MOLECULAR BIOLOGY AND BIOCHEMISTRY OF BRAIN TUMOURS

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Summary

Elucidating some molecular mechanisms and biochemistry of brain tumours is an important step towards the development of adjuvant medical therapies. The present study concentrates on cholecystokinin (CCK), a gut-brain peptide that has been described to be able to induce mitosis of rat gliomas as well as hormone secretion by the anterior pituitary, via the CCK-B receptor. The significance of a polymorphism in the growth hormone releasing hormone (GHRH) receptor (GHRH-R) gene was also determined. Finally, defects in the β-catenin gene, an important component of the developmental pathway, in a sub-set of craniopharyngiomas were investigated.

Reverse transcription-polymerase chain reaction (RT-PCR), restriction digestion analysis and direct sequencing demonstrated expression of CCK peptide itself and its A and B receptors by human gliomas, meningiomas and pituitary tumours. CCK peptides stimulated growth of cultured gliomas and meningiomas as well as *in vitro* hormone secretion [growth hormone (GH), luteinizing hormone (LH) and follicle stimulating hormone (FSH)] by human pituitary tumours. These biological effects were reduced or abolished by CCK antagonists. In addition, an antibody to CCK reduced mitosis by gliomas and meningiomas, and the same antibody inhibited hormone secretion by cultured human pituitary tumours. CCK peptides stimulated phosphatidylinositol (PI) hydrolysis, indicating coupling of the CCK receptors to phopsholipase C. Cyclic AMP was unaffected. In addition, caspase-3 activity was significantly and markedly increased, whilst proteasome activity was decreased. Taken together, these results may indicate an autocrine / paracrine role of CCK in the control of growth and / or functioning of gliomas, meningiomas and pituitary tumours.

Primer induced restriction analysis (PIRA) of a rarer and alternative polymorphism in the GHRH-R receptor, in which Thr replaces Ala at codon 57, in human GH-secreting pituitary tumours was investigated. Whilst the rarer form correlated with an increased response of the pituitary cells to GHRH *in vitro*, allele distribution studies revealed that it is unlikely that the polymorphism contributes to increased risk of developing GH-secreting tumours and therefore acromegaly.

Further findings of this study, using PCR and direct sequencing, were the demonstration of an association between β-catenin gene alterations and craniopharyngiomas of the adamantinomatous type. Since this gene product is involved with development, these results suggest that β-catenin mutations may contribute to the initiation and subsequent growth of congenital adamantinomatous craniopharyngiomas.

Keywords: cholecystokinin (CCK), CCK antagonists, U-87 MG, Growth hormone releasing hormone receptor (GHRH-R), β-catenin.

DEDICATION

To my beloved Father, a great Doctor, who has influenced my so far progress and inspired my decisions.

He will be forever missed but always remembered.

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ABBREVIATIONS

Abs	Absorbance
AMP	Adenosine monophosphate
AMV	Avian myeloblastosis virus
APC	Tumourtous polyposis coli
AR	Androgen receptor
ATP	Adenosine triphosphate
bp	Nucleotide base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CCK	Cholecystokinin
CCK-8s	Cholecystokinin-8 sulphate
CCK-A-R	Cholecystokinin A receptor subtype
CCK-B-R	Cholecystokinin B receptor subtype
cDNA	Complementary deoxyribonucleic acid
CKI	Casein kinase I
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
Dvl	Disheveled
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GHRH-R	Growth hormone releasing hormone receptor
G_i	G protein inhibitory
GPCRs	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase-3β
$G_s\alpha$	α subunit of the stimulatory G protein
GTP	Guanosine triphosphate
H_2SO_4	Sulfuric acid
HB	Homogenising buffer
HCl	Hydrochloride
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HUMARA	Human androgen-receptor
IC	Chloroform:isoamylalcohol
IGF	Insulin-like growth factor
IP	Inositol phosphate
IP_2	Inositol biphosphate
IP_3	Inositol triphosphate
KOH	Potassium hydroxide

LB	Lysis buffer
LEF / TCF	Lymphoid enhancer factor / T-cell-factor
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LiCl	Lithium chloride
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
OD	Optical density
oligo-dT	Oligo-deoxythymidine
PBS	Phosphate buffered saline
PBS-PSF	Phosphate buffered saline-penicillin-streptomycin-fungizone
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Phosphatidyl inositol
PIC	Phenol:chloroform:isoamylalcohol
PIRA	Primer-induced restriction analysis
PKC	Protein kinase C
PRL	Prolactin
RFLP	Restriction fragment length polymorphism
RIA	Radio immunoassay
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction

STR	Short tandem repeat
SD	Standard deviation
SDS	Sodium dodecyl lauryl sulfate
SFCS	Striped fetal calf serum
SMEM	Supplemented minimum essential medium
SSCP	Single stranded conformational polymorphism
ssDNA	Single stranded deoxyribonucleic acid
Taq	DNA polymerase from *Thermus aquaticus*
TBE	Tris-boric acid-ethylenediaminetetraacetic acid
TBP	TATA-box-binding protein
TE	Tris-ethylenediaminetetraacetic acid
TMB	3,3',5,'-tetramethylbenzidine
TSGs	Tumour suppressor genes
TSH	Thyroid stimulating hormone
VP-16	Etoposide

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CHAPTER ONE

INTRODUCTION

1.1 BRAIN TUMOURS

In broad terms, brain tumours can be divided into those of the parenchyma or other sustentation tissues (e.g. gliomas and meningiomas) and those of the hypothalamic region, such as craniopharyngiomas and pituitary tumours. The former are sustentation tissue tumours and cause serious health problems because of pressure effects on surrounding normal tissue. The latter, however, tend to be of epithelial cell origin and are often associated with endocrine defects either because of direct involvement of hormone producing cells (most pituitary tumours) or because of secondary defects due to disturbance of hypothalamic function (some pituitary tumours and craniopharyngiomas) (Walker *et al.*, 1985). Primary intracranial tumours can originate from brain parenchyma (intra-axial) or from 'outside' the brain parenchyma (extra-axial) such as those developing from the meninges. Tumours originating from glial cells (gliomas) are the most frequent intra-axial brain neoplasm. Among gliomas, approximately 60% of the glial tumours are represented by astrocytomas. Primary brain tumours represent approximately 2% of all different forms of cancer, being far less frequent than other forms of cancer in adults, while ranking second in incident after leukaemias in paediatric patients (Walker *et al.*, 1985). Metastasis of brain tumours occurs in 20-40% of all central nervous system (CNS) tumours (Bentson *et al.*, 1988).

Compared to extra-cranial tumours, brain tumours display markedly different biological characteristics. The traditional distinction between benign and malignant form has a limited value in the brain since histologically benign tumours can be fatal, by causing intracranial hypertension and compression of critical structures. In addition, histologically malignant brain tumours only rarely metastasise to extra-

cranial structures. Primary brain tumours present a high incidence of recurrence after initial surgical removal and may show progressive dedifferentiation in the course of the disease, with progression from benign to malignant forms (McComb and Bigner, 1984; Russel and Moss, 1986). The local growth of brain tumours is also of relevance since they tend to infiltrate the surrounding normal tissue, therefore limiting the possibility of radical resection and increasing the likelihood of recurrence after treatment. Among extra-axial brain tumours, meningiomas, similarly to gliomas, arise from arachnoidal cells in the meninges, especially in areas of the arachnoid villi and rarely metastasise but tend to compress locally. However, the aggressive type will infiltrate nearby structures and recur (20%) after surgery (McComb and Bigner, 1984).

Of all intracranial tumours, the most frequent are gliomas (50%), followed by meningiomas (15%), and acoustic nerve schwannomas (5-10%). Despite a wide variety of therapeutic options, including neurosurgical removal (the standard treatment), chemotherapy and radiation treatment (Prados *et al.*, 1996), survival rates of patients are poor (Walker *et al.*, 1985). This is often due to the inaccessible location and the aggressive nature of many brain tumours making successful treatment very difficult. Very often it is impossible to remove all of the neoplastic tissue and, despite postoperative chemo- or radiotherapy, there is tumour recurrence. These problems are particularly associated with gliomas, craniopharyngiomas and some types of pituitary tumour. In addition, gliomas, pituitary tumours and craniopharyngiomas, although all brain tumours, and primarily treated via neurosurgical removal (via the transcranial or transsphenoidal routes), present differing problems because of their differing locations, differing cellular

composition and variable clinical effects. Selection of treatment is presently based mainly on the definition of tumour extension, as defined with morphologic imaging techniques, and on histological examinations and histologic "grading", usually on tissue specimens obtained with stereotactic biopsy by appropriate selection of sites and possibly multiple samples from different tumour areas (Mosskin *et al.*, 1987; Burger and Kleihues, 1989). Because they are difficult to treat surgically it would be desirable to have available adjuvant medical therapies. The present research has thus been directed at elucidation of the underlying molecular and biochemical defects and growth control mechanisms which could account for development of primary malignant human gliomas, meningiomas, craniopharyngiomas and pituitary tumours. This attempt was carried out in collaboration with leading neurosurgeons and neurosurgical departments within the UK, Germany and Brazil, with whose help and present findings we aim to improve quality of life of patients suffering these brain tumours. Adjuvant or alternative therapies can be developed on the basis of elucidation of the factors which control tumour growth and function. Because of their differing nature, it must be predicted that differing optimal medical therapies could be developed, depending on tumour type.

The studies have focused mainly on the 'gut-brain' peptide, cholecystokinin (CCK) and the demonstration that it possibly comprises an internal system through which growth of gliomas and meningiomas and hormone secretion by pituitary tumours may be under autocrine / paracrine control. Additional studies show that defects in the β-catenin gene and its associated developmental pathway may be a cause of a sub-set of craniopharyngiomas. Finally, the significance of a polymorphism in the

growth hormone releasing hormone (GHRH) receptor (GHRH-R) gene was investigated and herein reported upon.

CHAPTER TWO

CCK & BRAIN

TUMOURS OF THE BRAIN

PARENCHYMA & MENINGES

2.1 INTRODUCTION

The conventional definition of "brain tumours" includes neoplasms originating from the brain parenchyma (gliomas), as well as from meninges (meningiomas) the hypothalamic region and of the osseous intracranial structures that can indirectly affect brain tissue (Zulch, 1979; Russel and Rubinstein, 1989; Kleihues *et al.*, 1993). Gliomas are tumours originating from supporting glial cells and are the most frequent intra-axial brain neoplasm. Glial cells include astrocytes, oligodendrocytes and ependymal cells, so gliomas can be astrocytomas, oligodendrogliomas, or ependymomas (McComb and Bigner, 1984). Approximately 60% of gliomas are represented by astrocytomas. Among extra-axial brain tumours, meningiomas, similarly to gliomas, rarely metastasise and tend to compress locally. However, the aggressive type will infiltrate nearby structures and recur after surgery (McComb and Bigner, 1984; Russel and Moss, 1986). Meningiomas account for 20% of intracranial neoplasms and originate from arachnoid cap cells of the meninges. Although more than 90% of meningiomas are pathologically benign, the tumour is often uncontrollable because of its location at the skull base or the involvement of cranial nerves or crucial vessels.

Gliomas are very serious brain tumours with life expectancy after diagnosis averaging only nine months. Conventional therapy involves neurosurgical removal via the transcranial route followed by radiotherapy and / or chemotherapy. Nevertheless, their inaccessible location and / or aggressive and recurrent nature often hinder complete removal by neurological procedures and it is tumour recurrence which leads to mortality. Because of this, research has centered on developing novel therapies. One approach has been the design of gene therapy

procedures in which a 'suicide' gene or a lethal virus is introduced into the glioma cells (McKie *et al.*, 1996; Rainov and Kramm, 2001). Unfortunately, the success rate by these approaches has been disappointing due to poor uptake by the neoplastic cells of the vehicles used. Another approach has been to decipher which factors may influence glioma cell growth and develop drugs or therapies which may interrupt these factors. For example, it is now well established that some gliomas produce growth factors and antagonists have proved to have an inhibitory effect on cell proliferation (Glick *et al.*, 1997).

Nevertheless, to date, the growth regulating mechanisms of meningiomas and gliomas are still far from being fully elucidated. Although early studies were confined to those related to gender-specific steroids, recent findings suggest that peptide growth factors are more centrally involved in tumour cell growth. Various growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1, and tumour growth factor-α are mitogenic to meningioma (Westphal and Herrmann, 1986; Weisman *et al.*, 1986; Kurihara *et al.*, 1989; Koper *et al.*, 1991; Nitta *et al.*, 1991), and glioma cells (Nagane *et al.*, 1996; Tang *et al.*, 1997). Some studies have proven the actual secretion of these peptide growth factors that may act in an autocrine / paracrine mechanisms and be important in tumourigenesis (Kaplan *et al.*, 1982; Sporn and Roberts, 1985; Goustin *et al.*, 1986).

In a similar approach, animal models have shown a potential role for brain peptides in controlling glioma growth suggesting that antagonists to these peptides may prove to be of benefit to patients. One of these peptides, CCK, is able to powerfully

stimulate growth of rat glioma cells *in vitro*, (Kaufmann *et al.*, 1998). It might therefore be anticipated that blockade of CCK receptors will have an inhibitory effect on glioma cell mitosis. In this chapter, results of similar experiments on human gliomas and meningiomas are reported. Both primary glioma and meningioma cells as well as a human glioma cell line were tested for their ability to express CCK and CCK receptors. Experiments using an anti-CCK antibody and CCK antagonists were designed to determine the possible significance of CCK and CCK receptor expression.

2.2 MATERIALS AND METHODS

2.2.1 Materials – Tissue Culture

Affinity Research Products Ltd. Exeter, UK, Lactacystin

Amersham Biosciences, Bucks, UK, [methyl-^{3}H]thymidine, Anionic Exchange Columns, cAMP Biotrak Enzymeimmunoassay (EIA) System, Myo-[2-^{3}H]inositol

AperTech, UK, Zapocyte

Aston University, UK, 7.4.5 CCK Antagonist, HSH CCK Antagonist

Bachem, Meyerside, UK, Cholecystokinin Octapeptide (sulfated) (human), Cholecystokinin-33 (human), Epidermal Growth Factor (EGF)

BD Biosciences, US, Ac-DEVD-AMC, Caspase-3 (CPP32) Fluorogenic Substrate, Z-VAD-FMK, General Caspase Inhibitor

Bio-Rad Laboratories GmBH, Munich, Germany, Bio-Rad Protein Assay, Bovine Serum Albumin (BSA), Dowex

Boerhinger, Germany, Collagenase

Coulter Electronics BMDH, Beds, UK, 'Isoton II' Electrolyte Solution, Coulter Counter ZM

European Collection of Cell Cultures, Salisbury, UK, U-87 MG Human Glioblastoma Astrocytoma

Feather, Japan, Scalpels (sterile)

Greiner, Germany, Petri Dish, Screw-Cap Centrifuge Tubes (15-50ml)

Invitrogen Life Technologies, Paisley, UK, Foetal Calf Serum (FCS) Heat Inactivated, Fungizone with Amphotericin B (250µg/ml), L-Glutamine 200MM (100X), Minimum Essential Medium (MEM)-Eagle (10X) with Hank's salts, without L-glutamine and Sodium Bicarbonate ($NaHCO_3$), Multiwell Tissue Culture Plates, Non-Essential Amino Acids (NEAAs) (100X), Penicillin (10.000units/ml)-Streptomycin (10.000µg/ml), Sodium Bicarbonate 7.5%, Trizol Reagent, Trypsin 2.5% (1:250)

Jencons Scientific Ltd., Bedfordshire, UK, Ultrasonic Processor

Minisart Sartorious, Goettingen, Germany, 0.2 / 0.5µm Syringe Filters

ML Laboratories PLC, London, UK, CCK-B Crud Substance (L365, 260), Devacade Drug Substance (L364 718 000 D023)

OMNI international, Warrenton, USA, Electric Tissue Grinder

Oxoid, Hampshire, UK, Phosphate Buffered Saline (PBS) Tablets

Packard Instrument Company, Meriden, USA, Liquid Scintillation Analyzer

Perkin Elmer, Life Sciences, Boston, USA, Ptiphase HiSafe 3 General Purpose Cocktail for Aqueous Samples

Sigma-Aldrich Company, Stirling, UK, 4-(2-hydroxyethyl)1-piperzine ethane sulfonic acid (HEPES) Free Acid, Acetic Acid, Brilliant Blue, Bromophenol Blue, Charcoal, Activated, Acid Washed, Cold Inositol (unlabeled), Dextran, Diethylpyrocarbonate (DTT), Dimethyl Sulphoxide (DMSO), Ethanol, Ethylenediaminetetraacetic acid (EDTA), Etoposide (VP-16), Formic Acid

Ammonium, Glycin, Hydrochloric Acid, Isopropanol, Lithium Chloride, Magnesium Chloride, N-Succinyl-Leu-Leu-Val-Tyr-Amino Methyl Coumarin Fluorigenic Substrate, Perchloric Acid, Phenylmethylsulfonyl Fluoride, Potassium Chloride, Potassium Hydroxide, Potassium Sodium Tartrate, Sodium Carbonate, Sodium Chloride, Sodium Deoxycholate, Sodium Dodecyl (lauryl) Sulfate (SDS), Sodium Hydroxide, Sodium Orthovanadate, Sodium Phosphate (monobasic), Sodium Pyrophosphate, Sulfuric Acid, Tris, Triton X100

Spectra Max Gemini XS, Molecular Devices, USA, Microplate Fluorometer System

Sterilin, UK, Tissue Culture Flasks

Strasted, UK, Semi-Micro Cuvette (10 x 4mm)

Thermo Labsystems, Vantaa, Finland, Black Microtiter Plate 96 Well

Materials – Molecular Biology

Aston University, UK, 70-1392bp Molecular Weight Markers

Bio-Rad Laboratories GmBH, Munich, Germany, Xylene Cyanol

Fermentas, USA, HinfI, PstI, PvuII

Hettich, USA, Microfuge

Invitrogen Life Technologies, Paisley, UK, Bromophenol Blue, PCR Reagent System, Ultra Pure Agarose Electrophoresis Grade

JeioTech Co. Ltd, Seoul, Korea, Shaking Water Bath (BS-10)

MWG AG Biotech., Germany, Primers

New England BioLabs, USA, BsaJI, FokI

Qiagen, Düsseldorf, Germany, QUAEX II Gel Extraction Kit

Roche, Mannheim, Germany, 1st Strand cDNA Synthesis Kit for RT-PCR (AMV), BSiWI

Sanyo, UK, Freezer Centrifuge

Sigma-Aldrich Company, Stirling, UK, Ammonium Chloride, Boric Acid, Calcium Chloride, Chloroform, Deionised Formamide, Dimethylformamide, Disodium Hydrogenophosphate, Ethidium Bromide Aqueous Solution, Formamide, Glucose, Glycerol, Isoamyl Alcohol, Magnesium Chloride, Magnesium Sulfate, Mineral Oil, Phenol: Chloroform: Isoamyl Alcohol 25:24:1, Potassium Phosphate (monobasic), Sodium Acetate

Strasted, UK, Microfuge Tubes

Stuart Scientific, UK, Vortex

Syngene, UK, UV Transilluminator

Thermo-Dux, Wertheim, Germany, Techne Progene Thermocycler

2.2.2 Buffers and Solutions – Tissue Culture

CCK non-selective antagonist (7.4.5) (1mM) (MW 250), 4mg 7.4.5 dissolved in 16ml DMSO.

CCK-33 (1μM), 0.1mg CCK-33 dissolved in 1.25ml 5% acetic acid, aliquoted in 50μl and stored at -20 C.

CCK-8s (20nM), 1mg CCK-8s dissolved in 2.1ml $NaHCO_3$ (7.5% $^{w}/_{v}$), aliquoted in 50μl and stored at -20 C.

CCK-A antagonist (HSH) (10mM) (MW 390), 11mg HSH dissolved in 2.8ml DMSO.

CCK-A antagonist (L-364,718) (200μM), 80mg CCK-A dissolved in 1ml DMSO.

CCK-B antagonist (L-365,260) (200μM), 80mg CCK-A dissolved in 1ml DMSO.

Charcoal stripping of FCS, 2g activated charcoal and 200mg dextran C were dissolved in 5ml distilled water and 2.5ml of the mixture were added to 200ml of FCS in a

500ml glass bottle. The serum-charcoal mix was shaken at 55 C for 30 minutes in a shaking water bath. The serum was centrifuged for 20 minutes at 3,000 rpm in 50ml centrifuge tubes. After the serum was removed from the charcoal the remaining charcoal-dextran mix was added and the serum was shaken again at 55 C for 30 minutes. The serum was centrifuged until most of the charcoal was removed. The remaining charcoal was removed by filter sterilization through a 1.2μm filter, which was performed twice under the laminar-flow hood. The above procedure removes small molecules such as steroids and inositols as well as small peptide growth factors.

EGF (5μg/vial), 0.2mg EGF dissolved in 50μl of sterile distilled water, aliquoted in 5μl and stored at -20 C.

FCS (10%) Supplemented Minimum Essential Medium (SMEM) (pH 7.5), 25ml of minimum essential medium (MEM) - eagle (10X) with Hank's salts, without L-glutamine and $NaHCO_3$, 400ml sterile distilled water, 50ml FCS*, 5ml NEAAs (100X), 5ml 2M HEPES, 5ml $NaHCO_3$ (7.5% $^{w}/_{v}$), 5ml L-glutamine (2mM), 5ml penicillin (100U/mL) / streptomycin (100μg/mL) solution, the pH was adjusted with 5M NaOH. *No serum was added when serum free SMEM was prepared.

HEPES (2M), 115.2g of HEPES were dissolved in 100ml distilled water. When dissolved the volume was made up with to 200ml with distilled water and autoclaved to sterilise.

PBS:EDTA, 2 BPS tablets and 20mg EDTA were dissolved in 100ml sterile distilled water.

SCFS (5%) SMEM (pH 7.5), 25ml of minimum essential medium (MEM) - eagle (10X) with Hank's salts, without L-glutamine and $NaHCO_3$, 400ml sterile distilled water, 25ml SFCS, 5ml NEAAs (100X), 5ml 2M HEPES, 5ml $NaHCO_3$ (7.5% $^{w}/_{v}$),

5ml L-glutamine (2mM), 5ml penicillin (100U/ml) / streptomycin (100µg/ml) solution, the pH was adjusted with 5M NaOH.

Trypsin, 5ml trypsin (2.5%) dissolved in 45ml PBS:EDTA solution.

Buffers and Solutions – Proteasome Assay

Homogenising Buffer (HB), 100mM Tris-HCl (pH 7.5) 4ml

100mM ATP 2ml

100mM DTT 2ml

50mM $MgCl_2$ 10ml

Made up to 10ml with distilled water, aliquoted in 10ml and stored at -20 C.

Lactacystin (100µM), 100µg lactacystin dissolved in 2.7ml PBS, aliquoted in 100µl and stored at -20 C.

N-Suc-LLVY-AMC (1mM), 1mg N-Suc-LLVY-AMC dissolved in 600µl DMSO. The stock was diluted 1/100 with 100mM Tris-HCl (pH 8.0) to give a working concentration of 1mM.

Buffers and Solutions – Caspase-3 Assay

Ac-DEVD-AMC (20µM), 1mg Ac-DEVD-AMC dissolved in 1ml protease assay buffer, aliquoted in 50µl and stored at -20 C.

Lysis Buffer (LB), 10mM Tris-HCl (pH 7.5), 10mM $NaPO_4$, 130nM NaCl, 1% triton X100 and 10mM $Na_4P_2O_7$ were dissolved in distilled water, filter sterilised thought 0.2µm filter and stored at 4 C.

Protease Assay Buffer, 20mM HEPES (pH 7.5), 10% glycerol and 2mM DTT* were dissolved in distilled water. *30mg DTT dissolved in 1000µl HEPES / glycerol

(200nM DTT)$^{+}$. $^{+}$100µl (200nM DTT) dissolved in 10ml HEPES / glycerol (2mM DTT). DDT was added to the protease buffer just before use.

Z-VAD-FMK (10mM), 1mg Z-VAD-FMK dissolved in 24µl DMSO.

Buffers and Solutions – Molecular Biology

Agarose gel (2%), 10g agarose dissolved in 500ml TBE (1x). 50µl ethidium bromide were also added.

Gel Electrophoresis Loading Dye, 0.1% bromophenol blue, 0.1% xylene cyanole and 40% glycerol were dissolved in 10mM Tris-HCl buffer (pH 8.0).

Molecular Weight Markers (70-1392bp), 60µl pGEM 3Zf(+) vector (300ng/µl) digested with an excess of 12µl HinFI and 18µl fuffer L (10x) and 90µl sterile water. The reaction was incubated for 2 hours to overnight at 37 C and killed at 65 C for 10 minutes.

Primer Preparation, 1ml of sterile distilled water was added to the primer vial and mixed thoroughly. The primer was diluted 1/20 with sterile distilled water and absorbance was read at 260nm. Single stranded DNA concentration (µM) was given by the formula: $\frac{A_{260} \times dilution\ factor \times 60}{no.\ of\ bases\ per\ \mathrm{Pr}imer}$.

TBE Buffer (5x), 0.25M Tris, 0.25M Boric Acid and 5mM EDTA were dissolved in 1L distilled water.

TE Buffer 10nM (pH 7.0), 0.5ml 1M Tris-HCl (pH 7.0) and 0.5ml 0.1M EDTA were dissolved in 49ml sterile distilled water.

Tris-HCl Buffer 10mM (pH 8.0), 0.5ml 1M Tris-HCl buffer (pH 8.0) dissolved in 49.5ml sterile distilled water.

Tris-HCl Buffer 10mM (pH 8.5), 0.5ml 1M Tris-HCl (pH 8.5) dissolved in 49.5ml sterile distilled water.

2.2.3 Methods

To further investigate the potential that CCK may be involved in human glioma and meningioma growth, reverse transcription-polymerase chain reaction (RT-PCR) was used to investigate the presence of CCK-receptor gene expression and CCK peptide itself. A neutralizing antibody against CCK was incorporated into growth experiments to investigate the possible effects of endogenous CCK on glioma cell growth. In parallel the effect of exogenously added CCK on glioma cell growth was assessed and antagonized *in vitro*.

2.2.3.1 Gene Expression

Total RNA Extraction

Surgically resected human gliomas and meningiomas (Department of Neurosurgery, University of Goettingen, Germany) were transferred to a 1.5ml eppendorf and 1ml of Trizol reagent was added. Tissues were homogenized on ice using an electric tissue grinder. For cells grown in monolayer, 1ml of Trizol reagent was added directly in the cell culture flask and lysed cells were scraped into a 1.5ml eppendorf. Homogenized samples were incubated at room temperature for 5 minutes and 200µl of chloroform were added to each sample. The tubes were vortexed for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged, at 3,000 rpm for 15 minutes at 4 C. Following centrifugation, the top aqueous phase was transferred to a fresh 1.5ml eppendorf. The extracted RNA was precipitated using 0.5ml of isopropyl alcohol and incubated at room temperature for 10 minutes, and subsequently centrifuged at 3,000 rpm for 10 minutes at 4 C. The supernatant was removed and the pellet was washed once with 1ml 75% ice-cold ethanol. The samples were vortexed and centrifuged at 3,500 rpm for 5 minutes at 4 C. The

supernatant was removed and tissue RNA was briefly dried for 10 minutes at room temperature. The dry RNA pellet was dissolved in RNAase-free water and its concentration was estimated as follows: A 1:100 dilution of the RNA solution was made and the absorbance was assessed at 260nm and 280nm. The ratio A_{260} / A_{280} should be within the 1.6-1.8 which would indicate that the RNA is free of protein contamination. RNA concentration (μg/ml) was calculated by the formula: $A_{260} \times dilution\ factor \times 40$.

Reverse Transcription (RT)

The extracted RNA (1-5μg) was reverse transcribed into cDNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV). RNA was denatured by incubation for 2 minutes at 95 C, followed by rapid cooling on ice for 5 minutes. Single stranded complementary DNA (cDNA) was synthesized by mixing the denatured RNA (8.2μl) with ribonuclease inhibitor (1μl), reaction buffer (2μl), $MgCl_2$ (4μl), deoxynucleotide triphosphate (dNTP) mix (2μl), oligo-(deoxythymidine) (oligo-dT) primer (2μl), and AMV reverse transcriptase (0.8μl) in a total volume of 20μl. The reaction was incubated for 10 minutes at room temperature, for 1 hour at 42 C, for 5 minutes at 95 C, and 5 minutes on ice.

Primers

RT-PCR and general PCR primers were designed using Primer3 software (Rozen and Skaletsky, 2000) based on the sequence data of the genes available in GenBank (table 1). In each case, the primers were directed against separate exons flanking at least one intron.

Table 1: Sequences of the primers used in RT-PCR and PCR reactions

GenBank accession no.	Forward primer	Reverse primer	Amplicon size (bp)
CCK (AH002739)	5'-AGCTGAGGGTAT CGCAGAGA-3'	5'-TGGGTCCTCTA GGAGGGGTA-3'	229
CCK-A-R (D85606)	5'-TTTGAAGGTGAT TGCTGCTACCTGG-3'	5'-GCTGACTTCTT CTGGCTAGCCTCAA-3'	276
CCK-B-R (D21219)	5'-CCGACCACTGCA GGCACGAGTGTGG-3'	5'-GCTGTCGCTGT CACTGTCGCCGTCA-3'	309

Polymerase Chain Reaction (PCR)

PCR was performed using the PCR reagent system from Invitrogen, UK. For RT-PCR amplification, the corresponding cDNA was added to a 100µl reaction containing 10µl reaction buffer (10x) plus $MgCl_2$, 10µl deoxynucleotide triphosphate (dNTP) mix (2mM), 5µl of each corresponding 20µM sense and antisense amplimers, 0.5µl *Taq* DNA polymerase (5U/µl), 64.5µl of sterile water, and overlaid with 100µl light mineral oil. Each DNA sample was amplified using a Techne Progene thermocycler and hot start conditions were used. Full details of each PCR as stated in table 2. Samples of the reaction products (10µl each) were electrophoresed at a constant 45mA through 2% agarose / TBE gel, (6 x 6 cm), with ethidium bromide (1µg/ml) staining, against molecular weight markers. Gels were visualized on a UV transilluminator. The remaining PCR products were salt-ethanol precipitated, redissolved in 15µl of 10mM tris buffer (pH 8.0) and subsequently run on a 2% agarose gel until the PCR band was well clear of the primers (amplimers). Under UV light, DNA bands were excised and placed into a microfuge tube. DNA bands were purified with a Quiaex II gel extraction kit according to the manufacturer's instructions. Of the resulting 40µl purified PCR DNA, 5µl were mixed

with water and ran on a 2% agarose gel against markers (5µl) to check for presence of DNA and quantify its concentration. Five to 10µl were used for sequencing (Functional Genomics Lab, Birmingham University, UK). Each experiment was done at least two times, including DNA extraction.

Table 2: Optimal PCR condition for RT derived cDNA

Primer	Conditions		Cycles
CCK	94 C	5 minutes	1 cycle
	.....		
	95 C	1 minutes	35 cycles
	53 C	2 minutes	
	95 C	3 minutes	
	.....		
	72 C	3 minutes	1 cycle
	4 C	10 minutes	
CCK-A-R	94 C	5 minutes	1 cycle
	.....		
	95 C	1 minutes	35 cycles
	55 C	2 minutes	
	95 C	3 minutes	
	.....		
	72 C	3 minutes	1 cycle
	4 C	10 minutes	
CCK-B-R	94 C	5 minutes	1 cycle
	.....		
	95 C	1 minutes	35 cycles
	65 C	2 minutes	
	95 C	3 minutes	
	.....		
	72 C	3 minutes	1 cycle
	4 C	10 minutes	

Restriction Enzyme and Direct Sequencing Analyses

Purified PCR DNA (16.5µl) was digested for 2 hours to overnight at 37 C with an excess of the corresponding restriction enzyme (table 3) and buffer in 25µl total volume. The predicted band size after digestion with the appropriate restriction enzyme is summarized in table 3. After digestion, the reaction was terminated by incubating the mixture at 65 C for 10min. Digested DNA was resolved on a 2% gel against molecular weight markers and visualised under the UV light.

Table 3: PCR products cleaved by restriction endonucleases

Primer	Amplicon size (bp)	Restriction Endonucleases	Predicted bands (bp)
CCK	229	PstI	105, 124
CCK-A-R	276	FokI	105, 171
CCK-B-R	309	PstI	12, 129, 168

For sequencing, 3-10ng/µl of purified DNA, as quantified against the DNA ladder, were calculated in amount (µl) and water was added to a final volume of 8µl followed by 2µl of 1.6µM of forward (table 4).

Table 4: Calculated mass (ng) of DNA in each of the bands in the 3200bp DNA ladder (500ng)

Fragment	Base Pairs (bp)	DNA Mass (ng)
1	1392	217
2	517	81
3	454	71
4	398	62
5	244	38
6	70	11

2.2.3.2 *In vitro* Methods

Glioma Cell Line Maintenance

The cell line used for the experiments described herein was the U-87 MG human glioblastoma astrocytoma obtained from Cell Collector Service (Porton Down, UK). All cell culture techniques were carried out aseptically. Cells were stored at -80 C in 10% FCS SMEM and 10% DMSO. Cells were resurrected in 10% FCS SMEM by rapid thawing at 37 C.

Cells were passaged prior to confluency every 5-6 days with re-seeding in 10% FCS SMEM. Briefly the cells were washed in 3ml sterile PBS and incubated for 1-2 minutes in 2ml sterile 0.25% trypsin in PBS / EDTA to disrupt the monolayer. Cells were centrifuged at 1,200 rpm for 3 minutes, resuspended in 10ml of 10% FCS SMEM and subcultured into sterile flasks, multiwell plates or glass tubes, as required, and incubated at 37 C. Cells were counted where necessary using an aliquot on a haemocytometer. The number of cells was counted in the two 1mm corner squares (figure 1). Each square of the haemocytometer with the cover slip in place represents a total volume of $0.1mm^3$. The cell concentration ($x10^4$ cells / ml) was given by the formula: $Average\ Count\ per\ Square \times 10^4$

Primary Cell Culture of Human Glioma and Meningioma Tissue

At operation a portion of each glioma and meningioma (Department of Neurosurgery, University of Goettingen, Germany) was placed into culture medium, transported to the laboratory and processed for cell culture. The freshly resected tissue was washed 2-3 times in PBS supplemented with penicillin (200U/ml), streptomycin (200µg/ml), and fungizone (amphotericin B 5µg/ml) (PBS-PSF). Under sterile conditions, the tissue was placed into a 50ml sterile centrifuge tube, and 20ml of PBS-PSF solution were added. The larger pieces of tissue were allowed to settle by gravity for about 1 minute and as much as possible of the PBS-PSF was carefully removed. The process was repeated until most of the contaminating red blood cells had been removed. The washed tissue was placed into a petri-dish together with a small amount of PBS-PSF (1-2ml). The tissue was divided into small pieces with scalpels, transferred into a 75cm^2 tissue culture flask, and incubated overnight at 37 C with 10ml collagenase (200U/ml) filter sterilized in of 10% FCS SMEM. The dispersed tissues were washed and then transferred to a fresh flask. The cells were allowed to equilibrate and attach in fresh 10% FCS SMEM during the following 24 hours, after which the cells were fed normally with periodic medium changes, until grown to confluence.

Cell Growth Experiments

Experiments were performed on primary glioma and meningioma tissues as well as on the U-87 MG cell line to determine the direct effects of CCK and CCK antagonists on growth. Mitosis was determined by either cell number or [^{3}H]thymidine uptake. For cell counting, 1-2x10^5 cells / flask were distributed into 12-24 25cm^2 tissue culture flasks. Cells were allowed to attach and equilibrate

during the following 24 hours after which medium containing varying doses of CCK peptides, anti-CCK antibody and CCK antagonists were added. In some experiments, the effect of combinations of CCK antagonists with growth factors was also examined. At least three flasks were used for treatments and controls. After growth for 6-12 days, cell numbers in each flask was determined by use of a Coulter Counter. Cells were washed with 3ml of sterile PBS and cells were trypsinised with 1ml of 0.25 % trypsin, prepared in sterile PBS containing 0.02% EDTA. The cells were incubated for 1 minute at 37 C. The flask was lightly tapped on the bench to detach any remaining cells and 1ml 10% FCS SMEM was added to the flasks. The flasks were vigorously shaken to disperse any cell clumps. Cell numbers were calculated by means of Coulter Counter ZM, where 200µl of cell suspension were mixed with 9.8ml of isoton and counted.

For [^{3}H]thymidine uptake, $2x10^4$ cells / well were distributed into 24-well plates and allowed to attach and equilibrate for 24 hours. Medium containing the various treatments were then added to the wells and the cells incubated for three days. At least three wells were used for treatments and controls. After incubation, rate of [^{3}H]thymidine uptake was determined as follows: The cells were labelled with 1µCi of [^{3}H]thymidine (81.2Ci/mM) for 3 hours at 37 C. The medium was then removed and cells were rinsed twice with 3ml of PBS, and lysed by addition of 200µl of 10mM HEPES buffer containing magnesium chloride ($MgCl_2$ 1.5mM) and 50µl zapocyte. Cells lysates were pipetted up and down and 200µl of cell suspension were added in each scintillation vial containing 10ml of scintillation cocktail. Incorporated [^{3}H]thymidine was quantified by scintillation counting.

2.2.3.3 Intracellular Second Messengers

Phosphatidylinositol (PI) Hydrolysis

Assessment of the effect of CCK agonists and antagonists on the rate of PI hydrolysis *in vitro* was investigated. U-87 MG cells were plated into glass cell culture tubes at a concentration of $2x10^5$ cells / tube and left to equilibrate overnight at 37 C. Equilibrated cells were incubated for 24 hours at 37 C in 1.5ml 0.5% FCS SMEM and 5μCi/ml [^{3}H]*myo*-inositiol to prelabel membrane-associated PI. After pre-labelling, cells were washed twice with 2ml serum-free SMEM containing 10mM LiCl and 1mM unlabeled inositol, followed by addition of serum-free SMEM without (controls) or with various combinations of CCK agonists (CCK-8s and CCK-33) and antagonists (L-365,260 and L-364,718). At least three cultures were used for each treatment. Cells were incubated for 15-60 minutes and 1-24 hours in serum free medium at 37 C. Following aspiration of test medium, release of accumulated inositol phosphates was achieved by incubation of each culture with 1ml 3.3% $^v/_v$ perchloric acid to extract the free inositol phosphates (mono-, bis-, and tris-). After 20 minutes incubation at 4 C, the perchloric acid extracts were collected and each mixed with 80μl 10M KOH, followed by centrifugation to remove the precipitates. The supernatants were applied to 1ml dowex anionic exchange columns, each suspended in 2ml of water. Columns were washed in a stepwise manner, first with 2ml of 0.1M formic acid to remove contaminants and then with 2ml of 1M formic acid to remove and IP_1, IP_2 and IP_3. Occasionally, the IP_2 and IP_3 fractions were eluted separately by adding 2ml of 0.5 M ammonium formate (IP_2) first and then 2ml of 1M ammonium formate (IP_3). The radioactive content of the later fractions (0.4ml), containing free inositol phosphates, was determined by

liquid scintillation counting. Fractions (0.4ml) were added in each scintillation vial containing 10ml of scintillation cocktail and counted.

Radioactivity remaining in the membranes was determined by dissolving the extracted cells with 1ml of 1M NaOH for 10 minutes at room temperature, which was subsequently neutralised by 1ml of 1M HCl. Radioactivity was assessed in 0.4ml of sample which were mixed with 10ml of scintillation cocktail and counted. Results are expressed as amount of radioactivity in the free (aqueous) inositol phosphate fractions as a percentage of the total radioactivity (membranes plus free) and are representative of rate of PI hydrolysis.

cAMP Production

U-87 MG cells were plated into glass cell culture tubes at a concentration of $2x10^5$ cells / tube and left to equilibration overnight at 37 C. Equilibrated U-87 MG cells were washed with serum-free supplemented growth medium followed by addition of fresh serum-free supplemented growth medium without (controls) or with CCK-8s (2-200nM) or CCK-33 (1-100nM). At least three cultures were used for each treatment. Cells were incubated at 37 C for 30 minutes, the media were removed, and 1.5ml ice-cold ethanol containing 0.1M HCl was added to each culture. The tubes were vortexed and incubated in the acidified ethanol for 24 hours at -20 C. The extracted cells were again vortexed and dried down under vacuum at 60 C for 1 hour. The cell extracts were reconstituted in assay buffer for cAMP content by using a RIA kit by Amersham Biosciences, according to the manufacturer's instructions.

2.2.3.4 Proteasome and Apoptotic Activity Induced by CCK Antagonists

U-87 MG cells were seeded into 6-well plates at a density of $3x10^5$ cells / 2ml per well and allowed to attach overnight in 10% FCS SMEM. After an overnight equilibration at 37 C, cells were first washed (3ml) and subsequently grown in the presence of 5% SFCS SMEM and varying concentrations of non-peptide antagonists to CCK (L-365,260 and L-364,718) (1-100nM) and etoposide (VP-16) (10ng/mL) for 24 hours. After treatment with CCK antagonists, cells were washed twice with 2ml ice cold PBS and subsequently lysed in 300µl homogenizing buffer. Cells were scraped into 1.5ml microfuge tubes and sonicated on ice, three times for 15 seconds at MHz with 10 seconds intervals. Samples were centrifuged at 15,000 rpm for 10 minutes at 4 C. The recovered supernatant was assayed at 10 and 100µl. To measure proteasome activity, the cell lysates were mixed with 100µl of 1mM N-Suc-LLVY-AMC substrate and incubated in the presence and absence of 10µM lactacystin. The volume of 100µM lactacystin required varied according to the amount of the cell lysate tested each time. Treatments were assayed in duplicate, including a reactant blank and lactacystin controls. The 96-well plate was wrapped in aluminium foil and incubated for 1 hour on ice.

The intensity of fluorescence of each sample was measured by a microplate fluorometer system at 360nm excitatory and 460nm emission wavelengths. All readings were standardized against the protein concentration in each sample. Values were expressed as % of untreated controls. Readings obtained from samples treated with 10µM lactacystin were subtracted from those without to give proteasome specific fluorescence. Specific fluorescence activity in each sample standardized per

protein concentration was given by the formula:

$$\frac{Fluorescence}{[\mathrm{Pr}otein]\times 100} = Fluorescence\ /\ \mu g\ .$$

To measure caspase-3 activity (an indicator of apoptosis), U-87 MG cells were seeded into 6-well plates at a density of $3x10^5$ cells / 2ml per well and allowed to attach overnight in 10% FCS SMEM. After an overnight equilibration at 37 C, cells were first washed (3ml) and subsequently grown in the presence of 5% SFCS SMEM and varying concentrations of non-peptide antagonists to CCK (L-365,260 and L-364,718) (1-100nM) and etoposide (10ng/ml) for 24 hours. After treatment with CCK antagonists, cells were washed twice with 2ml ice cold PBS and subsequently lysed in 0.5ml caspase lysis buffer and incubated for 30 minutes on ice. In a 96-well plate, 1.4µl of 20µM AC-DEVO-AMC substrate were mixed with 10 and 50µl of cell lysate respectively. The total volume per well was made up to 100µl with protease assay buffer. The 96-well plate was wrapped in aluminium foil and incubated for 1-2 hours at 37 C.

The intensity of fluorescence of each sample was measured after 1 hour of incubation and then again after 2 hours, by a microplate fluorometer system, at 380nm excitatory and 440nm emission wavelengths. All readings were standardized against the protein concentration in each sample and subtracted 1 hour from 2 hours readings were plotted.

Protein Concentration

Protein concentrations in cell lysates were determined by means of the Bio-Rad assay using a bovine serum albumin (BSA) standard curve. A top standard of

100µg/ml BSA was doubled diluted down for the purpose of constructing a standard curve. The standard curve ranged from 0.5-25µg/ml and was assessed in a 10x4mm semi-micro cuvette where 20µl of each treatment and BSA standard was mixed with Bio-Rad dye reagent 1:4 diluted with distilled water to a final volume of 1ml. Samples were incubated at room temperature for 10 minutes and absorbance was read at 595nm.

2.2.3.5 Statistical Analysis

All studies were performed in triplicate. The results are given as means ± the standard deviation of the mean (± SD). The statistical significances of all data were determined by t-tests.

2.3 RESULTS

2.3.1 Determination of mRNA Expression for CCK, CCK-A and CCK-B Receptor in Human Gliomas

Experiments were performed on tumour specimens obtained from a total of 14 human gliomas. Histologically, the tumour grades consisted of 8 gliomas, 5 glioblastomas and 1 gliosarcoma. The histological diagnoses were confirmed by a neuropathologist using a portion of the original tumour tissue as shown in table 5. High-grade (glioblastoma) U-87 MG human cell line was also used for the experiments.

