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Sonic hedgehog signaling pathway in normal

and adenomatous pituitary

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To Fiona

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Abbreviations

ACRO	Acromegaly
ACTH	Adrenocorticotrophic hormone
AP	Alkaline phosphatase
AP-1	Activator protein - 1
bp	base pairs
BCC	Basal cell carcinoma
cAMP	cyclic adenosine monophosphate, cyclic AMP
Ci	Cubitus interruptus
CK1	Casein kinase 1
CMV	Cytomegalovirus
CNS	central nervous system
Cos2	Costal 2
CRE	cyclic AMP regulatory element
CREB	cyclic AMP regulatory element binding protein
CRH	Corticotropin releasing hormone
CRH-R1	CRH receptor 1
Cush	Cushing tumor
DAB	Diaminobenzidine tetrahydrochloride
Disp	Dispatched
Dhh	Desert hedgehog
DMEM	Dulbecco´s Modified Eagle´s Medium
FCS	Foetal calf serum
FSH	Follicle-stimulating hormone
Fu	Fused
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GSK3	glycogen synthase kinase
Hh	Hedgehog
Hip	Hedgehog – interacting protein
IHC	Immunohistochemistry

Ihh	Indian hegehog
LH	Luteinizing hormone
NBCCS	Nevoid basal cell carcinoma syndrome
NFPA	Non-functioning pituitary adenoma
PFA	Paraformaldehyde
PKA	Protein kinase A
PNET	Primary neuro-ectodermal tumors
POMC	Pro-opiomelanocortin
Prl	Prolactin
Prol	Prolactinoma
Ptc	Patched 1 or Ptc1
RIA	Radioimunoessay
Shh	Sonic hedgehog
Shh -/-	Shh deficient mouse
Shh-C	Sonic hedgehog C-terminal polypeptide
Shh-N	Sonic hedgehog N-terminal polypeptide
Smo	Smoothened
Su(Fu)	Suppressor of Fused
TRH	Thyreotropin releasing hormone
TSH	Thyrotropin or Thyroid-stimulating hormone
Tvt	Tout-velu

1. INTRODUCTION

Signaling proteins function in a cellular environment to direct cells into a change of state, such as promotion of proliferation or differentiation.

Sonic hedgehog (Shh) is a signaling protein important in regulating patterning, proliferation, survival and growth in both embryo and adult mammalian systems (1).

Sonic hedgehog is absolutely required for pituitary development: Shh -/- (Shh deficient) mice do not have even a rudimentary Rathke's pouch (the embryonic structure that develops into the pituitary gland's anterior lobe). Shh is uniformly expressed throughout the oral ectoderm, but its expression is restricted to the Rathke's pouch as soon as it becomes morphologically visible. This restriction exerts effects on both pituitary cell proliferation and cell-type determination (2).

There are currently no studies on the expression and role of Shh pathway in the adult pituitary gland.

1. 1   The pituitary gland and pituitary tumors

1.1.1. The pituitary gland

The pituitary gland has a vital role in maintaining physiological homeostasis under basal and challenge conditions. It is roundish, weighs about 0,6 g and resides in the sella turcica, a saddle-shaped depression in the sphenoid bone, where it has important anatomic relations with the hypothalamus, cavernous sinus, carotid artery, optic tracts and other cranial nerves.

The pituitary gland in mammalian embryos originates through the interaction of the neural and the oral ectoderm. The oral ectoderm grows upward from the roof of the mouth and gives rise to the Rathke's pouch, which then develops into the anterior and intermediate pituitary gland, containing at least 6 distinct cell phenotypes (3, 4). At the same time, another finger of ectodermal tissue evaginates ventrally from the diencephalon of the developing brain. This extension of the ventral brain forms the posterior pituitary or neurohypophysis. Ultimately, the two tissues grow tightly into one another, but their structure remains distinctly different, reflecting their differing embryological origins (presented schematically in Fig.1).
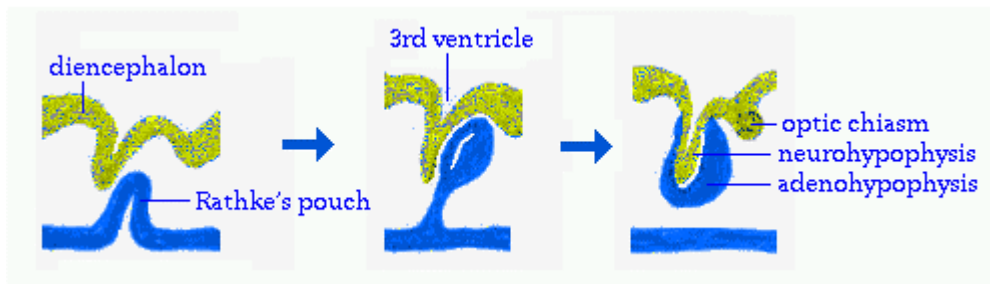
Fig. 1. *Pituitary gland embryology*

The pituitary gland in mammalian embryos is formed through the interaction of the neural and the oral ectoderm. The oral ectoderm grows upward from the roof of the mouth and forms the Rathke's pouch, the embryonic structure that gives rise to the pituitary gland's anterior lobe. At the same time, the neural ectoderm evaginates ventrally creating an extension of the brain which will give rise to the posterior pituitary gland.

The human pituitary gland is composed of two distinctive parts (Fig.2):

- the anterior pituitary (adenohypophysis) is a classical gland composed predominantly of cells that secrete protein hormones.

- the posterior pituitary (neurohypophysis) is an extension of the hypothalamus. It is

composed largely of the axons of hypothalamic neurons which extend downward as a large bundle behind the anterior pituitary. They also form the so-called pituitary stalk, which appears to suspend the anterior gland from the hypothalamus.
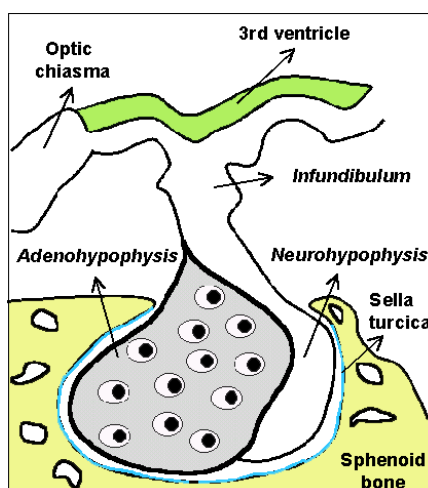
Fig. 2. *The pituitary gland*

The pituitary gland is shown resting in the sella turcica, a saddle-shaped depression in the sphenoid bone. The antomical location under the optic chiasma and the third ventricle is clearly seen.

The adenohypophysis contains different hormone producing cells. The neurohypophysis contains axons which mainly derive from the magnocellular neurons of the hypothalamic supraoptic and paraventricular nuclei.

The adenohypophysis is a complex gland containing different cell types. These cells produce six principal hormones: three peptide hormones (GH, Prl and ACTH) and three glycoprotein hormones (FSH, LH and TSH).

Advances in ultrastructural techniques have made possible a functional classification of these cells according to the hormones that they produce. This classification includes: somatotrophs (GH-secreeting cells, acidophils), lactotrophs (prolactin-secreting cells, mammotrophs, acidophils), mammosomatotrophs (few bihormonal cells, producing both GH and prolactin, acidophils), corticotrophs (ACTH-secreting cells, basophils), gonadotrophs (FSH/LH-secreting cells, basophils) and thyrotrophs (TSH-secreting cells, basophils).

The anterior pituitary also contains folliculostellate (FS) cells that appear in the pituitary at the age of 5 months and have unique morphologic features (5). Their role has become more evident recently. The network of nonendocrine FS cells helps in establishing an intrapituitary communication system: they respond to central and peripheral stimuli and communicate with endocrine cells via growth factors and cytokines, suggesting an important role in the paracrine regulation of hormone secretion (6, 7).

Hormone secretion by the anterior pituitary gland is under the positive-feedback control of hypothalamic releasing factors (CRH, GHRH, GnRH, TRH), hypothalamic inhibiting factors (dopamine, somatostatin) and under the negative-feedback control of peripheral hormones (thyroxin, glucocorticoids, etc).

The posterior lobe releases oxytocin and vasopressin from axon terminals that originate in cell bodies located in the hypothalamus.

The pituitary gland contains also an intermediate lobe, which is rudimentary in human beings. Recently it has been established that it produces several hormones with physiologic significance (reviewed in 8).

There is a large body of *in vitro* evidence that pituitary function is not only dependent on hormonal signals from the brain but also on paracrine signals produced in the tissue itself. These signals appear to be involved in the control of pituitary hormone secretion as well as in pituitary cell differentiation and development. The paracrine factors which have been identified in the pituitary belong to diverse biological molecules such as neuropeptides, acetylcholine, growth factors, cytokines (9) and posttranslationally modified derivatives of pituitary hormones.

1.1.2 Regulation of corticotroph cells by CRH

Hypothalamic corticotropin releasing hormone (CRH) stimulates pituitary ACTH secretion through interaction with CRH receptors type 1 (CRH-R1) localized in corticotrophs. CRH increases transcription of Pro-opiomelanocortin (POMC), the ACTH precursor (10). This effect is mainly mediated by the cyclic-AMP (cAMP) pathway. CRH stimulation of corticotroph cells results in an elevation of cAMP and activation of protein-kinase A (PKA) which in turn plays important roles in activating POMC expression. Other hormones and neuropeptides (e.g. glucocorticoids) also modify POMC transcription by acting on the second messengers: cAMP and Ca^{++} (the calcium/calmodulin pathway) (11).

The POMC promoter does not have the classical cAMP responsive element, although it was known that the effect of PKA activation on POMC was mediated through the cAMP regulatory element binding protein (CREB). A novel POMC-cAMP-responsive element (POMC-CRE) was identified in 1995 (12).

Activator protein-1 (AP-1) is another transcription factor induced by CRH. It binds directly to POMC and is formed and regulated by the Jun and Fos family of proteins (13, 14). However, deletion of the AP-1 binding site on POMC does not suppress completely the CRH stimulation of POMC (15), indicating that other transcription factors might also regulate POMC.

So, CRH stimulation of corticotroph cells involves different pathways and successive recruitment of activators and repressors. Some of these pathways have been studied but they do not fully explain the complex regulation of ACTH. Prompted by this and by the crucial importance of Shh for pituitary development, we decided to study the involvement of the Shh pathway in ACTH secretion and POMC transcription.

1.1.3 Pituitary tumors

Pituitary tumors are common neoplasms, reported to account for 10-15% of all intracranial tumors, being so the second common neoplasms after meningeomas (16). Their prevalence is 300 per 1000000 inhabitants. They are usually nonmetastasizing neoplasms, which exhibit a wide range of clinical signs and symptoms produced by hormone over secretion, hormone deficiency or mass effects.

The pathogenesis of pituitary adenomas is very complex. Studies examining X-chromosome inactivation in female patients have shown that pituitary adenomas are monoclonal. This is also supported by the lack of associated hyperplasia accompanying pituitary adenomas. Nevertheless, tumor progression from a single initiated cell requires stimulus for growth. In the pituitary gland, hypophysiotropic hormones and growth and differentiation factors are the obvious candidates to be implicated in the genesis and progression of these tumors. The above theory is supported by several animal models and unusual clinical cases. The current approach integrates these proposed theories: it is likely that the majority of pituitary adenomas develop from transformed cells that are dependent on hormonal and/or growth factor stimulation for tumor progression (reviewed in 17).

Recent studies of cell differentiation regulators in the adenohypophysis have led to a more sophisticated understanding of the mechanisms that determine the patterns of hormone production and cell proliferation in pituitary adenomas. There is evidence that differentiating factors like retinoic acid (RA) and BMP4 which take part in co-ordinating the control of progenitor cell identity, proliferation and differentiation in anterior pituitary, play important roles in pituitary adenomas (18, 19). Sonic hedgehog, one of the most important pathways involved in pituitary development and differentiation, has not been studied in adult pituitary or in pituitary adenomas.

1.2   Shh signal transduction pathway – an overview

Members of the hedgehog (hh) family encode a novel class of secreted proteins that act as intercellular signals. Hedgehog was first identified by a genetic screen in the fruit fly *Drosophila melanogaster*, described in the Nobel Prize winning work of C. Nüsslein-Volhard and E. Wieshaus (20).
As with other developmental pathways first elucidated through genetic studies in *Drosophila melanogaster*, the hedgehog pathway is also conserved in vertebrates. Several hedgehog homologues have been isolated from various vertebrate species (21, 22, 23). There are three mammalian hedgehog homologues: Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh).

Shh has the largest known range of biological actions and is the only hedgehog homologue present in the central nervous system (CNS). Shh is responsible for the major effects on development of the brain, spinal cord, axial skeleton, limbs, pituitary gland, lungs, gut, etc. Ihh has been implicated in the regulation of cartilage differentiation and growth of long bones. Dhh exerts its effect mainly in the developing germline and in Schwann cells of the peripheral nervous system (reviewed in 24). With the exception of the gut, in which both Ihh and Shh are expressed, the expression patterns of the hedgehog family members do not overlap.

The primary Shh translation product includes a signal peptide, which is cleaved to yield a 45-kDa precursor protein. Shh itself catalyses a second intramolecular cleavage that generates a 20-kDa N-terminal polypeptide (Shh-N) containing all known signaling functions of the molecule and a 25-kDa C-terminal polypeptide (Shh-C), which appears to have no function other than catalyzing the autoproteolytic cleavage (25, 26).

The Shh-N is modified by the addition of a cholesterol moiety at its C-terminus (27). The cholesterol modification is essential for the normal range of signaling (28) and it is thought to be required for the correct spatial restriction of the actions of Shh. Due to these modifications, Hedgehog proteins have been detected and act far from their sources. Additional proteins are involved in Shh release and diffusion (reviewed in 29). These include Dispatched (Disp) and Tout-velu (Tvt). Dispatched is a 12-transmembrane-domain protein with a sterol-sensing domain required for Hh release. Ttv regulates synthesis of proteoglycans and functions to allow movement of Hh.

Details of Shh signaling pathway are given in Fig. 3.

Hedgehog signaling at the cell surface occurs via the Ptc-Smo receptor complex, a multicomponent receptor complex involving Patched (Ptc, also known as Ptc1) and Smoothened (Smo). Ptc is a 12-transmembrane-domain protein that binds to Shh and Smo is a 7-transmembrane domain protein homologous to G-protein-coupled receptors (reviewed in 30). In the absence of Shh, Ptc represses Smo. Shh binding to Ptc releases the basal repression of Smo by Ptc and activates the pathway, Smo acts intracellularly to transduce the Hh signal to the nucleus.
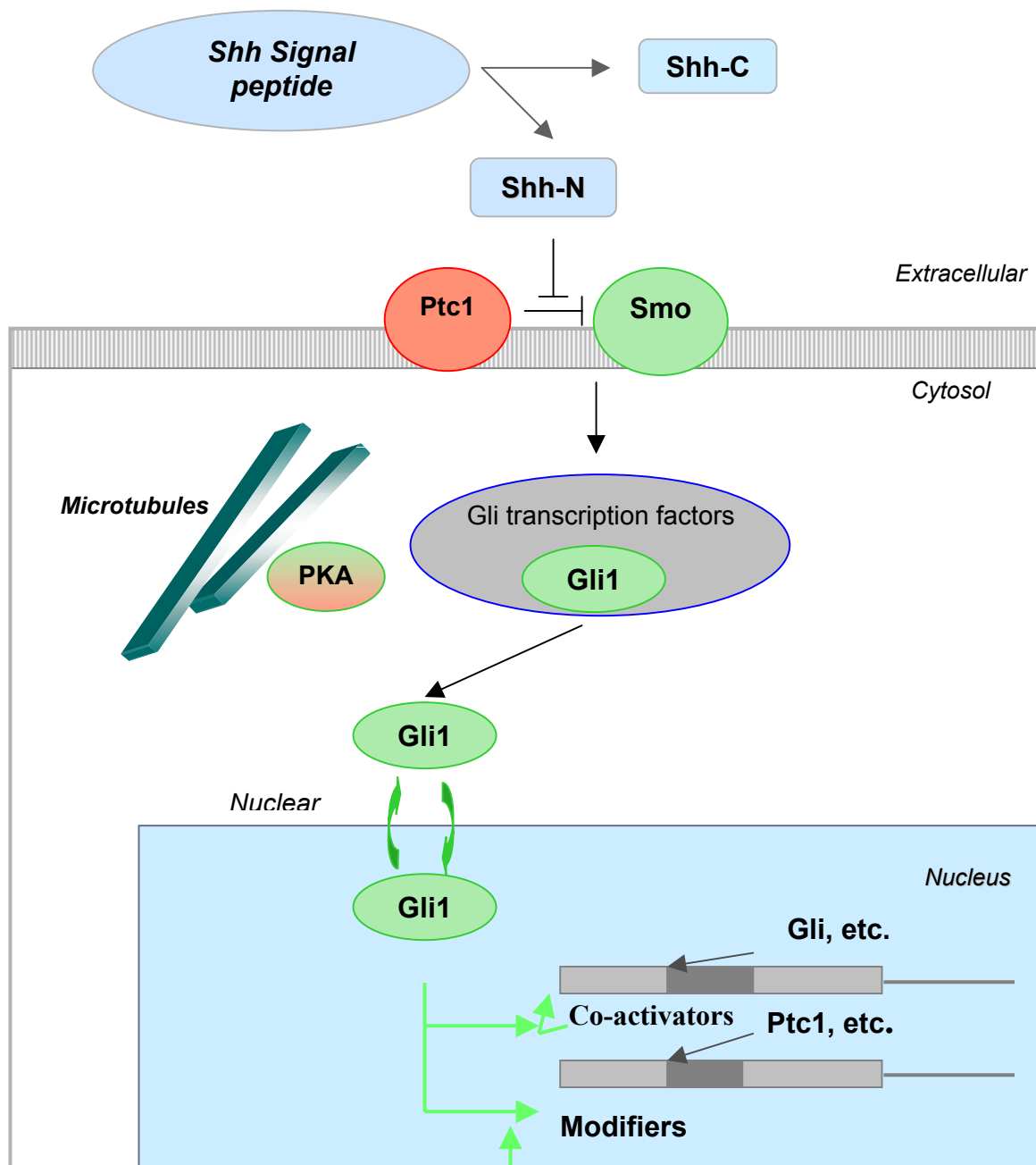
Figure 3.
Sonic hedgehog signaling pathway
Shh goes through an autocatalyzation that generates a C-terminal polypeptide and a N-terminal polypeptide (the last containing all the signaling functions of the molecule). Shh binding to Ptc releases the basal repression of Smo by Ptc and activates the pathway. Smo transduces the Shh signal to modify the Gli proteins. Gli1, the transcription factor (positive mediator) of the Shh pathway, translocates to the nucleus where it promotes the expression of target genes e.g. Ptc1, Ptc2, Gli1, etc.

A second murine Patched gene, Ptc2 (also known as Ptch2), was discovered in 1998 (31). It is expressed in the adult eye and in epithelial cells of developing hair follicles, teeth and whiskers. Both Ptc1 and Ptc2 are genuine hedgehog receptors capable of recognizing the various hedgehog ligands (Sonic, Desert, and Indian) with similar affinity (32).

Inside the cell and downstream of Smo, a large multimolecular network transduces the Hh signal to modify the Gli proteins, zinc-finger transcription factors that mediate Hh signaling (33). A very important protein involved in modulating the cytoplasmatic transduction of the Hh signal is protein kinase A (PKA). The classical action of PKA in development is a negative regulation of the PKA pathway (34). Later work identified the PKA binding site on the human Gli1 DNA and revealed that Gli1 can be both positively and negatively regulated by PKA (35).

