ^{3}H or ^{14}C isotopes of thymidine, have been used to identify dividing cells *in vivo* or *in vitro*. The introduction of a pyrimidine analogue of thymidine, 5-bromo-2-deoxyuridine (BrdU), in conjunction with the development of monoclonal antibodies to BrdU (anti-BrdU) has provided a successful alternative (Gratzner *et al.,* 1975).

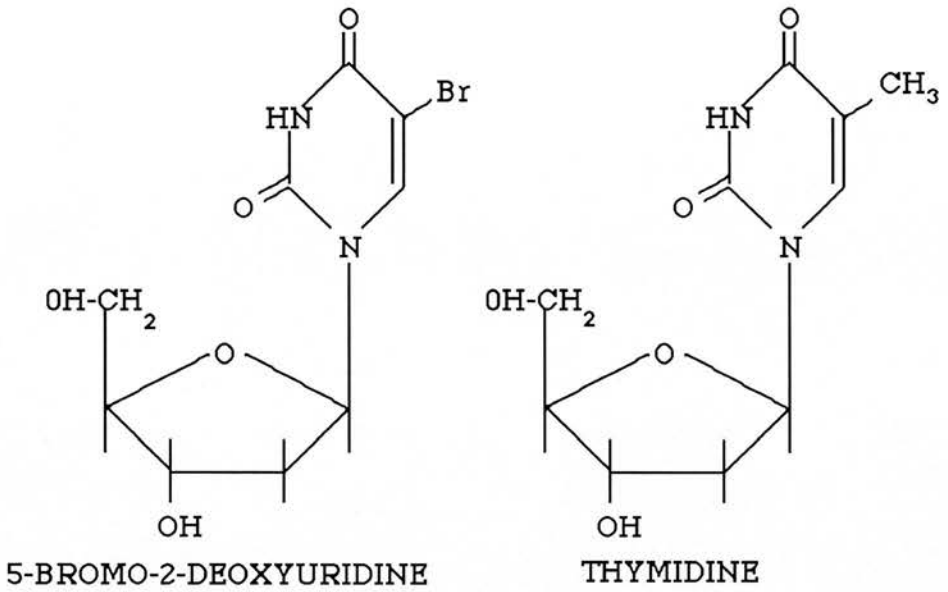


Figure 2.5 Structural comparisons of 5-bromo-2-deoxyuridine and thymidine.

The usual method for BrdU administration has been intramuscular or intravenous injection, however this technique is not very practical over a long period of time. A BrdU pellet had been designed (Innovative Research of America, Ohio, U.S.A.) to deliver a pre-determined dose of BrdU over a specified time period, which has been used in several species (Weghorst, 1991).

A Matrix-driven delivery (MDD) system controls the release of BrdU over a 21 day period. The calculated hourly release rates for the pellets were determined to be

approximately 20, 50, 100, 200 and 200µg per hour for the 5 doses of 10mg, 25mg, 50mg, 100mg and 200mg BrdU respectively (Fig. 2.7). The BrdU pellets were implanted into 5 groups of laying bantam hens, which were transferred to floor pens to induce broodiness.

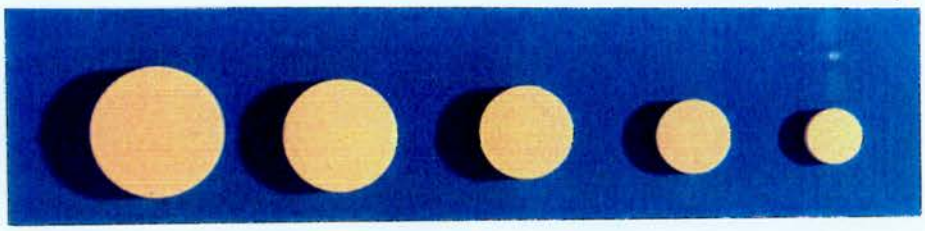


Figure 2.6 A photograph of the bromodeoxyuridine (BrdU) pellets which were implanted into the neck. The pellets are aligned in descending size and dosage; 200mg, 100mg, 50mg, 25mg and 10mg BrdU.

2.10.2 Pellet implantation

After an injection of local anaesthetic (lignocaine), the skin on the back of the birds neck was pulled and a small incision was made the size of the pellet, with a scalpel. A pocket was made horizontally with a pair of forceps, about 2 cm beyond the incision site. The pellet was placed in the pocket with a forceps. A stitch (cat gut) was used to close the pocket and an antibiotic (Aureomycin, Cynamid, Gosport, U.K.) was applied to the wound as a precaution. The bird was then monitored on a daily basis for any adverse affects, resulting from the pellet implantation procedure.

2.10.3 Experimental procedure

Thirty laying bantam hens were chosen at random, and allocated to a group (n=5), and administered a predetermined dose of BrdU, in the form of a 21 day release pellet (see Section 2.10.1). A small incision was made in the neck of control animals to mimic the implantation procedure. After pellet implantation, each group was transferred to a floor pen with 5 nest boxes to induce broodiness, and were monitored on a daily basis for any characteristic signs of broody behaviour. In addition, the birds were weighed and a blood sample taken on days 1, 7, 14 and 21 days of the study, and these samples were analysed by PRL radioimmunoassay

(see Section 2.9.3). The number of laying birds transferred to floor pens at any one time was staggered, to reduce the workload at the end of the experiment. Ten birds were placed into their respective floor pens on day 1, and then two further groups of 10 birds were transferred on subsequent days. The birds were terminally anaesthetised and perfused with heparin/saline (see Section 2.2.3), on day 21. The anterior pituitary glands were removed for microwave fixation and processing (see Section 2.2.4).

2.10.4 Blood smear

Blood samples were taken from the brachial wing vein with heparinised syringes, and a drop of blood was applied to a slide with a plastic Pasteur pipette. A blood smear was formed by pushing the drop of blood down the slide, and allowed to air dry. The slides were then stored in a dry box, until required for immunocytochemical staining.

2.10.5 Tissue collection

The experimental birds were perfused (Section 2.2.3) with 250-300ml (0.9% NaCl w/v) containing 750 IU heparin, for 10-15 min to remove the blood from the brain and then the anterior pituitary gland was dissected using the method described in Section 2.2.2.

2.10.6 Immunocytochemical detection of BrdU labelling in tissue sections

The anterior pituitary glands were subjected to microwave fixation and microtome sectioning as described in Section 2.2.5. The anterior pituitary tissue sections were processed for peroxidase staining (mouse, Vector ABC kit) (Section 2.2.7), except for the inclusion of a DNA denaturation step. Briefly, the sections were incubated successively in methanol (0.3% H_2O_2) PBS washes, 2M HCl for 1 hour at 37°C, and borate buffer (neutralisation) (0.1M, pH 8.5) for 10 mins. The acid treatment was required to relax the DNA and aid antibody accessibility (Dinjens *et al.*, 1992). A mouse monoclonal anti-BrdU (1:1 000) antibody was used to detect the incorporated BrdU in anterior pituitary sections. The peroxidase was visualised by immersing the sections in 0.025% DAB containing 0.03% H_2O_2 in 0.05M Tris, pH 7.5. The sections were dried, cover slipped in DPX mountant and viewed by light microscopy (Olympus).

2.10.7 Immunocytochemical detection of BrdU labelling in blood smears

The blood smears were fixed in methanol (6 mins) and air dried. The blood smears were subjected to the same acid hydrolysis and neutralisation treatments, described previously in Section 2.10.6. Then the sections were incubated overnight with a mouse monoclonal anti-BrdU (1:1 000) antibody. The primary antibody was visualised by incubating the sections with an anti-mouse FITC conjugate (1:50) second antibody (Sigma), mounted in aqueous mounting media and coverslipped for examination by blue epi-illumination (Nikon Optiphot) (see Section 2.2.7).

2.11 Tissue Culture

2.11.1 Reagents

All procedures were performed in a laminar air cabinet (Flow Labs) using aseptic conditions, and sterile solutions and equipment. The medium used for cell culture was medium 199 (M199), supplied as a sterile powder which was redissolved in sterile H_20, bicarbonate buffered (26.2M, $NaHCO_3$) and pH adjusted with HCl to 7.4. The medium was supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 4 mM L-glutamine and 2% (v/v) steroid stripped newborn calf serum (NBCS), and filtered with a 0.22µm bottle top filter (Costar Ltd, High Wycombe, U.K.). The steroids were stripped from NBCS by mixing with 1% (w/v) charcoal and 0.1% (w/v) dextran T-70 overnight at 4°C, followed by centrifugation (Beckman J2-21M/E, JA-20 rotor) at 20,000 x g for 30 mins. at 4°C, and sterilised by filtration.

2.11.2 Tissue collection and processing

The anterior pituitary glands were removed from the hen and placed in a sterile 30ml plastic Universal vial containing 10ml of medium M199 at room temperature. The vial was then transferred to the flow cabinet and the tissue placed onto a new recepticle containing supplemented medium M199 and the pituitary glands were washed 3-4 times in medium. The pituitary glands were then placed in a sterile glass petri dish and cut along the midline with a razor blade, to produce Hemi-pituitary glands. These were washed twice, in the manner described previously.

2.11.3 Culture method

The Hemi-pituitary glands from laying or broody bantam hens were randomly selected from each group and placed into a well containing 500µl medium, in a multi-well culture plate (24-well plate) (Costar Ltd, High Wycombe, U.K.) and cultured at 37°C in a humidified atmosphere of 5% CO_2: 95% air.

2.11.4 Pituitary tissue stimulation protocol

The Hemi-pituitary glands were incubated for 3 hours, which was considered a wash period. The 500µl medium was removed and replaced with 500µl of fresh medium in the presence or absence of treatment. After the incubation time had elapsed the medium was removed and frozen at -20°C for LH and/or PRL radioimmunoassay. The wet pituitary weight was then measured.

2.12 Data presentation and analysis

The data were processed by Apple Macintosh. The determination of statistical significance, of data obtained from radioimmunoassay was by ANOVA, followed by post hoc comparisons when appropriate, using the Dunnett's test. A Students t-test (StatWorks) was also used for the analysis of VIP extracted from the hypothalamic tissue, of laying and broody hens. The data was expressed as the mean ± standard error of the mean for each experimental group (SEM) (Cricket Software), where $P<0.05$ was considered significant.

Chapter 3: THE IMMUNOCYTOCHEMICAL LOCALISATION OF VIP IN THE ANTERIOR PITUITARY GLAND

3.1 Introduction

Vasoactive intestinal polypeptide (VIP) has been reported to be synthesised within the anterior pituitary gland of the rat (Arnaout *et al.,* 1986; Hagen *et al.,* 1986). A cell which expresses VIP immunoreactivity (VIP-ir) has been observed but the specific cell type remains uncertain. In the rat the lactotroph may synthesize intra-pituitary VIP (Morel *et al.,* 1982; Steel *et al.,* 1989), and regulate prolactin secretion in an autocrine manner (Nagy *et al.,* 1988). However this observation is not supported by similar immunocytochemical studies, where the VIP-cell type has been found to be distinct from that of lactotrophs and thyrotrophs (Lam *et al.,* 1989; Segerson *et al.,* 1989; Carrillo and Phelps, 1992). The aim of this investigation was to develop an immunocytochemical method, to localise VIP in the anterior pituitary of the bantam hen and to determine the cell types in which VIP occurs.

3.2 The immunocytochemical localisation of VIP in the anterior pituitary gland

3.2.1 Validation of immunocytochemical procedure

The immunocytochemical procedure was developed to allow the use of thin sections, which retained their morphology and resolution when viewed at a high magnification. Initial trials used a method devised by Fragioni and Borgioli (1986), and the fixative Kryofix (Merck) which was used for microwave fixation (Boon and Kok, 1987). The method was suitable for the immunolocalisation of VIP-ir and PRL-immunoreactivity (PRL-ir), but was not compatible with any anti-LH antibody. Therefore, the fixation was modified and a Bouin Hollande fixative was substituted for the Kryofix and was established as the standard immunocytochemical fixative throughout the studies. The VIP-ir was localised in the anterior pituitary gland of laying (n=6) and broody (n=6) bantam hens and was visualised using a donkey anti-sheep FITC (SAPU) conjugated antibody and viewed by fluorescent microscopy (Section 2.2.7)

The immunolabelling of VIP cells in the anterior pituitary was abolished (Fig. 3.1) by pre-absorbing the VIP antiserum with 20µg VIP/ml antiserum diluted 1:2000, No staining was observed when the sections were incubated with VIP primary antiserum only, or when the primary anti serum was replaced with normal donkey serum. Finally, sections of the mediobasal hypothalamus and median eminence region were used as a positive control tissue (Fig. 3.1), to confirm that pre-absorption of the VIP antiserum with VIP, abolished all specific immunostaining (Fig. 3.1). The characteristic labelling of gonadotrophs and lactotrophs using antibodies against luteinising hormone (LH) and prolactin (PRL) respectively, was achieved with a donkey anti-rabbit Lissamine conjugate (Jackson).

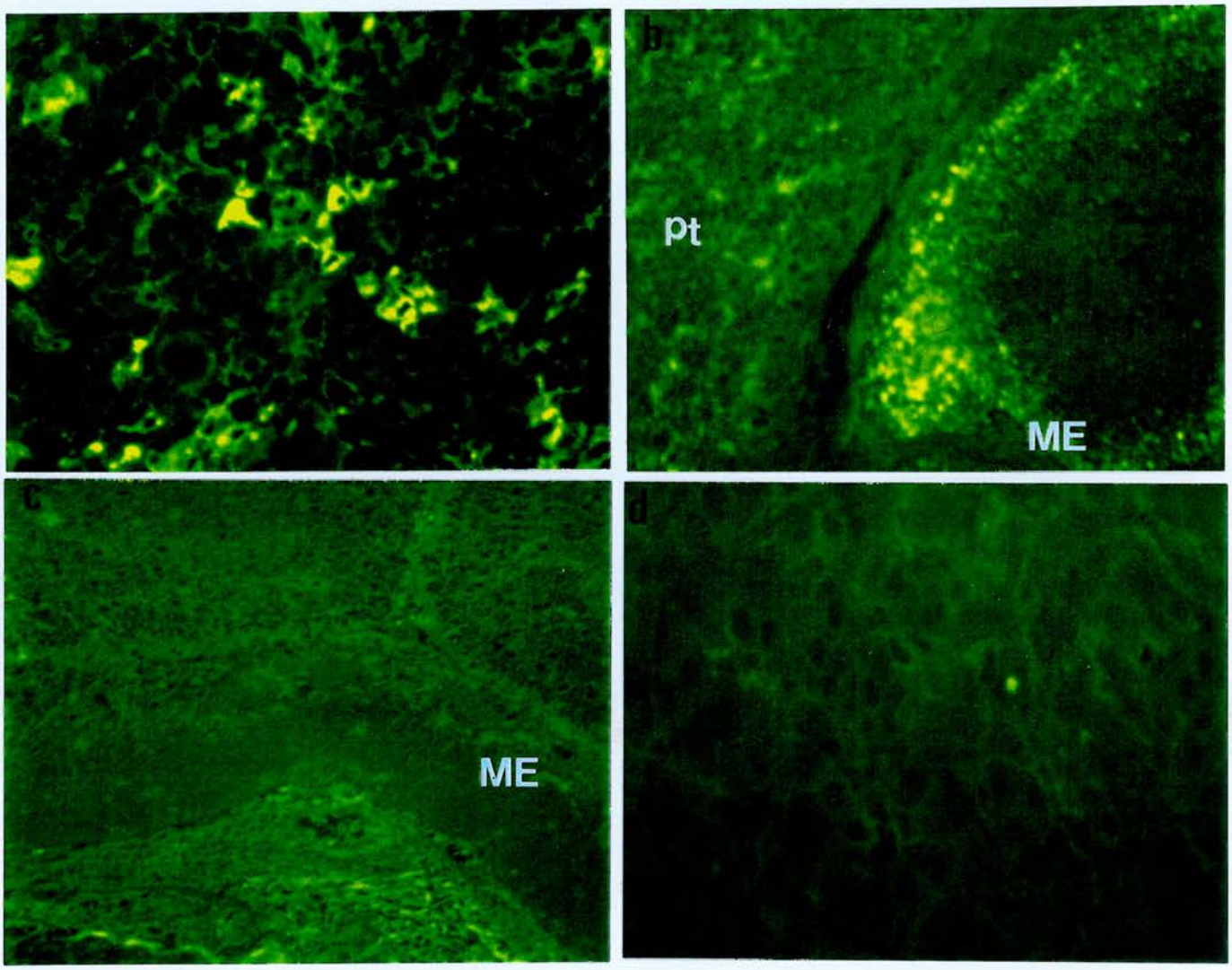


Figure 3.1 Photomicrographs illustrating the (a) distribution of VIP-immunoreactivity type in the anterior pituitary (pt) gland and the (Mag. x200) (b) VIP-immunoreactive terminal fibres in the median eminence (ME) (Mag. x100). The immunostaining was abolished in the (c) median eminence (Mag. x100) and the (d) anterior pituitary using the VIP antiserum diluted 1:2000 pre-absorbed with 20µg VIP/ml (Mag. x400).

3.2.2 Distribution of VIP, LH and PRL in the anterior pituitary

The VIP-ir was found in both the cephalic and caudal lobes of the anterior pituitary. An attempt was made to count VIP-ir cells in the pituitary gland using an image analysis programme, but was unsuccessful. The VIP-ir in the anterior pituitary of the laying bantam hen (Fig. 3.2) appeared to be more intense compared to that of the broody bantam (Fig. 3.2). The intensity of VIP-ir staining in the cephalic lobe in the pituitary of the broody bantam was less, than in the caudal lobe (Fig. 3.3). LH-immunoreactive (LH-ir) cells were distributed throughout the cephalic and caudal lobes in the anterior pituitary of both the laying (n=5) and broody (n=5) bantam hen, and was similar to the distribution of the VIP-ir cells (Fig. 3.2). The LH-ir appeared to be more intense in the pituitary of the laying hen compared to the broody hen; and the immunoreactive labelling in the cephalic lobe appeared to be less intense compared to that of the caudal lobe in the broody bantam (Fig. 3.2). The PRL immunoreactive (PRL-ir) cells were confined to the cephalic lobe in the anterior pituitary of the laying (n=5) and broody (n=5) bantam hen (Fig. 3.2). However, the PRL immunoreactive labelling in the cephalic lobe of the broody bantam hen was more extensive than in the cephalic lobe of the laying hen. (Fig. 3.2).

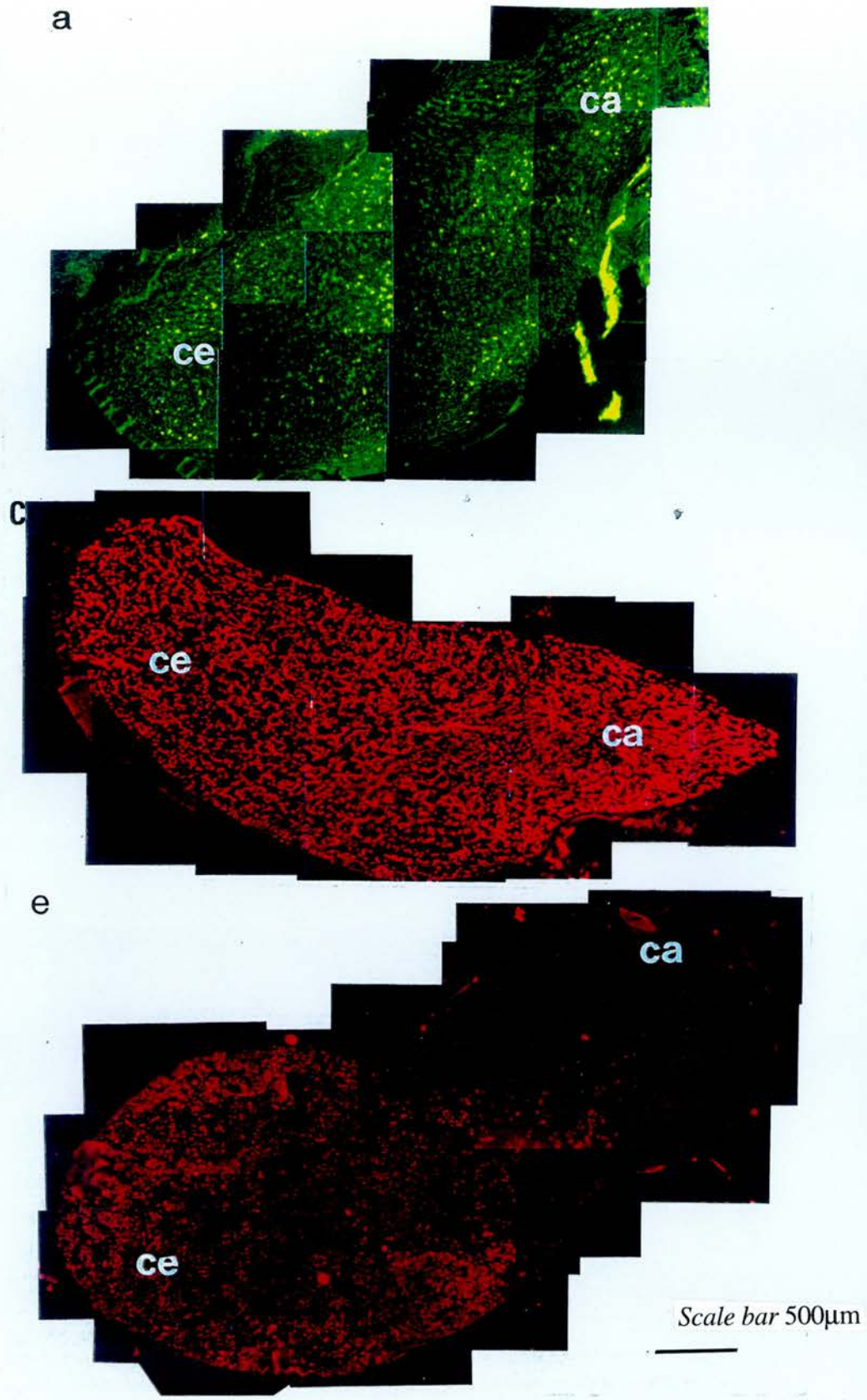
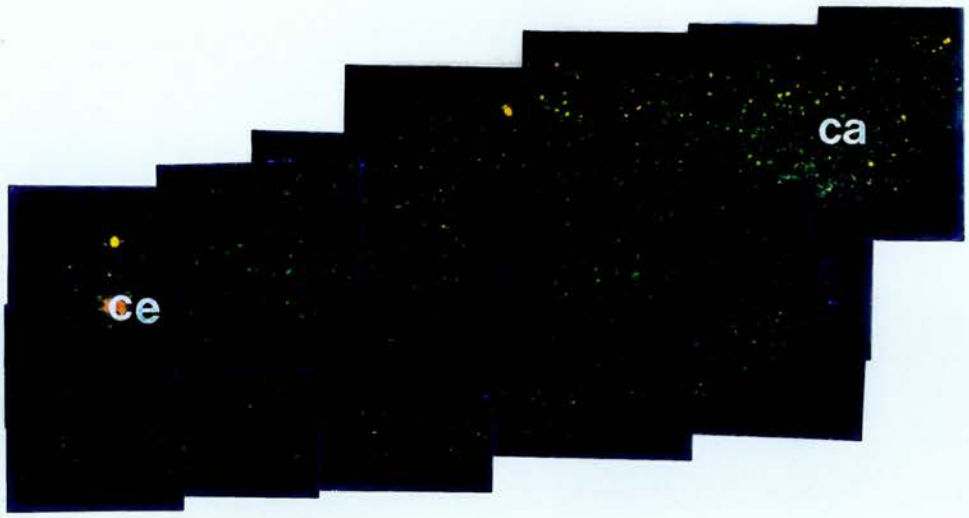


Figure 3.2 Composite photomicrographs showing the distribution of (a, b) VIP-ir cells, (c, d) LH-ir cells and (e, f) PRL-ir cells in the anterior pituitary gland of the (a, c, e, *left page*) laying bantam and (b, d, f, *right page*) broody bantam hen. (ce) Cephalic lobe, (ca) Caudal lobe.

b



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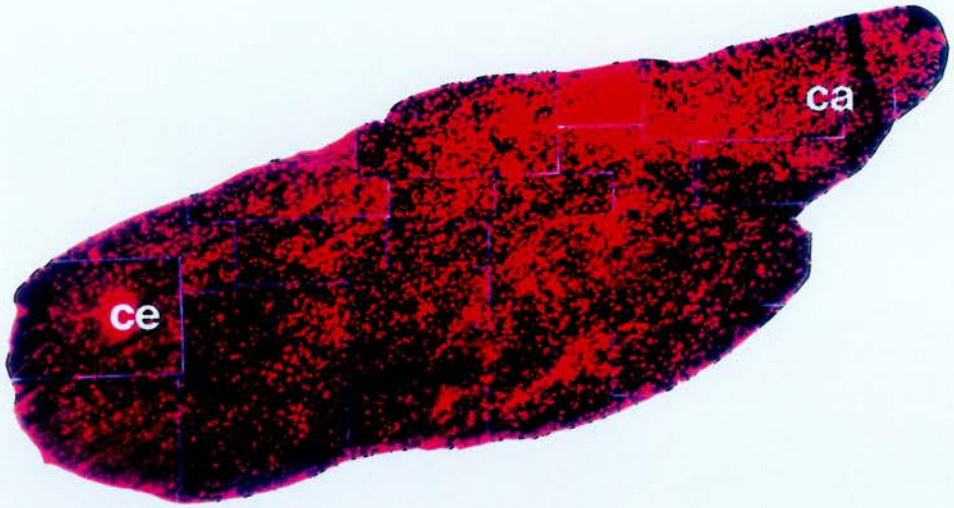

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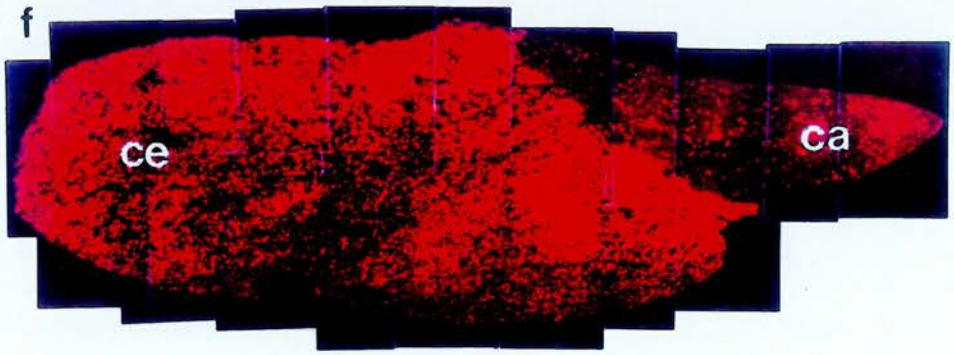

3.2.3 The VIP-cell type

The VIP-immunoreactivity was confined to the cytoplasm. There were two VIP-cell types which were arranged singly or in clusters of 2-6 cells. One cell type stellate in shape, with an irregular shaped nucleus, and long cytoplasmic projections which extended between neighbouring pituitary hormone secreting cells (Fig. 3.4).It was weakly immunolabelled and distinctive from that of the normal pituitary hormone secreting cell types (Fig. 3.4). The second VIP-cell was often larger than LH and PRL cells, with a large nucleus, polygonal shape and greater immunoreactive labelling (Fig. 3.4). In contrast, the LH- and PRL-cells were more rounded or oval in shape compared to that of the VIP-cell.

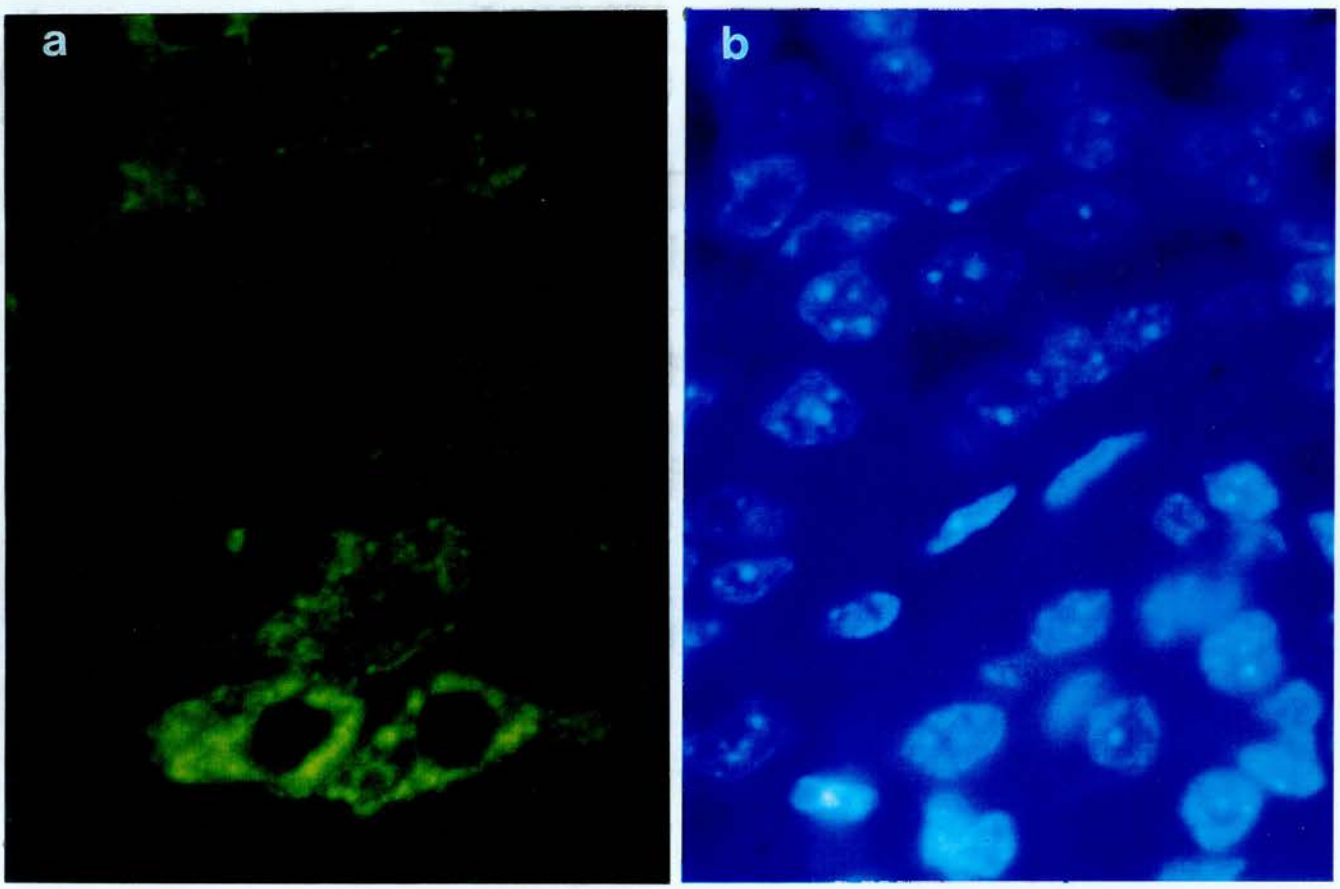

Figure 3.4 Photomicrographs illustrating the two types of VIP-immunoreactive cell types, visualised by an immunocytochemical procedure using antibodies raised against chicken vasoactive intestinal polypeptide. (a) One VIP-cell type (*S*) was more stellate in shape with long cytoplasmic projections, and the second VIP-cell (*V*) was larger but more polygonal in shape. The (b) nuclei were labelled with DAPI (Magnification x 1000).

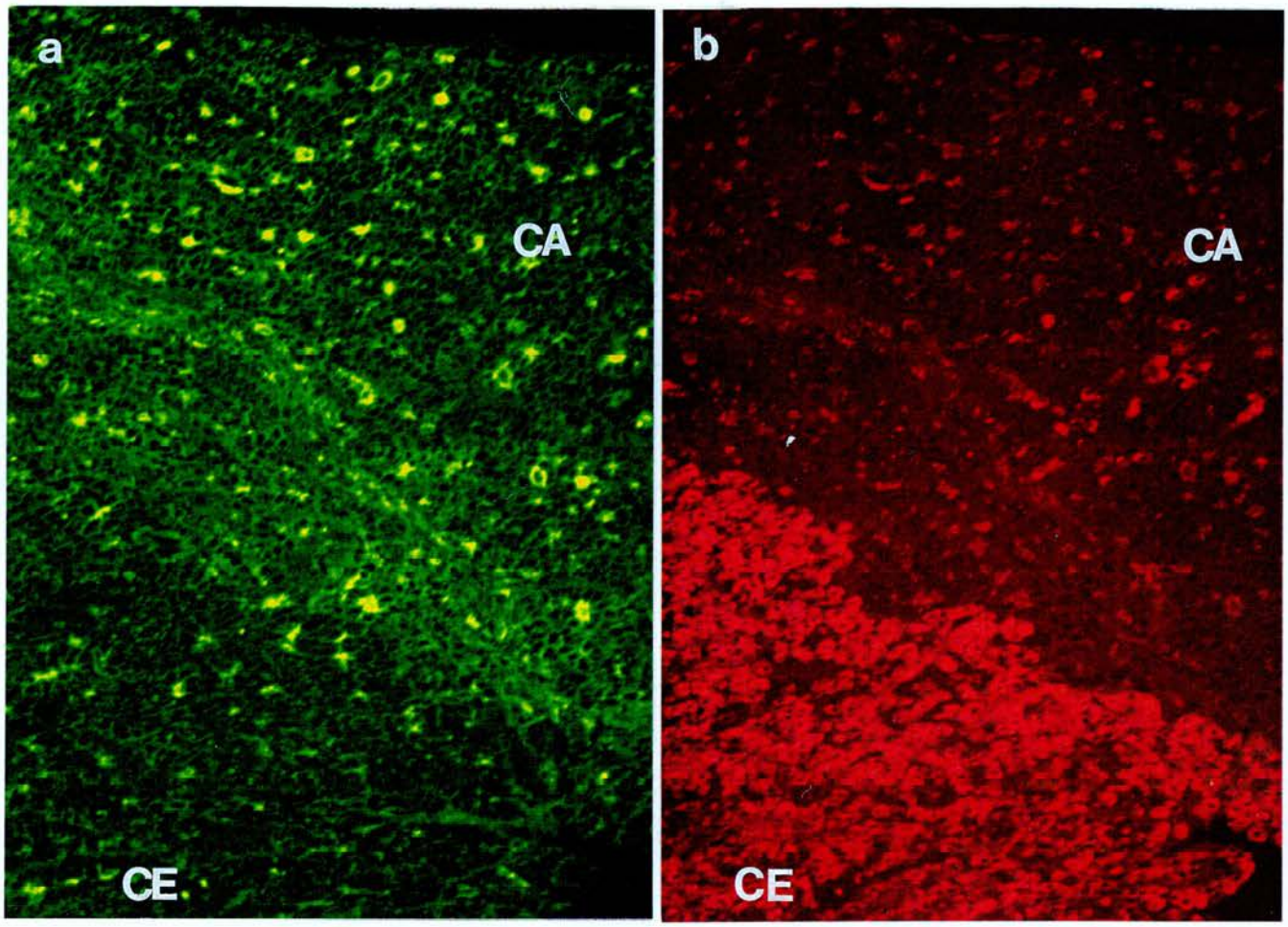

Figure 3.3 The distribution of VIP-immunoreactivity (VIP-ir) and PRL-immunoreactivity (PRL-ir) in the anterior pituitary gland of a broody bantam hen. The (a) VIP-ir appears greater in the caudal lobe compared to that of the cephalic lobe. The (b) PRL-ir is confined to the cephalic lobe (Mag. x100).