All 14 human gliomas as well as the U-87 MG cells were shown by RT-PCR analysis to express mRNA for CCK itself and CCK-B receptor. PCR bands of predicted size were yielded by the cDNA samples, and these bands were cut in the

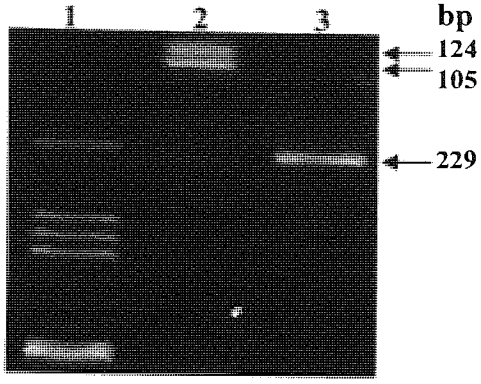
predicted pattern as described in the Materials and Methods. An example is shown in figure 2. By the same analysis, evidence for CCK-A receptor was detected in only 2 of the 14 gliomas.

Table 5: Presence of CCK, CCK-A / B Receptors in Primary Human Gliomas

Sample	Sex	Age	Diagnose	CCK-A	CCK-B	CCK
0			U-87 MG cell line	-	√	√
1	F	48	Glioblastoma	√	√	√
			Cell Culture (P*-6)	√	-	√
2	M	43	Glioma	-	√	√
			Cell Culture (P*-6)	-	-	√
3	F		Glioblastoma	-	√	√
4			Glioma	-	√	√
5			Gliosarcoma	-	√	√
6	F	40	Glioma	-	√	√
			Cell Culture (P*-6)	-	-	√
7	F		Glioma	-	√	√
208	M	11	Glioma	-	√	√
268	M	65	Glioma	-	√	√
271	F	38	Glioblastoma	-	√	√
314	F	78	Glioma	-	√	√
380	F	52	Glioblastoma	-	√	√
134	F	76	Glioblastoma	√	√	√
			Cell Culture (P*-6)	√	-	√
548	M	43	Glioma	-	√	√

(*) = Passage No. 6; (-) = Presence; (√) = absence

- **A: CCK**

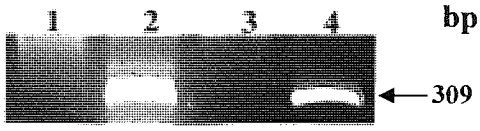


Lane 1 – DNA Ladder

Lane 2 – cDNA digested with PstI

Lane 3 – **CCK** cDNA

- **B: CCK-B**



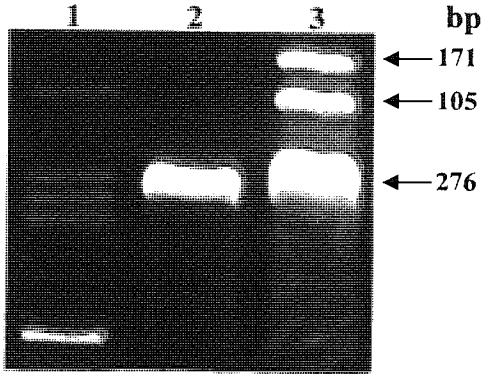
Lane 1 – Glioma **6** cell culture (P-6) derived cDNA

Lane 2 – Glioma **6** tissue derived cDNA

Lane 3 – Glioblastoma **134** cell culture (P-6) derived cDNA

Lane 4 – Glioblastoma **134** tissue derived

- **C: CCK-A**

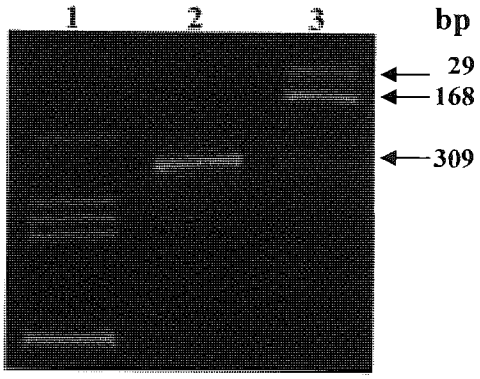


Lane 1 – DNA Ladder

Lane 2 – **CCK-A** PCR DNA

Lane 3 – cDNA digested with FokI

- **D: CCK-B**



Lane 1 – DNA Ladder

Lane 2 – **CCK-B** cDNA

Lane 3 – cDNA digested with PstI

Figure 2: 2% agarose gels of A) RT-PCR for CCK using RNA derived from a human glioma (lane 3) and cut by PstI (lane 2); B) RT-PCR for CCK-B-R using RNA derived from two different human glioma derived cells (lane 1 ,3) versus RNA derived from corresponding glioma tissue (lane 2, 4); C) RT-PCR for CCK-A-R using RNA derived from a human glioma (lane 2) and cut by FokI (lane 3); D) RT-PCR for CCK-B-R using RNA derived from a human glioma (lane 2) and cut by PstI (lane 3); bp, number of base pairs in marker bands (arrowed).

Direct sequence analysis of these bands confirmed that they were representative of both the human CCK peptide along with its receptor subtypes CCK-A and / B mRNA. A specific portion of sequencing is shown in figure 3. The present study provides solid evidence of mRNA expression of CCK peptide and its CCK-B receptor subtype by most, if not all, human gliomas. The mRNA sequences presented in figure 3 are without the intervening intronic regions, indicating that the source of template was cDNA (mRNA) rather than genomic DNA.

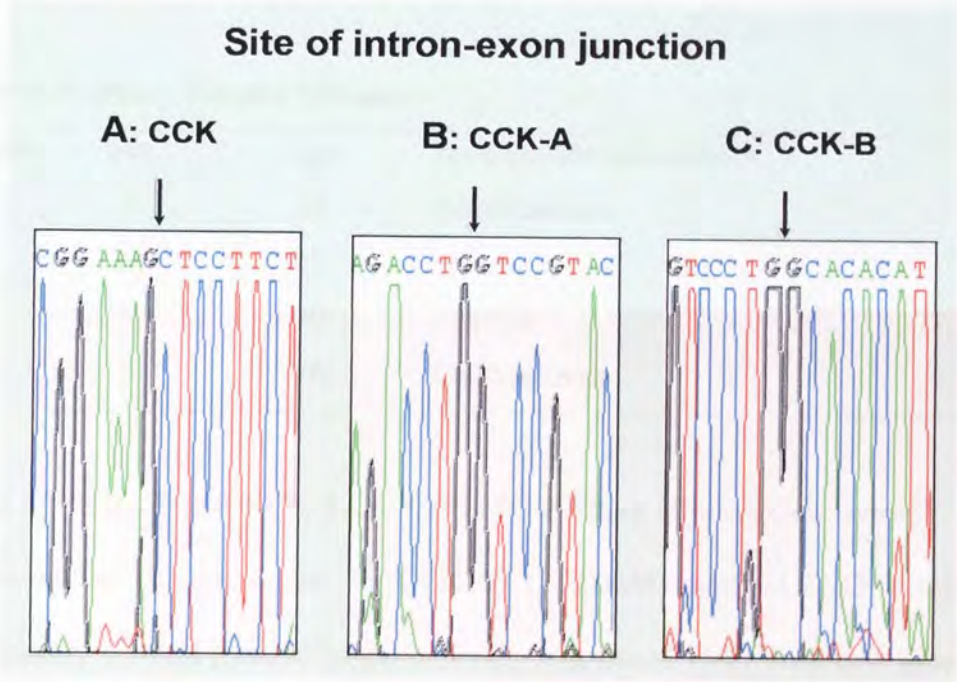


Figure 3: Partial sequence of RT-PCR DNA bands. A) The sequences read 5'-GCCCGGAAAGCTCCTTCTGG-3', and is identical to the CCK cDNA (mRNA); B) 5'-GCAGTCCCTGGCACACATTA-3', and is identical to the CCK-A receptor (mRNA); C) 5'-CCGCCAGACCTGGTCCGTACTG-3', and is identical to the CCK-B receptor (mRNA). All sequences are without the genes' intervening intronic region situated, as arrowed, between the underlined gene residues (GeneBank accession no. in table 1).

2.3.2 Effects of CCK Peptides and Growth Factors on Primary Human Glioblastoma and U-87 MG Cell Growth

Experiments were performed on tumour specimens obtained from a total of 4 human gliomas removed from 3 female and 1 male patients, age range 40-76. Histologically, the tumour grades consisted of 2 gliomas and 2 glioblastomas. The histological diagnoses were confirmed by a neuropathologist using a portion of the original tumour tissue as shown in table 6. U-87 MG cells were also used for the experiments.

Table 6: Primary Human Gliomas

Sample	Sex	Age	Immunohistochemistry
1	F	48	Glioblastoma
2	M	43	Glioma
6	F	40	Glioma
134	F	76	Glioblastoma

Effect of CCK-33 and CCK-8s on U-87 MG Human Glioma Cell Growth

As shown in figures 4 and 5, CCK-8s (2-200nM) and CCK-33 (1-100nM) significantly ($P<0.01$) and dose-dependently stimulated U-87 MG cell growth as determined by means of Coulter Counter where actual cell numbers were recorded. The magnitude of stimulation was a moderate 120-160%.

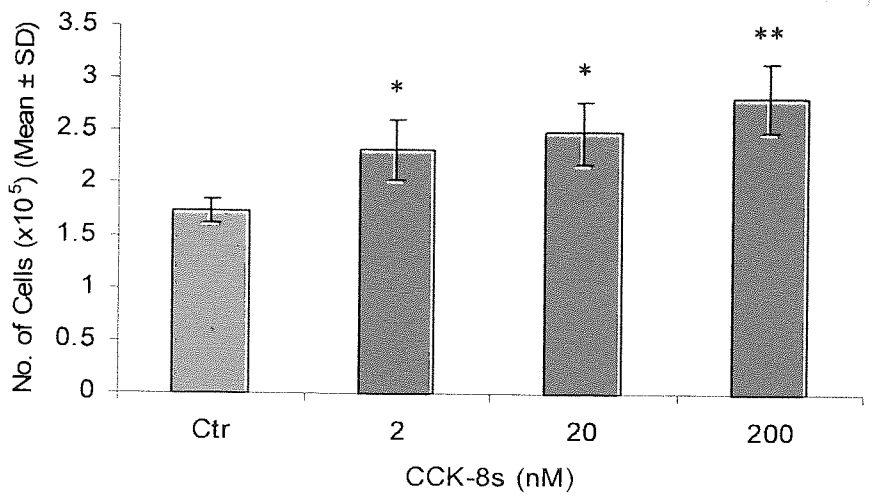


Figure 4: Effects of CCK-8s (2-200nM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01 vs. control.

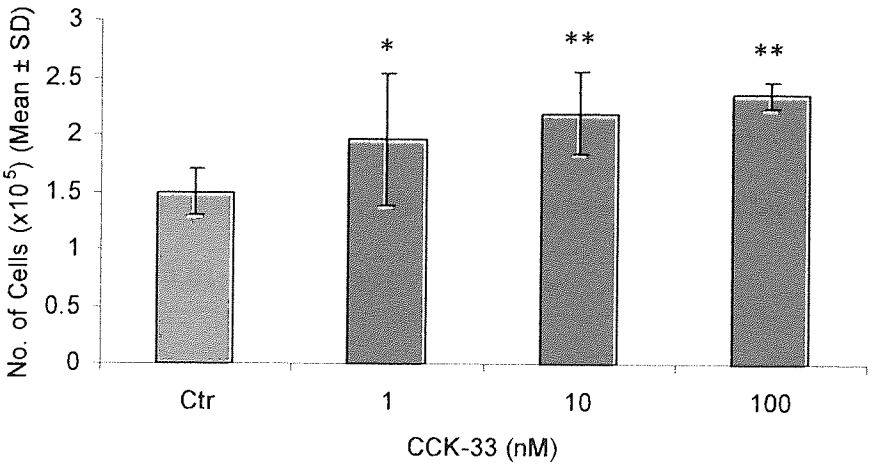


Figure 5: Effects of CCK-33 (1-100nM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01 vs. control.

Effects of CCK-8s (2-200nM) and CCK-33 (1-100nM) on U-87 MG cell growth was also assessed by thymidine uptake (figures 6 and 7). A significant (P<0.001)

stimulation was exerted by both peptides. The magnitude of stimulation for CCK-33 ranged from 120-160%, whereas for CCK-8s a more powerful 120-190% increase was observed.

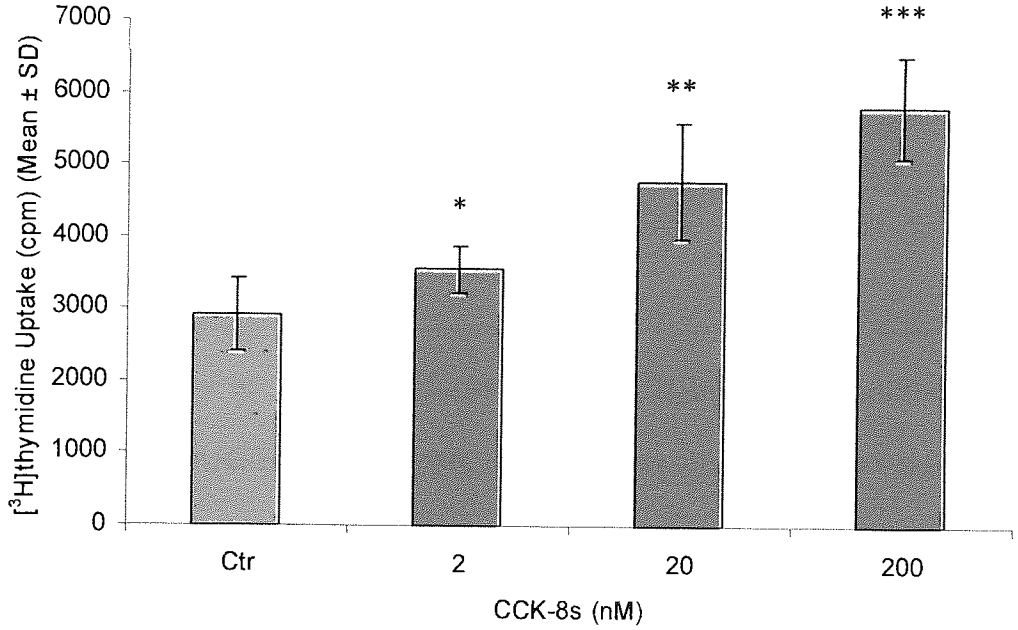


Figure 6: Effects of CCK-8s (2-200nM) on [^{3}H]thymidine uptake by cell cultures of U-87 MG cells. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01; ***P<0.001 vs. control.

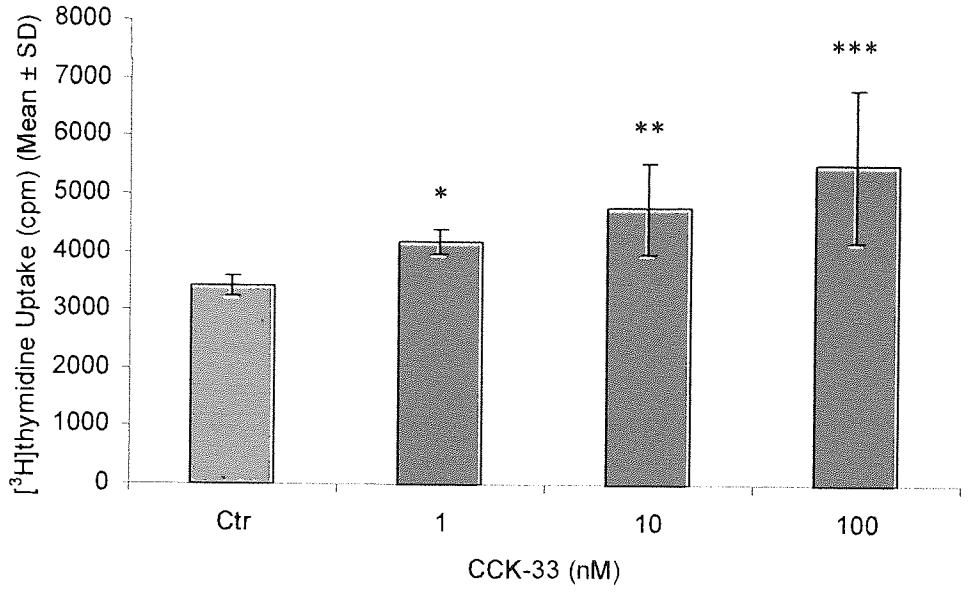


Figure 7: Effects of CCK-33 (1-100nM) on [^{3}H]thymidine uptake by cell cultures of U-87 MG cells. Triplicate cultures were used for each variable; n=3 *P<0.05; **P<0.01; ***P<0.001 vs. control.

Effect of CCK-33 on Primary Human Glioma Cell Growth

In a further study, primary glioblastoma cells were seeded at a concentration of $1x10^5$ / flask and cultured for 12 days in the presence of CCK-33 (100nM) after which the cells were counted. Significant stimulation was observed at day 6 and this continued throughout the 12 days of the culture (P<0.001) (figure 8).

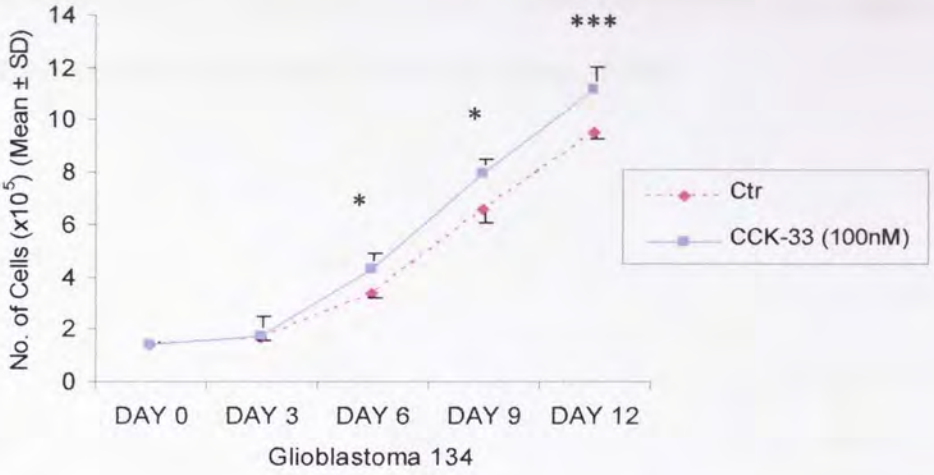


Figure 8: Stimulatory time course effects of CCK-33 (100nM) on *in vitro* cell growth of glioblastoma (134) during 12 days. Triplicate cultures were used for each variable; n=1. *P<0.05; ***P<0.001 vs. control.

2.3.3 Effects of CCK-33 and CCK-8s on PI Hydrolysis and cAMP Production by U-87 MG Human Gliomas

In culture the responses of receptor agonists, CCK-8s and CCK-33 were examined via second messengers. A time course effect of CCK-8s (2-200nM) and CCK-33 (1-100nM) on PI hydrolysis is presented in figures 9, 10 and 11. Significant (P<0.001) and powerful (2.1-fold) stimulation was observed at 30 minutes and this rose to a maximum at 1 hour (a 2.2-fold increase) after which the effects began to decline. A more detailed analysis of the effects of CCK-8s on inositol phosphate formation is also depicted in figures 9 and 10, over 24 hours with varying doses of CCK-8s (2-200nM). In this kinetic analysis, CCK-8s stimulated hydrolysis product formation indicated an initial large and dose-dependent increase in IP_3 by 15 minutes (figure 10) followed by a later increases in IP_1 and IP_2 (figure 9), reflecting the conversion

of the released IP_3 to these latter forms. These kinetics are very similar to those described in other cell systems (Patel and Schrey, 1990).

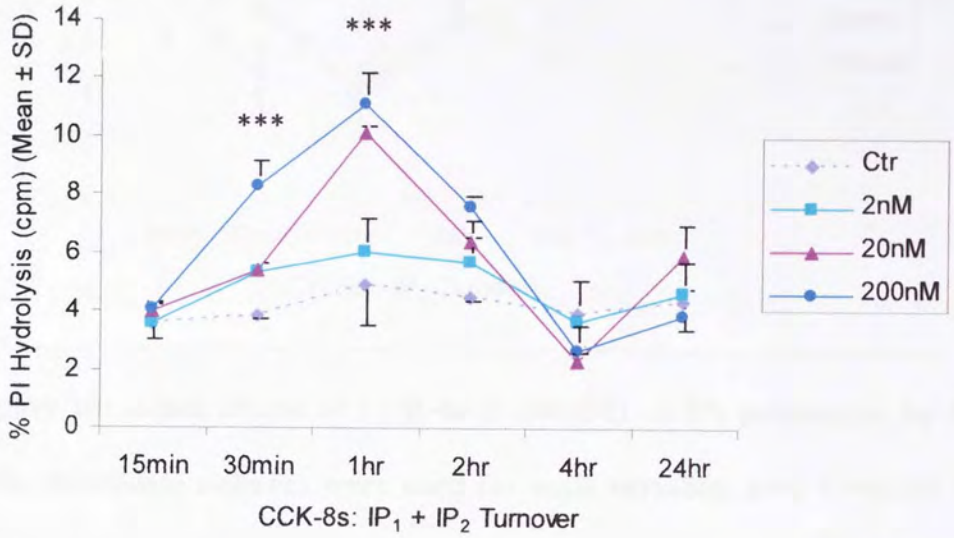


Figure 9: Time course effect of CCK-8s (2-200nM) on PI hydrolysis in U-87 MG cells. Acute effects of CCK-8s on IP_1 plus IP_2 production by U-87 MG cells. Triplicate cultures were used for each variable; n=2. ***P<0.001 vs. control.

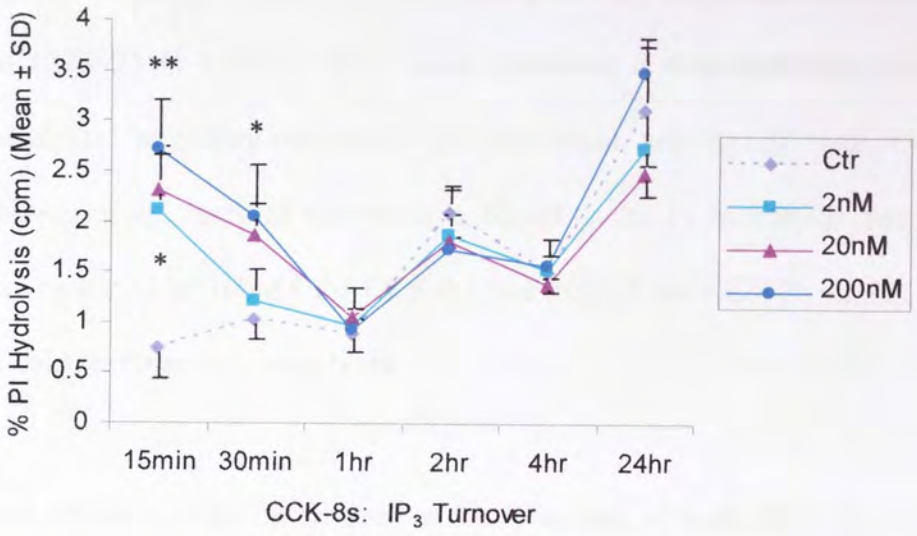


Figure 10: Acute effects of CCK-8s (2-200nM) on IP_3 production by U-87 MG cells. Triplicate cultures were used for each variable; n=2. *P<0.05; **P<0.01 vs. control.

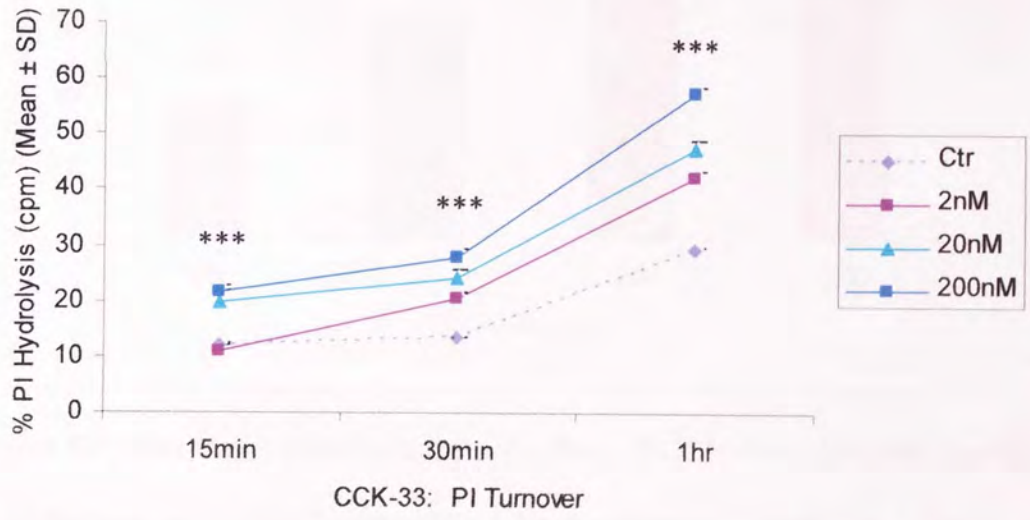


Figure 11: Time course effect of CCK-33 (1-100nM) on PI hydrolysis by U-87 MG cells. Triplicate cultures were used for each variable; n=2. ***P<0.001 vs. control.

As depicted in figures 12 and 13, exposure of U-87 MG cells to CCK-8s (2-200nM) and CCK-33 (1-100nM) for 1 hour produced a dose-dependent increase in PI hydrolysis, providing evidence for functional activity of both CCK receptor subtypes, since receptor activation is bound to the PI hydrolysis. Maximal effects were observed at 100nM for CCK-33 and 200nM for CCK-8s, which led to 2 and 2.7-fold stimulation respectively.

Total cAMP activity determined in the presence of both CCK-8s (2-200nM) and CCK-33 (1-100nM) peptides was not changed.

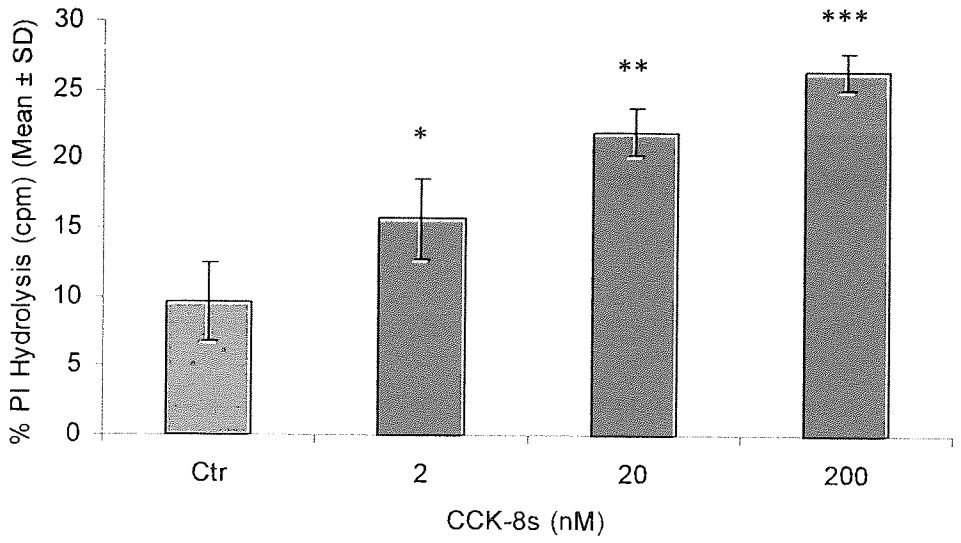


Figure 12: Dose-dependent stimulatory effect of CCK-8s (2-200nM) on rate of PI hydrolysis in U-87 MG cells after 1 hour exposure. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01; ***P<0.001 vs. control.

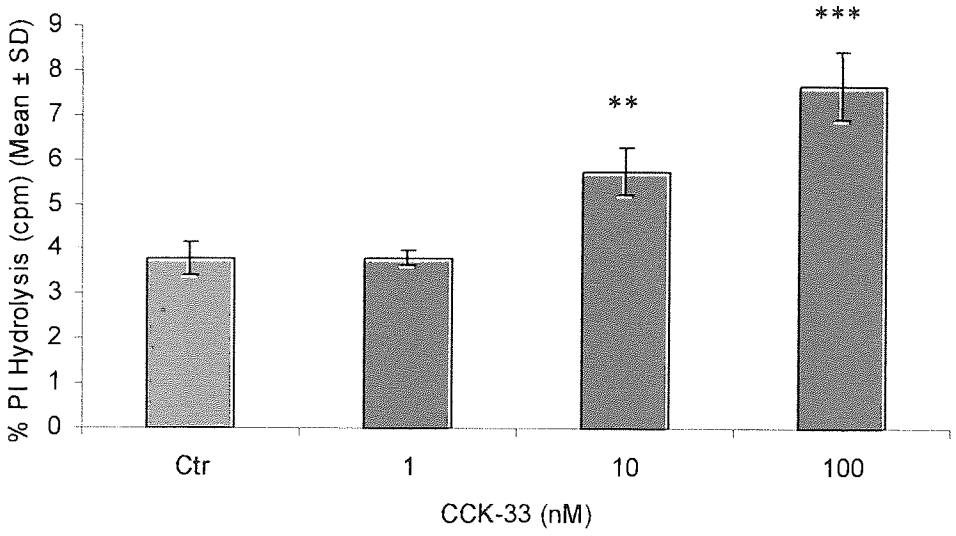


Figure 13: Dose-dependent stimulatory effect of CCK-33 (1-100nM) on rate of PI hydrolysis in U-87 MG cells after 1 hour exposure. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

2.3.4 Effect of a Neutralizing Antibody against CCK on Human Glioma Cell Proliferation

Since the presence of CCK receptors together with CCK peptide expression itself suggested that there might be an autocrine loop controlling glioma cell growth, the neutralizing effect of an antibody against the CCK peptide on endogenous CCK activity on cell proliferation was examined. A significant dose-dependent inhibition of cell growth was observed in U-87 MG cells, with the resultant decrease in [^{3}H]thymidine incorporation ranging from 30-50% of control (figure 14).

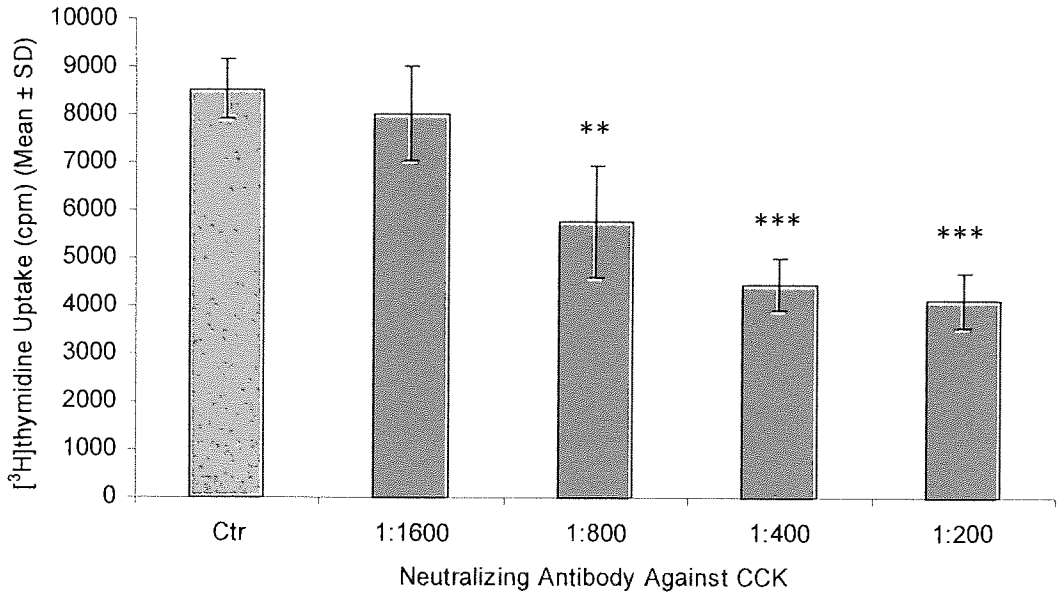


Figure 14: Inhibition of growth of U-87 MG cell growth by anti-CCK antibody. Triplicate cultures were used for each variable; n=1. **P<0.01, ***P<0.001 vs. control.

A cell growth study was also performed on cell cultures derived from glioblastoma 548, to examine the long term effect of a neutralizing anti-CCK antibody. Again a significant inhibition of cell growth after 6 days, resulting in 50-80% of control, was observed in the presence of the antibody against CCK and is depicted in figure 15.

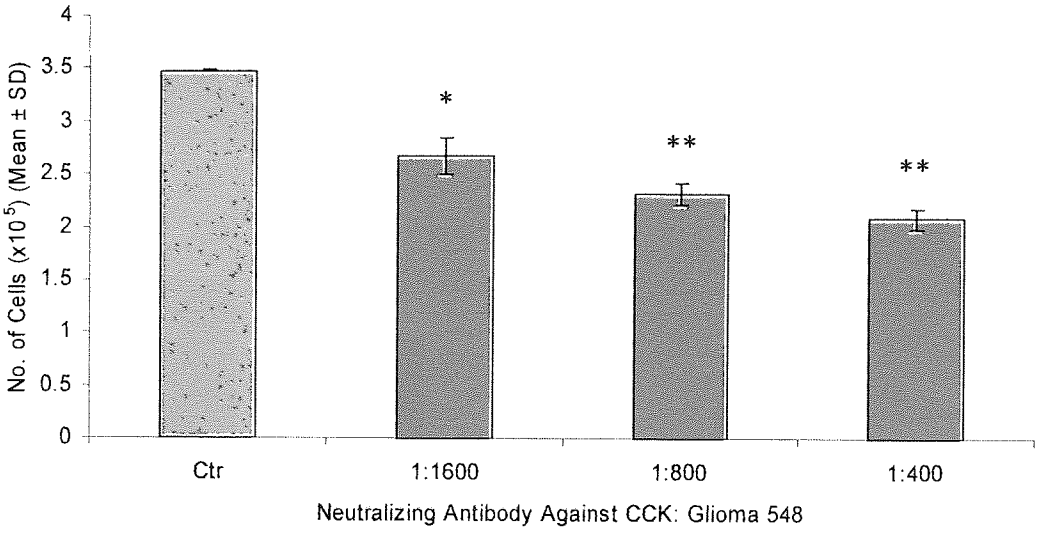


Figure 15: Inhibition of growth of primary (passage no. 1) human glioblastoma (548) cell growth after 6 days treatment with anti-CCK antibody. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01 vs. control.

2.3.5 Effects of CCK Antagonists on Human Glioma Cell Growth

Two groups of CCK antagonists were used in this study. Initially, the antagonistic effects of well established, specific antagonists to CCK-A and CCK-B receptors were explored. As a second approach in an attempt to antagonizing CCK stimulated glioma cell growth, novel non peptide CCK antagonists that were developed at Aston University (Lattmann *et al.*, 2001) were investigated on human gliomas cell growth using *in vitro* cell culture techniques.

Effects of CCK-8s and CCK-33 Peptides ± CCK-A and CCK-B Antagonists on U-87 MG Human Glioma Cell Growth

In figures 16 and 17, CCK-A (1-1000nM) and CCK-B (1-1000nM) receptor antagonists significantly (P<0.01) and dose-dependently inhibited U-87 MG cell

growth as determined by means of Coulter Counter where actual cell numbers were recorded. The magnitude of inhibition ranged from 80% down to 30% decrease in cell growth.

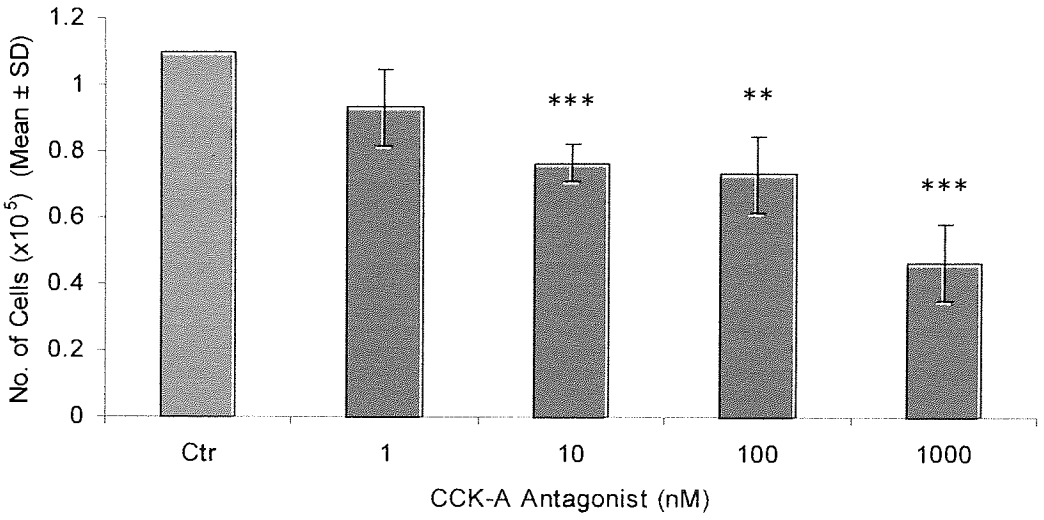


Figure 16: Inhibitory effect of CCK-A antagonist (1-1000nM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

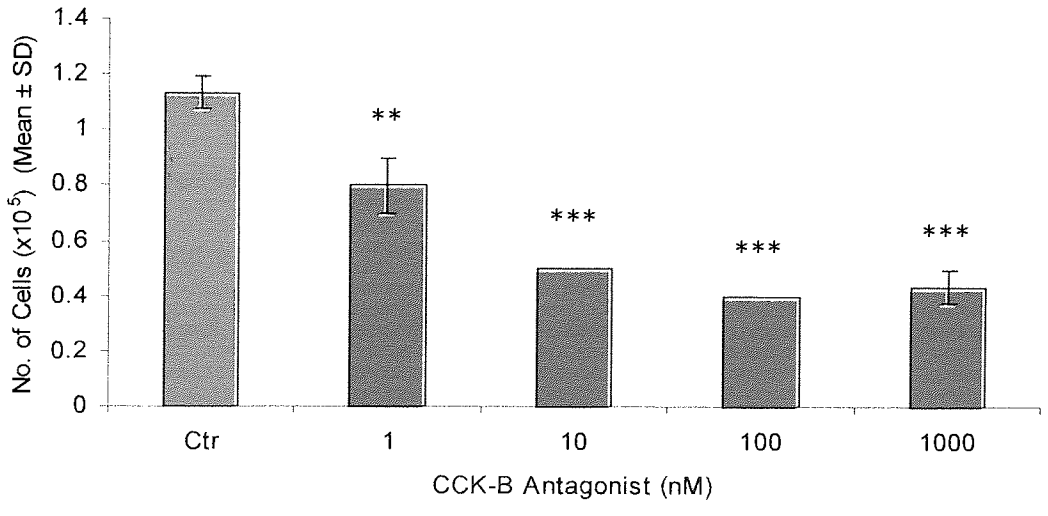


Figure 17: Inhibitory effect of CCK-B antagonist (1-1000nM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

Effects of CCK-A (100nM) and CCK-B (100nM) receptor antagonists alone and / or co-incubated with CCK-8s (200nM) on U-87 MG cell growth was also assessed by thymidine uptake. Addition of CCK-8s (200nM) to U-87 MG cells led to a significant (P>0.001) increase of [^{3}H]thymidine incorporation, in U-87 cells, ranging from 120-130% of control. The effects were antagonized by CCK antagonists (50-100nM) in a dose-dependent manner as shown in figures 18 and 19.

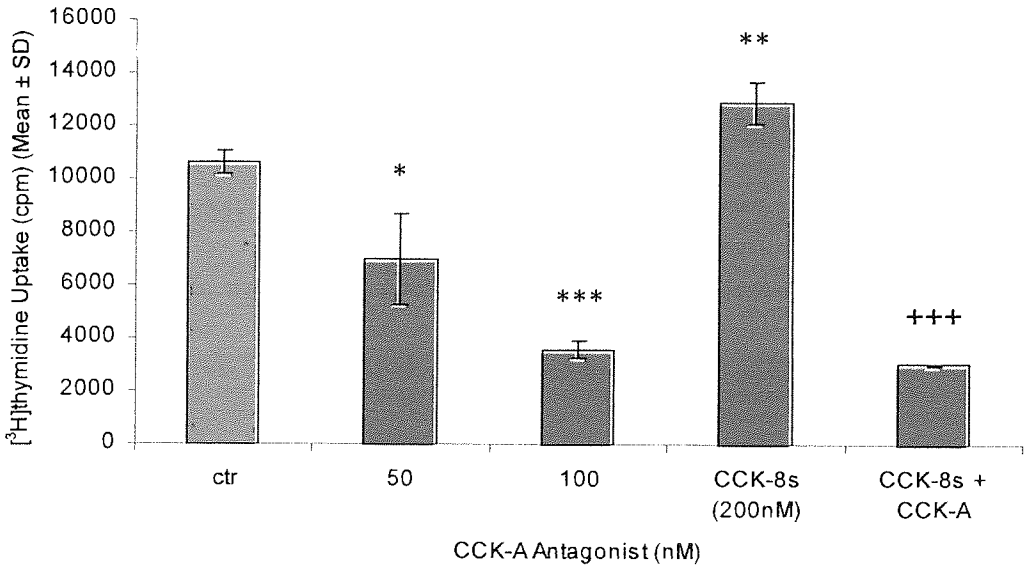


Figure 18: Inhibitory effect of CCK-A antagonist (50-100nM) as well as effects of CCK-8s (200nM) alone and in combination with CCK-A (100nM) antagonist on [^{3}H]thymidine uptake by U-87 MG cells. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-8s.

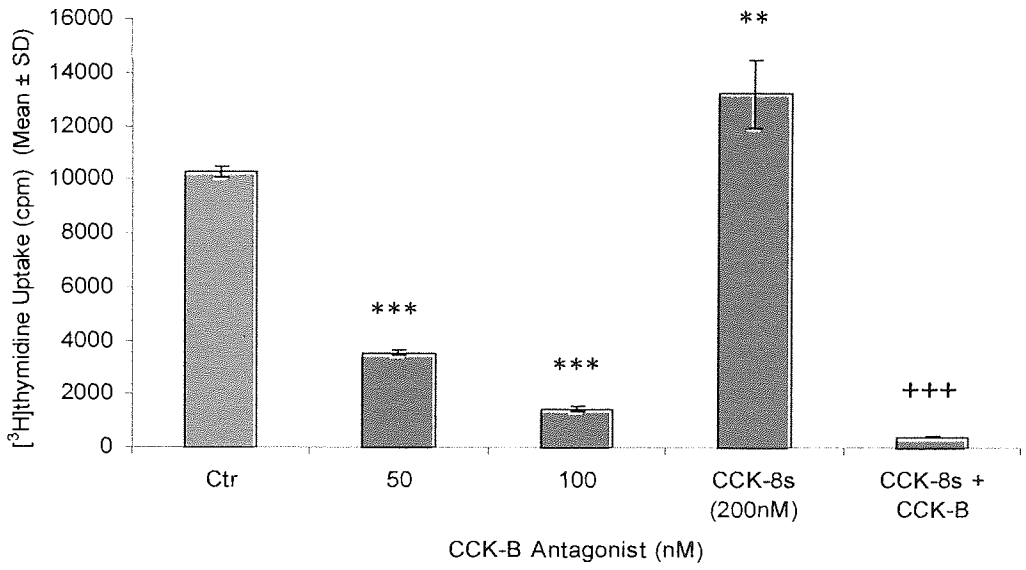


Figure 19: Inhibitory effect of CCK-B antagonist (50-100nM) as well as effects of CCK-8s (200nM) alone and in combination with CCK-B (100nM) antagonist on [^{3}H]thymidine uptake by U-87 MG cells. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-8s.

Effects of CCK-8s and CCK-33 Peptides ± CCK-A and CCK-B Antagonists on Human Glioma Cell Growth

The effects of epidermal growth factor (EGF) (1-100ng/ml) on U-87 MG cell growth were investigated in this part of the study. Because EGF is a potent stimulant of human glioma cell growth, the effects of the non peptide CCK antagonists can be further explored. EGF alone as tested on U-87 MG human gliomas caused a significant (P<0.001) increase in cell growth and maximal effects were observed at 10ng/ml, which led to a 1.7-fold stimulation (figure 20). A higher concentration of 100ng/ml suggests receptor saturation.