Gli was originally identified as an amplified gene in a human glioma (36) and is the analogue of the Drosophila cubitus interruptus (ci). There are three Gli proteins that participate in the mediation, interpretation of or in the response to the Shh signal. They reside in both the nucleus and the cytoplasm, where they are components of a multimolecular complex that is tethered to the skeleton. Gli1 is the main activator that acts to mediate and/or amplify the Shh response and is transcriptionally induced by Hh signaling in all contexts examined. Gli 3 antagonizes the function of Shh-Gli1 and Gli2 may have both activating and repressing functions (37). After translocating to the nucleus, Gli1 promotes the expression of target genes. Target genes are Ptc, Ptc2, Gli1 itself, as well as members of the WNT family, TGFB/BMP family, but not Shh.

1.3 Sonic hedgehog signaling pathway in human disease

Shh signaling has been shown to regulate cell fate specification, cell proliferation and cell survival in different target cells. Signaling can be short- and long-range, direct and indirect, as well as concentration-dependent (reviewed in 30).

Humans or mice lacking Shh develop holoprosencephaly and cyclopia due to a failure of separation of the lobes of the forebrain (38, 39).

The attention drawn to the Sonic hedgehog pathway in the recent years can be attributed to its involvement in human tumors. Inactivating mutations in the Patched gene are responsible for the inherited cancer predisposition disorder known as Gorlin or nevoid basal cell carcinoma syndrome (NBCCS), which comprises multiple basal cell carcinomas (BCC) and multiple Odontogenic keratocysts (40, 41). Inactivation of patched has been shown to be a major factor in sporadic BCC formation, with mutations detected in between 12 and 40% of them. Inactivation of the Shh pathway by cyclopamine has been proposed as a potential therapeutic tool in non-pregnant adults with BCCs (42).

The patched gene has also been implicated in the etiology of a range of other tumors including PNETs, medulloblastoma, squamous cell carcinomas of the esophagus, transitional cell carcinomas of the bladder, and the benign skin lesions trichoepitheliomas. Some of these tumors express also up-regulation of other members of the Shh pathway, such as Smo, Gli2 and Gli3. A review of diseases associated to specific Shh pathway components is given in Table 1 (modified from Ref. 43).

A recent review of the Sonic hedgehog pathway in human cancers (1) supports the theory that the potential for tumorigenesis resides in stem cells. The need for the body to remain morphologically plastic (and therefore evolutionary fit) by retaining the stem cells in the adult has a price – tumorigenesis.

Hedgehog pathway component	Role in the pathway	Diseases and malformations associated with increased function ↑	Diseases and malformations associated with decreased function ↓
Gli1	Regulator of Shh targets	Basal cell carcinomas in frogs and mice, brain tumors in frogs	
Gli2	Regulator of Shh targets	Basal cell carcinomas in mice	Lung, skeleton, limb, facial and other malformations in mice
Gli3	Regulator of Shh targets		Pallister-Hall syndrome, Greig cephalopolysyndactyly syndrome Exencephaly, limb, skeletal and other defects in mice
Su(Fu)	Regulates the state and activity of Gli proteins.		Medulloblastoma
Smo	Transducer of Hh signaling	Basal cell carcinomas Possibly brain tumors	Cyclopia and multiple effects (in heart and gut) in mice
Ptc	Hh membrane receptor Inhibits Smoothened	Possibly holoprosencephaly	Gorlin syndrome / basal cell nevus syndrome (skeletal defects, tumors...) Basal cell carcinomas, rhabdomyosarcomas, medulloblastomas Medulloblastomas and rhabdomyosarcomas in mice
Tout-velu	Transport of Hh		Hereditary multiple exostoses (benign bone tumors)
Cholesterol	Regulation of Shh activity, diffusion and potency		Smith-Lemli-Opitz syndrome (growth problems, retardation and holoprosencephaly in some cases)
Shh	Extracellular ligand	Basal cell carcinomas in mice	Holoprosencephaly Holoprosencephaly, cyclopia and multiple defects in many organs in mice
Dhh	Extracellular ligand		Abnormal testis and peripheral nerve sheath development in mice
Ihh	Extracellular ligand		Abnormal bone formation in mice

Table 1. _Highlights of diseases associated to hedgehog pathway components_
(modified from Mullor et al, Trends in Cell Biology, 2002)

Recent *in vivo* and *in vitro* studies in vertebrates have demonstrated that the Shh pathway, when present and activated in adult life, plays specific functions, such as:

The hedgehog signaling regulates insulin production and increases insulin gene transcription in INS-1 β-cells, suggesting that defective Shh signaling is implicated in the pathogenesis of type 2 diabetes (44).

Shh is expressed on resting and activated human peripheral CD4(+) T cells and modulates cytokine production by these cells (45).

Shh is also expressed by cholinergic neurons in the adult rat basal forebrain, where acting synergistically with NGF plays a role in the development of postmitotic cholinergic neurons, suggesting a therapeutic role in neurodegenerative disease (46).

1.4 Shh signaling and pituitary gland development

As mentioned above, the pituitary gland is formed through the interaction of the neural and the oral ectoderm. Recent work has begun to unravel the general mechanisms of pituitary organ induction based on defining the obligatory interactions between the neural and the oral ectoderm as a prerequisite for pituitary gland formation. BMP2, BMP4, FGF and Sonic hedgehog activity are required for the development of a definitive pouch (47).

Shh is uniformly expressed throughout the oral ectoderm, but its expression is restricted to the Rathke's pouch as soon as it becomes morphologically visible. Patched is expressed at high levels in all cells of Rathke's pouch suggesting that cells of the nascent pituitary gland are receiving a Hedgehog signal. The Shh -/- mice do not have a Rathke's pouch. In transgenic embryos over-expressing Shh inhibiting protein, only a cystic rudiment of the pituitary gland was found (2). These results provide strong evidence that Hedgehog signaling plays an essential role during pituitary organogenesis.

It has been observed that overall inhibition of Shh-Gli signaling in zebrafish leads the adenohypophysis anlage to transdifferentiate into lens. The earliest lens marker δ-crystallin is expressed abundantly in Rathke's pouch of chicken, suggesting a close relationship between the cell states of the adenohypophysis anlage and the early lens. Since the adenohypophysis is able to form lens tissue in certain mutants with attenuated Hh signaling, it is speculated that normally Hh signaling inhibits the

potential of lens differentiation present in the adenohypophysis anlage (48). The crucial role of hedgehog in adenohypophysis formation in zebrafish was recently also studied by another group, using loss- and gain- of function experiments (49) These studies provide further evidence for the absolute necessity of Shh in pituitary development. However, the expression of Shh in the adult pituitary has not been examined.

Aim of the work

The Shh pathway is a crucial developmental pathway which is found to be active in post-developmental life and important in human disease. Previous studies done in pituitary development show the necessity of Shh signaling for pituitary gland formation.

The aims of this study were the expression of the Shh pathway members (Shh, Ptc1, Ptc2 and Gli1) in the adult pituitary gland and the effect of the Shh pathway in the hormone-secreting function of the normal adult pituitary. Where applicable, the underlying mechanisms and the interactions with other known stimulants were studied.

At the same time, this work aimed to study the expression of Shh pathway members in pituitary adenomas and to compare their expression between normal and adenomatous pituitary. This could identify a possible role of Sonic hedgehog as a pathogenetic factor in pituitary adenoma formation.

The data on Shh impact in pituitary development, implicates this pathway with a role in adult pituitary gland.

3. Methods

3.1 Equipment

Cell culture bottles	Nunc, Denmark
Cell culture incubator	Cytoperm 8080, Heraeus GmbH, Hanau, Germany
Cell culture plates	Falcon, Heidelberg, Germany
Cryostat Leica	Leica Microsystems, Nussloch, Germany
Electrophoresis equipment	Bio-rad Laboratories, Hercules, CA, USA
Elisa – plate reader	Dynatech MR 5000, Dynatech, Denkendorf, Germany
Laminar flow	Typ UVF 6.18 S, BDK , Luft-und Reinraumtechnik GmbH, Genkingen, Germany
Luminometer and fluorometer	Wallac 1420, Wallac Distribution, Freiburg, Germany
Microscope	Axioskop 2, Carl Zeiss GmbH, Jena, Germany
Pipettes	Eppendorf, Hamburg, Germany
Spectrophotometer	Pharmacia, Freiburg, Germany
Table centrifuge	Eppendorf, Hamburg, Germany
Water bath	Köttermann Labortechnik, Uetze-Hänigsen, Germany

3.2.   Reagents

ABC kit	Vector Laboratories, Burlingane, CA, USA
ABC-AP kit	Vector Laboratories, Burlingane, CA, USA
Acetic acid	Merck, Darmstadt, Germany
Acridine orange	Sigma-Aldrich, St. Louis, MO, USA
Agar	Life Technologies, Paisley, Scotland, UK
Amphotericine	ICN Biomedicals, Irvine, CA, USA
Ampuwa water	Frisenius, Germany
cAMP (8-CPT)	Sigma-Aldrich, St. Louis, MO, USA
CRH	Bachem, Bubendorf, Switzerland
Collagenase	Worthington Biochemical Corporation, Lakewood, NJ, USA
Cyclopamine	Toronto Research Chemicals, Toronto, Canada
Diaminobenzidine	Sigma-Aldrich, St. Louis, MO, USA
DMSO	Sigma-Aldrich, St. Louis, MO, USA
Dulbecco's modified Eagle Medium (DMEM)	Gibco / Invitrogen, Carlsbad, CA, USA
Enthelan	Merck, Darmstadt, Germany
Ethidium Bromide	Sigma-Aldrich, St. Louis, MO, USA
Foetal Calf Serum	Gibco / Invitrogen, Carlsbad, CA, USA
Forskolin	Sigma-Aldrich, St. Louis, MO, USA
Glutamine	Sigma-Aldrich, St. Louis, MO, USA
H-89	Calbiochem, San Diego, CA, USA
Hybond ECL membrane	Amersham Biosciences, Bucks, UK
Hydrochlorid acid	Merck, Darmstadt, Germany
Lipofectamine	Invitrogen, Carlsbad, CA, USA
L-glutamine	Biochrom, Berlin, Germany
Luciferin	Roche, Mannheim, Germany
Lumi-light Western Blotting substrate	Roche, Mannheim, Germany
MEM-Vitamins	Biochrom, Berlin, Germany
mShh-N	R & D systems, Minneapolis, MN, USA
Optimem 1	Gibco / Invitrogen, Carlsbad, CA, USA

pAP1-Luc	Stratagene, La Jolla, CA, USA
Paraformaldehyde	Merck, Darmstadt, Germany
PBS	Gibco / Invitrogen, Carlsbad, CA, USA
pcDNA 3,1-His	Invitrogen, Carlsbad, CA, USA
pCre-Luc	Clontech Laboratories, Palo Alto, CA, USA
pEGFP-C2	Clontech, Palo Alto, CA, USA
Penicillin / Streptomycin	Biochrom, Berlin, Germany
Peptone	ICN Pharmaceuticals, Aurora, OH, USA
Protease inhibitor cocktail	Sigma-Aldrich, St. Louis, MO, USA
Qiagen Midi/Maxi Kit	Qiagen, Hilden, Germany
Reporter lysis buffer	Promega, Mannheim, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium peroxide (NaOH)	Merck, Darmstadt, Germany
Toluidin Blue	Sigma-Aldrich, St. Louis, MO, USA
Transferrine	Sigma-Aldrich, St. Louis, MO, USA
Triiodothyronine	Henning, Berlin, Germany
Tris pure	ICN Pharmaceuticals, Aurora, OH, USA
Trypsin / EDTA	Biochrom AG, Berlin, Germany
Tween 20	Sigma-Aldrich, St. Louis, MO, USA
WST-1	Roche Diagnostics, Mannheim, Germany
Xylol	Roth, Karlsruhe, Germany
Yeast extract	ICN Pharmaceuticals, Aurora, OH, USA

3.3 Solutions

Collagenase Mix 1000U/ml	For 100 ml solution: Collagenase: 4 g Trypsin inhibitor: 10 mg Hyaluronidase: 100 mg BSA: 400 mg Dnase: 500 µl
HDB buffer	For 1 l solution: Hepes 25mM: 5,95 g NaCl 137mM: 8 g KCl 5mM: 0,370 g $Na_2HPO_4.H_2O$ 0,7mM: 0,120 g Glucose 10mM: 1,982 g Amphotericine B 25µg/ml: 10 ml Penicillin / Streptomycin 10^5U/l: 10 ml Adjust pH to 7,3 with NaOH Store at +4°C
LB medium	For 1 l solution: Peptone: 10 g Yeast extract: 5 g NaCl: 5 g NaOH 1M: 2ml Adjust to pH 7,0
PBS 1x	To prepare 1 l solution: NaCl: 8g KCL: 0,2g $Na_2HPO_4.H_2O$: 1,44g KH_2PO_4: 0,2g Adjust to pH 7,4
PFA 4% (paraformaldehyde)	To prepare 100 ml solution: Paraformaldehyde: 4g PBS: 20ml Ampuwa water: 80 ml Add 1M NaOH to adjust pH 7,4

	Heat at 56°C to dissolve
	Filter and cool before usage
	Store at +4°C for not more than 2 days
TBS 1x (Tris Based buffer)	To prepare 1 l solution:
	Tris powder: 2,42 g
	NaCl: 8 g
	Adjust to pH 7,6
TB (Tris Buffer)	To prepare 1 l solution:
	Tris powder: 12,114 g
	Adjust to pH 7,6
Tris-HCl 1M	To prepare 1 l solution:
	Tris powder: 121,42 g
	Add 25% HCL to adjust pH 8,2

3.4 Human tissues

Experiments were performed after approval of the ethics committee of the Max Planck Institute, and informed consent was received from each patient or their relatives. 2 normal human pituitaries (1 male and 1 female) and 55 pituitary adenomas (29 males and 26 females) were included in the study.

Normal pituitary tissues were obtained from autopsies performed 12 to 16 hours post mortem in normal healthy subjects after accidental death. Surgically excised human pituitary tumor tissues were obtained from consecutive unselected patients after transsphenoidal surgery, performed in 3 neurosurgical departments in Germany and Italy.

Tissues were placed in DMEM and transferred to our laboratory on ice within 36 hours. After washing and removing debris fibers, one aliquot was snap-frozen on dry ice and kept at -80°C for Immunohistochemical staining (50). Snap-freezing is the most common method for preparing tissues for immunohistochemistry procedures. Quickly freezing tissue preserves the basic morphology and most antigens, and produces a hard block of material that can be cut into sections for immunostaining.

Tumors were diagnosed by clinical, biochemical, radiological and surgical findings. IHC for hormone staining were performed to certify the anatomo-pathological diagnosis (described later in 2.5.1). The pituitary adenomas used in this work, were classified into acromegaly-associated pituitary tumors (ACRO; 7 cases), prolactinomas (PROL; 3 cases), corticotropinomas (CUSH; 13 cases), TSH-secreting adenomas (TSH; 2 cases) and clinically nonfunctioning adenomas (NFPA; 30 cases); the later was divided after immunopathological examination into gonadotropinomas (17 cases), null cell adenomas (11 cases), 1 silent corticotroph adenoma and 1 silent GH/Prl secreting tumor.

3.5   Immunohistochemistry

Immunohistochemistry (IHC) is a technique for localizing and visualizing a protein in a tissue section by using an antibody (primary antibody) specific for the target antigen.

Tissue antigens are detected by a three-stage process: the binding of the primary antibody to its specific epitope, the binding of a biotinylated secondary antibody to the primary antibody and the subsequent detection by a colorimetric reaction. The secondary antibody binds the epitopes of the host animal in which the primary antibody was raised. The enzymatic complex used is the avidin and biotinylated horse-radish peroxidase macromolecular complex (ABC) containing the enzyme peroxidase. Avidin is a high molecular weight glycoprotein with an extraordinarily high affinity for the small molecular weight vitamin, biotin. The secondary antibody is conjugated to the biotin beads, which also bind to the avidin-biotin complex. The chromogen used to localise the peroxidase in the tissue sections was diaminobenzidine tetrahydrochloride (DAB). DAB produces a brown precipitate which is insoluble in alcohol and clearing agents, allowing sections to be permanently mounted.

Another method used in this study is the ABC-AP system. The principle is the same as above, but the enzyme used is alkaline phosphatase (AP). AP substrate produces a more translucent reaction product than peroxidase substrates and provides additional color choices, which are needed in double labeling (explained later in double immunohistochemistry). The enzymatic activity of AP can be localized by coupling a soluble product generated during the hydrolytic reaction with a "capture reagent". The chromogen used is vector red, which produces a red reaction that can be seen using either brightfield or fluorescent microscopy.

Snap-frozen normal and adenomatous pituitary tissues were cut in a cryostat. 8-µm sections were thaw-mounted onto sterile poly-L-lysine-coated slides and fixed in cold 4% phosphate-buffered paraformaldehyde (PFA). After fixation in PFA, the slides were washed twice in PBS for 3 minutes, immersed in 70% Ethanol for 4 minutes and then put in 96% Ethanol at +4°C for storage (as described in 51).

Antibodies

A detailed list of primary and secondary antibodies used in the study, containing the respective hosts and dilutions used, is given in Table 2.

Primary antibody	Source	Host	Dilution
Shh-N	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	goat	1:200
Shh-N	R&D systems (Minneapolis, MN, USA)	goat	1:200
Ptc1	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	goat	1:150
Ptc2	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	goat	1:100
Gli1	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	goat	1:200
ACTH	DAKO (Glostrup, Denmark)	mouse	1:1000
GH	Gift from Dr. C. J. Strasburger, Berlin, Germany	mouse	1:800
FSH	Immunotech (Marseille, France)	Mouse	1:800
LH	Immunotech (Marseille, France)	Mouse	1:1000
Prl	Immunotech (Marseille, France)	Mouse	1:1000
TSH	Immunotech (Marseille, France)	mouse	1:800

Secondary antibody	Source	Host	Dilution
biotinylated anti-goat			
	Vector Laboratories (Burlingame, CA, USA)	horse	1:300
biotinylated anti-mouse			
	Vector Laboratories (Burlingame, CA, USA)	goat	1:300

Table 2: _List of Primary and secondary antibodies used in the study_

3.5.1 Single Immunohistochemistry

For detecting the expression of Shh, Ptc1, Ptc2 and Gli1 at a protein level, the specific antibodies we used in combination with the ABC complex and DAB chromogen (as described in 52).

On the day of the experiment, the slides were briefly washed in TBS (pH 7.6) for 5 min and incubated for 30 minutes in the blocking serum. For blocking was used serum from the same animal species in which the respective secondary antibody was produced, in this case 10% horse serum. Afterwards the primary antibody was applied and the slides were left overnight at +4°C. The next day, after 3 washes (5 minutes each) in TBS, the secondary antibody was applied and the slides were incubated for 30 minutes in room temperature. The secondary antibody was

biotinylated anti-goat, made in horse. After three further washes, the slides were incubated with the ABC complex for 30 minutes at room temperature. The ABC complex was prepared at least 30 minutes in advance in Tris Buffer pH 7.6 (15µl Avidin + 15µl Biotin + 970µl TB). Then, after another three washes, the slides were immersed in freshly prepared DAB (2 ml of 1mg/ml DAB + 33 µl of 30% hydrogen peroxide + 98ml Tris Buffer). DAB incubation time was specific for each primary antibody and was decided by looking at the amount of the brownish precipitate under the microscope.

After the DAB, the slides were washed many times in TBS and counterstained with toluidine-blue for 10 minutes. This stains the nuclei pale blue, allowing an easy view of the tissue organization. Afterwards, the slides were briefly washed twice and dehydrated using progressive concentrations of Ethanol (30 seconds in 70% Ethanol + 2 drops/100ml acetic acid 100%, 1 minute in 96% Ethanol, 1 minute in 100% Ethanol). Slides were then fixed in rotihistol and cover-slipped with Enthelan.

For detecting the expression of pituitary hormones at a protein level (and subsequently localizing the different pituitary cells), we used the ABC-AP kit and Vector Red as a chromogen. The protocol is identical with the one explained above, some substances and substrates were different. The secondary antibody was biotinylated anti-mouse made in goat, as all primary antibodies against hormones were produced in mouse and the blocking was performed in 10% goat serum. The preparation of the ABC-AP complex was the same as above (15µl Reagent A + 15µl Reagent B+ 970µl TB, made at least 30 minutes in advance). The Vector Red was prepared according to the manufacturer's instructions, using 2 ml of Tris-Cl solution (100mM, pH 8,2-8,5), 500 µl Levamisol (for blocking endogenous alkaline phosphatase activity), 1 drop of compound 1, 1 drop of compound 2 and 1 drop of compound 3.