3.2.4 Relationship between gonadotrophs, lactotrophs and the VIP-cell

The VIP-ir cells were closely associated with both LH and PRL cells. The VIP-cells were often positioned centrally in groups of LH cells in the pituitary of a laying bantam (Fig. 3.5a), or closely surrounded adjacent LH cells in the pituitary of a broody bantam (Fig. 3.5b). There was no evidence for the colocalisation of VIP-ir in LH-ir cells. There was also a close relationship between VIP-cells and PRL cells in the cephalic lobe of a laying (Fig. 3.5c) and broody bantam (Fig 3.5d), but there was no evidence of VIP-ir and PRL colocalisation.

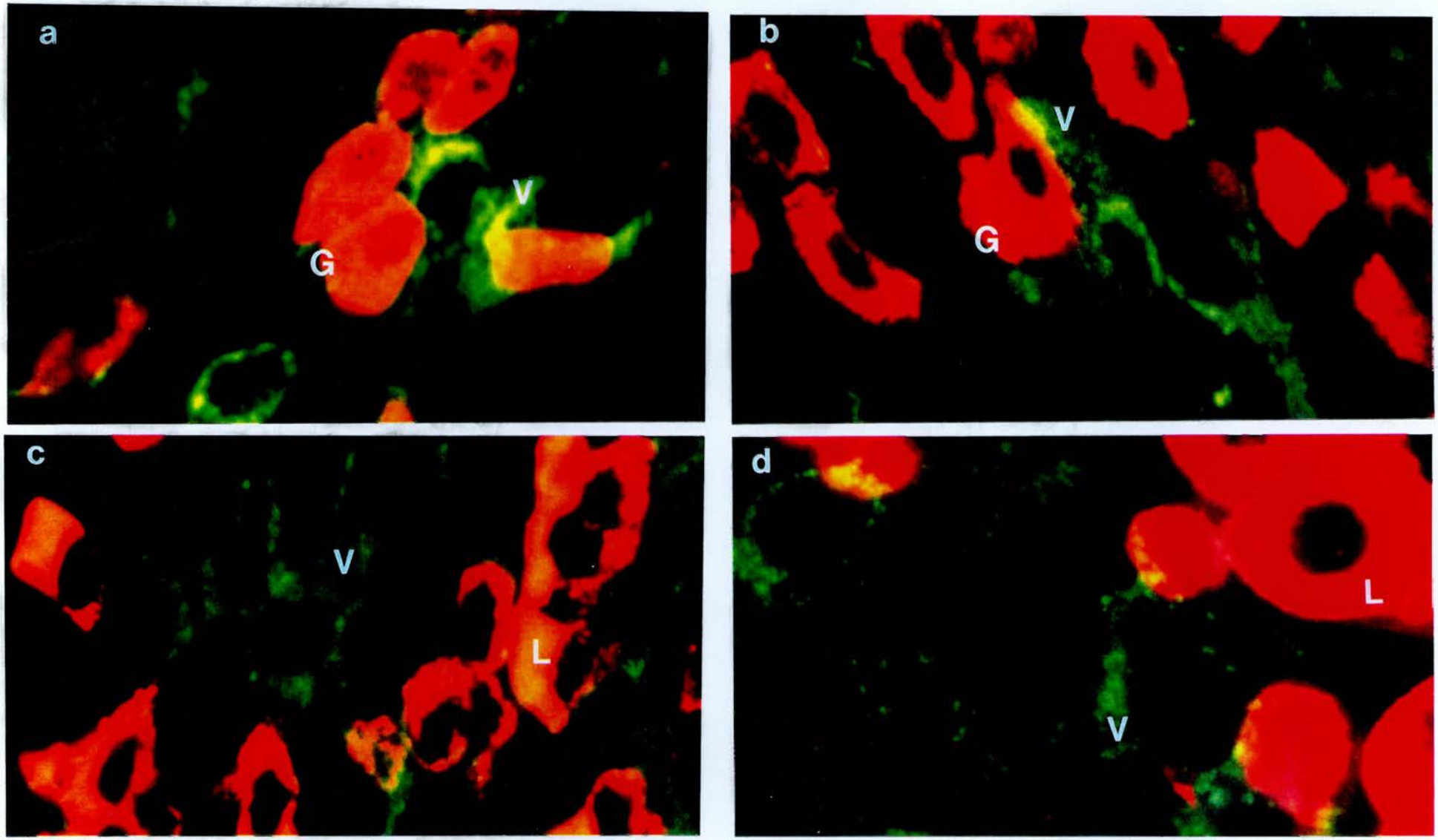

Figure 3.5 Sagital sections (2μm) of anterior pituitary tissue from a (a, c) laying and (b, d) broody bantam hen, showing the relationship between VIP-like immunoreactive (V) cells and gonadotrophs (G) or lactotrophs (L) in the cephalic lobe. After double labelling using FITC (*green*) to visualise VIP and Lissamine rhodamine (*red*) to visualise LH and PRL. The image was generated by superimposing the photographic image of LH or PRL cells onto a photographic image of VIP cells to form a composite image (Magnification x 1000).

3.2.5 The localisation of S-100 and GFAP immunoreactivity in the hypothalamus

The aim of this study was to establish the distribution of S-100 and GFAP in the hypothalamus, to confirm the specificity of the immunolabelling observed in the anterior pituitary gland (n=3). Both proteins are reported to occur in the hypothalamus of several species, avian brain (Goto *et al.,* 1988; Cameron-Curry *et al.,* 1991), and are specific markers for glial and folliculo-stellate cells..

S-100 and GFAP-immunoreactivity occurred in the basal hypothalamic region. S-100(+)-ir occurred in glial-like cells, which were irregular in shape with numerous fibre structures in the paraventricular area of the hypothalamus (Fig 3.6), but little or no immunoreactivity was observed along the third ventricle (Fig 3.6). The GFAP immunoreactive (GFAP(+)) cells were the same as those of the S-100(+) cells. They had an irregular shaped cell body with several projections, and occurred in the paraventricular hypothalamus (not shown).

Little or no S-100(+) or GFAP(+) immunoreactivity occurred elsewhere in the hypothalamus and appeared to be absent along the third ventricle (Fig 3.6). A DAPI stain was also used to identify the nuclei on the specimen, and confirm the S-100 and GFAP immunoreactivity was associated with specific cell structures. The S-100 and GFAP antibodies immunolabelled cells within the hypothalamus, which appeared to be glial like as described previously (Goto *et al.,* 1988; Cameron-Curry *et al.,* 1991).

3.2.6 The localisation of GFAP and S-100 protein in the anterior pituitary

The localisation of folliculo-stellate cells (FS-cells) in the chicken anterior pituitary gland was visualised using antibodies raised against S-100 protein, and glial fibrillary acidic protein (GFAP), which are specific markers for astroglia and FS-cells. The antibodies have been characterised, and used to demonstrate the distribution of S-100 immunoreactivity in the chick brain (Atoji *et al.,* 1990) and GFAP-immunoreactivity in the quail central nervous system (Cameron-Curry *et al.,* 1991).

In the present study, S-100-like immunoreactive (S-100(+)) cells were distributed throughout the anterior pituitary gland in both lobes of the laying and broody bantam hen. GFAP-immunoreactivity was not seen in the chicken anterior pituitary gland. The majority of S-100(+) cells were intensely immunoreactive and ,formed a dispersed network of stellate cells, linked by fine cytoplasmic processes, which

encircled numerous pituitary hormone secreting cells (Fig 3.7c). Less intensely stained S-100(+) immunoreactive cells were also observed. These were less stellate and had a large nuclear: cytoplasm ratio, and occurred in clusters (Fig. 3.7a). The morphological features of the heavily labelled S-100(+) cells were characteristic of folliculo-stellate cells (FS-cells) (see Section 1.9.1).

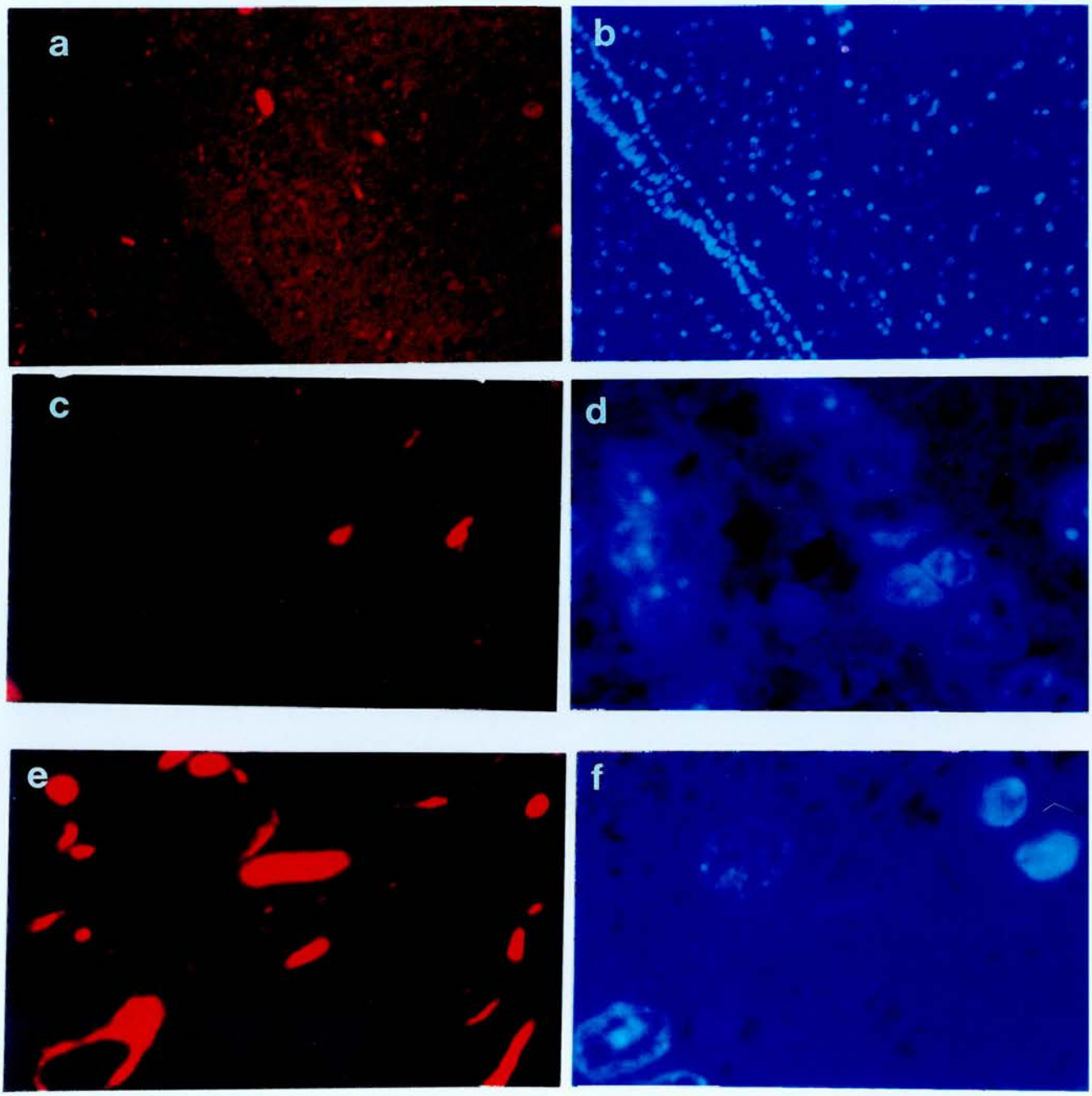

Figure 3.6 Photomicrographs showing the lack of (a, c) S-100 immunoreactivity (Mag. x100). The (e) S-100(+) immunoreactivity occurred in glial-like cells contained in the paraventricular region of the hypothalamus (Mag. x 1000). The nuclei (b, d, f) were immunolabelled with DAPI.

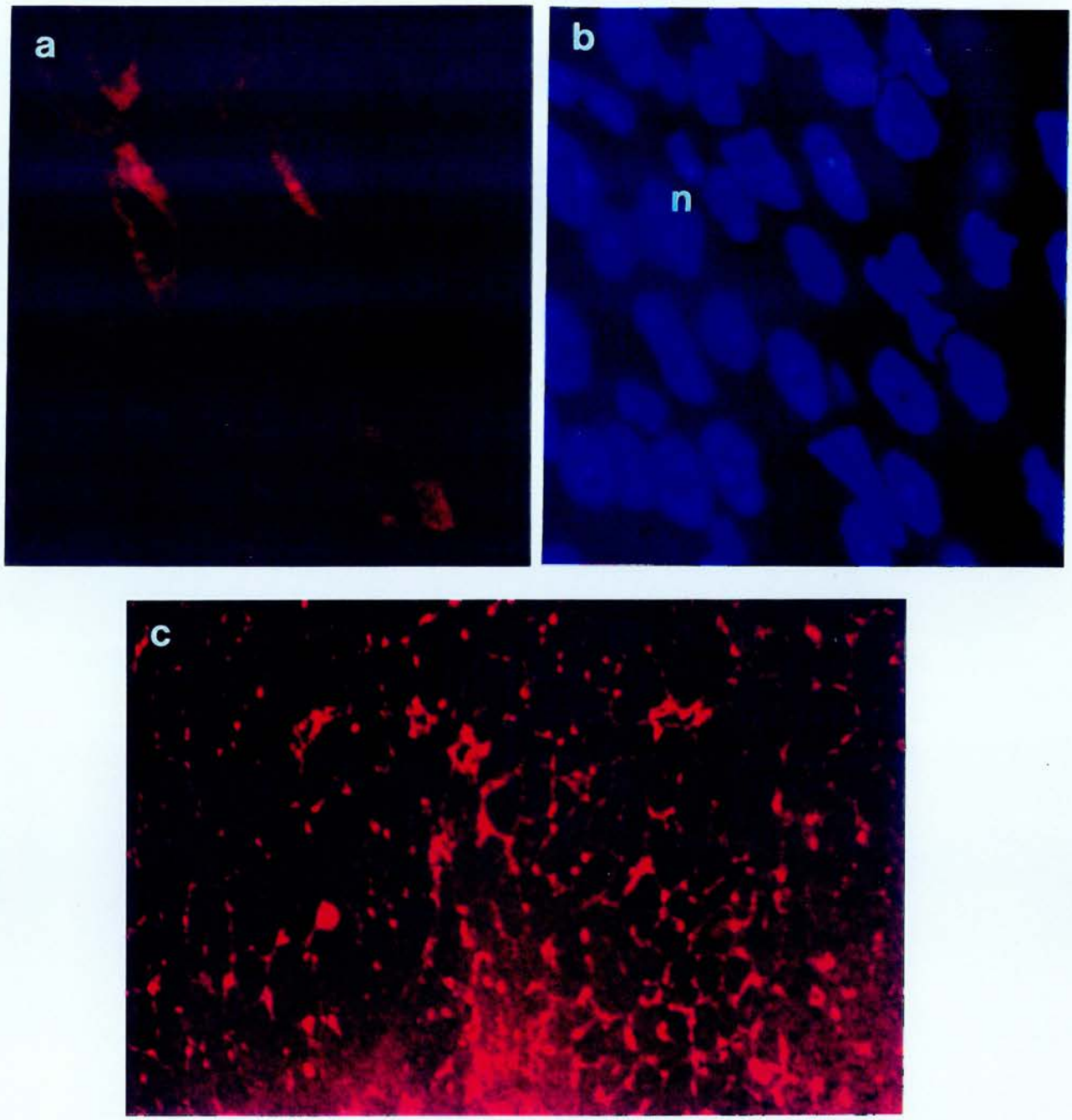

Figure 3.7 A photomicrograph of (a) the weaker S-100(+) stained cells (Mag. x1000) in the cephalic lobe of the anterior pituitary and (b) the nucleus visualised with a DAPI stain (Mag. x1000). The dispersed network of FS-cells is distributed (c) throughout the anterior pituitary gland, visualised by an immunocytochemical procedure using an antibody raised against S-100 protein (Mag. x100).

3.2.7 Co-localisation of VIP and S-100 in the pituitary

The morphology and distribution of VIP-cells and FS-cells was similar, and both occurred in the cephalic and caudal lobe of the anterior pituitary gland. VIP was co-localised with S-100 in many FS-cells (Fig. 3.8). However, not all VIP-immunoreactive cell types contained S-100 immunoreactivity, and these VIP-ir cells were larger and more intensely stained (Fig. 3.8).

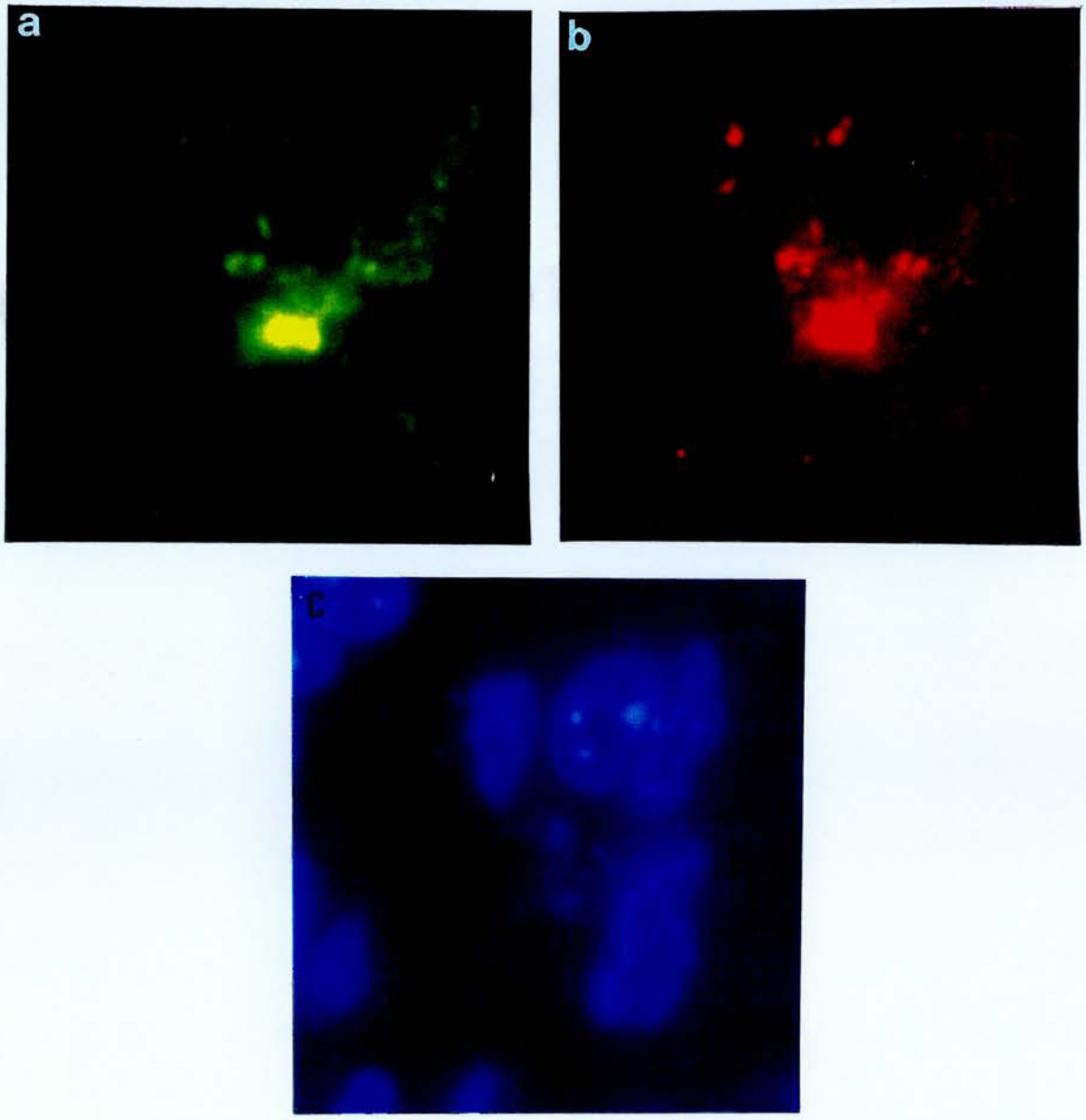

Figure 3.8 Co-localization of (a) VIP- and (b) S-100-positive cells in the anterior pituitary gland. After double labelling using FITC (green) to visualise VIP and Lissamine rhodamine (red) to visualise S-100 in FS-cells, (a) VIP-ir was seen in (b) FS-cells. The presence of (c) nuclei in VIP- and FS-cells was achieved by staining the section with DAPI (blue) (Magnification x 1000).

3.3 Distribution of PACAP-like immunoreactivity in the basal hypothalamus and anterior pituitary.

In the previous section (3.2.1) VIP-ir was demonstrated in the anterior pituitary gland and basal hypothalamus, however it was not confirmed whether or not the chicken VIP antibody cross reacted with the recently discovered neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP). The aim of this study was to confirm that the immunoreactivity observed in the anterior pituitary was due to the presence of VIP, and not due to a cross reaction with PACAP. PACAP-immunoreactivity is reported to occur throughout the hypothalamus and median eminence of several mammalian species (Koves *et al.,* 1990b; 1991; Vigh *et al.,* 1991). Sections of hypothalamus tissue from a laying bantam (n=3) were used as a positive control, in conjunction with the anterior pituitary gland (n=3). The localisation of PACAP-ir was determined using a specific mammalian anti-PACAP27 (mPACAP) antibody (a gift from A. Arimura). The specificity of the cVIP (code/6DL/31/4) and mPACAP antibodies, was determined by pre-incubating the primary antibodies with cVIP, cPACAP38 or ovine PACAP38.

3.3.1 The localisation of VIP and PACAP immunoreactivity in the hypothalamus and anterior pituitary gland

The optimal dilution of the mPACAP antibody used on free-floating hypothalamic sections was 1: 4000, when visualised by DAB staining (Section 2.2.8), and 1: 2000 when visualised by Lissamine rhodamine fluorescent labelling method (Section 2.2.7). The mPACAP antibody was used on thin (2μm) sections of the anterior pituitary gland (Section 2.2.5), with a range of dilutions (1: 100-2000), and also visualised with a Lissamine rhodamine fluorescent labelling method (Section 2.2.7). The antibody has been fully characterised, and shows no cross reaction with a number of related peptides, including mammalian VIP, and the density and distribution of PACAP-like immunoreactivity in the hypothalamus was similar regardless of treatment (Koves *et al.,* 1990b).

VIP-immunoreactivity was seen throughout the anterior pituitary gland, but PACAP-ir was absent (Fig. 3.9). The PACAP antiserum stained a discrete network of cell bodies and fibres in the medio-basal hypothalamus and median eminence, of the laying bantam hen. The PACAP-ir cell bodies and fibres were confined to the tuberal region, lining an area adjacent the wall of the 3rd ventricle (VIII) and

extending as far as the infundibular nuclei (Fig. 3.10a, b). In the median eminence a dense network of fibres were also present in the external zone (ME) but significantly less staining was present in the internal zone of the median eminence (Fig. 3.10c).

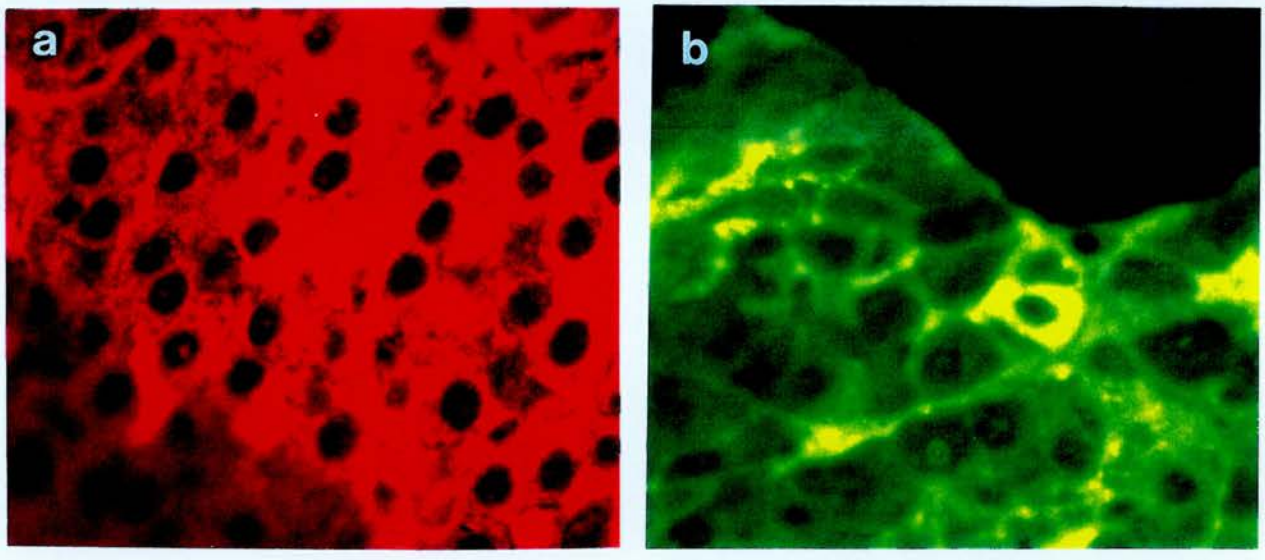

Figure 3.9 Photomicrographs showing the lack of (a) PACAP-immunoreactive labelling in the chicken anterior pituitary gland. The (b) VIP-ir labelling was not abolished by pre-absorption of the cVIP antiserum with cPACAP (Mag. x400).

3.3.2 Colocalisation of PACAP and VIP immunoreactivity in the hypothalamus

Double staining of the neurons in the basal hypothalamus (n=3) using the PACAP (1: 2000) and VIP (31/4; 1: 10 000) antibodies showed that both peptides were present in the same group of neurons (Fig. 3.11). The hypothalamic sections were also incubated with PACAP antiserum pre-absorbed with an excess amount (10µg per ml diluted antiserum) of chicken PACAP38, ovine PACAP38 (oPACAP38) or chicken VIP. The VIP antiserum was also tested for cross-reactivity by pre-absorbing the antibody with the same peptides, prior to incubation with adjacent sections of the basal hypothalamus. No cross-reactivity was demonstrated between the VIP antiserum and cPACAP or oPACAP peptides (*not shown*), but specific labelling was abolished by pre-absorption with cVIP (Fig. 3.11). The PACAP immunoreactive labelling was abolished by pre-absorption of the PACAP antiserum with both PACAPs and cVIP, and demonstrated the mammalian PACAP antiserum was unsuitable for the specific localisation of PACAP in the chicken brain.

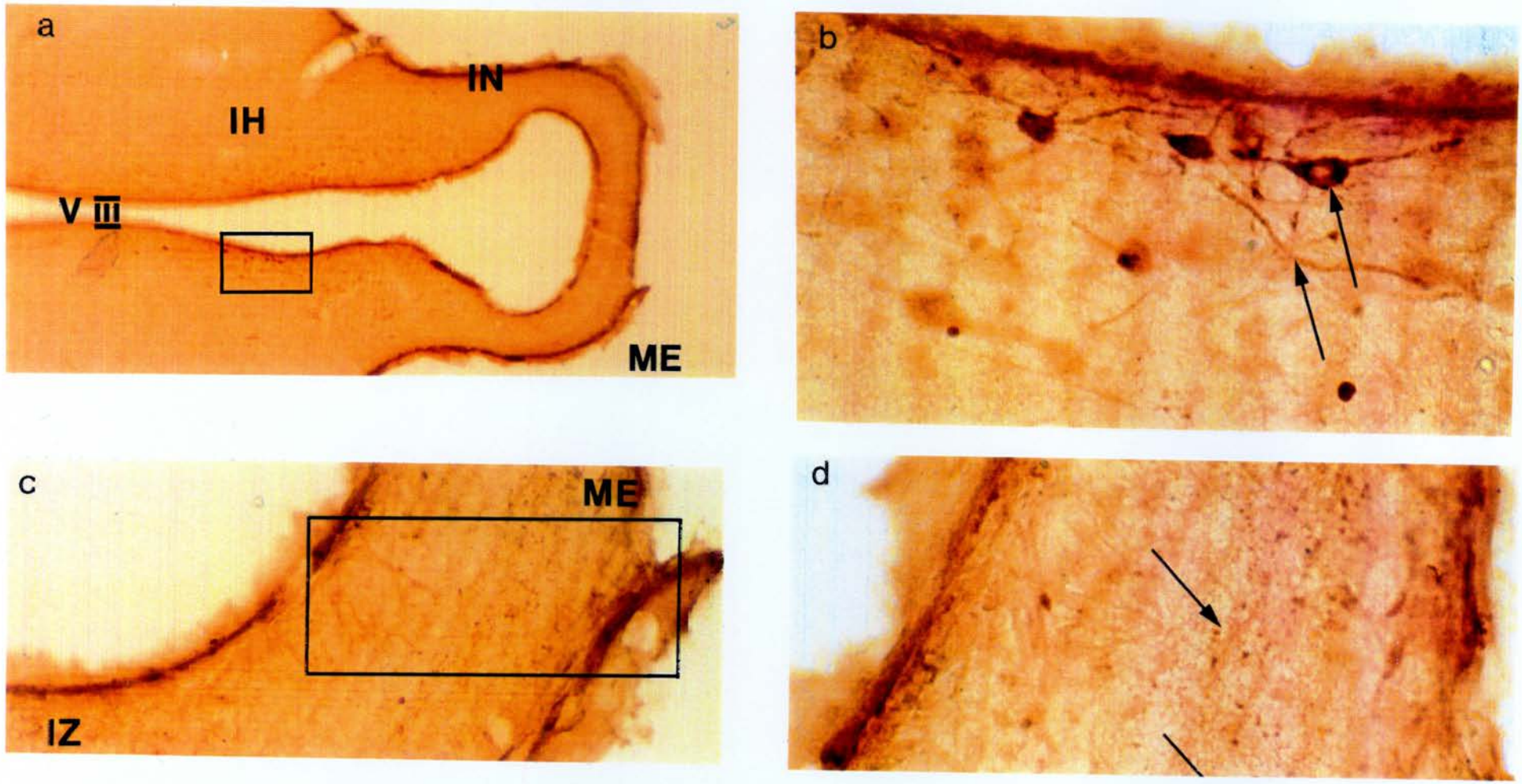

Figure 3.10 Photomicrographs showing the PACAP-immunoreactive (a) cell bodies and fibres in the medio-basal hypothalamus (Mag. x40) and the (c) median eminence (Mag. x200) stained with DAB. The photomicrographs (b) and (d) are high power images of the medio-basal hypothalamus and median eminence respectively (Mag. x400). The arrows indicate immunoreactive cell bodies or fibres. *Abbreviations*: *IH* inferior hypothalamic nuclei, *IN* infundibular hypothalamic nuclei, *ME* median eminence, *VIII* 3rd ventricle, *IZ* internal zone.