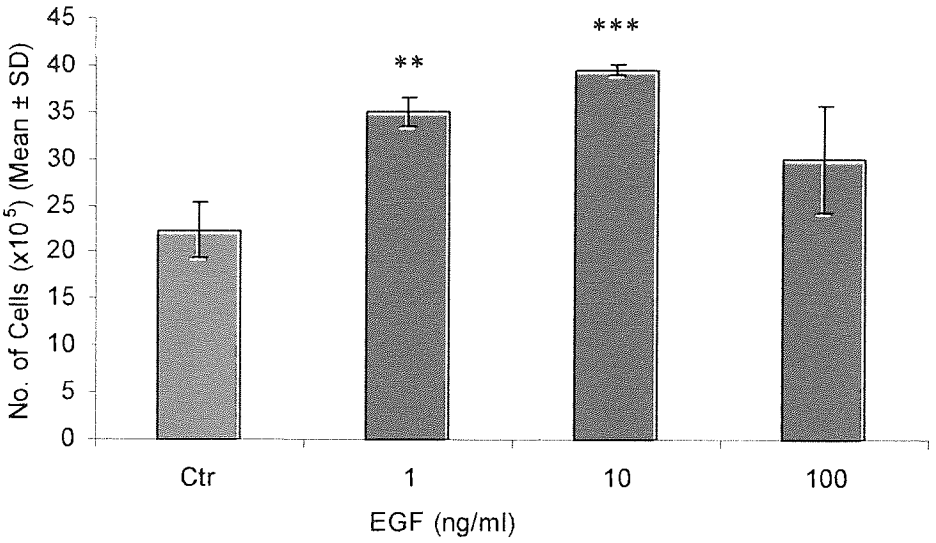


Figure 20: Effects of EGF (1-100ng/ml) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control.

Similar experiments on primary glioblastoma derived cells showed a significant increase in cell growth a rate after stimulation with CCK-8s (200nM) and EGF (20ng/ml) for 6 days. Primary glioblastoma cell growth was strongly inhibited by selective non-peptide antagonists to CCK-A receptor (100nM), which completely abolished the stimulatory effects of CCK-8s and EGF during co-incubation (figure 21).

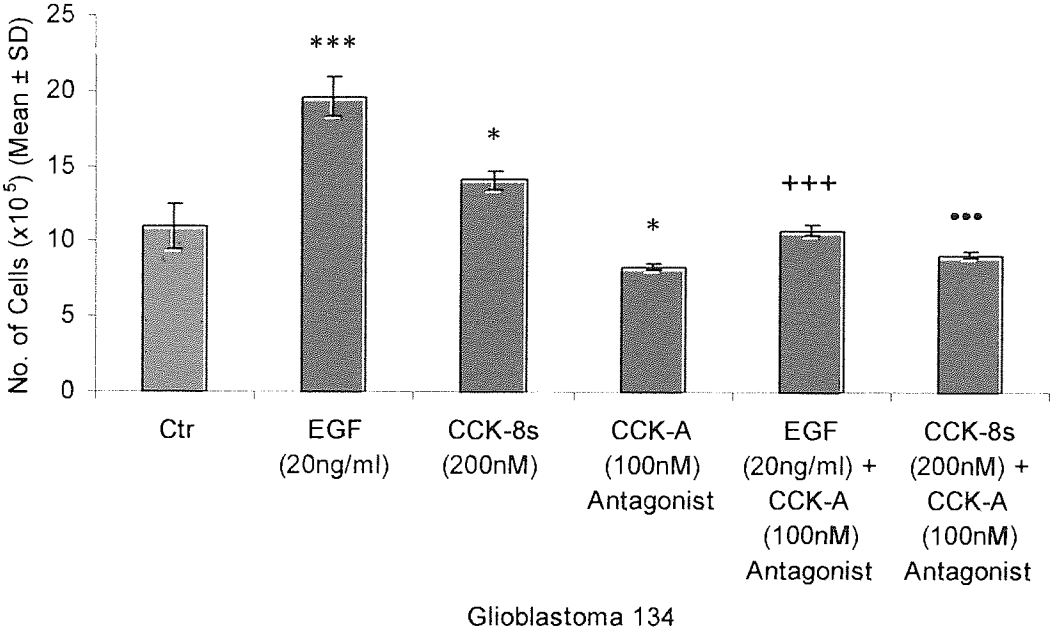


Figure 21: Stimulatory effects of CCK-8s (200nM) and EGF (20ng/mL) on *in vitro* cell growth of primary (passage no. 1) glioblastoma (134) after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. *P<0.05; ***P<0.001 vs. control, $^{+++}$P<0.001 vs. EGF; •••P<0.001 vs. CCK-8s.

Effect of CCK-33 and CCK-8s ± CCK-A and CCK-B Antagonists on PI Hydrolysis by U-87 MG Human Gliomas

In culture the responses of the two CCK receptor agonists (CCK-8s and CCK-33) alone and in combination with antagonists to CCK-A (100nM) and CCK-B (100nM) receptors were also examined via second messengers. Both CCK-33 (100nM) and CCK-8s (200nM) produced a significant (P<0.01 and P<0.001) and powerful (1.9-2.2-fold) increase in PI hydrolysis, providing evidence for functional activity of both CCK receptor subtypes, since receptor activation is bound to the PI

hydrolysis. In addition, both antagonists to CCK significantly inhibited production of PI hydrolysis and greatly reduced the stimulatory effects of CCK-8s and CCK-33. Results are presented in figures 22 and 23.

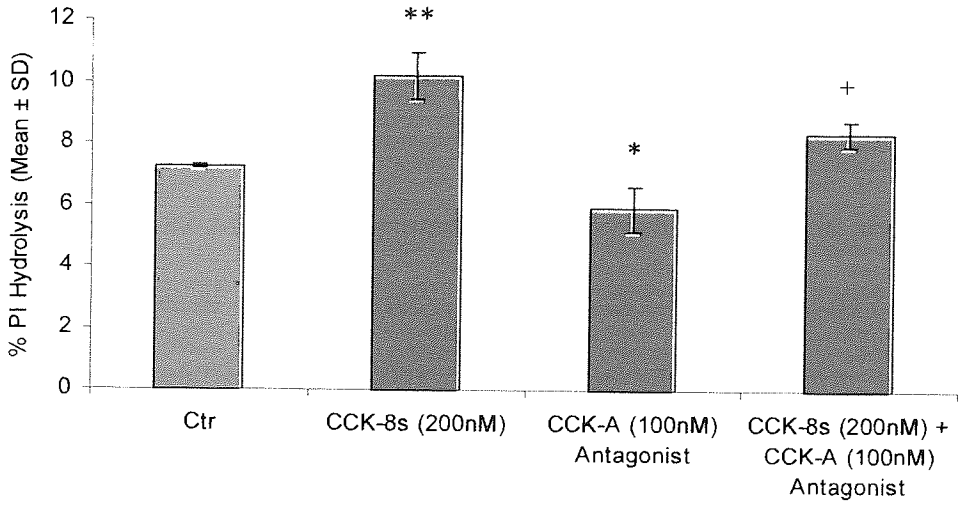


Figure 22: Effects of CCK-8s (200nM) and CCK-A (100nM) antagonist on rate of PI hydrolysis after 45 min, by U-87 MG cells. Triplicate cultures were used for each variable; n=3. *P<0.05; **P<0.01 vs. control; $^{+}$P<0.05 vs. CCK-8s.

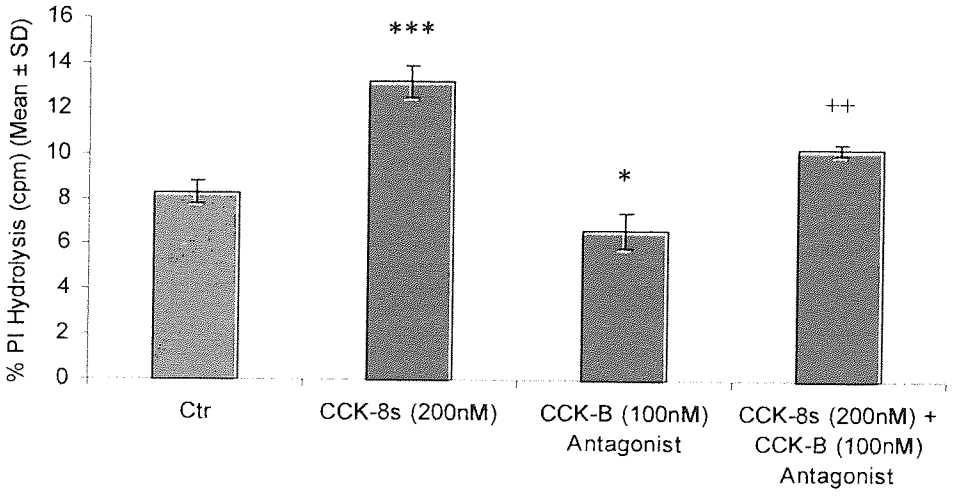


Figure 23: Effects of CCK-8s (200nM) and CCK-B (100nM) antagonist on rate of PI hydrolysis after 45 min, by U-87 MG cells. Triplicate cultures were used for each variable; n=3. *P<0.05; ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s.

Effects of Novel Non-Peptide CCK Antagonists on U-87 MG Human Glioma Cell Growth

The second part of the antagonistic experiments on CCK stimulated human glioma cell growth was investigated using the novel non peptide CCK antagonists developed at Aston University (Lattmann *et al.*, 2001).

For human U-87 MG gliomas grown in culture for 6 days in the presence of different dosages of the non-selective, non-peptide CCK antagonist 7.4.5 (0-10μM) there was a significant ($P<0.001$) inhibition of cell growth. The most dramatic inhibitory effect (6.3-fold) was observed by the 10μM concentrations. However they may not be the higher possible concentration that can have a significant effect on growth since highest concentrations have not been tested. Results are shown in figure 24.

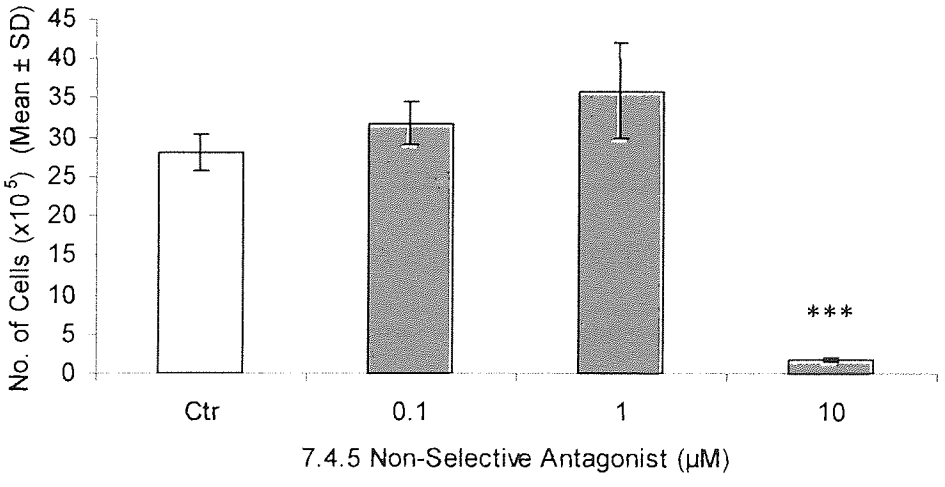


Figure 24: Inhibitory effect of 7.4.5 antagonist (0.1-10μM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. ***P<0.001 vs. control.

The suspected efficacy for non-peptide CCK antagonists 7.4.5 at lower concentrations was verified by a dose-dependent (1.25-20μM) inhibition of U-87 MG cells (figure 25). Cell growth was further inhibited in the presence of 20μM concentration.

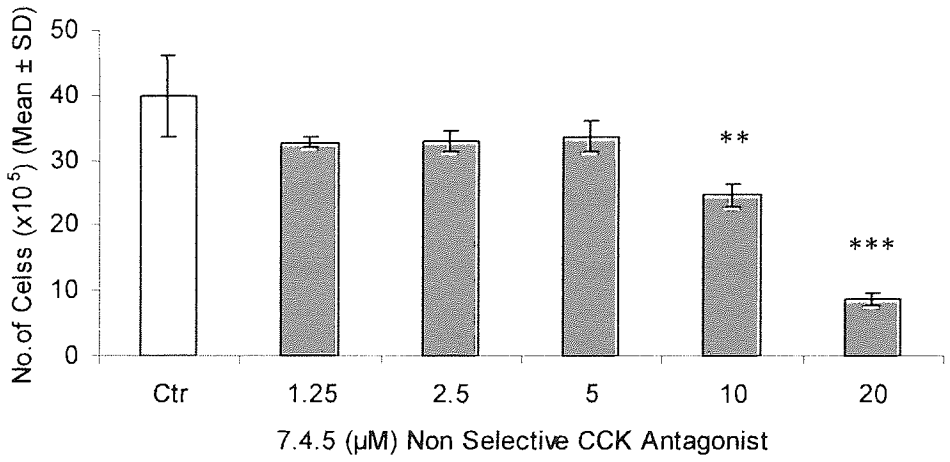


Figure 25: Inhibitory effect of 7.4.5 antagonist (1.25-20μM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

Experiments with the selective CCK-A (0.1-10μM) non-peptide antagonists HSH, on human U-87 MG cells, led to significant (P<0.001) inhibition of human glioma cell growth after 6 days in culture, with the highest concentration of 10μM having the most dramatic effect (70%) (figure 26).

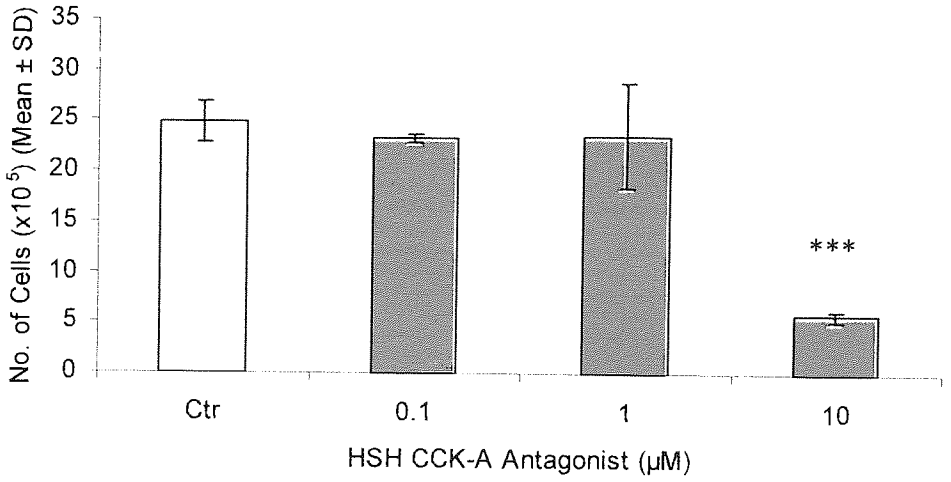


Figure 26: Inhibitory effect of HSH antagonist (0.1-10μM) on U-87 MG cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=3. ***P<0.001 vs. control.

Alternatively, inhibition of cell mitosis by HSH (25-100μM) in a dose-response manner was assessed by means of [^{3}H]thymidine uptake for 2 hours. The CCK antagonist HSH exhibited a dose-dependent significant (P<0.001) inhibition when compared to the control (figure 27).

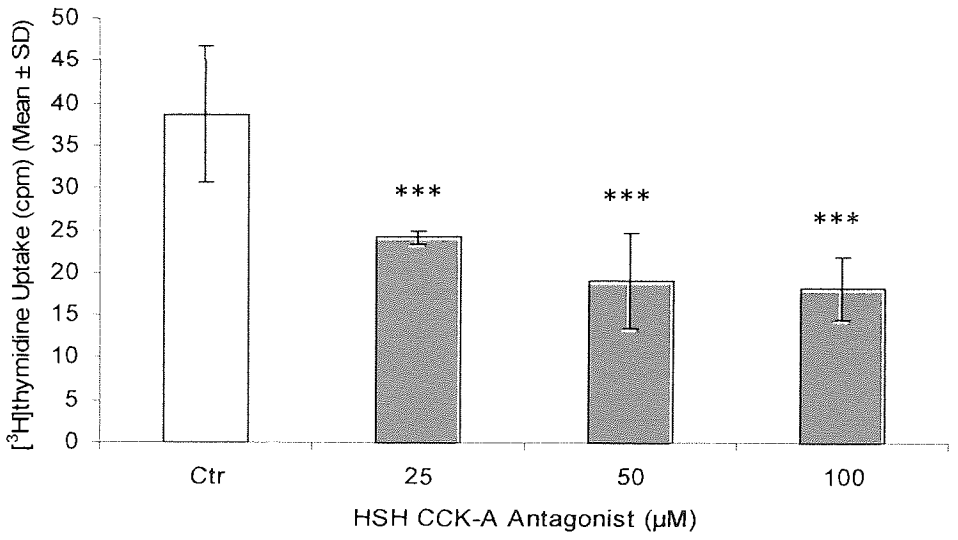


Figure 27: Inhibitory effects of HSH antagonist (25-100μM) on [^{3}H]thymidine uptake by U-87 MG cells. Triplicate cultures were used for each variable; n=3. ***P<0.001 vs. control.

A time course inhibitory effect of HSH (10-20μM) on human U-87 MG cells grown for 9 days *in vitro* is shown in figures 28 and 29. Significant (P<0.001) inhibition was observed as early as day 3 in the presence of 20μM, whereas a longer incubation of 9 days was required for a lower dose of 10μM to achieve as significant (P<0.001) inhibition.

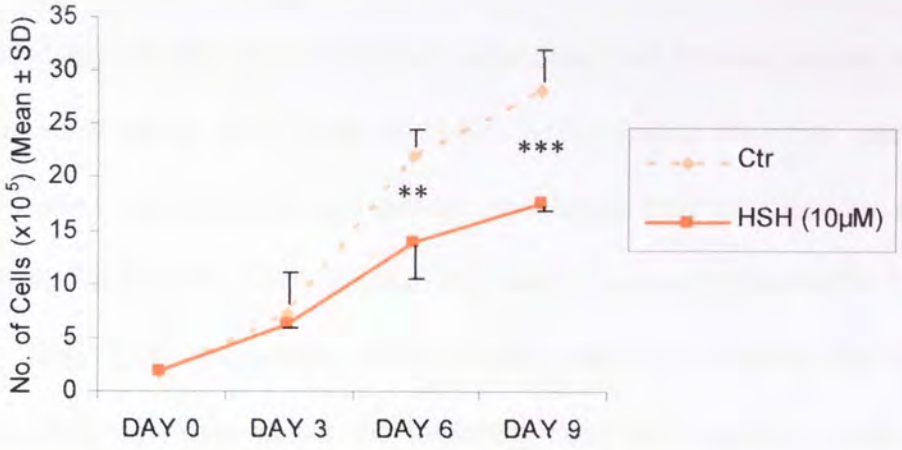


Figure 28: Inhibitory time course effects of HSH antagonist (10μM) on U-87 MG cell growth during 9 days. Media were changed every 3rd day. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

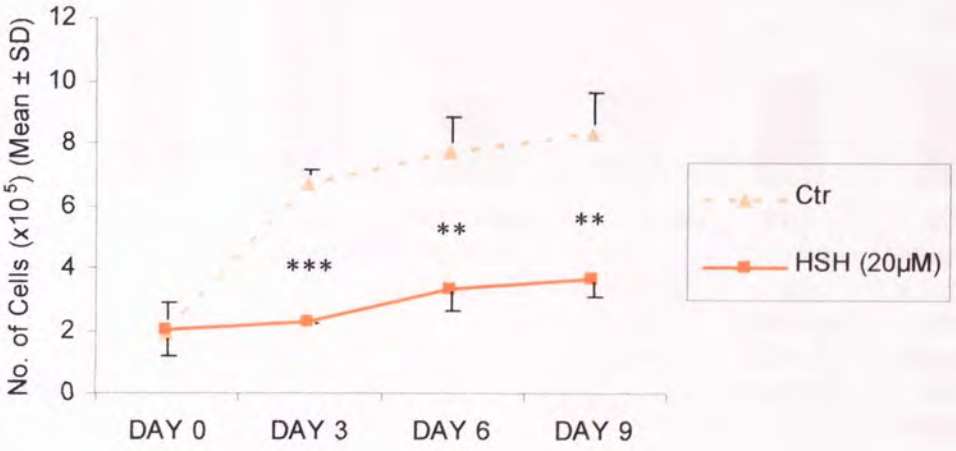


Figure 29: Inhibitory time course effects of HSH antagonist (10μM) on U-87 MG cell growth during 9 days. Media were changed every 3rd day. Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control.

Effects of EGF ± Effects of Novel Non-Peptide CCK Antagonists on U-87 MG Human Glioma Cell Growth

EGF (10ng/ml) was used to treat the cells alone and in combination with the CCK antagonists (HSH and 7.4.5) in order to investigate to what extent the CCK antagonists can inhibit the cell growth stimulation. EGF significantly stimulated U-87 MG cell growth as compared to the control human gliomas after 6 days (figure 30). Both CCK antagonists HSH (10μM) and 7.4.5 (10μM) had a significant ($P<0.001$) inhibitory effect on human glioma cell growth, which completely abolished the stimulatory of effect of EGF.

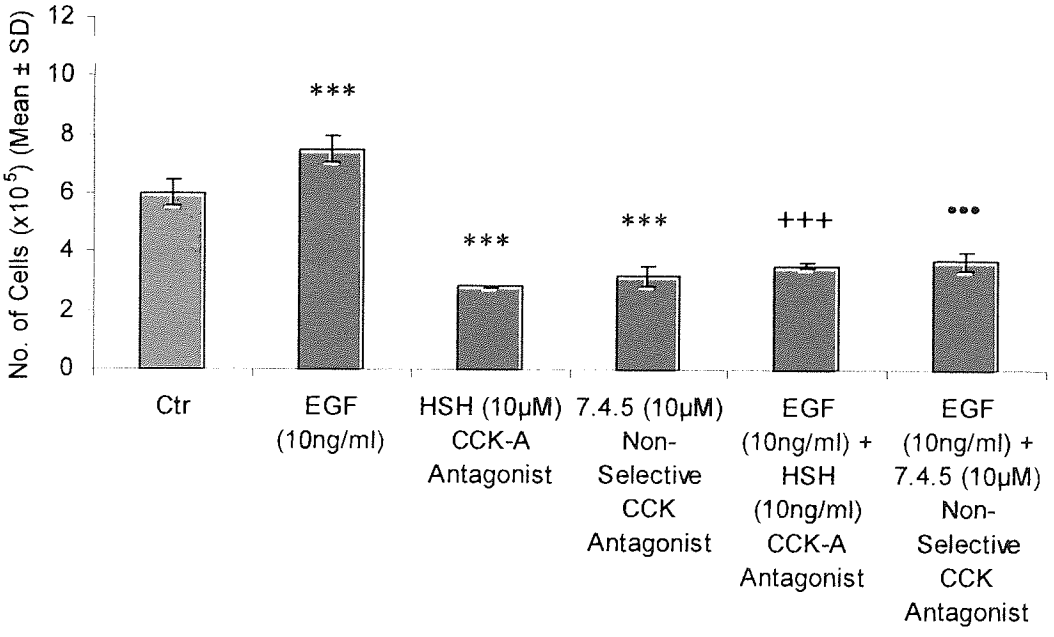


Figure 30: Stimulatory effects of EGF (10ng/ml) on U-87 MG cell growth after 6 days. Cells were incubated in the absence (control), and presence of HSH (10μM) and 7.4.5 antagonist (10μM). Media were changed every 3rd day. Triplicate cultures were used for each variable; n=3. ***P<0.001 vs. control; +++P<0.01 vs. EGF; •••P<0.001 vs. EGF.

The effect of EGF (50ng/ml) alone and in combination with the CCK antagonist HSH (10μM) was also assessed by [^{3}H]thymidine uptake (figure 31). Results obtained verified the stimulatory effect previously described for EGF, as well as the inhibitory effect of HSH alone and co-incubated with EGF, as presented above in figure 30.

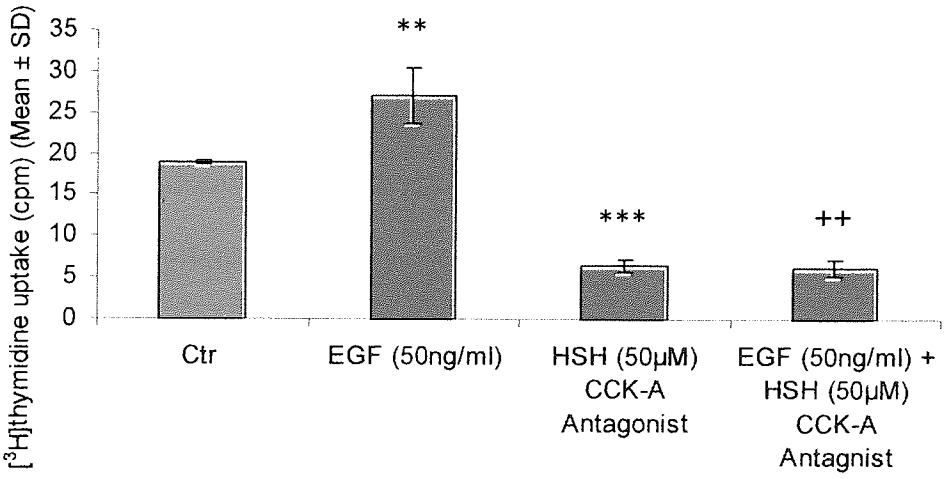


Figure 31: Effects of EGF (50ng/ml) on [^{3}H]thymidine uptake by U-87 MG cells. Cells were incubated in the absence (control), and presence of EGF and / or HSH (50μM). Triplicate cultures were used for each variable; n=3. **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.01 vs. EGF.

Effects of CCK-8s and CCK-33 Peptides ± Novel Non-Peptide CCK Antagonists on Human Glioma Cells

Having investigated the effects of the novel non peptide CKK antagonists on U-87 MG cell growth, the experiments were expanded to primary human glioma cells derived from surgically resected tumours. Primary glioblastoma cell growth was strongly inhibited (P<0.001) by selective non-peptide antagonists to CCK-A receptor (HSH) (100μM) as shown in figure 32.

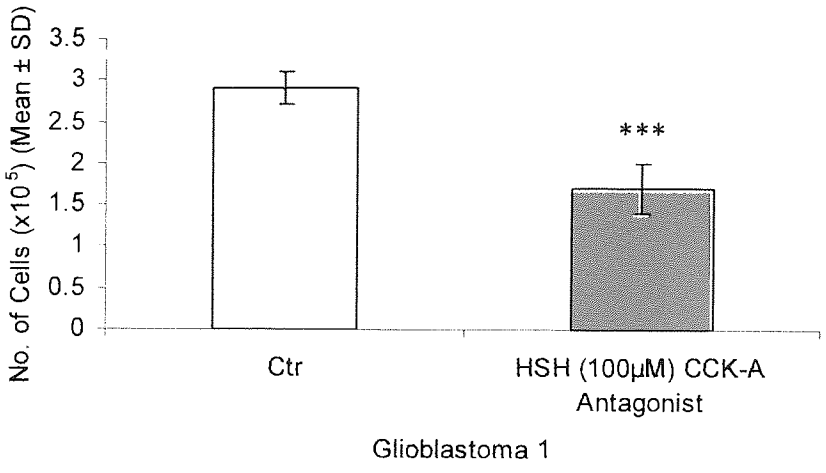


Figure 32: Inhibitory effect of HSH antagonist (100μM) on primary (passage no. 1) human glioblastoma (1) derived cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. ***P<0.001 vs. control.

As illustrated in figure 33, experiments were conducted with EGF (50ng/ml) on a human glioma. EGF powerfully (52%) stimulated primary human glioma cell growth and this effect was inhibited (P<0.001) by co-incubation with HSH (10μM). However effects of HSH alone in this specific human glioma specimen were not significant.

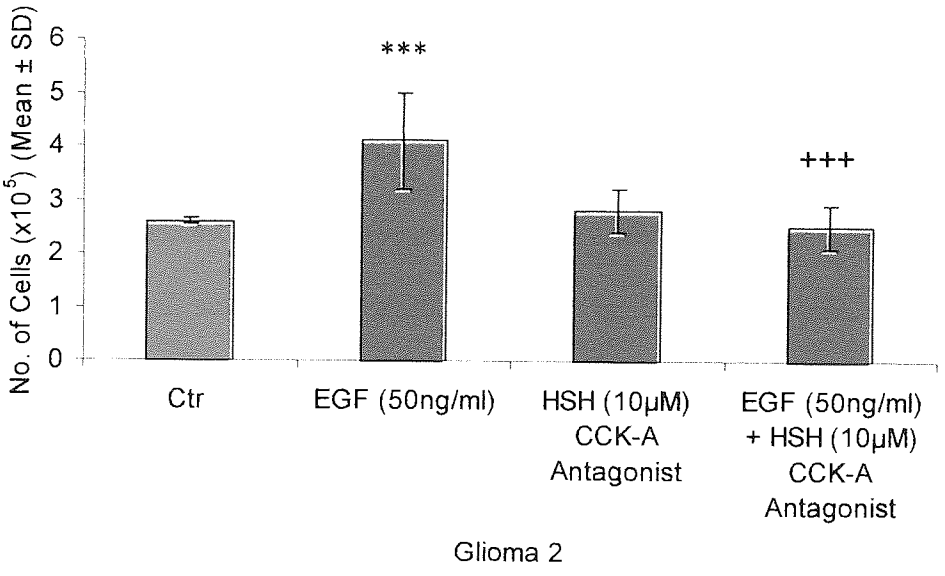


Figure 33: Effects of EGF (50ng/ml) and HSH (10μM) in the presence and absence of EGF on primary (passage no. 1) human glioma (2) derived cell growth after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. ***P<0.001 vs. control; $^{+++}$P<0.001 vs. EGF.

2.3.6 Proteasome and Apoptotic Activity Induced by CCK Antagonists

Proteasome Activity

To further investigate the effects of CCK antagonists on U-87 MG cells, the proteasome functional activity of the β-subunits of the proteasome was determined. Suc-LLVY-AMC is a peptide substrate of the 'chymotrypsin-like' enzyme activity of the proteasome. This is the most dominant catalytic activity and the one most widely used in the literature as an indicator of proteasomal function. Once cleaved the fluorescence AMC (amino methyl coumarin) is released and is proportional to the level of proteasome activity.

The effects of CCK antagonists and the antiglioma agent etoposide on the chymotrypsin-like' enzyme activity of the proteasome in U-87 MG cells are

presented in figure 34. The activity was significantly inhibited in a dose-dependent manner in cells treated with the CCK antagonists and antiglioma agent etoposide suggesting possible apoptotic activity for the CCK antagonists. Lactacystin is a proteasomal inhibitor which was included to differentiate any fluorescence from non-proteasomal sources. Co-treatment with lactacystin in culture also led to apoptosis.

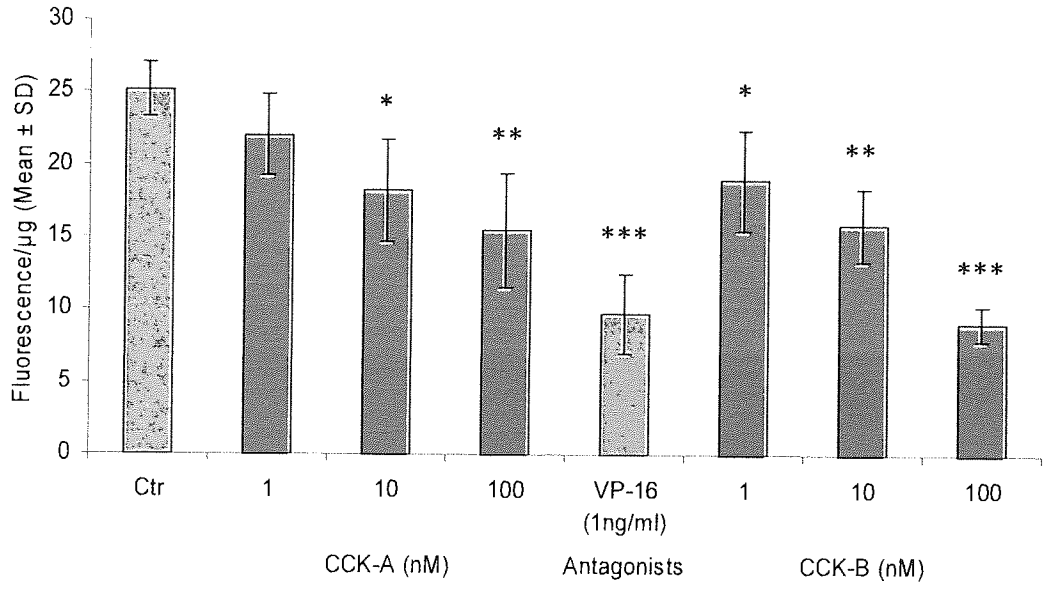


Figure 34: Effects of varying doses of CCK-A and / B antagonists (1-100nM) and etoposide (VP-16) (10ng/ml) an antiglioma drug in progressive decrease on the "chymotrypsin-like" enzyme activity of the proteasome in U-87 MG cells. Readings obtained from samples treated with lactacystin (10µM) were subtracted from those without to give proteasome specific fluorescence. Triplicate cultures were used for each variable; n=3. *P<0.05, **P<0.01, ***P<0.001 vs. control.

Caspase-3 Activity

To provide further evidence for the apoptotic action of the CCK antagonists, activation of caspases in U-87 MG human glioblastoma cells treated with antagonists to CCK was determined by utilizing a fluorigenic tetrapeptide substrate, AC-DEVO-AMC, which has been shown to be specific for caspase-3. Both CCK antagonists significantly stimulated caspase-3 activity in a dose-dependent manner. The same significant elevated caspase-3 activity was observed for the antiglioma positive apoptotic agent etoposide. Results obtained from 10 and 50µl of treated cell lysate, are depicted in figures 35 and 36.

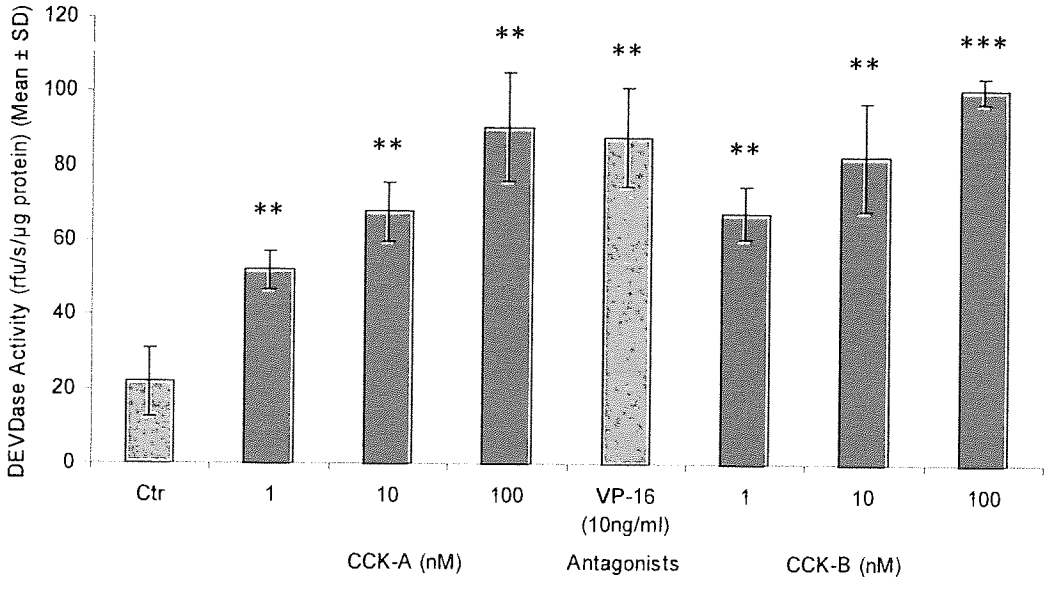


Figure 35: Caspase-3 induction in subconfluent U-87 MG cells treated with varying doses of CCK-A and / B antagonists (1-100nM) and etoposide (VP-16) (10ng/ml). Extracts (10µl) from control and treated cells were used to determine DEVDase (caspase-3) activity. Triplicate cultures were used for each variable; n=3. **P<0.01, ***P<0.001 vs. control.

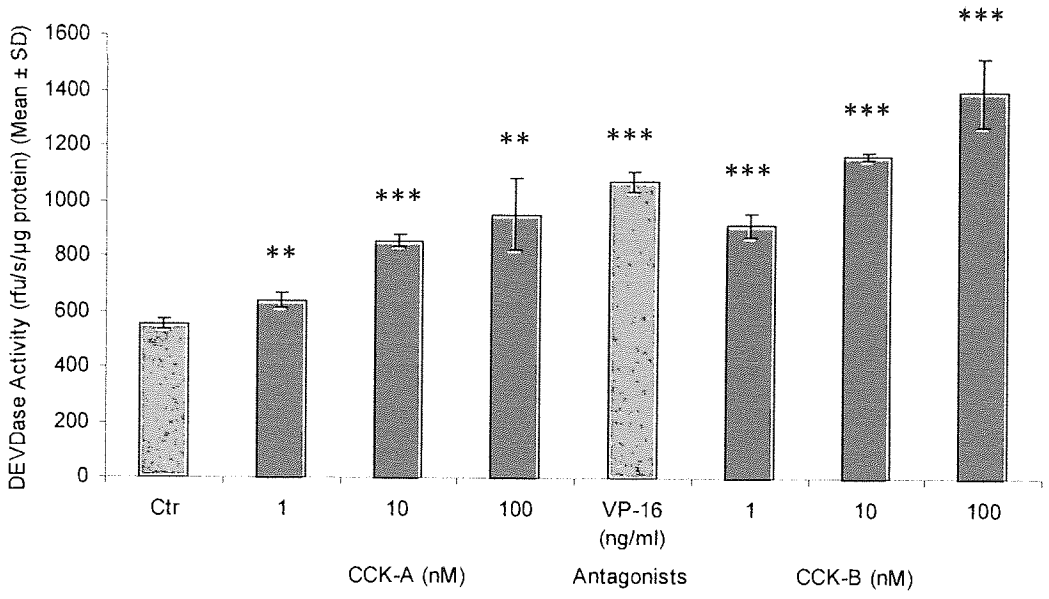


Figure 36: Caspase-3 induction in subconfluent U-87 MG cells treated with varying doses of CCK-A and / B antagonists (1-100nM) and etoposide (VP-16) (10ng/ml). Extracts (50µl) from control and treated cells were used to determine DEVDase (caspase-3) activity. Triplicate cultures were used for each variable; n=3. **P<0.01, ***P<0.001 vs. control.

2.3.7 CCK and Primary Human Meningiomas

Experiments were performed on tumour specimens obtained from a total of 6 human meningiomas removed from 3 female and 3 male patients, age range 38-69. The histological diagnoses were confirmed by a neuropathologist using a portion of the original tumour tissue as shown in table 7.

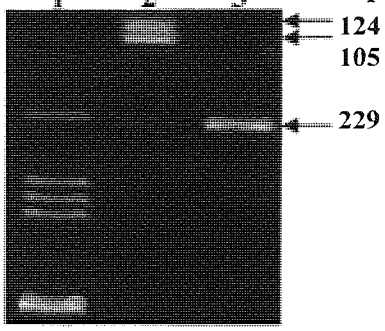
Table 7: Primary Human Meningiomas

Sample	Sex	Age	Diagnose	CCK	CCK-A	CCK-B
232	M	43	Meningioma	√	√	√
			Cell Culture(P*-2)	√	-	-
275	M	69	Meningioma	√	-	√
310	F	57	Meningioma	√	√	√
374	F	46	Meningioma	√	√	√
			Cell Culture(P*-2)	√	-	-
377	M	38	Meningioma	√	-	√
384	F	40	Meningioma	√	√	√
			Cell Culture(P*-2)	√	-	-

(*) = Passage No. 2; (-) = Presence; (√) = absence

All 6 human meningiomas were shown by RT-PCR analysis to express mRNA for CCK itself and CCK-B receptor. PCR bands of predicted size were yielded by the cDNA samples, and these bands were cut in the predicted pattern as described in the Materials and Methods. An example is shown in figure 37. By the same analysis, evidence for CCK-A receptor was detected in 4 of the 6 meningiomas (66%). In culture none out the three meningiomas retained expression of either CCK receptor subtype after two or more passages.

- **A: CCK**

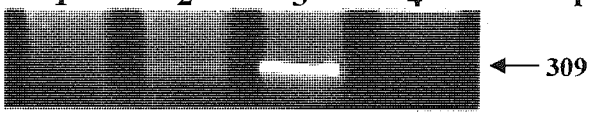


Lane 1 – 1392bp Ladder

Lane 2 – cDNA digested with PstI

Lane 3 – **CCK** cDNA

- **B: CCK-B**



Lane 1 – Meningioma **374** cell culture (P-2) derived cDNA

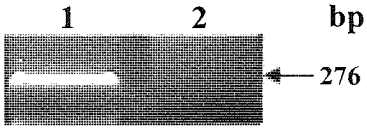
Lane 2 – Meningioma **374** tissue derived cDNA

Lane 3 – Meningioma **384** tissue derived

Lane 4 – Meningioma **384** cell culture (P-2) derived cDNA

cDNA

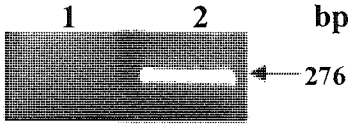
- **C: CCK-A**



Lane 1 – Meningioma **374** tissue derived DNA

Lane 2 – Meningioma **374** cell culture (P-2) derived cDNA

- **D: CCK-A**



Lane 1 – Meningioma **384** cell culture (P-2) derived cDNA

Lane 2 – Meningioma **384** tissue derived cDNA

Figure 37: 2% Agarose gels of A) RT-PCR for CCK using RNA derived from a human meningioma (lane 3) and cut by PstI (lane 2); B) RT-PCR for CCK-B-R using RNA derived from two different human meningioma derived cells (lane 1, 4) versus RNA derived from corresponding meningioma tissue (lane 2, 3); C) RT-PCR for CCK-A-R using RNA derived from corresponding meningioma tissue (lane 1) versus RNA derived from two different human meningioma derived cells (lane 2); D) RT-PCR for CCK-A-R using RNA derived from two different human meningioma derived cells (lane 1) versus RNA derived from corresponding meningioma tissue (lane 2); bp, number of base pairs in marker bands (arrowed).

Direct sequence analysis of these bands confirmed that they were representative of both the human CCK peptide along with its receptor subtypes CCK-A and / B mRNA. A specific portion of sequencing is shown in figure 38. The mRNA sequences presented in figure 38 are without the intervening intronic regions, indicating that the source of template was cDNA (mRNA) rather than genomic DNA.

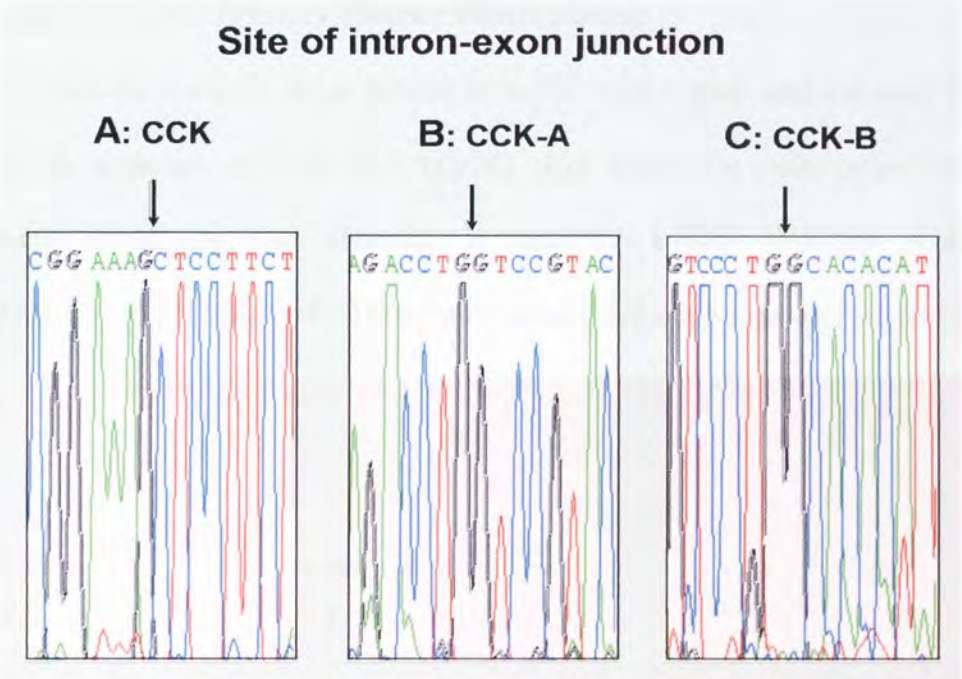


Figure 38: Partial sequence of RT-PCR DNA bands. A) The sequences read 5'-GCCCGGAAAGCTCCTTCTGG-3', and is identical to the CCK cDNA (mRNA); B) 5'-GCAGTCCCTGGCACACATTA-3', and is identical to the CCK-A receptor (mRNA); C) 5'-CCGCCAGACCTGGTCCGTACTG-3', and is identical to the CCK-B receptor (mRNA). All sequences are without the genes's intervening intronic region situated, as arrowed, between the underlined gene residues (GeneBank accession no. in Table 1).