3.5.2   Double Immunohistochemistry

This method facilitates the localization of two different antigens in the same tissue section. It was performed to co-localize the expression of Shh, Ptc1, Ptc2 and Gli1 in different hormone-producing pituitary cells.

For this were used two different enzyme systems (peroxidase and alkaline phosphatase) and their chromogens (DAB for ABC and Vector Red for ABC-AP),

performing sequentially the staining of each primary antibody (as explained in 53). The experiment was started as a single immunohistochemical staining for detecting one of the primary antigens (Shh, Ptc1, Ptc2 and Gli1) using ABC and DAB. Immediately after followed the second immunohistochemical staining for detecting the second primary antigen (one of the hormones produced by the pituitary gland), using ABC-AP and Vector Red. Counterstaining was performed only at the end of the second immunohistochemistry.

3.6 Cell culture
All the cell culture work was performed under a sterile laminar flow.

3.6.1 Rat pituitary primary cell culture
The pituitary primary cell culture was obtained (as explained in 54) from adult male Sprague-Dawley rats (180-250 g). They were kept for 5 days in our animal house in standard conditions: 12 hours light/dark rhythm, temperature 21°C, water and standard food. Pituitary glands were obtained after decapitation performed quickly after CO_2 narcosis. The tissue was washed throughout with HDB+ buffer [137mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4, 10mM glucose, 15mM HEPES (pH 7.3)]. Sliced fragments were inserted in a preparation buffer containing 4 g/l collagenase, 10 mg/l DNAse II, 0.1 g/l soybean trypsin inhibitor, and 1 g/l hyaluronidase and were enzymatically dispersed in 37°C for nearly 45 minutes. Dispersed cells were centrifuged and resuspended in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 2 mM essential vitamins, 40U/l insulin, 20 mg/l natrium selenate, 5 mg/l transferrin, 30 pM triiodothyronine (T_3), 10% foetal calf serum, 2 mmol/l L-glutamine, 2.5 ng/l Amphotericine B and 10^5 U/ml penicillin-streptomycin. Cell viability was determined by fluorescence microscopy by staining with acridin orange and ethidium bromide. Acridin orange enters the membranes of normal cells, yielding green fluorescence in viable cells. Ethidium bromide does not pass the healthy cell membrane and enters only in dead cells with damaged membranes, yielding a red fluorescence. Cell viability of pituitary cells was determined as the percentage of green cells in the total number of cells (counted in a Neubauer measuring chamber) and was over 95%. Cells were distributed in 96-well plates and incubated at 37°C under 5 % CO_2. The stimulation was performed 48 hours after preparation.

3.6.2 Immortalised pituitary cell lines

The immortalized cell lines used in this study were the corticotroph cell line AtT-20 and the lactosomatotroph cell line GH3 obtained from the American Type Culture Collection (Rockville, MD, USA). The AtT-20 cell line originates from a murine ACTH-secreting pituitary tumor (55). The GH3 cell line is originally obtained from a radiation induced pituitary tumor in 7-month old Wistar-Furth female rats (56).

The medium used for their culture was DMEM supplemented with 10% foetal calf serum, 2 mmol/l L-glutamine, 2.5 ng/l Amphotericin B and 10^5 U/ml penicillin-streptomycin. They were kept in 75 cm^2 cell culture bottles, in incubators at 37°C in the presence of 5% CO_2. When confluent, the cells were washed with PBS, trypsinized, centrifuged at 1200g for 4 min and re-dispersed in fresh medium. They were numbered and re-plated in new cell culture bottles and in different well-plates according to the experiments done (as explained in 57).

For hormone measurement and cell proliferation experiments, the cells were plated in 96-well plates. The amounts were: 2000/well AtT-20 and GH3 cells for cell proliferation assays, 2000/well AtT-20 and 4000/well GH3 cells for hormone measurement assays. All stimulation experiments were carried out in quadruplicates.

For transfections, the AtT-20 cells were plated in 6-well plates, at a density of 300,000 / well and all experiments were carried out in triplicates.

3.6.3 Stimulations

24 hours prior to stimulation, cells were serum-deprived to stop their growth in the G0 phase. This step helps in putting all the cells in the same cell cycle phase, making them so more sensitive to all further stimuli.

Stimulants

The stimulants used in the cells culture studies are as follows:

mShh-N is a recombinant mouse Sonic hedgehog amino-terminal peptide, coding the amino acid residues 25-198 of the mouse Shh (21). A search in the NCBI databank on Sonic hegehog mouse-rat (mus musculus – rattus norvegicus) homology maps resulted in a full homology, so the same substance was used for experiments in

mouse and in rat cells. The lyophilized sample was stored at –20°C. 250 µl sterile PBS was added to the vial containing 25 µg Shh-N, giving a stock dilution 100 µg/ml. According to the manufacturer's instructions, the product upon reconstitution was stored at +4°C for at most one month.

Cyclopamine is an inhibitor of the Shh pathway. The vial containing 1 mg product was stored originally at -20°C and was reconstituted in 480 µl DMSO, yielding so a stock dilution of 5mM. The stock dilution was aliquoted and stored in -20°C.

CRH, human, mouse and rat corticotropin was diluted in PBS to obtain 10µl aliquots of 200µM, which were stored at -20°C.

Forskolin was used in hormone measurement experiments as an agonist of the PKA (protein kinase A) pathway. It was diluted in 100% ethanol to a stock dilution of 10mM and stored at -20°C. In all experiments containing Forskolin, the same amount of 100% ethanol was added to the other wells (containing control medium or other stimulants).

pCPT-cAMP was used as a stronger agonist of the PKA pathway. It was dissolved in PBS to a stock dilution of 10mM, was aliquoted and stored at –20°C.

Stimulations for RIA experiments

The cells were serum deprived for 24 hours before each experiment. Then, the medium was removed and replaced by 100µl stimulant medium, containing stimulants dissolved in serum deprived medium (the serum hormones interfere with our hormone measurements). 24 hours afterwards, the experiment was stopped and the supernatant was transferred to 0,5ml Eppendorf tubes for RIA measurements.

Stimulations for WST-1 experiments

The medium was removed from the cells in the 96-well plates and was replaced by the stimulant medium, containing stimulants dissolved in medium containing 2% FCS (in order to measure the effect on cell growth, the cells should proliferate and this can happen only in the presence of serum). Another stimulation was performed after 48 hours. The experiments were stopped after 4 days, by putting WST-1 inside the wells (explained in the WST-1 measurement).

3.7   Hormone measurement by RIA

Radioimmunoassay (RIA) is a highly sensitive and quantitative technique used for the measurement of substances such as enzymes, proteins, hormones, that exist in very low concentrations. In our study, we used RIA to measure the concentration of rat and mouse ACTH, rat GH and rat prolactin secreted in culture by pituitary primary cultures or stable cell lines.

RIA uses radiolabeled Antigenes (Ag) to detect Ag:Ab reactions. The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of specific antibody binding sites. The Ags are labeled with the I^{125} (iodine-125) isotope, and the presence of Ag:Ab reactions is detected using a gamma counter.

So the first step for starting the RIA is developing an antibody that is highly specific for the hormone being measured. An N-terminal specific antibody against mouse and rat ACTH was raised in rabbits using an antigen produced by the two-step carbodiimid method (explained in 58). Standarts were purchased from Bachem (Bubendorf, Switzerland). The rat GH and rat Prolactin antibodies were included in the specific RIA reagent kits provided by the National Hormone and Peptide Program (Baltimore, MD), containing the specific antigens, antiserums and standards.

A small quantity of the antibody was mixed with a certain quantity of the sample (supernatant) containing the hormone to be measured. At the same time, a certain amount of tracer (standard Antigen labeled with the radioactive isotope I^{125}) was added to the mixture. The samples were incubated 1 hour at 37°C, allowing time for the hormone (Ag) to bind to the antibody. The mixture was prepared in such quantities that there was not enough antibody to bind with both the labeled hormone and with the hormone to be measured, so the natural hormone and the labeled hormone had to compete for binding sites. The quantity of each hormone bound was proportional to their concentration and the amount of labeled hormone (tracer) bound to the specific antibody was inversely proportional to the concentration of the natural hormone. After binding had reached an equilibrium, the quantity of radioactive hormone bound to the antibody was measured in a gamma counter. As explained above, the amount of radioactivity present in the test was inversely proportional to the amount of hormone in the sample.

Quantification of the unknown free hormone in the sample was achieved by comparing their activity with a standard curve prepared by using increasing amounts of known concentrations of the hormone.

3.8 Cell Proliferation measurement by WST-1

WST-1 is one of the assays available for analyzing the number of viable cells by measuring the cleavage of tetrazolium salts added to the culture medium.

The WST-1 added to the cell is converted by the mitochondrial dehydrogenases into a dye that can be quantified by a scanning multiwell spectrophotometer (ELISA reader). An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of the dye formed, which directly correlates to the number of metabolically active cells in the culture.

The stimulation experiment was stopped after 96 hours by adding 10µl WST-1 reagent to each well containing 100µl of cells in medium. After 1 hour, the results were measured in the ELISA reader at 450 nm (as explained in 59).

In each experiment, the background control was measured. This consisted of 4 wells containing 100µl culture medium plated at the same time with the stimulation medium, where 10µl WST-1 was added. Slight spontaneous absorbance occurs when WST-1 is added to the medium in the absence of the cells. This background absorbance depends on the culture medium, the incubation time and the exposure to light (WST-1 is a light sensitive substance). The value of the background absorbance (usually between 0,1 and 0,2 absorbance units) were subtracted from all our control and stimulation samples.

3.9   Western Blotting

Western blot analysis can detect one protein in a mixture of any number of proteins, giving also information about the size of the protein. The method is dependent on the use of a high-quality antibody directed against a desired protein. This antibody is used as a probe to detect the protein of interest. The protein is detected through visualization of the specific antibody.

For the Western Blot experiment we prepared 2 x 6-well plates containing 400 000 AtT-20 cells per well. The cells were plated in normal DMEM containing 10% FCS the next day they were about 70-80% confluent. Then, they were stimulated with Shh,

CRH or the combination of both for 24 hours. Three wells were used for each condition. After the stimulation time finished, the protein was extracted using a protease inhibitor cocktail diluted 1:100 in PBS (200µl/well) and pipeting up and down a few times with a very small (insulin) syringe.

The mixture of proteins obtained in the solution was separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This separates the proteins by size. The gel density was 10% for CRH-R1 and 15% for SHH.

Afterwards, a nitrocellulose membrane (Hybond ECL) was placed on the gel and, using electrophoresis, the protein bands were driven onto the nitrocellulose membrane. The negative charge was on the side of the gel and the positive charge on the side of the nitrocellulose membrane, driving so the negatively charged proteins over to the positively charged nitrocellulose membrane. This gives a nitrocellulose membrane that is imprinted with the same protein bands as the gel.

The nitrocellulose membrane was blocked for 2 hours in a solution containing 5% milk and 0,1% TWEEN. Then, it was incubated for 1,5 hours with the primary antibody (s. Tab. 3) diluted in 2,5% milk and 0,1% TWEEN. The primary antibody (the two primary antibodies we used were specific against Shh-N and CRH-R1), sticks to the specific protein and forms an antibody-protein complex with the protein of interest. After the incubation with the primary antibody, the membrane was washed 3 times, 10 minutes each in PBS containing 0,1% TWEEN.

Primary antibody	Source	Host	Dilution
Shh-N	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	rabbit	1:500
CRH-R1	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	rabbit	1:250

Secondary antibody	Source	Host	Dilution
Anti-rabbit Horseradish peroxidase	Amersham Biosciences (Bucks, UK)	donkey	1:2000

Tab. 3 *List of antibodies used for Western Blot analysis*

Then, the nitrocellulose membrane was incubated for 1 hour with the secondary antibody diluted 1:2000 in a solution containing 2,5% milk and 0,1% TWEEN. The secondary antibody should be directed against the primary antibody. As both the primary antibodies were made in rabbit, the secondary antibody was anti-rabbit. At the same time, the secondary antibody was an antibody-enzyme conjugate. The conjugated enzyme was there to allow visualization of the reaction. The secondary antibody was conjugated to Horseradish Peroxidase. Three further washes for 10 minutes each, were then performed as explained above.

The Lumi-light Western Blotting Substrate was used to detect the enzyme and was prepared according to the manufacturer's instructions.

An x-ray film was put together with the membrane in an autoradiography cassette, to detect the flash of light given off by the enzyme. After 30 seconds, the film was removed and developed to visualise the immunoreactivity. The bands were present wherever there was a protein-primary antibody-secondary antibody-enzyme complex, or, in other words, wherever the specific protein was.

3.10   Transfections

3.10.1   Plasmids

Plasmids are circular DNA molecules, nowadays very much used for studying the control of gene expression and investigating the regulatory elements and cell signaling.

Plasmids used in molecular biology usually contain an antibiotic resistance gene. This helps during the transformation step (described below) for selecting only the host bacteria resistant to a certain antibiotic. There are expression and reporter plasmids.

The expression plasmid introduces into the cell the cDNA of the gene that we want to overexpress, driven by a constitutively active promoter (in our case the cytomegalovirus promoter). This increases the transcription of this gene and makes possible the study of its effects on other genes. The expression plasmids used in this

study are pcDNA3-mouseGli (60) (gifts from Dr. H. Sasaki, Center for developmental biology, Riken, Kobe, Japan) and its control plasmid pcDNA 3,1-His (Invitrogen).

pcDNA3-mouseGli1 contains the full sequence of the mouse Gli1 cDNA inserted in a pcDNA 3,1-His plasmid (Invitrogen) containing the ampicillin resistance gene. The mouse Gli1 cDNA is here under the control of the CMV (cytomegalovirus) promoter. For easy use terminology, this plasmid will be called mGli1 and the control plasmid used in all the experiments will be pcDNA 3,1-His (pcDNA).

The reporter plasmids contain reporter genes sensitive to the activation status of endogenous genes. Endogenous gene expression is monitored through creation of a fusion gene in which the promoter of an endogenous gene is coupled to the reporter gene, which is easier to detect. If the endogenous gene promoter is 'turned off', neither the endogenous gene nor the reporter gene is transcribed. Similarly, if the promoter for the endogenous gene is activated, then the reporter gene is transcribed. All our reporter plasmids contain the reporter luciferase. Luciferase is an enzyme found in the firefly beetle (Photinus pyralis) which interacts with its substrate luciferin to produce a light emission peaking at 562nm. This emission is easily measured in a luminometer. The luciferase activity correlates with the transcription of the gene promoter coupled to luciferase in a certain plasmid.

The reporter plasmids used in this study are POMC-Luc, 8 x 3'Gli-BC-Luc, 8 x mut3'Gli-BS-Luc, AP1-Luc and Cre-Luc.

POMC-Luc contains the luciferase gene under the control of 770bp of the rat POMC (proopiomelanocortin). These 770bp contain all the sequences necessary for the correct in-vivo POMC expression in the mouse pituitary (18). The plasmid contains the ampicillin resistance gene.

8 x 3'Gli-BC-Luc and 8 x mut3'Gli-BS-Luc are a gift from Dr. H. Sasaki (Center for developmental biology, Riken, Kobe, Japan). 8 x 3'Gli-BC-Luc contains 8 directly repeated copies of 3'Gli-BS (unique nucleic acid sequence that binds to Gli1) bound to the luciferase gene (61). 8 x mut3'Gli-BS-Luc is the control plasmid to be used in all experiments: a sequence alteration within the 8xGli-BS makes the binding to Gli1 impossible, so the reporter levels are constantly off, despite increased or reduced Gli1 transcription.

pAP1-Luc contains tandem repeats of the transcription factor complex AP1, the firefly luciferase gene and the ampicillin resistance gene.

pCre-Luc is destined to monitor the activation of cAMP binding protein (CREB) and cAMP mediated signal transduction pathways. The plasmid contains multiple copies of the CRE-binding sequence, the firefly luciferase gene and the ampicillin resistance gene.

Parallel control plasmid

The control plasmid used to check the transfection efficiency in all our experiments is pEGFP-C2, encoding a variant of the *Aequorea Victoria* green fluorescent protein (GFP) that has been optimized for brighter fluorescence and higher expression in mammalian cells. The respective protein, when excited with a wave length of around 490 nm, becomes fluorescent and emits at 510 nm. This fluorescence / emission can be measured in a fluorometer. The plasmid contains the kanamycin resistance gene.

3.10.2   Transformation

All the plasmids were received in small amounts. To obtain large quantities of plasmid DNA, one easy way is to place the desired DNA into bacteria, grow the bacteria, then harvest the bacteria, and isolate the DNA. The bacteria used are treated so that they take the plasmid up into their cells. These are called competent cells. We used competent Escherichia Coli. Because the plasmid DNA contains an antibiotic resistance gene, the bacteria with the plasmid inside can grow also in the presence of that specific antibiotic. The process of transformation consists of mixing competent bacteria with plasmid DNA and then selecting bacteria containing the plasmid using agar plates that contain an antibiotic.

For this, 10µl Competent E. Coli cells were thawed on ice and mixed with 1µl of the transforming plasmid. The mixture was left on ice for 30 minutes, and then briefly heat-shocked for 35-45 seconds in a +42°C waterbath. 500µl LB medium (non-selective medium, suitable for bacterial growth) was added to permit the bacteria to recover and express the antibiotic resistance gene and the mixture was left at 37°C for 45 minutes. The cells were then spread on petri plates that contain a solid LB agar medium and the specific antibiotic. The plates were incubated at 37°C overnight to permit bacterial growth. An individual bacterium that takes up a plasmid DNA molecule grows on the plate and gives rise to small, cell-dense colonies. All of the

cells in one colony are clones of the original transformant cell and so contain the same plasmid DNA.

Identification of the transformed bacteria is facilitated by the presence of the antibiotic resistance gene on the plasmid. Only cells that have taken up plasmid DNA and express the antibiotic resistance gene will grow on plates containing the antibiotic. Ampicillin, being a bacteriostatic antibiotic does not directly kill bacteria, but inhibits their growth. Agar plates containing kanamycin were used for the pEGFP control plasmid that expresses the kanamycin resistance gene.

For each plasmid, one single colony was picked up from the agar plate and put in 200 ml LB medium supplemented with the specific antibiotic, at 37°C to grow overnight. The next day, the medium was centrifuged. The supernatant was discarded and the precipitate containing bacteria and plasmid DNA was treated following the Qiagen Midi/Maxi Kit protocol and the manufacturer's instructions. At the end of the protocol, the plasmid DNA was isolated as a small pellet, which was dissolved in 200 µl TE solution (pH 8.0). The concentration of the plasmid in this solution was measured in a photometer.

3.10.3   Transfection

Transfection is the introduction of foreign molecules such as DNA into a recipient eukaryotic cell. By transfecting with an expression plasmid, we overexpress a certain gene and can study its consequences. By introducing a reporter plasmid into a cell, one can measure the transcriptional activity of the endogenous gene linked to the promoter. In this study were transfected AtT-20 cells.

The day before the experiment, nearly 400 000 cells were plated in each well of a 6-well plate, in normal DMEM containing 10% FCS. The next day, the cells were 70-80% confluent.

To make possible the entry of the plasmid into the cell, was used the Lipofectamine 2000, a cationic lipid-based reagent designed to help the plasmid DNA enter the cell membrane (as explained in 62). Necessary for the high efficiency of transfection and normal entry of the plasmid DNA into the cells is the lack of serum. Therefore, a special medium containing all the nutrients, but no serum was used: Optimem 1.

For each well was prepared a mixture of 1,4 µg plasmid DNA, 0,6 µg pEGFP control DNA and 10µl Lipofectamine in Optimem. The cells were left in Optimem for 6 hours.