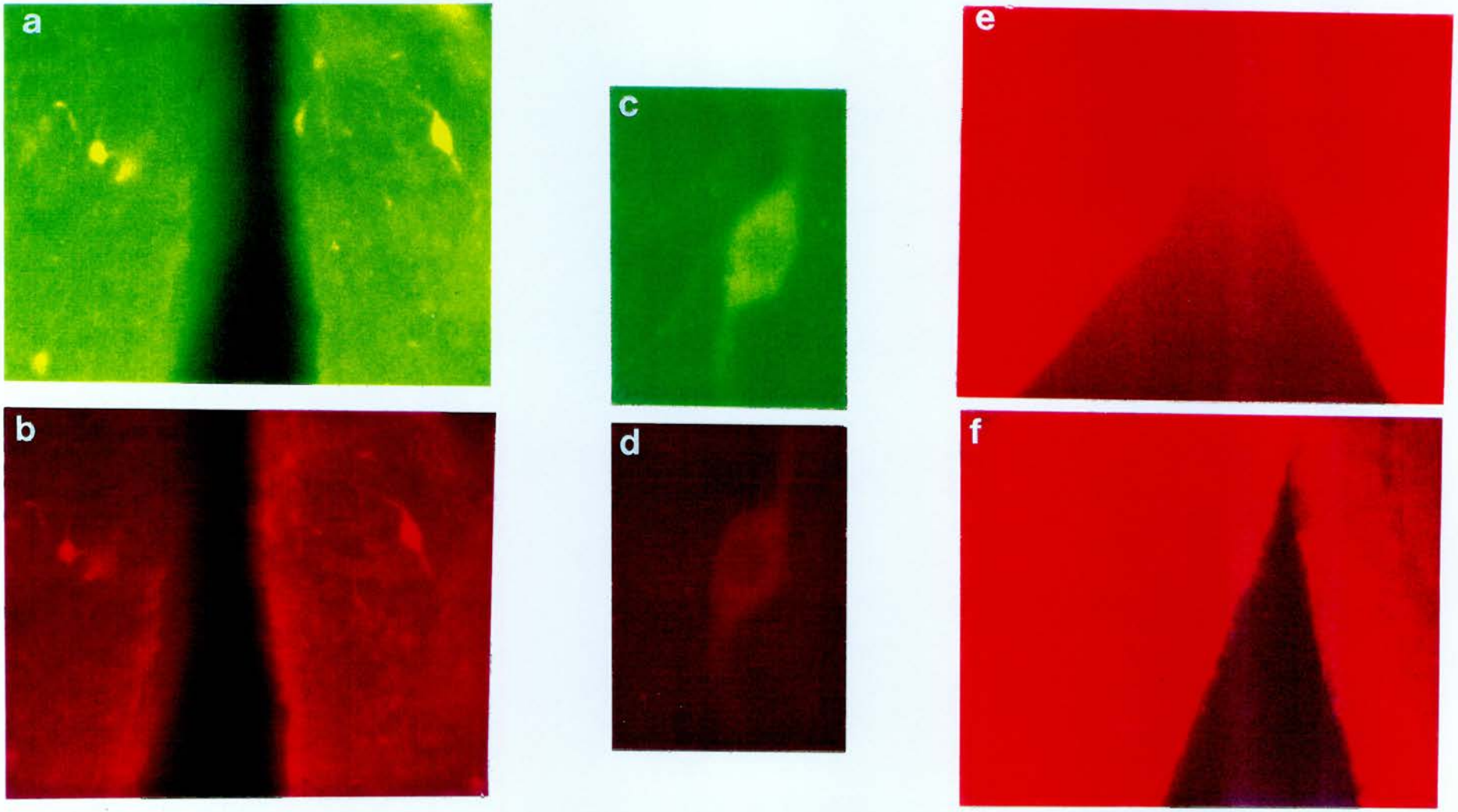

Figure 3.11 The co-localisation of PACAP and VIP in neurons of the basal hypothalamus in the laying bantam. The (a) VIP immunoreactivity (*yellow fluorescence*) was visualised with a second antibody conjugated with FITC (Mag. x100) and the (b) PACAP immunoreactivity (*red fluorescence*) was visualised with a second antibody conjugated with Lissamine rhodamine (Mag. x100). A high power image of a (c) VIP-ir and (d) PACAP-ir cell body (Mag. x400). The PACAP antiserum pre-incubated with (e) chicken PACAP (Mag. x100) and (f) chicken VIP, abolished specific immunoreactive labelling (Mag. x200).

3.4 The analysis of anterior pituitary extracts by HPLC and radioimmunoassay

The aim of this study was to confirm that VIP immunoreactivity extracted from the anterior pituitary gland of laying and broody hens, has the same HPLC elution profile as synthetic chicken VIP. A positive control was also incorporated into the study, by including VIP immunoreactivity extracted from the medial basal hypothalamus of both laying and bantam hens, which contains VIP cell bodies and VIP mRNA (Sharp *et al.,* 1989; Talbot *et al.,* 1995).

3.4.1 Column characterisation

A reverse-phase (RP) column (Vydac 218TP5415) was set up using conditions which were described previously (Section 2.8.3). In brief, the samples were subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on a Vydac 218TP5415 column using a linear gradient of acetonitrile (ACN) with 0.1% TFA at a flow rate of 1.5 ml/min for 40 min. The elution profile of synthetic chicken VIP (cVIP) was characterised as a single peak (see Section 3.3.2), with a retention time of 14-15 min (Fig. 3.12a).

3.4.2 Production and purification of iodinated VIP [^{125}I]-VIP.

The same conditions were applied to separate cVIP from that of iodinated cVIP ([^{125}I]-VIP) after the iodination reaction (Fig. 3.12b, c). Initial studies established the concentration of chloramine T necessary to iodinate 5µg of cVIP, was 1 mg/ml; by replacing Na^{125}I with NaI during the reaction. The major peak of cVIP was eluted after 14-15 min and the cold iodinated VIP ([I]-VIP) was eluted after 16-17 min. The separation of [I]-VIP from cVIP, by HPLC is shown in Figure 3.12. These conditions were then used for the separation and purification of [^{125}I]-VIP, which was collected and used in the VIP radioimmunoassay (Fig. 3.13).

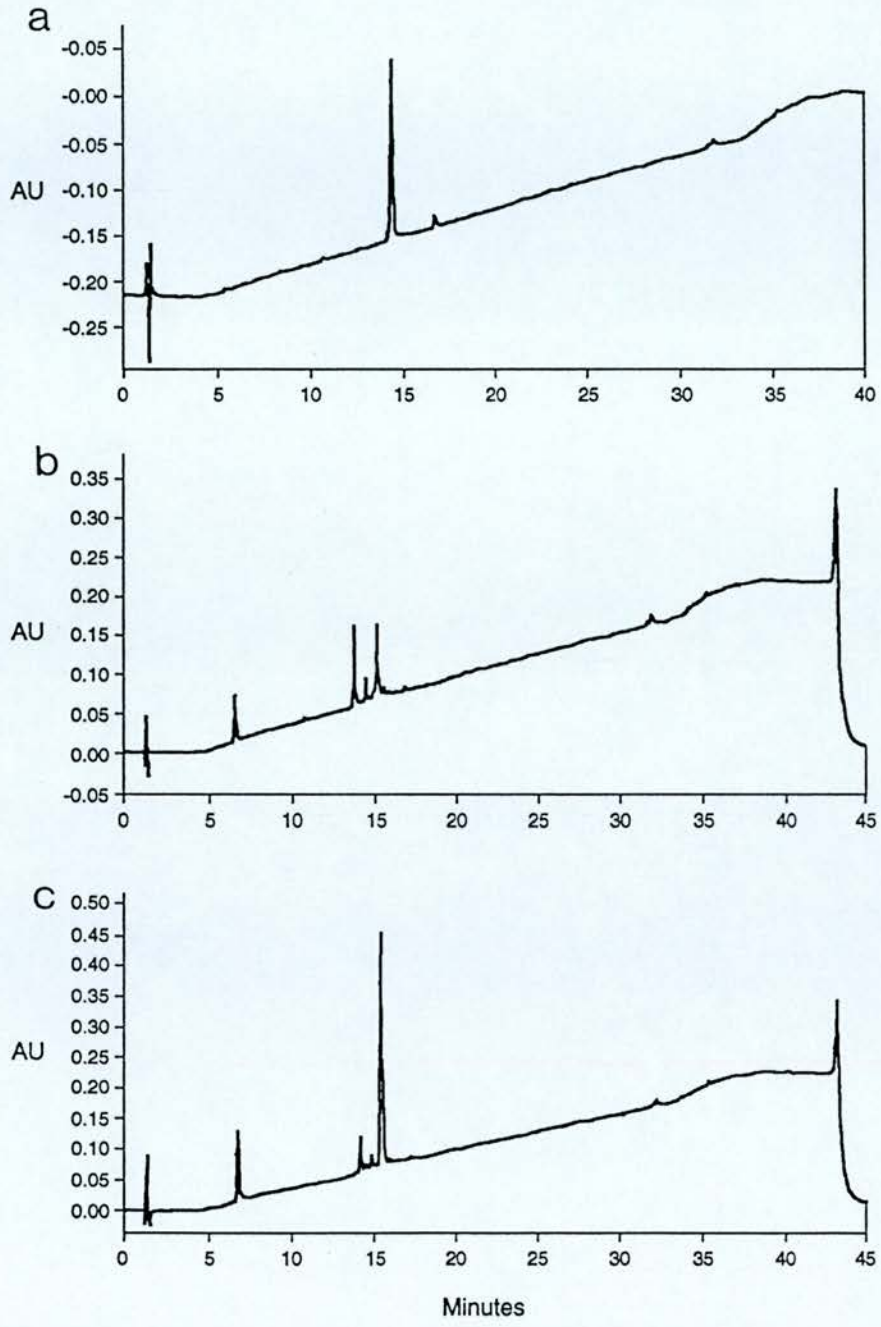

Figure 3.12 The elution profile of (a) synthetic VIP (cVIP) as recorded by U.V. absorbance at 280 nm (Waters). cVIP had a retention time of 14-15 min, Iodinated-VIP (I-VIP) was eluted when cVIP was incubated with (b) 0.2 mg chloramine T and (c) 1 mg chloramine T for 1 min in the iodination reaction. The column flow rate was 1.5 ml / min. Solvent system: linear gradient elution from (A) to (B) for 40 min. H_2O: CH_3CN: 10%TFA= (A) 90:10:1, (B) 10:90:1.

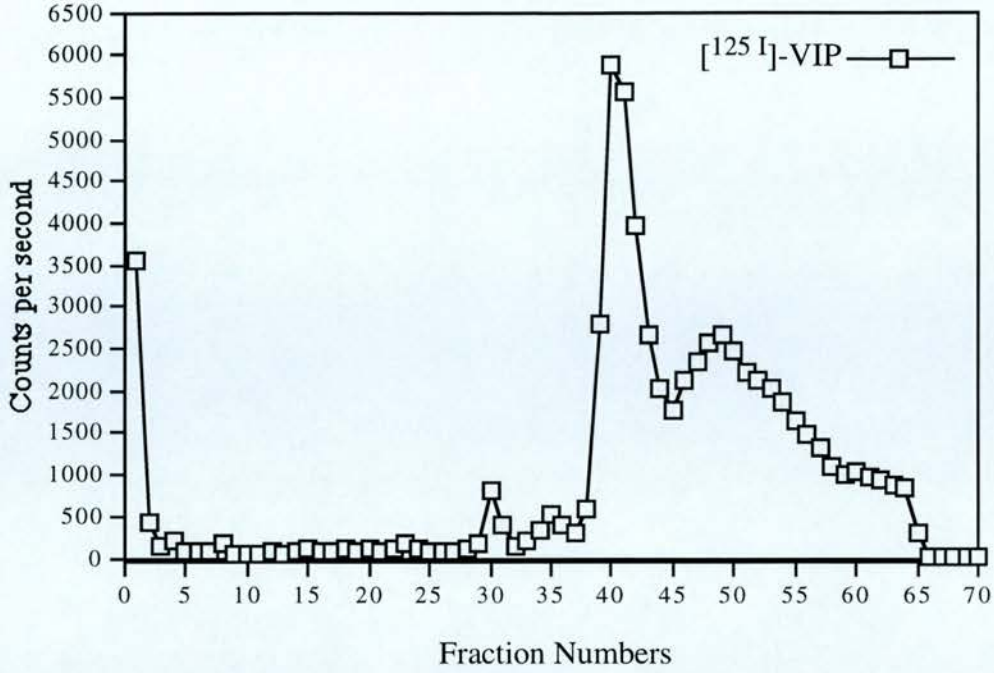

Figure 3.13 The HPLC purification of iodinated VIP ([^{125}I]-VIP), which was loaded onto a HPLC column (Vydac 218TP5415), eluted in 0.75 ml fractions, and collected in fractions number 41-43. The column flow rate was 1.5 ml / min. Solvent system: linear gradient elution from (A) to (B) for 40 min. H_2O: CH_3CN: 10%TFA, (A) 90:10:1, (B) 10:90:1.

3.4.3 Radioimmunoassay of cVIP

The anti-VIP antibodies show no cross reaction with a wide range of VIP-like neuropeptides (Sharp *et al.,* 1989), but have not been tested with the recently discovered polypeptide chicken PACAP (cPACAP). The specificity of both VIP antibodies 6DL/33/4 (RIA) and 6DL/31/4 (ICC), was confirmed and did not show any cross reaction with cPACAP either (Fig. 3.14). The cVIP displaced the binding of [^{125}I]-VIP, from both VIP antibodies, but cPACAP did not cross react with either antibody and therefore did not inhibit the binding with the antibody. This result confirmed the observation that the VIP-immunoreactive product localised in the anterior pituitary gland (Section 3.2.8) and detected by VIP radioimmunoassay, was chicken VIP and not cPACAP.

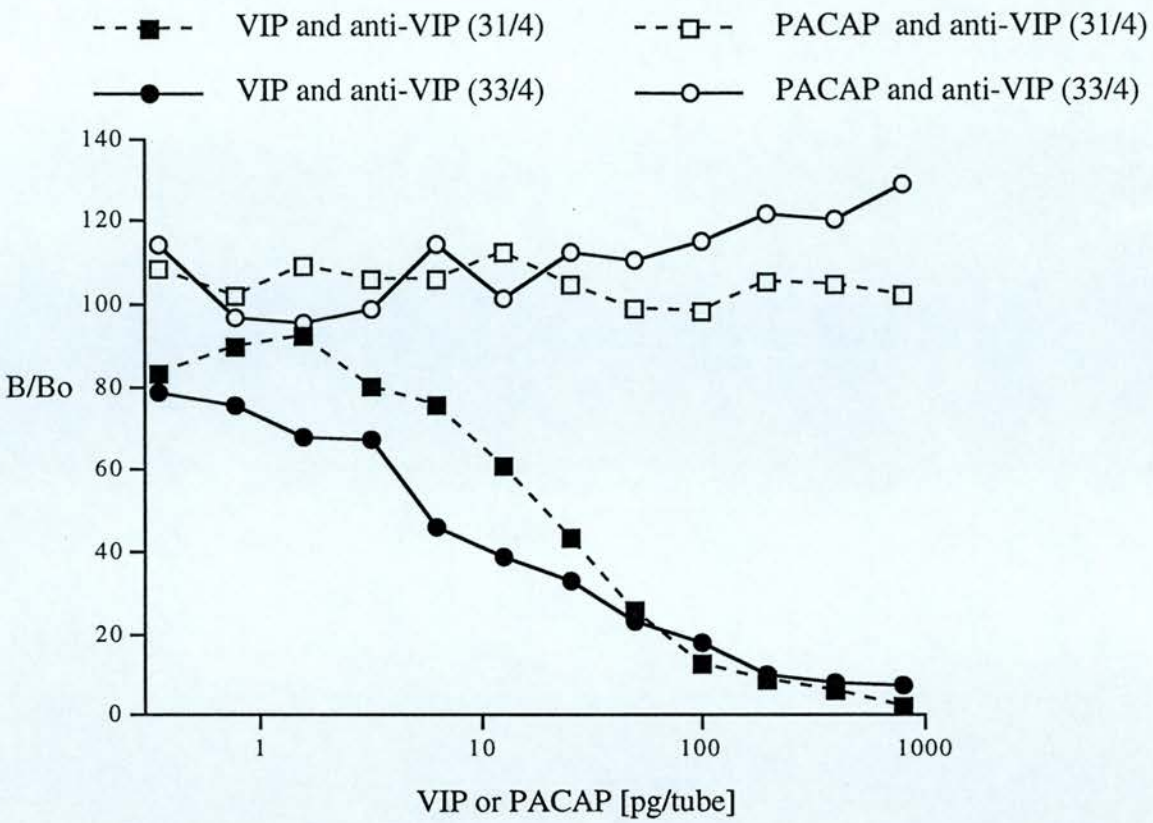

Figure 3.14 A radioimmunoassay for chicken VIP (cVIP) using two antibodies (codes/6DL/31/4 & 33/4) showing the displacement of [^{125}I]-VIP by the cVIP standard (*filled symbol*) and cPACAP (*empty symbol*). The cVIP displaced the binding of [^{125}I]-VIP from both VIP antibodies, but cPACAP was ineffective.

3.4.4 Measurement of VIP extracted from pituitary tissue

Vasoactive intestinal polypeptide immunoreactivity was extracted (Section 2.8.1) from the basal hypothalami and anterior pituitary glands from laying (n=10) and broody (n=10) bantam hens and subjected to RP-HPLC, and analysis by chicken VIP radioimmunoassay (VIP-RIA). The VIP-RIA detected VIP immunoreactive material from the hypothalamus, pituitary and synthetic VIP (standard 5μg), eluted in fraction number 29-30 which corresponds to a retention time of 14-15 minutes (Fig. 3.15). The immunoreactive material extracted from hypothalamic and anterior pituitary tissues was cVIP, because it shared the same elution profile and retention time as that of synthetic cVIP.

Figure 3.15 The reverse-phase HPLC of (a) synthetic cVIP chicken (b, d) anterior pituitary and (c, e) hypothalamic extracts from (b, c) broody and (d, e) laying bantam hens. The VIP immunoreactivity was observed in fraction number 29-30. (*next page*). The solvent system was described previously in Fig. 3.12.

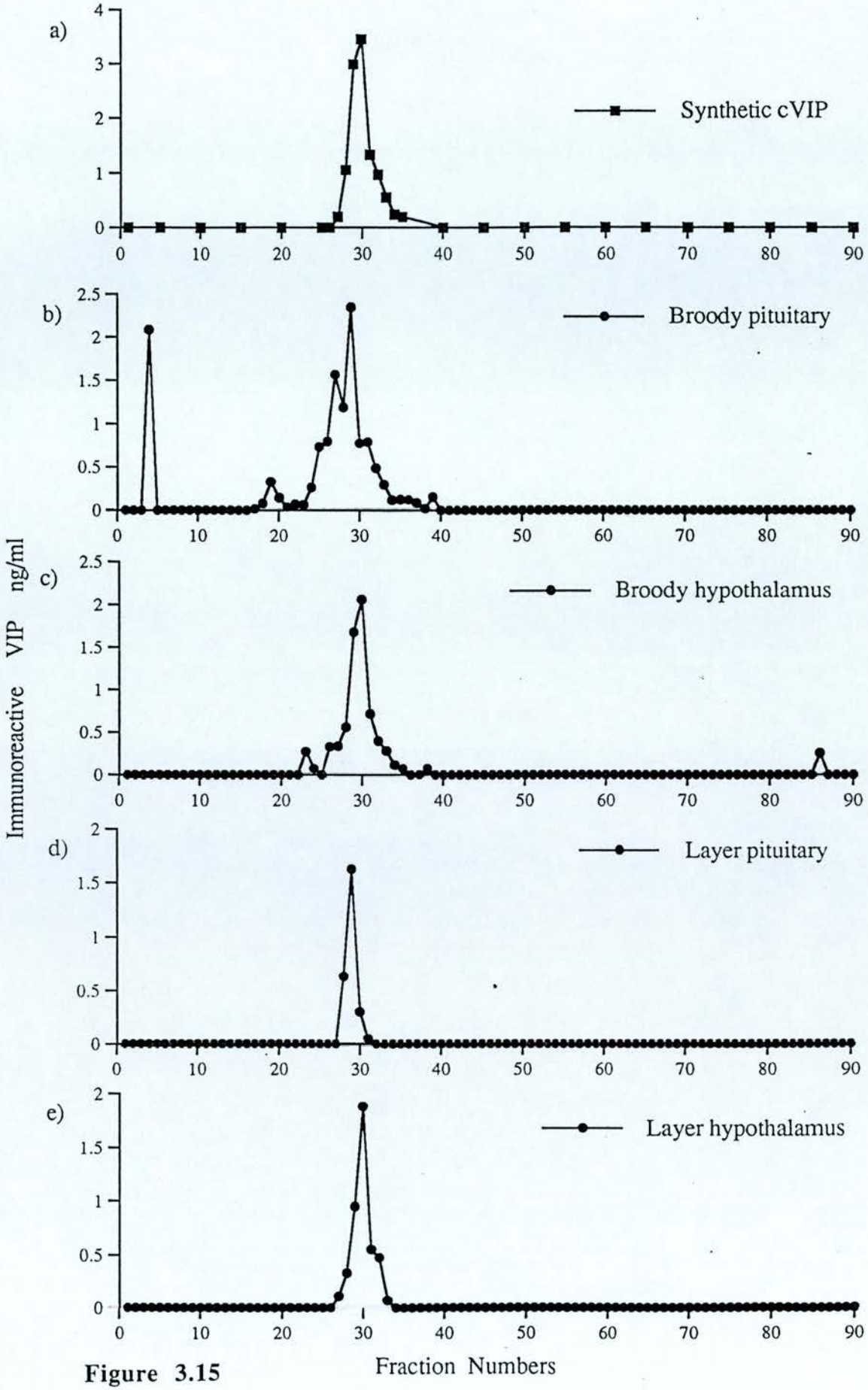
a)
Synthetic cVIP
b)
Broody pituitary
c)
Broody hypothalamus
d)
Layer pituitary
e)
Layer hypothalamus
Immunoreactive VIP ng/ml
Fraction Numbers

Figure 3.15

The VIP content of the hypothalamus and anterior pituitary gland, from both laying and broody bantam hens was determined by VIP radioimmunoassay (Table 3.1). There was a small but insignificant difference between the VIP content of medio-basal hypothalamus from a laying (n=5) and broody (n=5) bantam hen. Any discrepancy may have been due to an inaccurate dissection procedure, as the birds selected for this broody group exhibited all the characteristic features of an incubating bantam hen for at least 1-2 weeks. The VIP tissue content of the laying anterior pituitary glands (n=10) was greater than that of the VIP tissue content of broody hen anterior pituitary glands (n=10) (Table 3.1). However, because these values were determined from two pooled tissue samples, it was not possible to determine the statistical significance of this latter difference.

Table 3.1 A comparison between incubating and laying bantam hens of the amount of chicken vasoactive intestinal polypeptide (VIP) in the medio-basal hypothalamus and anterior pituitary. The hypothalamic values are means ± SEM (n=5) (Student's t-test), and the anterior pituitary gland values were determined from a pooled laying or broody sample (n=10).

	VIP Content [ng/ml/mg tissue]		
Brain region	Laying hen		Broody hen
Hypothalamus	262.4 ± 39.4		391.1 ± 74.8
Anterior Pituitary	120.36		74.01

3.5 Discussion

This study demonstrated the presence of VIP-immunoreactivity in the anterior pituitary gland of laying and broody bantam hens. The RP-HPLC separation and VIP-radioimmunoassay analysis of pituitary extracts confirmed that the major immunoreactive product identified was VIP. The VIP antibodies used for immunocytochemistry (6DL/31/4) studies and RIA analysis (6DL/33/4) are highly specific and showed no cross reaction, with a wide range of VIP related peptides (Talbot *et al.,* 1989), including cPACAP which shares approximately 60% homology with chicken VIP. In addition, VIP-immunoreactive labelling in the anterior pituitary and hypothalamus was not affected by pre-incubation of the VIP antibody (6DL/33/4) with cPACAP, and therefore confirmed the specificity of VIP-immunoreactive labelling in both tissues, with this antibody.

Vasoactive intestinal polypeptide immunoreactive (VIP-ir) cells occurred in the cephalic and caudal lobes of the anterior pituitary gland of both laying and broody hens. A number of authors have reported that only a few VIP-ir cells occur in the anterior pituitaries of intact male rats (Koves *et al.,* 1990a), and a few or no VIP-ir cells occur in the pituitaries of intact female rats (Lam *et al.,* 1989; Koves *et al.,* 1990a; Steel *et al.,* 1990). These authors reported that VIP-ir was colocalised in a small population of lactotrophs (Morel *et al.,* 1982), after long term oestrogen treatment (Koves *et al.,* 1990a; Steel *et al.,* 1990) and may regulate prolactin secretion in an autocrine manner (Nagy *et al.,* 1988). In contrast, this study showed that no VIP-ir was localised in lactotrophs or gonadotrophs, in the chicken anterior pituitary gland. This observation is consistent with the reports of some authors, that VIP-ir in the anterior pituitary of the rat occurs in cells other than the lactotroph or thyrotroph (Lam *et al.,* 1989; Steel *et al.,* 1990; Carrillo and Phelps, 1992).

The discrepancies between our results and others, may be due to different experimental protocols or VIP antibody specificity and variability. This study used a Bouin's fixative, but other studies using a paraformaldehyde (4%) fixation method (Lam *et al.,* 1989; Koves *et al.,* 1990a) were not as successful, and detected limited numbers of VIP-ir cells. Immunoreactive VIP is susceptible to damage from fixation and processing, and in particular the C-terminal and mid-portions of VIP are affected during paraffin processing (Larsson, 1982). Therefore, the microwave processing used in this study, combined with a Bouins fixation may

enhance the conditions required to visualise VIP-immunoreactivity in the anterior pituitary. The attempt to count the number of VIP-ir cells was not successful, because the intensity of immunostaining often faded after prolonged illumination, and the image analysis system (Magiscan 2, Joyce Lobel Ltd., Gateshead, Tyne and Wear, U.K.) used to quantify the cell number, was unable to discriminate weakly stained stellate VIP-ir cells from the background.

One VIP-ir cell type was stellate in appearance, with an irregular shaped nucleus and cytoplasmic projections between unstained pituitary secretory cells. A stellate VIP-ir cell has been reported by several groups, which differs from classical pituitary hormone cell types (Lam *et al.,* 1989; Segerson *et al.,* 1989; Carrillo and Phelps, 1992). The morphological features of the VIP-ir cells were similar to those of the folliculo-stellate cells (FS-cell) (Harrison *et al.,* 1982). The FS-cells were identified throughout the anterior pituitary of the chicken, with an antibody raised against S-100 protein, which is a molecular marker for FS-cells of the anterior pituitary of mammals and birds (Cocchian and Miani, 1980; Atoji *et al.,* 1990). The S-100 protein occurs in the anterior pituitary gland of most mammalian species. The glial cell marker GFAP (glial fibrillary acidic protein) is also used to identify FS-cells in the human anterior pituitary gland (Velasco *et al.,* 1982; Trojanowski *et al.,* 1984), but is not detected in the anterior pituitary glands of the bull, goat or sheep (Shimada, 1992). This study also failed to demonstrate GFAP immunoreactivity in the chicken anterior pituitary gland, which supports the findings of the latter study (Shimada, 1992).

A number of FS-cells contained both S-100- and VIP-immunoreactivity, but the larger VIP-ir cells which were more polygonal in shape did not appear to contain S-100 immunolabelling. A number of S-100(+) cells in the pituitary were weakly immunolabelled and were also polygonal in shape. There are reports of round or oval S-100 immunoreactive cells in the quail anterior pituitary gland (Van Nassauw *et al.,* 1987; Atoji *et al.,* 1990). This suggests that the VIP-ir cells which do not contain S-100 protein, may be a differentiated FS-cell, or possibly a separate category of pituitary cell (Segerson *et al.,* 1989).

The VIP-ir visualised immunocytochemically in the anterior pituitary gland of an incubating hen, seemed less than the VIP-ir occurring in the laying hen, which was processed at the same time. Although, there was no significant difference between the VIP content measured by HPLC-RIA, in the anterior pituitaries of the laying

and incubating hens. VIP-ir was found in the caudal and cephalic lobes, but the lactotrophs only occurred in the cephalic lobe. The topographical relationship between VIP-ir cells and lactotrophs in the cephalic lobe and lack of association between these cell types in the caudal lobe, suggests that intra-pituitary VIP may not be an important regulator of PRL secretion in the chicken. It may be the case, that in the chicken, hypothalamic VIP is the main factor which regulates PRL secretion associated with incubation behaviour, and that the role of intra-pituitary VIP in the chicken is mediated by the action of oestrogen.

This view is supported by the observation that increased plasma PRL in the rat suppresses intra-pituitary VIP concentrations, but is overridden by oestrogen treatment (Prysor-Jones *et al.,* 1987). In the ovariectomised rat oestrogen treatment increases pituitary VIP cell number (Steel *et al.,* 1989) and VIP mRNA (Reichlin, 1988; Lam, 1991). In the broody hen ovarian steroids are depressed (Cogger *et al.,* 1979; Sharp *et al.,* 1979) and hypothalamic VIP levels are increased (Sharp *et al.,* 1989), which may explain the apparent reduction of VIP-immunolabelling in the anterior pituitary.

The distribution of cells containing VIP-ir and LH immunoreactivity was essentially the same, and there was also a close association between the two cell types. The intensity of VIP immunostaining appeared greater in the anterior pituitary of the laying hen than of the incubating hen, and there was a close relationship between VIP and LH cells. Indeed, a number of VIP-cells appeared to envelop neighbouring gonadotrophs with cytoplasmic projections. VIP may be acting in a paracrine manner within the chicken pituitary to regulate gonadotroph function. This hypothesis is supported by the observation that VIP potentiates the LH releasing activity of LHRH in man (Hammond *et al.,* 1993).

PACAP-immunoreactive (PACAP-ir) cells were localised in the nucleus infundibularis of the medial basal hypothalamus and PACAP-ir co-expressed with VIP in the same neurons. However, pre-absorption of the PACAP antisera with chicken VIP abolished PACAP-immunostaining, but VIP-immunolabelling was not abolished by the pre-absorption of the cVIP antiserum with cPACAP. Therefore this study was not able to confirm localisation of PACAP-immunoreactivity in the hypothalamus or anterior pituitary gland of the chicken. PACAP-immunoreactive fibres are localised in the ovine hypothalamus, posterior pituitary (Koves *et al.,* 1990b) and is reported to occur in the chicken brain (Yasuhara *et al.,* 1993).

PACAP mRNA was also detected in the anterior pituitary of the rat (Wuttke *et al.*, 1994), but independent studies failed to detect PACAP-immunoreactivity or PACAP mRNA in the rat anterior pituitary (Mikkelsen *et al.*, 1995), or human pituitary adenomas (Vertongen *et al.*, 1995b). This study cannot discount the possibility that PACAP is produced and expressed by the anterior pituitary of the chicken, but has confirmed that the VIP-immunolabelling in the anterior pituitary was not due to PACAP.

Further studies are needed to establish whether PACAP is expressed in the hypothalamus, ME or the anterior pituitary gland of the chicken using antibodies which are more specific for chicken PACAP. The distribution of PACAP in the hypothalamus is similar in several mammalian species (Koves *et al.*, 1990b; Tamada *et al.*, 1994), and is reported to occur in the chicken (Yasuhara *et al.*, 1992). Therefore, PACAP, like VIP may play an important role as a hypothalamic factor released in birds and regulate hormone secretion in the anterior pituitary gland.

In conclusion, VIP-immunoreactivity which cannot be accounted for by cross reaction with PACAP occurs in the anterior pituitary gland of the chicken. It occurs in a folliculo-stellate-like cell, and not in lactotrophs or gonadotrophs. The functional significance of intra-pituitary VIP in relation to PRL secretion remains to be determined. However, the close relationship between VIP-ir cells and LH cells in the anterior pituitary gland suggests VIP may be acting as an important paracrine factor, which modulates LH secretion. The possibility that intra-pituitary VIP may regulate other pituitary hormone cell types cannot be discounted, and needs further investigation.

Chapter 4: THE EXPRESSION OF VIP mRNA IN THE ANTERIOR PITUITARY GLAND

4.1 Introduction

In the previous chapter it was shown that VIP was detectable in the chicken anterior pituitary gland using immunocytochemistry and HPLC analysis of pituitary extracts, but this does not provide evidence that VIP is produced by the anterior pituitary and not sequested by cells. The mRNA encoding VIP occurs in the anterior pituitary gland of the rat (Reichlin, 1988; Lam *et al.,* 1990; 1991) and human (Byrne *et al.,* 1992). There is evidence to suggest that changes in expression of VIP in the pituitary are influenced by the alterations in the steroid environment (see Section 1.6.1). Furthermore, intrapituitary VIP is believed to play a role of a paracrine/autocrine factor modulating the function of pituitary hormone secreting cells. The aim of this study was to demonstrate VIP gene expression in anterior pituitary tissue of the chicken, using RT-PCR and a solution hybridisation RNase protection assay. An *in situ* hybridization technique was used to establish whether the expression of VIP mRNA was specifically localised in the VIP-cell type, identified in Chapter 3. A positive control was incorporated into the study by including the VIP cell bodies located in the medial basal hypothalamus adjacent to the anterior pituitary gland.

4.2 VIP gene expression in pituitary tissue using RT-PCR

RNA was isolated from anterior pituitary (n=10) to confirm the presence of VIP mRNA in this tissue. The primers 251L and 275Z were based on the sequence of the VIP cDNA clone VIP19 (Talbot *et al.,* 1995) and span the region homologous to exon 3 and exon 6 (Section 2.4.1). The RNA was reverse transcribed and the cDNA amplified by PCR.

A major fragment of 275 bp and a minor fragment of 380 bp was generated from pituitary cDNA (Fig. 4.1), but no bands were observed in the control samples, which included a water control, mouse mRNA, cDNA and pituitary mRNA. The RT-PCR products were digested by a restriction endonuclease (*Bst* B1), which generated two fragments of 185 bp and 93 bp from the 275 bp product and 290 bp and 93 bp from the 380 bp product. These fragment sizes result from the digestion

of the 275bp and 380bp fragments which contain a Bst B1 restriction site in the cDNA sequence and correspond to the fragment size predicted from the VIP cDNA sequence (Fig. 4.2).

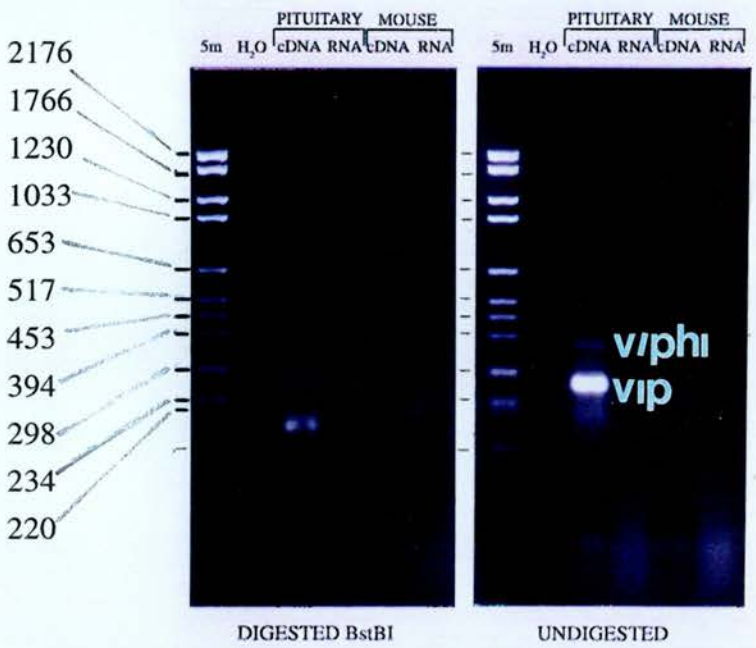

Figure 4.1 The detection of VIP mRNA by RT-PCR in the anterior pituitary gland of a laying hen, using reverse transcribed total RNA (cDNA) or RNA from the chicken anterior pituitary, using primers 215L and 275Z. The 'VIP only' PCR product was 275bp, and the 'VIP/PHI' (V/phi) PCR product was 380bp. The RT-PCR products digested by Bst B1. The size markers were a digest of pBR328 with *Bg*1 and *Hinf* 1.