2.3.8 Effects of CCK Peptides and Growth Factors on Primary Human Meningioma Cell Growth

Experiments were performed on tumour specimens obtained from a total of 3 human meningiomas removed from 2 female and 1 male patients, age range 40-46. The histological diagnoses were confirmed by a neuropathologist using a portion of the original tumour tissue as shown in table 7.

Effect of CCK-33 on Primary Human Meningiomas

Primary meningioma cells were seeded at $1x10^5$ cells / flask and cultured for 12 days in the presence of CCK-33 (100nM) after which the cells were counted. Significant stimulation was observed at day 3 (1.8-fold) and this continued throughout day 6 (1.1-fold) ($P<0.001$) after which effects began to decline (figure 39).

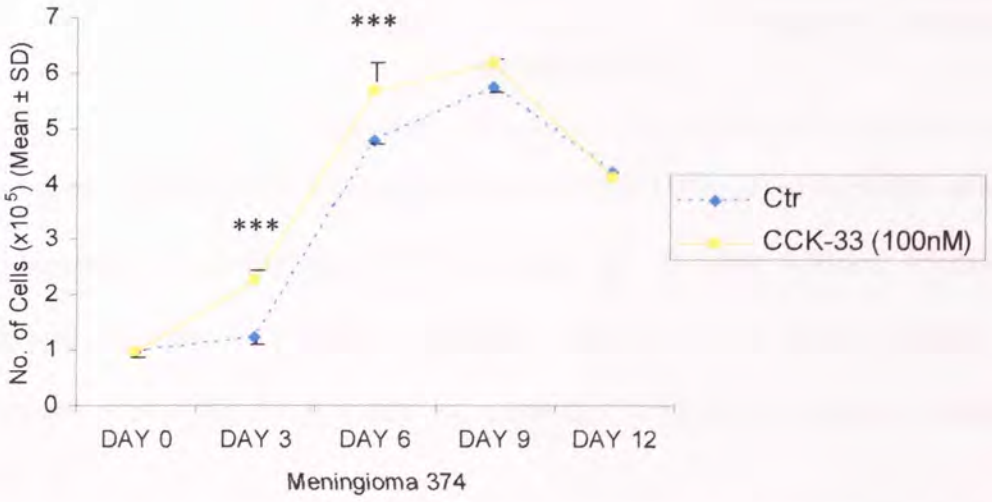


Figure 39: Stimulatory time course of CCK-33 (100nM) on *in vitro* cell growth of meningioma (374) (passage no. 1) after 12 days. Triplicate cultures were used for each variable; n=1. ***P<0.001 vs. control.

Effect of CCK-33, CCK-8s and EGF ± CCK-A and CCK-B Antagonists on Primary Human Meningiomas

Figure 40, shows a significant increase in meningioma derived cell growth stimulated with EGF (10ng/ml) after 6 days. Primary meningioma cell growth was strongly inhibited by selective non-peptide antagonists to CCK-B receptor (100nM), which completely abolished the stimulatory effects of EGF during co-incubation.

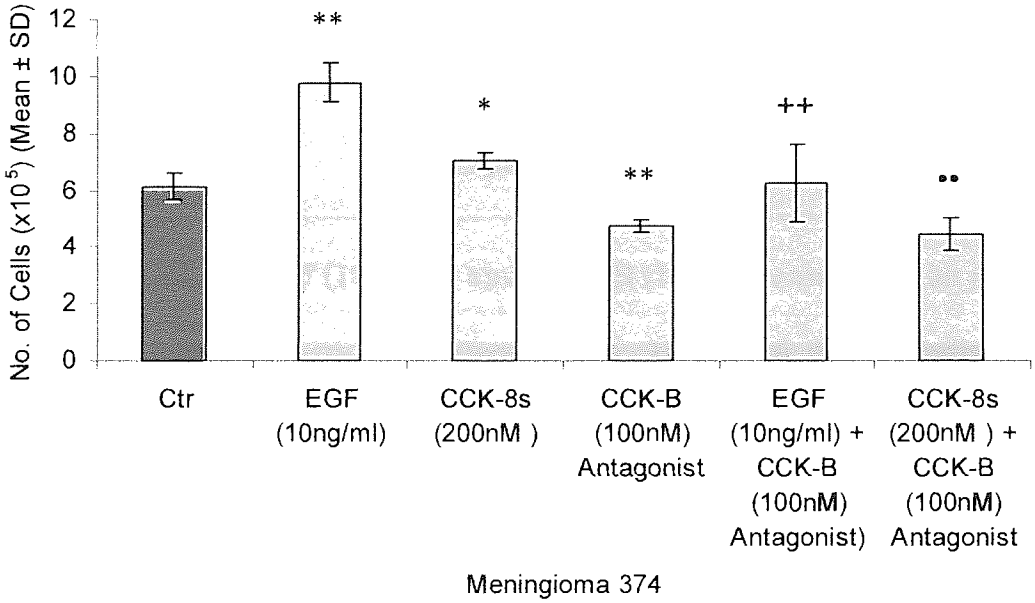


Figure 40: Effects of EGF (10ng/ml) and CCK-B (100nM) antagonist on *in vitro* cell growth of meningioma (374) (passage no. 2) after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.001 vs. EGF; ••P<0.01 vs. CCK-8s.

Figure 41, shows a significant increase of meningioma derived cell growth when stimulated with EGF (10ng/ml) after 6 days. Primary meningioma cell growth was

strongly inhibited by selective non-peptide antagonists to CCK-A receptor (100nM), which completely abolished the stimulatory effects of EGF when co-incubated.

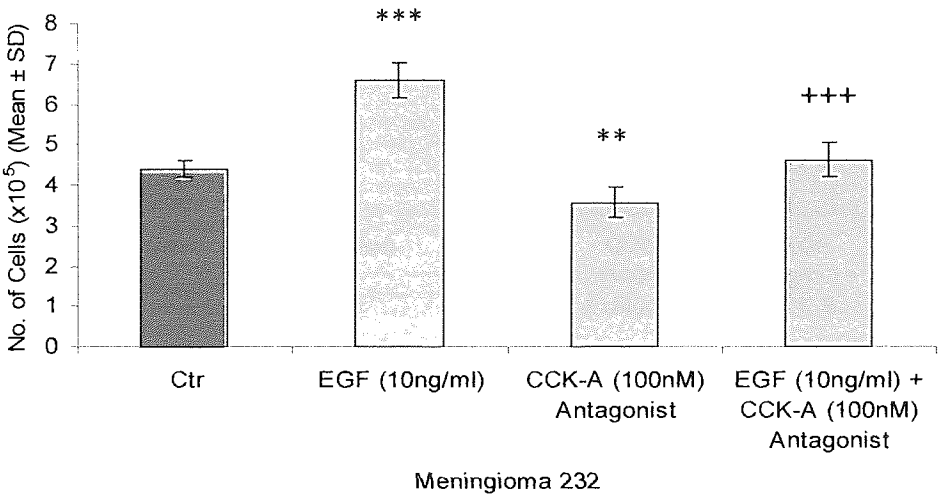


Figure 41: Effects of EGF (10ng/ml) and CCK-B (100nM) antagonist on *in vitro* cell growth of meningioma (232) (passage no. 1) after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. EGF.

Similar experiments on a different meningioma derived cells showed a significant increase in cell growth rate when stimulated with CCK-8s (200nM) and EGF (10ng/ml) after 6 days. Primary meningioma cell growth was strongly inhibited by selective non-peptide antagonists to CCK-B receptor (100nM), which completely abolished the stimulatory effects of CCK-8s and EGF when co-incubated as shown in figure 42.

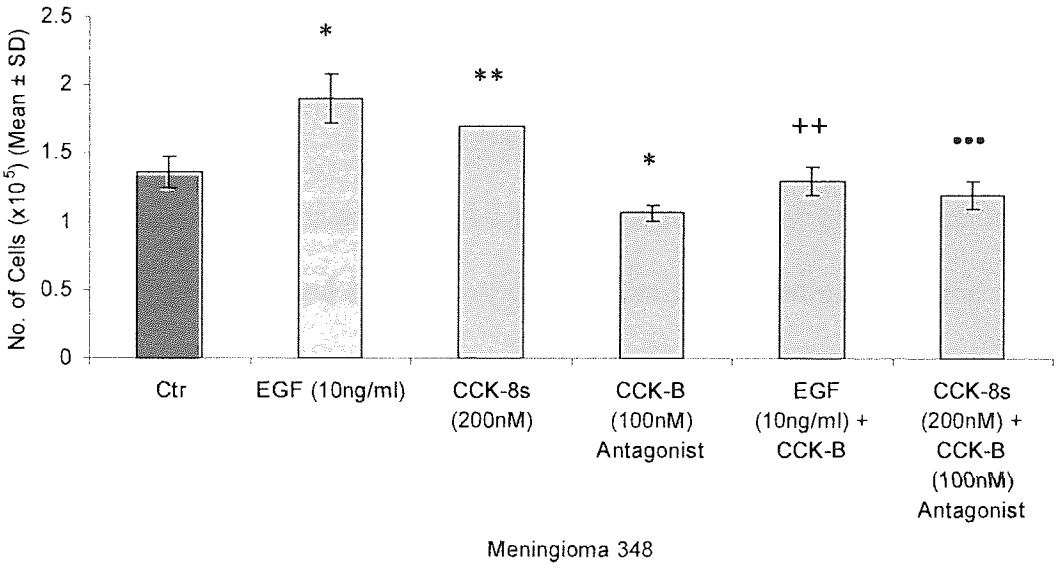


Figure 42: Stimulatory effects of EGF (10ng/ml) and CCK-8s (200nM) alone and in combination with CCK-B (100nM) antagonist on *in vitro* cell growth of meningioma (348) (passage no. 1) after 6 days. Media were changed on day 3. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; vs. control; $^{++}$P<0.001 vs. EGF; •••P<0.001 vs. CCK-8s.

2.4 DISCUSSION

2.4.1 CCK, Gliomas and Meningiomas

Malignant brain tumours such as glioblastoma multiform are very difficult to successfully treat and inevitably prove to be lethal. About 80% of patients die within the first year of diagnosis, despite extensive surgical excision and adjuvant radio- and chemotherapy (Lefranc *et al.*, 2002). In contrast, meningiomas, even those that are malignant, do not usually prove to be lethal, but do cause pressure effects that lead to secondary disorders. The pressure of a growing meningioma can sometimes be lethal. Their treatment resistance is related to their exceptional

migratory nature and ability to insinuate themselves seamlessly and extensively into neuronal tissue. Incidence of gliomas is around 1% and 0.8% for meningiomas in the general population (Behin *et al.*, 2003).

Recent studies have revealed a crucial role for a number of growth factors and cytokines in regulation and invasion of gliomas into surrounding structures. Their function lies on the one hand in the autocrine stimulation of the tumour cells themselves and on the other hand the growth factors and cytokines seem to play a major role in the paracrine activation of the tumour by surrounding stroma. Gastrin has been described as such a growth factor (Lefranc *et al.*, 2002). In addition, CCK closely related and of the same family of amidated peptide hormones as gastrin, was shown to powerfully stimulate growth, and induce Ca^{2+} mobilization of rat glioma C_6 cells *in vitro* (Orino *et al.*, 1991). These effects are by a protein kinase C (PKC)-dependent mechanism via the CCK-B receptor (Kaufmann *et al.*, 1995a; Kaufmann *et al.*, 1998). As the tumour infiltrating progresses, islands of host tissue are incorporated within the neoplasm (Russel and Rubinstein, 1977; Zulch, 1986) and normal brain elements such as astrocytes and even neurons are found more or less deeply within the tumour (Cox, 1933; Scherer, 1940). It is a very remarkable feature of the silver stains, as applied to glial tumours that neurones seem to be well preserved in the neoplasm without evidence of degradation (Cox, 1933). Demonstration of CCK immunoreactivity in the neurones enclosed in the tumour provides the identification that the cytoskeleton is not the only neuronal element to be preserved (Przedborski *et al.*, 1988). It is likely that the persistence of neuropeptide-containing neurons in all anaplastic astrocytomas could be the result of a less aggressive interaction between the tumour and the host tissue. Indeed, the

anaplastic astrocytoma and meningiomas exhibit a marked tendency for extensive infiltration without any major reaction of the normal tissue. Therefore it has been suggested that the presence of neurons within the anaplastic astrocytomas may be regarded as a source of CCK which could modulate the behaviour of these tumours and facilitate their progression into higher grades (Przedborski *et al.*, 1988). For some time, CCK has been suggested to contribute to glioma proliferation and recent studies have suggested that these peptides, in addition to their physiological role may play an important role in tumour growth regulation (Smith and Solomon, 1988; Rehfeld and van Solinge, 1994).

Gastrin and CCK are both members of the amidated peptide hormone family and are characterized by an identical carboxyl-terminal pentapeptide sequence (-Gly-Trp-Met-Asp-Phe-NH_2). Both are widely distributed throughout the CNS and the digestive tract (Noble *et al.*, 1999). CCK is a regulatory peptide which exists in several molecular forms varying in length from 4 to 58 amino acid residues (Rehfeld, 1978; Cantor and Rehfeld, 1987; Carton, 1989). All these peptides share the carboxyl terminal region, displaying different lengths of their amino-terminal extension. The carboxyl-terminal octapeptide (CCK-8) is common to most members of the CCK / gastrin family and retains the whole biological activity. Carboxyl terminal amidation and sulphation are essential for CCK function (Vinayek *et al.*, 1987; Huang *et al.*, 1989) and it appears that sulphation of the tyrosine residue at position 27 is required for biological activity (Noble *et al.*, 1999). CCK is produced by both nerves and endocrine cells in the gut. CCK is found in the I cells of intestinal mucosa with higher concentrations in duodenum and upper jejunum (Buffa *et al.*, 1976) and CCK immunoreactive nerve fibres have been demonstrated

in the pancreas of several species (Larsson, 1979). However, CCK is found in higher concentrations in the brain than in the gut, where it serves as a neurotransmitter or a neuromodulator (Beinfeld, 1997). This peptide, initially characterized as a 33-amino-acid sequence, is present in a variety of biologically active molecular forms derived from a 115-amino-acid precursor molecule (prepro-CCK) (Deschenes *et al.*, 1984) that passes through the regulated secretory pathway where it is processed to smaller bioactive forms after cleavage by trypsin-like enzymes (Straus and Yalow, 1978; Malesci *et al.*, 1980) and is secreted in response to specific stimuli (Beinfeld, 1997). The brain specializes in producing the small forms, such as CCK-8, while the intestine makes larger forms like CCK 58, 33, and 22. Even though all these forms contain CCK-8 amide and share the same biological activity, they differ in their amino terminal extension (Beinfeld and Wang, 2002). In plasma, after a meal, the most abundant circulating CCK molecules are the large forms such as CCK-58, CCK-39, CCK-33, CCK-22, and CCK-8 (Liddle *et al.*, 1985). However, in the CNS, short fragments of the peptide like CCK-4 and CCK-8 sulphated (CCK-8s) and non-sulphated (CCK-8ns) are predominant (Dockray, 1982; Dockray *et al.*, 1985; Rehfeld *et al.*, 1985). In general, CCK-8 is the predominant form in the CNS, whereas CCK-33 is present in gut tissues (Saito *et al.*, 1980; Wank *et al.*, 1992). A variety of physiological roles are attributed to CCK as a gastrointestinal hormone and / or neurotransmitter (Lewis and Williams, 1990). These include stimulation of pancreatic enzyme secretion, inhibition of gastric emptying, potentiation of amino acid-induced insulin secretion in humans, and direct stimulation of insulin secretion by activation of pancreatic β-cell CCK receptors, regulation of gall bladder contraction and food intake. In the CNS, CCK can produce antinociception (Hill *et al.*, 1987).

On the basis of ligand binding and molecular biology studies, two main classes of receptors have been identified, namely, the CCK-A and CCK-B / gastrin receptors (Moran *et al.*, 1986; Jensen *et al.*, 1994). Despite sharing 50% sequence identity, CCK-A and CCK-B receptors can be clearly identified by using a number of selective CCK agonists and antagonists (Noble *et al.*, 1999). CCK-A receptors bind sulphated CCK far more efficiently than non-sulphated forms (table 8). In contrast, CCK-B receptors bind both forms as well as gastrin (Saito *et al.*, 1980; Moran *et al.*, 1986; Hughes *et al.*, 1990) (table 8). Both CCK-A and CCK-B receptors are members of the seven transmembrane G protein-coupled receptors. CCK-A receptors predominate in the periphery and mediate actions such as pancreatic enzyme secretion, gall bladder contraction and gut motility. CCK-B receptors are predominant in the CNS (Shulkes and Baldwin, 1997). CCK and gastrin also regulate growth of normal tissues as well as of gastrointestinal cancers (Townsend *et al.*, 1986). It might therefore be anticipated that blockade of CCK receptors will have an inhibitory effect on tumour cell mitosis.

Table 8-a: Inhibition of specific ^{125}I-CCK binding to CCK-B and CCK-A receptors by CCK agonists

	IC_{50} (nM)	
Ligand	**CCK-A (Rat Pancreas)**	**CCK-B (Mouse Cortex)**
CCK-8	59	2.6
CCK-8s	0.12	0.27
Gastrin	845	51

Hughes *et al.*, 1990; ^{125}I-CCK (50pM)

Table 8-b: Inhibition of specific ^{125}I-CCK-8 binding to human CCK-A and CCK-B receptors by CCK agonists

	IC_{50} (nM)	
Ligand	**CCK-A (Human Gallbladder)**	**CCK-B (Human Cerebral Cortex)**
CCK-8	900 *	Limited previous data on human tissue
CCK-8s	10 *	9.7 +

* Tokunaga *et al.*, 1993; ^{125}I-CCK-8s (150pM)

+ Hill and Wooddruff, 1990; ^{125}I-CCK-8s (50pM)

A wealth of findings during the past decade on the expression of (proto)oncogenes and the nature of their protein products has given impetus to the concept of autocrine growth factors and their involvement in neoplastic growth (Sporn and Roberts, 1985; Goustin *et al.*, 1986). *In vitro* studies have shown that many types of tumour cells secrete polypeptide growth factor-like molecules which bind to membrane receptors on the same cells, thereby eliciting a mitogenic response. The present findings provide strong evidence that an analogous system operates within gliomas and meningiomas. The data suggest that proliferation of gliomas and meningioma cells may be under autocrine control, since CCK stimulated growth of freshly passaged cells themselves, which was significantly inhibited by novel and established CCK antagonists.

Conflicting data have been published on whether tumours of the central and peripheral nervous system in general, and gliomas in particular, express CCK receptors (Kaufmann *et al.*, 1995a; Reubi, 2003). The present study provides solid evidence of mRNA expression of CCK peptide and its CCK-B receptor subtype by most, if not all, human gliomas. RT-PCR studies revealed that CCK-B receptor subtype along with CCK mRNA was expressed in all tumours tested, whereas CCK-A receptor (14%) was limited to the glioblastoma-type tumours whereas CCK-A receptor was far more frequently (66%) expressed in human meningiomas. Since G-protein-coupled seven-transmembrane domain receptors are over expressed in gliomas (Roettger *et al.*, 1997) these results may be of high significance to tumourigenesis. CCK receptor expression was also investigated in primary glioma and meningioma cell cultures and it was observed that both glioma and meningiomas cells in culture tend to lose expression for CCK-B but not for CCK-A receptor. Only 2 tumours were shown to express CCK-A receptor even after the ninth passage. In contrast meningiomas cultured as monolayer quickly lost their ability to express either the CCK-A or B receptor as early as the second passage. The loss of CCK receptor expression and a high proliferation rate in meningiomas cultured as monolayer indicate that the cells might dedifferentiate when cultured. Because of this loss, growth experiments were performed only on primary freshly passage cells. The present novel finding may comprise valuable information for experimental studies on primary gliomas and meningiomas.

The presence of CCK-receptors together with CCK peptide expression itself may well suggest the presence of a paracrine / autocrine loop controlling glioma cell growth. In support of this conclusion, experiments with a neutralizing antibody

against the CCK peptide significantly inhibited non-stimulated glioma growth, which suggests that anti-CCK antibody can neutralize extracellular CCK, being secreted by the cells (Kaufmann *et al.*, 1995b). This is the first time that a paracrine / autocrine role of CCK in human glioma cell proliferation has been demonstrated. It was only recently (Xu *et al.*, 1996) that the first evidence for an autocrine / paracrine role for CCK was described for the GH3 rat pituitary tumour cell line thought to be mediated by the CCK-B receptor. Therefore, it is evident that uncontrollable growth of gliomas is, at least in part, due to abnormal secretion of these growth factors. Evidence of CCK peptide and CCK-B receptor mRNA expression in human gliomas provides further evidence for these findings. From these early studies we were able to form a hypothesis for the action of the CCK system that involves growth stimulation and tumour progression of human gliomas due to the probable autocrine / paracrine role of CCK in the CNS (figure 43).

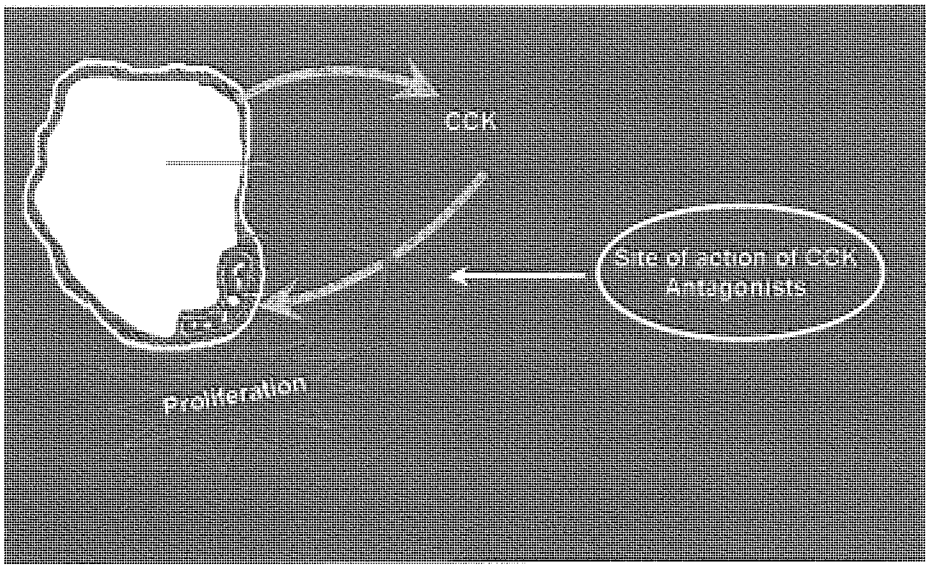


Figure 43: Proposed autocrine / paracrine stimulation of CCK hormone secretion in human gliomas and meningiomas.

Moreover, expression of CCK itself along with its type B receptor on human meningiomas introduces the concept of autocrine regulation by CCK for this type of tumour as well. Some studies (Adams *et al.*, 1991) have shown that uncontrollable growth of meningiomas is, at least in part, due to abnormal secretion of various growth factors. It is of interest that despite the increasing research of CCK peptide involvement in brain tumours and cancer related conditions, there are only two studies (Reubi *et al.*, 1998; Lefranc *et al.*, 2003), where meningiomas were looked at only in terms of CCK receptor expression. The present study provides clear evidence of CCK and CCK-B subtypes expression on meningiomas and proposes that CCK is one peptide that is normally expressed in brain tissue since it has been abundantly found in the CNS, which abnormally promotes meningiomas cell growth possibly in an autocrine / paracrine manner. Whether the CCK system is involved in the oncogenesis of these neoplasms remains to be elucidated. However, the fact that CCK is involved in the maintenance of these tumours as shown *in vitro* supports the concept. To further test the hypothesis that gliomas and meningiomas are under autocrine control by CCK, studies were directed to determine whether CCK peptides could stimulate human glioma cell growth and could be antagonised by CCK-A and B specific antagonists. In addition PI and cAMP dependent second messenger systems were investigated to establish functionality of both CCK receptor subtypes.

As demonstrated in the present studies and previously been reported, activation of human glioma CCK receptors leads to PI hydrolysis but not cAMP formation (Iversen *et al.*, 1996). It is therefore concluded that CCK-B is the receptor via which CCK carries out its effect on human gliomas. However, it is of interest that PI

hydrolysis rapidly declined within 1 hour of agonist stimulation. One possible explanation is rapid de-activation of the receptor may be as a result of receptors desensitization due to accumulation of CCK from endogenous paracrine / autocrine secretion as well as additive CCK ligand. The CCK receptor is regulated via the three principal mechanisms that are important for regulating all G-protein coupled receptors (GPCRs): desensitization, sequestration, and down regulation. Cell surface receptor internalization and trafficking are essential in the latter two forms of receptor regulation. Desensitization of cellular responses to CCK stimulation followed by compartmentalization of the agonist occupied CCK receptor has been demonstrated (Roerig *et al.*, 1996) and it is believed to be via clathrin dependent and independent pathways, but independent of G protein activation and receptor phosphorylation (del Valle, 1999). Since the phosphoinositide / PKC pathway is not involved in CCK receptor trafficking, it remains unclear whether the moderate PI hydrolysis is due to receptor down regulation as a result of receptor desensitization. CCK receptor desensitization may provide a cellular mechanism to assure rapid desensitization and carefully regulated resensitization (Roerig *et al.*, 1996). Therefore adding CCK to a system that controls its normal secretion may initiate a positive growth regulation in gliomas in order to protect the cell from potentially damaging overstimulation. Roettger *et al.* (1997) demonstrated that an antagonist to CCK-A (L-364,718) stimulated receptor internalization, which may not be independent of a possible agonist-induced internalization. Both CCK-8s and CCK-33 directly stimulated human glioma and meningioma cell growth *in vitro*. It may be that CCK is another growth factor contributing to glioma and meningioma growth and possibly the invasion process (Lefranc *et al.*, 2002). However, the effect exerted by CCK-8s was not as potent as previously described in rat glioma C_6 cells

(Orino *et al.*, 1991; Lefranc *et al.*, 2003). In addition, the presence of an autocrine / paracrine loop in the CCK system might explain the weak stimulatory effects of exogenously added CCK peptides on glioma and meningioma growth as assessed by both cell numbers and thymidine incorporation studies.

2.4.2 Effect of Novel and Established Non-Peptide CCK Antagonists on Human Glioma Cell Growth

Having established that glioma and meningioma growth may be under autocrine / paracrine control of CCK which is mediated through the CCK-B receptor subtype it was of prime importance to explore the possibilities of disrupting this autocrine mechanism that operates within these neoplasms. The approach involved novel and well established non-peptide CCK antagonists that if proven to be potent antagonists may comprise novel adjuvant modes of therapy as an alternative treatment to primary neurosurgery and radiotherapy. Combining experiments to include novel versus the well established available CCK antagonists gave an insight on the potency of these newly developed antagonists. The ultimate aim is to synthesize non-peptide orally active antagonists that can be ultimately used as a therapy for brain tumours.

Subtype specific CCK receptor antagonists can be used to discriminate between the CCK-A and CCK-B receptor subtypes. In addition, the affinities for the C-terminal CCK peptides can also conveniently by used to discriminate between CCK-A and CCK-B receptors. CCK-8s binds selectively and with 100 fold higher affinity than the CCK-8 to the CCK-A receptor subtype. The CCK-B receptor has similar high affinity for CCK-8s and CCK-8 (Hughes *et al.*, 1990). The discovery of Asperlicin

(Chang *et al.*, 1985), a potent, non-peptide, benzodiazepine-like metabolite of *Aspergillus alliaceus*, was the starting point for scientists to develop specific and potent CCK antagonists of the multiple stimulatory effects of CCK-8s on islet tissue. The development of a number of selective, potent antagonists, some of which penetrate the blood-brain barrier, has greatly enhanced our understanding of the pharmacology and physiology of CCK. These antagonists have been derived from cyclic nucleotides, amino acids, CCK and gastrin, benzodiazepines, quinazolinone, and diphenylpryzolidinone derivatives (Presti and Gardner, 1993). The CCK-A (L-364,718) antagonist is chemically a 3-(acylamino)benzodiazepine analogue whereas, the CCK-B antagonist (L-365,260) is chemically a 3-(benzoylamino)benzodiazepine compound. These compounds provided the first tools for the pharmacological discrimination between the peripheral CCK-A and the central CCK-B receptor subtypes. L-364,718 was extremely potent at antagonising the binding of ^{125}I-CCK to pancreatic CCK-A receptor (IC_{50} 0.19nM), whereas L-365,260 was found to be a selective antagonist of the brain CCK-B receptor (IC_{50} 5.2nM) (table 9). Similarly, the novel non-peptide CCK antagonists (HSH and 4.7.5) also exploited in this study are natural product-like products also based on the Asperlicin structure. Thus, the natural lead compound provided a chemical target which was easy to synthesize and biologically active.

Table 9-a: Inhibition of specific ^{125}I-CCK binding to CCK-A and CCK-B receptors by two established benzodiazepine CCK antagonists

	IC_{50} (nM)	
Ligand	**CCK-A (Rat Pancreas)**	**CCK-B (Mouse Cortex)**
L-364,718	0.19	31.7
L-365,260	240	5.2

Hughes *et al.*, 1990; ^{125}I-CCK (50pM)

Table 9-b: Inhibition of specific ^{125}I-CCK-8 binding to CCK-A and CCK-B receptors by two novel benzodiazepine CCK antagonists

	IC_{50} (nM)	
Ligand	**CCK-A (guinea-pig pancreas)**	**CCK-B (guinea-pig brain)**
HSH	10	4500
7.4.5	20	25

Lattmann *et al.*, 2001; ^{125}I-CCK-8s (25pM)

Table 9-c: Inhibition of specific ^{125}I-CCK-8 binding to human CCK-A and CCK-B receptors by two established benzodiazepine CCK antagonists

	IC_{50} (nM)	
Ligand	**CCK-A (Human)**	**CCK-B (Human Cerebral Cortex)**
L-364,718	Limited previous data on	6.9
L-365,260	human tissue	8.2

Hill and Wooddruff, 1990; ^{125}I-CCK-8s (50pM)

In the present studies, both novel (HSH and 7.4.5) and commercially available (L-364,718 / CCK-A and L-365,260 / CCK-B) non-peptide CCK antagonists had powerful inhibitory effects on human glioma and meningioma cell growth. The novel CCK antagonist, HSH and 7.4.5 strongly inhibited growth of the human glioma cells in a dose-dependent manner *in vitro*. In addition both antagonists completely abolished the significant stimulation exerted by EGF on primary human glioma cell growth. A time course study revealed that these inhibitory effects remained consistent for at least two weeks in culture. Similar findings were

obtained using [^{3}H]thymidine uptake performed on both U-87 MG cells and on primary gliomas in culture. These results suggest that these novel CCK antagonists are able to inhibit human glioma cell growth.

The specificity of the novel and established CCK antagonists was not addressed in the growth inhibition experiments described here since reversibility was not tested. However, published experiments demonstrate that the established CCK antagonists are reversible. The *in vivo* pharmacological activity of the CCKB antagonists on Chinese hamster ovary (CHO) cells expressing the human CCK-B receptor revealed a Ca^{2+} mobilization pattern consistent with that expected for a competitive CCK-B antagonist in the presence of CCK-4 agonist (0.1-1000nM) (Dunlop *et al.*, 1997). In a further study, increasing concentrations (30-10,000nM) of L-365,260 produced progressive rightward shifts in the concentration effect curve for CCK-4 without affecting the maximum response to the antagonist. In addition, three successive exposures to CCK-4 were applied with co-application with L-365,260 during the second agonist stimulation. Antagonist activity was clearly demonstrated and the cells were fully responsive to CCK-4 after a 1 hour washout period demonstrating reversible antagonist activity of this compound (Dunlop, 1998). Considering the above and the fact that all the antagonists incorporated in this study, including the novel ones, share the same non-toxic natural-like structure it is hypothesised that all the antagonists will behave as competitive antagonists. The inhibitory effect observed in the present studies is most likely to occur via antagonism of the CCK-B receptor since earlier studies have described high affinity CCK-B type receptors and their stimulation of inositol metabolism, calcium signalling and protein phosphorylation, as well as a CCK-8s-induced increase in [^{3}H]thymidine

incorporation in rat glioma C_6 cells (Kaufmann *et al.*, 1998). In addition RT-PCR analyses where CCK-B receptor was far more abundantly expressed on these neoplasms compared to the CCK-A receptor subtype strongly supports this hypothesis. Although both CCK-A (L-364,718) and CCK-B (L-365,260) antagonists inhibited cell growth dose-dependently and both had powerful effects, it was noted that potency of compound L-365,260 to inhibit CCK-8s induced cell growth, [^{3}H]thymidine incorporation and PI hydrolyses in U-87 MG cells was higher than estimated for compound L-364,718. This probably means that the CCK-A antagonists are not strictly selective and able, at least to an extent, to inhibit the CCK-B receptor as well, since RT-PCR analysis failed to provide any evidence for CCK-A receptor expression on U-87 MG cells. In a similar study where both CCK-A and B antagonists were used to antagonise CCK-8s and BC 264 CCK agonists, in rat glioma C_6 cells, which are known to possess CCK-B but not CCK-A receptor binding sites, the CCK-B antagonist was more effective than the CCK-A antagonists which also had an effect on [^{3}H]thymidine incorporation (Kaufmann *et al.*, 1995a). Therefore, it is concluded that effects of CCK-peptides on U-87 MG cell growth are mediated by activation of CCK-B type receptors and it is suggested that CCK-B receptors may play an important role in brain cancer cell proliferation. In addition results reported for the canine parietal gastrin receptor (CCK-B-R) showed a 7-fold greater affinity for the CCK-A receptor antagonists L-364,718 than for the gastrin receptor antagonists L-365,260 (Kopin *et al.*, 1992). This divergence in canine gastrin receptor reversal in affinity for the CCK-A and CCK-B antagonists is of great significance in this present study. Alternatively, although the RT-PCR analysis failed to reveal the CCK-A receptor in many cases they may be a technical problem in which false negative results were yielded. As far as the novel non-

peptide antagonists (HSH and 7.4.5) are concerned, they are relatively new compounds and it is still uncertain whether HSH is highly selective for the A receptor to comment on its antagonistic effects on U-87 MG cells that lack CCK-A receptor binding sites. Further experiments are required to demonstrate the involvement of PKC activation in CCK antagonist action. In addition, it will be important to demonstrate binding of the antagonists to CCK receptors and to determine the primary receptor involved. This is significant since recent data suggest that the several of forms of gastrin and CCK and the several types of CCK receptors may be responsible for tumour growth stimulation. Whereas both amidated gastrin and CCK have growth promoting properties, these endogenous peptides may not only act through CCK-A and CCK-B receptors to mediate this growth stimulation, but perhaps also through CCK-C receptors or through receptors selective for glycine-extended gastrin similar to the receptor described on the rat pancreatic carcinoma cell line AR4-2J (Schaer and Reubi, 1999).

In this case, because of its non-peptide nature, HSH may prove to be an orally active agent against human gliomas. Recently, selective non-peptide antagonists have been developed which supports both the CCK-A and CCK-B receptor subtype classifications. Two of the most potent and selective antagonists are L-364,718 for CCK-A receptors and L-365,260 for CCK-B receptors (Pisegna *et al.*, 1992) that strongly inhibited endogenous and exogenous CCK stimulated glioma and meningioma cell growth. PI hydrolysis performed on antagonistic experiments revealed full functionality for both of the CCK-A and CCK-B receptors via which both CCK antagonists actively antagonised the exogenous stimulatory effects of CCK peptides on glioma and meningioma cell growth *in vitro*. Such a therapy may,

on one hand, prevent gastrin / CCK-induced symptoms in healthy tissue, whereas, on the other hand, it may prevent a further growth of those tumours that express CCK receptors in addition to the peptide itself.

2.4.3 Antagonists to CCK Inhibit Glioma Growth via Apoptosis

Recently a new group of antitumour agents, proteasome inhibitors, has been characterized (Drexler, 1997; Held-Feindt *et al.*, 2000). Proteasome inhibitors induce apoptosis in several tumour cell types such as pancreatic and lung cancers. Caspases, mediators of apoptosis, are often activated although the primary trigger of apoptosis is still unclear (Lopes *et al.*, 1997). In the present study caspase-3 activity was increased by the antagonists, the end mediator of apoptosis, and proteasome inhibition occurred after treatment of U-87 MG with antagonists to CCK. Since the ubiquitin-proteasome system, which is fundamental non-lysosomal tool that cells use to process or degrade a variety of short-lived proteins, is involved in apoptosis (Yu *et al.*, 2002), mechanisms for apoptosis may be cell-specific considering the different proteins targeted for degradation in different cells types (Lopes *et al.*, 1997). The proteasome mediated step(s) in apoptosis is located upstream of mitochondrial changes and caspase activation and can involve different systems, such as NF-κB, Bax and Bcl-2 (Meng *et al.*, 1999; Drexler *et al.*, 2000; Tani *et al.*, 2001). The ubiquitin/proteasome system regulates protein turnover by degrading polyubiquitinated proteins. To date, all studies on the relationship of apoptosis and the proteasome have emphasized the key role of the proteasome in the regulation of apoptosis, by virtue of its ability to degrade regulatory molecules involved in apoptosis. Some studies demonstrated that proteasome inhibitor itself induced apoptosis of certain cells including human glioma cells (Sadou *et al.*, 1996;

Orlowski, 1999), whilst others reported that apoptotic stimuli induced apoptosis by inhibiting proteasome activity of the target cells (Choi *et al.*, 2003). According to recent studies, this caspase-mediated cleavage inhibits the proteasomal degradation of ubiquitin-dependent and –independent cellular substrates, including proapoptotic molecules such as Smac, so facilitating the execution of the apoptotic program by providing a feed-forward amplification loop (Sun *et al.*, 2004). The present study supports previous findings and clearly demonstrates how CCK antagonists induce apoptosis by strongly inhibiting proteasome activity in human gliomas. Apoptotic activity on U-87 MG cells was also confirmed by etoposide which is one of the more effective drugs in the treatment of human glioma (Yin *et al.*, 2000). Etoposide has been shown to induce apoptotic cell death in various cell types, including human glioma U-87 MG cell line (Mesner *et al.*, 1997; Yin *et al.*, 2000). In addition, proteasome is selectively involved in the pathway used by etoposide to induce cell suicide (Stefanelli *et al.*, 1998). However, proteasome-induced apoptosis in U-87 MG by lactacystin was found to be a mitochondrial-independent activation of caspase-3. One possible mechanism for anti-growth effects by the CCK antagonists may be via induction of apoptosis, and may be caspase- and mitochondrial- dependent. Elucidation of the changes that occur in mitochondria during CCK antagonist induced apoptosis is essential. In addition, the mechanism underlying the proteolytic cascade also needs to be investigated. Proteasome inhibitors, as single or combined with other anticancer drugs agents, are suggested to be a new class of potential anticancer agents (Orino *et al.*, 1991; Kaufmann *et al.*, 1995a).

2.5 Conclusion

The precise molecular mechanisms underlying the regulation of brain tumour growth remains unresolved. Recent studies have revealed involvement of a number of growth factors and cytokines in regulating invasion of gliomas in surrounding structures. Gastrin has been described as one, and found to act as an endogenous modulator of glioma progression and invasion to surrounding structures (Lefranc *et al.*, 2002). This study provides evidence of an autocrine / paracrine growth stimulatory loop by CCK and its receptor(s) in glioma and meningioma cells. However, it should be noted that although we have established the status of CCK in human brain tumours (gliomas and meningiomas) it has not been possible to gather any information or examine normal brain elements and whether they can also secrete mitogenic factors in culture. In addition, it is becoming evident that implementation of antagonists to CCK, L-365,260 and L-364,718 in the antiglioma / anticancer is a distinct possibility.

CHAPTER THREE

HUMAN PITUITARY TUMOURS: CCK, HORMONE SECRETION AND SIGNIFICANCE OF A GHRH-R POLYMORPHISM

3.1 INTRODUCTION

The pea-sized pituitary gland or "master endocrine gland" is extremely important in regulating the function of many other endocrine glands and organs of the body. It lies behind and between the eyes at the base of the brain, just beneath the hypothalamus to which it is connected by a thin stalk. The pituitary gland is controlled by substances (releasing and inhibitory factors) produced by the hypothalamus. The pituitary gland, in turn, secretes hormones into the bloodstream, being released from five different types of cells that the adenohypophysis consists of (gonadotrophs, lactotrophs, corticotrophs, thyrotrophs and somatotrophs). These secreted hormones (growth hormone, prolactin, adrenocorticotropin, thyroid-stimulating hormone, luteinizing hormone and follicle stimulating hormone) (Horvath and Kovacs, 1991) are chemical messengers that instruct various glands to secrete their own hormones or cause cellular division and differentiation. Unlike other brain neoplasms, pituitary tumours arise from functional epithelial cells and are often associated with an endocrine disease because of excessive hormone secretion. Thus, they may secrete excessive amounts of prolactin (PRL) (prolactinomas) and thereby cause infertility. Likewise, tumours of the GH-secreting cells (somatotrophinomas) cause either gigantism or acromegaly as a consequence of tissue overgrowth.

The true incidence of pituitary tumours has not been established with certainty, but it is suggested that they occur in approximately 20% of the general population, but are not diagnosed in the majority of cases (Kovacs and Horvath, 1986). Human pituitary tumours can be divided into two broad groups: overt secretory tumours, which lead to endocrine disorders such as acromegaly and Cushing's disease

because of excessive hormone production, and those apparently not associated with obvious endocrine dysfunction (Adams and Mashiter, 1985; Miura *et al.*, 1985). The latter are classically referred to as 'functionless' pituitary tumours and their diagnosis is usually as a result of pressure effects leading to presenting symptoms such as headache and visual field defects (Losa *et al.*, 2001). However, with the application of additional and improved techniques, together with increasing knowledge of the anterior pituitary hormones, it is becoming clear that some 'functionless' pituitary tumours exhibit secretory characteristics and thus can themselves be subdivided on the basis of hormone production (Mashiter *et al.*, 1981; Snyder *et al.*, 1985). Thus, by means of pituitary 'explant culture', a technique developed by Adams and Mashiter in the 1980s (1985), a subset of 'functionless' pituitary tumours has been demonstrated to be associated with gonadotrophin secretion. Subsequent studies confirmed these findings (Snyder *et al.*, 1985; Chaidarun and Klibanski, 2002) and further demonstrated that the luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion could be modulated by hypothalamic factors (Petersen *et al.*, 1996). It is now proposed that 'functionless' pituitary tumours which secrete LH / FSH should be regarded as gonadotrophinomas (Losa *et al.*, 2001) and that, originally, many of these were not diagnosed as such because they often occurred in post-menopausal women in whom serum gonadotrophin levels were physiologically elevated (Chaidarun and Klibanski, 2002). However, the concept that 'functionless' pituitary tumours are gonadotrophinomas can be questioned and instead be attributed to limited transdifferentiation (Childs, 2002).

Based on the understanding of the factors which control pituitary hormone secretion, non-invasive medical therapies for pituitary tumours have been developed, eliminating the need for neurosurgical intervention. The prime example is prolactinoma. PRL secretion and growth of PRL-secreting cells is inhibited by dopamine and this has led to development of dopamine agonists which are able to completely control prolactinomas (Molitch, 2001). For example, bromocriptine (a dopamine agonist) normalizes serum PRL levels and shrinks the pituitary tumour, thus eliminating the need of neurosurgical intervention. In contrast, the primary treatment of choice for GH-secreting and 'functionless' tumours remains transsphenoidal removal, but this method is by no means completely successful in restoring GH levels to normal (Melmed, 1993). Clearly a better understanding of the biochemistry and molecular biology of these latter types of pituitary tumours may lead to development of medical therapies in much the same way that medical therapy of prolactinomas has been designed.

There is an on-going controversy regarding the basis of pituitary tumourigenesis. Two prevailing theories have predominated: external hormonal stimulation or intrinsic pituitary defect at the gene level. Several animal studies provide support for the role of hormonal stimulation in the development of these neoplasms and there is evidence for intra-pituitary production of hypothalamic hormones that may be responsible for excess stimulation. In contrast, the monoclonal nature of pituitary tumours and the lack of associated hyperplasia accompanying pituitary tumours strongly argue for an intrinsic somatic defect(s) as the principal etiology contributing to the genesis of these lesions. Numerous factors have been shown to govern pituitary cell proliferation. These various hypophysiotropic hormones and

growth factors likely play a role as promoters of tumour cell growth in genetically transformed cells (figure 44). In some instances, abnormal forms of growth factor receptors may be important in the early stages of cell transformation consistent with the clonal composition of pituitary tumours (Ezzat, 2001).