Afterwards, the supernatant was removed and normal DMEM without FCS was added to the cells (some serum hormones interfere with POMC-Luc basal activity). Stimulations (when applicable) were performed 24 hours after the beginning of the experiment (or 18 hours after the removal of Optimem). The stimulants were added to the cells in serum deprived medium and left for 6 hours. Afterwards, the cells were washed once with PBS and lysed with the protein preserving lysis buffer and scraped. The lysates were transferred into Eppendorf tubes and briefly centrifuged. 20 µl of the supernatant were plated in a nontransparent 96-well plate, the plate was put in a Berthold Luminometer, 50µl luciferin was automatically added to each well by the machine and the luciferase activity was measured.

In parallel, 60 µl supernatant was plated in a 96-well plate and the GFP fluorescence was measured in a fluorometer. The program used was 285nm/315nm Fluorescence and consists in illuminating the GFP protein inside each well with a wave length of 285nm, and measuring at the same time the emission of the fluorescent protein at 315nm. The values were corrected, so that they correspond to the 20 µl supernatant used for the luminiscence measurement.

At the end, the luciferin values were divided by the GFP fluorescence values, to normalize the results obtained according to the transfection efficiency in each well. All experiments were carried out in triplicate and repeated at least three times independently.

All experiments where 2 plasmids (other than pEGFP) were introduced into the cells (for example the co-transfection of mGli1 and POMC-Luc) lasted 24 hours (6 hours on Optimem and 42 hours in Normal DMEM).

3.11 Statistics

Hormone secretion and cell proliferation experiments were all performed in quadruplicate (four wells having identical conditions). The result graphs contain the mean of 4 values for a certain condition and the standard error.

For Hormone measurement experiments, a standard curve was created from samples with known hormone concentration and kept as reference. For WST-1 experiments, the background values were subtracted from all the OD-450 values. To determine the significance of our stimulants' effects on cell proliferation and hormone secretion, the mean values were compared by one-way ANOVA. P-values smaller

than 0,05 were considered significant. The significance grades are marked with stars as follows: * for p<0,05, ** for p<0,005, *** for p<0,001.

Transfection experiments were all performed in triplicate (three wells having identical conditions). A parallel control was run inside each well. The values for each well were corrected according to the control and the result graphs contain the mean of 3 values for a certain condition and the standard error. The statistical significance was determined using one-way ANOVA in combination with Scheffe's test. P-values smaller than 0,05 were considered significant (stars meaning explained above).

4. Results

4.1 Members of the Sonic hedgehog pathway are expressed in the pituitary gland
(Single and Double Immunohistochemistry results)

Using immunohistochemistry, the expression of Sonic hedgehog pathway members in the normal adult pituitary was studied.

Shh was expressed in the normal pituitary at the protein level. The stainings for Shh were performed with 2 different antibodies and the results were similar and consistent: cytoplasmatic staining in epithelial-type cells, evident around 5% of all the anterior pituitary cells (Fig.4). Shh proteins were localized close to each other and in groups.

Double IHC revealed which anterior pituitary cells express Shh. For this 2 stainings were performed one after the other (double immunohistochemistry), first the Shh and second the specific hormone (ACTH, GH, Prol, FSH, LH and TSH). Parallel negative controls were run for each case. The Shh staining co-localizes 98% with that of ACTH, showing that most Sonic hedgehog proteins produced inside the pituitary gland originate from corticotroph cells. There were no co-localizations with lactotrophs, gonadotrops and TSH-producing cells, while only 1-2 % of Shh cells correlated with GH (Fig.4).

To test whether Shh produced in corticotrophs exerts an effect on corticotroph and other pituitary cells, we searched for the presence of Shh receptors patched 1 and patched 2. The staining for Ptc1 and Ptc2 were evident in nearly half of the anterior pituitary cells (Fig. 5,6). Both receptors were expressed in all the normal pituitaries tested.

The double IHC for Patched 1 was run in the same way as for Shh. It was evident that the majority of Ptc1 containing cells co-localize with gonadotrophs (double stain with LH), as well as with TSH-producing cells (Fig. 5).

Double ICH for Patched 2 was performed as the ones above (Fig. 6). Ptc2 is mainly localized in corticotroph cells (double staining with ACTH) and in somatotroph cells (double staining with GH). To a lesser degree there is co-localization with prolactin. There was no co-staining with LH or TSH.
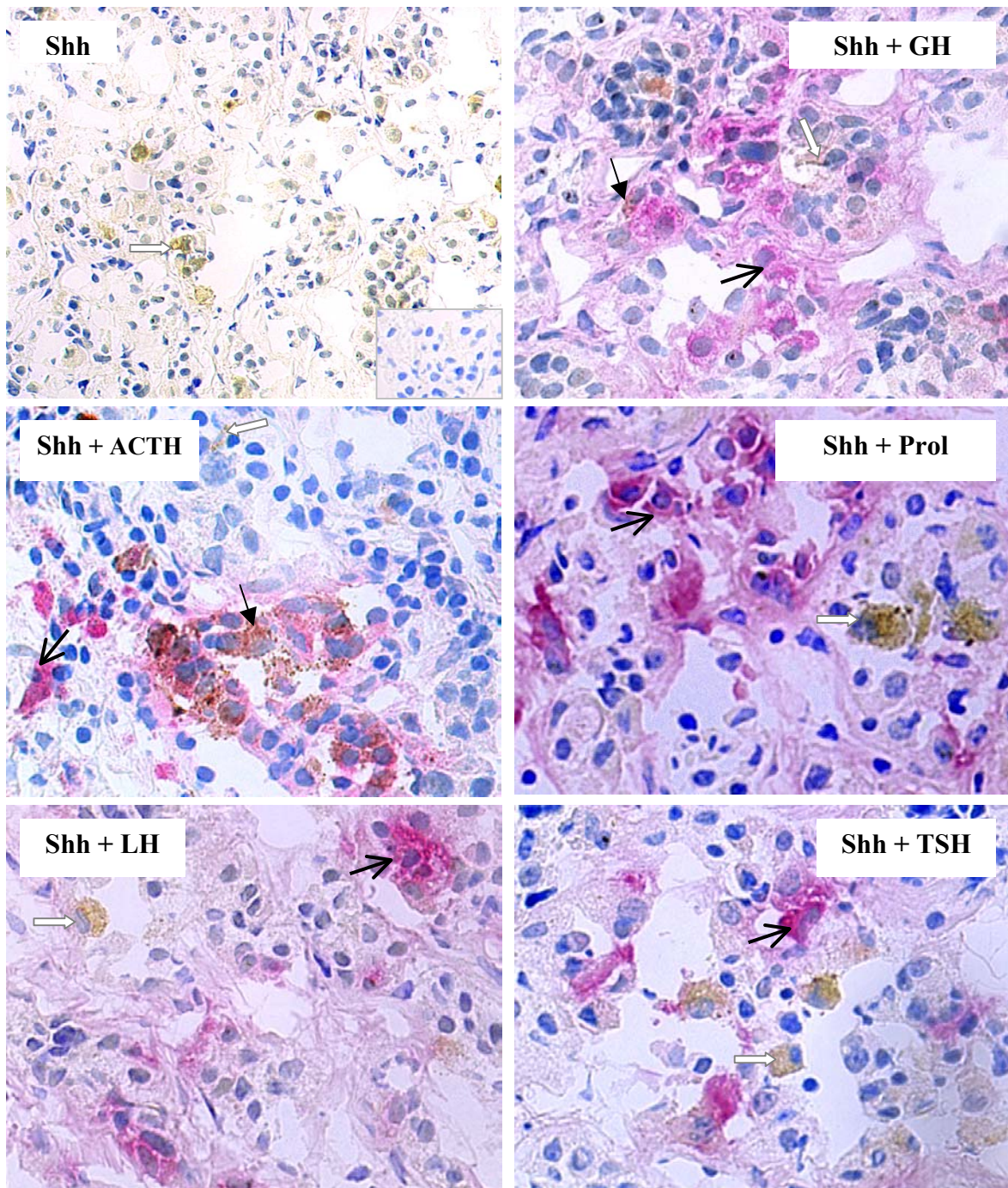
Figure 4.

Sonic hedgehog protein expression in the human anterior pituitary gland.

Shh single staining is shown separately (x20 magnification). Shh protein (brown) is marked with a white block arrow. Double IHC staining was performed separately for each hormone (pictures in x40 magnification). The presence of the specific hormone stains the cytoplasm red and is marked with an open arrow. The co-localization of Shh and one specific hormone in the same cell (double staining) is brown + red and marked with a closed arrow. The nuclei are stained in blue.

The majority of Shh producing cells (over 98%) are corticotrophs, only 1 co-localizes with GH and there is no co-localization with Prolactin, LH, FSH or TSH.
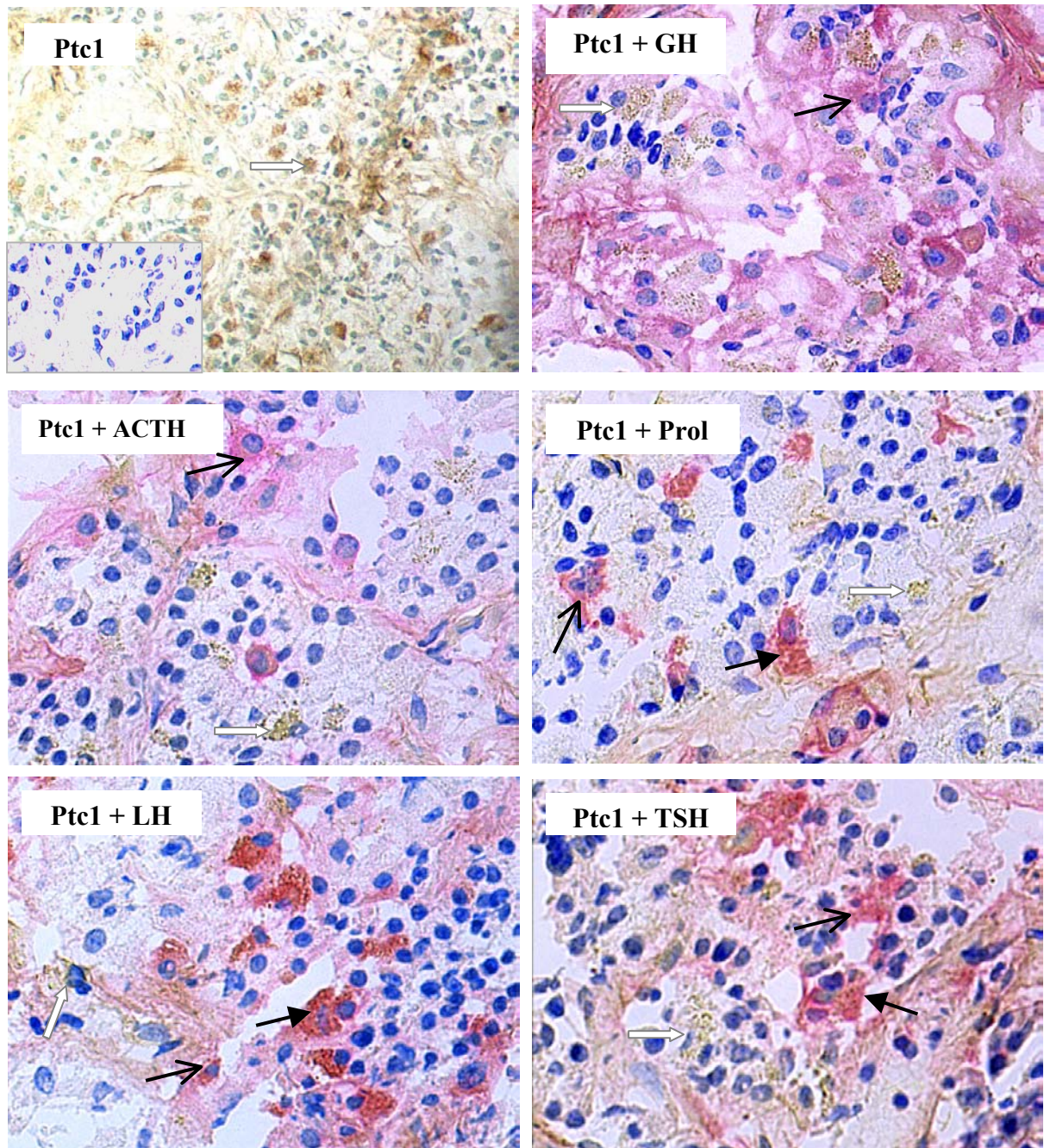
Figure 5.

Patched-1 protein expression in the human anterior pituitary gland.

Ptc1 single staining is shown separately (x20 magnification). Ptc1 presence stains the cell membrane and cytoplasm brown and is marked with a white block arrow. Double IHC staining was performed separately for each hormone (pictures in x40 magnification). The presence of the specific hormone stains the cytoplasm red and is marked with an open arrow. The co-localization of Ptc1 and one specific hormone in the same cell (double staining) is brown + red and marked with a closed arrow.

The nuclei are stained in blue.

The majority of pituitary cells possessing Ptc1 are gonadotrophs and thyreotrophs.
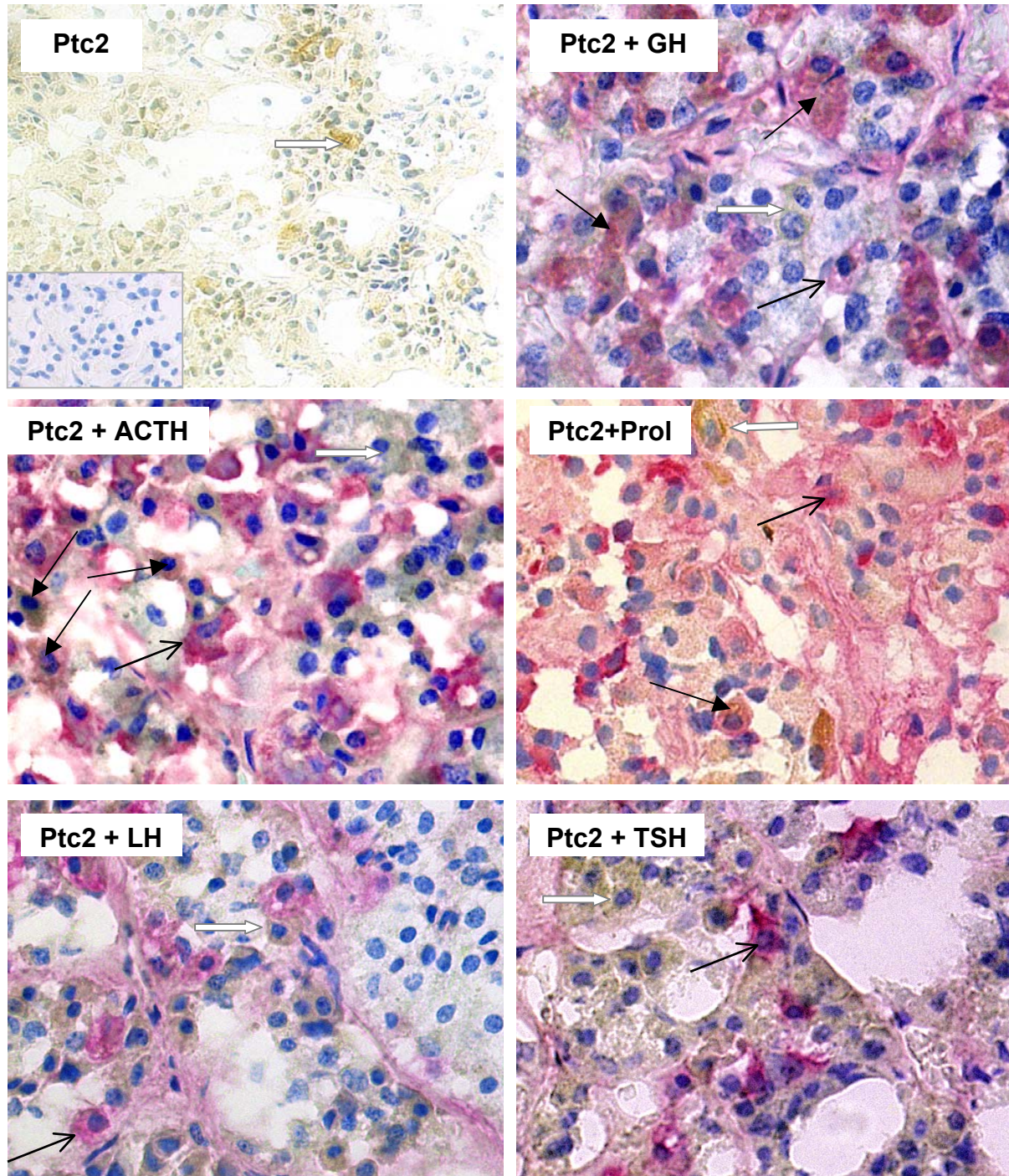
Figure 6.

Patched-2 protein expression in the human anterior pituitary gland.

Ptc2 single staining is shown separately (x20 magnification). Ptc2 presence stains the cell membrane and cytoplasm brown and is marked with a white block arrow. Double IHC staining is performed separately for each hormone (pictures in x40 magnification). The presence of the specific hormone stains the cytoplasm red and is marked with an open arrow. The co-localization of Ptc2 and one specific hormone in the same cell (double staining) is brown + red and marked with a closed arrow. The nuclei are stained in blue. The majority of pituitary cells possessing Ptc2 are corticotrophs, sommatotrophs as well as some lactotrophs.
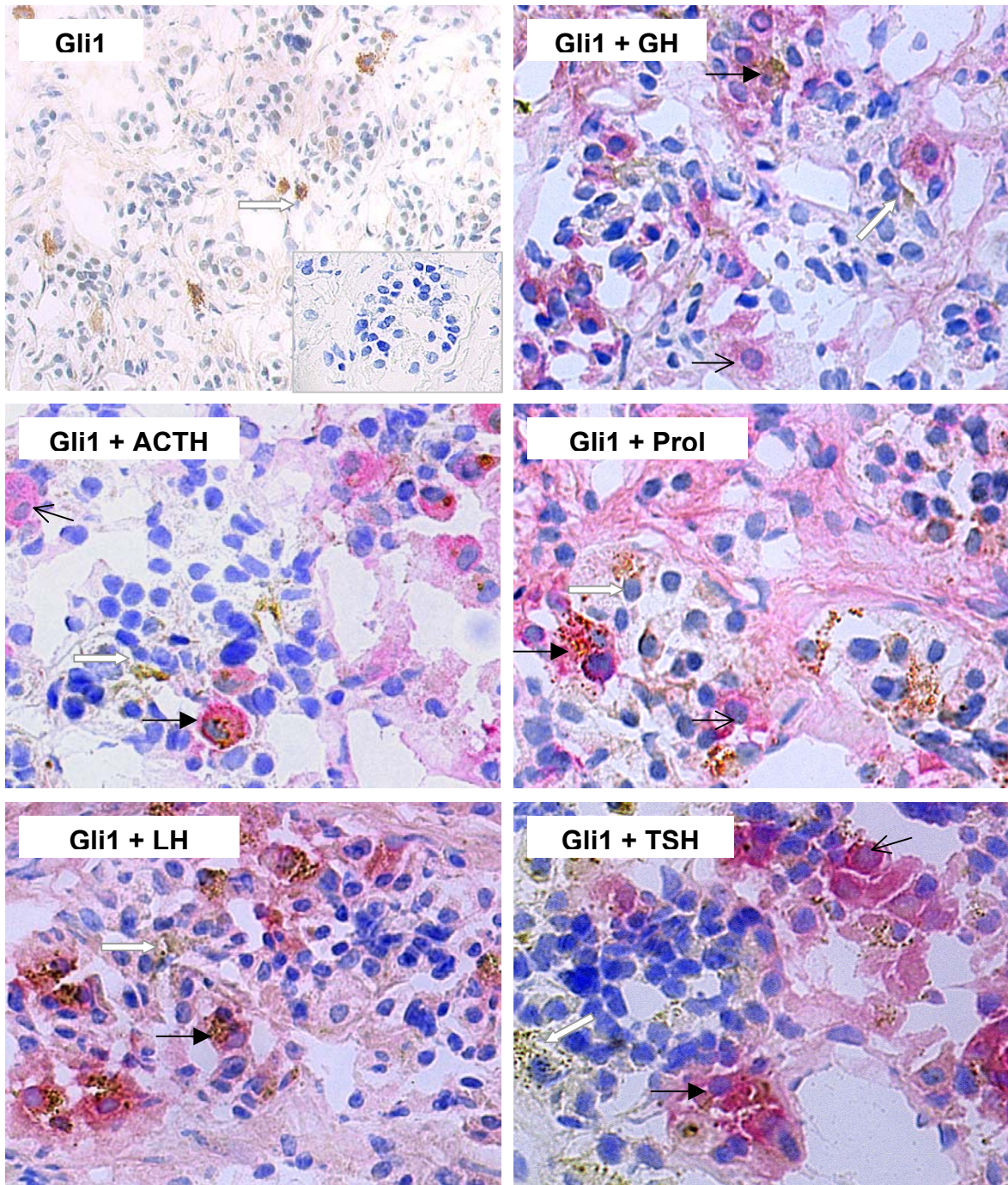
Figure 7.