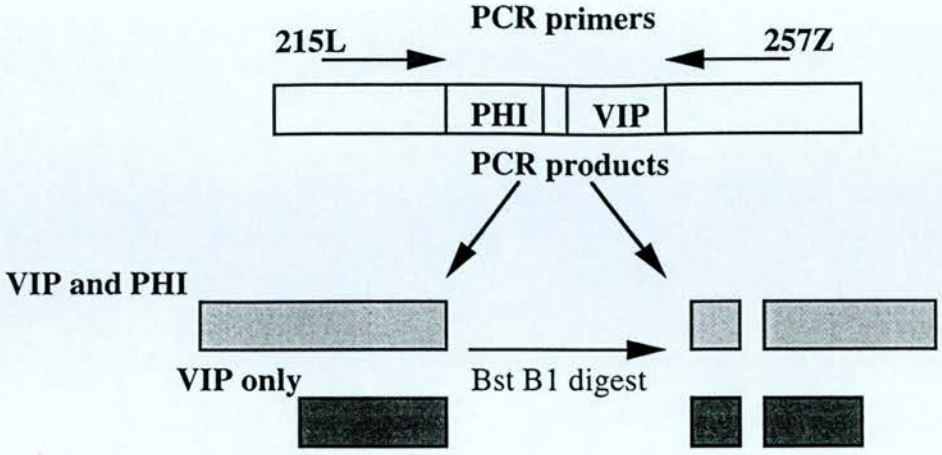

Figure 4.2 The relative position of PCR primers 215L and 257Z and the predicted size of PCR products. The digestion of the PCR products by the restriction enzyme *Bst* B1(arrow), results in the production of 185bp and 93bp fragments from the 275 bp PCR product and 290 bp and 93 bp fragments from the 380 bp PCR product, which were visualised in Fig. 4.1.

4.3 An *in situ* Hybridisation (ISH) histochemical investigation

The aim of this study was to establish the cell type which synthesizes VIP in the chicken anterior pituitary gland. A 45 base oligonucleotide end-labelled with ^{35}S-dATP by terminal transferase, was used for *in situ* hybridisation of VIP mRNA in the basal hypothalamus, which contains cell bodies synthesising VIP (Sharp *et al.*, 1989; Talbot *et al.*, 1995). However, this oligonucleotide produced unacceptable levels of non-specific background product in the anterior pituitary which was not seen in the hypothalamus. Therefore it was decided to abandon this approach and use a non-radioactive RNA probe which might be more sensitive and produce less background product.

Initial trials established the conditions for *in situ* hybridisation using a digoxigenin-labelled (DIG-labelled) riboprobe (Section 2.6.1) on coronal cryostat sections of the basal hypothalamus, from the laying bantam hen. Several approaches were adopted to optimise the *in situ* hybridization of chicken pituitary VIP mRNA.

4.3.1 Probe design

The first antisense probe to be used was transcribed from the plasmid VF48 (Section 2.6.1), which was linearised with *Nco* 1 and was referred to as VF48N. This probe spanned bases 477-169 of the VIP cDNA sequence (Fig. 4.3), and included most of exon 5, encoding the VIP peptide. A complementary sense riboprobe was also generated by transcribing the plasmid VF48 which was linearised with *Eco* R1, and termed VF48E (Fig. 4.3).

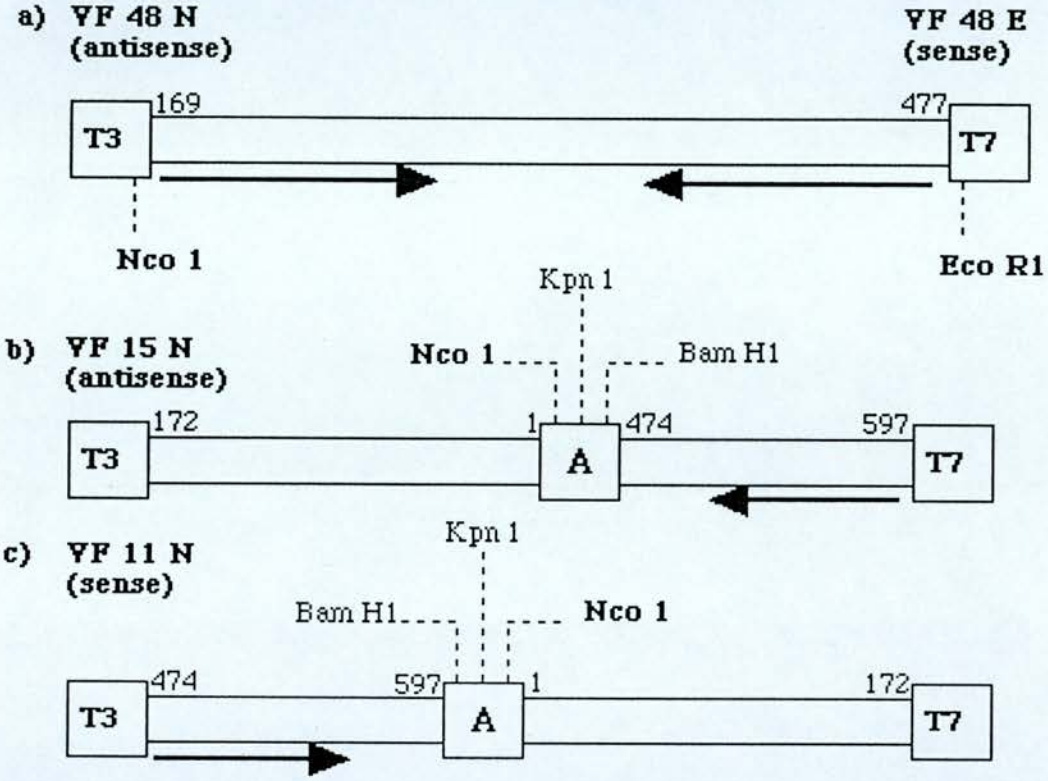

Figure 4.3 A schematic representation of the DNA clones used to generate sense and antisense probes for *in situ* hybridization. The restriction enzyme in **bold** was used to linearise the plasmid. *Abbreviations*: *A Eco* R1 adapter; *arrow* indicates direction of transcription by RNA polymerase; *T3* and *T7* RNA polymerases used for RNA transcription.

In subsequent studies (Section 4.3.3), an additional non-overlapping antisense probe was generated and combined in the hybridisation buffer. The second plasmid VF15 was linearised with *Nco* 1 and RNA transcription generated an antisense riboprobe termed VF15N (spanning 474-597) (Fig. 4.3). A third clone VF11, which contained the same sequence but in the reverse orientation, was used to produce a sense probe for a control, termed VF11N (Fig. 4.3). The probe quality was analysed after purification by gel electrophoresis (Section 2.5.2) and Northern blotting (Section 2.5.3).

4.3.2 Hybridisation conditions for hypothalamic tissue

The antisense and sense probes (VF48N and VF48E) were used at a concentration of 170ng/slide (1.7ng/µl) and hybridized at 55°C overnight, using the conditions described previously (Section 2.6.4) These conditions were used to saturate the target VIP mRNA. The hybridisation signal was visualised with an anti-DIG peroxidase detection kit (Boehringer Mannheim), which allowed amplification of the signal. The sections were incubated in a DAB solution for 0.5 hr to 1.5 hrs without adversely affecting the signal : noise ratio. The labelling of cells seen using DIG-labelled VF48N was confined to the basal hypothalamus region known to express VIP neurons (Sharp *et al.,* 1989). The sense probe VF48E used as a negative control did not generate any specific labelling on adjacent sections (Fig. 4.4).

The DAB reaction product was concentrated in the cytoplasm of cell bodies and absent in the nucleus. The labelling of cells identified in the hypothalamus of the laying hen (n=3) was less than that observed in the hypothalamus of the broody (n=3) bantam (Fig. 4.5a, b).

4.3.3 Hybridisation conditions for anterior pituitary tissue

The hybridisation conditions used to localise VIP mRNA in cells of the chicken anterior pituitary gland (n=4), were the same conditions used to confirm VIP mRNA in neurons in the basal hypothalamus. The initial studies were unsuccessful, and no specific signal was visualised in the anterior pituitary after 1 hour in DAB solution. The staining periods were extended for the pituitary sections, and incubations times of 2-3 hours yielded minimal or no staining. However, the control sections of the basal hypothalamus, were expressing the expected specific staining, after 1 hour (Fig. 4.6).

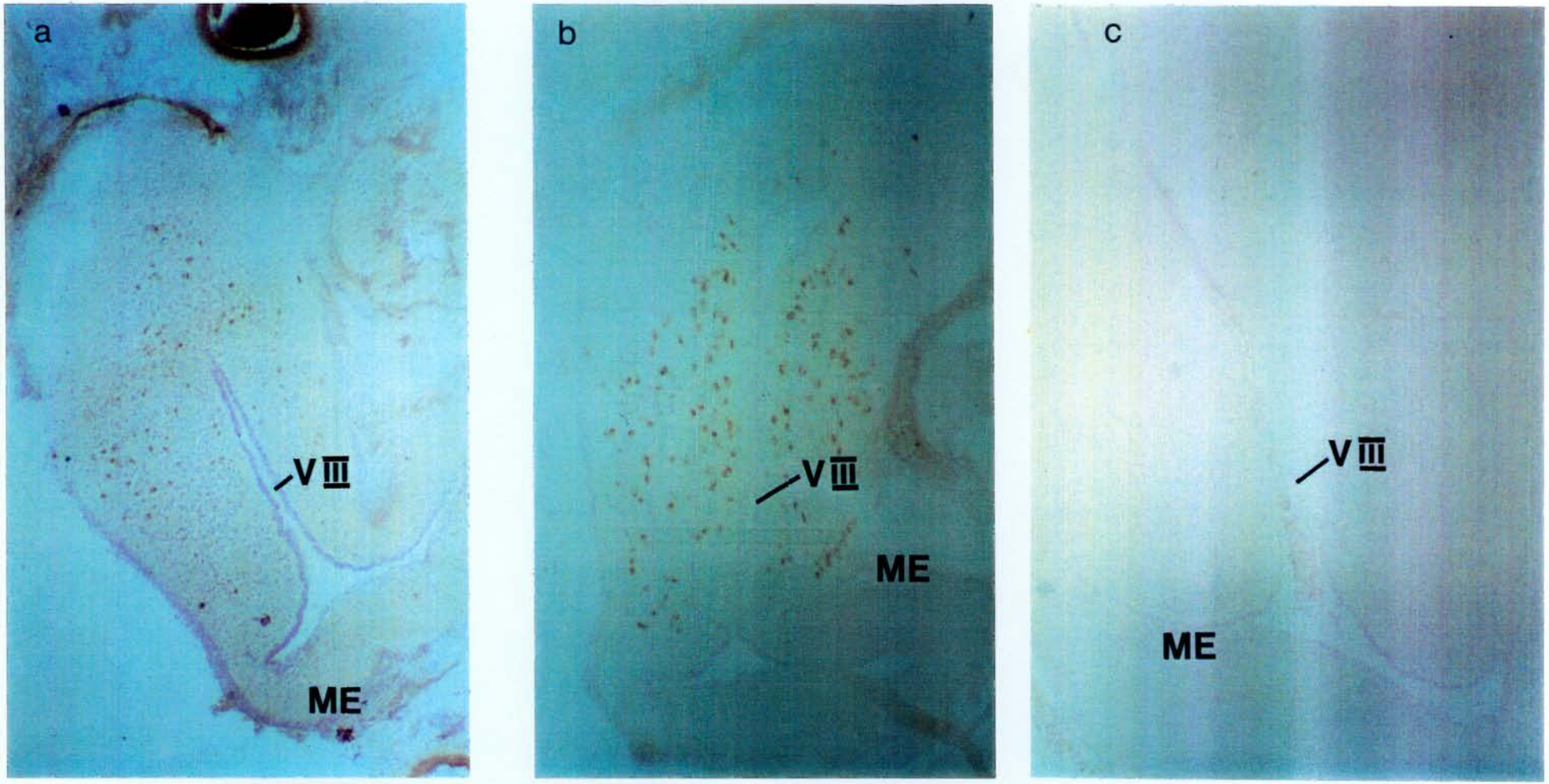

Figure 4.4 The localisation of VIP mRNA by *in situ* hybridisation using the DIG-labelled (antisense) probe VF48N, in cryostat coronal sections through the basal hypothalamus of a (a, c) laying and (b) broody hen. The specificity of the antisense probe was verified by the (c) lack of staining on an adjacent section with the DIG-labelled (sense) probe VF48E. (Mag. x40).

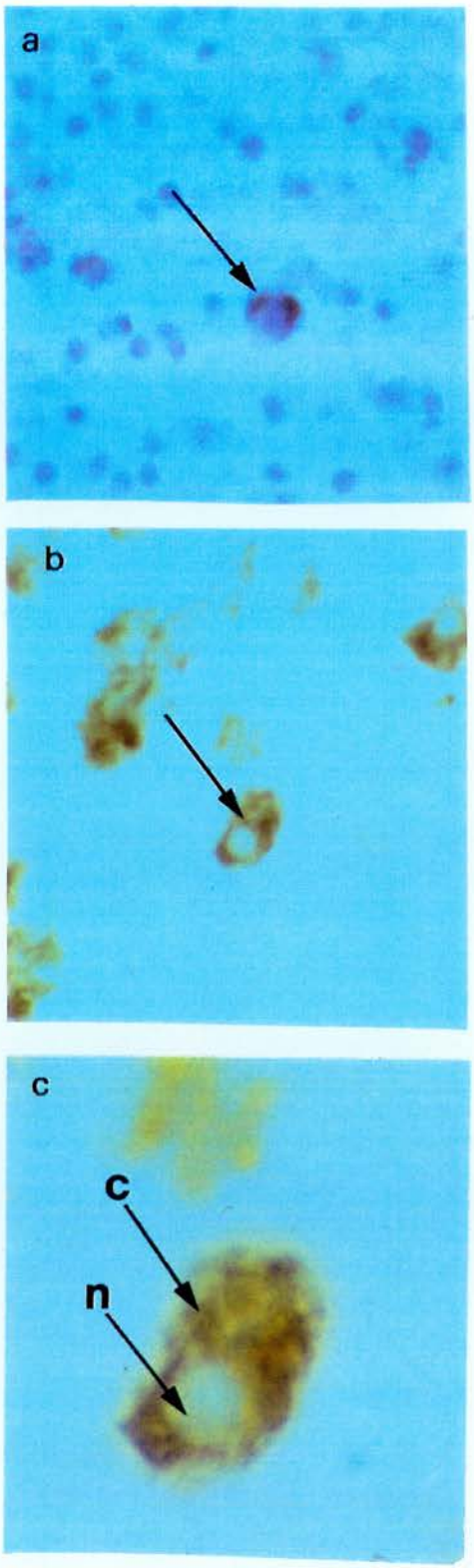

Figure 4.5 The detection of VIP mRNA in neurones of the basal hypothalamus of the (a) laying and (b) broody hen (Mag. x400). The staining (brown precipitate) was confined to the (c) cytoplasm (*arrow, c*) and absent in the nucleus (*arrow, n*) (Mag. x1000). The cresyl violet counterstain (b) demonstrates the lack of DAB staining in the nucleus, of the neuron expressing VIP mRNA.

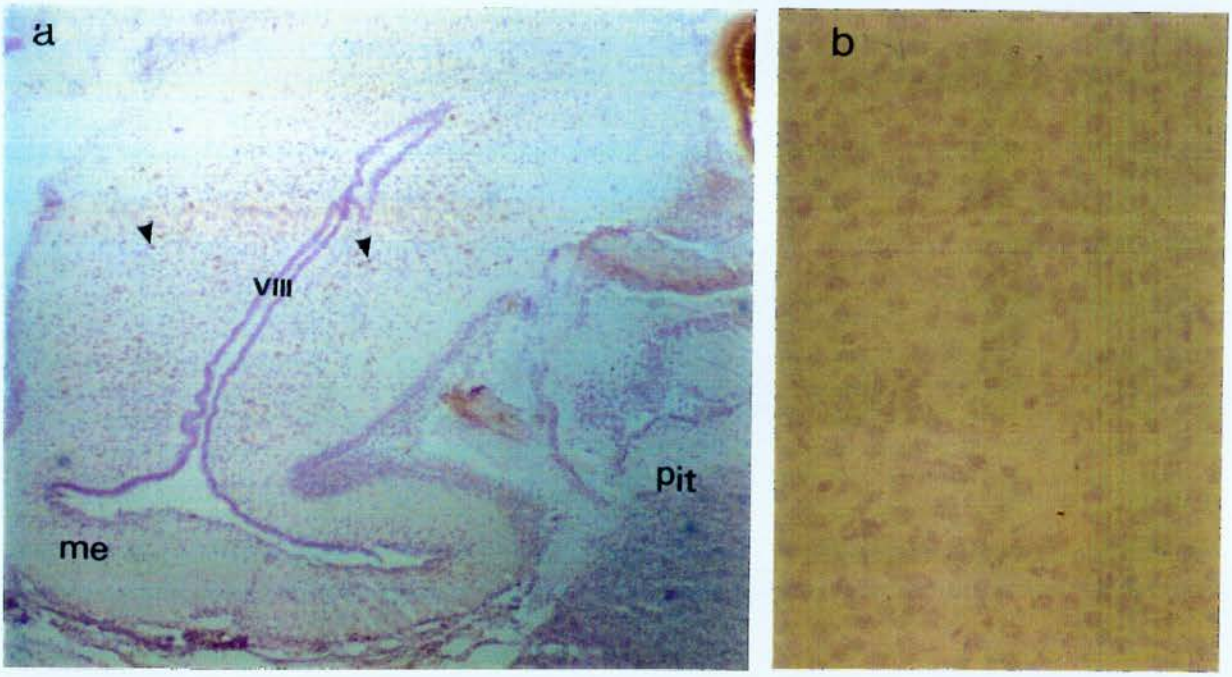

Figure 4.6 The visualisation of VIP mRNA in the basal hypothalamus (*arrow head*) of the (a) laying hen (Mag. x40) and of staining in the (b) anterior pituitary (400). The riboprobe VF48N was used to localise VIP mRNA. *Abbreviations*, *me* median eminence, *pit* pituitary and *viii* 3rd ventricle.

An attempt was made to increase the sensitivity of the ISH method applied to the anterior pituitary gland by doubling the concentration of VF48N probe in the hybridization mix. However this was not successful. Trials with the antisense probe VF15N showed that it was suitable for the *in situ* localisation of VIP mRNA in the hypothalamus (Fig. 4.7a) and no labelling was observed using the corresponding sense probe, VF11N (Fig. 4.7b). The two complementary riboprobes VF48N and VF15N were combined in a further attempt to increase the

sensitivity of the hybridisation signal in the anterior pituitary. The resulting amount of label seen in the pituitary was inconsistent and probably non-specific (Fig. 4.7c).

4.3.4 Effect of proteinase K treatment

In a further attempt to localise pituitary VIP mRNA, the hybridisation protocol was modified to increase the permeability of the pituitary tissue. Proteinase K (PK) treatments (1, 5 or 10 µg/ml, 15 min at 37°C) were used on the hypothalamic (n=3) and pituitary (n=3) cryostat sections previously fixed with 4% PFA (see Section 2.6.3). The PK treatment increased the detection of labelling in the anterior pituitary, without adversely affecting the specific staining in the basal hypothalamus. However, a number of artefacts were observed in the pituitary with the sense probe (Fig. 4.8), and the morphological integrity of the labelled cells expressing VIP mRNA in the hypothalamus was compromised by PK treatment (10µg/ml).

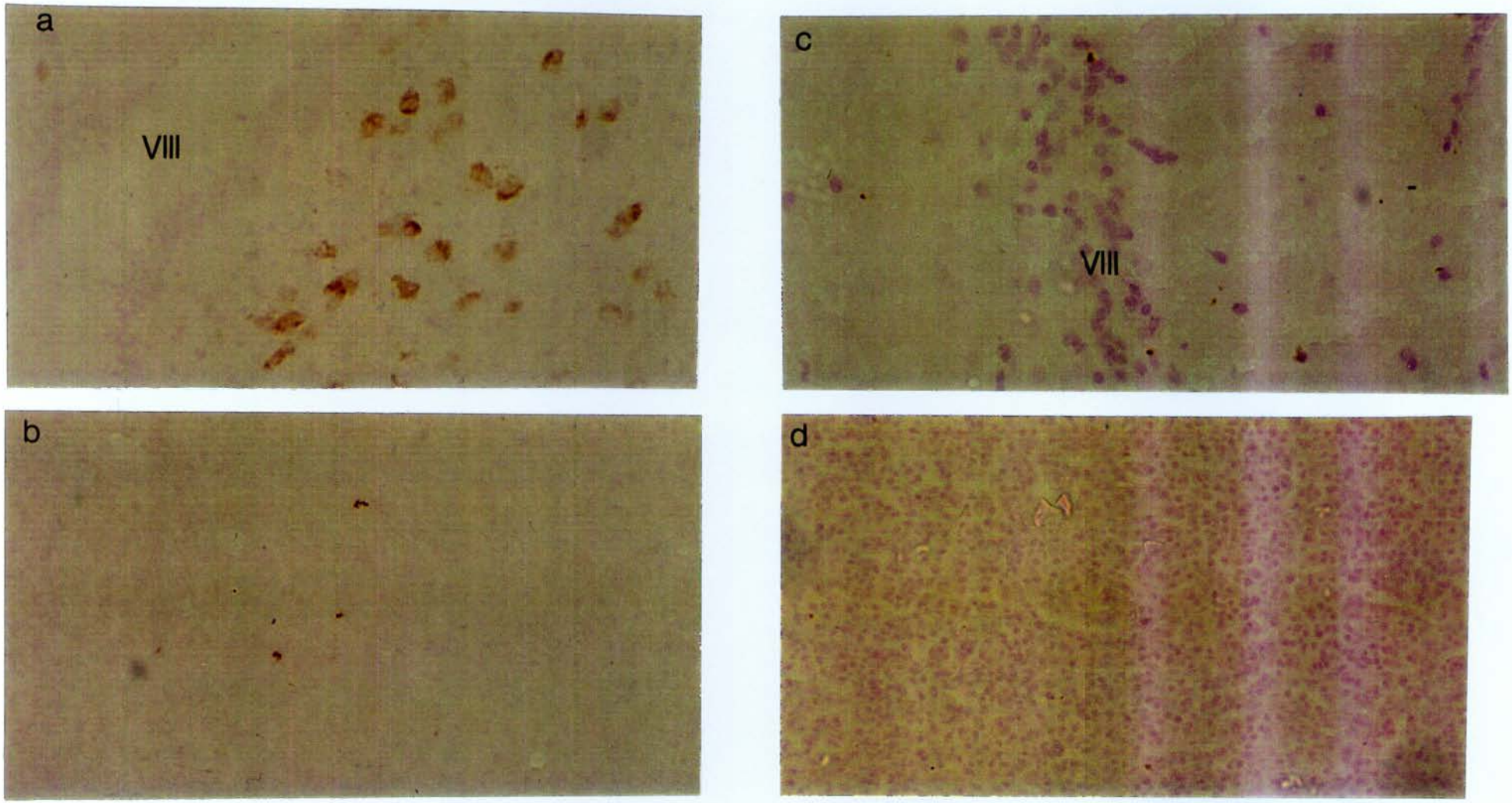

Figure 4.7 The localisation of VIP mRNA by *in situ* hybridization on cryostat sections of the basal hypothalamus from a laying hen, with a complementary riboprobe VF15N (Mag. x200). No specific labelling was seen with this probe (VF15N) in the (b) anterior pituitary gland (Mag, x200). No labelling was seen with the antisense or sense probe VF11N, in the (c) basal hypothalamus or (d) anterior pituitary gland (Mag. x200).

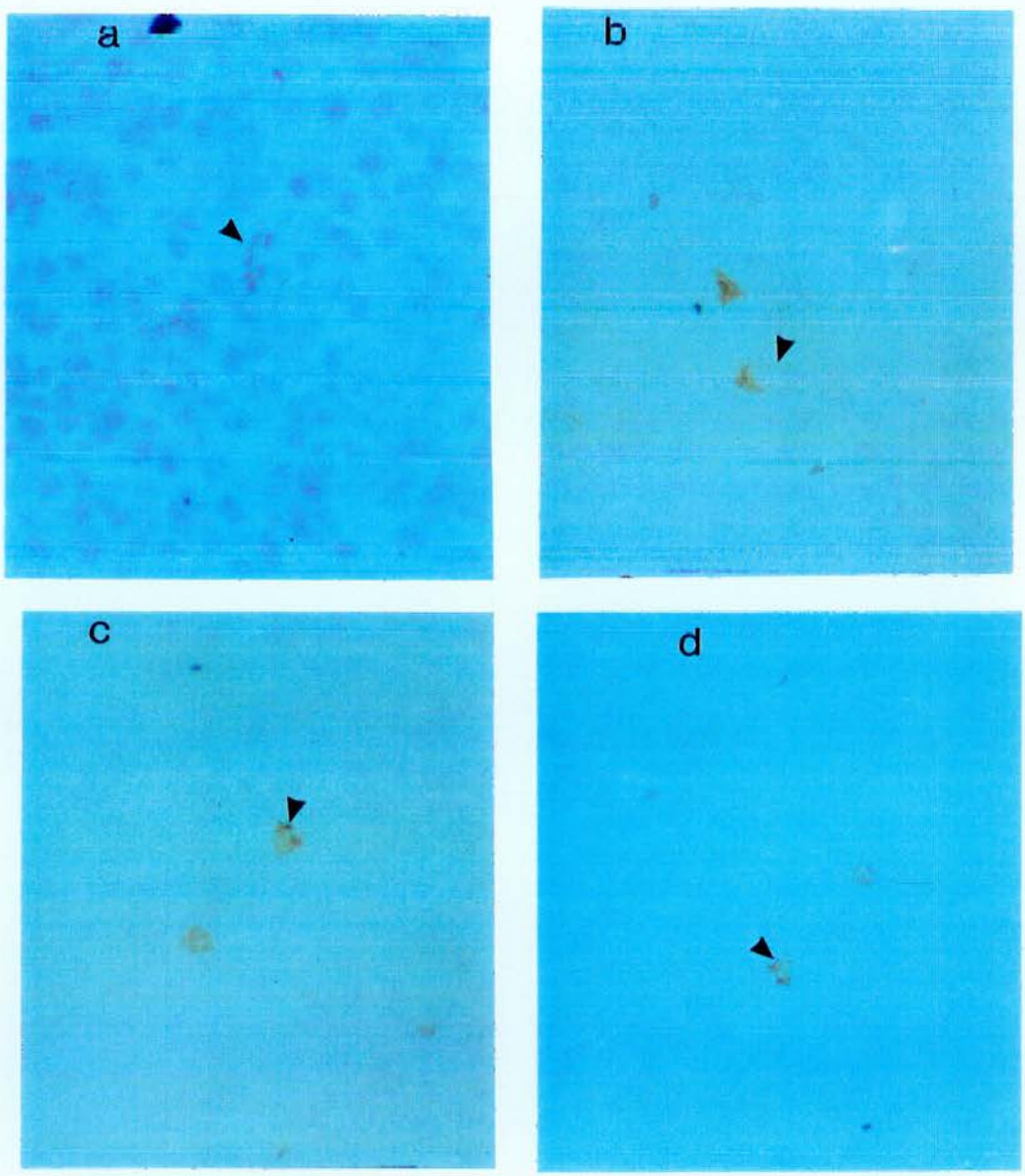

Figure 4.8 Photomicrographs illustrating the labelling visualised in the (a, b) anterior pituitary of a laying hen using the DIG-labelled VF48N probe, after proteinase K treatment (5µg/ml for 15 mins), and the artefacts (c, d) observed using the control probe VF48E. (Mag. x400).

The specificity of the VIP mRNA visualised in the chicken anterior pituitary gland, by DIG-labelled RNA probes could not be established. Therefore, the ISH study was abandoned and a solution hybridisation method was used to demonstrate the expression of mRNA encoding VIP in the anterior pituitary gland.

4.4 VIP gene expression using Solution hybridization and RNase protection assay

This technique has a greater degree of sensitivity because it uses a high-specific-activity probe which is complementary to VIP mRNA, and is not affected by the problem of penetration, which is common in fixed tissue sections used for *in situ* hybridisation

Total RNA was isolated from the gut (n=2), hypothalamus (n=3) and anterior pituitary gland of a laying hen (n=10). A high specific activity RNA probe was generated which was complementary to part of exon 4 (102 bp), exon 5 and some of exon 6 (Section 2.7.1). The RNA probe protected two fragments of 260 bp and 160 bp in total RNA from the gut, hypothalamus and one 160 bp RNA fragment in the pituitary (Fig. 4.9). The 260 bp fragment corresponded to the predicted size for an RNA encoding both VIP and PHI. The 160 bp protected RNA fragment in total RNA from the gut, hypothalamus and pituitary was predicted for a VIP-only encoding RNA (Fig. 4.9). The VIP/PHI mRNA transcript was not detected in the anterior pituitary, and no specific bands were found in the tRNA, hepatocyte RNA or water controls.

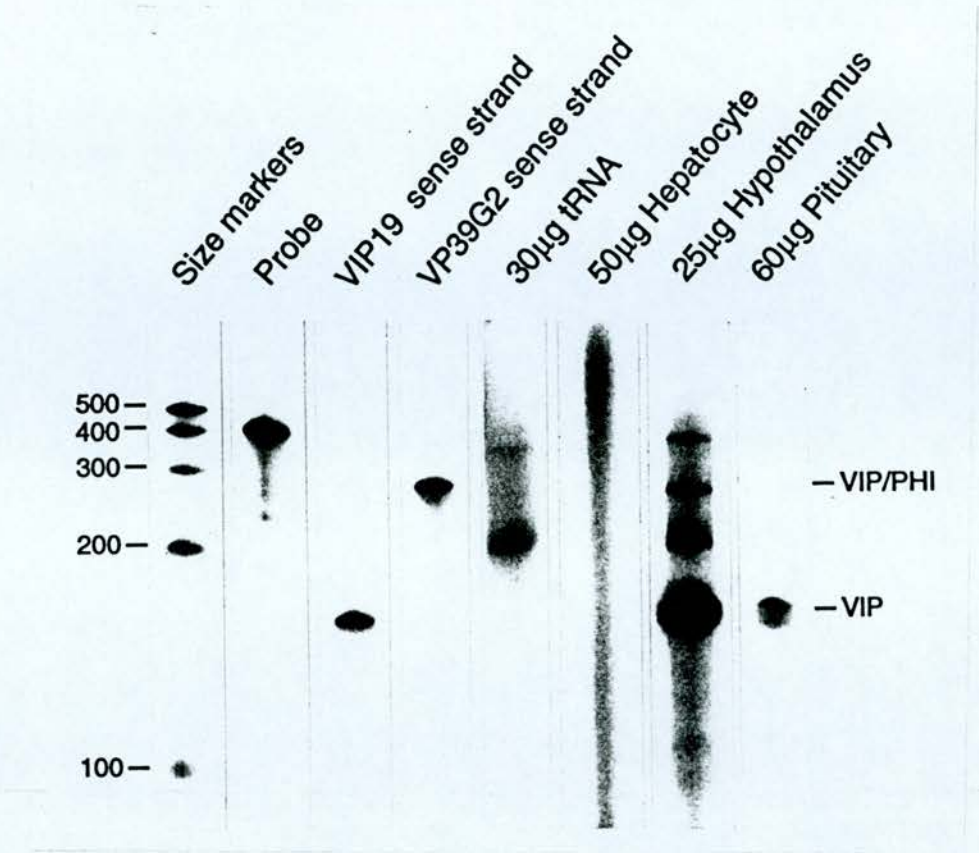

Figure 4. 9 The solution hybridization and RNase protection demonstrates that the complementary probe has protected VIP mRNA of the predicted size from RNase digestion. The visualisation of protected bands confirmed that mRNA encoding chicken VIP occurs in the chicken hypothalamus and anterior pituitary gland.

4.5 Discussion

The RT-PCR and solution hybridisation studies showed for the first time that mRNA encoding the chicken VIP gene was found in the anterior pituitary gland of the laying hen. The solution hybridisation study also showed that this method was not sensitive enough to detect the larger VIP/PHI transcript, but it was detected by RT-PCR. The smaller VIP-only transcript was the predominant form in the pituitary tissue of the laying bantam hen. This observation supports the findings that the two VIP mRNAs are also present in unequal amounts with the VIP only transcript dominating in the anterior pituitary, hypothalamus and gut of the chicken (Talbot *et al.,* 1995) and turkey (You *et al.,* 1995). This observation shows that VIP is synthesised in the anterior pituitary gland and is not merely sequestered by the gland from other sources. Furthermore, it is consistent with the indirect (Hagen *et al.,* 1986; Nagy *et al.,* 1988) and direct evidence (Arnaout *et al.,* 1986; Koves *et al.,* 1990a; Morel *et al.,* 1982; Lam *et al.,* 1989) that VIP is produced by the anterior pituitary of the rat. The expression of VIP/PHI mRNA in the anterior pituitary gland was suggested by the RT-PCR analysis, but at a level significantly less than that of VIP mRNA, since pituitary VIP/PHI mRNA could not be demonstrated using solution hybridisation. Prolactin secretion in the turkey is stimulated by mammalian PHI, although less effectively than VIP (Proudman and Opel, 1990). PHI may therefore have a role to play in the function of the anterior pituitary, but the results of this study are not detailed enough to draw any conclusions.

The *in situ* hybridisation study was not able to localise VIP mRNA in cells of the anterior pituitary. However, the specific hybridisation in the basal hypothalamus was localised to individual neurons, and the distribution was similar to the pattern described for VIP neurons localised by immunocytochemistry (Sharp *et al.,* 1989) and by *in situ* hybridisation using a ^{35}S-labelled (45 base) oligonucleotide probe (Talbot *et al.,* 1995). The sensitivity of the DIG-labelled RNA probes was not able to detect the low level of VIP mRNA in the pituitary gland.