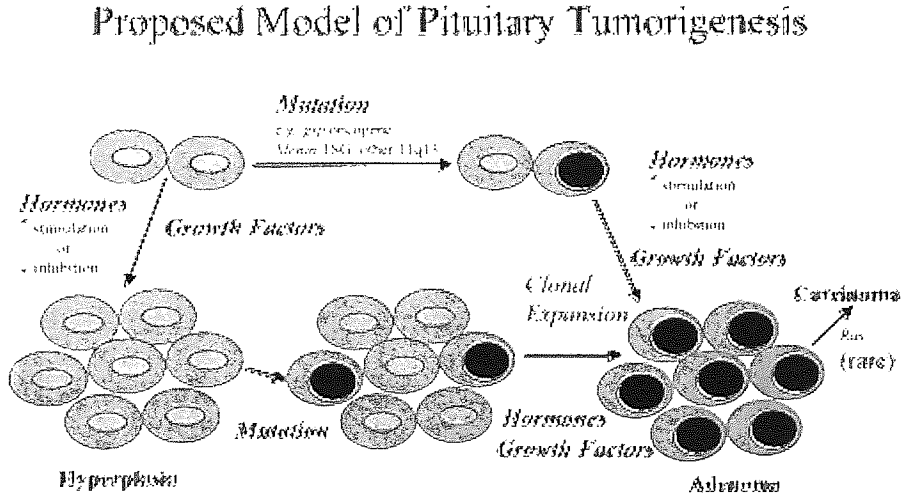


Figure 44: Proposed model of pituitary tumourigenesis. An integrated approach incorporates both the hormonal stimulation theory and the intrinsic pituitary defect theory of tumourigenesis. Animal models and patients with hypophysiotrophic hormone excess, suppressive hormone insufficiency, or growth factor excess develop hyperplasia (left pathway); the increased proliferation predisposes the cells to mutation (dark nuclei) and subsequent tumour formation. Most human pituitary tumours are unassociated with hyperplasia and likely result from a genetic event that alters a cell (dark nucleus, top right) that is the target for promotion by hormones or growth factors (right) (Asa and Ezzat, 1998).

3.1.1 GH-Secreting Pituitary Tumours

Considerable progress has been made in recent years into the understanding of the aetiology of GH-secreting tumours and there is some evidence that this may help in developing medical modes of therapy (Adams *et al.*, 1995). There is compelling evidence that the vast majority of human pituitary tumours are monoclonal in origin, suggesting that they arise as a consequence of somatic mutations (Herman *et al.*, 1990; Adams *et al.*, 2000). At least some secretory tumours are associated with activating mutations in genes controlling intracellular transduction pathways. The best understood are *gsp* oncogenes in GH-secreting somatotrophinomas, members of a subset of pituitary tumours that possess alterations in the $G_s\alpha$ gene. Such mutations lead to amino-acid substitutions which abolish the intrinsic guanosine triphosphatase (GTPase) activity of these G-protein subunits which ultimately lead to constitutive activity of the $G_s\alpha$-adenylyl cyclase-cAMP transduction system (Landis *et al.*, 1989). The constitutive production of cAMP is thought to be the cause of excessive GH secretion and pituitary tumour formation via excessive cell proliferation (Vallar *et al.*, 1987; Landis *et al.*, 1989). It appears that these tumours are amenable to medical therapy with analogues of somatostatin, the hypothalamic factor that inhibits GH secretion, presumably because of activation of G_i, which, in turn, inhibits cAMP production (Adams *et al.*, 1995).

This subset comprises about 40% of all somatotrophinomas but the molecular aetiology of excessive GH secretion in the remaining 60% of tumours remains unknown. However, there is some evidence that a polymorphism in the GH-releasing hormone (GHRH)-receptor (GHRH-R) gene may predispose subjects to development of GH-secreting tumours because it apparently confers increased

sensitivity to the stimulatory effects of GHRH (Adams *et al.*, 2000). Thus, it is possible that at least some of the remaining group of somatotrophinomas which lack *gsp* oncogenes may arise because of this polymorphism. It is hoped that discoveries like this will ultimately lead to novel modes of medical therapy for pituitary tumours based on targeting this GHRH-R subtype. In the present study the clinical significance of this GHRH-R polymorphism has therefore been further examined. Specifically, the incidence of the polymorphism in GH-secreting tumours was compared with that found in normal subjects and its influence on the effect of GHRH on GH secretion *in vitro* was determined.

3.1.2 Functionless Pituitary Tumours

Because they are not usually associated with hormone hypersecretion, functionless pituitary tumours are often not detected until they have grown considerably in size and begin to cause pressure effects such as headaches and visual field defects. The primary clinical problem with functionless pituitary tumours is thus caused by the tumour overgrowth *per se,* rather than hormone alterations. Because they are not associated with hormone hypersecretion, much less research has been directed at determining the receptor status of functionless pituitary tumours and the factors which might control their growth. Thus, in contrast to secretory tumours in which specific hormone lowering drugs can be used as adjuvant or primary therapy, a similar medical therapy for functionless tumours has not yet been developed. Neurosurgical intervention remains the treatment of choice for functionless pituitary tumours. However, it is clear from the progress made with GH and PRL-secreting tumours that an understanding of the receptor status and factors controlling pituitary cell function can lead to novel modes of therapy. For these reasons, the purpose of

the present work was to investigate the potential significance of the gut-brain peptide, CCK, on functionless human pituitary tumour biology.

A number of previous studies suggest that CCK is able to influence pituitary hormone secretion (Morley *et al.*, 1979; Smith and Freedman, 1996). In particular, direct stimulatory effects on growth hormone (GH) and PRL secretion have been described (Vijayan *et al.*, 1979; Imura *et al.*, 1981; Malarkey *et al.*, 1981; Matsumura *et al.*, 1984; Calogero *et al.*, 1993) indicating that CCK-receptors (CCK-R) are present on pituitary cells, although this has not yet been directly demonstrated. Early *in vivo* studies on rats have produced conflicting data on the effects of CCK on gonadotrophin (LH and FSH) secretion, with both stimulatory and inhibitory influences reported (Kawakam *et al.*, 1979; Hashimoto and Kimura, 1986). To date, there is a paucity of data on the effects of CCK on human pituitary hormones, especially on LH and FSH. In the present studies the fact that many functionless human pituitary tumours secret small amounts of LH and FSH was exploited. Cell cultures of functionless pituitary tumours were used to examine the effects of CCK on gonadotrophin secretion. In addition, similar studies were performed on GH-secreting tumours in order to confirm and extend earlier findings that CCK may play a role in controlling pituitary somatotrophs. In parallel to the *in vitro* cell culture studies, and in view of the growing evidence of stimulatory autocrine loops via peptides in human tumours, molecular techniques were used to determine whether human pituitary tumours produce CCK itself and express CCK receptors. Antagonists to CCK were also examined since these might prove to form the basis of developing novel medical therapies.

3.2 MATERIAL AND METHODS

Materials and Methods and the preparation of solutions are as previously described (refer to chapter two, pages 33-39) and only the extra additions are listed here.

3.2.1 Materials – Tissue Culture

Ferring, Kiel, Germanyu, Growth Hormone Releasing Hormone (GHRH) Somatorelin (human)

Jencons Scientific Ltd., Bedfordshire, UK, Ultrasonic Processor

Labco, Marlow, UK, Glass Culture Tubes

Netria, St. Bartholomew's Hospital, London, UK, Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), Growth Hormone (GH), and Prolactin (PRL) Enzyme Labelled Assay

Sigma-Aldrich Company, Stirling, UK, Estradiol (E2), Luteinizing Hormone Releasing, Hormone (LHRH) Human Acetate, Pronase E

Materials – Molecular Biology

MWG AG Biotech., Germany, Primers

New England BioLabs, USA, BsaJI, FokI

Qiagen, Düsseldorf, Germany, QIAamp DNA Blood Mini Kit

3.2.2 Buffers and Solutions – Tissue Culture

E2 (10μM), 2.7mg dissolved in 10ml sterile distilled water (1mM). 50μl of 1mM dissolved in 5ml 5% SFCS SMEM (10μM).

Ghrelin (1μM), 500mg dissolved in 7.4ml PBS, aliquoted in 50μl and stored at -20 C.

GHRH (1μg/50μl), 50μg GHRH dissolved in 2.5ml PBS, aliquoted in 50μl and stored at -20 C.

LHRH (10μg/50μl), 5mg LHRH dissolved in 5ml PBS (1mg/ml). 1ml of 1mg/ml dissolved in 4ml PBS (10μg/50μl), aliquoted in 50μl and stored at -20 C.

Buffers and Solutions – Tissue Culture

DNA extraction buffer, 10mM Tris and 1mM EDTA (pH 8.0) dissolved in sterile distilled water and containing 1% SDS. 0.1ml pronase E (50mg/ml, prepared in sterile distilled water) was added to 1ml of DNA extraction buffer just before use.

3.2.3 Methods

3.2.3.1 DNA Extraction

Surgically resected pituitary tumours (Department of Neurosurgery, University of Goettingen, Germany) were dissected using sterilized scalpels in petri dishes. The dissected tissues were transferred to a 15ml screw-cap centrifuge tube and incubated in 1ml of DNA extraction buffer at 48 C for 2 hours. The tubes were mix vortexed and protein fractions were removed from the DNA by phenol / chlorophorm extraction.

The phenol:chloroform:isoamylalcohol (PIC) protein extraction mixture came prepared in the ratio of 25:24:1. 1ml of PIC was added to the tissues and the tubes were mix vortexed for 30 seconds and subsequently centrifuged at 4000 rpm at 37 C for 10 minutes. After centrifugation the top aqueous layer, containing the DNA, was transferred into a fresh 15ml tube. Proteins remain in the phenol (PIC) layer. The extraction on the aqueous fraction was repeated with 1ml of fresh PIC mixture. The

top aqueous layer was transferred again to a fresh 15ml tube. An equal volume of chloroform:isoamylalcohol (IC) prepared in the ratio of 24:1 (chloroform), (isoamyl alcohol) was added to the aqueous layer removed and mix vortexed for 10 seconds in order to extract any contaminating phenol. Samples were centrifuged again at 4,000 rpm at 37 C for 10 minutes and the aqueous phase was transferred to a fresh tube. On some occasions DNA yield was increased by adding water to the IC phase and re-centrifuging and combining the two aqueous phases. DNA samples were salt-ethanol precipitated overnight at -20 C by adding 3M sodium acetate (pH 5.2) to a final concentration of 0.3M and 2.5 volumes of ice-cold ethanol. The next day samples were centrifuged at 4 C for 10 minutes at 1,300 rpm. The supernatant was removed and the DNA pellet was gently washed with 1ml of ice-cold 70% ethanol. The pellet was dried at room temperature for 10 minutes and redissolved in 300-500µl of water and its concentration was estimated. A 1:100 dilution of the DNA solution was made and the absorbance was assessed at 260nm and 280nm. The ratio A_{260} / A_{280} should be within the 1.6-1.8 range, indicating that the DNA is free of protein contamination. In case of protein contamination, PIC and IC extraction was performed again. DNA concentration (µg/ml) was given by the formula: $A_{260} \times dilution\ factor \times 50$.

3.2.3.2 GHRH-R Gene Polymorphism in Somatotrophinomas

The more common allele at codon 57 reads GCG (Ala) and the alternative form, believed to confer increased sensitivity to GHRH, is ACG (Thr). To test the hypotheses that the GHRH-R form possessing ACG (Thr) confers hypersensitivity to GHRH and may be associated with increased susceptibility to developing a pituitary tumour, the distribution of both alleles in normal and acromegalic populations was determined and correlated to *in vitro* effect of GHRH on GH

secretion by somatotrophinomas in culture. Expression of the GHRH receptors and possible sequence alterations was investigated by means of PCR and primer-induced restriction analysis (PIRA) analyses and confirmed by direct sequencing.

Analysis of codon 57 of the GHRH-R gene was carried out on DNA samples extracted from 51 human pituitary somatotrophinomas removed from acromegalic patients and 21 further DNA samples obtained from non-acromegalic individuals. Identification of the GCG to ACG (Ala57Thr) polymorphism at codon 57 was achieved by the PIRA technique, in which the forward PCR primer was modified such that a BsiWI restriction enzyme site was introduced into the PCR product DNA in cases where the polymorphism was in the template. The forward and reverse primer sequences, targeted to intronic regions flanking exon 3, were as follows:

5'-GGGCTGAGTCTCTGCTGCTCCTGGCTCTCTATCCAGGCTGCCG<u>C</u>T-3' and

5'-CACCCCTCACCTGACTTCTGAGCTGA-3'

The highlighted (underlined) base in the forward primer represents the modification (C in the wild type), which introduces the BsiWI site (CGTACG) into the PCR DNA if the polymorphism is present in the template where Ala replaces Thr at codon 57. The primers were designed to yield a PCR product of 154bp in length. Purified PCR DNA was digested overnight at 37 C with an excess BsiWI as previously described (refer to chapter 2, page 44). Digestion with BsiWI will give rise to two bands of 110 and 44bp in case of homozygous whereas three bands will be generated in case of heterozygosity where one of the two alleles (154bp) will

remain undigested. Each DNA sample was amplified using a Techne Progene thermocycler PCR with the corresponding optimal condition as described in table 10. The fragments were resolved on a 2% agarose gel against molecular weight markers and undigested DNA, and visualized under the UV light.

Table 10: Optimal PCR Condition for PIRA Analysis		
GHRH-R **GenBank accession no.:** AC005155	95 C 1 minutes 65 C 2 minutes 95 C 3 minutes ..... 72 C 3 minutes 4 C 10 minutes	35 cycles 1 cycle

Primary Cell Culture of Human Pituitary Tumours

At operation, a portion of each pituitary tumour (Department of Neurosurgery, University of Goettingen, Germany) was placed into culture medium, transported to the laboratory and processed for cell culture as previously described (refer to chapter 2, page 46). For pituitary derived tissues, divided pieces were incubated at 37 C in an orbital incubator shaker for 2-3 hours and the dispersed tissues were washed. For each tumour studied, equal aliquots of $1\text{-}2x10^5$ pituitary cells were distributed into at least 12 glass culture tubes and allowed to attach and equilibrate during the following 24 hours in 2ml fresh 10% FCS SMEM, after which experiments were commenced. For human pituitary tumours, appreciable mitosis does not occur. Nevertheless, for the secretory tumours (e.g. GH, PRL), hormone secretion can be maintained for up to one month in cell culture. However, the present experiments were performed within a few days of establishing the cultures. In addition, the dispersed cells may or may not adhere to the culture tubes. In the latter case, all

experiments required that the cells were first centrifuged before removing culture medium.

Effects of GHRH on cAMP

Experiments were performed on four out of the seventeen pituitary somatotrophinomas available for this part of the study. Tumourous samples were removed from 3 male and 1 female acromegalic patients, aged 33-61 (table 11). All patients had elevated serum GH; basal preoperative levels ranged from 6.5-212mlU/L. Following surgical removal, tissues were immediately processed for cell culture. Following equilibration of the cells for 24 hours at 37 C, culture medium was removed and the cells first washed (2ml) and subsequently incubated with 5% SFCS SMEM with no additive (control) or containing 2nM of GHRH. These doses were used because they are maximally effective in the cell culture system (Adams *et al.*, 1984; Adams *et al.*, 1994). At least three cultures were used for controls or GHRH treatments. After 30-minute incubation at 37 C, the cells were extracted with ice-cold acidified ethanol and cAMP in the extracts was measured by ELISA (Amersham Biosciences, UK).

Effects of GHRH on GH Secretion *in vitro*

Seventeen somatotrophinomas were removed from 11 male and 6 female acromegalic patients, age range 33-63 (table 11), and assessed by cell culture for the effects of GHRH on GH secretion. All patients had elevated serum GH; basal preoperative levels ranged from 6.5-212mlU/L. Dispersed somatotrophinoma cells were incubated with 5% SFCS SMEM with no additive (control) or containing 2nM of GHRH. At least three cultures were used for controls or GHRH treatments. After

4-hour incubations at 37 C, media were then collected and assayed for GH content, by ELISA using kits obtained from NETRIA.

Table 11: Primary Human Somatotrophinomas

Sample	Sex	Age	Immunohistochemistry
923	M	33	GH +ve
930	M	59	GH +ve
938	F	61	GH +ve
942	M	51	GH +ve
943	F	60	GH +ve
947	F	34	GH +ve
358	M	56	GH +ve
957	M	60	GH +ve
958	F	58	GH +ve
978	M	54	GH +ve
984	M	52	GH +ve
985	M	38	GH +ve
1005	M	57	GH +ve
1033	F	59	GH +ve
1060	F	39	GH +ve
1167	M	37	GH +ve
1170	M	42	GH +ve

3.2.3.3 Effect of CCK-8s and CCK-33 on Hormone Secretion by Human Pituitary Tumours

To investigate the direct effects of CCK peptides on gonadotrophin and GH secretion by human functionless and somatotrophic pituitary tumours, freshly resected tissues were cultured with varying doses of CCK peptides alone and in combination with various agonists and antagonists of pituitary function. In parallel, RT-PCR was used to demonstrate the presence of CCK-receptor gene expression

and to determine whether these types of pituitary cell may also produce CCK peptide itself, whereas receptor functionality was assessed by means of phosphatidylinositol hydrolysis and cAMP production in the presence of CCK peptides. A neutralizing antibody against CCK was incorporated into hormone secreting experiments to investigate the possible effects of endogenous CCK whereas, antagonists to CCK were used to determine the effects of exogenously added CCK on hormone secretion by human pituitary tumours *in vitro*.

Experiments were performed on 2 somatotrophinomas (acromegalic patients) and 7 functionless pituitary tumours. Both acromegalic tumours stained exclusively for GH (table 12) whereas, none of the patients with the functionless tumours presented with evidence of pituitary hormone hypersecretion except for mildly elevated PRL levels in 2 cases due to tumour compression of the pituitary stalk. According to immunohistochemical analysis, the tumours stained positive for gonadotrophin secreting cells (table 12).

Dispersed somatotrophinoma (acromegalic) cells were incubated with 5% SFCS SMEM for 4 hours in order to assess basal secretion. Media were collected and cultures were incubated with 5% SFCS SMEM and varying concentrations of CCK-33 (1-100nM) and CCK-8s (2-200nM), non-peptide antagonists to CCK (L-365,260 and L-364,718) (1-100nM) and a neutralizing antibody (1:1600-1:400 dilutions) against CCK for 4 hours. In addition, the effects of GHRH and Ghrelin (for GH secreting tumours) as well as LHRH and E2 (for functionless pituitary tumours) alone and in combination with CCK-33 and CCK-8s were determined. Similar experiments were performed on functionless pituitary derived cells where

incubations lasted for 24 hours for basal and treatment secretion periods. Media were then collected and assayed for GH, PRL, LH and FSH content, by ELISA using kits obtained from NETRIA (London). At least three replicate cultures were used for each treatment, including controls. Cell cultures were re-used after a wash (2-3) stage with and overnight equilibration in 10% FCS SMEM.

Table 12: Primary Human Pituitary Tumours

Human Functionless Pituitary Tumours			
Sample	**Sex**	**Age**	**Immunohistochemistry**
400	M	54	FSH +ve
48	F		FSH & LH +ve
422	M	63	FSH & LH +ve
528	M	55	FSH & LH +ve
529	F	55	FSH & LH +ve
536	M		FSH +ve
545	F	55	FSH +ve
Human Pituitary Somatotrophinomas			
402	M	65	GH +ve
47	M		GH +ve

3.2.3.4 ELISA of Anterior Pituitary Hormones

Hormone concentrations in the collected media were determined by enzyme-linked immunosorbent assay (ELISA) using kits obtained from NETRIA. ELISA is based on use of two monoclonal antibodies, one directed against the N-terminus of the human hormone investigated, and the other against the C-terminus. The second anti-body is conjugated to horse radish peroxidase (HRP). The 96-well plates supplied were pre-coated with the first antibody.

Standard or samples were pipetted into the wells and the hormone was allowed to bind to the first antibody during a 2-hour to overnight incubation. The plates were thoroughly washed and the second antibody added. The amount of bound conjugate, which is directly proportional to amount of hormone in the samples, was quantified by reaction with the enzyme substrate, TMB (3,3',5,'-tetramethylbenzidine). The reaction was stopped by addition of sulphuric acid and absorbance was measured at 450nm with 620nm as reference.

Standards were prepared by addition of 1ml distilled water to the top lyophilized standard (200mIU/L for GH and PRL) (100mIU/L for LH and FSH). Doubling dilutions of the top standard provided the rest of the standards (100, 50, 25, 12.5, 6.15, 3.12 and 0mIU/L). A 50µl aliquot of standard or unknown sample was pipetted into each well, in duplicate followed by 200µl assay buffer. The 96-well plate was incubated from 2 hours to overnight, depended on the type of hormone assayed, at room temperature on a horizontal shaker at 60 rpm. For GH and PRL, overnight incubation was required, whereas for LH and FSH, the plate was incubated for 2 hours. The plate was washed three times with 300µl 1x wash buffer. Between each wash, the plate was blotted on a tissue paper to ensure complete removal of residual volume. The 1ml of corresponding conjugate provided was added to 25ml of corresponding assay buffer from which 200µl were added to each well. The plate was incubated for 4 hours at room temperature on a horizontal shaker at 60 rpm. The plate was washed again three times with 300µl 1x wash buffer and, 200µl of K-blue substrate were added to each well. The plate was incubated for 20 minutes at room temperature on a horizontal shaker at 60 rpm. The reaction was terminated by addition

of 50μL 2.5M sulfuric acid (H_2SO_4) and the plate was read on a plate reader at 450nm with as reference 620nm.

3.2.3.5 Intracellular Second Messengers

Effects of CCK-8s and CCK-33 on PI Hydrolysis

Assessment of the effect of CCK-8s (2-200nM) and CCK-33 (1-100nM) on the rate of PI hydrolysis *in vitro* was investigated. PI hydrolysis of human pituitary tumour derived cells was assessed by measuring accumulation of [^{3}H]*myo*-inositol phosphates in the presence of 10mM lithium chloride (LiCl) as previously described (refer to chapter 2, pages 48-49).

Effects of CCK-8s and CCK-33 on cAMP Production

Human pituitary tumour derived cells were exposed to CCK-8s (2-200nM) and CCK-33 (1-100nM) for the purpose of determining cAMP production as previously described (refer to chapter 2, page 49).

3.2.3.6 Statistical Analyses

Statistical significances were determined by unpaired t-test using pooled estimates of error.

3.2.3.7 Gene Expression

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Expression CCK peptide and CCK-A and / B receptors by human pituitary tumours was examined using identical methods as previously described for gliomas (chapter 2, pages 40-44). A total of 5 somatotrophinomas and 17 functionless pituitary

tumours were studied. Clinical and immunohistochemistry analysis of these tumours and patients are presented in tables 13 and 14.

Total RNA was extracted from a portion of tissue (Department of Neurosurgery, University of Goettingen, Germany) and quantified by spectrophotometry as previously described. RNA was also extracted from pituitary derived fibroblasts. These were obtained by serial passages of the tumour cultures, which eventually allows purified fibroblasts to be grown (Honegger *et al.*, 1997). The extracted RNA (1-5μg) was reverse transcribed into cDNA and subjected to PCR using primers specific for CCK peptide, CCK-A and CCK-B receptor mRNA expression. For the full analysis, RT-PCR products were salt-ethanol precipitated and subsequently purified. Purified PCR DNAs were digested overnight at 37 C with an excess of respective enzymes and the cut and uncut PCR DNAs were resolved on a 2% agarose gel. DNA bands were visualized under UV light. In addition, purified PCR DNAs were directly sequenced.

Table 13: Human Pituitary Somatotrophinomas

Sample	Sex	Age	Immunohistochemistry	CCK	CCK-A	CCK-B
3	F	72	Acromegaly GH +ve	√	-	√
402	M	65	Acromegaly GH +ve	√	-	√
388	M	26	Acromegaly GH +ve	√	-	√
393	F	22	Acromegaly GH & PRL +ve	√	-	√
47	M		Silent Secret GH +ve	√	-	√

(-) = Presence; (√) = absence

Table 14: Primary Human Functionless Pituitary Tumours

Sample	Sex	Age	Immunohistochemistry	CCK	CCK-A	CCK-B
4	F	45		√	-	√
11	M	26		√	-	√
12	F	65		√	-	√
14	M	66		√	√	√
15	M	35		√	-	√
18	F	43		√	-	√
22	M	76		√	-	√
32				√	-	√
33	F	56		√	-	√
400	M	54	FSH +ve	√	√	√
42	M	54		√	-	√
48	F	63	FSH & LH +ve	√	-	√
422	M		FSH & LH +ve	√	-	√
528	M	55	FSH & LH +ve	√	√	√
529	F	55	-ve	√	-	√
536	M		FSH +ve	√	-	√
545	F	55	-ve	√	-	√

(-) = Presence; (√) = absence

3.3 RESULTS

3.3.1 GHRH-R Gene Polymorphism and Somatotrophinomas

The primer-induced restriction analysis (PIRA) is predicted to yield bands of 110 and 44bp from BsiWI (5'-CGTACG-3') digested PCR product if codon 57 of the GHRH-R gene is ACG. A typical result is shown in figure 45. It is clear that the predicted band pattern was yielded by the PCR DNA samples. The presence of the 154bp band in the sample (patient 1) generated by the DNA containing the polymorphism reflects heterozygosity for both alleles (GCG and ACG).

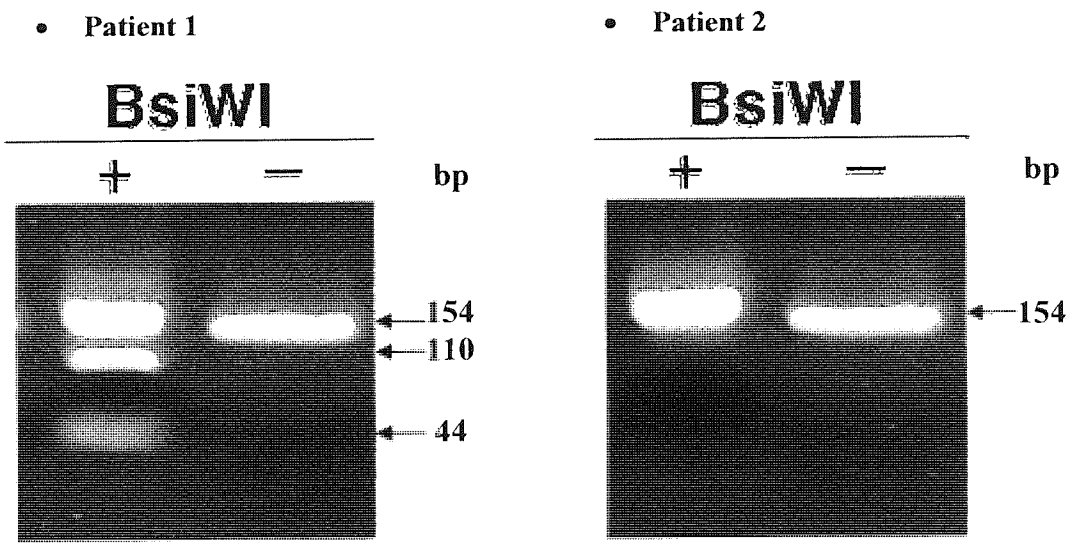


Figure 45: PIRA analysis of DNA derived from 2 human pituitary somatotrophinomas (patient 1 and patient 2). The band pattern in patient 1 indicates heterozygocity (GCG / ACG) genotype whereas for patient 2 homozygocity for the GCG genotype.

Figure 46 shows direct sequencing results obtained from two somatotrophinoma DNA samples, one (patient 1) possessing the polymorphism and the other (patient 2) homozygous for the more common allele (GCG).

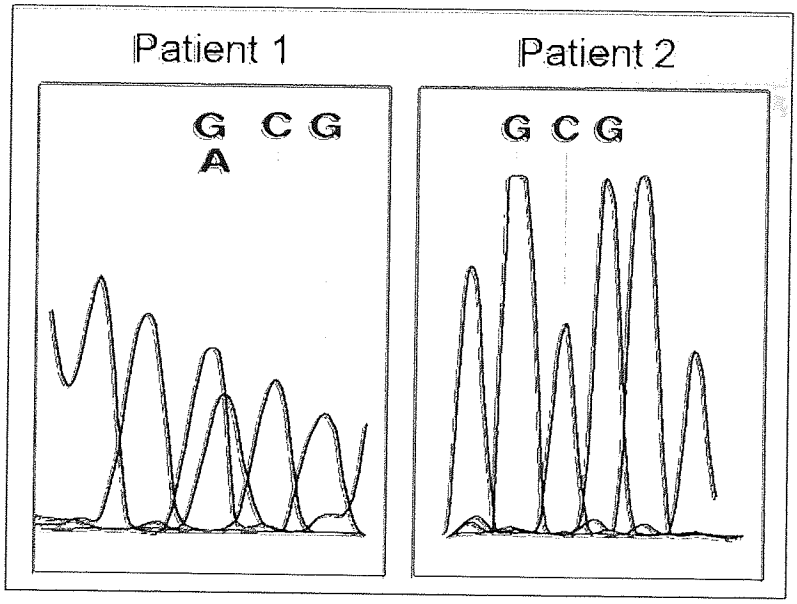


Figure 46: Sequence of codon 57 of the GHRH-receptor gene in DNA derived from patient 1 a human pituitary somatotrophinoma (left panel) and patient 2 another human pituitary somatotrophinoma (right panel). The double peak in the 5' base of this codon observed somatotrophinoma-derived DNA represents two allelic forms, GCG (alanine) and AGC codon results from a G ⇒ A normal polymorphism, since this base alteration is also present in the blood-derived DNA.

Table 15 summarizes the distribution of the different genotypes of DNA removed from non-acromegalic and acromegalic subjects. The somatotrophinoma (acromegalic) group is divided into *gsp*-negative and *gsp*-positive tumours. Of the 51 somatotrophinomas, 10 (19.6%) were heterozygous for both alleles at codon 57. This incidence did not significantly differ between *gsp*-negative and *gsp*-positive

tumours, being 18.8% and 21%, respectively. Likewise, the distribution of the genotypes was very similar in the non-acromegalic DNA samples, in which 23.8% were heterozygous at codon 57 as shown in table 15. Homozygosity for the alternative GHRH-R gene was not observed in any of the 72 samples.

Table 15: Distribution of GHRH-R Codon 57 Polymorphism in Acromegalic and Non-Acromegalic Subjects

	Codon 57 genotype		
	GCG / GCG (Ala / Ala)	**GCG / ACG (Ala / Thr)**	**ACG / ACG (Thr / Thr)**
Acromegalic (*gsp*-negative)	26 (79%)	6 (21%)	0 (0%)
Acromegalic (*gsp*-positive)	15 (81%)	4 (19%)	0 (0%)
Non-acromegalic	16 (76%)	5 (24%)	0 (0%)

Effects of GHRH on cAMP

The effect of GHRH on cAMP production by 4 of the tumours was examined *in vitro*. Tumour 923 and 930 did not possess the alternative codon 57 and exhibited a 2-fold increase in cAMP production in response to GHRH (figure 47).

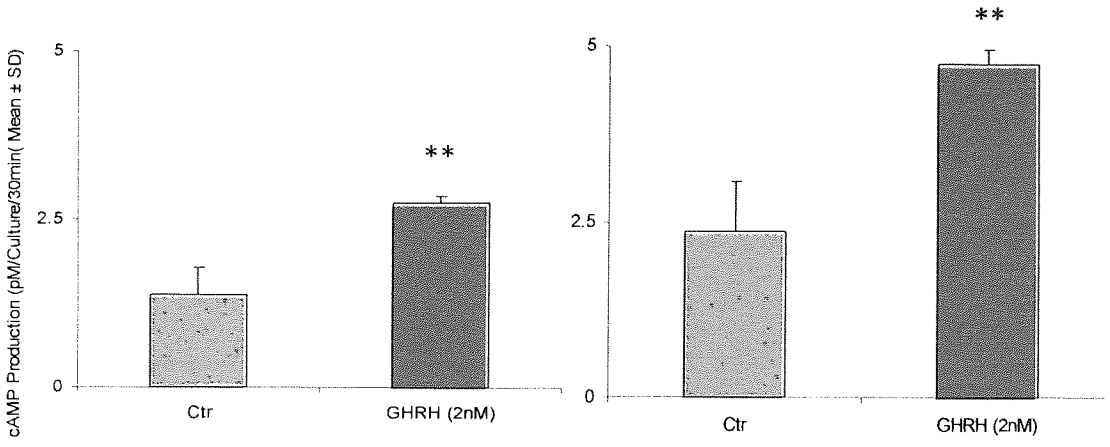


Figure 47: Stimulatory effect of GHRH on intracellular cAMP production by *in vitro* cultures on human pituitary somatotrophinomas (923 and 930) without the Ala → Thr alteration in residue 57 of the GHRH-receptor gene. N=1; **P<0.01 vs. control.

In contrast, cAMP production was greatly stimulated (40 and 200-fold) by GHRH when added to cultures of tumours 938 and 942 as shown in figure 48. Both of these tumours were shown to possess the Ala → Thr alternative form of codon 57. Interestingly, although tumour 942 exhibited the highest and very powerful (20-fold), response to GHRH, it possessed a *gsp* oncogene (data not shown).

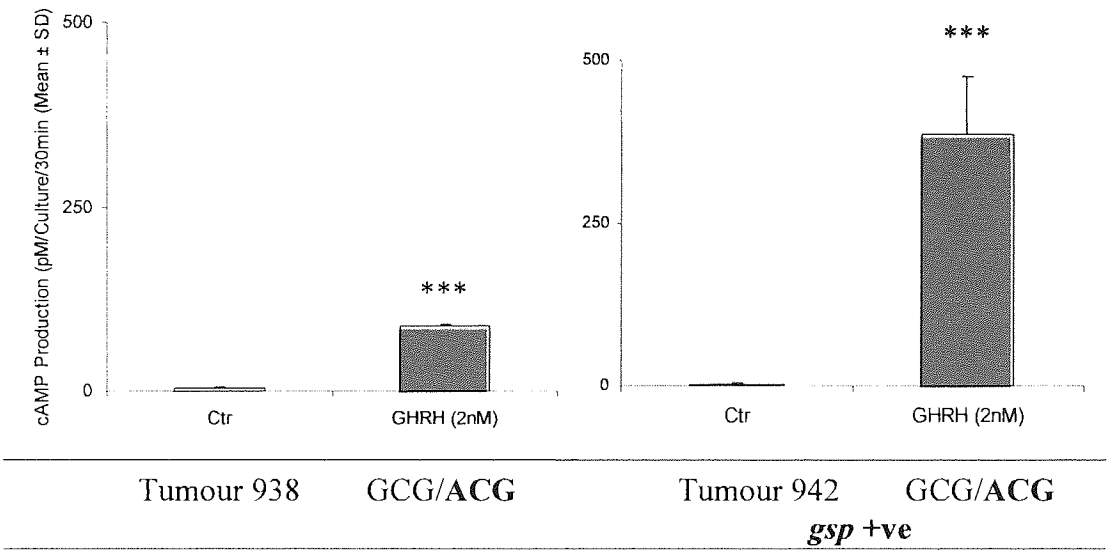


Figure 48: Stimulatory effect of GHRH on intracellular cAMP production by *in vitro* cultures on human pituitary somatotrophinomas (938 and 942) with the Ala → Thr alteration in residue 57 of the GHRH-receptor gene. N=1; ***P<0.001 vs. control.

Effects of GHRH on GH Secretion *in vitro*

Sixteen of the tumours were tested for response of GH secretion to GHRH *in vitro* and results are presented in figure 49. Three out of 17 tumours were shown by the PIRA analysis to possess both allelic forms of the GHRH-R in terms of codon 57. The average GH response of these tumours to GHRH was significantly ($P<0.05$) greater than the 13 tumours which were homozygous for the more common GCG allele.

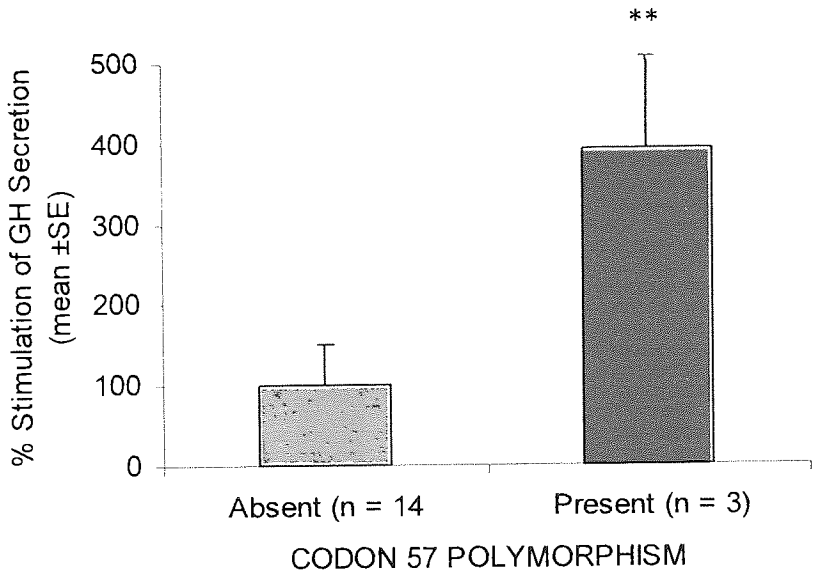


Figure 49: Stimulatory effect of GHRH on GH secretion by *in vitro* cultures on human pituitary somatotrophinomas with (left bar) and without (right bar) the Ala 57 Thr polymorphism. n=1; **P<0.05 vs. control.

3.3.2 Effect of CCK-33 and CCK-8s on LH and FSH Secretion by Functionless Pituitary Tumours

CCK-33 (1-100nM) significantly (P<0.01) and dose-dependently stimulated LH and FSH secretion by cell cultures of a functionless human pituitary tumour positive for FSH secreting cells (figure 50). The magnitude of stimulation ranged from 120-170%. In addition, E2 powerfully stimulated LH secretion and enhanced (3-fold) the stimulatory effect of CCK-33.

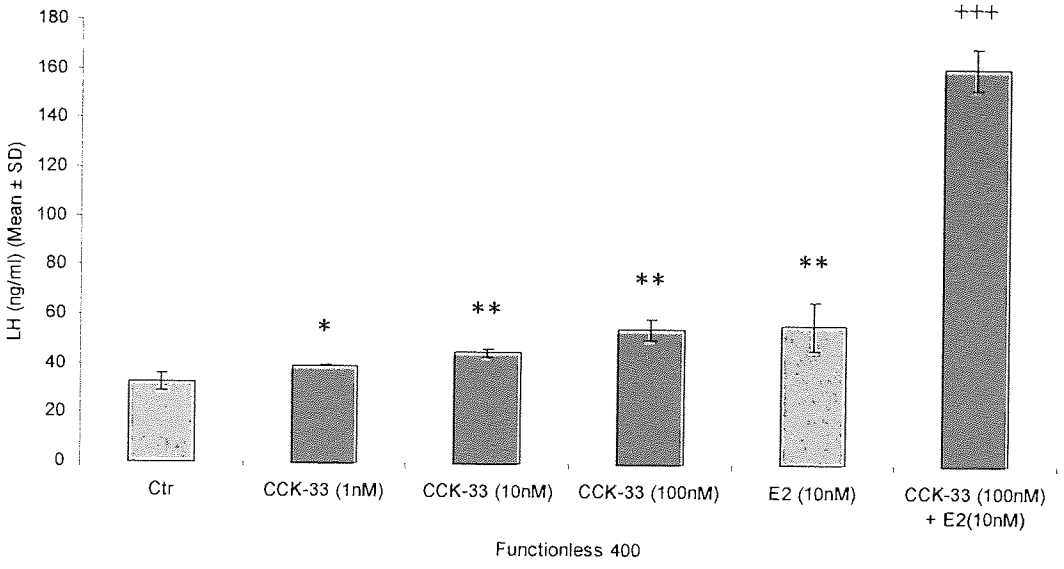


Figure 50: Stimulatory effect of CCK-33 (1-100nM) and E2 (10nM) on LH secretion by a human functionless pituitary tumour (400) after 24 h. Triplicate cultures were used for each variable; n=2. *P<0.05; **P<0.01 vs. control; $^{+++}$P<0.001 vs. E2.

In case of FSH secretion, E2 caused a rather modest stimulation. Co-incubation of E2 with CCK-33 contributed to a marked and significant increase of FSH in both cases by 0.9-2-fold respectively, as shown in figure 51.

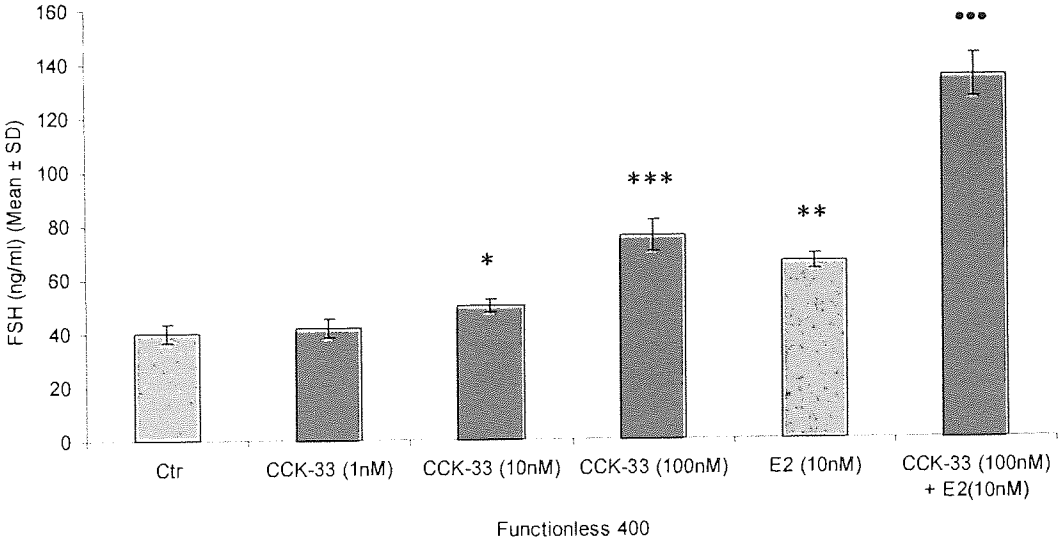


Figure 51: Stimulatory effect of CCK-33 (1-100nM) and E2 (10nM) on FSH secretion by a human functionless pituitary tumour (400) after 24 h. Triplicate cultures were used for each variable; n=2. *P<0.05; **P<0.01; ***P<0.001 vs. control; •••P<0.001 vs. E2.

Figure 52 shows that CCK-8s (2-200nM) yielded very similar stimulatory effects on LH and FSH. Different growth factors known to induce GH secretion were incorporated into the experiment this time to investigate their involvement in gonadotrophin secretion. GHRH (10ng/ml) and Ghrelin (10ng/ml) caused stimulation of LH secretion, an effect significantly altered by co-incubation with CCK-8s by 1.2-1.5-fold, respectively.

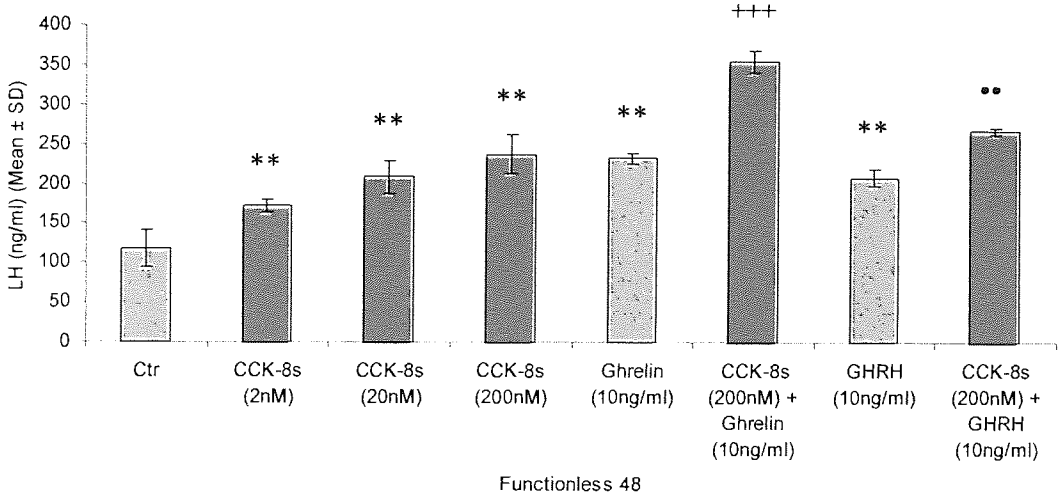


Figure 52: Stimulatory effect of CCK-8s (2-200nM), GHRH (10ng/mL) and Ghrelin (10ng/ml) on LH secretion by a human functionless pituitary tumour (48) after 24 h. Triplicate cultures were used for each variable; n=2. **P<0.01 vs. control; $^{+++}$P<0.001 vs. Ghrelin; ••P<0.01 vs. GHRH.