Gli1 protein expression in the human anterior pituitary gland.

Gli1 single staining is shown separately (x20 magnification). Gli1 presence stains the cell nucleus and cytoplasm brown and is marked with a white block arrow. Double IHC staining is performed separately for each hormone (pictures in x40 magnification). The presence of the specific hormone stains the cytoplasm red and is marked with an open arrow. The co-localization of Gli1 and one specific hormone in the same cell (double staining) is brown + red and marked with a closed arrow. The nuclei are stained in blue.

Gli1 is the main transcription factor of the Shh pathway. The aim of testing for the presence of Gli1 was to find out whether the Shh pathway is active in the pituitary gland.

The immunohistochemistry experiments revealed that Gli1 is also present in the human pituitary, having a partially cytoplasmic – partially nuclear staining, expected by the fact that it translocates to the nucleus to promote the expression of target genes (Fig. 7).

The double immunohistochemistry finds co-expression of Gli1 with all anterior pituitary hormones, showing that the Gli1 is present at a protein level in all hormone secreting adenohypophysis cells.

All together, the expression of Shh, Ptc1, Ptc2 and Gli1 in human anterior pituitary cells suggests that this pathway is active and important for the normal functioning of the adult pituitary. Corticotrophs are Shh producing and target cells at the same time, implying a crucial role of Shh in corticotroph cell function.

4.2 Sonic hedgehog increases ACTH and GH secretion in the normal rat pituitary

The control of pituitary hormone secretion is dependent not only on the classical hormone releasing factors, but also on paracrine factors such as neuropeptides, growth factors, cytokines, etc which have been identified in the pituitary.

Shh is produced in corticotroph cells, but being a signaling protein it could be able to induce effects also in distance. As also presented in 4.1, the presence of Shh receptors and the transcription factor Gli1 indicates an active Shh pathway in anterior pituitary hormone secreting cells.

Therefore, it was investigated whether Shh has any role in the major function of corticotrophs, sommatotrophs and lactotrophs, which is respectively ACTH, GH and prolactin secretion. For this a primary culture of rat pituitary cells was prepared. The cells were stimulated with Shh in concentrations from 0,2 to 5 µg/ml, CRH 100nM and the combination of both Shh and CRH at maximal concentrations. The results are presented in Fig. 8.

It results that Shh increases ACTH production in a concentration dependent manner and the effects at 1µg/ml and at 5 g/ml are statistically significant. Shh at 5 µg/ml increases ACTH production nearly 2,5 fold. At the same time, CRH at 100nM increases ACTH production nearly 170% while the combination Shh 5µg/ml and CRH 100nM increases ACTH secretion over 425%, so leading to a synergistic effect.
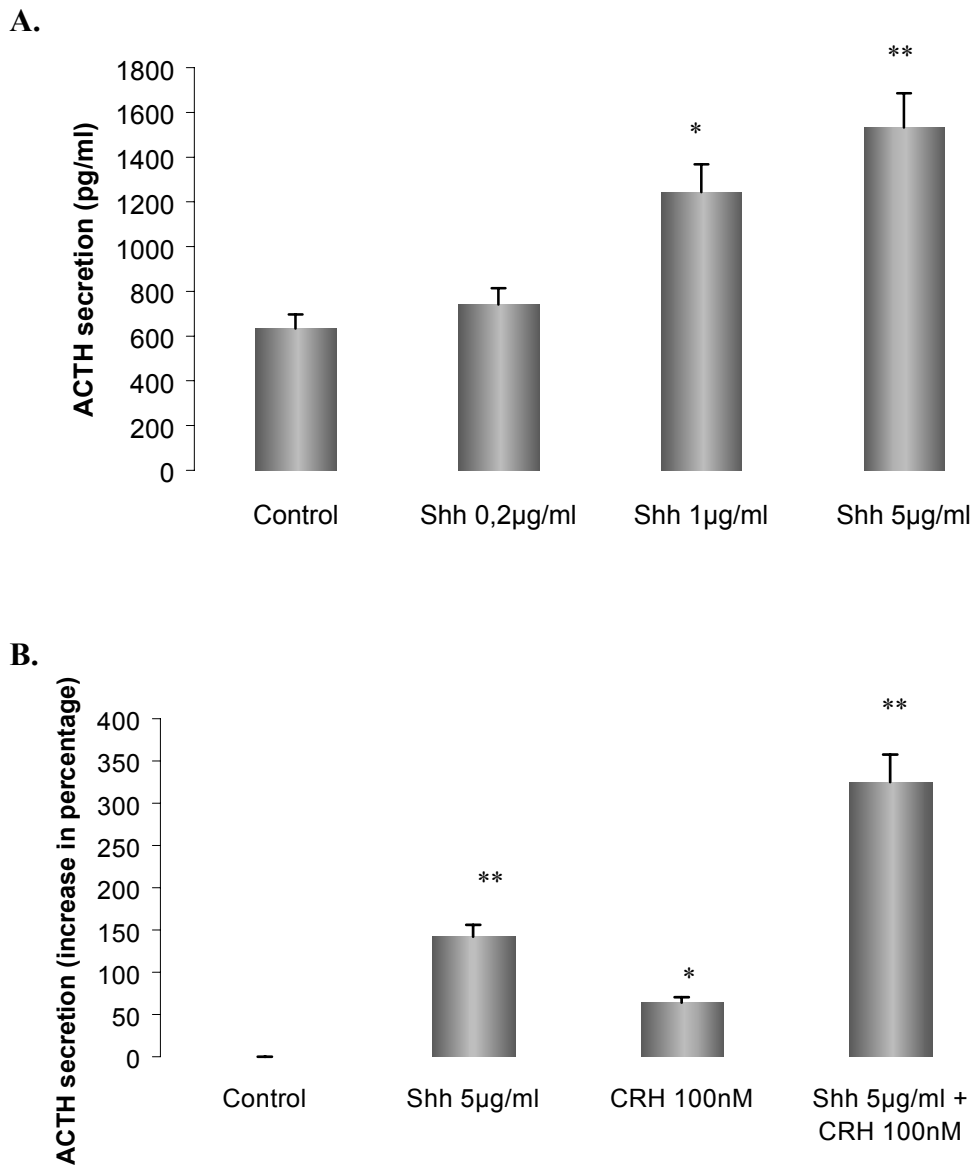
A.

B.

Figure 8.

A. Shh stimulation of ACTH secretion in normal rat pituitary cells

Rat pituitary cells were plated in 96 well plates at a concentration 10k/well and in 10% FCS medium. After 2 days under basal culture conditions, they were stimulated with Shh in concentrations 0,2 – 5µg/ml using stimulation medium containing 0% foetal calf serum (FCS). Each stimulation was performed in quadruplicates and negative controls were run in parallel. The supernatant was collected 24 hours after stimulation and ACTH secretion was measured by RIA.

B. CRH and Shh have synergic effects in ACTH stimulation in normal rat pituitary cells

The experiment was performed in identical conditions as 5A. The cells were stimulated with Shh 5µg/ml, CRH 100 nM and the combination of both. The results are presented in increase (in percentage) compared to the control. The absolute value of the control corresponding to 100% is 633 ng/ml.

To examine the effects of Shh on GH and prolactin secretion on normal rat pituitary cells, an identical experiment was performed, stimulating rat pituitary cells in primary culture with Shh in concentrations from 0,2 to 5 µg/ml. The results are shown in Figures 9 and 10.
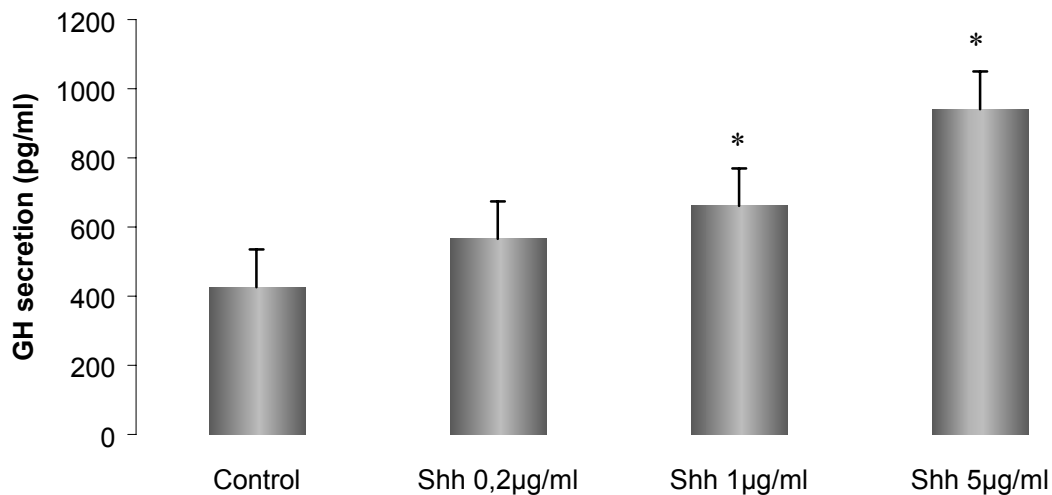

Figure 9.

Regulation of GH secretion by Shh in normal rat pituitary cells

Fresh rat pituitary cells were plated in 96 well plates at a concentration 10k/well and in 10% FCS medium. After 2 days in culture, they were stimulated with Shh in concentrations 0,2 – 5 µg/ml using stimulation medium containing 0% FCS. Each stimulation was performed in quadruplicates and negative controls were run in parallel. The supernatant was collected 24 hours after stimulation and GH secretion was measured by RIA.

As seen, increasing doses of Shh stimulate GH secretion in a linearly progressive way. The effects of Shh at 1µg/ml and at 5 µg/ml are statistically significant. The maximum concentration of Shh increases GH secretion by 220% of the control values.
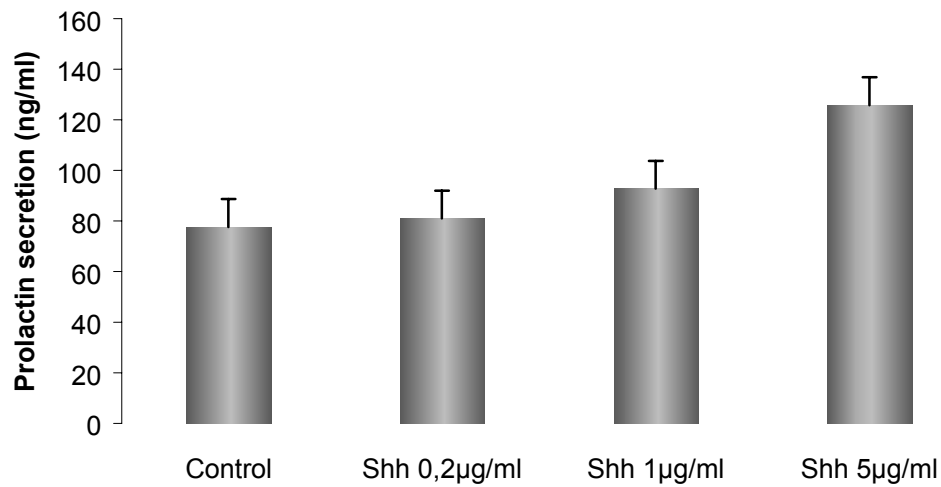
Figure 10.

Rat pituitary cells were plated in 96 well plates at a concentration 10k/well and in 10% FCS medium. After 2 days in culture, they were stimulated with Shh in concentrations 0,2 – 5µg/ml using stimulation medium containing 0% foetal calf serum (FCS). Each stimulation was performed in quadruplicates and negative controls were run in parallel.. The supernatant was collected 24 hours after stimulation and Prolactin secretion was measured by RIA.

A dose dependent slight increase in prolactin secretion is also seen following stimulation with Shh (especially at 5 µg/ml) but this effect is not statistically significant.

The Shh localization in corticotrophs speaks for a more important role in these cells. The Shh stimulation experiments performed in normal rat pituitary in primary culture reveal that Shh is a secretagogue of ACTH and has a synergistic effect with CRH.

4. 3. Sonic hedgehog effects on ACTH secretion in the AtT-20 cell line

4.3.1 Shh increases ACTH secretion in AtT-20 cells

The increase in ACTH secretion caused by Shh in rat pituitary cells, prompted us to study in more details the effect of Shh on hormone secretion in the AtT-20 murine corticotroph cell line that secretes ACTH.

For this, AtT-20 cells were plated in 96-well plates and stimulated with Shh in concentrations from 0,2 to 5 µg/ml.

It results that Shh at 5µg/ml induces ACTH secretion 4-5 times and this effect is statistically very significant (Fig. 11.)
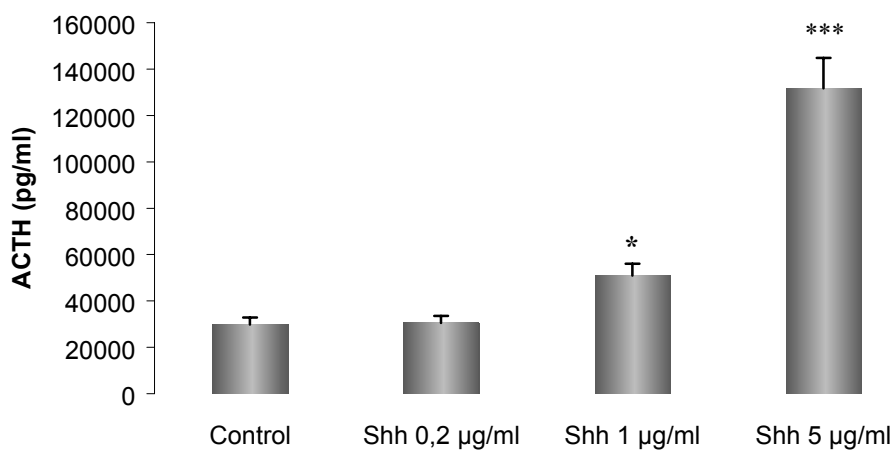

Figure 11.

Shh stimulation of ACTH secretion in the AtT-20 corticotroph cell line

AtT-20 cells were plated in 96-well plates at a concentration of 2000 cells/well and in 10% FCS medium. After 1 day in culture, the cells were serum-deprived for 24 hours and afterwards stimulated in 0% FCS medium with three Shh doses from 0,2 to 5 µg/ml. The supernatant was collected 24 hours after stimulation and ACTH secretion was measured by RIA.

The specific Sonic hedgehog antagonist cyclopamine was also used in cell culture experiments for hormone measurements. The original powder was diluted according to the manufacturer's instructions in DMSO to a stock dilution concentration of 5mM. Cyclopamine is usually used in concentrations from 0,2 to 20 µM. Given the fact that this is a veratrum alkaloid and potentially toxic, the toxicity of different doses in the AtT-20 cell line was studied at the very beginning.

For this, AtT-20 cells were plated in 96-well plates at a concentration of 2000 cells/well and in 10% medium. After 1 day in culture, the cells were serum deprived for 24 hours and then stimulated with Cyclopamine in doses 0,2µM, 1µM, 5µM, 10 µM and 20 µM. DMSO was added to control and stimulation wells, in such amounts that all wells included in the experiment contained the same amount of DMSO (coming from cyclopamine stock dilution or from DMSO original tube).

After 24 hours, the cell viability was estimated using acridin orange and ethidium bromide. Only wells having cyclopamine in concentrations lower or equal to 5µM had the same viability as the control wells. Cyclopamine in doses 10 and 20µM was very toxic, causing cell death.

As a result, AtT-20 cells were stimulated with cyclopamine 5µM to check its effect on basal ACTH secretion and on Shh stimulated ACTH secretion (Fig. 12).

Cyclopamine at 5µM has no significant effect on basal ACTH production, but it reduces by 30% the ACTH increase by Shh 5µg/ml, and this effect is statistically significant. Nevertheless, the interpretation of these results should take into consideration the limitation of not being able to use higher doses because of toxic effects in the AtT-20 cell line (Cyclopamine is generally used in concentrations from 5 to 20µM in cell culture studies performed in other cell lines).
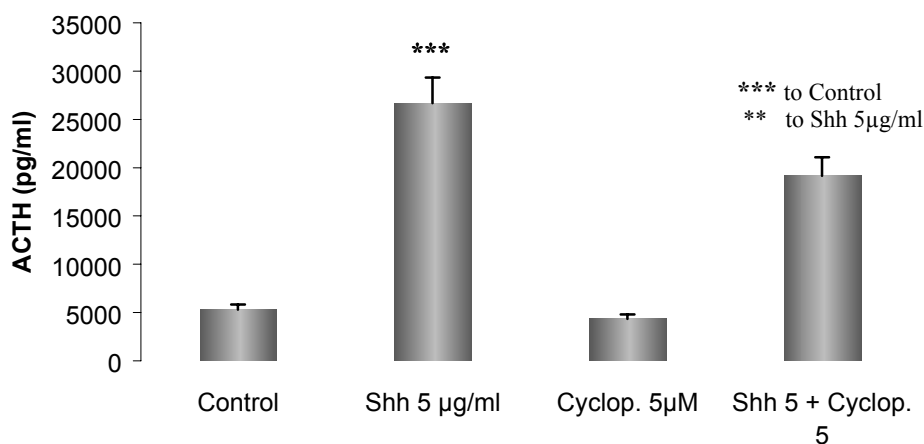
Figure 12.

Cyclopamine effect on basal and stimulated ACTH secretion in AtT-20 cells.

AtT-20 cells were plated in 96-well plates at a concentration of 2000 cells/well and in 10% FCS medium. After 1 day in culture, the cells were serum-deprived for 24 hours and afterwards stimulated in 0% FCS medium with Shh 5µg/ml, Cyclopamine 5µM and the combination both. The supernatant was collected 24 hours after stimulation and ACTH secretion was measured by RIA.

4.3.2. Shh and CRH / Forskolin have synergistic effects on ACTH secretion

Sonic hedgehog is produced locally in the pituitary and acts as a major ACTH secretagogue. The mechanisms involved were studied by starting with the effect of the co-stimulation of AtT-20 cells with Shh and CRH, the physiological stimulus for ACTH secretion. The experiment was designed exactly as the ones in paragraph 4.3.1, using 4 different wells for each condition in 96-well plates (Fig. 13).

The co-stimulation with Shh and CRH increases ACTH secretion more than 11 times the control values. This effect is over 200% more than the sum of the Shh and CRH effects separately, being a synergistic effect.

Figure 13.

Shh and CRH have synergistic effects on ACTH secretion

The experiment was performed using the same protocol as the one explained in Fig. 8. After serum deprivation, the cells were stimulated with Shh 5µg/ml, CRH 100nM and the combination of both. After 24 hours in stimulation medium, the supernatant was collected and ACTH secretion measured by RIA. The results are presented in increase (in percentage) compared to the control. The absolute value of the control corresponding to 100% is 6936 ng/ml.

The same experiment was performed using another stimulator of ACTH secretion, forskolin. Forskolin is a phosphodiesterase inhibitor that increases the intracellular levels of cAMP, which stimulates the PKA pathway, one of the most important pathways by which CRH exerts its ACTH-secreting effect.