There may be several reasons why this approach was unsuccessful. This may have been a reflection of the quantity of VIP mRNA expressed by both tissues, or the inability of long riboprobes to penetrate the pituitary tissue to hybridise with the target mRNA. The concentration of probe may have been too high, and the saturation of the tissue with probe appeared to result in the formation of artefacts,

which were not removed by stringent washing or RNAse treatment. The DIG-labelled probe is likely to lack the sensitivity required for the detection of pituitary VIP mRNA.

VIP mRNA occurs in stellate cells of the anterior pituitary gland of the hypothyroid rat, as demonstrated by *in situ* hybridisation with an ^{35}S-labelled RNA probe, but it is not present in the normal pituitary (Segerson *et al.,* 1989). The detection of VIP mRNA in the normal chicken pituitary may not be possible using non-radioactive probes. Indeed, the VIP mRNA demonstrated in the pituitary by solution hybridisation RNAse protection assay was only possible using a high specific activity ^{32}P-labelled RNA probe. Therefore, endocrine manipulation, including hypothyroidism, adrenalectomy or oestrogen administration, which increases VIP gene expression in the rat anterior pituitary (see Section 1.6.1), may be necessary to increase VIP gene expression in the chicken pituitary in order to detect it using *in situ* hybridisation.

An alternative method of hybridisation may have been to use a non-radioactive or radioactive oligonucleotide (oligos). The oligos have a greater sensitivity than non-radioactive probes and are better able to penetrate tissue sections without pretreatment with proteinase K, which may damage the structural morphology. An ^{35}S-labelled probe was used in trials, but was replaced by a non-radioactive probe because of high background in pituitary sections, which may be a feature of using radio labelled probes on this tissue. This is supported by the following observation. The detection of vasopressin (VP) mRNA in the posterior pituitary of the rat is hampered by the non-specific signal in the anterior pituitary with the use of ^{33}P-labelled oligos, but not with hapten-labelled oligos (Trembleau and Bloom, 1995). No VP mRNA occurs in the anterior pituitary (Mohr *et al.,* 1991), and it is suggested that the background may be an artefactual product of excessive probe concentration, or cross-hybridisation of the probe with unknown sequences.

Recent research also shows that the use of multiple non-overlapping non-radioactive oligonucleotide probes, significantly increase the sensitivity and resolution of *in situ* hybridisation detection in which the mRNA is present at low concentrations (Trembleau and Bloom, 1995). This technique may be a suitable approach to identify VIP mRNA in the chicken anterior pituitary, and confirm that the stellate cells identified in Chapter 3, synthesize VIP.

In conclusion, the RT-PCR and solution hybridisation and RNAse protection assay confirmed VIP *de novo* synthesis in the anterior pituitary gland of the chicken. Further work is needed to identify unequivocally the specific cell type which produces VIP in the pituitary, but it is likely VIP produced by a folliculo-stellate cell type, may function as a paracrine regulator of pituitary hormone function.

Chapter 5: THE IMMUNOCYTOCHEMICAL LOCALISATION OF VIP RECEPTORS IN THE ANTERIOR PITUITARY AND HYPOTHALAMUS

5.1 Introduction

It has been suggested that the VIP receptor is heterogeneous (Laburthe *et al.*, 1989; Robberecht *et al.*, 1990). High and low affinity VIP binding sites have been characterised, in mammalian (Laburthe *et al.*, 1989) and avian species (Meunier *et al.*, 1991; Rozenboim and El Halawani, 1993), and more than one pharmacologically distinct class of VIP receptor occurs in rat tissues (Robberecht *et al.*, 1986; Gozes *et al.*, 1991a, b; Blum *et al.*, 1992). Two VIP receptor subtypes have been cloned from the rat (Ishihara *et al.*, 1992; Lutz *et al.*, 1993) and human (Sreedharan *et al.*, 1993; Xia *et al.*, 1996), which are referred to as the subtype-I (VIP-RI, see Section 1.7.4) and subtype-II (VIP-RII, see Section 1.7.5) VIP-receptor, respectively.

Hypothalamic vasoactive intestinal polypeptide stimulates prolactin secretion and prolactin gene expression in birds (Talbot *et al.*, 1991; Youngren *et al.*, 1994), and its mode of action is mediated by a specific VIP binding site (Rozenboim and El Halawani, 1993; Gonzales *et al.*, 1994b). In the previous chapters it was shown that VIP is synthesised in the anterior pituitary gland suggesting that it may act as a paracrine factor to regulate pituitary hormone secretion. The aim of this chapter was to localise immunocytochemically VIP receptors in the chicken anterior pituitary cell types using antibodies raised against the predicted amino acid sequence of the human subtype I (Goetzl *et al.*, 1994) and II VIP receptors (a gift from E.J. Goetzl). VIP receptor subtypes were also localised in the hypothalamus to gain evidence for specificity and compare with published information on the distribution of VIP binding sites in the avian brain (Hof *et al.*, 1994).

5.2 The localisation of the subtype-I VIP receptor

5.2.1 Characterisation of subtype-I VIP receptor (VIP-RI) antibodies

The distribution of VIP-R subtype I in the anterior pituitary sections (Section 5.2.6) was visualised using a donkey anti-rabbit conjugated rhodamine antibody or an ABC peroxidase staining kit. Two primary antibodies were used (Table 5.1)

the first was raised against a peptide sequence from the first extracellular loop (Peptide *A*, 32 amino acids) and the second raised against the carboxyl terminus (Peptide *B*, 67 amino acids) (Section 1.7.4). The distribution of immunoreactive labelling in the anterior pituitary was the same for both anti-peptide antibodies (*A* and *B*) (*not shown*), but the intensity of labelling was greater when using the antibody raised against peptide *A*. Most experiments were therefore carried out using this VIP-R antibody (1:100).

Table 5.1 Human subtype-I VIP-receptor antibodies used for immunocytochemistry. *Abbreviations* +++, excellent staining, ++, acceptable staining.

Antibody	**Amino acid no. from N-terminus**	**Epitope location**	**Labelling Intensity**
VIP-RI(A)	191-222	1st extracellular loop	+++
VIP-RI(B)	391-457	Carboxyl terminal	++

5.2.2 The localisation of the VIP receptor subtype-I (VIP-RI)

Initial studies showed that VIP-RI immunolabelling was confined to the cephalic lobe of the anterior pituitary gland of laying (n=4) and broody (n=4) bantams (Fig. 5.1). Immunoreactive labelling visualised using a rhodamine conjugate second antibody, was in isolated cells and cell clusters throughout the cephalic lobe, in the cytoplasm but not in nuclei (Fig. 5.1). Immunoreactive labelling in the cephalic lobe in the broody hen was weaker than that in the laying hen (*not shown*). The specificity of antibodies VIP-R(A) and VIP-R(B) was confirmed, by the observation that immunolabelling in the pituitary was absent when antibodies had been passed through a column containing Sephadex coupled to the peptide used to raise the antibodies (Goetzl *et al.,* 1994). (Fig. 5.1d).

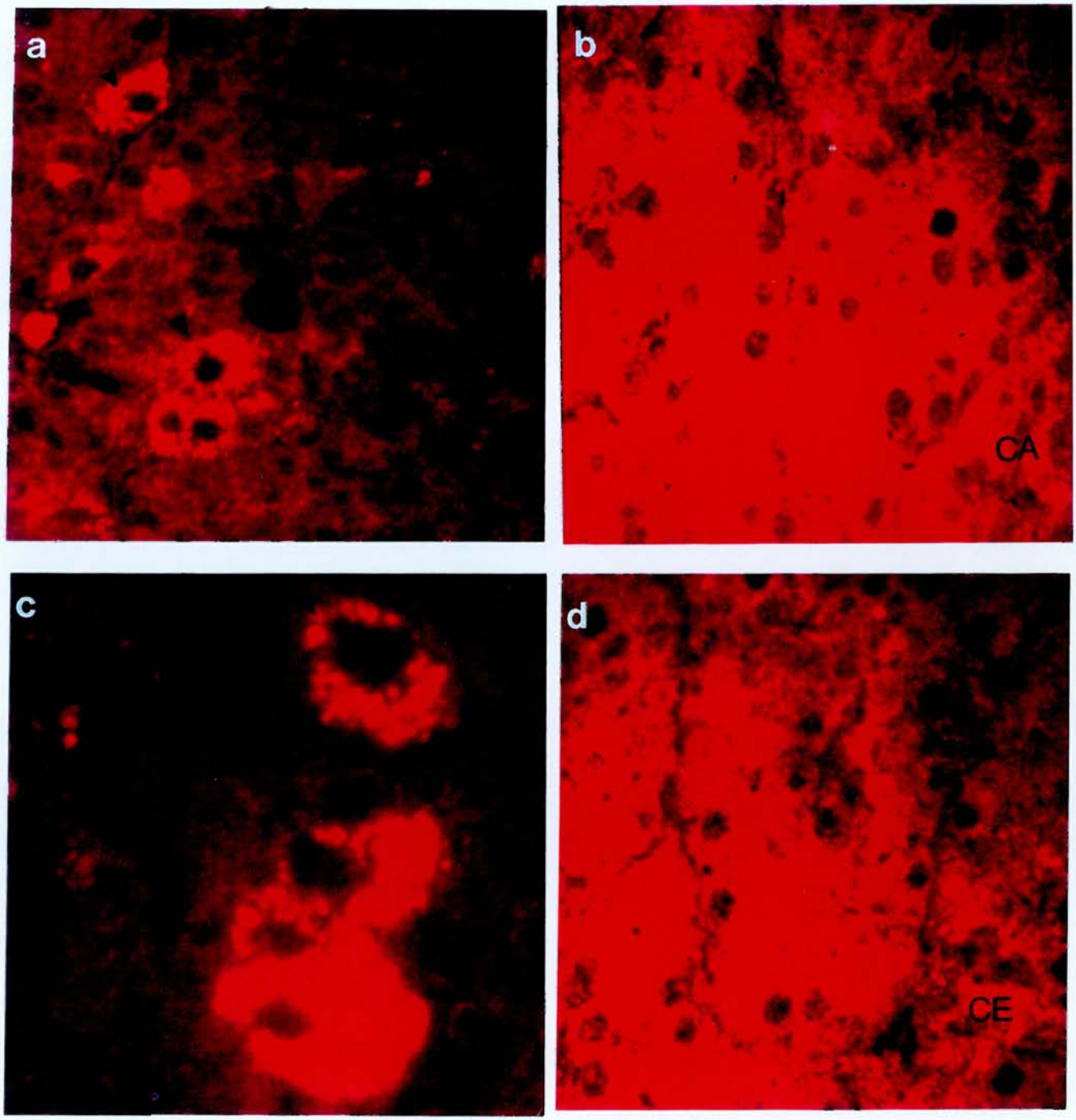

Figure 5.1. The distribution of VIP-RI immunoreactivity in the (a) cephalic lobe (CE) and (b) lack of staining in the caudal lobe of the anterior pituitary (CA), demonstrated using a rhodamine conjugated second antibody (Mag. x400). The VIP-RI immunoreactive cells were found in (c) isolated cells and cell clusters (Mag. x1000). The immunolabelling was (d) abolished by absorbing the VIP-RI antibody, with the peptide used to produce it (peptide *A*) (Mag. x400).

5.2.3 The distribution of PRL, LH, GH and ACTH cells using the adjacent sections

A series of experiments were carried out to localise the VIP-RI in cells of the anterior pituitary gland from a laying hen (n=4), using adjacent sections. Thin (2μm) sections were mounted in groups of three, on consecutive slides, and one slide was labelled for VIP-RI and the other slide was immunolabelled using an antibody to either PRL (code/31/1), LH (code/783) ACTH (code/5.81) or GH (see Section 2.2.7). Immunolabelling was visualised using a biotin-avidin peroxidase staining kit. The distribution of PRL and LH cells is described previously in Chapter 3 (Section 3.2.1). The distribution of ACTH immunoreactive cells was similar to that of PRL and VIP-RI expressing cells, occurring in the cephalic lobe of the anterior pituitary gland. In contrast, the GH immunoreactive cells were found only in the caudal lobe.

5.2.4 The localisation of the VIP-RI in pituitary hormone secreting cells using adjacent sections

Prolactin-producing cells and cells containing VIP-RI were localised in the cephalic lobe (Fig 5.4), but there was no evidence of colocalisation of PRL and VIP-RI in the same cell (Fig. 5.4). LH-producing cells did not contain VIP-RI immunoreactivity in the cephalic lobe (Fig. 5.5), and the GH cells were also not associated with the cells containing VIP-RI immunoreactivity except along the cephalic/caudal boundary (*not shown*). However, the distribution of ACTH cells and VIP-RI was essentially the same (Fig. 5.6), and the majority of ACTH cells identified on adjacent sections throughout the cephalic lobe of the anterior pituitary contained VIP-RI immunolabelling (Fig. 5.6).

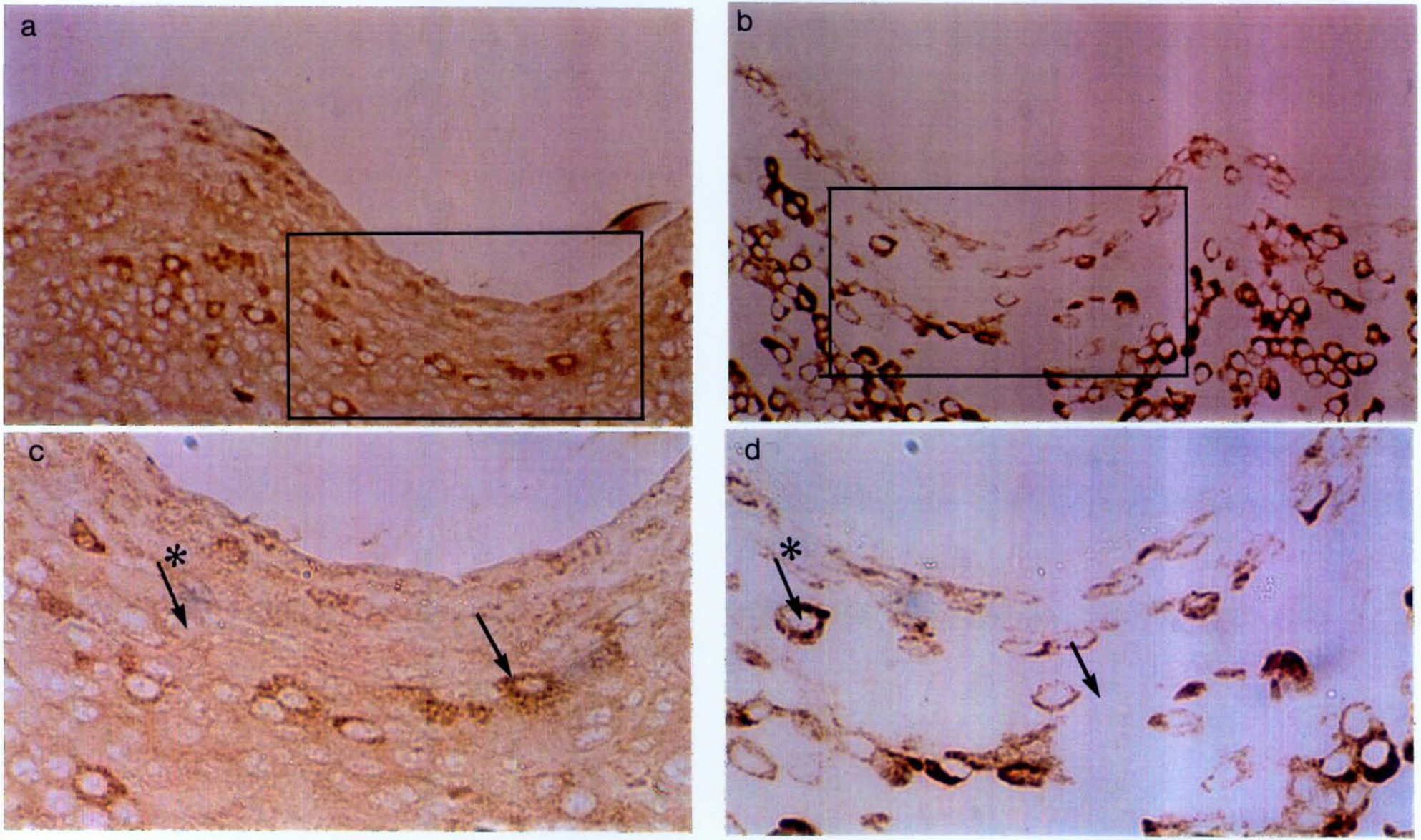

Figure 5.3 Immunocytochemical labelling of cells containing (b, d) PRL immunoreactivity and (a, c) the VIP-receptor subtype-I immunoreactivity (VIP-RI) on adjacent sections (a, b; Mag. x200). PRL-containing cells (e.g. *, *arrow*) did not contain VIP-RI (e.g. *, *arrow*). Conversely cells containing VIP-RI immunoreactivity (e.g. *arrow*), did not contain PRL immunoreactivity (e.g. *arrow*) (c, d; Mag. x1000).

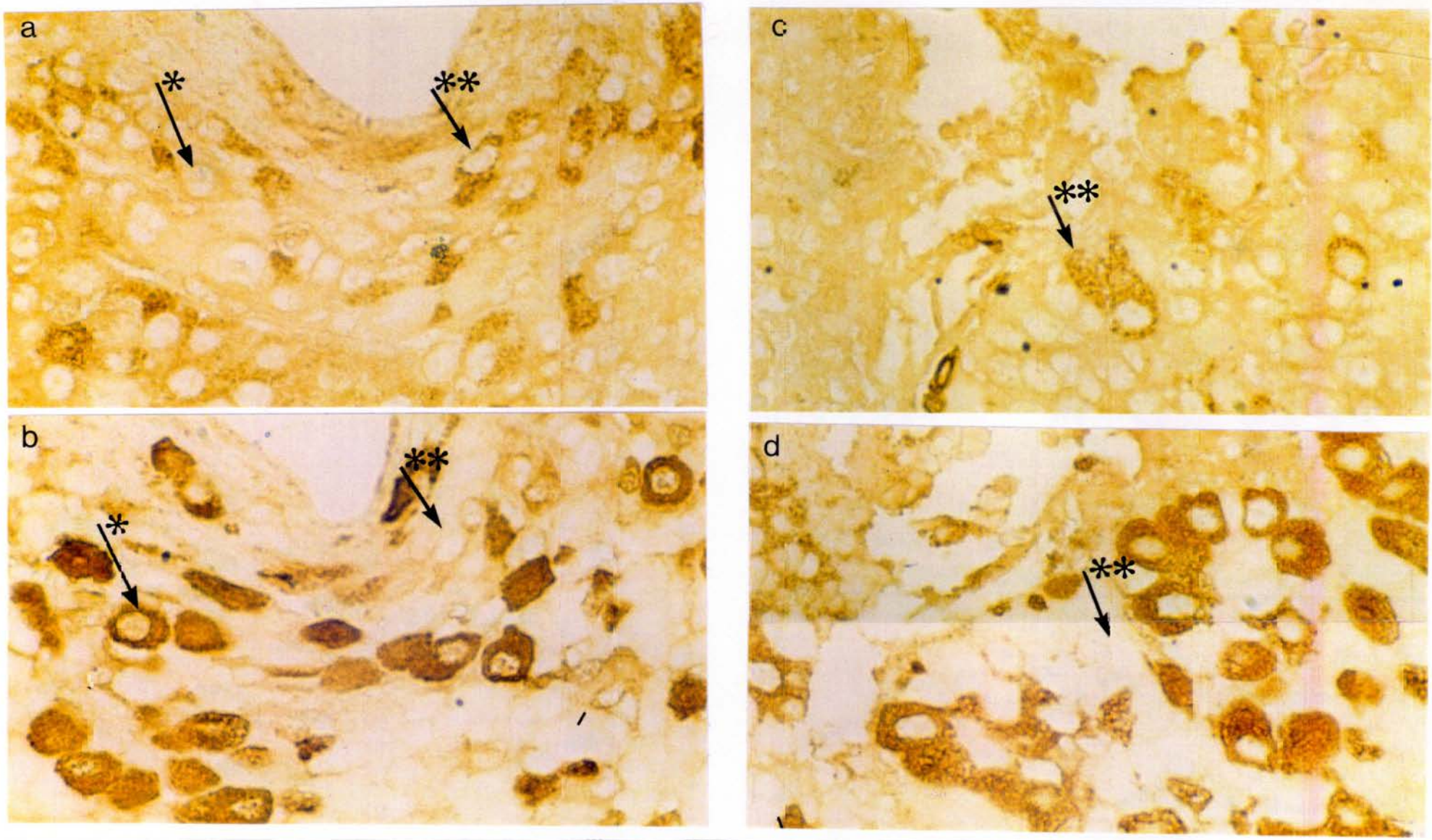

Figure 5.4 Immunocytochemical labelling of cells containing (a, c) the VIP-receptor subtype-I immunoreactivity (VIP-RI) and (b, d) LH immunoreactivity on adjacent sections.(a, b; Mag. x1000). LH cells (e.g.*, *arrow*) did not contain VIP-RI immunoreactivity (e.g.*, *arrow*) in the same cell, on the adjacent section. VIP-RI cells (e.g. **arrow*) did not contain LH immunoreactivity (e.g. **arrow*) (c, d; Mag. x1000).

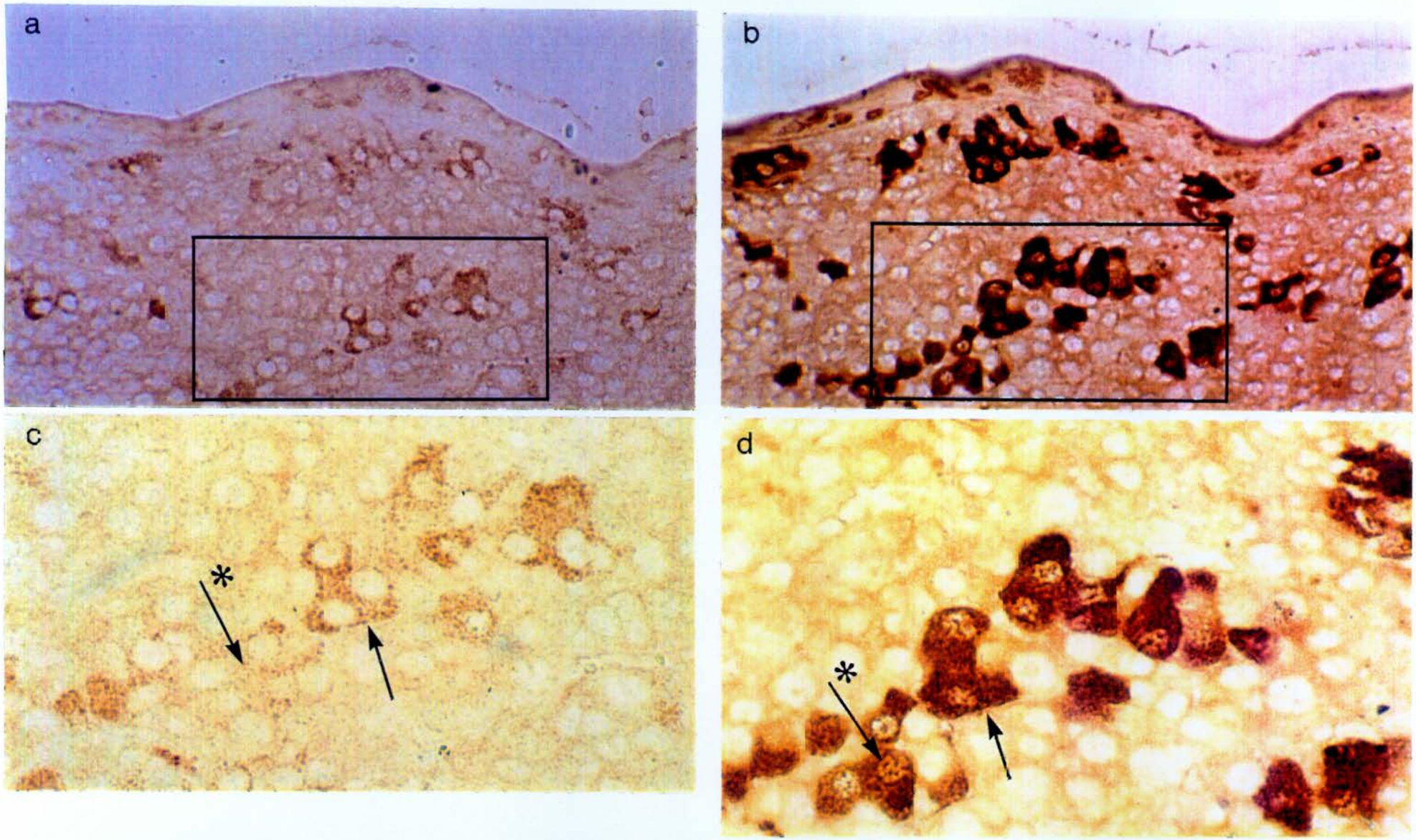

Figure 5.5 Immunocytochemical labelling of cells containing (a, c) the VIP-receptor subtype-I (VIP-RI) immunoreactivity and (b, d) ACTH immunoreactivity on adjacent sections (a, b; Mag. x200). ACTH cells (e.g. arrow) containing VIP-RI immunoreactivity (e.g. arrow). A few ACTH cells (eg *, arrow) were weakly immunolabelled with VIP-RI immunoreactivity (eg, *, arrow) (c, d; Mag x 1000).

5.3 The localisation of the VIP receptor subtype-II (VIP-RII) in the anterior pituitary

The distribution of VIP-RII immunoreactivity was visualised on sagittal sections of the anterior pituitary gland from laying (n=3) and broody (n=3) bantam hens, using an ABC peroxidase staining kit (see Section 2.2.2). The antibodies used in this study (Table 5.2), were generated by immunizing rabbits with five different peptide sequences deduced from the nucleotide sequence of the recently cloned subtype-II VIP-R (VIP-RII) (Goetzl *et al.*, 1996).

Table 5.2 Human subtype-II VIP-receptor antibodies used for immunocytochemistry. *Abbreviations* : +++ good clear staining, ++ acceptable staining, with high background, + very little or no staining.

Antibody	Amino acid no. from N-terminus	Epitope location	Labelling Intensity
VIP-RII(4)	54-70	Extracellular amino terminal	+
VIP-RII(5)	105-122	Carboxyl terminal	+++
VIP-RII(6)	174-195	1st Extracellular loop	++
VIP-RII(22)	19-37	Extracellular amino terminal	+
VIP-RII(23)	67-83	Extracellular amino terminal	+

5.3.1 The characterisation of the VIP-RII antibodies

Of the five antibodies investigated (Table 5.2) only the VIP-RII(5) and VIP-RII(6) antibodies were suitable for the localisation of VIP-RII immunoreactivity. The visualisation of the subtype-II VIP receptor (VIP-RII) was difficult because of a high non-specific background labelling (Fig. 5.6).

The immunolabelling of cells in the anterior pituitary with the VIP-RII(6) antibody, was also observed in the cephalic and caudal lobes, but the intensity and pattern of immunolabelled cells differed from that of the VIP-RII(5) (Section 5.3.2). The VIP-RII(6) immunolabelled cells in the caudal lobe were more intensely labelled and more distinct than the cells labelled in the cephalic lobe (Fig. 5.6). It was apparent, that although immunolabelled cells occurred in both lobes of

the pituitary the antibodies were labelling either different cells or the intensity of immunolabelled cells varied.

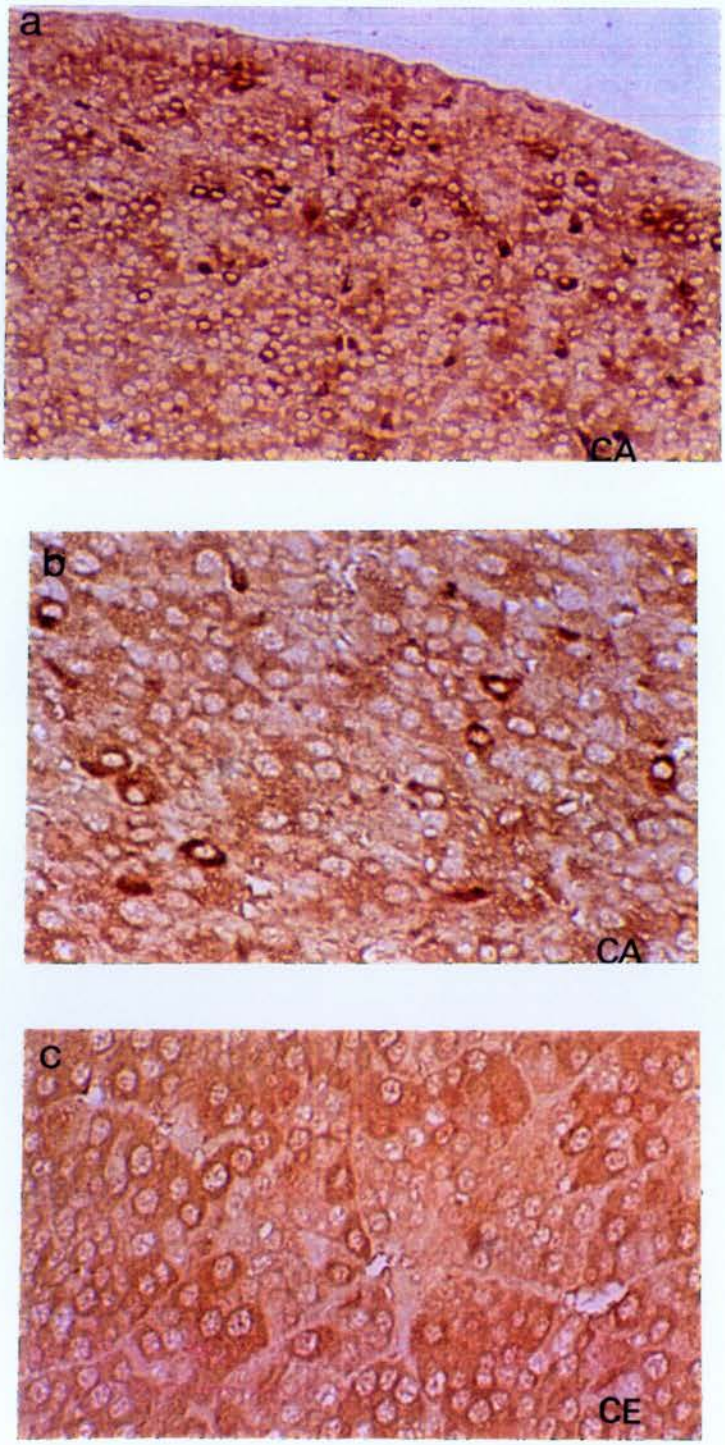

Figure 5.6 The immunolabelling of cells in the cephalic and caudal lobes of the chicken anterior pituitary gland, using the (a, b, c) VIP-RII(6) antibody. (Mag. x200, c, d; Mag, x400). Note the different labelling of cells in the cephalic and caudal lobes. *Abbreviations*, *CA* caudal lobe, *CE* cephalic lobe.

5.3.2 The localisation of the VIP-RII in cells of the anterior pituitary

Several attempts were made to identify the cell types containing VIP-RII immunoreactivity, but variability of immunolabelling made it difficult to distinguish between labelled and unlabelled cells. The immunolabelling of cells in the anterior pituitary (n=3) using the VIP-RII(5) antibody was observed in both cephalic and caudal lobes, and localised in individual cells (Fig. 5.7), and was similar to that of GH-producing cells. Furthermore, the analysis of adjacent sections immunolabelled with antibodies raised against VIP-RII(5) or GH, showed suggested colocalisation (Fig. 5.7), but this could not be established.

5.4. The localisation of the VIP receptors in the hypothalamus

VIP binding sites have been demonstrated in the turkey (Rozenboim and El Halawani, 1993) and chicken anterior pituitary gland (Gonzales *et al.*, 1994) by radioligand binding methods. VIP binding sites were mapped in the avian brain using autoradiography (Hof *et al.*, 1994; Kuenzel *et al.*, 1996). The aim of this experiment was to localise VIP receptors in the chicken hypothalamus and compare it with the studies of ligand binding sites.

5.4.1 The localisation of the VIP-RI in the hypothalamus

The distribution of VIP-RI immunoreactivity was localised using free-floating coronal sections through the hypothalamus of laying hens (n=3), in the plane of the Kuenzel and Masson (1988) stereotaxic atlas, and staining was visualised using an avidin-biotin peroxidase staining kit (see Section 2.2.7). VIP-RI immunoreactivity was found in the internal zone of the median eminence (Fig. 5.8), and the nucleus periventricularis (PHN) (Fig. 5.9), but was not seen in the nucleus paraventricularis magnocellularis (PVN) (Fig. 5.9) or the nucleus supraopticus (SO) (*not shown*). VIP-RI immunoreactivity was localised in the cytoplasm of fusiform and multipolar cell bodies.