3.3.3 Effect of Antagonists to CCK on LH and FSH Secretion by Functionless Pituitary Tumours

CCK-33 (100nM) significantly stimulated LH secretion by cell cultures of a functionless human pituitary tumour positive for gonadotrophs (figure 53). The magnitude of stimulation was a significant 150%. An antagonist to the CCK-B (L-366,260) (100nM) receptor significantly (P<0.001) inhibited the secretion of LH in culture while in combined treatment with CCK-33, antagonists to CCK-B receptor completely abolished the stimulatory effect of CCK-33.

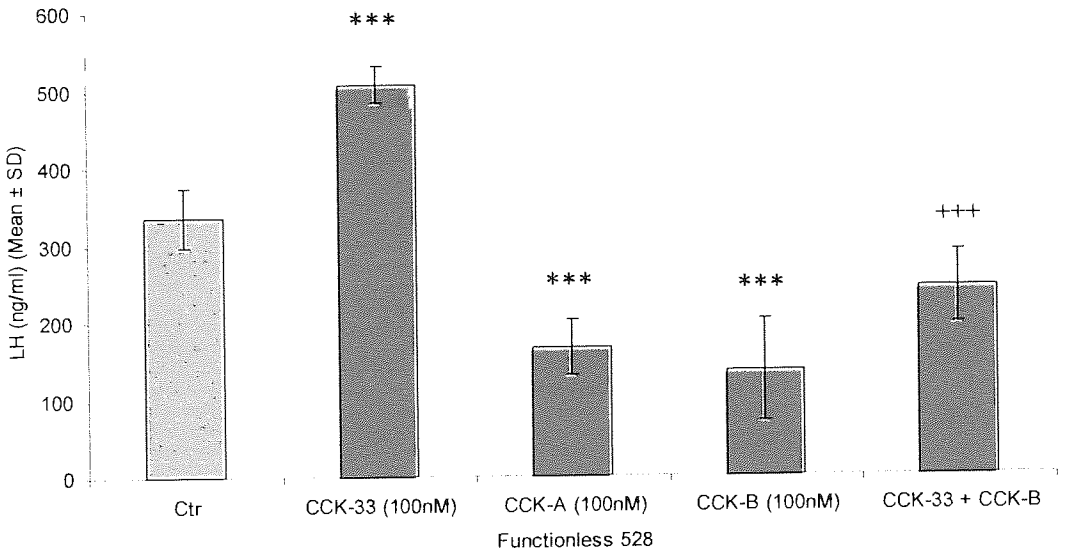


Figure 53: Stimulatory effect of CCK-33 (100nM) on LH secretion by a human functionless pituitary tumour (528) after 24 h. Triplicate cultures were used for each variable; n=1. ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-33.

Similarly, as shown in figures 54 and 55, both CCK-8s and CCK-33 (100nM) significantly stimulated LH and FSH secretion by cell cultures of a functionless human pituitary tumour positive for FSH secreting cells. The magnitude of stimulation was a significant 120-170%. Antagonists for the CCK-A (L-364,718) and B (L-366,260) (100nM) receptor significantly inhibited the secretion of LH and FSH in culture and when co-incubated with the CCK peptides (CCK-8s and CCCK-33), their stimulatory effects was completely abolished. In addition, LH secretion was greatly enhanced (280%) by LHRH (100ng/ml) suggesting active secreting status for the functionless pituitary tumour.

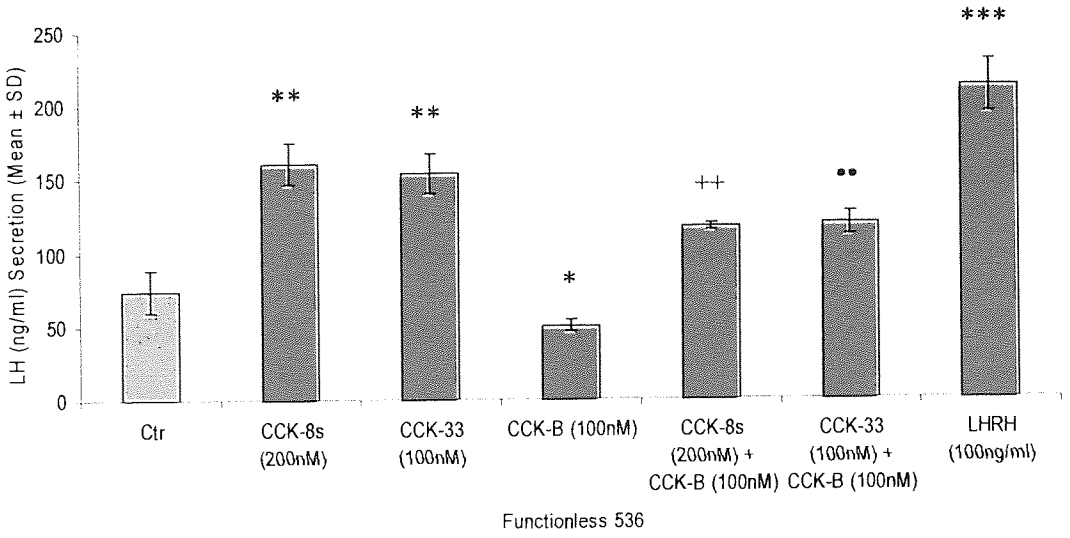


Figure 54: Stimulatory effect of CCK-8s (200nM) and CCK-33 (100nM) on LH secretion by a human functionless pituitary tumour (536) after 24 h. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s; ••P<0.01 vs. CCK-33.

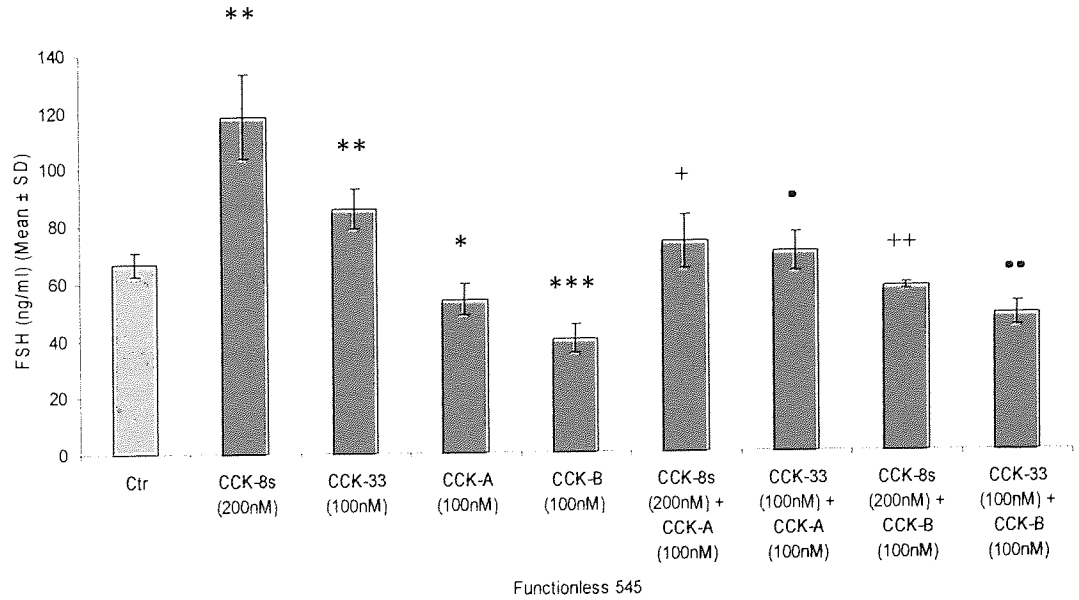


Figure 55: Stimulatory effect of CCK-8s (200nM) and CCK-33 (100nM) on LH secretion by a human functionless pituitary tumour (545) after 24 h. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{+}$P<0.05; $^{++}$P<0.01 vs. CCK-8s; •P<0.05; ••P<0.01 vs. CCK-33.

CCK-8s (200nM) significantly (P<0.01) stimulated FSH secretion by cell cultures of a functionless human pituitary tumour positive for FSH secreting cells. The magnitude of stimulation was a significant 170%. In addition, E2 (10nM) and LHRH (10ng/ml) powerfully stimulated FSH secretion (figure 56). Only the stimulatory effect of CCK-8s was powerfully inhibited by the CCK-B (100nM) antagonist, whereas no effect was exerted on either the E2 or LHRH.

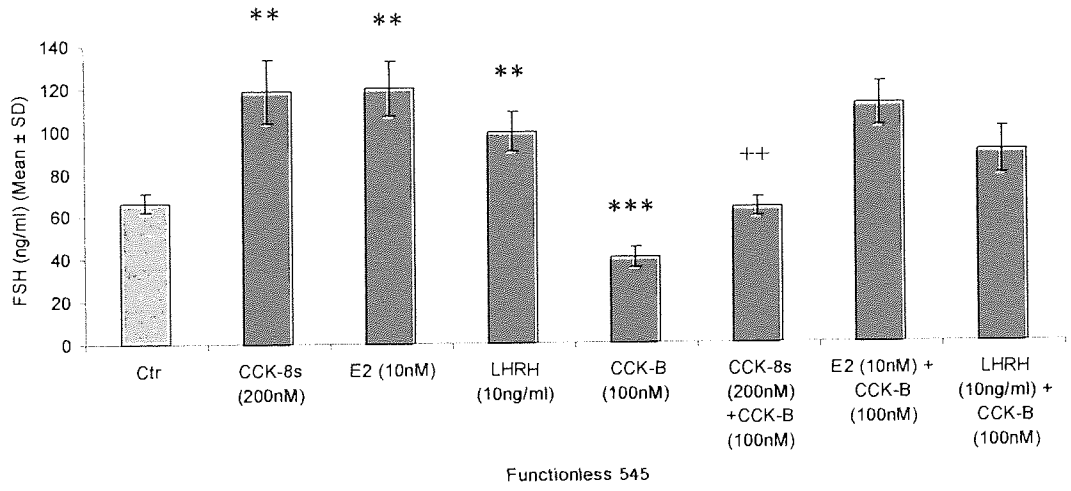


Figure 56: Stimulatory effect of CCK-8s (200nM) on FSH secretion by a human functionless pituitary tumour (545) after 24 h. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s.

3.3.4 Effect of CCK-33 and CCK-8s on GH and PRL Secretion by Human Somatotrophinomas

Both CCK-33 and CCK-8s significantly (P<0.01) stimulated GH secretion by the somatotrophinomas in culture (figure 57). The amount of stimulation ranged from 20-50% respectively.

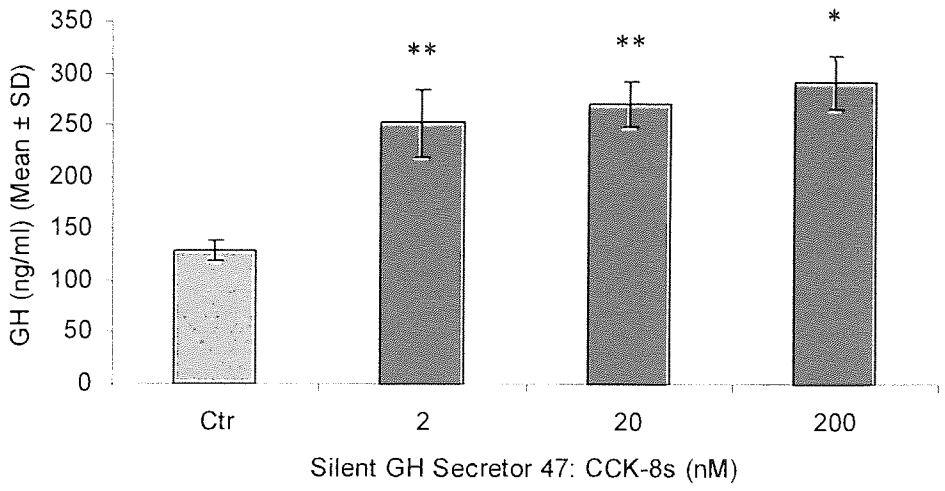


Figure 57: Stimulatory effects of CCK-8s (2-200nM) and CCK-33 (100nM) on GH secretion by a human GH silent secretor pituitary tumour (47) after 4 h. Triplicate cultures were used for each variable; n=2. *P<0.05; **P<0.01 vs. control.

Similarly, CCK-8s (200nM) caused a significant (P<0.01) stimulation on GH secretion as shown in figures 58 and 59. GHRH (10ng/ml) and Ghrelin (10ng/ml), both increased GH release. In combination with CCK-8s, the effect of Ghrelin, but not GHRH, was enhanced.

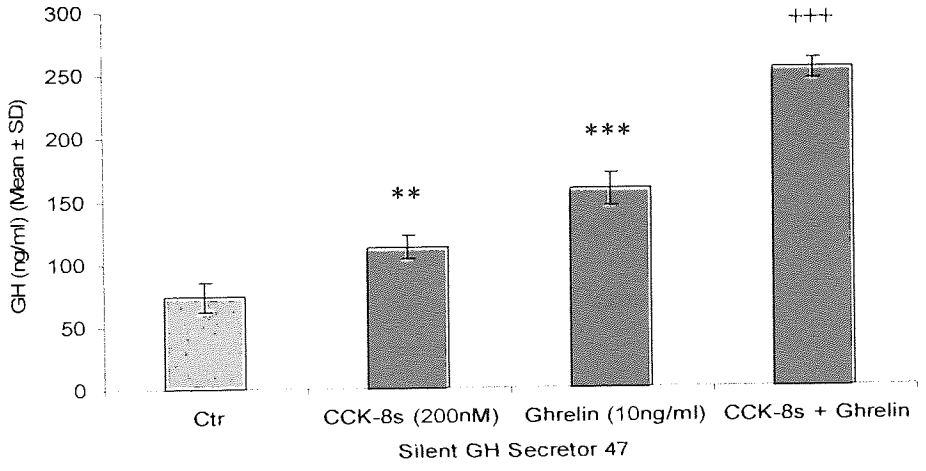


Figure 58: Stimulatory effects of CCK-8s (200nM) and Ghrelin (10ng/ml) on GH secretion by a human GH silent secretor pituitary tumour (47) after 4 h. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. Ghrelin.

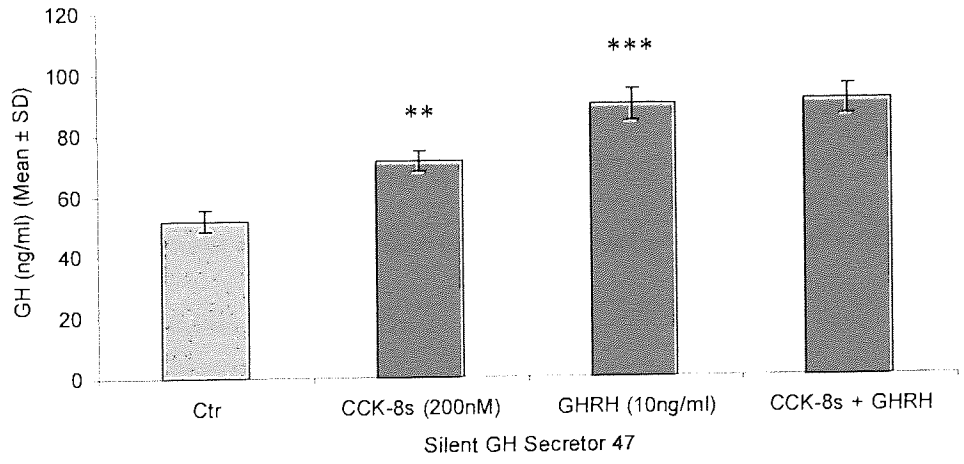


Figure 59: Stimulatory effects of CCK-8s (200nM) and GHRH (10ng/ml) on GH secretion by a human GH silent secretor pituitary tumour (47) after 4 h. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control.

3.3.5 Effect of Antagonists to CCK on GH Secretion by Human Somatotrophinomas

Antagonists to the CCK-A (L-364,718) (100nM) and CCK-B (L-366,260) (100nM) receptors both significantly ($P<0.001$) inhibited the secretion of GH by somatotrophinomas in culture as shown in figures 60 and 61. Additionally, the antagonists completely blocked or greatly reduced the stimulator effects of CCK-8s.

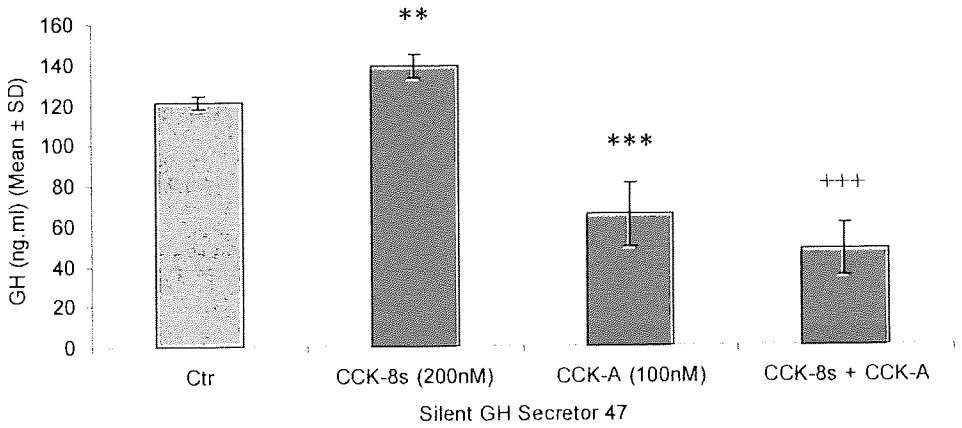


Figure 60: Effects of CCK-8s (200nM) and CCK-A (100nM) antagonist on GH secretion by a human GH silent secretor pituitary tumour (47) after 4 h. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-8s.

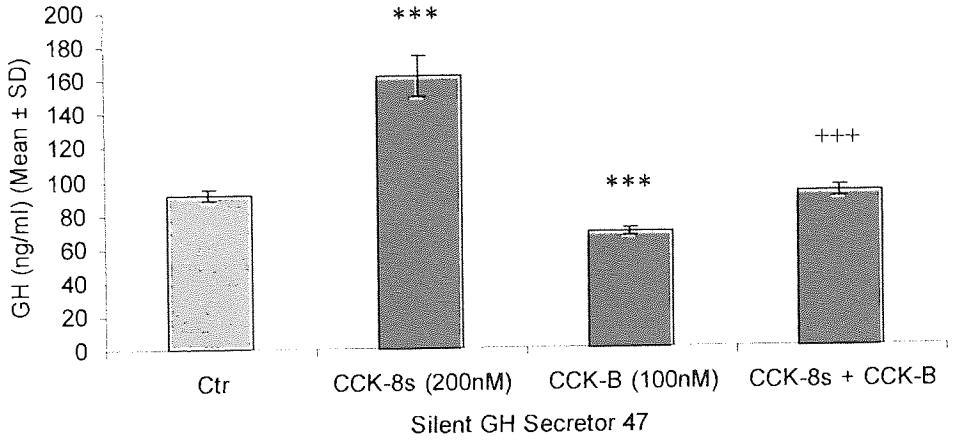


Figure 61: Effects of CCK-8s (200nM) and CCK-B (100nM) antagonist on GH secretion by a human GH silent secretor pituitary tumour (47) after 4 h. Triplicate cultures were used for each variable; n=1. ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-8s.

3.3.6 Effect of CCK-33 and CCK-8s on PI hydrolysis and cAMP Production

In culture, the responses to the two CCK receptor agonists (CCK-8s and CCK-33) were examined via second messengers. Both CCK-8s (1-100nM) and CCK-33 (2-200nM) produced dose-dependent increases in PI hydrolysis (figures 62 and 63), providing evidence for functional activity of both CCK receptor subtypes, since receptor activation is bound to the PI hydrolysis.

Total cAMP production determined in the presence of both CCK-33 and CCK-8s peptides was not changed.

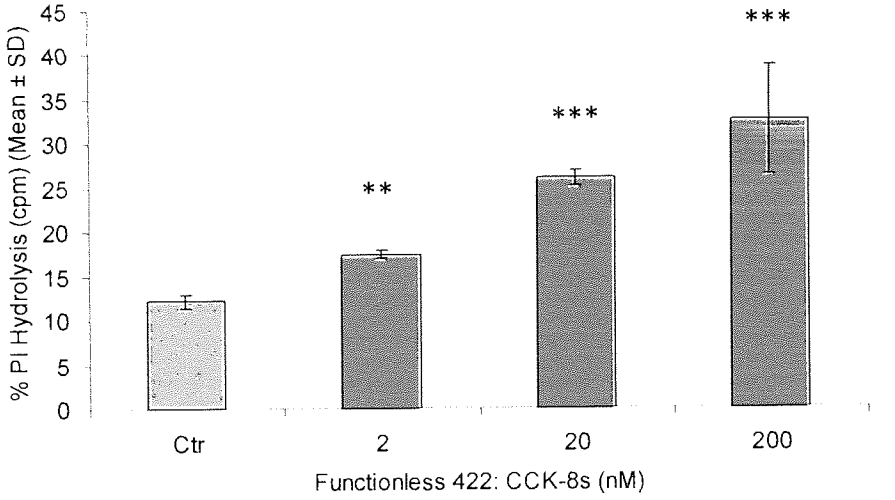


Figure 62: Dose-dependent stimulatory effect of CCK-8s (2-200nM) on rate of PI hydrolysis after 6-hour incubations by a human functionless pituitary tumour. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control.

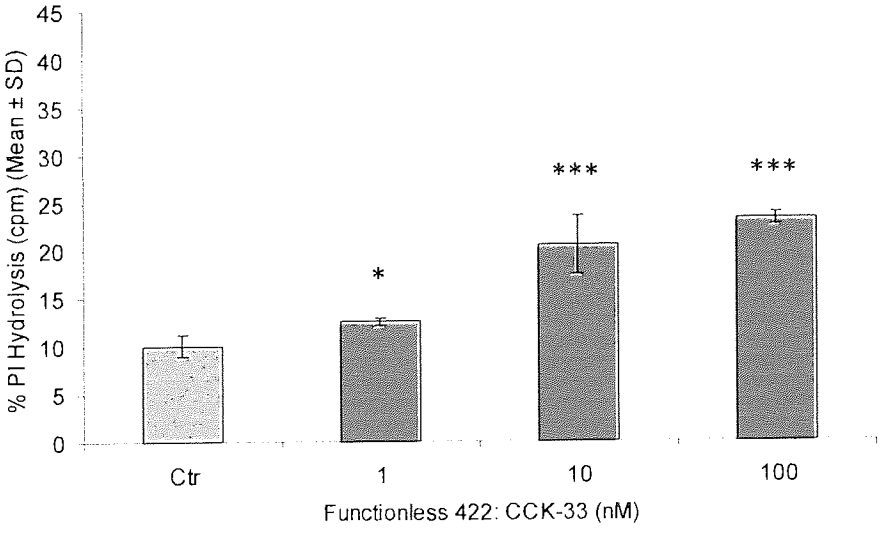
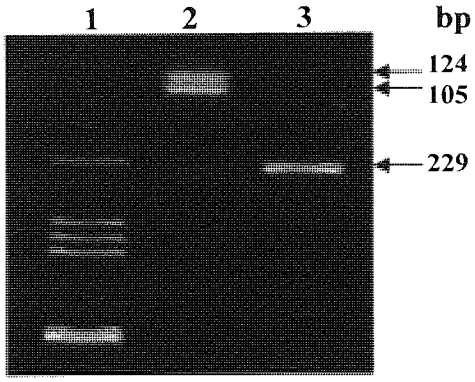


Figure 63: Dose-dependent stimulatory effect of CCK-33 (1-100nM) on rate of PI hydrolysis after 6-hour incubations by a human functionless pituitary tumour. Triplicate cultures were used for each variable; n=1. *P<0.05; ***P<0.001 vs. control.

3.3.7 Gene Expression of CCK and CCK Receptors by Human Pituitary Tumours

All 22 human pituitary tumours (17 functionless and 5 somatotrophinomas) were shown by RT-PCR analysis to express mRNA for CCK itself and CCK-B receptor. PCR bands of predicted size were yielded by the cDNA samples, and these bands were cut in the predicted pattern as described in the Materials and Methods. An example is shown in figure 64. By the same analysis, evidence for CCK-A receptor was detected in only 3 of the functionless tumours (figure 64). Similar analyses on pituitary derived fibroblasts yielded negative results for CCK, CCK-A and CCK-B receptor mRNA, suggesting that they are only expressed by tumourous cells (figure 64).

- **A: CCK**

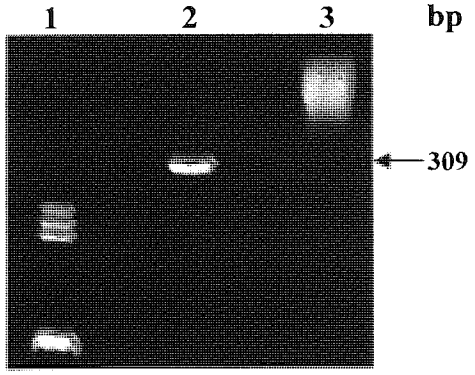


Lane 1 – DNA Ladder

Lane 2 – cDNA digested with PstI

Lane 3 – **CCK** cDNA

- **B: CCK-B**

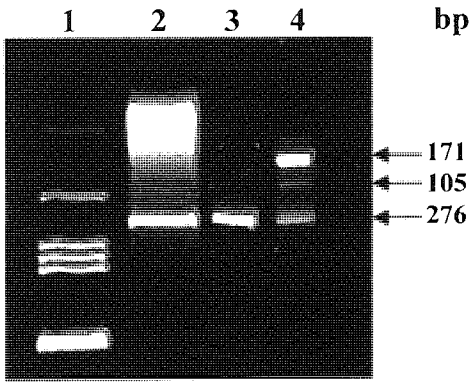


Lane 1 – DNA Ladder

Lane 2 – **CCK-B** cDNA (+ve Ctr)

Lane 3 – **Fibroblast** cDNA (-ve)

- **C: CCK-A**



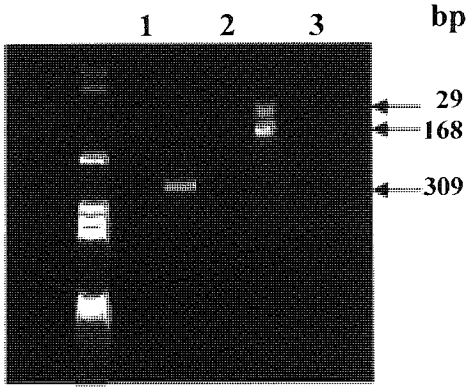
Lane 1 – DNA Ladder

Lane 2 – **CCK-A** PCR DNA

Lane 3 – **CCK-A** cDNA (Purified)

Lane 4 - cDNA digested with FokI

- **D: CCK-B)**



Lane 1 – DNA Ladder

Lane 2 – **CCK-B** cDNA

Lane 3 – cDNA digested with PstI

Figure 64: 2% agarose gels of A) RT-PCR for CCK using RNA derived from a human pituitary tumour (lane 3) and cut by PstI (lane 2); B) RT-PCR for CCK using RNA derived from a human pituitary tumour (lane 2) and cultures pituitary derived fibroblasts (lane 3); C) RT-PCR for CCK-A-R using RNA derived from a human functionless pituitary tumour (lane 2), same purified DNA sample (lane 3) and cut by FokI (lane 4); D) RT-PCR for CCK-B-R using RNA derived from a human pituitary tumour (lane 2) and cut by PstI (lane 3); bp, base pairs in marker bands (arrowed).

Direct sequence analysis of these bands confirmed that they were representative of both the human CCK peptide along with its receptor subtypes CCK-A and / B mRNA. A specific portion of sequencing is shown in figure 65. The mRNA sequences presented in figure 38 are without the intervening intronic regions.

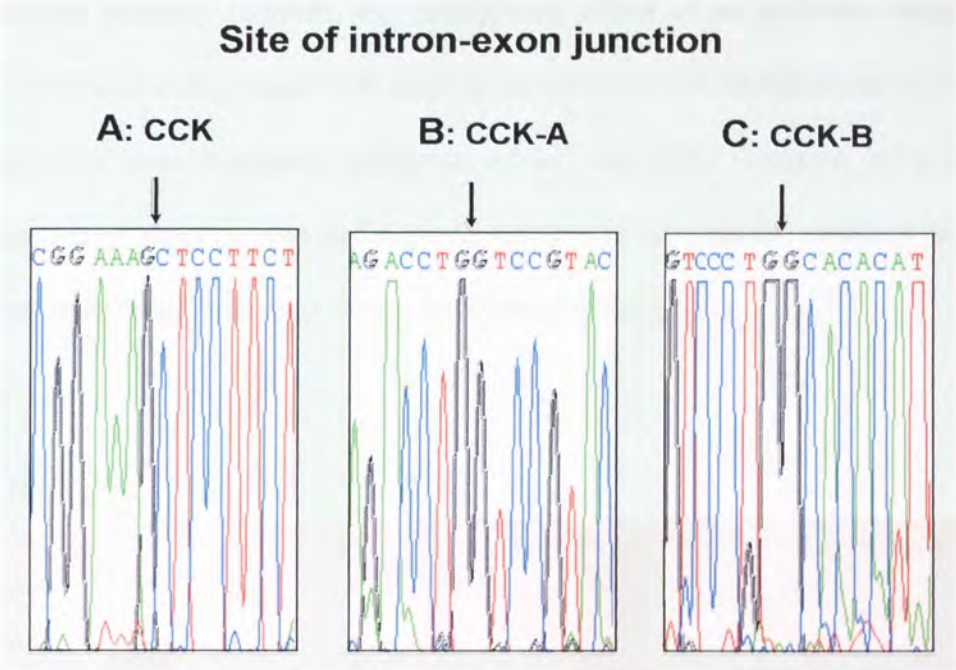


Figure 65: Partial sequence of RT-PCR DNA bands. A) The sequences read 5'-GCCCGGAAAGCTCCTTCTGG-3', and is identical to the CCK cDNA (mRNA); B) 5'-GCAGTCCCTGGCACACATTA-3', and is identical to the CCK-A receptor (mRNA); C) 5'-CCGCCAGACCTGGTCCGTACTG-3', and is identical to the CCK-B receptor (mRNA). All sequences are without the genes's intervening intronic region situated, as arrowed, between the underlined gene residues (GeneBank accession no. in table 1).

3.3.8 Effect of a Neutralizing Antibody against CCK on Gonadotrophin Secretion by Human Functionless Pituitary Tumours

Since the presence of CCK receptors together with CCK peptide expression itself suggested that there might be an autocrine loop (see later in discussion of gene expression, page 160) controlling anterior pituitary hormone secretion by human functionless pituitary tumours, the neutralizing effect of an antibody against the CCK peptide on endogenous CCK activity on LH and FSH secretion was examined. A significant dose-dependent inhibition of LH and FHS secretion by a human functionless pituitary tumour derived cells was observed, with the resultant decrease of about 50-60% of control as shown in figures 66 and 67.

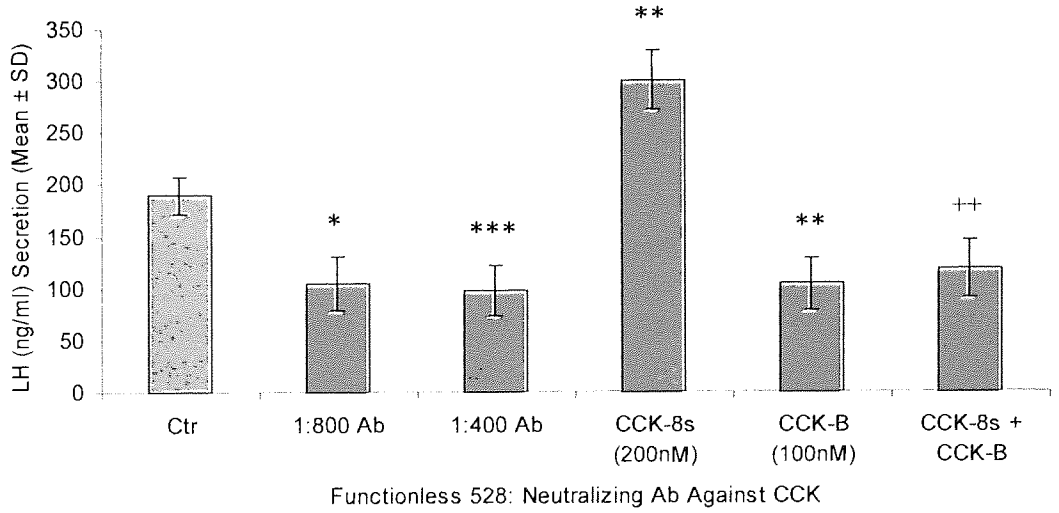


Figure 66: Inhibition of non-stimulated LH secretion by anti CCK antibody by a functionless pituitary tumour (528). Effects of CCK-8s and / or CCK-B-R specific antagonist after 24 h. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s.

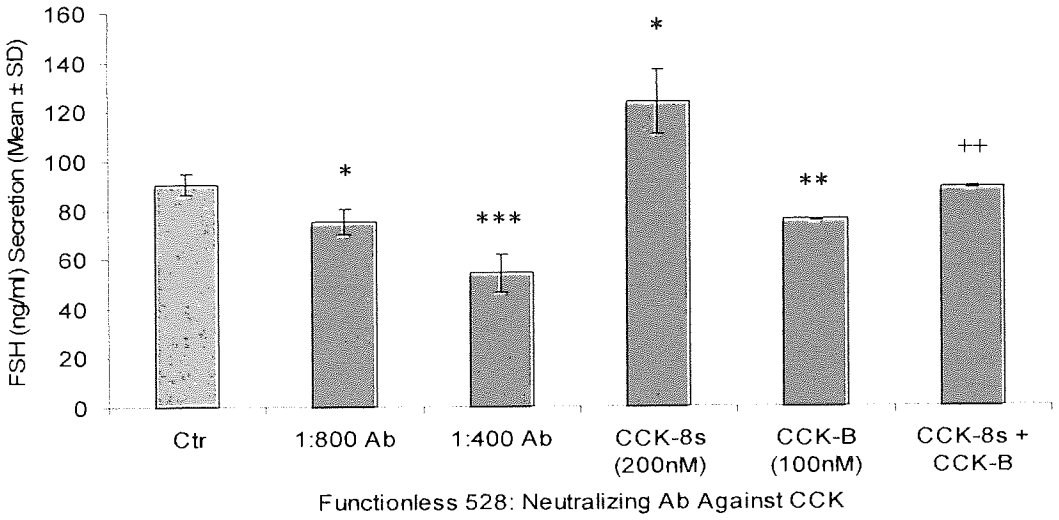


Figure 67: Inhibition of non-stimulated FSH secretion by anti CCK antibody by a functionless pituitary tumour (528). Effects of CCK-8s and / or CCK-B-R specific antagonist after 24 h. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01; ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s.

A hormone secretion study was performed in one cell culture derived from a functionless pituitary tumour to examine the long term effect of a neutralizing anti-CCK antibody on LH and FSH secretion. A significant inhibition of LH and FSH secretion by a human functionless pituitary tumour derived cells was observed after 24 hours, resulting in 30-120% reduction of control (figures 68 and 69).

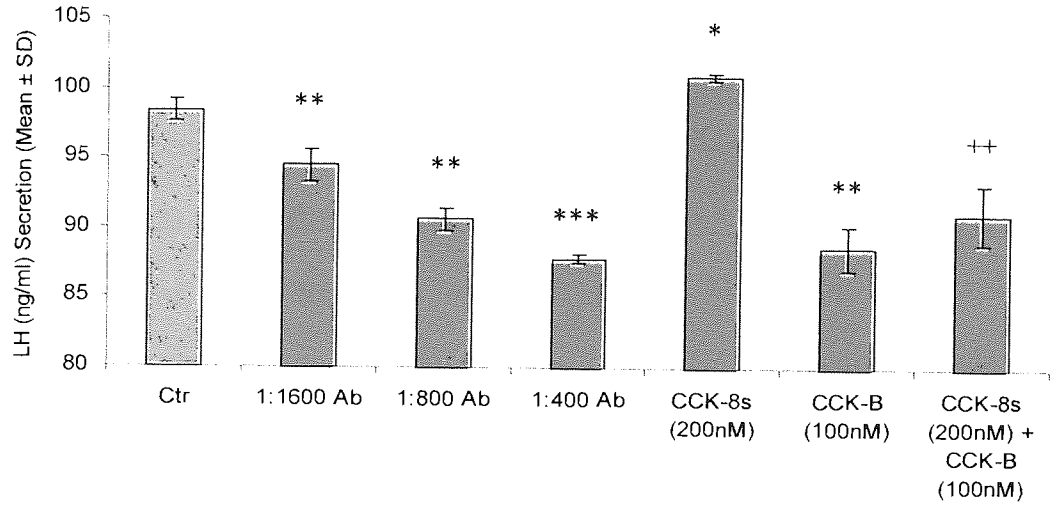


Figure 68: Inhibition of non-stimulated LH secretion by anti CCK antibody by a functionless pituitary tumour (529). Effects of CCK-8s and / or CCK-B-R specific antagonist after 24 h. Triplicate cultures were used for each variable; n=1. *P<0.05; **P<0.01, ***P<0.001 vs. control; $^{++}$P<0.01 vs. CCK-8s.

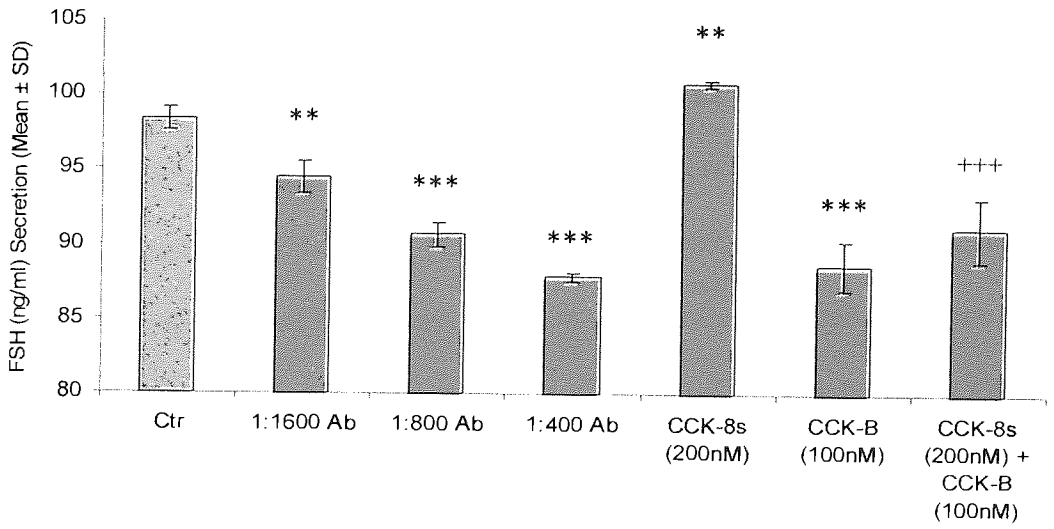


Figure 69: Inhibition of non-stimulated FSH secretion by anti CCK antibody by a functionless pituitary tumour (529). Effects of CCK-8s and / or CCK-B-R specific antagonist after 24 h. Triplicate cultures were used for each variable; n=1. **P<0.01; ***P<0.001 vs. control; $^{+++}$P<0.001 vs. CCK-8s.

3.4 DISCUSSION

3.4.1 Somatotrophinomas and GHRH-R Gene Polymorphism

The GHRH receptor is a typical G_s-protein coupled, 7-transmebrane receptor consisting of 423 amino (Mayo *et al.*, 1995). Binding of its ligand, GHRH, leads to displacement of GDP from the α-subunit of the G_s-protein ($G_s\alpha$) and its replacements with GTP (Faglia, 1993; Adams *et al.*, 1995). Subsequently, adenylyl cyclase is activated and the resultant increase in intracellular cAMP production leads to GH secretion. Tight control of these levels is exerted by intrinsic GTPase activity of the $G_s\alpha$ subunit, which soon results in removal of a phosphate group from the bound GTP and a return to the basal state of the GHRH-adenylyl cyclase-cAMP transduction cascade.

Since experimentally-induced constitutive activation of this pathway leads not only to excessive GH secretion, but also pituitary somatotroph hyperplasia and tumour development in animals (Mayo *et al.*, 1985; Burton *et al.*, 1991), it is not surprising that activating mutations with similar effects are associated with somatotrophinomas in humans (Landis *et al.*, 1989). The discovery of *gsp* oncogenes in a subset of human pituitary somatotrophinomas represents one of the most important advances in the elucidation of pituitary tumour biology (Vallar *et al.*, 1987; Landis *et al.*, 1989). The effect of these oncogenes is to disrupt the GHRH receptor-G_s-protein-adenylyl cyclase pathway (Vallar *et al.*, 1987; Faglia, 1993). Specifically, point mutations, which abolish the GTPase activity of $G_s\alpha$-GTP subunit (*gsp* oncogenes) are the presumed major etiological factor in about 40% of these pituitary tumours (Landis *et al.*, 1989). Biochemically, *gsp* oncogenes lead to constitutive adenylyl cyclase activity and excessive cAMP production, properties

which originally led to the suspicion of, and the search for, a defect in the G_s-protein (Vallar *et al.*, 1987). The single base missense mutation that can occur in codon 201 or 227 of the G_sa gene results in amino acid substitutions that abolish the intrinsic GTPase activity of the mature $G_s\alpha$ polypeptide, and consequently, adenylyl cyclase remains permanently active (figure 70)

Figure 70: Upon ligand (GHRH) binding the $G_s\alpha$ subunit bound to GTP will go on to stimulate cAMP production. While GDP-GTP exchange, GTPase activity that tightly regulates cAMP production releases an inorganic phosphate (Pi) and causes the GHRH-R to return to its inactive state until another GHRH molecule binds the GHRH-R and the cycles commences again. The mutation in the $G_s\alpha$ subunit will stop the intrinsic phosphatase activity leading to continuous cAMP production.

The effect of *gsp* oncogenes clearly demonstrate that abnormally elevated cAMP levels can lead to pituitary tumour development. Because of these findings, it is logical to suspect that defects elsewhere in the GHRH-adenylyl cyclase-cAMP pathway could be another molecular factor in the development of at least some somatotrophinomas. An obvious target for investigation is the GHRH-R, particularly since activating mutations in other G-protein-coupled receptors have been described (Spiegel, 1996). For these reasons, the present study investigated the potential role of the alternative GHRH-R form, containing Thr at position 57 as opposed to the more commonly found Ala, in the function of GH-secreting pituitary cells and acromegaly. This is particularly important to study since transfection experiments indicate that the alternative form confers increased responsiveness to GHRH (Petersenn *et al.*, 2000). The present study shows that about 20% of acromegalic patients are heterozygous for the two forms of the GHRH-R gene. The present results indicate that the somatotrophinomas possessing the alternative GHRH-R form respond, on average, more strongly to GHRH in terms of GH secretion and cAMP formation than tumours homozygous for the more common allele. These latter results confirm both the increased effect of GHRH on cAMP formation and the transfection studies mentioned above (Landis *et al.*, 1989). Perhaps significantly, the effect of i.v. GHRH on serum GH levels is highly variable in both normal and acromegalic subjects with some individuals showing a very large response (Wood *et al.*, 1983). Based on the present observations, it is possible that alternative forms of GHRH-R may at last partially explain this variability of response to GHRH.