The co-stimulation with Shh and forskolin increases ACTH secretion more than the sum of both Shh and forskolin stimulations separately, being a synergistic effect (Fig. 14).
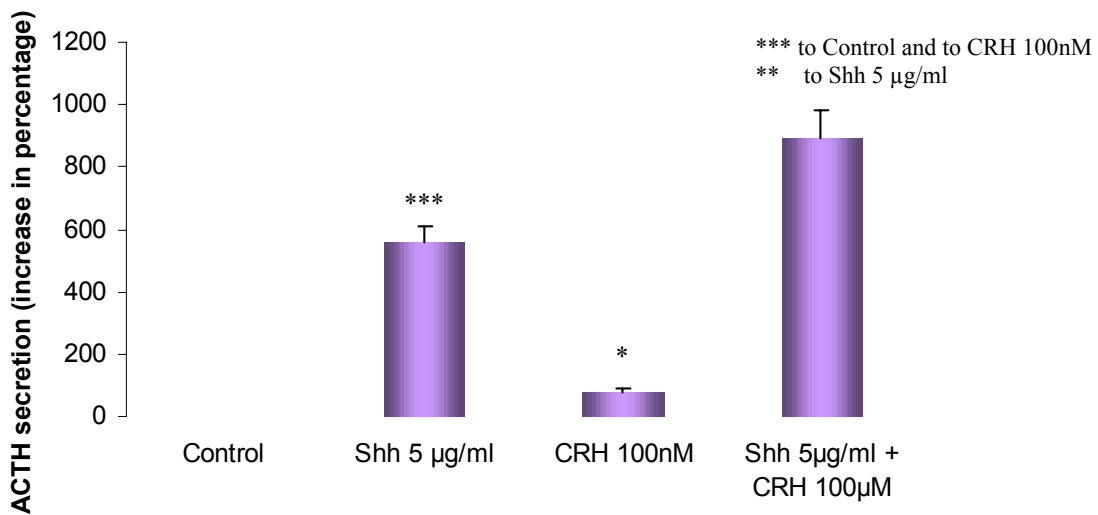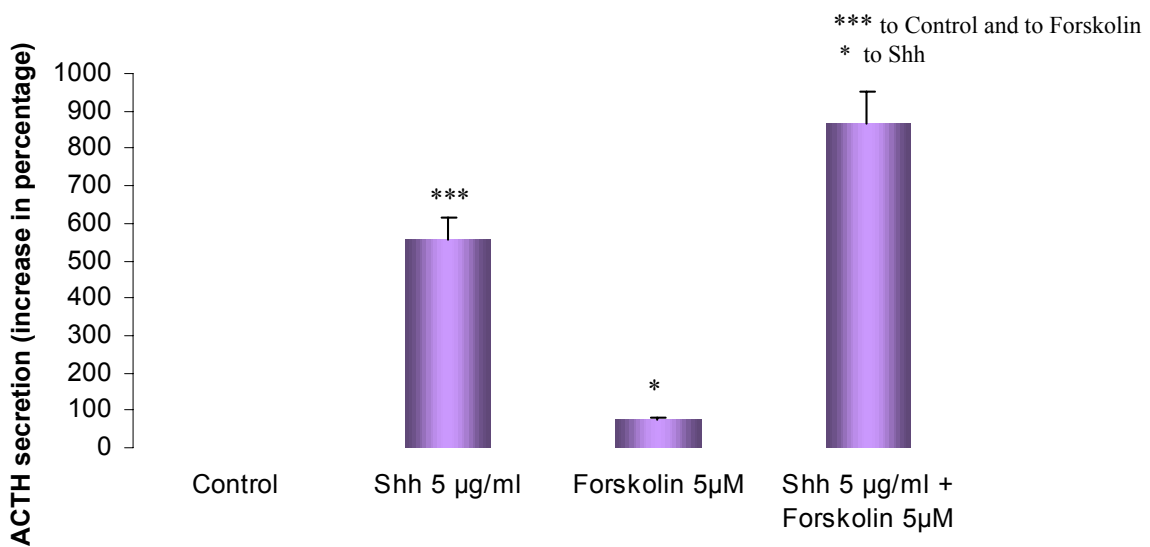

Figure 14.

Shh and Forskolin have synergic effects on ACTH secretion

The experiment was performed using the same protocol as the one explained in Fig. 8. After serum deprivation, the cells were stimulated with Shh 5µg/ml, Forskolin 5 µM and the combination of both. After 24 hours in stimulation medium, the supernatant was collected and ACTH secretion measured by RIA. The results are presented in increase (in percentage) compared to the control. The absolute value of the control corresponding to 100% is 6936 ng/ml.

4.4 Sonic hedgehog effect on hormone secretion in the GH3 cell line

4.4.1 Sonic hedgehog increases Growth Hormone secretion

The mammosomatotroph GH3 cell line secretes growth hormone and prolactin, being so a classical model for studying the regulation of these two hormones.

Given the fact that Sonic hedgehog is produced locally in the pituitary gland and stimulates GH secretion in the normal rat pituitary, the effects of Shh in hormone production by the GH3 cell line were studied as well.

For this, GH3 cells were plated in 96-well plates and stimulated with Shh in concentrations from 0,2 to 5 µg/ml. It results that the increase in GH secretion is linearly progressive with increasing doses of Shh and the effects are statistically significant. Shh at maximal dose increases GH secretion more than 4,5 fold (Fig. 15).

Figure 15.

Sonic hedgehog increases growth hormone secretion in the GH3 cell line

GH3 cells were plated in 96-well plates at a concentration of 4000 cells/well and in 10% FCS medium. After 1 day in culture, the cells were serum-deprived for 24 hours and afterwards stimulated in 0% FCS medium with Shh in doses from 0,2 to 5 µg/ml. The supernatant was collected 24 hours after stimulation and GH secretion was measured by RIA.

4.4.2 Sonic hedgehog effect on Prolactin secretion

For studying the effect of Shh on prolactin secretion in GH3 cells, a similar experiment to the one explained in 4.4.1 was performed. GH3 cells were plated in 96-well plates and stimulated with Shh in concentrations from 0,2 to 5 µg/ml (Fig.16). Shh stimulation results in a slight increase in prolactin secretion (about 140% increase when stimulated with Shh 5µg/ml), but this effect is not statistically significant.
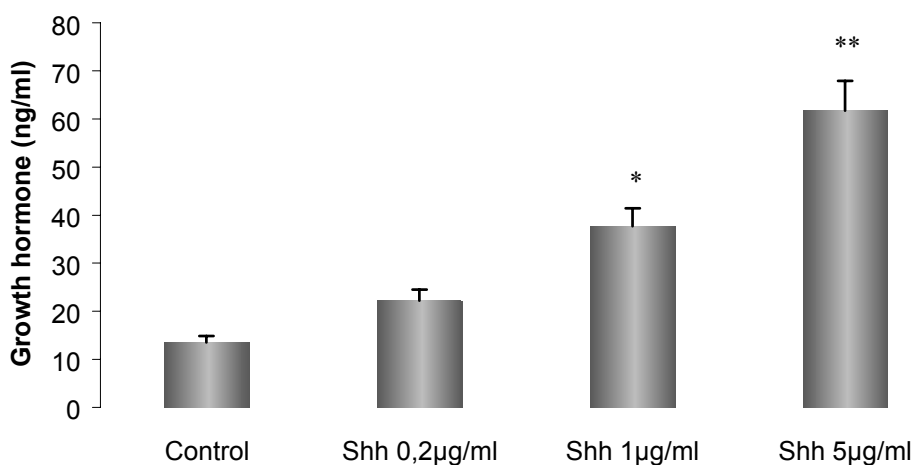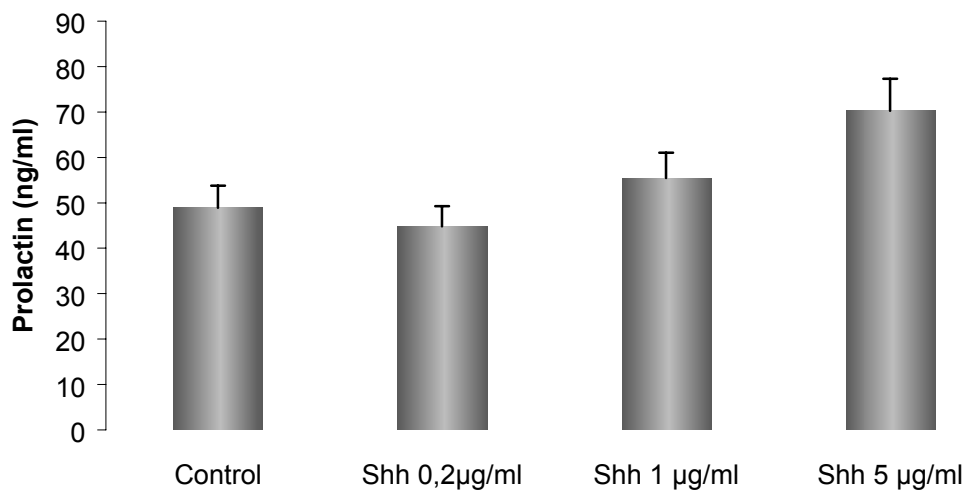

Figure 16.

Sonic hedgehog effect on Prolactin secretion in the GH3 cell line

GH3 cells were plated in 96-well plates at a concentration of 4000 cells/well and in 10% FCS medium. After 1 day in culture, the cells were serum-deprived for 24 hours and afterwards stimulated in 0% FCS medium with Shh in doses from 0,2 to 5 µg/ml. The supernatant was collected 24 hours after stimulation and Prolactin secretion was measured by RIA.

Shh stimulation results in an increase in the Prolactin secretion (about 140% increase when stimulated with Shh 5µg/ml), but this effect is not statistically significant.

In summary, cell culture stimulation experiments show a positive role of Shh in stimulating ACTH and GH secretion. Especially the ACTH secretion seems remarkably increased and this effect is synergistic with CRH and Forskolin. These results are in line with the effects observed in normal pituitary cells.

So, the Shh effect on corticotrophs seems to be more important, as it was expected by the co-localization of Shh in these cells, meaning that the Shh signal they receive is by themselves regenerated and quantitatively stronger as compared to the effects in other neighboring cells.

4.5   Sonic hedgehog and CRH cross-talk at the protein level

The results shown in 4.2 and 4.3 show a major effect of Shh as an ACTH secretagogue, as well as a synergistic effect of Shh and CRH on ACTH secretion. In order to identify the mechanisms of this synergism, the co-regulation of the Shh and CRH pathways in the AtT-20 cell line was investigated.

CRH is produced by the hypothalamus and acts as a major regulator of ACTH secretion in corticotroph cells through the CRH-R1 receptors (Muller et al, 2001). An upregulation of CRH-R1 by Shh could make the cells more sensitive to CRH and explain the synergy. At the same time, as demonstrated in sections 4.1, 4.2 and 4.3.1, corticotrophs secrete and respond to Shh, indicating an autocrine or paracrine loop. An increase of Shh protein levels by CRH could also explain the synergism on ACTH secretion.

 For this, AtT-20 cells were seeded in 6-well plates and treated for 24 hours with CRH 300 nM, Shh 5µg/ml and the combination of both. Afterwards, the protein lysate was taken and used to perform a Western Blot for both Shh and CRH-R1 (Fig. 17).
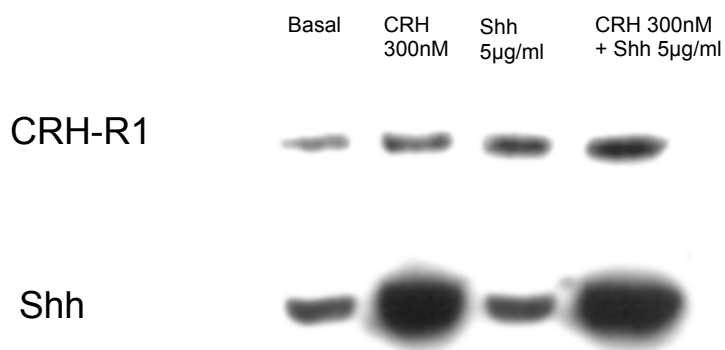
Figure 17.

Shh increases CRH-R1 protein level and CRH stimulation increases Shh protein level

300000 AtT-20 cells per well were seeded in a 6-well plate in DMEM medium containing 10% FCS. The next day, when the cells were around 70-80% confluent, they were stimulated with CRH 300nM, Shh 5µg/ml and the combination of both. A parallel control was run and the experiment was performed in triplicates. 24 hours after stimulation, the protein lysate was taken and frozen immediately in –80°C. The next day, a Western Blot experiment was run, using both anti-CRH-R1 and anti-Shh antibodies.

It results that both CRH and Shh increase basal protein levels of CRH-R1, the combination of both leads to an additive effect.

At the same time, CRH stimulation up-regulates significantly the Shh protein levels, while Shh itself induces no changes.

The above experiment gives data about a cross-talk between Shh and CRH pathways in the AtT-20 cell-line. This cross-talk may be one reason for the synergy of Shh and CRH on ACTH secretion.

4.6 Shh pathway up-regulates POMC transcriptional activity

4.6.1 Shh increases Gli1-Luc transcription
Gli1 is a target of itself in the ATT-20 cell line

It is known that ACTH secretion in corticotrophs is a consequence of the alternate processing of its precursor peptide: pro-opiomelanocortin (POMC) (White and Gibson, 1998). In order to find additional mechanisms explaining the effect of Shh on ACTH stimulation, the effect of Shh pathway on POMC transcription was studied.

For this, AtT-20 cells were transfected with a POMC-Luc reporter plasmid, expressing all the sequences necessary for the correct in-vivo POMC expression in mouse pituitary. The transfected cells were stimulated with 5 µg/ml Shh, using 10% FCS medium. A parallel negative control was run and the experiment was run in triplicate (Fig. 18).

As seen below Shh increases POMC-transcriptional activity over 80%.

Figure 18.

Shh increases POMC transcriptional activity

300 000 AtT-20 cells/well were seeded in 6-well plates. 24 hours after, the medium was replaced with transfection medium which contained the POMC-Luc plasmid and the pEGFP control plasmid (1,5µg POMC-Luc plus 0,5µg pEGFP plasmid per well, total 2µg plasmid/well) and the transfection reagent Lipofectamine (30µl/well) in serum-free Optimem medium. After 6 hours in transfection medium, the cells were put in 0% FCS medium for 18 hours. The next day, the cells were stimulated with Shh 2 µg/ml diluted in 10% FCS medium for 6 hours and the protein lysate was extracted afterwards. The luciferin and GFP fluorescein values were respectively read in a luminometer and in a fluorometer. The luciferase activity results were corrected according to the control GFP fluorescein values.

It is known that Shh effects are mediated by the activation of the transcription factor Gli1. To check whether this mechanism is also present in the AtT-20 cell line, AtT-20 cells were transfected with the Gli1 reporter plasmid (Gli1-Luc) or the mutant Gli1 reporter plasmid (mutGli1-Luc) and stimulated with Shh (Fig. 19). The pEGFP plasmid encoding a variant of the green fluorescent protein was used as a parallel control. All conditions were run in triplicate.

As seen, Shh increases Gli1 transcriptional activity about 50% in the AtT-20 cell line. This experiment verifies that Gli1 is also activated by Shh in ATT-20 cells.

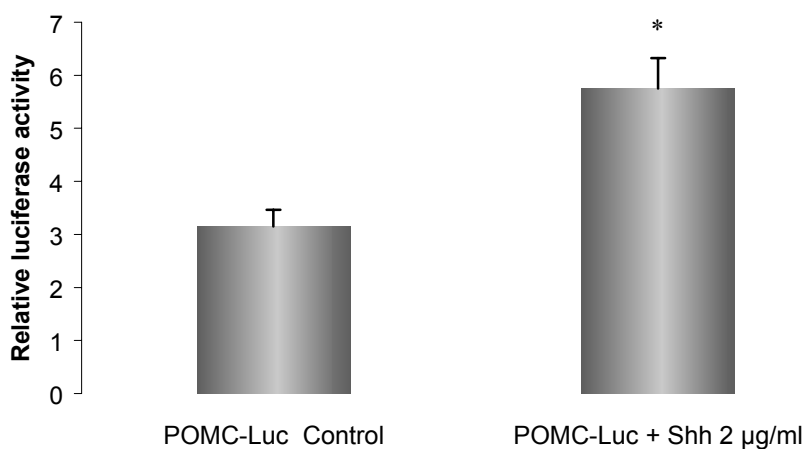| Gli1-Luc | + | + | - | - |
| mutGli1-Luc | - | - | + | + |
| Shh 5µg/ml | - | + | - | + |
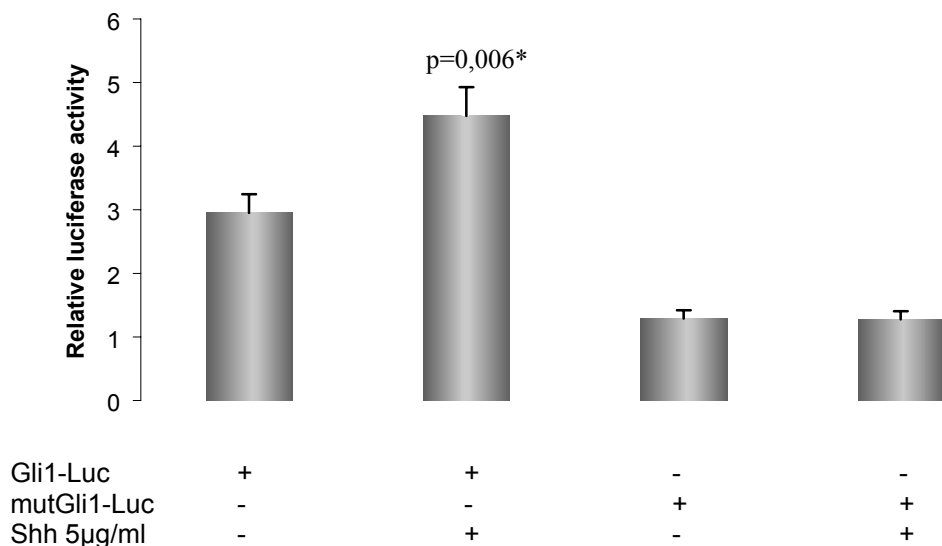
Figure 19.

Shh increases Gli1 transcriptional activity

300 000 AtT-20 cells/well were seeded in 6-well plates. 24 hours after, the medium was replaced with transfection medium which contained the plasmids shown above (total 2µg plasmid per well) and the transfection reagent Lipofectamine (30µl/well) in serum-free Optimem medium. After 6 hours in transfection medium, the cells were put in 0% FCS medium for 18 hours. The next day, the cells were stimulated with Shh 5µg/ml for 6 hours and the protein lysate was extracted afterwards. The luciferin and GFP fluorescein values were respectively read in a luminometer and in a fluorometer. The luciferase activity results were corrected according to the control GFP fluorescein values.

It is known that Gli1 translocates to the nucleus and increases the expression of target genes, including itself. We checked, whether Gli1 stimulates its own expression in the AtT-20 cell line. AtT-20 cells were transfected with either the Gli1 reporter plasmid (Gli1-Luc) or the mutant Gli1 reporter plasmid (mutGli1-Luc) and either the Gli1 expression plasmid (mGli1) or the respective control plasmid (pcDNA). All experiments were run in triplicates. The results are presented in Figure 20.