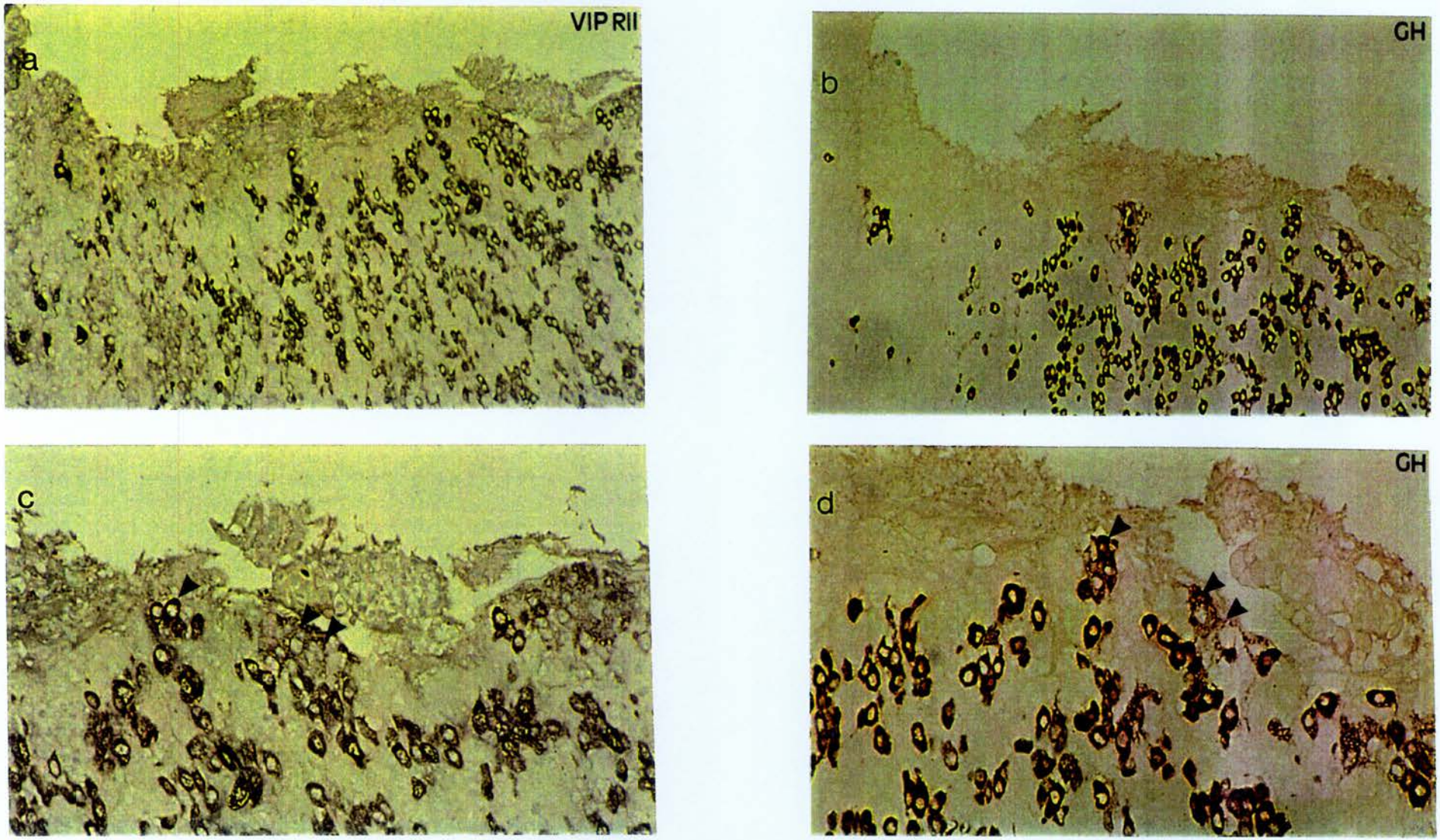

Figure 5.7 A representation of the immunocytochemical labelling of cells containing (a, c) the VIP-receptor subtype-II (VIP-RII) and (b, d) GH immunoreactivity on an adjacent section (a, b; Mag. x200). GH cells (e.g. *arrow*) containing VIP-RII immunoreactivity (e.g. *arrow*) (c, d; Mag. x400).

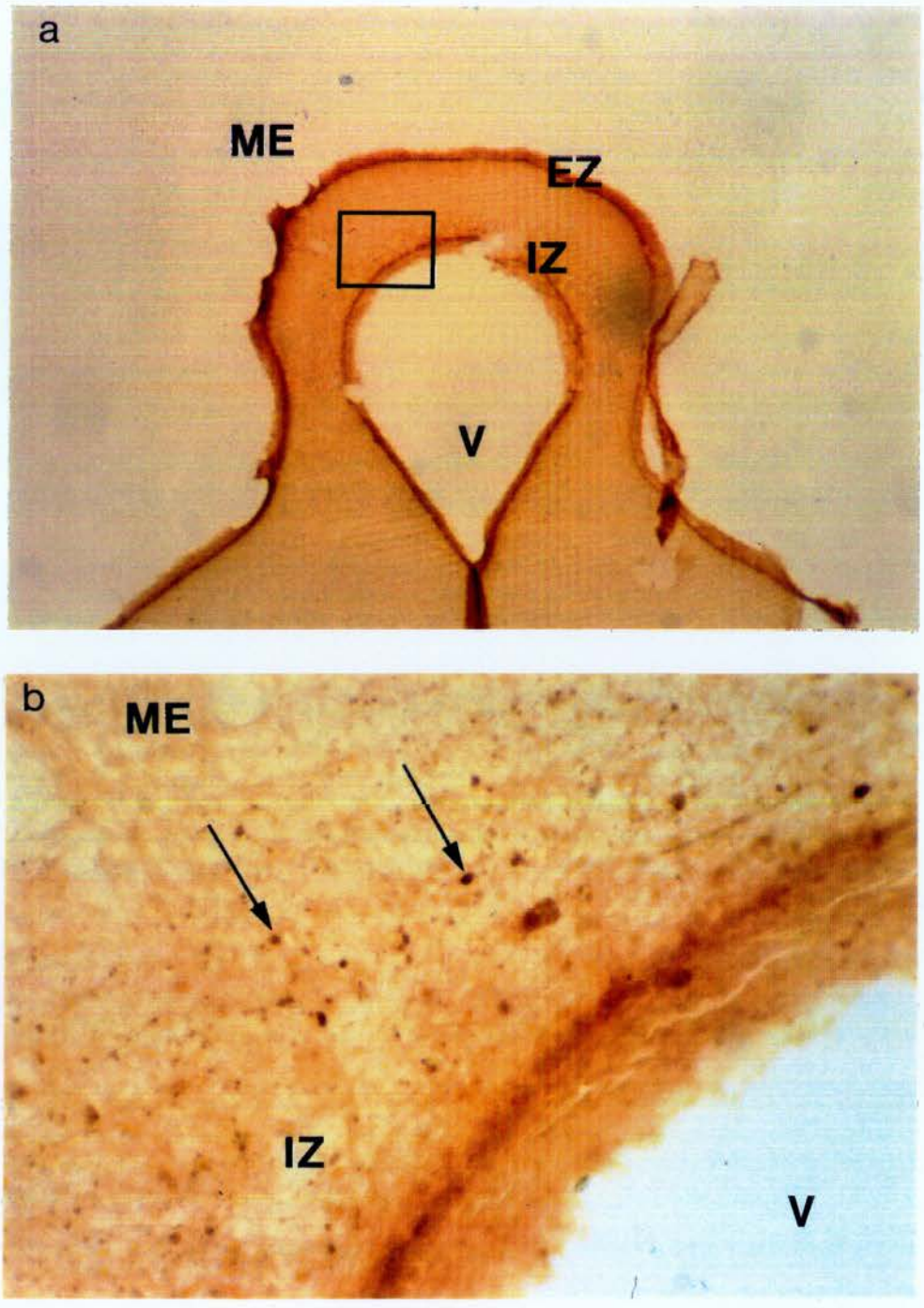

Figure 5.8 The immunocytochemical localisation of VIP-receptor subtype-I (*VIP-RI*) in the (a) internal zone of the median eminence (Mag. x40) and (b) high power image (Mag. x1000). A VIP-RI immunoreactive cell body and fibres are indicated by *arrows*. *Abbreviations: EZ* external zone; *IZ* internal zone; *ME* median eminence; *V* third ventricle.

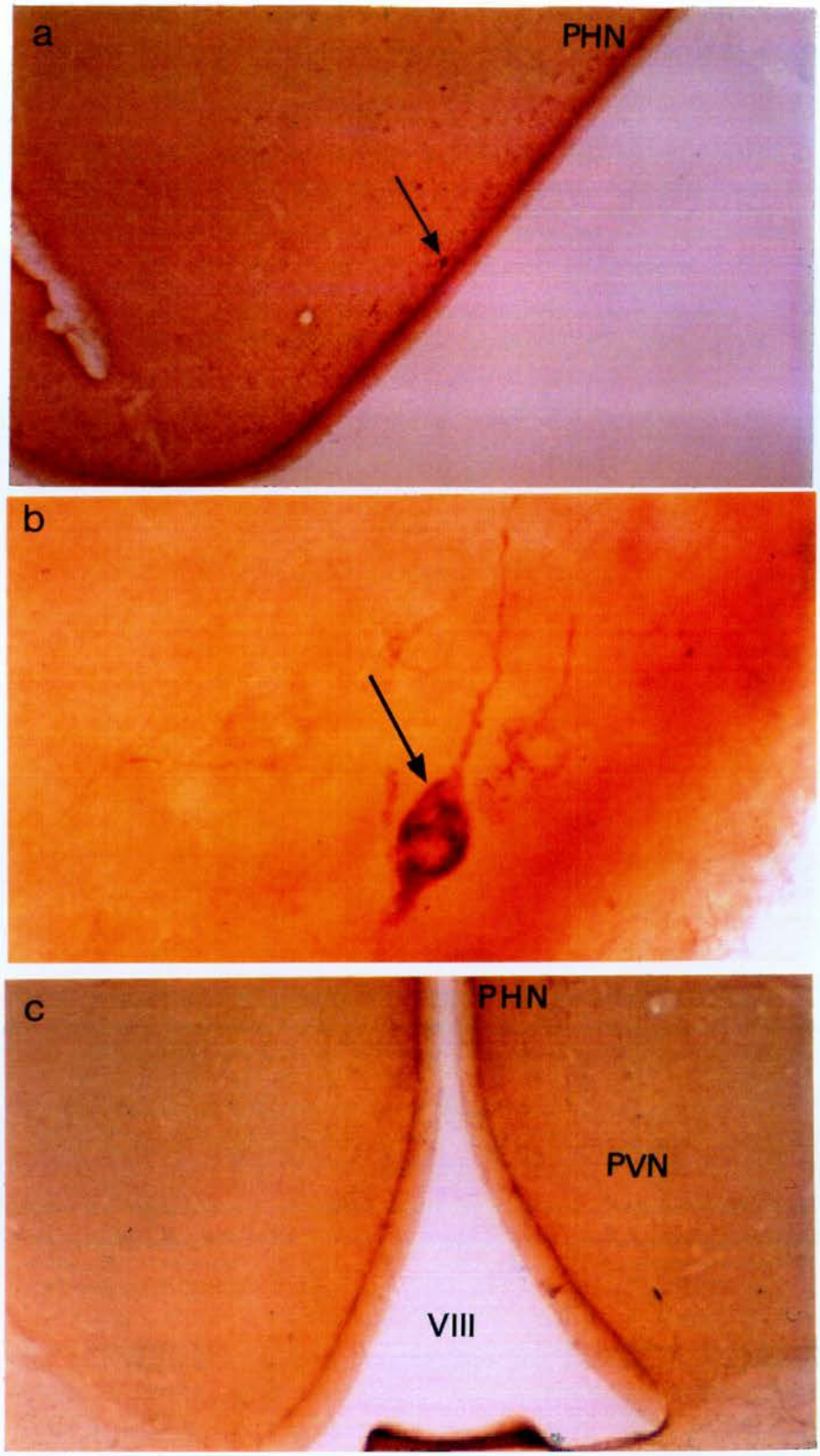

Figure 5.9 The immunocytochemical distribution of VIP-receptor subtype-I (*VIP-RI*) in the (a) periventricular nucleus (Mag. x100) and (b) an immunoreactive fusiform cell body (Mag. x1000). The VIP-RI immunoreactivity was absent in the (c) nucleus paraventricularis Mag. x100). *Abbreviations*: *PHN* nucleus periventricularis hypothalami, *PVN* nucleus paraventricularis magnocellularis, *VIII* third ventricle.

5.4.2 The localisation of the VIP-RII in the hypothalamus

The distribution of VIP-RII immunoreactivity was visualised in free-floating coronal sections through the brain of a laying chicken (n=4), using the method described previously (Section 5.4.2). The distribution of VIP-RII immunoreactivity in the hypothalamus was more widespread than of VIP-RI, and was localised in the preoptic area and middle and posterior hypothalamic areas. Immunolabelling occurred in the cytoplasm and fibres of cell bodies, but was not seen in the cell nuclei. VIP-RII labelled cells were seen in the magnocellular hypothalamic cells of the nucleus paraventricularis and supraopticus. The lateral nucleus and parvocellular portion of the nucleus periventricularis also contained VIP-RII immunoreactivity. The VIP-RII labelled cell bodies were observed in the nucleus habenularis-lateralis, -medialis, and the anterior hypothalamic nucleus.

5.4.3 A comparison of the VIP-RII and vasotocin distributions in the hypothalamus

The distribution of VIP-RII immunoreactive cells was similar to the reported distribution of vasotocin (VT) neurons in the preoptic-anterior hypothalamus (Gray and Simon, 1983). A study was carried out to determine whether the distribution of VIP-RII immunoreactivity corresponded to the distribution of AVP-immunoreactivity using adjacent coronal free-floating sections.

The vasotocin labelled cells were localised in the nucleus paraventricularis (Fig. 5.11) and periventricularis, supraopticus (SOe, SOv) and preopticus (Fig. 5.10), which also expressed the VIP-RII immunoreactivity on adjacent sections (Fig. 5.11). No attempt was made to confirm localisation of the VIP-RII immunoreactivity, in the vasotocin neurons using double labelling but the distribution of VIP-RII and vasotocin immunolabelling was consistent with co-localisation.

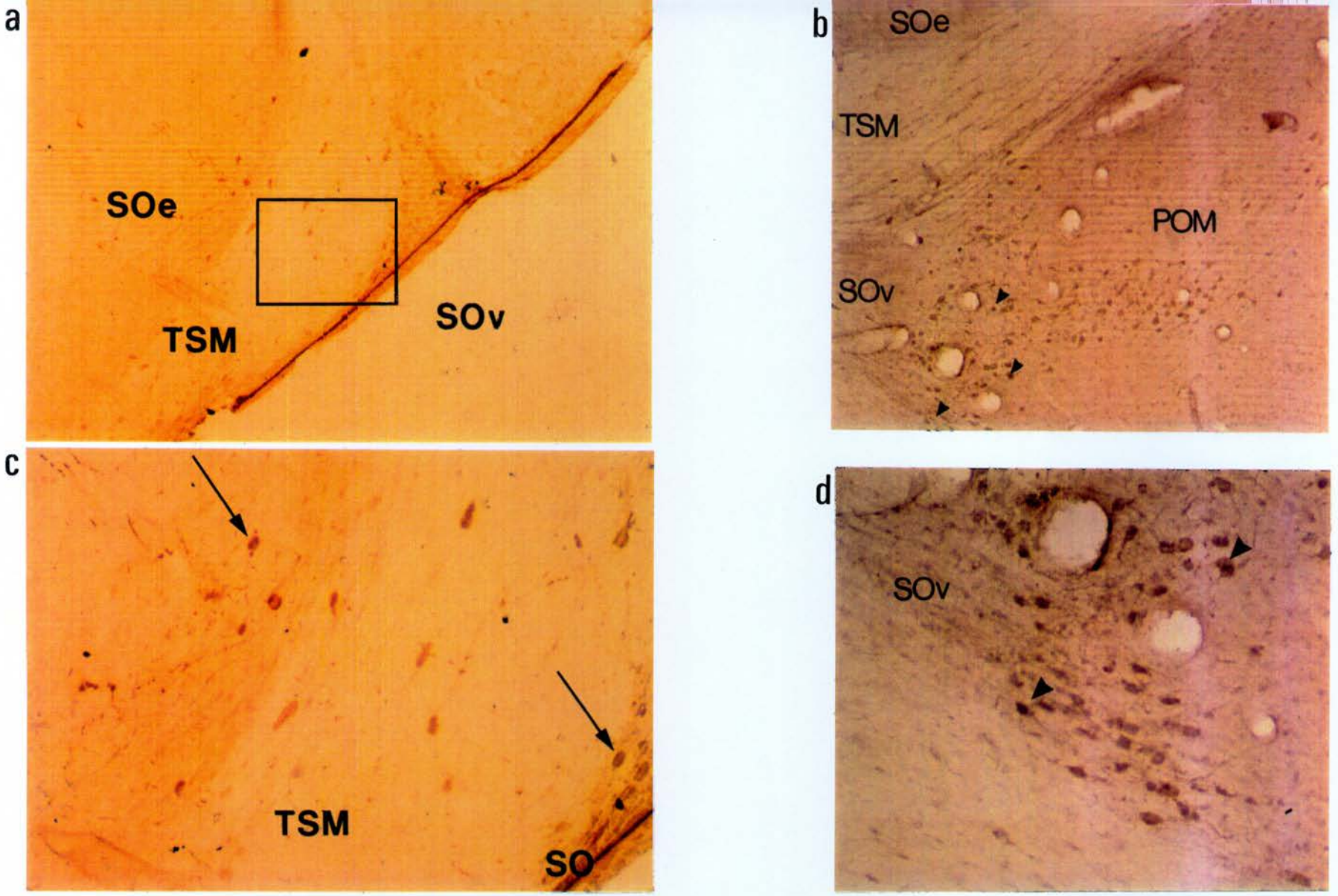

Figure 5.10 The distribution of (a, c) vasotocin and the (b, d) VIP-receptor subtype-II (VIP-RII) in the (a, b) nucleus preopticus, on an adjacent section. *Abbreviations*: *SOe* nucleus supraopticus; *SOv* nucleus supraopticus, pars ventralis; *TSM* tractus septomesencephalicus; *VIII* third ventricle, *POM* nucleus preopticus.

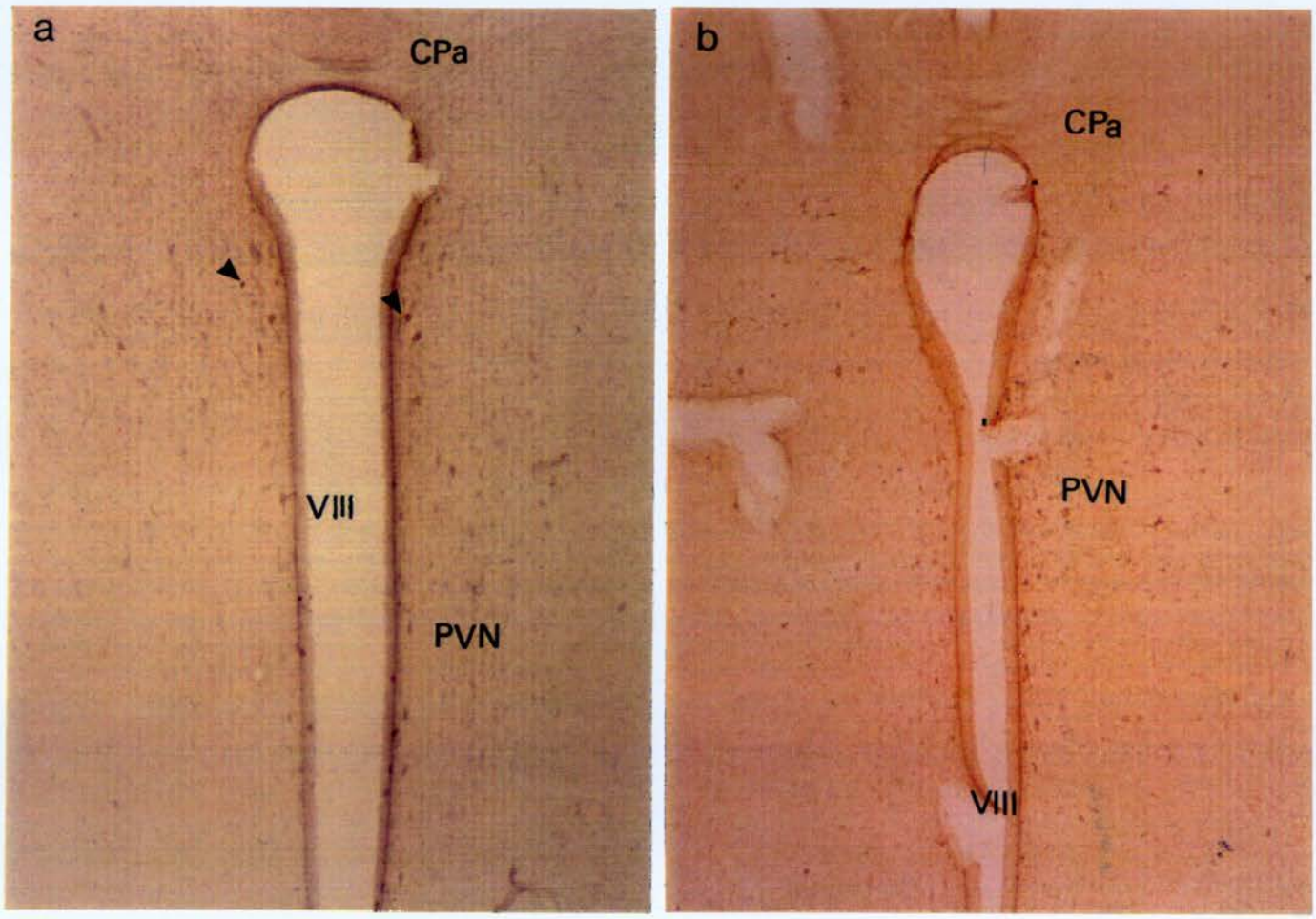

Figure 5.11 The distribution of (a) vasotocin and (b) VIP-receptor subtype-II (VIP-RII) in the nucleus paraventricularis, on an adjacent section. *Abbreviations*: *PVN* nucleus paraventricularis magnocellularis; *VIII* third ventricle.

5.5 Discussion

This study demonstrated the localisation of vasoactive intestinal polypeptide receptors in the anterior pituitary gland of the chicken, using antibodies raised against portions of the deduced amino acid sequence of the human subtype-I (Goetzl *et al.,* 1994) and subtype-II VIP receptors (a gift from E.J. Goetzl).

The subtype-I VIP-R was localised exclusively in ACTH cells. This is consistent with the evidence that the VIP-RI mRNA occurs in low levels in the rat anterior pituitary gland (Usdin *et al.,* 1994; Rawlings *et al.,* 1995), and also occurs in the corticotroph-like AtT20 cell line (Journot and Rawlings, unpublished results). The VIP-RI was not seen in PRL, LH and GH cells, but may occur in these cell types at a concentration too low to be detected using immunocytochemistry.

The mRNA encoding the VIP-RI is reported not to be present in the lactotroph/somatotroph-like (GH_4C_1) or the gonadotroph like (αT3-1) cell lines (Rawlings *et al.,* 1995). However, VIP stimulates cAMP production and a Ca^{2+} influx response in these cell lines. This observation suggests that the actions of VIP in these cell lines are mediated by a VIP receptor other than the subtype-I VIP-R (Rawlings *et al.,* 1995).

This immunocytochemical study was not able to identify the cell type or types in the chicken anterior pituitary gland which contained the subtype-II VIP receptor, because of high non-specific background immunolabelling. VIP binding sites occur in both cephalic and caudal lobes of the chicken, and the specific binding in the cephalic lobe of an incubating hen is reported to be greater than in a laying hen (Gonzales *et al.,* 1994a). VIP stimulates PRL secretion and PRL gene expression in the chicken (Talbot *et al.,* 1991), and VIP receptors are localised on lactotrophs in the rat anterior pituitary gland (Wanke and Rorstad, 1990). In the rat the subtype-II VIP receptor is the main VIP neuroendocrine receptor type in the anterior pituitary gland. This has been demonstrated using *in situ* hybridization and RT-PCR (Lutz *et al.,* 1993; Usdin *et al.,* 1994). On the basis of the rat studies it was anticipated that VIP-RII immunoreactivity would occur in lactotrophs of the chicken anterior pituitary gland.

The VIP receptors subtype-I and -II are both members of the G-protein-linked receptor family, which bind to a seven transmembrane spanning receptor and share

a high degree of homology with each other (Segre and Goldring, 1993). The human VIP-RI (Sreedharan *et al.,* 1993) shares a 84% homology with the rat VIP-RI (Ishihara *et al.,* 1992), and the human VIP-RII (Xia *et al.,* 1996), shares a 87% homology with the rat VIP-RII (Lutz *et al.,* 1993). It is therefore likely that the chicken VIP-RII is structurally similar to that of the human VIP-RII.

The apparent lack of discrete immunolabelling of individual cells in the chicken anterior pituitary gland using the VIP-RII antibodies may be due to several factors. Firstly, the VIP-RII may occur in more than one cell type in the chicken anterior pituitary gland, which may account for the widespread immunolabelling observed in the pituitary. This view is supported by the observation that mRNA encoding the VIP-RIIoccurs in both the gonadotroph-like αT3-1 and the PRL secreting somatotroph-like GH_4C_1 cell lines (Rawlings *et al.,* 1995). Secondly, this VIP-RII96) antibody is a polyclonal and may not have the specificity required for use in different species, which resulted in the lack of discrete immunolabelling in the chicken anterior pituitary gland.

The failure to obtain immunolabelled cells in the anterior pituitary using VIP-RII antibodies, contrasted with the clear identification of VIP-RII immunolabelled cells in specific hypothalamic nuclei. The distribution of VIP-RII immunoreactivity in the hypothalamus was similar to that reported for distribution of VIP binding sites in the pigeon hypothalamus (Hof *et al.,* 1991) and VIP-RII mRNA in the rat brain as determined using *in situ* hybridisation (Lutz *et al.,* 1993; Usdin *et al.,* 1994). The VIP-RII antibody used in this study may recognise a VIP-like receptor in the chicken anterior pituitary and hypothalamus. However, this VIP-receptor may be different to the specific VIP binding site which has been identified in anterior pituitary gland of an incubating hen, and is predominantly expressed in the ceplahlic lobe (Gonzales et al., 1994b,c)

The localisation of VIP-RII immunoreactivity in the nucleus paraventricularis and supraopticus is consistent with the observation that VIP binding sites are reported to occur in the nucleus paraventricularis of the pigeon (Hof *et al.,* 1994). Vasoactive intestinal polypeptide-like immunoreactivity also occurs in the nucleus magnocellularis preopticus, nucleus supraopticus and periventricularis and paraventricularis of the chicken (Peczley and Kiss, 1988) and quail (Aste *et al.,* 1995). This observation suggests that VIP may stimulate the release of neuropeptides from these nuclei in the chicken hypothalamus. In the rat, VIP

infused into the cerebral ventricle or PVN, stimulates the release of arginine vasopressin (AVP), which is an ACTH releasing factor (Alexander and Sander, 1994). It appears that in mammals, VIP indirectly affects the ACTH secretion from the rat anterior pituitary gland by stimulating the release of oxytocin and or AVP (Bardum *et al.,* 1987; 1988). It is therefore likely that hypothalamic VIP may act through VIP receptors in the nucleus paraventricularis or supraopticus (Hof *et al.,* 1994), and stimulate the release of vasotocin in the chicken.

There was limited or a complete lack of immunolabelling using the VIP-RI antibody code B (VIP-RI(B)) raised against the carboxyl terminal (CT), and the VIP-RII antibody codes 4, 22 and 23 (VIP-RII(4), (22), (23)), raised against the extracellular amino terminal (AT) (See Table 5.2). This suggests that the accessibility of the CT and AT regions to the VIP-RI or VIP RII antibodies maybe reduced by fixation, or that these regions are susceptible to enzymatic degradation. Although, the VIP-RII antibody codes 22 and 23, are raised against epitopes on the human VIP-RII which are not found in the rat VIP-RII, and do not immunolabel the VIP-RII in the rat (E.J. Goetzl, personnel communication). These specific epitopes are probably not highly conserved and are not found on the chicken VIP-R, and this may account for the lack of immunolabelling, in the chicken anterior pituitary and hypothalamus.

In contrast, immunolabelling was achieved using the VIP-RI antibody code A and VIP-RII antibody code 6 (VIP-RII(6)). These antibodies were raised against an amino acid sequence that occurs on the 1st extracellular loop (ECL-1), of the human and rat VIP-RI (Goetzl *et al.,* 1994), and the human and rat VIP-RII (E.J. Goetzl, personnel communication). These epitopes are likely to be found on the ECL-1 of the chicken VIP-RI and VIP-RII-like receptors, and this is consistent with the observation that immunolabelling was demonstrated in the chicken anterior pituitary gland and hypothalamus.

In conclusion, a VIP receptor antibody raised against a specific epitope, which occurs on the human VIP-RI, allowed the immunocytochemical identification of specific cells in the pituitary and hypothalamus. The VIP-RI was in ACTH cells of the chicken anterior pituitary gland, and not localised in PRL, LH or GH cells. The identity of pituitary hormone cells which contain the VIP-RII was not established, although widespread labelling occurred throughout the anterior pituitary. This suggests that lactotrophs are not the only pituitary cell type which

contain the VIP-RII, since these cells are found predominantly in the cephalic lobe. Therefore, the human VIP-RII antibodies used in this study may lack the specificity required to identify the chicken VIP-binding site, which is positively correlated with the expression of PRL producing cells (Gonzales *et al.,* 1994b,c), and elevated PRl secretion during incubation behaviour.

Chapter 6: VIP AS A PITUITARY MITOGENIC FACTOR

6.1 Introduction

VIP is a neurotrophic factor playing a major role in the development and growth of the nervous system (Gozes and Brenneman, 1989; Gozes and Brenneman, 1993). It acts as a mitogenic factor to stimulate DNA synthesis in embryonic neurones (Pincus *et al.,* 1990), and increases the rate of mitosis in neural and non-neural tissues in cultured mouse embryos (Gressens *et al.,* 1993). The aim of this experiment was to determine whether the increase in prolactin secretion in incubating hens is associated with a proliferation rather than differentiation of cells producing prolactin. Any such proliferation might be due to increased hypothalamic release of VIP at the onset of incubation and or to changes in the function of the VIP-cell type in the anterior pituitary gland.

Cell proliferation both *in vivo* and *in vitro* can be measured by the use of a non-radioactive thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which is incorporated into the DNA of replicating cells. A monoclonal antibody (Mab) raised against BrdU, makes it possible to detect BrdU in mitotic cells immunocytochemically (see Section 2.10.1). The sensitivity of the BrdU technique depends on the dose of BrdU administered, and length of time the animal is exposed to BrdU (Boswald *et al.,* 1990). Typically the animal is exposed to a single or multiple-pulse BrdU injection, a few hours before being sacrificed, which only allows a small 'window' of time to demonstrate DNA replication within the tissue. Since the development of incubation behaviour occurs gradually over a 2-3 week period in the chicken, any changes in mitotic activity within the anterior pituitary gland may not be detected using a short exposure to BrdU. The development of slow-release pellets, makes it possible for BrdU to be delivered continuously for weeks or months, and thereby create a 'window' of exposure, of sufficient duration to detect an increase in pituitary mitotic activity (Weghorst *et al.,* 1991).

Slow-release pellets (Section 2.10.1) were used to deliver a pre-determined dose of BrdU over a 21 day period to laying hens (Section 2.10.3), which were transferred from individual cages to floor pens, to induce incubation behaviour. The BrdU-

labelled cells were detected using an immunocytochemical technique (Section 2.10.6).

6.2 The development of incubation behaviour in birds implanted with BrdU

The birds were not adversely affected by the implantation of the BrdU pellets. The number of birds that showed incubation behaviour was less than predicted (Table 6.1). This may have been because the birds were young and had not previously undergone a cycle of incubation and return to egg production. Additionally a drop in room temperature during the experiment may have inhibited the expression of incubation behaviour.

Table 6.1 The incidence of incubation behaviour in groups of laying bantam hens (*n*=5) transferred from individual cages to floor pens for 21 days, and implanted with slow release BrdU pellets containing different doses of BrdU. *Abreviation*, *C.* control, *Gp* group, *Inb.* incubating,*O/R* ovarian regression.

	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5	Gp 6
Pellet dose	10mg	25mg	50mg	100mg	200mg	C.
Inb.	0	1	1	2	2	2
Laying	5	4	4	3	3	3
O / R	0	1	1	2	2	2

The birds in groups 3, 4, 5 and 6 showed a significant decline in body weight, consistently over the three week period (Fig. 6.1). The body weight of birds in group 1 also showed a decline in the first week ($P<0.005$) but was not significant over weeks 2 ($P=0.06$) and 3 ($P=0.21$), and may be due to the fact that no birds in group 1 became broody. It can therefore be concluded that BrdU treatment has no adverse effect on body condition.

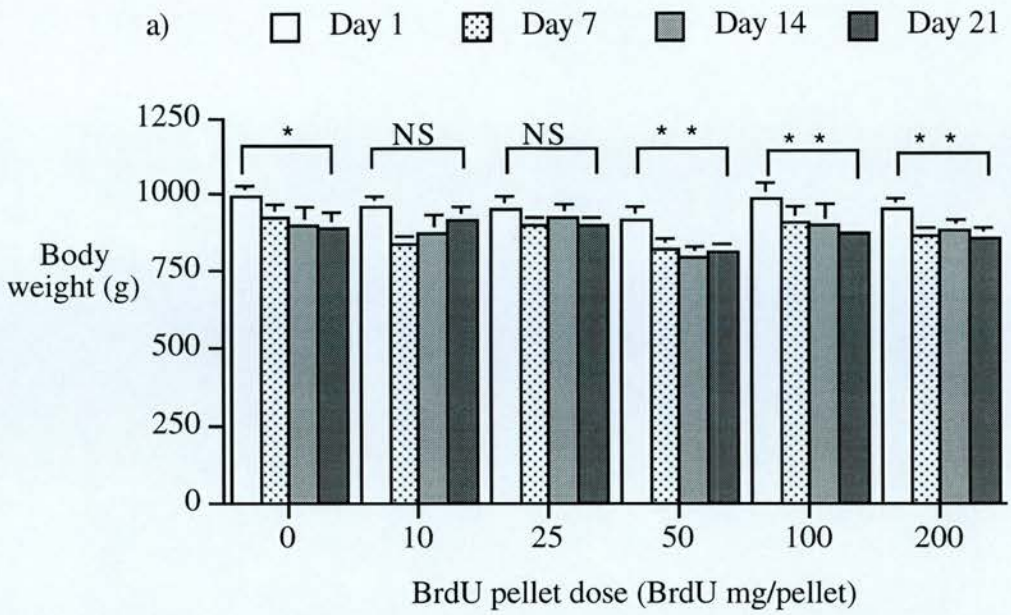

Figure 6.1 The effect of inducing incubation behaviour (n=5) on the body weight of laying birds, over a 3 week period. Five groups of five birds were implanted with a 10, 25, 50, 100 or 200mg BrdU slow release pellet, and transferred to floor pens containing nest boxes.