Given the above described and previous findings, it is reasonable to suggest that possession of the rarer polymorphism may predispose subjects to development of GH-secreting pituitary somatotrophinomas and therefore acromegaly. If this were the case, it might be anticipated that the alternative form possessing Thr at position 57 would be more common in the acromegalic population in comparison to non-acromegalics. The present findings on distribution of the polymorphism, however, show no significant difference in the distribution of the polymorphisms in the two groups of subjects. They therefore do not, in fact, support the concept that the alternative allele may predispose to developing acromegaly since the alleles were present in the same frequencies in both populations. Indeed, the distribution was also identical in the acromegalic group irrespective of presence or absence of *gsp* oncogenes whereas it might be expected that the alternative allele would be less frequent in the *gsp*-positive sub-group, as would be the case if the allele, via other biochemical mechanisms, were involved with the development of acromegaly. The lack of homozygosity for the alternative receptor form is also probably of little significance since, based on the allele distribution of the 72 samples studied, the Hardy-Weinberg equilibrium predicts that only about 1% of the population would possess both rarer alleles. It should also be stressed that the amino acid 57, which is present in the N-terminal extracellular domain of the full receptor, is neither conserved nor semi-conserved between species (Mayo *et al.*, 1985; Chan *et al.*, 1998). In rats and mice, Gly is found at residue 57, the porcine and bovine receptors possess Arg, and Thr has been found in goldfish. Nevertheless, an uncharged but hydrophilic amino acid has the correct properties to induce a new conformation in the extracellular domain of the GHRH receptor, perhaps leading to enhanced receptor activation. The extracellular domain of family B GPCRs is important in

ligand binding and residue 57 in the GHRH receptor is between conserved Cys and Asp residues that have been suggested to be important in this process (Hasmar, 2001).

3.4.2 Effects of CCK on Hormone Secretion

It is well established that the control of anterior pituitary hormone secretion is exerted by hypothalamic factors and feedback mechanisms (Harris and Green, 1947). In general, the hypothalamus produces specific inhibiting and releasing factors for each of the anterior pituitary hormones. In turn, target gland products exert a negative feedback, which inhibits further hormone release (Harris and Green, 1947). It is becoming clear, however, that these control mechanisms, especially those exerted by the hypothalamus, are far more complex and involve interactive effects of several factors. For example, although initially believed to be specific for one particular pituitary hormone, some hypothalamic factors actually modulate the secretion of several hormones (Adams *et al.*, 1979). Thus, TRH stimulates not only thyroid stimulating hormone (TSH) secretion but also PRL secretion (Adams *et al.*, 1979). Perhaps more significantly, the hypothalamus has been shown to contain many peptides originally thought to be limited to other locations in the body. This is particularly true for the so-called 'gut-brain' peptides. That is, some peptides thought to be restricted to the gut system are found throughout the CNS including the hypothalamus (Rehfeld, 1978; Larsson and Rehfeld, 1979). One of the most prominent of these 'gut-brain' peptides is CCK.

As mentioned in chapter two, although CCK was originally described as a gut peptide able to induce pancreatic secretions and gall-bladder contraction (Ivy and

Oldberg, 1928), subsequent studies, following its identification as a 33-amino acid peptide have demonstrated that it is also present in various forms throughout the CNS as a gastrin-like immunoreactive material (Vanderhaeghen *et al.*, 1975; Dockray, 1976).

CCK has been identified in the pituitary and the rat hypothalamus (Beinfeld *et al.*, 1980; Vanderhaeghen *et al.*, 1981), the latter having a role in pituitary regulation, controlling release of hormones of the anterior lobe by means of regulating factors. Binding densities for CCK receptors vary across the hypothalamus and several sites have a peripheral type profile. In 1985, it was shown that CCK parvicellular neurons are potentially involved in the regulation of adenohypophyseal hormone secretion through projections to the external zone of the median eminence and neurohypophysis and a pathway from dorsal root ganglia to the dorsal horn of the spinal cord. CCK immunoreactivity was also identified in the magnocellular-neurohypophyseal system, exclusively in oxytocinergic neurons (Reiner and Beinfeld, 1985). The evidence that CCK-8s and CCK-33 along with its receptors exist in high concentrations in the hypothalamus and adenohypophysis suggests that CCK might act as a neurohormone or neurotransmitter in regulating pituitary function (Rehfeld, 1978; Imura *et al.*, 1981; Innis and Snyder, 1980). A human embryonic pituitary cell line, Flow 2000, has been shown to possess CCK receptors of the peripheral subtype (CCK-A) (Lo and Hughes, 1987), whilst the GH3 rat anterior pituitary tumour line appears to express CCK-B receptors (Kuwahara *et al.*, 1993). In addition, previous studies have indicated that activation of the CCK-B receptor on GH3 cells leads to Ca^{2+} mobilisation in association with stimulation of PI turnover and IP_3 production (Malesci *et al.*, 1980). In support of this concept,

previous studies have shown that CCK and related peptides can modulate gonadotrophin secretion *in-vivo* in animals (Vijayan *et al.*, 1970; Malarkey *et al.*, 1981; Matsumura *et al.*, 1984), and to directly stimulate GH and PRL secretion by human pituitary cells in culture (Calogero *et al.*, 1993).

The present results confirm and extend to human tissue these preliminary findings. Both CCK-8s and CCK-33 directly stimulated GH secretion by somatotrophinomas in culture and these effects were enhanced by Ghrelin, suggesting that interactive effects may also occur *in-vivo.* However, a similar interaction with GHRH was not observed. This is of interest considering the intracellular mechanism of action of the three factors. All three bind to G-protein coupled 7 transmembrane receptors (Smith *et al.*, 1994; Mayo *et al.*, 1995; Adams *et al.*, 1996). As demonstrated in the present studies, activation of pituitary CCK receptors leads to PI hydrolysis but not cAMP formation. The PI second messenger system is also activated by Ghrelin, a recently discovered hypothalamic factor involved in controlling pulsatile GH secretion (Adams *et al.*, 1998). In contrast, the GHRH-R is coupled to adenylyl cyclase and cAMP generation (Mayo *et al.*, 1995). It is therefore surprising that the present studies revealed interactive effects of CCK with Ghrelin but not GHRH, since it might be expected that addition of stimulatory factors which activate different second messenger systems would lead to an additive or synergistic effect on GH secretion (Adams *et al.*, 1996). Conversely, since CCK and Ghrelin both activate PI hydrolysis, an additive effect of the two might not be expected. One possible explanation for these results is that different intracellular pools of GH are being affected by the three factors. There is compelling evidence that Ghrelin and GHRH stimulate the secretion of independent pools of GH (Adams *et al.*, 1996). It

is possible that CCK stimulates the same pool of GH as GHRH, albeit via a different second messenger system, whereas Ghrelin stimulates release of another intracellular pool of GH. Further experiments are required to confirm this hypothesis.

In terms of gonadotrophins, previous studies on animals *in-vivo* have yielded conflicting results, with some suggesting a stimulatory effect of CCK whilst others suggest an inhibitory effect (Kawakam *et al.*, 1979; Hashimoto and Kimura, 1986). The present results demonstrate, for the first time, a direct stimulatory effect of CCK peptides on both human LH and FSH secretion. As such, these results indicate that CCK may play a crucial role in controlling human LH and FSH secretion, especially since synergistic effects were observed in combination with E2. However, it should be noted that tumourous cells have been used and it is possible that these respond differently to CCK than normal human gonadotrophs.

Extending these findings, RT-PCR analyses clearly revealed that CCK peptide and CCK-B receptor subtype mRNA was always and abundantly expressed by pituitary cells. Moreover, as demonstrated in the present studies, activation of pituitary CCK receptors leads to PI hydrolysis but not cAMP formation, consistent with previous findings (Kuwahara *et al.*, 1993), suggesting a possible involvement of PI breakdown and calcium mobilization in the transduction system of CCK-B receptor in human pituitary tumours. The role of inositol phosphates as intracellular second messengers is well established, with activation of cell surface receptors promoting hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate and diacylglycerol. The former mobilises Ca^{2+} from

intracellular stores and the latter can activate the PKC enzyme family, bringing about protein phosphorylation. Many other inositol phosphates have also been identified although their precise roles are still under investigation. In the present study, we have examined CCK-B receptor signal transduction. The results suggest that PI turnover is not a signalling mechanism exclusive to CCK-A receptors and demonstrate that CCK-B receptors on cells extracted from human pituitary tumours are part of the family of receptors which employ inositol phosphates in cell signalling (Smith and Freedman, 1996). It is therefore concluded that CCK-B is the receptor via which CCK carries out its effect on human pituitary tumours since is the most abundant receptor subtype to be expressed.

These results suggest that CCK stimulate GH and gonadotrophin hormone secretion through CCK-B-R. However it is still unknown whether CCK is able to stimulate hormone secretion *in vivo* either directly or indirectly through other trophic substances. Since there has been no evidence of other factors involved, it seems more plausible to postulate that an activation of CCK-B receptor by CCK causes a direct stimulation of GH and gonadotrophin hormone release. This hypothesis can be supported by evidence showing that CCK-8s induced tyrosine phosphorylation and subsequent, c-fos and c-myc expression, suggesting that CCK-B receptor might transmit mitogenic signals by cross talking with tyrosine kinase cascades (Xu *et al.*, 1996). There is considerable evidence that CCK receptors are coupled to a mitogenic response (Larsson and Rehfeld, 1979). In addition, PI hydrolysis is coupled to hormone secretion (Saito *et al.*, 1980; Wank *et al.*, 1992) and thus it is conceivable that CCK may modulate the effects of hypothalamic releasing and

inhibiting factors. These two observations together with the present findings raise the possibility that CCK may play a role in human pituitary tumourigenesis.

In the present study we have also characterised CCK receptors on human pituitary tumours using RT-PCR analysis and functional studies using the PI hydrolysis turnover. The antagonism profile of selected agonists and selective non-peptide antagonists to CCK-A and CCK-B receptors was consistent with the CCK receptor in the human pituitary tumours being of the CCK-B subtype. The finding that CCK mRNA, in addition to its B receptor, is expressed raises the possibility that pituitary cells secrete CCK itself, which may act in an autocrine manner to modulate hormone secretion. On the other hand, CCK-A receptor was rarely expressed and was limited to 20% of the functionless pituitary tumours. In support of these findings, the CCK-B receptor selective antagonist L-365,260 displayed higher potency compared to the CCK-A receptor selective antagonist L-364,718), whereas, a neutralizing antibody against CCK significantly inhibited the autocrine / paracrine induced hormone secretion alone.

Finally, it is worth mentioning the possible clinical significance of the results of the present studies. The discovery that CCK may have a role in stimulating hormone secretion by human pituitary cells may have relevance in developing novel drugs suitable for suppressing hypersecretory conditions. Stimulatory effects of CCK peptides on pituitary hormone secretion were strongly inhibited by selective non-peptide antagonists to CCK-A and CCK-B receptors. The present results also demonstrate that antagonists to CCK do indeed directly inhibit hormone secretion and block the effects of CCK peptides. The results also extend the findings that the

same antagonists block the stimulatory effects of CCK on PI hydrolysis in rat pituitary cells (Smith and Freedman, 1996). It is conceivable therefore that the antagonists could reduce the hyperactivity of human pituitary tumours, especially if an autocrine loop is involved. It will also be important to determine whether the antagonists have an effect on mitotic activity of human pituitary tumours, similar to that exhibited by other anti-pituitary tumour drugs such as bromocriptine and octreotide (Gruszka *et al.*, 2001).

3.5 Conclusion

The distribution of the two allelic forms of the GHRH-R did not significantly differ between acromegalic and non-acromegalic subjects. However, the alternative GHRH-R form may confer increased sensitivity of GH-secreting pituitary cells to GHRH, whereas, possession of the alternative form of the receptors dose not appear to be associated with increased risk of developing acromegaly.

In terms of CCK, it is of interest whether an activation of autocrine / paracrine axis of CCK–CCK-B-R plays some role in an induction of pituitary tumour or its progression (Hoosein *et al.*, 1990; Remy-Heintz *et al.*, 1993). The present findings provide strong evidence that an autocrine / paracrine system may operate within human pituitary tumours. It was only recently (Xu *et al.*, 1996) that the first evidence for an autocrine / paracrine role for CCK was described on stimulation of GH3 rat pituitary tumour cell line thought the CCK-B receptor. It is conceivable, therefore, that the uncontrollable hormone secretion of pituitary tumours is, at least partly, due to abnormal secretion of this peptide. It must be emphasized, however, that we have not examined whether normal tissue in culture can also secrete this

mitogenic factor. The data show directly that CCK may act on stimulating cell growth of human pituitary tumours by directly inducing hormone secretion via the CCK-B receptor. The CCK-B receptor is PKC dependent (figure 43) and was abundantly expressed by human pituitary tumours along with the CCK peptide itself. However, it remains unclear whether the CCK-CCK-B-R axis, which is not ubiquitous in all of the neoplastic tissues, but rather specific for certain tumours including GH3 cells (Matsumori *et al.*, 1995), is activated before or after the transformation of the normal pituitary.

CHAPTER FOUR

CRANIOPHARYNGIOMAS: CLONAL ANALYSIS AND β-CATENIN MUTATIONS

4.1 INTRODUCTION

Craniopharyngiomas are congenital epithelial lesions and arise in the subarachnoidal spaces of the suprasellar region and the hypothalamic / optic chiasm region (Adamson *et al.*, 1990; Miller, 1994). Although their origin is not firmly established, they are generally thought to arise from embryonic remnants of Rathke's pouch and sac, representing a maldevelopment event. They clinically manifest themselves as a local mass effect, after a steady growth period that commences in foetal life (Shin *et al.*, 1999). These are relatively rare tumours and predominately diagnosed during childhood and there appears to be a peak incidence at puberty (Banna, 1976; Sorva and Heiskanen, 1986). They represent 9% of all childhood brain tumours but just 3% of all intracranial brain tumours. Because of their location, symptoms include severe headaches, vomiting, visual disturbances and endocrine defects. The latter is due to the close proximity of the lesions to the hypothalamus and includes failure to grow or enter puberty. Behavioural defects and eating disorders are also common.

The macroscopic appearance of the tumour might be cystic, solid or mixed. Clinically and histologically, two basic types have been identified (Adamson *et al.*, 1990). One is the epithelial or adult form with nests, papillary structures or trabeculae of squamous epithelial cells embedded in a loose connective tissue stroma. The other is the adamantinomatous or childhood type, wherein aggregates of stellar cells are surrounded by a layer of single or pseudostratified columnar epithelium resting upon a basement membrane, frequently associated with calcification and keratin nodule formation. The majority of craniopharyngiomas of

the adamantinomatous variant, usually appear in the first two decades of life, have a high rate of recurrence and are calcified (Miller, 1994; Shin *et al.*, 1999). These tumours are composed of neoplastic epithelial cells, bearing a striking resemblance to odontogenic lesions found in the jaw, particularly the ameloblastoma (adamantinoma) and the calcifying odontogenic cyst (Paulus *et al.*, 1997).

Craniopharyngiomas are difficult to treat because of their adverse location and 'sticky' nature that cause them to adhere to crucial intracranial structures, hindering radical surgical removal. Aggressive surgical procedures are associated with a high risk of damaging the brain (Hoffman *et al.*, 1992; Honegger *et al.*, 1992). Moreover, craniopharyngiomas are prone to recur (figure 71). In almost all cases, postoperative endocrine defects develop requiring life-long replacement management. Alternative novel adjuvant therapies for craniopharyngiomas are therefore desirable. An understanding of the molecular mechanisms underlying craniopharyngiomas growth would help in the development of such therapies. It is surprising, therefore, that very little research has been conducted on the biochemistry and molecular biology of craniopharyngiomas. Indeed, there have been only a handful of published papers, reporting very briefly on the receptor status, clonality and the possibility of abnormalities in the Wnt developmental signalling pathway. The purpose therefore of this part of the study was to further investigate these molecular aspects of craniopharyngiomas.

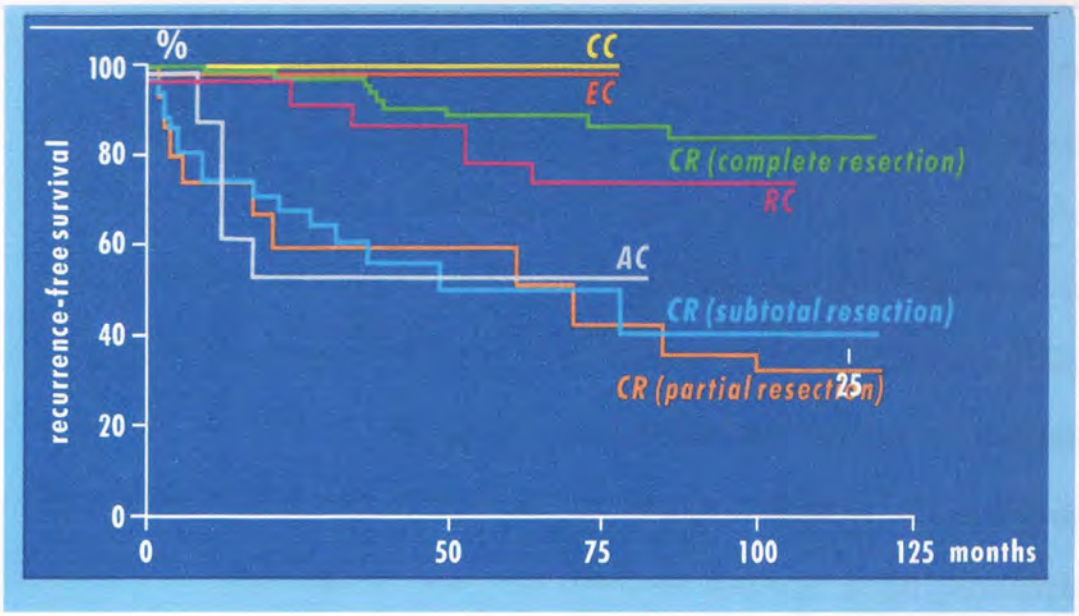


Figure 71: Recurrence-free survival time after primary surgery of craniopharyngiomas and sellar cysts in general.

4.1.1 Clonality

The determination of whether a tumour is mono- or polyclonal in origin provides clues to its aetiology. The finding that neoplasms are monoclonal indicates that they are derived from a single progenitor cell and suggests that an intrinsic gene defect (s) is the cause of excessive proliferation. Polyclonality, on the other hand, might be an indication that external factors, such as proliferation-inducing hormones or growth factors, play a major role in excessive growth (Honegger *et al.*, 1997). In a previous study, clonality of a small group of craniopharyngiomas was determined (Sarubi *et al.*, 2001). The results showed that 2 of 6 tumours were monoclonal while 4 were found to be polyclonal, immediately suggesting that craniopharyngiomas may have varying aetiologies.

To shed further light on this, the present studies were intended to investigate the clonality of a large group of craniopharyngiomas. The clonality of tumours and cancers can be determined by analysis of polymorphisms in X-linked genes and by exploiting X-chromosome inactivation (Lyon, 1962; Sarubi *et al.*, 2001). X-Chromosome inactivation is maintained in daughter cells by differential methylation of deoxycytosine residues in the DNA of X-chromosome genes, and differential methylation patterns of X-chromosome genes have been used to examine clonality of tumours in females heterozygous for a particular X-linked polymorphism. Previous methods have used somewhat cumbersome techniques involving complex radioactive methodology coupled with restriction fragment length polymorphism (RFLP) analysis. In the present studies, an attempt was made to develop and use an alternative and simplified non-radioactive method based on the PCR amplification of the highly polymorphic X-linked human androgen receptor (HUMARA) gene in combination with single stranded conformational polymorphism (SSCP) as determined by silver stain

4.1.2 β-Catenin Mutations and the Wnt Signalling Pathway

The protein β-catenin, also known as cadherin-associated protein, was first identified in humans as an important cell-cell adhesion molecule (Kemler and Ozawa, 1989). Cooperative studies of signalling pathways in *Xenopous* and *Drosophila* subsequently led to the discovery of a second role for β-catenin in human cells; this cell-signaling role involves translocation of the protein from the cytoplasm into the nucleus (Morin, 1999; Kikuchi, 2000). Genetic and embryonic studies have revealed that β-catenin is a component of the Wnt pathway (Kispert, 1998). This intracellular signalling pathway is highly conserved and regulates cell

proliferation, differentiation, morphology, motility, fate, axis formation, and organ development (Takeichi, 1991). Wnt signalling plays a central role in the development of the CNS, kidneys, placenta, reproductive tract, and limbs in vertebrates (Kengaku *et al.*, 1998; Kispert, 1998; Parr and McMahon, 1998; Patapoutian and Reichardt, 2000).

Many molecules interact with β-catenin. The functional consequences of some of these interactions are well established [e.g cadherins, (lymphoid enhancer factor / T-cell-factor (LEF / TCF), or the tumourtous polyposis coli (APC) / axin degradation machinery), whereas the significance of some other interactions is still incompletely understood. Among these various molecules involved in the Wnt pathway, alterations in APC, axin and β-catenin genes have been frequently found in several human epithelial type carcinomas (Clevers, 2000; Webster *et al.*, 2000). This increase in β-catenin levels results mostly from mutations in exon three of the β-catenin that affect serine / threonine residues in the glycogen synthase kinase-3β (GSK-3β) phosphorylation sites critical for β-catenin degradation. The result of such mutations is abnormal accumulation of β-catenin in the nucleus and activation of β-catenin:LEF / TCF-dependent transcription (Kikuchi, 2003). Studies have shown that this may contribute to uncontrolled cell proliferation and tumour progression in pituitary tumours (Semba *et al.*, 2001) and craniopharyngiomas (Sekine *et al.*, 2002).

The mechanisms by which these lesions appear are not understood and as down-regulation of cadherin-catenin complexes has been observed in various human tumours including odontogenic tumours, which have been shown to harbour β-

catenin mutations (Sekine *et al.*, 2003). It has been well recognised that adamantinomatous craniopharyngiomas show a histological resemblance to some odontogenic tumours (Love and Marshall, 1950; Gorlin and Chaudhry, 1959; Bernstein and Buchino, 1983; Paulus *et al.*, 1997). This histological resemblance between some odontogenic tumours and adamantinomatous craniopharyngiomas, as well as the critical role of Wnt signaling pathway in the CNS led to the present study to evaluate whether β-catenin mutation are also present in adamantinomatous craniopharyngiomas and what is the importance of the this pathway in development of craniopharyngiomas. In this present study the presence of somatic mutations in exon three of the β-catenin gene, where the vast majority of mutations have so far been described, was investigated in a large number of craniopharyngiomas of both the adamantinomatous and papillary type by means of PCR amplification and direct sequencing analysis.

4.2 MATERIALS AND METHODS

Materials and Methods and the preparation of solutions are as previously described (refer to chapter two, pages 35-36, 39) and only the extra additions are listed here.

4.2.1 Materials

Amersham Biosciences, Bucks, UK, DNA Silver Staining Kit, ExcelGel DNA analysis Kit

Fermentas, USA, Hin6I (HhaI), HpaII

New England BioLabs, USA, AluI

Qiagen, Düsseldorf, Germany, QIAamp DNA Blood Mini Kit

4.2.2 Buffers and Solutions

SSCP Loading Dye, 0.1% bromophenol blue, 0.1% xylene cyanole and 40% glycerol were dissolved in sterile distilled water.

4.2.3 Methods

4.2.3.1 Clonal Analysis

During the process of embryogenesis in the female, either the maternally derived or the paternally derived X-chromosome in each cell is randomly and permanently inactivated (Lyon, 1962) by DNA methylation and is thus protected from digestion with methylation sensitive restriction enzymes. Analysis of clonality in the present study is based on the pattern of X-chromosome inactivation which was assessed by PCR amplification with oligoprimers for the HUMARA gene locus and resolved by means of SSCP followed by non-radioactive silver staining. The method utilizes the highly polymorphic HUMARA gene, because it localizes to the X-chromosome (X-linked) and contains a short tandem repeat (STR) consisting of a polymorphic CAG sequence. The HUMARA gene also contains restriction enzyme sites for the methylation sensitive enzymes HpaII and HhaI in close proximity to the STR region (figure 72). Consequently, digestion with HpaII and HhaI of the HUMARA gene and subsequent PCR amplification using primers flanking the STR region will yield one allele (inactivated / protected) while the other will be eliminated (non methylated) in heterozygous monoclonal tissues of the female subjects. In monoclonal derived tumours, according to the X-chromosome inactivation phenomenon, the same X-chromosome will always be inactivated, whereas in polyclonal tumours a mixture of inactivated X-chromosomes will be present (detailed overview given in the discussion, pages 184-186).

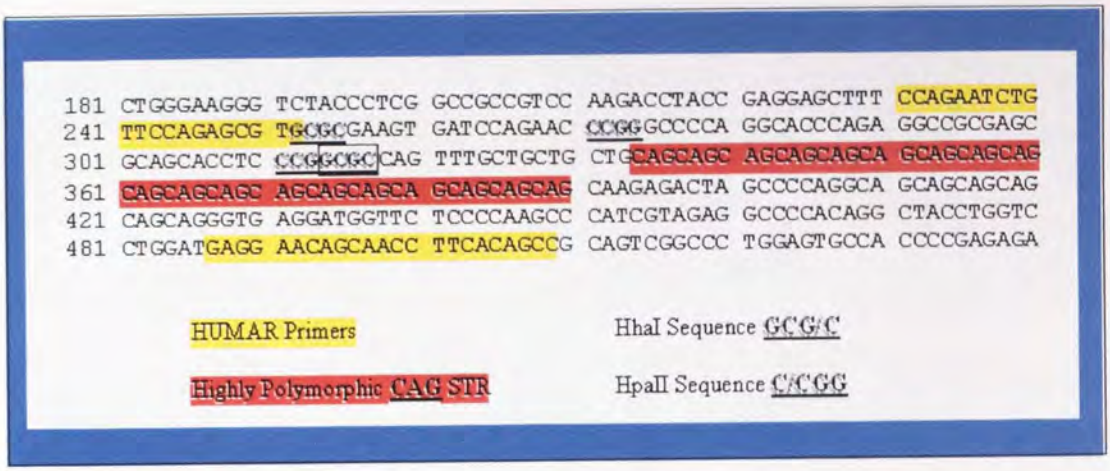


Figure 72: The HUMARA gene. The HUMARA primers (yellow) designed to encompass the highly polymorphic STR CAG sequence (red) and the methylation sensitive enzyme sites for HpaII and HhaI enzymes (bold and underlined).

To establish the method, experiments were performed using pituitary tumours from female subjects, since there is strong evidence that these are monoclonal in origin (Alexander *et al.*, 1990), and they will always yield 4 bands prior digestion and 2 bands afterwards when resolved by SSCP on polyacrylamide gels (figure 73). As a control DNA derived from male subjects will yield 2 bands before digestion and no bands afterwards. Therefore monoclonal tumours will have one X-chromosome inactivated and yield two bands (figure 73) after digestion whereas polyclonal tumours will have a mixture of alleles that are inactivated and will continue to yield four bands regardless of prior digestion with HpaII and HhaI, when resolved by SSCP analysis (figure 73), (detailed overview given in the discussion, pages 184-186). PCR products were resolved by means of SSCP followed by non-radioactive silver staining.

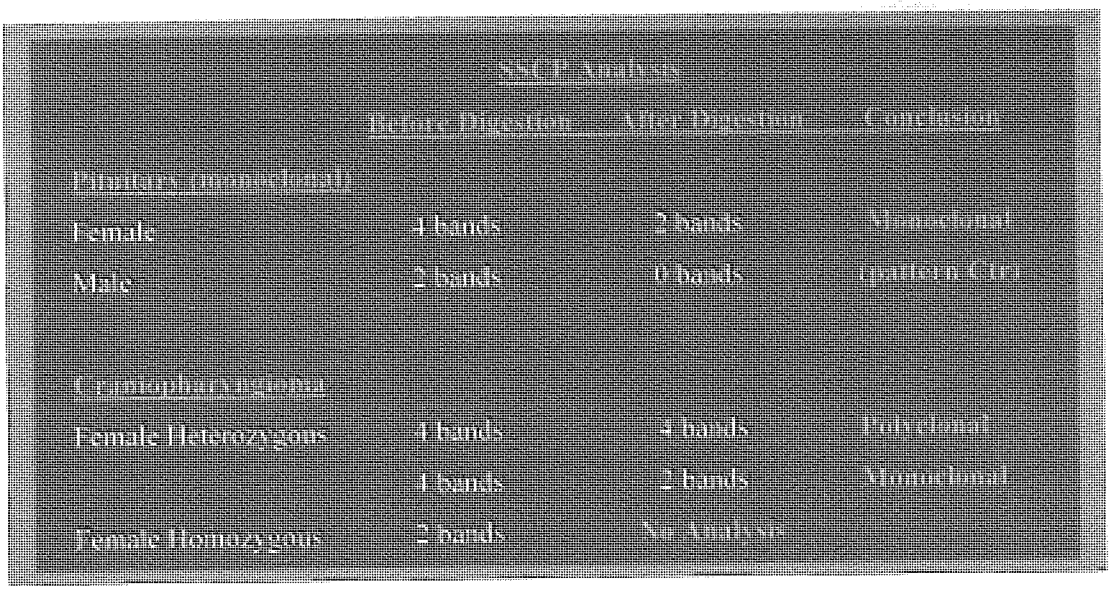


Figure 73: X-chromosome inactivation patterns of the amplified HUMARA gene before and after digestion with methylation sensitive enzymes (HpaII and HhaI) when resolved by SSCP analysis.

Genomic DNA extracted from human pituitary tumours as previously described (refer to chapter three, page 116) was digested overnight at 37 C with or without an excess of HpaII / HhaI in 25µl total volume. After digestion, the reaction was terminated by incubating the mixture at 65 C for 10min. The digested DNA was then phenol-chloroform extracted and salt-ethanol precipitated as previously described (refer to chapter three, pages 116-117). Purified samples were subjected to PCR with a previously described primers (sense: 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'; and antisense: 5'-GGCTGTGAAGGTTGCTGTTCCTCA-3') for the amplification of the HUMARA gene (Allen *et al.*, 1992). PCR was carried out in a reaction mixture of 100µl total volume, which was added directly to the precipitated DNA pellet. The primers were designed to flank the STR performed on native and HpaII / HhaI digested samples, and yield a PCR product of 280bp in length. Each DNA sample was amplified using

a Techne Progene thermocycler PCR with the corresponding optimal condition as described in table 16.

Table 16: Optimal PCR Condition for SSCP Analysis		
HUMARA **GenBank accession no.:** M35844.1	95 C 1 minutes 65 C 2 minutes 95 C 3 minutes ..... 72 C 3 minutes 4 C 10 minutes	35 cycles 1 cycle

Single Stranded Conformational Polymorphism (SSCP)

Five µl of PCR digested DNA products were mixed with 5µl of chilled SSCP gel-loading buffer and denatured at 95 C for 5 minutes and thereafter immediately placed on ice to prevent re-annealing of the single strand product. The whole mixture was loaded on a renaturing 10% polyacrylamide gel, and electrophoresis was performed at 4 C, for 1-2 hours at 1.2-1.5W constant current using a multiphore electrophoresis unit. After electrophoresis the gel was silver stained using a DNA Silver Staining Kit (Amersham Biosciences, UK) according to the manufacturer's instructions.

4.2.3.2 β-Catenin Mutations

DNA was extracted from 17 craniopharyngiomas of the adamantinomatous and 8 of the papillary type as previously described (refer to chapter three, page 116) and examined for possible β-catenin gene mutations. Following PCR with previously described primers (sense: 5'-GATTTGATGGAGTTGGACATGG-3'; and antisense: 5'-TGTTCTTGAGTGAAGGACTGAG-3') encompassing glycogen

synthase kinase-3β (GSK-3β) phosphorylation sites of the β-catenin gene (Kajino *et al.*, 2001).

Blood samples for most of the tumourous samples were also provided (Department of Neurosurgery, University of Goettingen, Germany) for which DNA was also extracted to allow comparative studies. DNA extraction from blood samples was performed using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions. To increase DNA yield, the QIAamp spin column loaded with buffer was incubated for 5 minutes at room temperature. PCR was carried out in a reaction mixture of 100µl total volume and full details of the condition used are stated in table 17.

Table 17: Optimal PCR Condition for β-Catenin Mutations

β-Catenin	95 C 1 minutes	35 cycles
GenBank accession no.:	55 C 2 minutes	
HSRNABECA	95 C 3 minutes	
	.....	
	72 C 3 minutes	1 cycle
	4 C 10 minutes	

The primers yielded a PCR product of 118bp. To ensure amplification of the required sequence, purified PCR DNA was digested with an excess of AluI that cleaves at one site and gives rise to two bands of 88 and 30bp. Bands were resolved on a 2% gel against molecular weight markers and visualised under the UV light. Possible mutations were revealed by direct sequencing (Functional Genomics Lab, Birmingham University, UK) as previously described (refer to chapter two, page

44). When apparent sequence abnormalities were found, these were confirmed by a second round of DNA extraction, PCR and direct sequencing.

4.3 RESULTS

4.3.1 Clonal Analyses

To establish the techniques and check that they could, at least in theory, be applied to the determination of clonality of craniopharyngiomas, DNA samples extracted from a male subject and a pituitary tumour from a female patient were employed. Since males possess only one X-chromosome, the analysis should yield 2 bands before digestion and no bands following digestion. Since pituitary tumours are monoclonal in origin, the DNA from the pituitary tumour of the female subject should also yield 2 and 4 bands respectively (figure 73). The results of analysis of DNA extracted from 2 pituitary tumours (derived form 1 male and 1 female) are shown in figure 74. The predicted band pattern prior to digestion was observed. It was therefore possible to apply the technique to craniopharyngiomas before and after digestion.

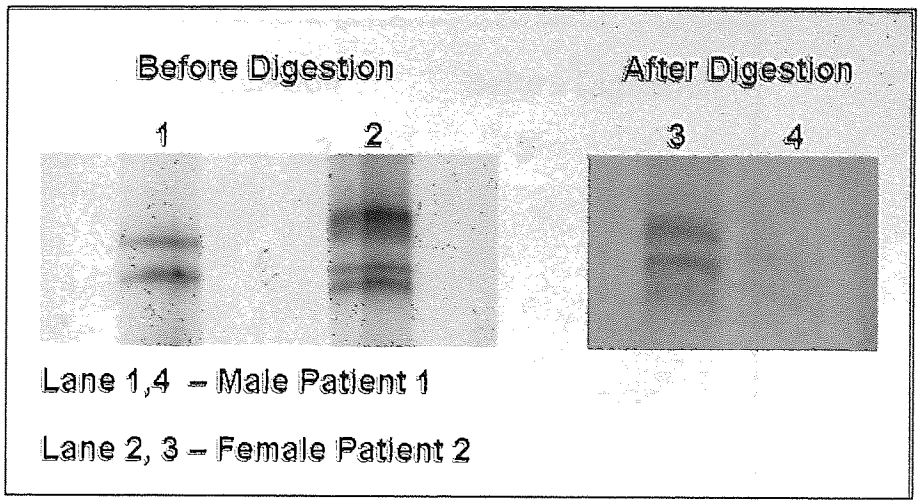


Figure 74: PCR amplification of the HUMARA gene using DNA extracted from 2 human pituitary tumours (derived from 1 male and 1 female) followed by SSCP analysis prior to digestion. Patient 1 (male) presents the 2-band pattern expected in the case of monoclonality (lane-1); whereas the 4-band pattern for patient 2 (female) represents the scenario of polyclonality prior to digestion. After digestion the monoclonal female subject yields 2 bands and the male none.

Even though a large number of craniopharyngiomas were analysed, success was limited to a single occasion where two craniopharyngiomas of the adamantinomatous type were analysed. The two tumours derived from 2 female subjects, displayed a digestion pattern consistent with a monoclonal composition as shown in figure 75. Prior to digestion, SSCP analysis yielded 4 bands for each patient and verified heterozygosity of the subjects analysed (figure 75). In the remainder of cases, various band patterns were generated preventing any conclusions to be drawn. The reason for this could not be explained.

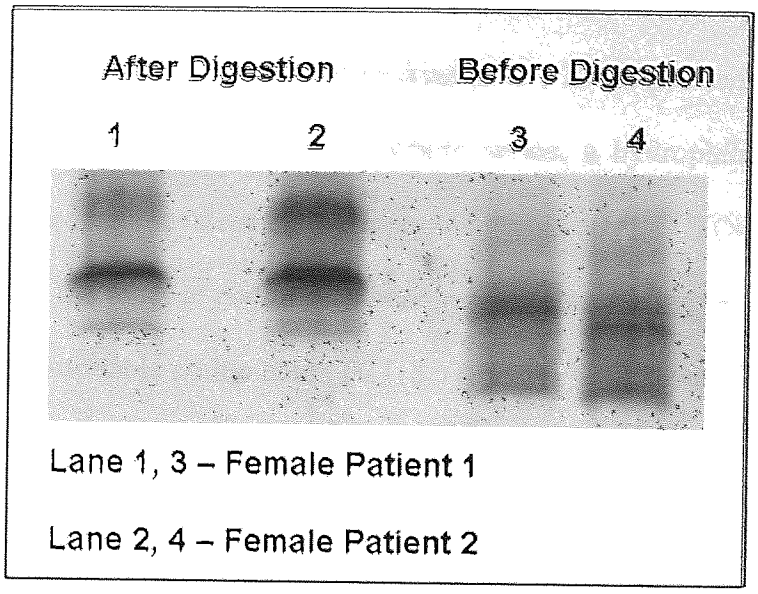


Figure 75: SSCP analysis based on the X-chromosome inactivation phenomenon. PCR amplification of the HUMARA gene using DNA extracted from 2 female craniopharyngiomas. Before digestion 4 bands (lane 3 and 4) were generated for both female subjects confirming heterozygosity. After the DNA samples were digested with HpaII and HhaI. The reduced two bands (lane 1 and 2) observed in both occasions indicated monoclonality.

4.3.2 β-Catenin Mutations

Sequencing analyses performed on all PCR products from 17 craniopharyngiomas of the adamantinomatous and 8 of the papillary type for possible β-catenin gene mutations showed a total of 6 alterations in 17 craniopharyngioma derived DNA samples when compared against the wild type sequence available from the NCBI database (GenBank accession no.; HSRNABECA).

In 6/17 (30%) of the adamantinomatous craniopharyngiomas sequence alterations were found, harbouring a mutation in new and previously described codons as shown in table 18. All were heterozygous as evidenced by the presence of the

double peak pattern yielded by the tumour DNA in the relevant codon (figure 76). Two previously described (Sekine *et al.*, 2002) β-catenin missense mutation were observed, one at codon 33 where serine, a hydrophilic-neutral amino acid, was replaced by cysteine, a hydrophobic amino acid [TCT(Ser)>TGT(Cys)] (figure 76), and the other at codon 37 where again serine was replaced by phenylalanine, also a hydrophobic amino acid [TCT(Ser)>TTT(Phe)] (figure 76) (table 18). In addition, a novel missense mutation was found at codon 33 where serine was this time replaced by tyrosine, an amino acid of the same family [TCT(Ser)>TAT(Tyr)] (figure 76). The most frequent sequence alteration, observed in 3/17 (18%) of the adamantinomatous craniopharyngiomas, was found in codon 41, harbouring a novel mutation where threonine, a hydrophilic-neutral amino acid, was replaced by isoleucine, a hydrophobic amino acid [ACC(Thr)>ATC(Ile)] (figure 76). All alterations are summarized in table 18.

In order to ensure that the observed mutations were genuine rather than polymorphisms, the patient's corresponding blood extracted DNA was also screened. Sequence analyses showed no evidence of sequence alterations (figure 76) suggesting that all sequence alterations were missense mutations affecting the serine / threonine residues at GSK-3β phosphorylation sites.

Table 18: Sequencing analysis for β-catenin gene mutations in 6 out of 17, and frame shift alterations in 6 out of 17 adamantinomatous craniopharyngiomas.

β-Catenin Missense & Frame Shift Mutations						
Case	Age/Sex	Codon	Mutation		Amino Acid Change	
2E	F	41	ACC	ATC	Threonine	Isoleucine
4E	F	37	TCT	TTT*	Serine	Phenylalanine
12E	M	41	ACC	ATC	Threonine	Isoleucine
13E	F	41	ACC	ATC	Threonine	Isoleucine
3G	F	33	TCT	TAT	Serine	Tyrosine
11G Tissue	M	33	TCT	TGT*	Serine	Cysteine
11G Blood	M	-				
3G	F	34	GGA	GGGA	Frame Shift Mutation	
3G	F	32	GAC	GGAC	Frame Shift Mutation	
10E	M	32	GAC	GGAAC	Frame Shift Mutation/ Double insertion	
14E	M	32	GAC	GGAAC	Frame Shift Mutation/ Double insertion	
6G	F	35	ATC	AATC	Frame Shift Mutation	
10G	M	32	GAC	GGAC	Frame Shift Mutation	
11G Tissue	M	32	GAC	GGAC	Frame Shift Mutation	
11G Blood						

* Previously described mutations (Sekine *et al.*, 2002)

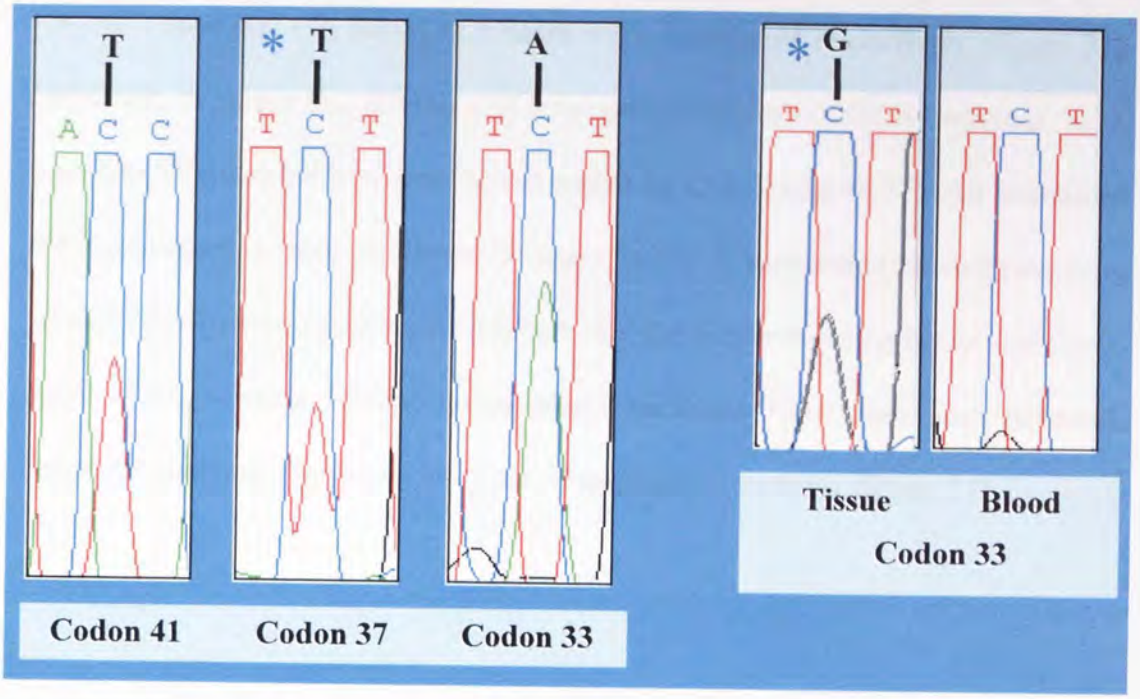


Figure 76: Direct sense sequencing of the β-catenin gene in adamantinomatous craniopharyngiomas. Six out of seventeen (30%) tumours harboured missense mutations affecting one threonine, an aspartic acid residue at phosphorylation-dependent interaction of β-catenin residue at codon 41 and two serine residue at codons 37 and 33. Double peaks in the relevant codon indicate heterozygosity. *Missense mutation previously described (Sekine *et al.*, 2002).

Furthermore, sequence analyses revealed some sequence alteration that resembled the pattern of frame shift mutations, where additional bases (insertions) were observed. Three possible frame shift mutations were observed in 7/17 (41%) of the adamantinomatous type craniopharyngiomas, whereas none was present in the 12 papillary craniopharyngiomas analyzed (table 19). All apparent frame shift mutations affected amino acid residues at GSK-3β phosphorylation sites flanking the first serine residue. There were four different apparent frame shift patterns affecting three different codons. Codon D_{32} (GAC) was the most frequent 3/17 (18%) affect and presented one single and one double base insertion were a G

(GGAC) base and GA (GAGAC) bases were introduced respectively (figure 77). Moreover, at codon G_{34} (GGA) a G base was introduced (GGGA) whereas, an A base (AATC) was the new insertion at codon I_{35} (ATC) (figure 77). All alterations are summarized in table 18. However, upon further examination (detailed reasoning given in the discussion), it seems unlikely that these alterations represent real frame shifts. The patient's corresponding blood extracted DNA was also screened. Sequence analyses showed no evidence of sequence alterations (figure 77).

Table 19: Sequencing analysis for β-catenin gene mutations in 12 papillary craniopharyngiomas where none of the analyzed tumours presented sequence alterations.