As seen in the graph, Gli1 over-expression increases the values of the Gli1 reporter plasmid above 100 fold. The fact that Gli1 increases its own transcription, means that the Shh stimulation on Gli1 gets amplified over 100 times, in order to insure a greater effect on target genes.

|				
Gli1-Luc	+	+	-	-
mutGli1-Luc	-	-	+	+
mGli1	-	+	-	+
pcDNA	+	-	+	-
pEGFP	+	+	+	+
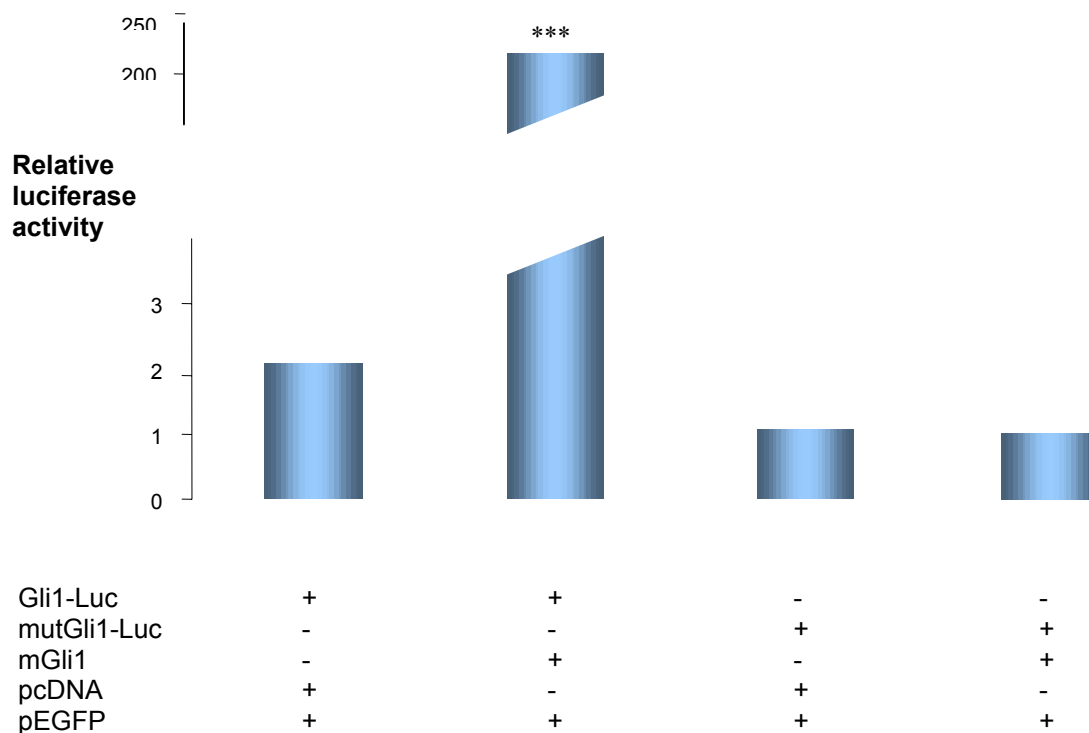
Figure 20.

mGli1 increases Gli1-Luc transcription

300 000 AtT-20 cells/well were seeded in 6-well plates. 24 hours after, the medium was replaced with transfection medium which contained the plasmids shown above (total 2µg plasmid per well) and the transfection reagent Lipofectamine in antibiotic-free Optimem medium. After 6 hours in transfection medium, the cells were put in 0% FCS medium. The protein lysate was taken after 24 hours. The luciferin and GFP fluorescein values were respectively read in a luminometer and in a fluorometer. The luciferase activity results were corrected according to the control GFP fluorescein values.

4.6.2. Gli1 increases POMC promoter transcription

In this work has been shown so far that Shh stimulates both Gli1 transcriptional activity and POMC transcription. In order to further elucidate the mechanisms involved in the strong ACTH stimulating effects of Sonic hedgehog in the AtT-20 cell line and corticotroph cells, the effect of Gli1 on POMC transcription was studied.

For this, AtT-20 cells were transfected with the POMC reporter plasmid (containing POMC-promoter linked to the luciferase gene) and either the Gli1 expression plasmid (mGli1) or the respective control plasmid (pcDNA). The pEGFP plasmid was used as a parallel transfection efficiency control. All experiments were run in triplicates (Fig. 21).

Gli1 over-expression increases the POMC-Luc values about 6 times compared to the control. These results reveal that a new transcription factor : Gli1 can activate POMC. This transcription factor mediates the Shh up-regulating effects on POMC transcription and consequently on ACTH secretion.

Figure 21.

mGli1 increases POMC transcriptional actvity

300 000 AtT-20 cells/well were seeded in 6-well plates and transfected according to a protocol similar to the one explained in Fig.18. The transfection medium contained the plasmids shown above (total 2µg plasmid per well) and the transfection reagent Lipofectamine in antibiotic-free Optimem medium. The cells were left 6 hours in Optimem, afterwards 42 hours in 0% FCS medium and the protein lysate was taken 48 hours after the start of the experiment. The luciferase activity results were corrected according to the GFP control fluorescein values.

4.7 Sonic hedgehog and CRH pathway cross-talk at the transcription level

4.7.1 Gli1 is upregulated by CRH and cAMP

The Gli1-induced increase of POMC transcription (4.6.2), the well known CRH increase of POMC transcription (63), the synergism of Shh and CRH on ACTH secretion (4.2 and 4.3.2) and the cross-talk between Shh and CRH pathways at a protein level (4.5), prompted us to study a possible cross-talk of these two pathways on the regulation of POMC transcription.

To start with, the effect of CRH on Gli1 gene transcription was studied . AtT-20 cells were plated in 6 well-plates, transfected with the Gli1 reporter plasmid and then stimulated with CRH 300nM (Fig. 22). As seen, CRH increases Gli1-Luc transcriptional activity about 2,6 times.
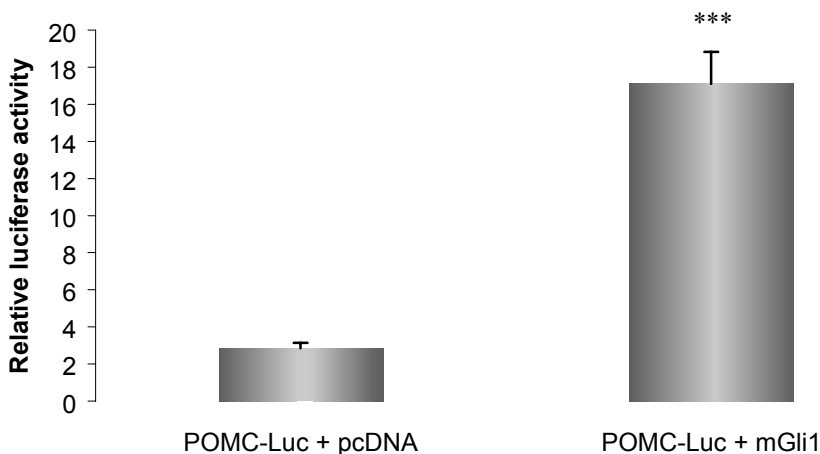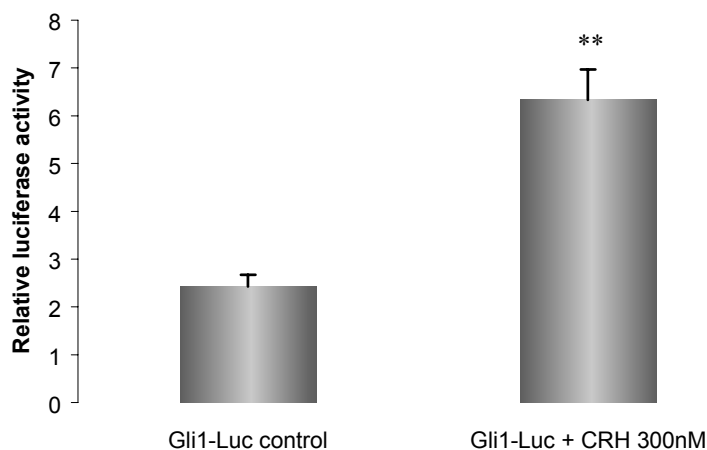

Figure 22.

CRH increases Gli1 transcriptional activity

300 000 AtT-20 cells/well were seeded in 6-well plates and transfected according to a protocol similar to the one explained in Fig.18. The transfection medium contained the Gli1 reporter plasmid (1,5µg/well), the pEGFP control plasmid (0,5µg/well) and the transfection reagent Lipofectamine (30µl/well) in antibiotic-free Optimem medium. The cells were left 6 hours in Optimem, afterwards put in 0% FCS medium for 18 hours and then stimulated with 300nM CRH. Three wells were used for each specific condition. After 6 hours in stimulation medium, the protein lysate was obtained and the luciferin and GFP fluorescein results were read. The luciferase activity results were corrected according to the respective GFP fluorescein values.

Cyclic AMP (cAMP) is a crucial mediator in both CRH and Shh pathways. It has a well-known strong effect on POMC transcription activated by the CRH pathway. At the same time, is a important regulator of the Shh pathway, known to exert positive or negative effects in mammalian cells (see 1.2).

The effect of cAMP on Gli1 transcriptional activity was studied in order to further elucidate a possible cross-talk between Shh and CRH pathways on POMC regulation. AtT-20 cells were plated in 6 well-plates, transfected with the Gli1 reporter plasmid and then stimulated with cAMP (Fig. 23/A). The effect of cAMP on POMC was tested in parallel in a similar experiment (Fig. 23/B).

As seen in the graph, cAMP stimulation increases Gli1 transcriptional activity about 3 times. The parallel increase of POMC transcription is shown as a positive control of the cAMP effects.

A.

B.
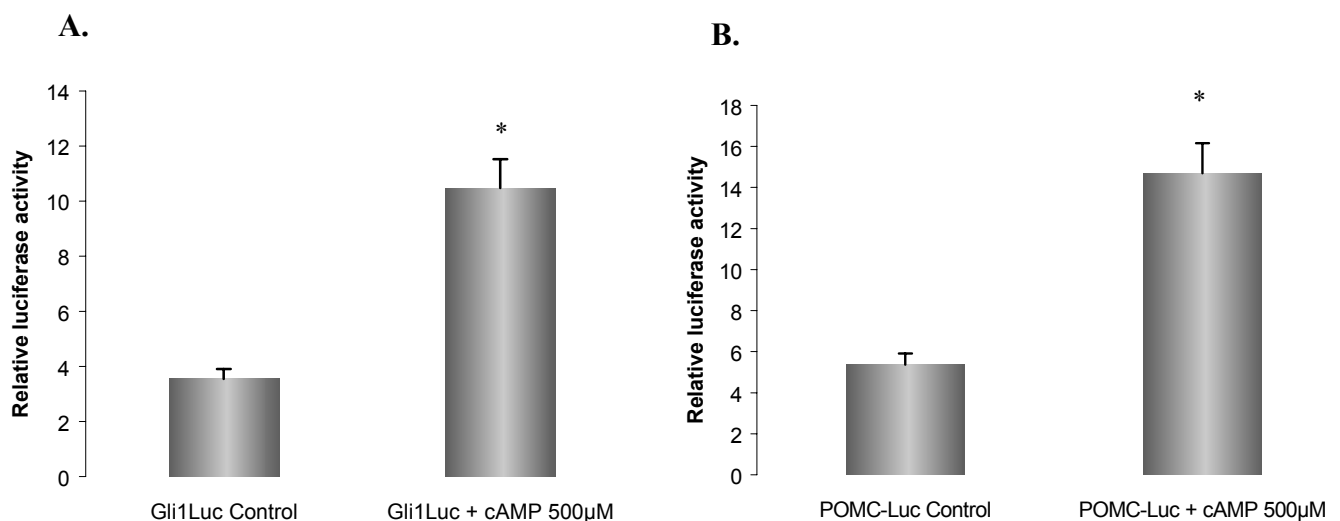
Figure 23.

cAMP increases Gli-Luc (A) and POMC-Luc(B) transcriptional activity

A. _The experiment was performed identically as the one explained in Fig. 22. The AtT-20 transfected cells were stimulated with 500µM cAMP._

B. _The experiment is identical to the one explained in Fig. 23/A. The AtT-20 cells were transfected for 6 hours in Optimem medium containing the POMC reporter plasmid (1,5µg/well), the pEGFP control plasmid (0,5Mg/well) and the transfection reagent Lipofectamine (30µl/well). Afterwards, they were put in 0% FCS medium for 18 hours and then stimulated with 500µM cAMP for 6 hours._

4.7.2 Gli1 increases AP-1 and Cre transcriptional activity

This part of the study aims to further elucidate the mechanisms of Shh / Gli1 stimulation of POMC and the cross-talk between Shh and CRH pathways. The effect of Gli1 on two transcription factors known to be upstream and up-regulate POMC: CREB and AP1 (see 1.1.2) was studied.

For this AtT-20 cells were transfected with either the Gli1 expression plasmid or the respective control and either the Cre reporter plasmid or the AP-1 reporter plasmid. The experiment contained triplicates for each specific condition and the pEGFP control plasmid was co-transfected in all the wells (Fig. 24).

As seen, mGli1 increases both AP-1 and Cre transcriptional activity in a statistically significant way (respectively, $p<0,05$ and $p=0,001$). Therefore, Gli1 can act upstream of AP-1 and CREB.
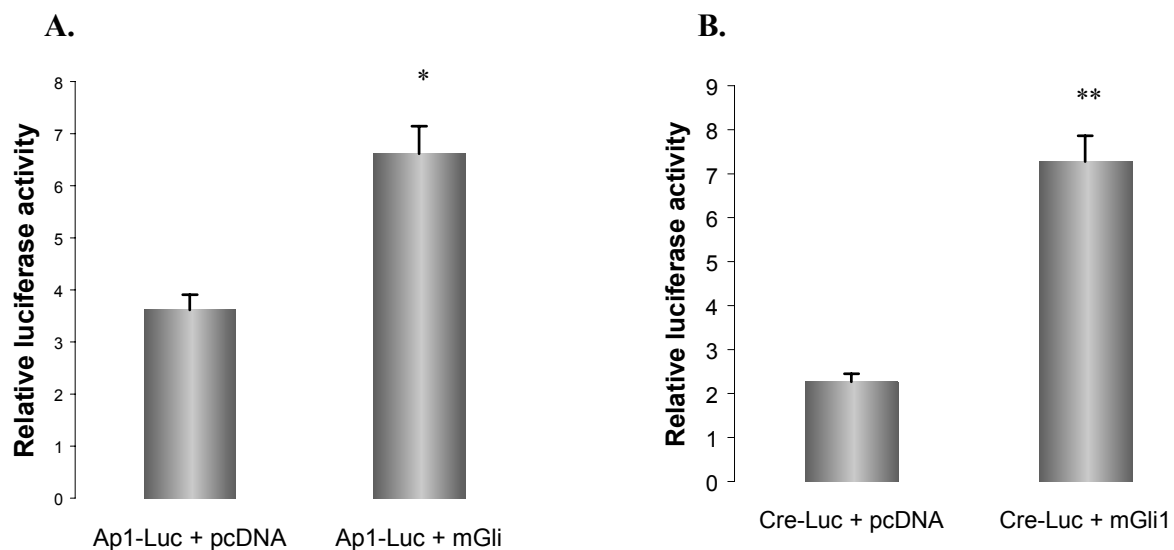
Figure 24.

A. mGli1 increases AP1 transcriptional activity

The experiment was performed using an identical protocol as the one explained in Fig. 18. The cells were transfected with AP1-Luc, mGli1 or pcDNA and the control plasmid pEGFP. The protein lysate was taken 48 hours after the start of the experiment and the luciferase activity values were corrected according to the relevant GFP fluorescence values.

B. mGli1 increases Cre-Luc transcriptional activity

The experiment was performed identically as the one explained in Fig. 16A. The cells were transfected with Cre-Luc, mGli1 or pcDNA and the control plasmid pEGFP and the luciferase activity values were corrected according to the relevant GFP fluorescence values.

4.8 Sonic hedgehog pathway members are downregulated in pituitary tumors

The experiments presented in 4.1 - 4.7 elucidate an important role for Shh pathway in normal pituitary. Previous work done in development has shown that Shh plays a role in the differentiation and growth of Rathke's pouch (2). Therefore, it was interesting to study the members of the Shh pathway in different human pituitary tumors.

For this, an extensive screening of 56 tumors was performed. At the beginning, each tumor was screened for the hormone content analysis (single immunohistochemistry for detecting the expression of pituitary hormones at a protein level). This determined the cell type each tumor possessed. Tumors that contained many different cell types, indicating that part of normal pituitary is resected adjacent to the tumor tissue, were excluded from this study.

The screening of tumors for Shh, Ptc1 and Gli1 was performed by Immunohistochemistry. The 55 pituitary adenomas included in the study were classified into acromegaly-associated pituitary tumors (ACRO; 7 cases), corticotropinomas (CUSH; 13 cases), prolactinomas (PROL; 3 cases), TSH-secreting adenomas (TSH; 2 cases) and clinically nonfunctioning adenomas (NFPA; 30 cases); the later was divided after immunopathological examination into gonadotropinomas (17 cases), null cell adenomas (11 cases), 1 silent corticotroph adenoma and 1 silent GH/Prl secreting tumor. Each IHC experiment was run with one primary antigen only and included in parallel 15 tumor slides and one normal pituitary slide used as control. The IHC results were evaluated by two different persons and then compared.

The intensity of the staining was classified in three different grades: 0, 1 and 2.
0 stands for no immunoreactivity, 1 stands for less than 1% of pituitary cells positive and 2 means more than 1% of pituitary cells positive. An extra classification was added for immunoreactivity in the connective tissue. It was marked with x and, if present, added to the main classification grade. This classification was decided by the fact that the immunoreactivity for each of the three proteins studied, especially Shh and Gli1 was very low compared to normal pituitary.

Details of each tumor, including patient data, clinical diagnosis and grade, hormone IHC characteristics and all immunoreactivity results for Shh, Ptc1 and Gli1 are given in Tab. 4.

Patient number	Age	Sex	Clinical Diagnosis	Hormone IHC Characteristics	Grade	Shh ir	Ptc1 ir	Gli1 ir
1	31	M	ACRO	GH	II	0	1	0
2	28	F	ACRO	GH	II	0	1 + x	0
3	51	F	ACRO	GH	III	0	1	0
4	24	M	ACRO	GH	II	2	2	2
5	67	M	ACRO	GH	III	0	1	0
6	50	M	ACRO	GH / Prl	II	0	0	0
7	69	M	ACRO / PROL	GH / Prl	III	0	0 + x	0
8	33	M	CUSH	ACTH	II	0	0	0
9	32	F	CUSH	ACTH	II	0	1	0
10	18	F	CUSH	ACTH	II	0	0	0
11	36	F	CUSH	ACTH	III	0	0	0
12	45	F	CUSH	ACTH	III	0	0	0
13	14	M	CUSH	ACTH	III	0	0	0
14	40	F	CUSH	ACTH	III	0	0	0
15	31	M	CUSH	ACTH	II	0	0	0
16	52	F	CUSH	ACTH	II	0	0	0
17	59	M	CUSH	ACTH	III	0	0	0
18	63	F	CUSH	ACTH	III	0	0 + x	0
19	20	F	CUSH	ACTH	II	0	1	0
20	47	F	CUSH	ACTH	II	0	1 + x	0
21	50	F	NFPA	ACTH	III	0	0 + x	0 + x
22	49	M	NFPA	FSH	III	0	1	0
23	29	F	NFPA	FSH	III	0	0 + x	0
24	64	M	NFPA	FSH	III	0	0	0
25	52	F	NFPA	FSH	III	0	1	0
26	76	M	NFPA	FSH	III	0 + x	0 + x	0 + x
27	34	M	NFPA	FSH	II	0	0 + x	1
28	61	F	NFPA	FSH	III	0	0 + x	0
29	35	M	NFPA	FSH	II	0	0	0
30	52	M	NFPA	FSH	III	0	1	0
31	64	M	NFPA	FSH	III	0	0	0
32	36	F	NFPA	FSH / LH	II	0	2	0
33	42	M	NFPA	FSH / LH	III	0	0 + x	0 + x
34	55	M	NFPA	FSH / LH	II	0	0 + x	0
35	28	F	NFPA	GH / Prl	II	1	1	0
36	28	M	NFPA	LH	II	0	1	0
37	53	M	NFPA	LH	III	0	1	0
38	59	M	NFPA	LH	II	0	1	0
39	60	M	NFPA	LH	III	0	2	0
40	51	F	NFPA	None	III	0	0	0
41	49	M	NFPA	None	II	0	1	0
42	68	M	NFPA	None	III	0	1	0
43	59	F	NFPA	None	III	0 + x	1	0
44	54	M	NFPA	None	III	0 + x	1	0
45	47	F	NFPA	None	III	0	2	x
46	47	F	NFPA	None	II	1 + x	1 + x	0 + x
47	57	M	NFPA	None	II	0	1	x
48	61	M	NFPA	None	III	0 + x	0 + x	0
49	52	F	NFPA	None	II	0	2	0
50	52	F	NFPA	None	II	0	0 + x	0
51	18	F	PROL	Prl	II	0	0	0
52	26	F	PROL	Prl	II	1	0	0
53	41	M	PROL	Prl / GH	II	0	0	0
54	56	M	TSH	TSH / α-subunit	II	2	2	1 + x
55	31	F	TSH / ACRO	TSH / α-subunit	III	0	0	0

Table 4.