6.2.1 Effect of different BrdU doses on incorporation into blood and anterior pituitary cells

6.2.1.1 Analysis of blood smears

Blood smears were used to confirm that the marker of cellular replication BrdU, was incorporated into the nuclei DNA of red blood cells, and to optimise the conditions for BrdU immunolabelling (Section 2.10.7). The BrdU-positive nuclei were visualised with an anti-mouse FITC conjugate second antibody. The labelling of red blood cell nuclei was dose dependent, and increased with dose (Fig. 6.2). Nuclear labelling was present in the groups which received 50, 100, and 200mg pellets, and absent in the control group, and in birds that received 10 and 25mg pellets. The immunolabelling was also abolished when the primary antibody was omitted, and when the monoclonal BrdU antibody was preabsorbed with BrdU (10µg/ml).

Immunolabelling was localised around the periphery of the nucleus, in the groups which received a 50mg pellet (Fig. 6.2). However, the immunolabelling was distributed more evenly throughout the nucleus in the birds which received the higher doses of 100mg and 200mg BrdU pellets (Fig. 6.3).

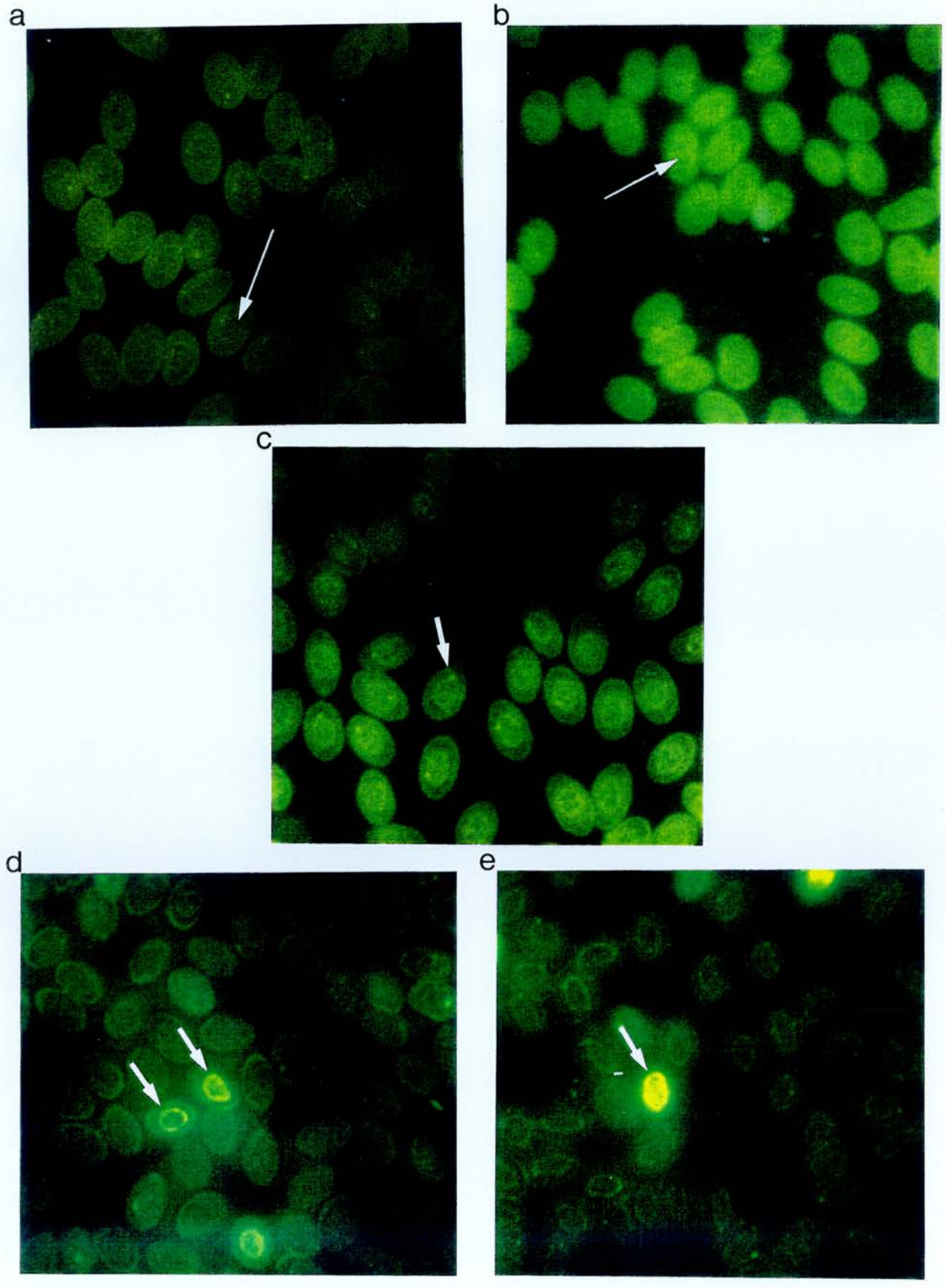

Figure 6.2 The immunolabelling of incorporated BrdU into the nuclei of red blood cells. The birds in each group (n =5) received either a (a) 10, (b) 25, (c) 50, (d) 100 or (e) 200mg BrdU pellet (Mag. x400).

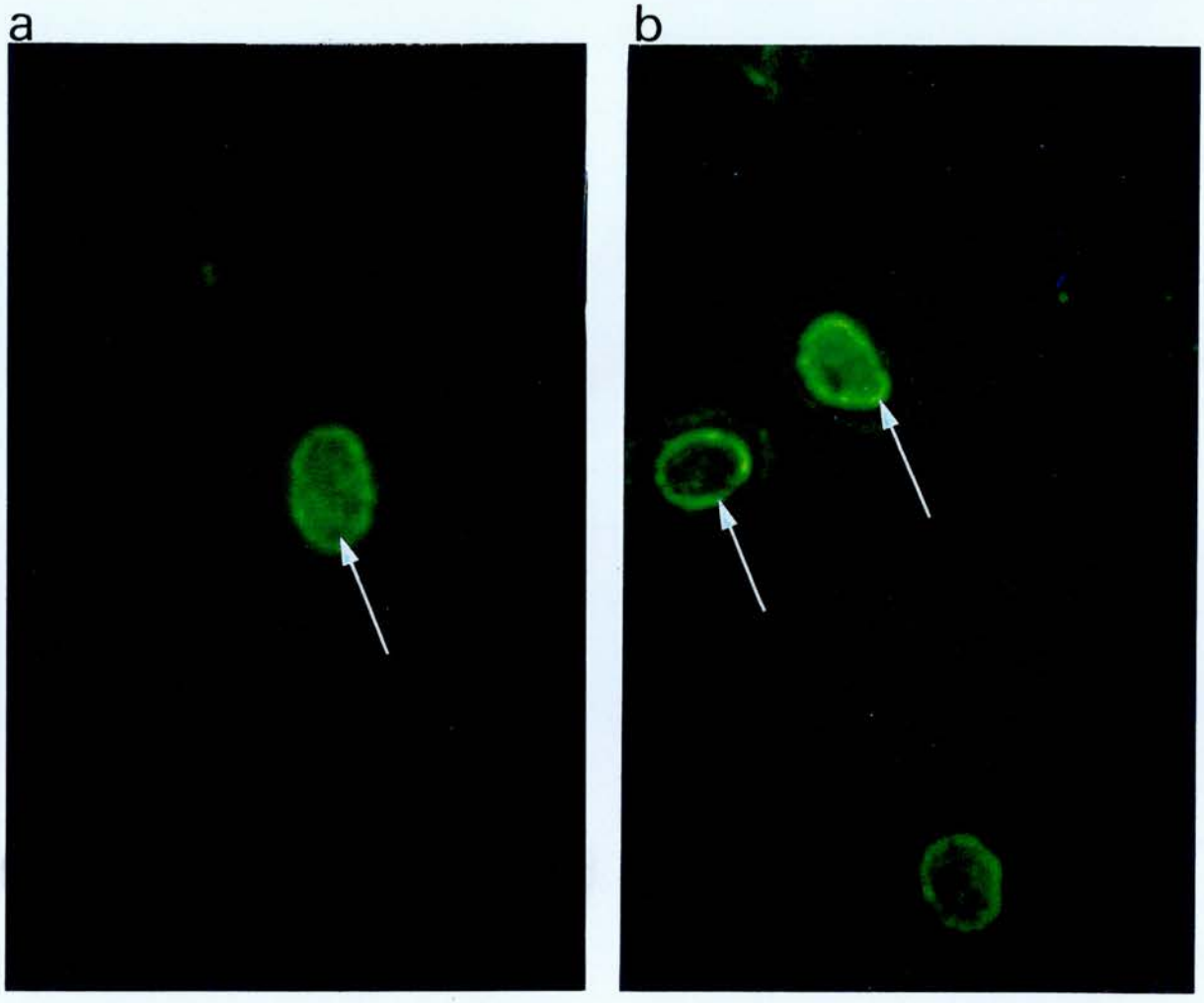

Figure 6.3 The immunolabelling of BrdU incorporated into the nuclei of red blood cells, in birds which received (a) 200 and (b) 100mg BrdU pellet was localised around the periphery, and dispersed diffusely through out the nucleus (Mag. x1000).

6.2.1.2 BrdU-labelled nuclei in the chicken anterior pituitary

Bromodeoxyuridine-labelled nuclei were localised in the chicken anterior pituitary using the monoclonal BrdU antibody (1:1000) and visualised using an avidin-peroxidase kit (ABC-Vector), because labelling of BrdU-labelled cells with the FTIC-conjugate (Section 6.2.1) was not sensitive enough to localize BrdU-labelled cells in the anterior pituitary.

The labelling of mitotic cells in the anterior pituitary was dependent on the amount of BrdU in the implanted pellet. No BrdU-labelled nuclei were observed in the anterior pituitary of control birds or those implanted with a 10, 25, or 50mg pellet . Immunolabelled nuclei were observed in the anterior pituitary of birds which were implanted with pellets containing the higher doses of BrdU (100mg and 200mg), after prolonged incubation of sections with the DAB substrate (Fig. 6.4). The immunolabelling was localised in the nucleus, and no specific labelling occurred in the cytoplasm.

The BrdU-labelled nuclei occurred in cells in the cephalic and caudal lobe of the anterior pituitary gland (Fig. 6.5), in both incubating and laying hens. There was no suggestion that BrdU-labelled cells in broody hens were more abundant than in laying hens. Attempts to co-localise the BrdU-labelled nuclei in the pituitary hormone secreting cells, using a double immunocytochemical procedure were not successful, and the identity of the mitotic cells was not established.

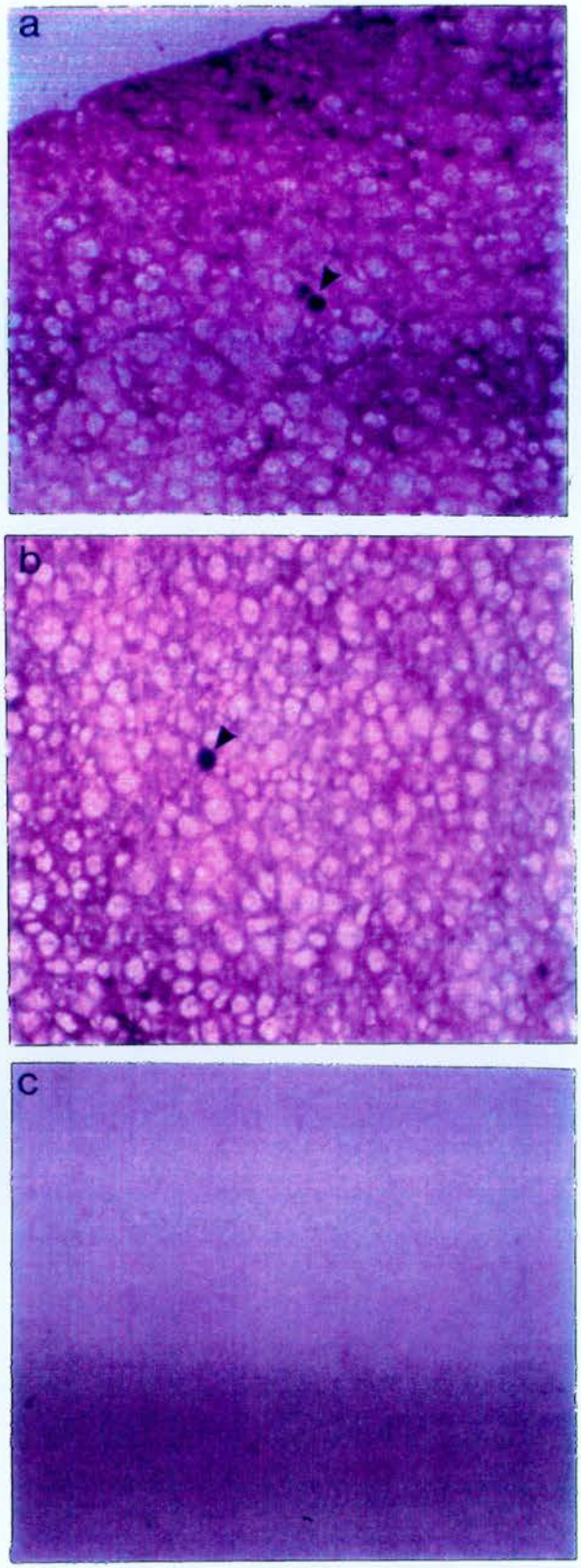

Figure 6.4 The immunolabelling of BrdU cells in the anterior pituitary of a hen which was implanted with a (a) 100, (b) 200mg slow release BrdU pellet, and (c) sham operated .

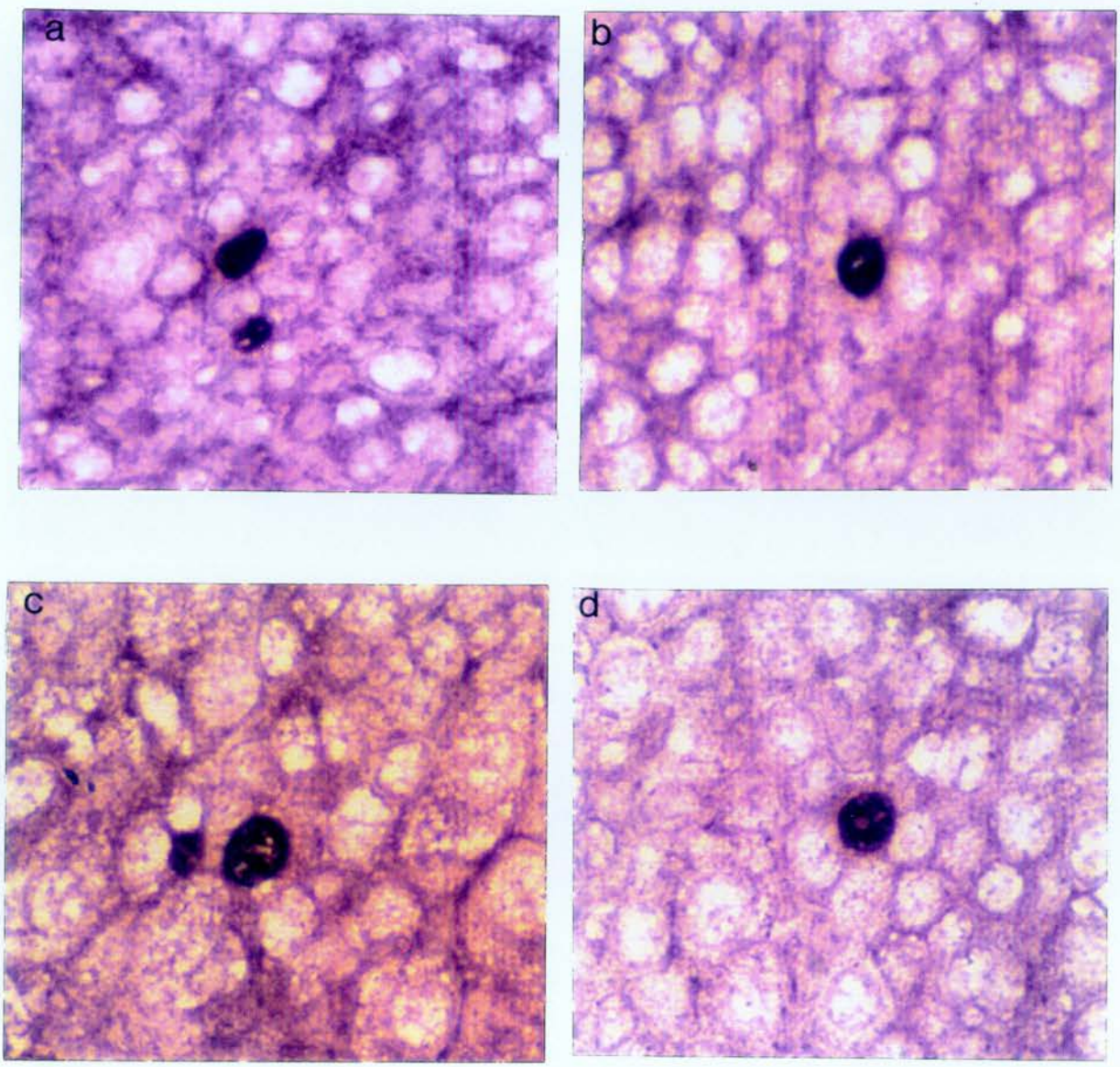

Figure 6.5 The distribution of BrdU-labelled cells within the chicken anterior pituitary gland. The immunolabelled cells were found in the cephalic (a,c) and (b,d) caudal lobes of both (a,b) laying and (c,d) broody hens.

6.3 Discussion

This experiment demonstrated that BrdU immunocytochemistry can be used to detect cell replication within the chicken anterior pituitary gland. The slow release BrdU pellets were designed to chronically deliver a dose of BrdU over a 21 day period. This experiment also showed that the dose of BrdU released by the pellets was critical, and affected the detection of BrdU-labelled cells in the blood and anterior pituitary. Proliferating cells in the anterior pituitary gland were only observed when implanted with a 100 or 200mg BrdU pellet.

Immunocytochemical labelling with BrdU *in vivo* and *in vitro*, is widely used to study cell proliferation, including cultured anterior pituitary cells (Carbajo-Peres *et al.*, 1990; Carbajo *et al.*, 1992). The denaturation of the DNA, and the fixation of the sample (Dolbeare *et al.*, 1985; Moran *et al.*, 1985; Hayashi *et al.*, 1988) are important parameters which affect the detection of BrdU incorporated into a tissue sample. Factors which affect the incorporation of BrdU into the S-phase nuclei are also important. For example, the concentration and period of exposure to BrdU influences the detection of cellular proliferation. The amount of BrdU needed for the detection of BrdU-labelled cells in tissue sections is greater (Carbajo *et al.*, 1995) than that required to detect BrdU-labelled cells in single cell suspensions by flow cytometry (FCM) (Dolbeare *et al.*, 1983). Therefore, the detection of BrdU-labelled cells in the chicken anterior pituitary gland may be an underestimation, and a consequence of inadequate doses of BrdU administered to the birds.

The dose of BrdU used to detect cell proliferation *in vivo*, varies greatly in the literature and ranges from 50 to 200mg/kg body weight (Dolbeare *et al.*, 1983; De Fazio *et al.*, 1987; Carbajo-Perez and Watanabe, 1990). The use of high concentrations of BrdU did not appear to cause an adverse reaction, even though it is reported to be mitogenic (Davidson *et al.*, 1988; Kaufman, 1988) and carcinogenic in rats (Napalkov *et al.*, 1989), and to inhibit cell differentiation and DNA synthesis (Chwalinski *et al.*, 1988; Morris *et al.*, 1989).

Since the number of lactotrophs in the pituitary of the incubating hen is much greater than in the laying hen, it was predicted that there might be a greater number of proliferating prolactin cells in the anterior pituitary of the incubating hen. This experiment indicates that there is no obvious difference in the number of mitotic cells in the anterior pituitary gland of the broody and laying bantam hens. It seems

therefore likely that another cell type may differentiate into a lactotroph during the induction of incubation behaviour, which would account for the increase in the number of PRL producing cells.

Although it is generally accepted that fully differentiated cells rarely undergo mitotic division, differentiated (granulated) cells in the male rat anterior pituitary gland have been observed to undergo cell replication (Zambrano and Deis, 1970; Kurosumi, 1971). It is not known which cell type differentiates into the pituitary hormone secreting cell. It might be mitotic (18%) cells identified in the rat anterior pituitary which do not contain PRL, LH, ACTH, TSH, GH or FSH immunoreactivity, and are referred to as immunonegative (agranular) cells (Oishi *et al.,* 1993). It is suggested that the immunonegative cells may be a folliculo-stellate cell (Oishi *et al.,* 1993), and the number of proliferating immunonegative cells in the rat decreases concomitantly with an increase in the percentage of GH and PRL cells (Shirasawa and Yoshimura, 1982). It is suggested that the FS-cells (Oishi *et al.,* 1993) may play an important role in the proliferation and differentiation in the rat anterior pituitary gland. It is therefore possible that FS-cells may differentiate into PRL cells at the onset of incubation behaviour in the domestic chicken.

Finally, it might be more interesting to establish whether VIP stimulates proliferation *in vitro*. A chicken anterior pituitary cell culture system would produce a controlled environment where VIPs effect on BrdU-incorporation could be established in the presence of a defined steroid environment. Furthermore, this steroid environment could be designed to mimick the steroid milieu of the laying or incubating bantam hen, to determine whether VIP and oestrogen interact to affect pituitary cell proliferation.

Chapter 7: VIP AS A PARACRINE REGULATOR OF ANTERIOR PITUITARY FUNCTION

7.1 Introduction

VIP is the main hypothalamic PRL releasing hormone in birds, (Mauro *et al.,* 1988; 1989; Sharp *et al.,* 1989), but there is little or no evidence showing an effect of VIP or PACAP on the secretion of other chicken pituitary hormones. However in the rat there is evidence that VIP (Section 1.4.4) and PACAP (Section 1.8.2) may regulate the secretion of several pituitary hormones. A specific PACAP receptor occurs in the mammalian anterior pituitary gland, and its affinity for PACAP is significantly greater than that for VIP (Gottschall *et al.,* 1990). In addition, VIP and PACAP are reported to share a similar affinity for the neuroendocrine VIP receptor (VIP-RI/II) (Christophe, 1994) which occurs in the rat anterior pituitary gland. These observations support the view that VIP and PACAP may also play an important role in the regulation of pituitary hormone secretion within the chicken anterior pituitary gland.

Therefore, the effects of VIP and PACAP on pituitary hormone secretion were determined *in vitro*. PACAP's mechanism of action is not fully understood, since it appears to affect pituitary hormone release when added to a perfusion system, but is not effective when applied to dispersed anterior pituitary cell cultures (Miyata *et al.,* 1989). For this reason hemi-pituitaries were used in this study, to maintain the cellular integrity of the tissue which may be important for the action of VIP or PACAP. The anterior pituitary glands were collected from laying and broody bantam hens, and the neural lobe removed. The pituitaries were cut along the midline to form hemi-pituitaries, randomly selected for each experimental group and exposed to VIP or PACAP concentrations ranging from 0.001 to 1000nM.

The incubation medium was replaced after 1hr and again after 24hrs for hormone analysis. These two incubation times were used to distinguish between the immediate releasing effects of VIP/PACAP on pituitary hormone release, from their long-term trophic effects on basal production of pituitary hormones.

7.2 The effect of VIP and PACAP on ACTH secretion

VIP and PACAP had no significant effect on ACTH secretion after 1 or 24 hrs from hemi-pituitaries of laying hens (Fig. 7.1). The release of ACTH in response to VIP [1nM] was suppressed, but was not significantly different from that of the control. VIP at high concentrations also had no effect on ACTH secretion after 1 or 24hrs when incubated with hemi-pituitaries from broody hens (*not shown*).

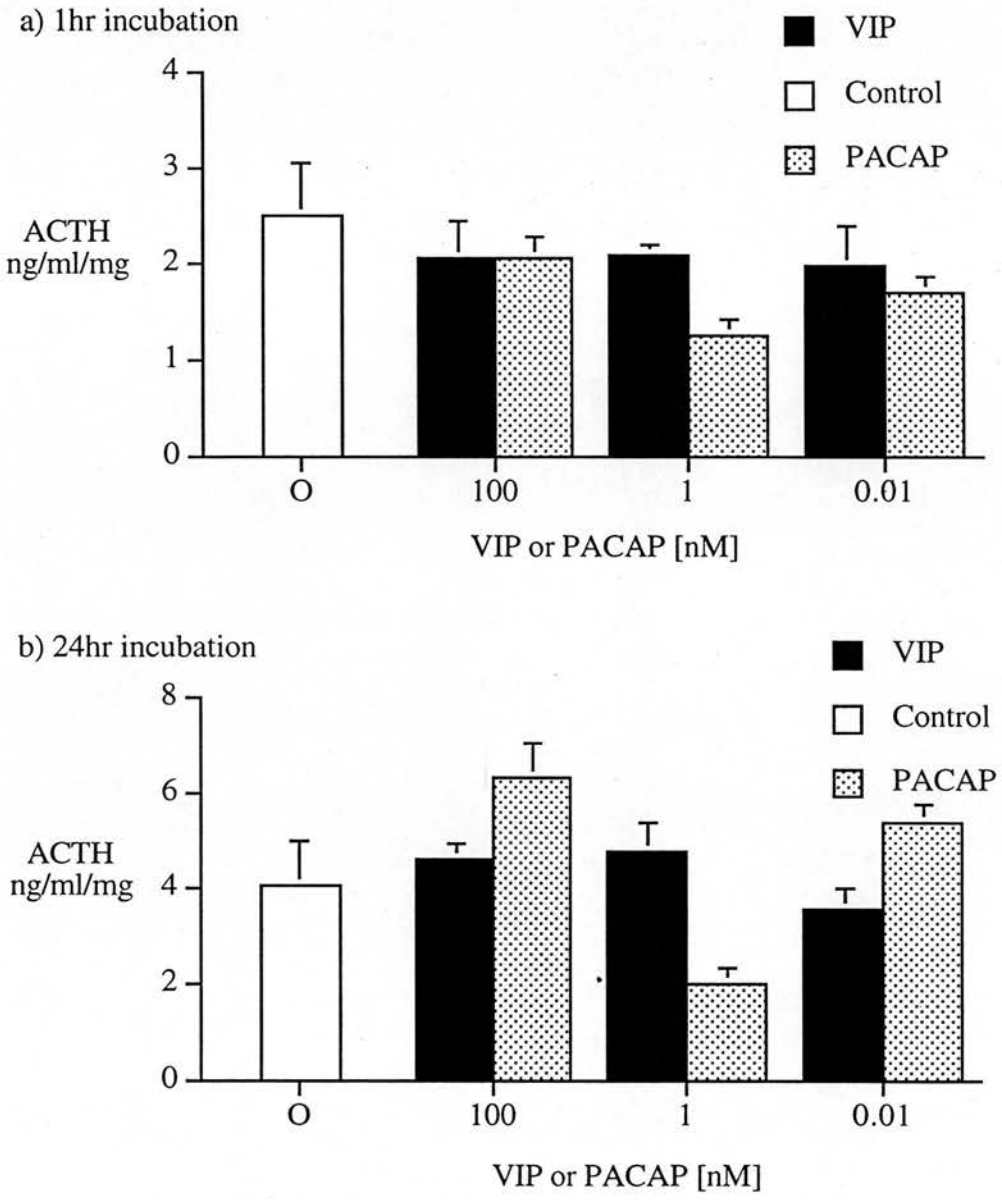

Figure 7.1 The dose response relationship between VIP or PACAP and ACTH release from the hemi-pituitaries of laying hens. Hemi-pituitary glands from laying bantam hens were incubated with either VIP or PACAP for (a) 1 hr and (b) 24 hrs. The bars represent mean ± SEM of 4 replicate wells.

7.3 Effect of VIP and PACAP on GH secretion

VIP and PACAP did not stimulate GH secretion from the hemi-pituitaries of laying hens at high concentrations (1-100nM) during a 1hr incubation (Figure 7.2), and were also ineffective after 24 hrs (Figure 7.2). VIP had no effect on GH secretion from broody hens, regardless of incubation time (PACAP was not studied) (Fig. 7.3).

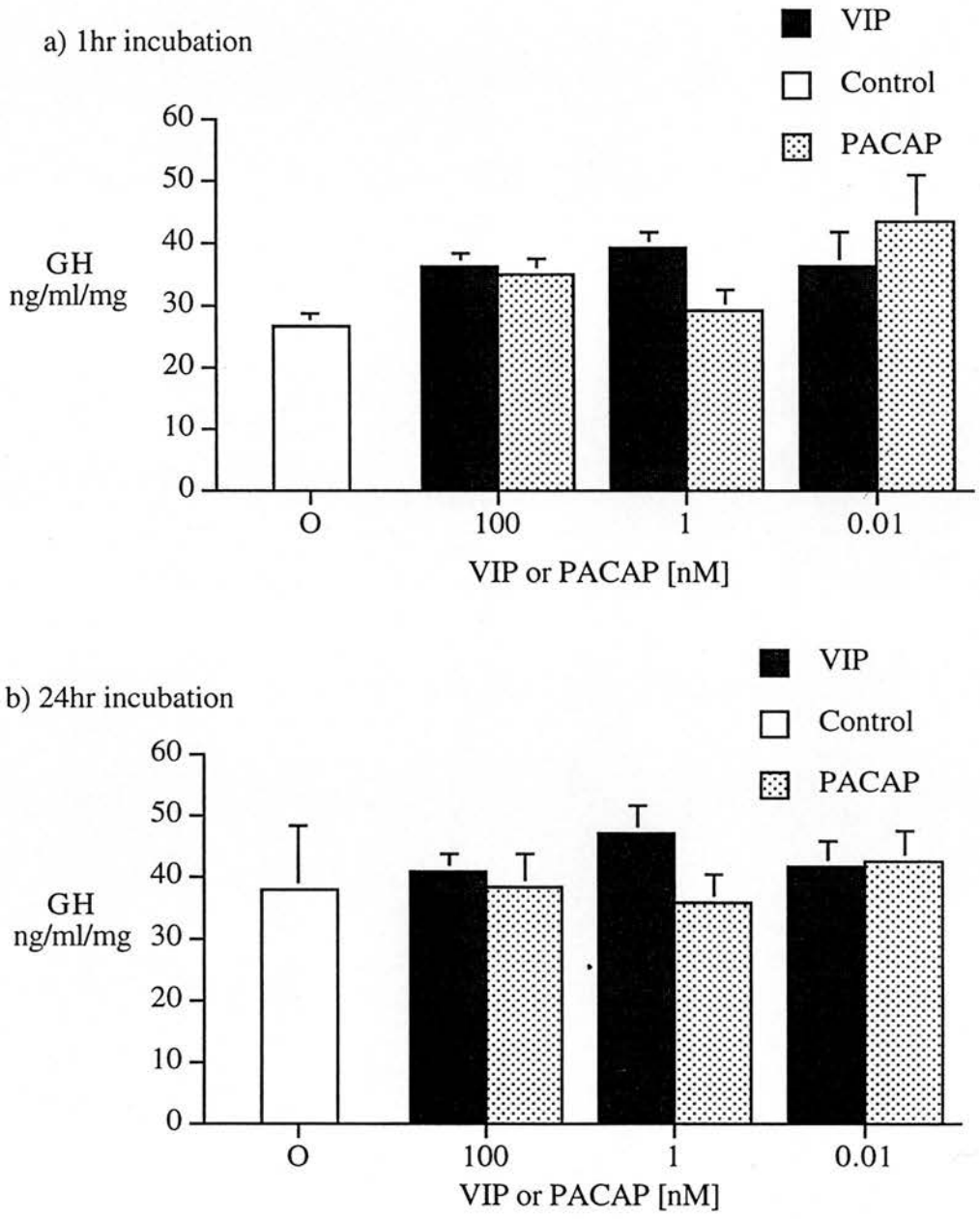

Figure 7.2 The dose response relationship between VIP, PACAP and GH release from the hemi-pituitaries of laying hens. Hemi-pituitary glands from laying bantam hens were incubated with either VIP or PACAP for (a) 1 hr and (b) 24 hrs. The bars represent mean ± SEM of 4 replicate wells.

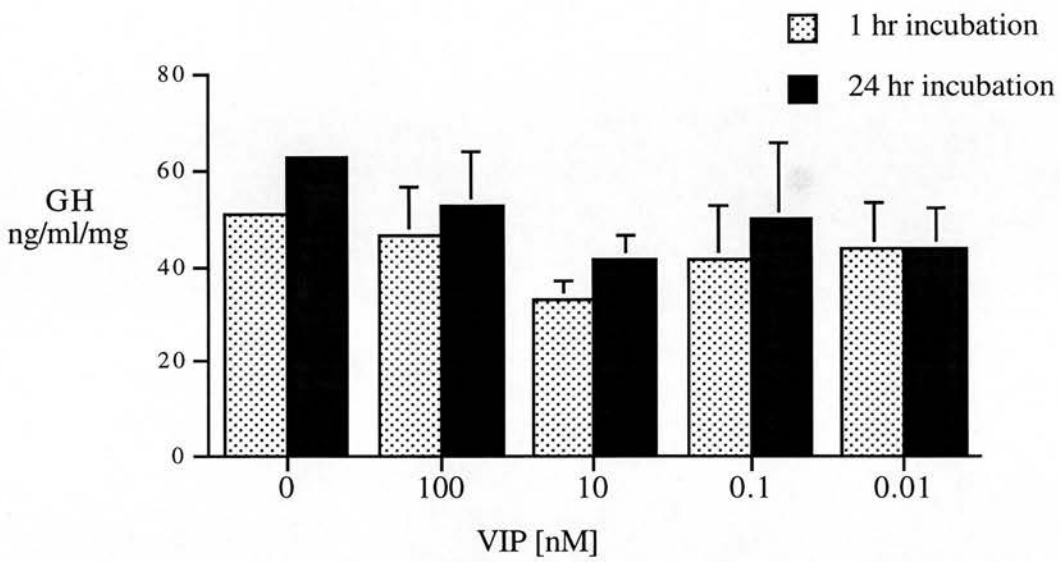

Figure 7.3 The dose response relationship between VIP and GH release from the hemi-pituitaries of broody hens. Hemi-pituitary glands from broody bantam hens were incubated in the presence of VIP, for 1hr and 24hrs. The bars represent mean ± SEM of 4 replicate wells.