Absence of Mutations in the Papillary Type				
Case	Age/Sex	Codon	Mutation	Amino Acid Change
1E	F		-	
3E	M		-	
6E	M		-	
7E	M		-	
8E	F		-	
11E	M		-	
1G	M		-	
2G	F		-	
4G	F		-	
7G	M		-	
8G	M		-	
5G Tissue	F		-	
5G Blood			-	

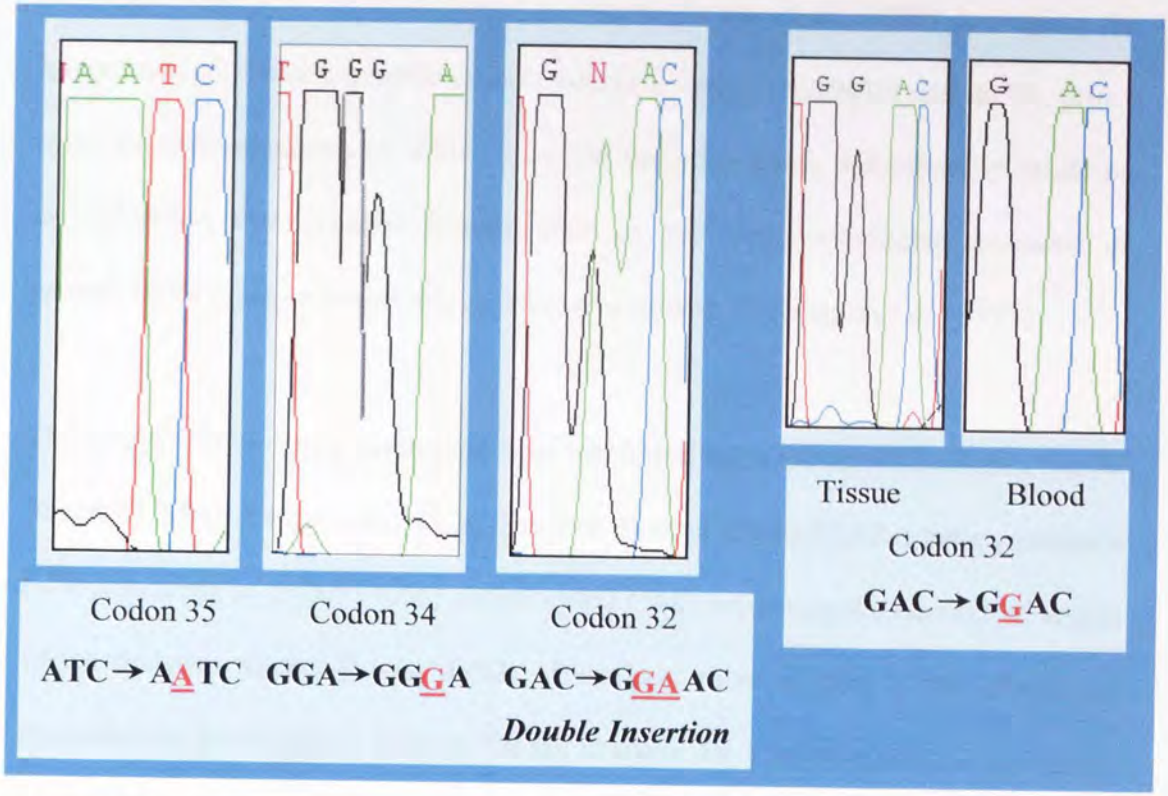


Figure 77: Direct sense sequencing of the β-catenin gene in adamantinomatous craniopharyngiomas. Seven out of seventeen (41%) harbour frame shift mutations affecting amino acids I_{35}, G_{34} and D_{32} that flank the first serine residue on GSK-3β phosphorylation site.

4.4 DISCUSSION

4.4.1 Clonal Analyses

The molecular mechanisms involved in craniopharyngioma growth remain elusive. Only ten craniopharyngiomas were ever reported in the literature to be analysed by karyotyping and only one displayed complex structural anomalies (Gorski *et al.*, 1992; Karnes *et al.*, 1992; Vagner-Capodano *et al.*, 1992). In addition, it has been suggested that craniopharyngiomas, a predominantly sporadic tumour, may also occur in a familial context and follow an autosomal recessive inheritance pattern (Boch *et al.*, 1997). It was only recently established that a subset of

craniopharyngiomas are monoclonal in origin (Sarubi *et al.*, 2001) conveying the importance of somatic genetic defects within a single progenitor cell as the prime cause of tumourigenesis in some cases. On the other hand, polyclonality might be an indication that external factors, such as proliferation-inducing hormone or growth factors, play a major role in excessive growth (Honegger *et al.*, 1995).

The analysis of clonality performed here was based upon the amplification of the X-linked HUMARA gene using PCR. The first exon of the HUMARA gene contains a STR consisting of highly (95%) polymorphic CAG sequences the number of which varies between alleles (Lyon, 1962). Therefore, there is only a 5% chance of encountering homozygous subjects for the analysis. In close proximity to the highly polymorphic CAG repeat, HpaII and HhaI enzyme cleavage sites allow the use of restriction digestion and PCR techniques for the clonality assay (figure 72). The basis of clonal analysis is the phenomenon of X-chromosome inactivation in female subjects where one of the two X-chromosomes becomes randomly inactivated early in tissue development and remains constant throughout the life of the cells (figure 78). Daughter cells inherit the same inactivated (maternal or paternal) X-chromosome. Hence, in monoclonal neoplasms derived from females, all cells contain the same inactivated X-chromosome, whereas polyclonal tissue posses a mixture of inactivated maternal and paternal X-chromosomes. Inactivation is achieved by DNA methylation, and thus the inactive alleles are protected from digestion by methylation sensitive enzymes. Changes in methylation patterns that accompany inactivation of X-chromosomes can be delineated with methylation sensitive enzymes (Vogelstein *et al.*, 1985).

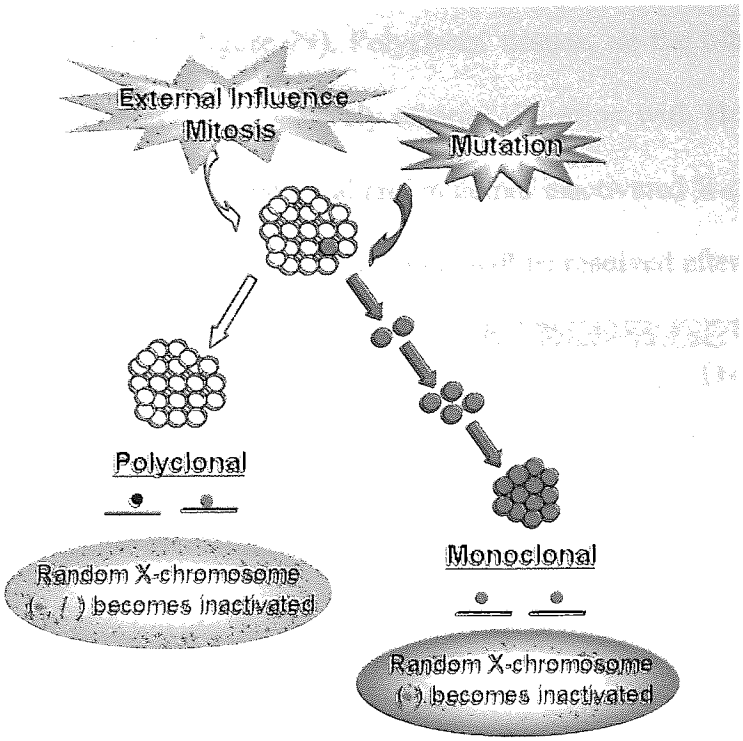


Figure 78: Pattern of monoclonality versus polyclonality. In female subjects one of the two X-chromosomes becomes randomly inactivated early in tissue development and remains constant throughout the life of the cell. In monoclonal neoplasm derived from females, all cells contain the same inactivated X-chromosome.

Therefore, this analysis is restricted to heterozygous female subjects since males have only one X-chromosome. PCR of exon 1 of the HUMARA gene using non-digested DNA from heterozygous females will yield four bands of different size, when resolved by SSCP (figure 79). Similarly, DNA extracted from monoclonal neoplasms, digested with HpaII and HhaI methylation sensitive restriction enzymes will eliminate one allele, and subsequent PCR using primers flanking the CAG repeat of the HUMARA gene will yield products in which two bands have been greatly or completely eliminated. SSCP analysis will therefore resolve two single

stranded bands (figure 79). Polyclonal tissues, on the other hand, will continue to yield four bands irrespectively of prior digestion with HpaII or HhaI because they contain a mixture of paternal and maternal inactivated X-chromosomes (Allen *at al*, 1992) and four single stranded bands will be resolved after SSCP analysis.

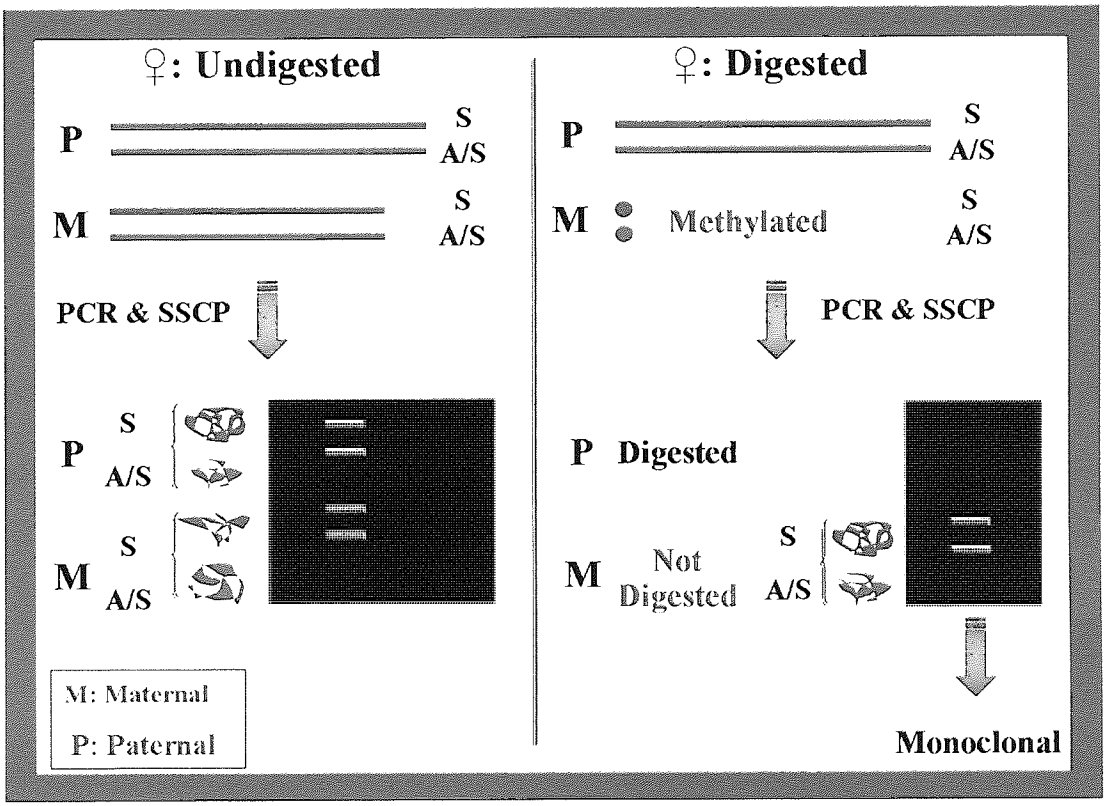


Figure 79: Heterozygous female prior and after digestion with HpaII and HhaI (methylation sensitive restriction enzymes). Distribution of sense (S) and anti-sense (A/S) X-chromosomal alleles after SSCP analysis in case of monoclonality.

SSCP, first described by Orita *et al.* (1989a), is in theory a rapid and sensitive method for the detection of base changes in given sequences of genomic DNA. This technique is based on the facts that specific regions of genomic sequences can be amplified using PCR and then analysed in non-denaturing polyacrylamide gels. The electrophoretic mobility of single-stranded nucleic acid depends not only on its size

but also on its sequence. Parameters regulating SSCP sensitivity comprise of environmental variables with a direct impact on DNA conformation, such as temperature, ionic strength, pH, presence of denaturants, and the gel properties (Orita *et al.*, 1989b). Despite the fact that SSCP can be used as a mutation detection method, it is also widely used as a screening tool for unknown sequence variations thanks to its sensitivity and simple use. Although other authors have underlined advantages of this method as a screening tool, there are significant disadvantages to be considered (Hayashi, 1991). The classic version of SSCP includes both high costs and the use of radioactive isotopes or silver staining to detect ssDNA bands, whereas the sensitivity of silver stain is approximately 1 pg/mm^2, and is comparable to radioactive methods (Bassam *et al.*, 1991). Nevertheless, there are also limitations to the non-radioactive SSCP methodology as applied to clonality studies. The method can be only performed on samples from heterozygous females.

Although the results obtained in the method adaptation stage using pituitary tumours suggested that the system was working since pituitary tumours removed from female subjects of monoclonal in origin (Allen *et al.*, 1992) yielded four bands prior to digestion, its application to the craniopharyngioma samples proved to be of limited success. The SSCP analysis of a great number of different digested PCR DNA failed to provide sufficient results to shed light into the clonality of craniopharyngiomas. Evidence of polyclonality was obtained on a single occasion where two samples yielded four bands irrespective of prior digestion with HpaII and HhaI. The initial idea was to develop a simple, rapid and sensitive method for detecting clonality of craniopharyngioma to compete with the radioactive methods, by reducing processing time, exposure, and biohazard, and by showing no reduction

to sensitivity. The SSCP method was initially thought to be adequate for this purpose, but its sensitivity proved unsatisfactory. In most experiments, too many bands were yielded suggesting incomplete denaturation and re-natured DNA. Attempts were made to overcome this problem where temperature and current were fluctuated. However the problems remained and it was not possible to apply the method to a large series of craniopharyngiomas.

The use of a temperature controlled run (Hongyo *et al.*, 1993) was based on the concept of variable gel concentration in respect to fragment length, as well as the composition of denaturing and loading sample buffers. It has been reported in several previous studies that there is a clear correlation between product size and the capacity of gels of different concentrations to resolve ssDNA migration shift due to sequence alterations (Hayashi, 1991; Varesco *et al.*, 1993). Although it is known that the concentrations of acrylamide need to be adjusted according to product size, in practice this parameter is rarely modified in the course of the screening. Instead, concentrations of acrylamide are chosen based on fragments of similar size that have been grouped (<200bp, 10%; 200-300bp, 8%; >300bp, 5-6%), (Yap and McGee, 1994). Precise temperature control during SSCP runs has been put forward as a tool to increase reliability (Hongyo *et al.*, 1993), and as an easily modifiable parameter in repeatable, non-isotopic experiments that may increase sensitivity. All the runs done in this study were performed at 4 C (plus 10 C). Temperature was maintained by means of a water circulator control of the temperature in the electrophoresis chamber. Glycerol has been reported to be a useful supplement for increasing sensitivity in SSCP acrylamide gel. Its action is probably due to a weak denaturating activity that could modify the concentration of single-strand fold DNA

molecules, revealing migration shifts that are lost in totally non-denaturing gels. However, use of glycerol in the present studies failed to yield clear cut results despite running at a variety of temperatures and the use of glycerol as a facilitator of band separation.

4.4.2 Craniopharyngiomas Harbouring β-Catenin Mutations

Several years ago attention became focused on mutations on exon 3 of the β-catenin gene, which affect serine / threonine phosphorylation by GSK-3β and protect the β-catenin protein from degradation through the ubiquitin-proteasome pathway, and it was only recently that genetic alterations of β-catenin were reported for the craniopharyngiomas of the adamantinomatous type (Sekine *et al.*, 2002). A year later, studies by the same group (Sekine *et al.*, 2003) presented genetic alteration that caused amino acids substitutions of serine / threonine residues of GSK-3β phosphorylation sites or residues flanking the first serine residue of the phosphorylation sites in human odontogenic tumours that histologically resemble adamantinomatous craniopharyngiomas (Love and Marshall, 1950). These genetic alterations were similar to the previously reported somatic mutation in various tumours (Polakis, 2000) including adamantinomatous craniopharyngiomas (Sekine *et al.*, 2002), which resulted in β-catenin stabilization by inhibiting GSK-3β-dependent phosphorylation and subsequent proteasomal degradation (Polakis, 2000). This is of interest, since ameloblastomas, a tumour also described by several early reports to have similar characteristics with the adamantinomatous craniopharyngiomas (Love and Marshall, 1950; Gorlin and Chaudhry, 1959) are found to bear no β-catenin mutations when analysed by the same study (Sekine *et al.*, 2003). Although ameloblastoma closely resembles odontogenic tumours

histologically, the two have genetically distinctive features. Therefore, β-catenin mutations are considered to be a characteristic genetic feature of odontogenic tumours and may play a critical role in its histogenesis. Likewise, they may be directly involved with the development of adamantinomatous craniopharyngiomas considering the similarities that these two neoplasms share. Mutations in the β-catenin gene have also been frequently found in several other human cancers, including colon, gastric medulloblastoma, melanoma, ovarian cancer, prostate cancer (Polakis, 2000), and pituitary tumours (Semba *et al.*, 2001). These mutations occur in residues in the GSK-3β phosphorylation site (S_{33}, S_{37}, T_{41}, S_{45}) (figure 80) and / or residues flanking one of these phosphorylation sites (D_{32}, G_{34}, I_{35}, H_{36}) and they abrogate the phosphorylation-dependent interaction of β-catenin with Fbw-1, thereby stabilizing β-catenin, which accumulates in the nucleus and activates TCF / LEF promoters (Kikuchi, 2003). An increase in free β-catenin might also result from a loss of E-cadherin expression. This is characteristic of epithelial type carcinomas, including thyroid, and correlates to increased invasiveness of the tumours (von Wasielewski *et al.*, 1997; Walgenbach *et al.*, 1998).

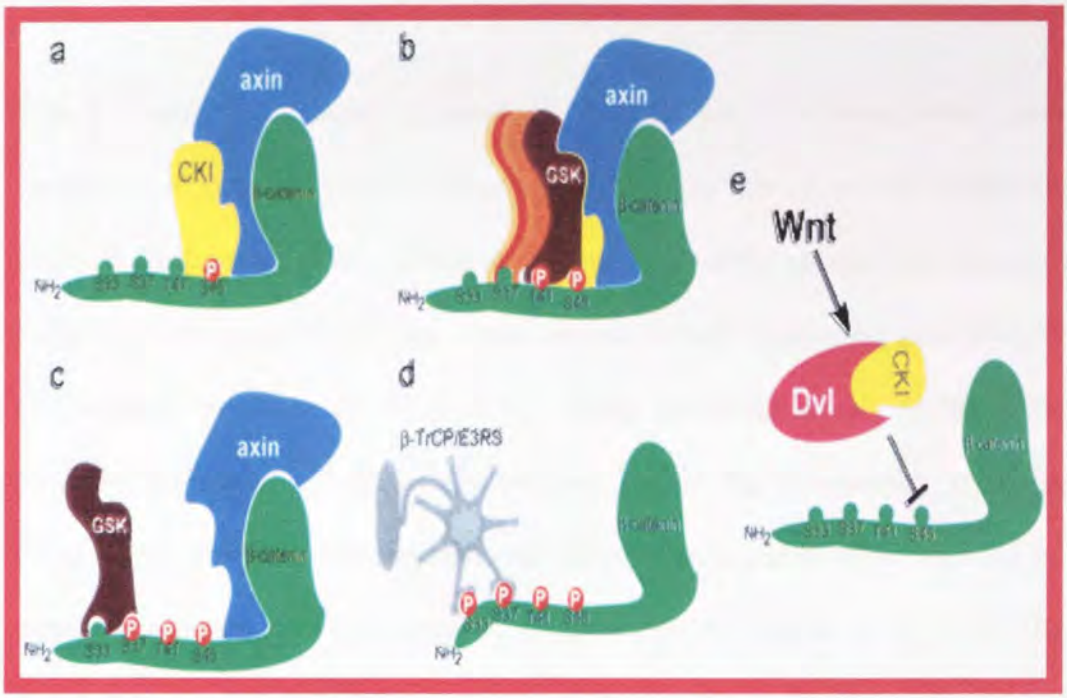


Figure 80: A model depicting the β-catenin phosphorylation-degradation cascade. (a) Axin recruits casein kinase I (CKI) to β-catenin at S45. (b, c) S_{45} phosphorylation primes β-catenin for the succeeding GSK-3β phosphorylation cascade, finally hitting the $S_{33/37}$ sites. (d) Phosphorylation at $S_{33/37}$ creates a docking site for βTrCP / E3RS, promoting the ubiquitination and degradation of β-catenin. (e) Wnt signalling, possibly through Dvl and CKI, regulates S_{45} phosphorylation. (Sharon *et al.*, 2002).

B-Catenin may be regarded as existing in three different subcellular forms: membrane-bound (as part of the adherence complex), cytosolic, and nuclear (Morin, 1999; Kikuchi, 2000). Binding of β-catenin to other members of the adherens complex, like E-cadherin and α-catenin, leads to tyrosine phosphorylation which causes β-catenin to dissociate form the adherens complex (Muller *et al.*, 1999) and probable transfer of the protein to the cytosol where it exists in a soluble, monomeric state (Morin, 1999; Kikuchi, 2000). Cytosolic β-catenin may subsequently be degraded or be translocated to the nucleus.

Free β-catenin is constantly targeted for degradation by a multiprotein complex consisting of axin, the tumour suppressor APC, and the serine / threonine kinase GSK-3β (Nakamura *et al.*, 1998). Axin and APC serve as scaffold proteins that bring GSK-3β and β-catenin into close contact thereby facilitating phosphorylation of β-catenin by GSK-3β (Hart *et al.*, 1998; Sakanaka *et al.*, 1998). GSK-3β phosphorylates multiple conserved residues within the N-terminus of β-catenin (Yost *et al.*, 1996). B-Catenin becomes ubiquitinated and is then degraded in the proteasome resulting in low cytosolic β-catenin levels (Aberle *et al.*, 1997) (figure 81).

Recently, a new family of proteins, Wnt factors, has been shown to closely control levels of free β-catenin. Wnt factors are secreted glycolproteins that bind to the cell surface receptor Frizzled. Wnt binding to the Frizzled receptor induces the membrane recruitment and phosphorylation of the cytosolic protein Disheveled (Dvl) (Axelrod *et al.*, 1998; Rothbacher *et al.*, 2000). Wnt signalling antagonizes by a mechanism not fully elucidated the activity of GSK-3β. The multiprotein complex disintegrates (Willert *et al.*, 1999). As a result, β-catenin is no longer targeted for degradation, but accumulates in the cytosol and enters the nucleus. There, β-catenin binds to members of the T cell factor / lymphoid-enhancing factors and activates expression of target genes, including *c-jun*, *c-myc*, fra-1 and cyclin D1 (He *et al.*, 1998; Mann *et al.*, 1999; Tetsu and McCormick, 1999) (figure 81).

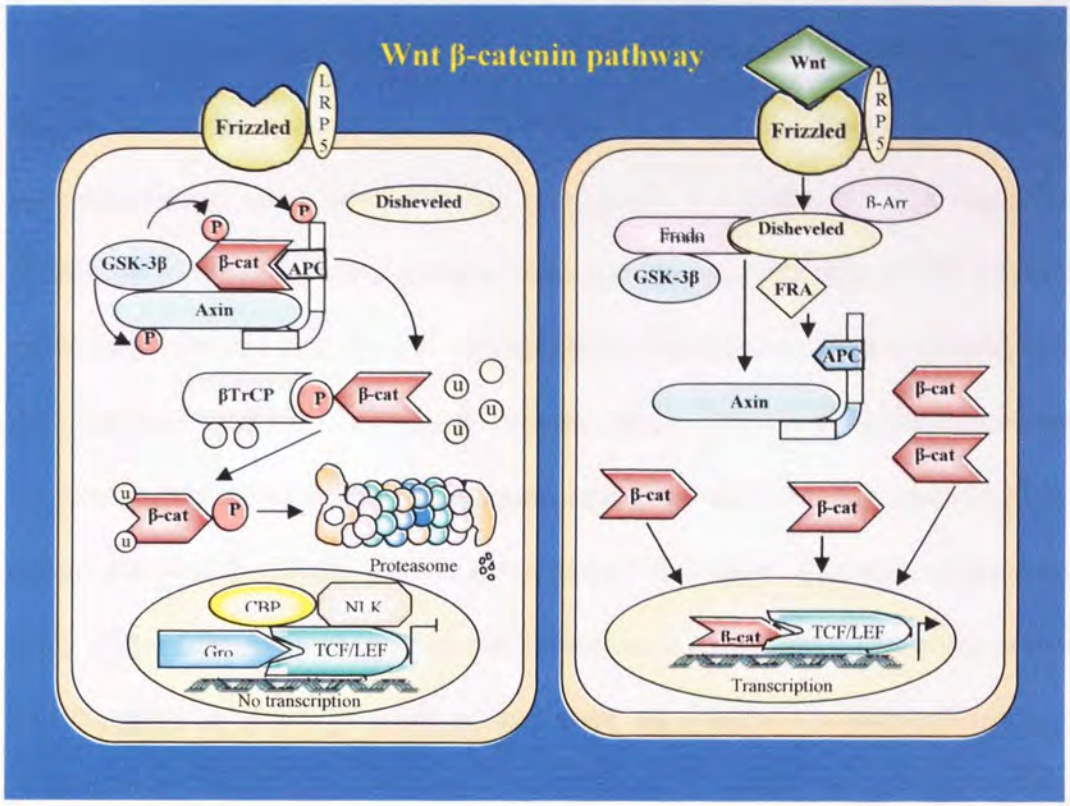


Figure 81: Schematic representation of the Wnt / β-catenin pathway. In the absence of Wnt GSK-3β phosphorylates β-catenin and targets it for ubiquitination by β-TrCP resulting in the proteasomal degradation of β-catenin. In contrast, in the presence of Wnt signalling, Dvl and other proteins inhibit phosphorylation of β-catenin by GSK-3β. A free pool of β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds TCF / LEF family of the transcription factors and activates transcription of target genes.

Missense mutations in the β-catenin gene were present in 6/17 (30%) of the adamantinomatous craniopharyngiomas studied. Our findings support the findings of Sekine *et al.* (2002) who found that β-catenin gene mutations were present in all 10 adamantinomatous craniopharyngiomas that showed a high nuclear expression of

β-catenin. All mutations (table 18) caused amino acid substitutions in three out the four serine / threonine residues of the GSK-3β phosphorylation sites (S_{33}, S_{37}, T_{41}) (figure 80), while none of the residue (D_{32}, G_{34}, I_{35}, H_{36}) flanking one of these phosphorylation sites was affected, compared to mutations that have been previously reported for these residues on other tumours (Polakis, 2000). Mutations cause β-catenin stabilization and cytoplasmic accumulation which eventually enters the nucleus and it has been shown that cases of adamantinomatous craniopharyngiomas harbouring β-catenin mutations show nuclear and cytoplasmic accumulation of β-catenin when immunostained (Sekine *et al.*, 2002). In the present study none of the tumours studied was subjected to immunohistochemistry analyses and therefore it is not possible to comment on β-catenin concentrations in the nucleus and cytoplasm. Presence of β-catenin mutations of the GSK-3β phosphorylation sites was determined by comparison of the sequence results to the wild type and the veracity of these was based on the sequence results from blood extracted DNA from each of the patients analyzed. In contrast, none of the papillary type tumours harboured mutations. These findings are consistent with reports by Shigeki *et al.* (2002) where exclusively membranous staining pattern was seen in non-neoplastic epithelial cells along with the absence of sequence alterations in papillary craniopharyngiomas.

Therefore, β-catenin gene mutations, as shown from the present study and by the overexpression shown by others, are considered to be characteristics of some adamantinomatous craniopharyngiomas and may serve to provide a molecular basis of the clinical and histopathologic differences between adamantinomatous and papillary craniopharyngiomas. This constitutive activation of TCF / LEF dependent

transcription by β-catenin stabilization seems to be directly involved in the tumourigenesis of these neoplasms. Because craniopharyngioma is a complex epithelial tumour exhibiting a variable histological appearance and clinicopathological differences could not distinguish between them, there was a need for a subdivision based on their heterogeneity (Thapar and Kovacs, 1998). Furthermore, the presence of mixed variant showing transitional features between the adamantinomatous and papillary types has also been characterized (Weiner *et al.*, 1994; Crotty *et al.*, 1995). However, the present study examined only samples of the two main types. Examination of craniopharyngiomas of the transitional type would provide useful information about the identity and help understand their relationship with the main two variants.

Uncontrollable activation of several target genes by the β-catenin:LEF / TCF complex has been implicated in the oncogenic effect conferred by β-catenin signalling and is suggested to contribute to tumour progression (figure 82). Induction of the genes that encode MYC and cyclin D1 major regulators of cell proliferation (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999), whose promoters contain LEF / TCF-binding sites, might provide the molecular basis for growth regulation by β-catenin signalling (Tetsu and McCormick, 1999), whereas the induction of matrilysin expression could promote cell invasion (Crawford *et al.*, 1999). In addition, PPARδ, a transcription factor involved in colon cancer, is also a target for the β-catenin-TCF complex (He *et al.*, 1999).

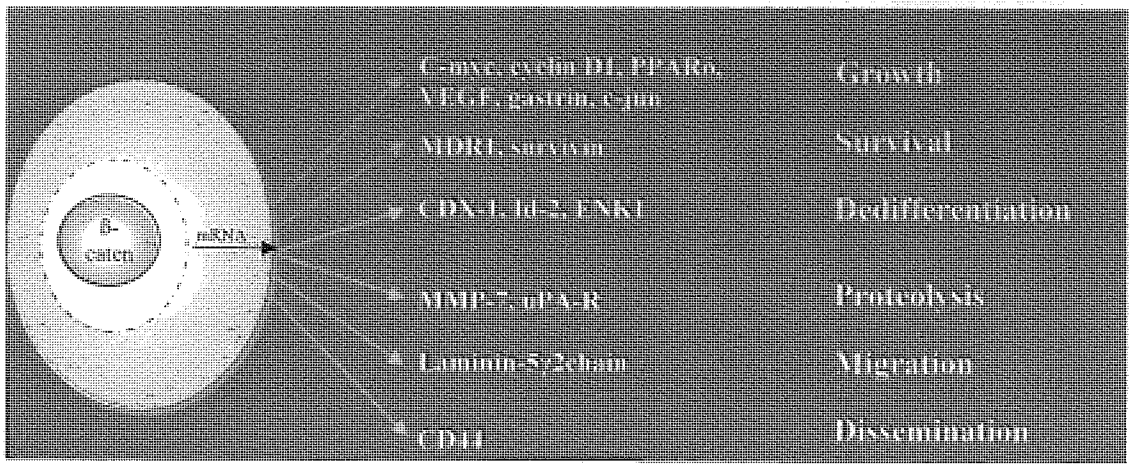


Figure 82: Summary of the oncogenic potential of nuclear β-catenin deduced from known target genes.

Interestingly, the overexpression of β-catenin in cells expressing normal p53, a major tumour suppressor, results in increased levels of transcriptionally active p53 (Dalamas *et al.*, 1999). This response may present a balancing cellular response to the oncogenic activity of elevated β-catenin, and this might drive a selective pressure during tumour progression for mutations in p53. Even though, craniopharyngiomas are composed of distinctive sheets of epithelial cells showing adamantinomatous or squamous-papillary histologic types, the reactivity of an archetypal tumour suppressor p53 a transcription factor known to be expressed in various human epithelia, was occasional and weak in craniopharyngiomas as determined by immunohistochemistry (Momota *et al.*, 2003).

Further analysis of the sequence results bearing possible frame shift mutations, established using a software analysis program available by the Biology Workbench (version 3.2) showed no evidence of a premature stop codon being introduced. Additionally, it is doubtful that the sequencing results represent frame shifts because there were no double peaks after the points of the apparent alterations. In reality,

such a result would only be observed in homozygous samples, which is a rather remote possibility in the current series of experiment where a high number of samples have been investigated. All the above point to the same conclusion of that these 'frame shift mutations' are clearly artefacts of the sequence procedure rather than real frame shift mutations. However, literature has shown homozygous deletion of a part of the β-catenin gene caused an identical in-frame mRNA deletion in a cell carcinoma cell line of the stomach (Hirohashi and Kanai, 2003). Although the truncated β-catenin was co-precipitated in immunoprecipitation experiments using an anti-human E-cadherin monoclonal antibody, α-catenin was not, suggesting the possibility that the interaction between α-catenin and E-cadherin is not direct, but mediated by β-catenin (Hinck *et al.*, 1994). These observations suggest the possibility that suppression of E-cadherin activity triggers the release of cancer cells from primary cancer nests. Therefore this might be one consequence frame shift mutation may have on human carcinogenesis.

Alternatively, and under the speculation that these are real frame shift mutations, the results would be most likely represent loss of protein function. To verify this, further functional analyses is required along with staining the nucleus for β-catenin being accumulated. A characteristic structural feature of β-catenin is a central armadillo (arm) repeat domain flanked by the C- and N- terminal domains (Hatzfeld, 1999) (figure 83). Although all three catenin domains can mediate protein-protein interactions, the great majority of partners bind to the arm repeat region. At least five arm repeats are needed to maintain protein-protein interactions (Hsu *et al.*, 1998). Although the nature of the binding site on the arm repeat dictates mutually exclusive binding of the various proteins to the region (Kishida *et al.*,

1998) the molecular interactions formed by each protein partner are unique. Thus, it is possible selectively to disturb the binding of certain proteins to the arm repeat by mutations within the arm domain, but maintain the binding of other partners (Prieve and Waterman, 1999). In the nucleus, both the N- and the C-termini of β-catenin link the β-catenin:LEF / TCF complex to the basal transcription machinery (van de Wetering *et al.*, 1997; Hsu, 1998;) via interactions with TATA-box-binding protein (TBP) (Hecht *et al.*, 1999) and CREB-binding protein (CBP) (Hecht *et al.*, 2000; Takemaru and Moon, 2000).

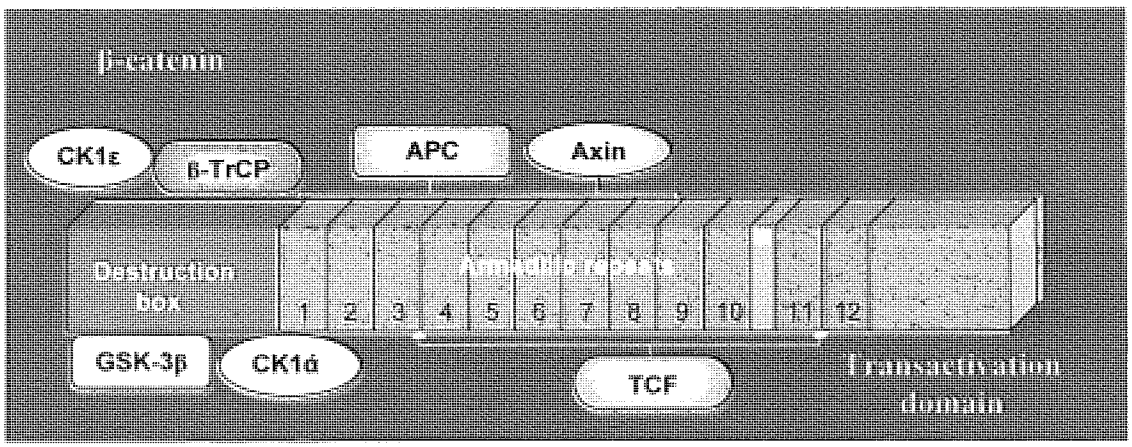


Figure 83: Schematic presentation of β-catenin protein structure and its interaction with other components of Wnt signalling. The central domain of β-catenin consists of 12 armadillo repeats that interact in an overlapping manner with APC, axin and TCF. The amino terminus contains multiple phosphorylation sites which are required for proteasome-mediated destruction. The carboxyl terminus contains a transcriptional activation domain.

In addition the N-termini where the GSK-3β phosphorylation site is situated and targeted for analyses, consists of 150 amino acids whereas, the arm repeat consists of 550 amino acids (Hatzfeld, 1999), therefore it is a long way downstream of the

detected frame shifts for other frame shifts to occur and correct the introduced mistakes in the sequence. If that does occur after a binding site has already been affected, it may still leave binding activity and function of other proteins unaffected as previously mentioned. It is the over-activity from accumulated β-catenin that activates transcription rather than its under-production.

Secondly, if TCF / LEF successfully binds the arm repeat of β-catenin gene, in the nucleus both the amino (N-) and the carboxyl (C-) termini of β-catenin are required to link β-catenin:LEF / TCF complex to the transcriptional machinery (van de Wetering *et al.*, 1997; Hsu *et al.*, 1998;), thus if frame shifts do ultimately cause a functional defect of the C-termini there is no evidence so far to suggest that linkage via the N-termini alone can't bring about transcription. Correlating presence of frame shift mutation and accumulation of β-catenin in the nucleus of the same sample would mean that there is no loss of protein function since β-catenin would have escaped phosphorylation due to the frame shift of the sequence and have accumulated in the nucleus as a consequence, justifying the above speculations.

4.4.3 Conclusion

Attempts for developing a novel non-radioactive technique for investigating clonality of craniopharyngiomas were proven unsuccessful. Even though the technique was adapted to an extent to the analysis of pituitary tumours, the outcome was unsuccessful with the craniopharyngiomas. As a result we were unable to obtain enough results throughout this project to provide an insight into the clonality of craniopharyngiomas.

In contrast, the other aspect of investigating genetic abnormality of the Wnt pathway, demonstrated that β-catenin gene mutations were an exclusive characteristic of the adamantinomatous variant. The results suggest that adamantinomatous and papillary craniopharyngiomas are distinctive not only clinically and histologically, but also genetically.

CHAPTER FIVE

CONCLUSIONS AND PERSPECTIVES

5.1 Peptides and Cancer

Peptide targeting of tumours is currently a fast moving field. There is increasing evidence that the most promising developments are linked to the targeting with radiolabel (or possibly cytotoxic) peptides as *in vivo* scintigraphy, radiotherapy, or cytotoxic therapy. Cancer research on peptides is currently dominated by two active fields: one is the search for new peptide receptors overexpressed in specific tumours, i.e., suitable peptide targets. The second field consists of the search and discovery of new radiopeptides and cytotoxic peptides, their development for potential clinical use in the previously defined targets and the resulting clinical efforts to optimise peptide receptor targeting. A prime example is the rapid progress being made in the search for the optimal conditions for a successful radiotherapy of somatostatin receptor-positive neuroendocrine tumours (Hejna *et al.*, 2002). However, in parallel to these clinical activities, more basic information must be gathered on peptide receptor biology and pathobiology in cancer, allowing a better understanding of the molecular mechanisms underlying the *in vivo* peptide receptor targeting. Some areas that need to be further explored are the mechanisms triggering the expression of peptide receptors in cancer tissue and whether the presence of peptide receptors in the tissue of origin is a prerequisite for the expression of tumoural receptors. In addition, light needs to be shed into the importance of the mutated peptide receptors detected occasionally in tumours.

Receptors can be expressed in tumours originating from either peptide receptor-expressing or peptide receptor-lacking tissues. Neoplastic transformation can result in a marked increase in the number of peptide receptors that occur physiologically in a tissue, as has been shown before for somatostatin receptors in acromegalics. In

the present study we have demonstrated that all tumours investigated expressed CCK receptors that most likely originated from peptide receptor expressing tissues considering evidence of CCK-A receptors expression in the peripheral systems and some discrete brain regions and CCK-B receptors that are widely present in the brain. However in the present study we were unable to provide a satisfactory answer, as to whether the receptor expression is comparable in normal brain tissues, on the basis of the availability of *in vivo* data, partially because of the inaccessible normal brain tissue in each of the tumour cases investigated.

5.2 CCK and Brain Tumours

Since the original discovery of CCK by Ivy and Oldberg in 1928, followed by the isolation and sequencing of this hormone (Jorpes and Mutt, 1966), and its detection in the CNS (Vanderhaeghen *et al.*, 1975), considerable advances have been made in the knowledge of the role of this neuropeptide. The actions of CCK have been extended to include endocrine secretion, motility and growth in the gastrointestinal system along with growth and other behavioural actions in the CNS. These actions are mediated by at least two distinct and heterogeneous receptors, which have been pharmacologically characterised and subsequently cloned. Several potential clinical applications concern the treatment of brain disorders and / or pain with CCK-B receptors agonists or antagonists. Taken together, past and present studies and increasing amount of information gathered about the involvement of CCK in CNS and brain tumours in particular, it is conceivable that adjuvant therapies evolved around the molecular and biochemical profile of this peptide may provide a promising area of further investigation.

Another important aspect of peptide involvement in cancer is whether transformed cells actually express significant concentrations of endogenous peptides, and if so, the degree of interference with tumoural peptide receptors. Numerous tumours can express both the peptide and its receptor in large amounts and the present study is a good example of such a system, where both CCK peptide and both its type A and B receptors were expressed in human gliomas, meningiomas and pituitary tumours. The combination of peptide and its receptor may regulate tumour growth and / or function via autocrine feedback mechanism (Moody *et al.*, 2003). Moreover, it may be worthwhile knowing whether an excess of endogenous peptides would prevent an adequate targeting of these tumour due to dilution of the exogenous radiopeptide at the tumour site because most of the peptide receptors have been internalised in tumour cells after binding of the corresponding endogenous peptide. An answer to these questions is crucial for the planning of diagnostic and therapeutic procedures of these neoplasms that present the autocrine / paracrine system of CCK that most probably results to receptor internalisation. A significant discovery has been the homo- and heterodimerization of peptide receptors in primary human tumours (Rocheville *et al.*, 2000a; Rocheville *et al.*, 2000b; Pfeiffer *et al.*, 2001), which raises the question about the impact it might have on receptor binding, on receptor internalisation, on the development of new analogues and, more generally, on receptor targeting strategies.

5.3 Pituitary Tumours

Pituitary tumours are common neoplasms that exhibit a wide range of biological behaviour in terms of hormone production and tumour growth. Classification of these neoplasms is now more appropriately based on functional measures and is

likely to develop with growing knowledge of pathways of normal adenohypophysial cytodifferentiation. Numerous factors have been shown to govern adenohypophysial cell proliferation. These various hypophysiotropic hormones and growth factors likely play a role as promoters of tumour cell growth in genetically transformed cells. It is demonstrated here that CCK peptide confers increased hormone secretion in human pituitary tumours may by acting in an autocrine / paracrine mechanism on these neoplasms. The clonal composition of pituitary tumours attests to the molecular basis of pituitary tumourigenesis, however, the oncogenes and / or tumour suppressor genes (TSGs) that are implicated in the transformation process for the vast majority of pituitary tumours remain unknown. Mutations that have been identified in other human malignancies are restricted to a very small subset of pituitary tumours, if they are identified at all. It would appear that novel genetic alterations are implicated (Daniely *et al.*, 1998). The molecular mechanism underlying dysregulated cell growth in the pituitary remains the subject of investigation. Maybe, the polymorphism in the GHRH-R reported in the present study can provide important information in the drug designing of optimal medical therapies against acromegaly and excessive GH production, but the results presented do not suggest that it is involved in tumourigenesis.

5.4 Craniopharyngiomas Harbouring β-Catenin Mutations

Studies on the dual role of β-catenin in cell adhesion and signal transduction have opened a unique opportunity to begin understanding, at the molecular level, the interplay and integration of cell and tissue morphogenesis with the control of gene expression. These studies have also provided new insights into novel mechanisms regulating embryonal development and tumour progression. While β-catenin is now

believed to be involved in the regulation of both cell adhesion and transcriptional activity of specific target genes, the relationship *in vivo* between these two differential functions of β-catenin is still under debate. Further research is needed to define the conditions in a cell's life under which a regulated release of β-catenin from the adhesion pool in the submembranal plaque is linked to activation of β-catenin / TCF target gene expression in the nucleus. In addition, the ever-increasing number of new molecular partners of β-catenin poses further challenges to our way of understanding the different roles of these new interactions involving β-catenin.

If β-catenin-directed interventions are to be developed, much more, however, needs to be learned about the physiological role of nuclear β-catenin signalling in human tissues, particularly if side effects are to be avoided. Learning more about β-catenin's function in other tissues may, in turn further expand our understanding of craniopharyngiomas and related intracranial tumours carcinogenesis. An important area of future study is the regulation of β-catenin gene transcription. Aside from the rare example of butyrate (Barshishat *et al.*, 2000), little is known about molecules and mechanisms that may influence the actual synthesis of β-catenin. Exploring whether and, if so, how β-catenin gene transcription interacts with its subcellular movements and its degradation is likely to further complicate what we know of the protein in carcinogenesis, but may eventually reveal other possible targets for therapeutic and / or preventative intervention.

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APPENDIX

ABSTRACTS AND PAPERS SUBMITTED IN SUPPORT OF THIS THESIS