7.4 The effect of VIP and PACAP on LH secretion

VIP and PACAP had no significant effect on LH secretion from the hemi-pituitaries of laying hens during a 1hr or 24 hr incubation period (Fig. 7.4). PACAP and VIP were also ineffective on LH secretion from the hemi-pituitaries of broody hens after 1hr (Fig. 7.5). Although, VIP did increase LH secretion from the hemi-pituitary glands of broody hens after a 24hr incubation period, the increase was not significant (P=0.055, ANOVA) (Fig. 7.5).

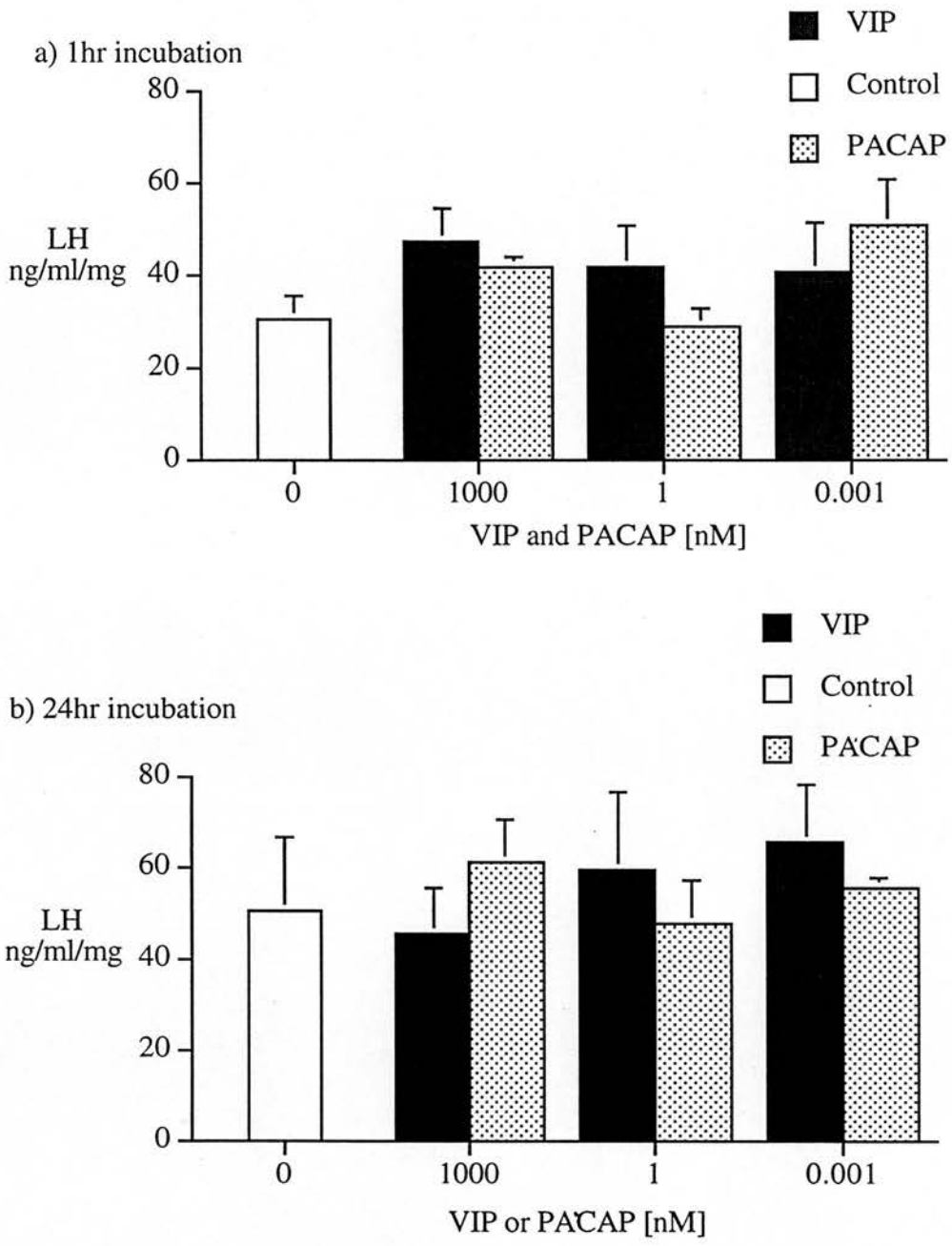

Figure 7.4 The dose response relationship between VIP and PACAP and LH release from hemi-pituitaries from laying hens. Hemi-pituitary glands from laying bantam hens were incubated with either VIP or PACAP for (a) 1 hr and (b) 24 hrs. The bars represent mean ± SEM of 5 replicate wells.

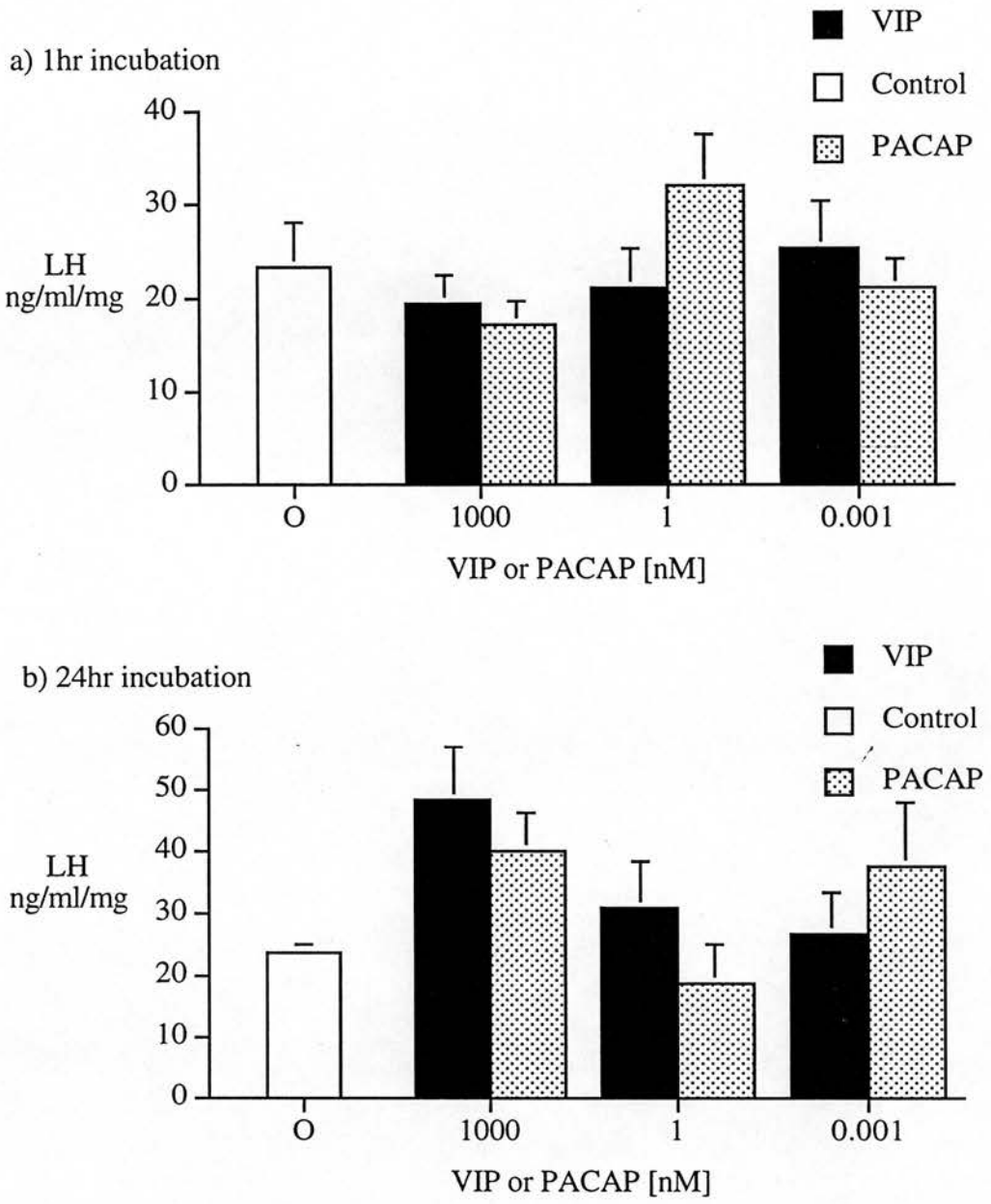

Figure 7.5 The dose response relationship between VIP and PACAP and LH release from hemi-pituitaries from broody hens. Hemi-pituitary glands from laying bantam hens were incubated with either VIP or PACAP for (a) 1 hr and (b) 24 hrs. The bars represent mean ± SEM of 5 replicate wells.

7.5 The effect of VIP and PACAP on PRL secretion

PRL secretion, from the hemi-pituitary glands of both laying and bantam hens was significantly stimulated by both VIP and PACAP. The VIP-stimulated PRL response from the broody hen pituitary glands was greater than that from laying hens (Figure. 7.6). The VIP-stimulated PRL response was increased in a dose

dependent manner, and was 2.7 fold greater in broody than in laying hens. PACAP also stimulated a 1.9 fold increase in PRL release from hemi-pituitaries from broody hens compared to that from laying hens (Fig. 7.6).

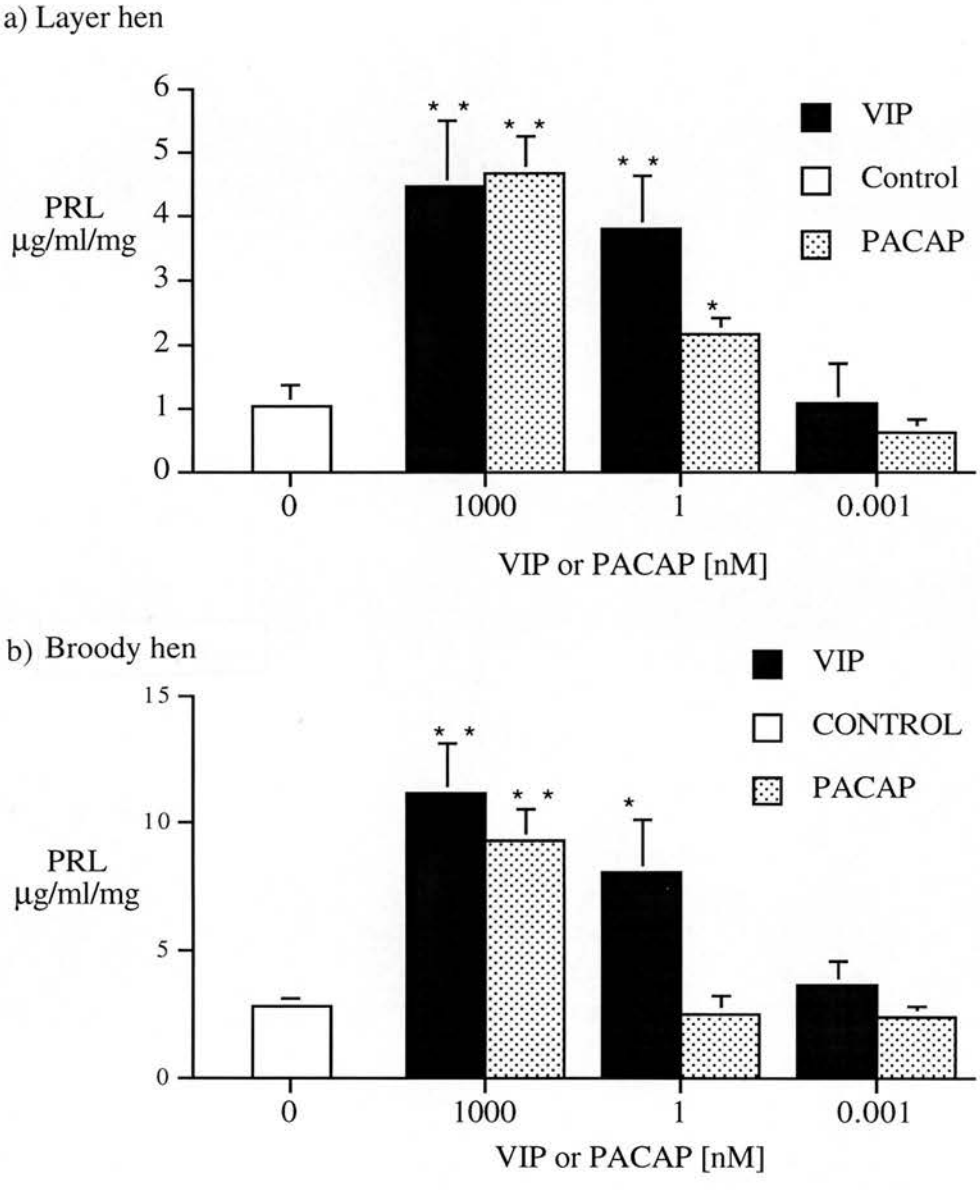

Figure 7.6 The dose response relationship between VIP and PACAP and PRL release from hemi-pituitaries from laying and broody hens. Hemi-pituitary glands from (a) laying and (b) broody bantam hens were incubated with either VIP or PACAP for 1 hr. The bars represent mean ± SEM of 5 replicate wells, *$P<0.05$, ** $P<0.01$.

7.6 Discussion of studies *in vitro*

VIP and PACAP stimulated prolactin secretion from hemi-pituitary glands *in vitro* from laying and incubating hens. VIP and PACAP did not stimulate GH *in vitro* from laying hen pituitaries and LH secretion from laying and incubating hen pituitaries. These results confirm VIP as the main specific prolactin releasing factor in the chicken (Macnamee *et al.,* 1986; Sharp *et al.,* 1989; Talbot *et al.,* 1991). In addition, this experiment suggests that pituitary adenylate cyclase activating polypeptide may also play a role in the regulation of PRL secretion.

The inability of VIP and PACAP to stimulate GH release *in vitro* suggests that these two peptides do not directly regulate the secretion of GH from the anterior pituitary gland. There is evidence that PACAP stimulates GH secretion and GH mRNA in a static incubation of rat anterior pituitary culture which contained a high proportion of GH cells (Velkeniers *et al.,* 1994). However, the reported effects of PACAP on GH release in a static rat anterior pituitary cell culture appear to be time dependent. The maximal effect of PACAP on GH release occurs after 15 min, but prolonged exposure to PACAP desensitises the pituitary (Wei *et al.,* 1993). This observation may also explain why PACAP has been reported to have no effect on GH release from the rat anterior pituitary gland after 3hr incubation (Miyata *et al.,* 1989; Culler and Paschal, 1991). The effects of VIP and PACAP on GH secretion in the chicken anterior pituitary gland are therefore consistent with the effect on GH release in the rat.

The absence of a stimulatory effect of VIP and PACAP on ACTH secretion *in vitro* after long and short term cultures makes it difficult to explain the functional significance of the VIP-RI localised on ACTH cells (Chapter 5). VIP is reported to stimulate ACTH from normal rat anterior pituitary glands, but only at high concentrations (1-3μM) (Westendorf *et al.,* 1983). However, the mouse corticotrophine-like AtT20 cell line and human corticotropinoma cells are responsive to lower doses of VIP, and the effects of co-incubation with VIP and corticotrophin releasing factor (Reisine *et al.,* 1982) or arginine vasopressin (White *et al.,* 1982) are additive to those of CRF or AVP alone. Therefore, VIP and PACAP may modulate the responsiveness of the chicken ACTH cell to releasing factors such as AVP.

This experiment showed that *in vitro* LH release was stimulated from the anterior pituitary gland of the broody hen after a long term exposure to VIP [1000nm]. However, the increase in LH secretion was not significantly different to that of the control value when the Dunnett's test was used following a one-way analysis of variance. This observation if significant may have suggested that VIP released by a pituitary VIP-cell type may modulate pituitary hormone secretion, and VIP may have a trophic action on the gonadotroph.

The absence of a stimulatory effect on LH release from the pituitary gland from laying hens after 24hr incubation with VIP/PACAP suggests that the action of VIP in the chicken anterior pituitary may be mediated by oestrogen *in vivo.* The absence of a stimulatory effect of VIP on LH release from the laying hen pituitary *in vitro*, may be because intra-pituitary VIP is already providing the maximum trophic support to the gonadotroph. Intra-pituitary VIP in the broody hen may be lower because plasma oestrogen concentrations are lower in the broody hen than in the laying hen (Cogger *et al.,* 1979). Long term incubation with VIP may replace the trophic support provided by VIP to the gonadotroph which is normally reduced in the anterior pituitary of the broody hen. In support of this view, a stimulatory interaction between VIP and oestrogen has been demonstrated in the rat. The administration of oestrogen to ovariectomised rats increases the VIP mRNA in the anterior pituitary gland (Lam *et al.,* 1991; Kasper *et al.,* 1992).

In conclusion, VIP and PACAP stimulate prolactin secretion, which is consistent with the role of an avian PRL-releasing factor. VIP and PACAP failed to stimulate ACTH and GH secretion from the hemi-pituitary of a laying hen at very high concentrations. After a 24 hr not a 1 hr incubation, VIP stimulated LH release, but not significantly from pituitary gland from the incubating hen *in vitro*. These observations support the view that VIP may be released in a paracrine manner from the VIP cell in the anterior pituitary and provide trophic support for the neighbouring gonadotroph population.

CHAPTER 8: GENERAL DISCUSSION

It is established that prolactin but not LH secretion in birds is stimulated by the hypothalamic releasing factor, vasoactive intestinal polypeptide (VIP) (see Section 1.5). However, active immunization against VIP suppresses both prolactin and luteinising hormone secretion (Sharp *et al.,* 1993). This observation suggested that VIP may facilitate the function of LH-producing cells. This Thesis provides biochemical, molecular and anatomical evidence that VIP produced as a paracrine secretion by folliculo-stellate-like cells in the anterior pituitary gland, may regulate the function of gonadotrophs (Fig. 8.1).

The presence of VIP within the chicken anterior pituitary gland was demonstrated using high performance liquid chromatography (HPLC), radioimmunoassay and immunocytochemistry. VIP mRNA was also detected in the anterior pituitary, using RT-PCR and solution hybridization RNase protection assay. A subtype-I VIP receptor (VIP-RI) was localised immunocytochemically in the anterior pituitary, and was co-localised in cells containing ACTH but not PRL, LH or GH. The subtype-II VIP receptor (VIP-RII), identified immunocytochemically, was also distributed throughout the anterior pituitary but the specific cell type(s) containing VIP-RII immunoreactivity were not identified. Finally, it was shown *in vitro* that VIP and a VIP-like peptide, pituitary adenylate cyclase activating polypeptide (PACAP) induced a rapid release of prolactin, but not LH, GH or ACTH. After a twenty-four hour incubation with VIP or PACAP, LH but not ACTH secretion was enhanced *in vitro*, from pituitary glands from broody hens.

VIP immunoreactivity was found in a distinct cell type which was distributed throughout both cephalic and caudal lobes of the anterior pituitary gland. One VIP-cell type was stellate, with cytoplasmic projections which extended between neighbouring pituitary hormone cells. The morphological features of this VIP-cell were similar to those of the folliculo-stellate cell (FS-cell), and most contained the Ca2+ binding protein S-100, a specific marker for FS-cells. A second larger VIP-cell type was more intensely labelled, polygonal in shape, and did not appear to contain S-100 protein.

Ultrastructural and histochemical evidence suggests that FS-cells are agranular and do not secrete hormones, but some small granules have been observed in FS-cells

(Vila-Porcile and Olivier, 1984). It is possible that the larger polygonal VIP-cell type in the chicken is not of folliculo-stellate cell origin, since it appears to lack S-100 immunoreactivity. There are a number of likely candidates for the cell type which contains VIP-immunoreactivity. For example, the corticotroph is described as stellate shaped (Nakane, 1970; 1975), and like the chicken pituitary VIP-cell has a characteristic polygonal/stellate shape, and is reported to be found at the centre of a glandular cell cord and forms cytoplasmic projections between neighbouring cells (Siperstein and Miller, 1970). Further, thyrotrophs in the rat anterior pituitary gland are reported to be polygonal in shape, and form clusters at the centre of the gland with cytoplasmic projections (Nakane, 1970; Baker, 1974). However, in the chicken it is unlikely that the VIP occurs exclusively in TSH or ACTH cell types, since these are confined to the cephalic lobes and VIP-immunoreactivity was distributed throughout both lobes of the anterior pituitary gland.

There is no consensus concerning the identity of the pituitary cell type containing VIP in mammals (Section 1.6). In the chicken, the VIP-cell type was distinct from that of lactotrophs and gonadotrophs (Fig. 8.1), which is consistent with the findings in the rat (Lam *et al.,* 1989; Steel *et al.,* 1990; Carrillo and Phelps, 1992). The VIP-cell identified in the chicken was localised in both lobes of the anterior pituitary, as is the gonadotroph, and was seen to envelop a number of adjacent LH cells with cytoplasmic projections. This cell-to-cell relationship suggests that the VIP-cell type may regulate gonadotroph function within the chicken anterior pituitary gland. A similar situation occurs in the rat, where the FS-cell has been suggested to have a 'housekeeping role' for the gonadotroph (Section 1.9.5). This view is supported by the observation that FS-cells closely surround gonadotrophs and not thyrotrophs after gonadectomy (Shirasawa *et al.,* 1983). Furthermore, the regulation of gonadotroph function may be modulated by FS-cells which release the FSH inhibiting peptide, follistatin (Gospodarowicz and Lau, 1989).

The RT-PCR and solution hybridisation RNase protection assay confirmed that the chicken anterior pituitary gland contains VIP mRNA. These observations demonstrate that the VIP detected by immunocytochemistry and radioimmunoassay is synthesised by the pituitary, and is not all sequestered from another source. It was not possible to confirm that VIP mRNA occurs only in the FS-cell type, because the *in situ* hybridisation (ISH) technique was not sensitive enough.

It might be possible to increase the amount of VIP mRNA in the anterior pituitary sufficiently for it to be detected by ISH, by endocrine manipulation. For example, oestrogen treatment following ovariectomy (Carrillo and Phelps, 1992), thyroidectomy (Lam *et al.,* 1989) or adrenalectomy (Lam *et al.,* 1992) have been reported to increase the amount of VIP mRNA in the rat anterior pituitary gland. Oestrogen treatment may also increase VIP gene expression in the chicken anterior pituitary gland (Fig. 8.1). This view is supported by the observation that the apparent VIP immunoreactivity in the anterior pituitary of the incubating hen was less than that in the laying hen, and that plasma oestrogen in the incubating hen is lower than in the laying hen (Cogger *et al.,* 1979).

The presence of PACAP could not be demonstrated immunocytochemically in the chicken anterior pituitary gland, using a high titre of an antibody which is specific for mammalian PACAP. This antibody used at low titre labelled cells in the medio-basal hypothalamus, although specificity was not confirmed, because the PACAP antibody cross-reacted with chicken VIP. Although, the chicken brain contains PACAP (Yasuhara *et al.,* 1992), there are no published reports describing the distribution of PACAP immunoreactivity in the chicken hypothalamus or anterior pituitary.

On the basis of these observations, it is likely that PACAP is not present in the chicken anterior pituitary gland, and that intra-pituitary VIP rather than PACAP is a paracrine factor which modulates pituitary hormone cell function. This view is consistent with the observation that PACAP mRNA is not present in the rat anterior pituitary gland (Mikkelsen *et al.,* 1995) or in human pituitary adenomas cells (Vertongen *et al.,* 1995b). However, specific high affinity PACAP binding sites occur in specific cell types in the rat anterior pituitary (Gottschall *et al.,* 1990; Vigh *et al.,* 1991). This suggests that hypothalamic PACAP in the rat, may play an important role in the regulation of anterior pituitary cell function. Further investigation is required to establish the neuroendocrine role of PACAP in birds. A specific chicken PACAP antibody and a chicken PACAP cDNA is needed to establish the presence of PACAP in the hypothalamus and the anterior pituitary. A specific chicken PACAP antiserum would also be valuable for passive immunization studies to determine the effect of PACAP on the secretion of pituitary hormones.

A specific VIP-R occurs in the lactotroph of the rat anterior pituitary gland (Wanke and Rorstad, 1990a), and it is likely that the stimulatory effect of VIP on PRL secretion and gene expression in the chicken (Talbot *et al.,* 1991) is also mediated by a specific VIP receptor (Gonzales *et al.,* 1994b, c) in the lactotroph. This study used antibodies raised against the subtype-I and subtype-II human VIP, to establish whether VIP receptors occur in lactotrophs and other anterior pituitary cell types. The VIP-RI was found only in ACTH cells, and not in PRL-, LH- or GH-cells (Fig. 8.1). This is consistent with the observation that only small amounts of VIP-RI mRNA occur in the rat pituitary gland (Usdin *et al.,* 1994; Rawlings *et al.,* 1995) and that VIP-RI mRNA occurs in the corticotroph-like AtT20 cell line, but not in the LH-like αT3-1 or PRL/GH-like GH_4C_1 cell lines (Rawlings *et al.,* 1995).

VIP-RII immunoreactivity was localised throughout both lobes of the chicken anterior pituitary gland, but the VIP-RII antibody did not clearly identify individual cells. It is predicted that there should be significant homology between the chicken high affinity VIP-R and the human VIP-RII for the antibody to recognise a chicken subtype-II-like VIP receptor. This view was based on the observation that the amino acid sequences of the human and rat VIP-RII, share approximately 87% sequence homology (Xia *et al.,* 1996), and it is predicted that a similar homology should occur between the chicken and mammalian VIP-RII receptors.

The stimulation of PRL secretion by VIP *in vitro* was expected, since it is established that VIP is the hypothalamic prolactin releasing factor in birds. The effect of PACAP on PRL secretion was not unexpected, because of the high degree of structural homology between cVIP (Nilsson. 1974) and cPACAP (Yasuhara *et al.,* 1993). It is likely that VIP and PACAP mediate their effects through the same receptor. The major neuroendocrine VIP receptor localised in the rat anterior pituitary is the VIP-RII (Lutz *et al.,* 1993; Usdin *et al.,* 1994), which also shares an equal affinity for VIP and PACAP and occurs principally in lactotrophs (Vertongen *et al.,* 1995b). The results of this study are consistent with the view that a specific VIP binding site in the avian anterior pituitary gland (Rozenboim and El Halawani, 1993; Gonzales *et al.,* 1994a), mediates the prolactin-releasing action of VIP and PACAP.

The release of ACTH was not stimulated by VIP or PACAP. This observation is consistent with the view that ACTH release from the rat anterior pituitary gland (*in*

vitro) is not stimulated by VIP, except at high micromolar concentrations (Westendorf *et al.,* 1983). Further, PACAP does not modify ACTH release in the rat *in vitro*, (Miyata *et al.,* 1989) in combination with the ACTH releasing factor, CRF (Culler and Paschall, 1991). Since VIP and PACAP do not appear to affect ACTH secretion directly the functional significance of the VIP-RI found exclusively on ACTH cells is uncertain.

Luteinising hormone release was stimulated *in vitro* from the hemi-pituitary glands of broody hens, by both VIP and PACAP after long (24hr) but not short term incubation (1hr). This observation may support the view that the VIP-cells identified in this study, may release VIP to modulate the function of gonadotrophs in the chicken anterior pituitary gland. The close association between VIP- and LH-cells, allows cell-to-cell communication (Fig. 8.1), and suggests a route by which paracrine factors produced in the VIP-cell, could control the function of the LH cell. These results are consistent with the hypothesis suggesting that VIP may act as a paracrine factor, providing trophic support for the gonadotroph.

VIP did not affect LH release *in vitro* from chicken hemi-pituitary glands, after a 1 or 24hr incubation. LH secretion from the broody hen hemi-pituitary was enhanced but not significantly, suggesting that the stimulatory effect of VIP on LH secretion may depend on the physiological state of the bird. In support of this view, in the ovariectomised rat, VIP mRNA is increased by oestrogen treatment (Lam *et al.,* 1991; Kasper *et al.,* 1992), and oestrogen may have a similar effect on pituitary VIP gene expression in the chicken. In the laying hen plasma oestrogen concentration is higher than in the broody hen (Cogger *et al.,* 1979). Increased plasma oestrogen may be the factor responsible for the inability of VIP to provide further trophic support *in vitro* to LH cells from laying hens. This view is consistent with the observation that the VIP-immunoreactivity appears greater in the anterior pituitary of a laying than in broody hens. It is therefore possible that in laying hens pituitary VIP supplies maximal trophic support to LH cells and prolonged incubation with VIP *in vitro* does not further stimulate basal LH secretion. In contrast, in the broody hen pituitary, VIP gene expression may be lower as a consequence of lower oestrogen. The long term incubation of hemi-pituitary gland of broody hens with VIP may have mimicked the paracrine action of intra-pituitary VIP and resulted in enhanced basal LH release.

The proposed interaction between oestrogen and VIP in the paracrine control of the gonadotroph, may explain why active immunization of out-of-lay bantam hens on short days depressed plasma LH and delayed the onset of egg laying after photostimulation (Section 1.5.4) (Sharp *et al.,* 1993). While on short days the VIP antibody may attenuate the paracrine action of VIP produced by folliculo-stellate-like cells acting on the gonadotroph, and result in a depressive response to GnRH. After transfer to long days, the initial GnRH induced release of LH might not have been sustained until oestrogen is produced by the ovary, stimulated sufficient VIP synthesis in the VIP folliculo-stellate-type cells to attenuate the immunosuppresive effect of circulating VIP antibody on gonadotroph function.

Intra-pituitary VIP may stimulate LH mRNA synthesis in the gonadotroph, and this would account for the increase in basal LH release from the pituitary of the broody hen *in vitro*. Future studies are necessary to establish whether VIP stimulates LH α– or β-subunit mRNA expression in the chicken anterior pituitary gland. It is reported that PACAP stimulates LH α and β mRNA expression in rat anterior pituitary cells when administered in a pulsitile manner *in vitro* (Tsujii *et al.,* 1994; 1995), and therefore VIP and PACAP may act in a similar manner to affect LH synthesis and secretion in the chicken gonadotroph.

In conclusion, this study shows that vasoactive intestinal polypeptide is produced by the chicken anterior pituitary gland and occurs in a folliculo-stellate cell type. The VIP-cell type is closely associated with pituitary hormone cells, in particular the gonadotroph. Vasoactive intestinal polypeptide also enhances basal LH release from the anterior pituitary gland (*in vitro*) of broody hens after a prolonged incubation. These observations are consistent with the view that in the chicken intra-pituitary may VIP act in a paracrine manner to provide trophic support for the gonadotroph (Fig. 8.1).

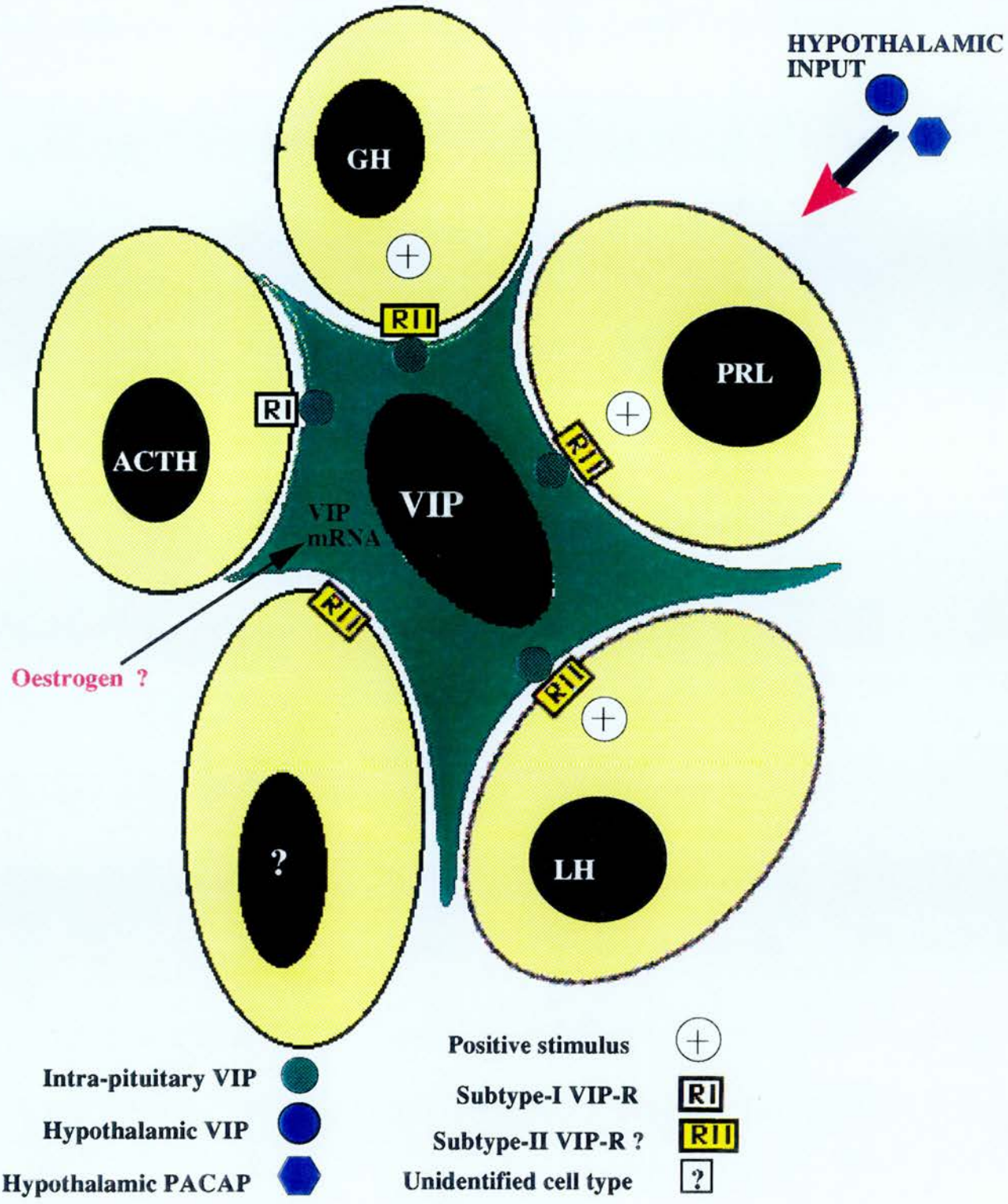

Figure 8.1 A summary diagram showing the proposed interaction between intra-pituitary VIP, and hormone secreting pituitary cell types. VIP occurs in a folliculo-stellate-like cell, which is closely associated with gonadotrophs. VIP may be secreted in a paracrine manner to modulate basal LH secretion.

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