Characteristics of 55 tumors used in the study and their IHC results to Shh, Ptc1 and Gli1.
NFPA = non functioning pituitary adenoma, ACRO = acromegaly, PROL = prolactinoma,
TSH = TSH-oma, ir = immunoreactivity, 0 = no immunoreactivity, 1= <1% of cells positive,
2 = >1% of cells positive, x = immunoreactivity in the fibres.

Patient details, clinical diagnosis and tumor grade were obtained from the neurosurgical departments where the tumor resection was performed. Hormone expression analysis and Shh, Ptc1 and Gli1 immunoreactivity were performed in our department.

The IHC staining of different tumors for Shh, Ptc1 and Gli1 compared to the normal pituitary as control, are shown respectively in Figures 25, 26 and 27.

NP   normal pituitary
ACRO   acromegaly
CUSH   cushing
NFPA   non-funtioning
           pituitary adenoma
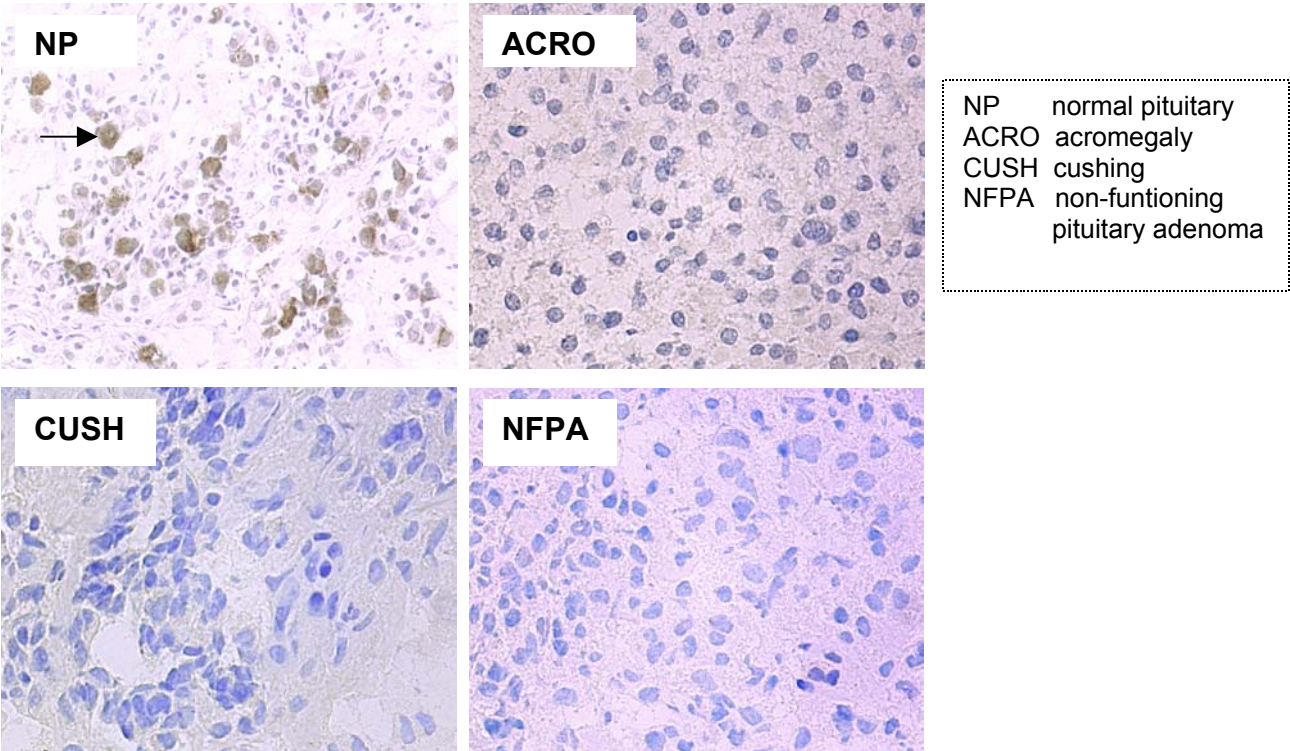
Figure 25.

Shh protein expression in the anterior pituitary gland and in different pituitary adenomas, detected by immunohistochemistry.

Shh expression stains the cell cytoplasm brown and is marked with an arrow. The normal pituitary picture is shown in x20 magnification and the pituitary adenoma pictures are shown in x40 magnification. A marked reduction of Shh staining is seen in pituitary adenomas as compared to the normal pituitary.
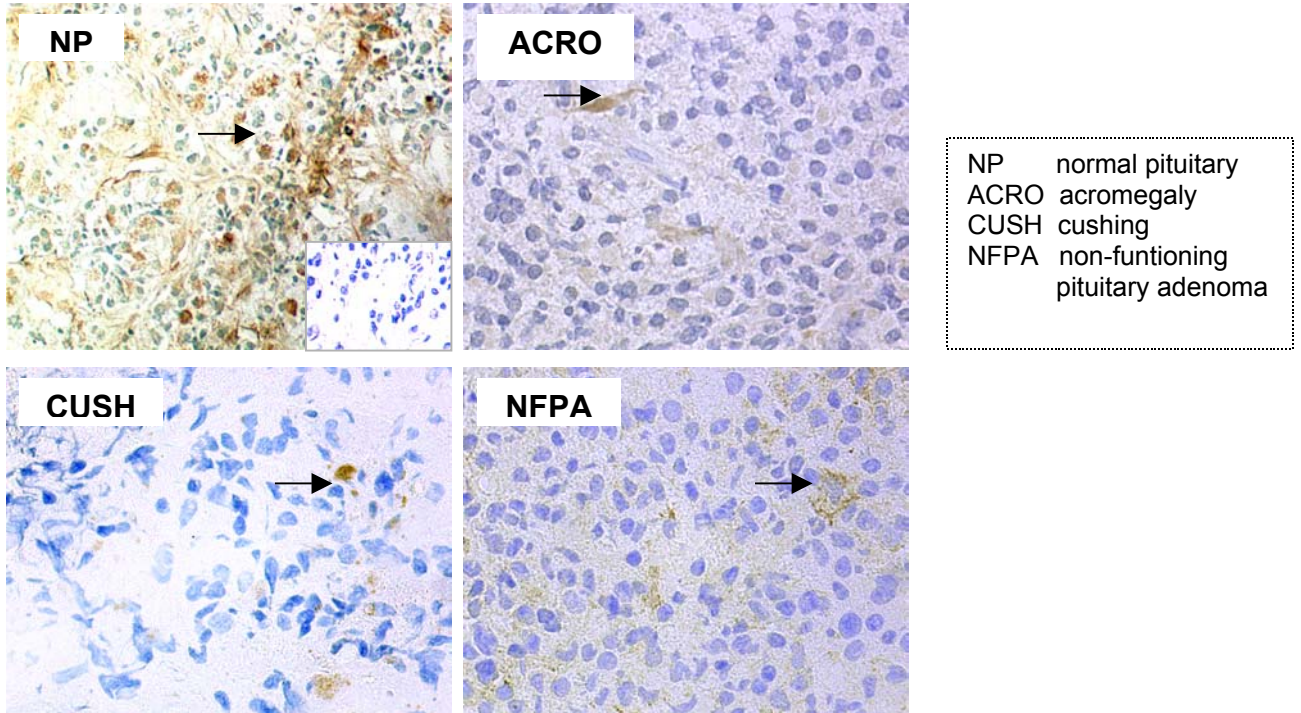
Figure 26. *Ptc1 staining in different pituitary adenomas compared to the normal pituitary as control*
Ptc1 protein (brown) is marked with an arrow. The normal pituitary picture is shown in x20 magnification and the pituitary adenoma pictures are shown in x40 magnification. A reduction in Ptc1 expression as compared to normal pituitary is noticeable in all pituitary adenomas.

Figure 27. *Gli1 staining in different pituitary adenomas compared to the normal pituitary as control*
Gli1 protein (brown) is marked with an arrow. The normal pituitary picture is shown in x20 magnification and the pituitary adenoma pictures are shown in x40 magnification. A significant reduction of Gli1 expression as compared to normal pituitary is noticeable in all pituitary adenomas.

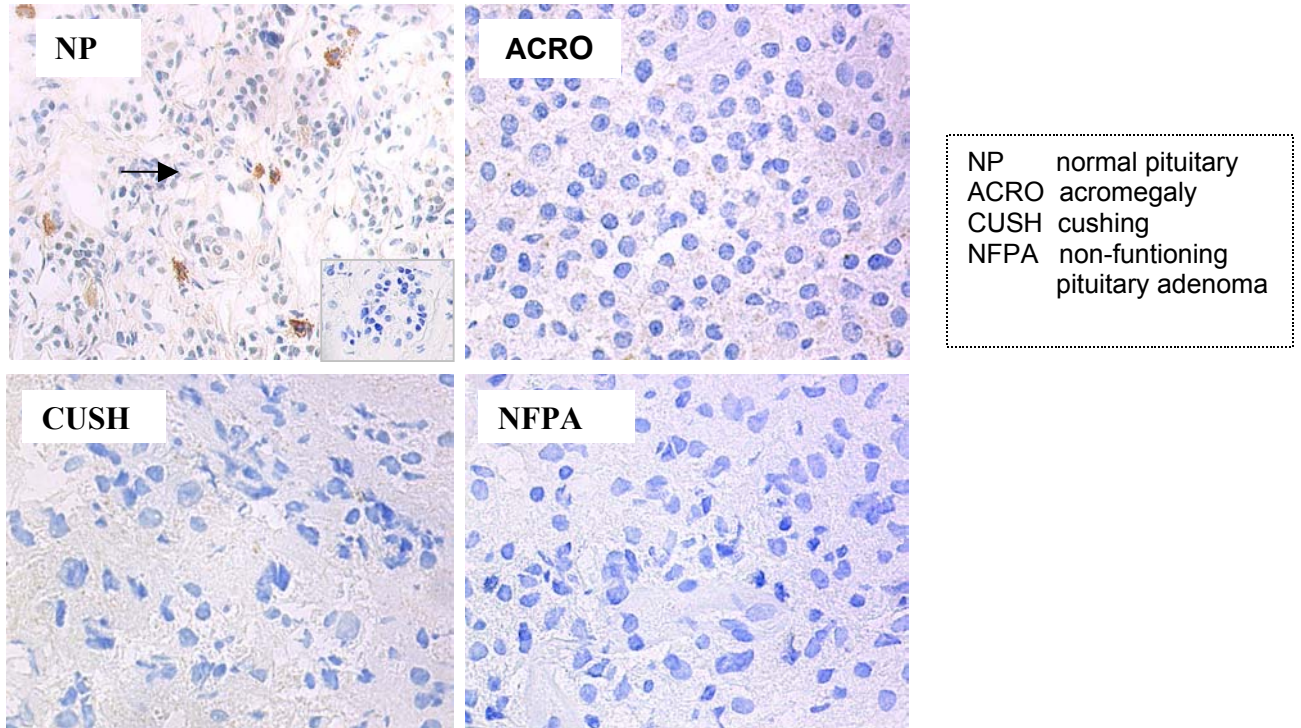The immunohistochemistry results shown above present a marked reduction of Shh and Gli1 in pituitary tumors as compared to the normal pituitary, meaning a downregulation of the Shh pathway in pituitary tumors.

Another table containing the Shh, Ptc1 and Gli1 expression in hormone-secreting cells of different tumors, summarizes the evaluation of the IHC results presented in Tab. 3. These results are given in percentages of tumors having different intensities of expression (Tab. 5).

	Sonic hedgehog grade			Patched-1 grade			Gli-1 grade		
	0	1	2	0	1	2	0	1	2
Cushing (%)	100	0	0	77	23	0	100	0	0
Acromegaly (%)	88	0	12	38	50	12	88	0	12
Non-functioning tumor (%)	93	7	0	43	43	13	97	3	0
Prolactinoma (%)	80	20	0	100	0	0	100	0	0
TSH-secreting tumor (%)	50	0	50	50	0	50	50	50	0

Table 5.

Shh, Ptc1 and Gli1 expression in pituitary adenomas.

NFPA = non functioning pituitary adenoma, ACRO = acromegaly, PROL = prolactinoma, TSH = TSH-oma, ir = immunoreactivity, 0 = no immunoreactivity, 1= <1% of cells positive, 2 = >1% of cells positive, x = immunoreactivity in the fibres.

The table presents the percentage of Cushing, Acromegaly, Prolacting secreeting, TSH secreeting and non-functioning tumors that contain IHC staining for Shh, Ptc1 and Gli1 in different levels (nothing, less than1% or more than 1%).

No Shh and Gli1 expression was found in 100% of Cushing tumors studied, indicating a downregulation of the Shh pathway.

All results presented in 4.8 show a reduction of Shh pathway members (especially Shh and Gli1) in pituitary adenomas. Given the fact that corticotrophs normally produce Shh themselves, the disappearance of Shh in all Cushing tumors studied is certainly to be considered further.

4.9 Sonic hedgehog effect on cell proliferation in the AtT-20 and GH3 cell lines

The obvious reduction of Shh and Gli1 expression in all pituitary adenomas as compared to the normal pituitary suggests that Shh plays a role in pituitary adenoma development. Therefore, the effect of Shh on cell proliferation in the corticotroph pituitary cell line AtT-20 and in the mamosommatotroph pituitary cell line GH3 was studied.

4.9.1 Shh reduces cell proliferation in the AtT-20 cell line

AtT-20 cells were seeded in 96-well plates, serum deprived and stimulated every 48 hours with Shh in doses from 0,5 to 5 µg/ml. 96 hours after the start of stimulation, the cell number in each well was measured by the WST-1 method (Fig. 28).

It results that Shh decreases cell proliferation, up to 40% and this result is statistically significant.
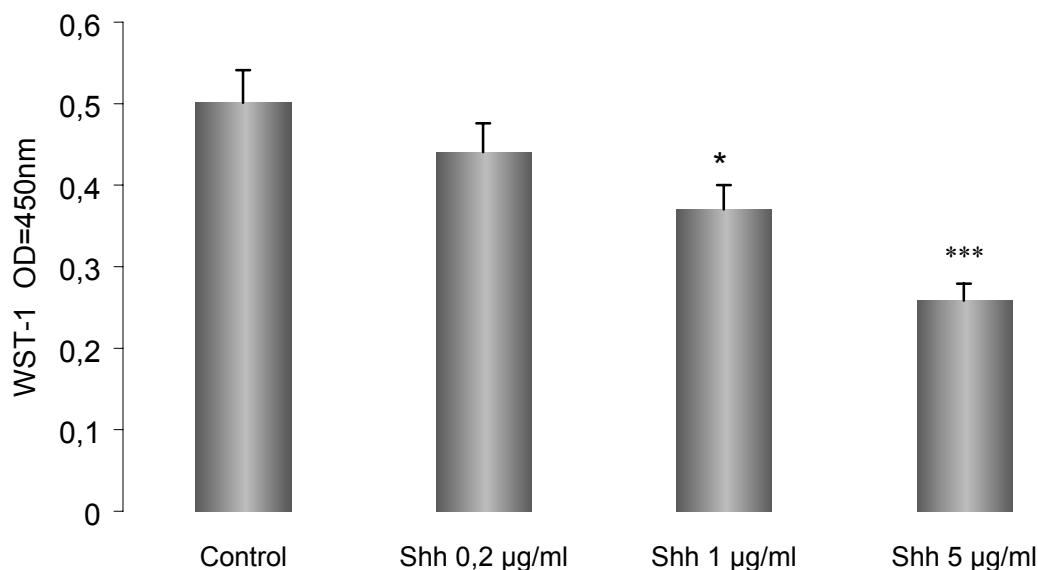
Figure 28.

Shh reduces AtT-20 cell proliferation

AtT-20 cells were seeded in 96-well plates, putting 2000 cells per well in 10% FCS medium. The next day, they were serum deprived for 24 hours and afterwards stimulated with different doses of Shh diluted in stimulation medium containing 2% FCS. The experiment was run in quadruplicates, using as stimulation control cells in medium only and medium only without cells. Due to the potential degradation at 37°C, the stimulant was added after 48 hours. After 4 days in stimulation medium, WST-1 was added and 45 minutes afterwards, the results were measured in the Elisa reader, using a wave-length of 450 nm. The OD values were corrected after substracting the values of medium without cells.

To test the effect of cyclopamine on this Shh induced decrease of AtT-20 cell proliferation, we performed an experiment identical to the one explained in Fig. 24, but stimulating with the maximal dose of Shh and the combination of Shh plus Cyclopamine (maximal non toxic dose as explained in 4.3.1 is 5µM). The results are presented in Fig. 29.

Cyclopamine at 5µM partially reverses the Shh induced decrease in AtT-20 cell proliferation and this result is statistically significant. The use of higher doses of Cyclopamine was not possible because of cytotoxicity in the AtT-20 cell line.
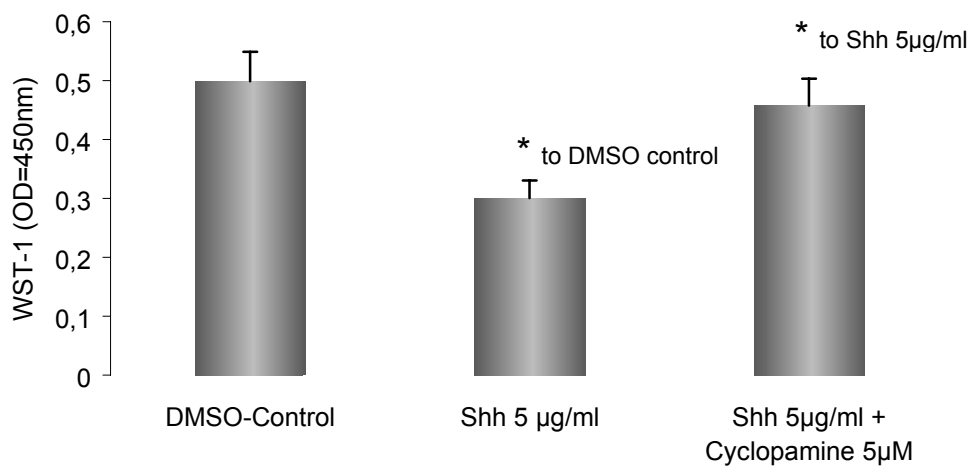
Figure 29.

Cyclopamine reverses the Shh induced decrease in AtT-20 cell proliferation

The experiment was performed as the one explained in Fig. 27. The only difference consists in adding to the control and Shh only containing wells the same dose of DMSO present in the Cyclopamine treated wells. 96 hours after the start of the stimulation, WST-1 was added to each well and 45 minutes after the results were read in the Elisa reader.

4.9.2 Shh has no impact on GH3 cell proliferation

For studying the effect of Shh on GH3 cell proliferation, GH3 cells were seeded in 96-well plates, serum deprived for 24 hours and then stimulated with Shh in doses from 0,2 to 5 µg/ml, cyclopamine 5µM (maximal non-toxic dose for GH3 cells as well, identified in the same way as explained in 4.3.1 for ATT-20 cells) and the combination of maximal doses of Shh and Cyclopamine (Fig. 30).

As seen in the graph, Shh at different doses, Cyclopamine in the maximal non-toxic dose and the combination of both have no statistically significant effect on GH3 cell proliferation.
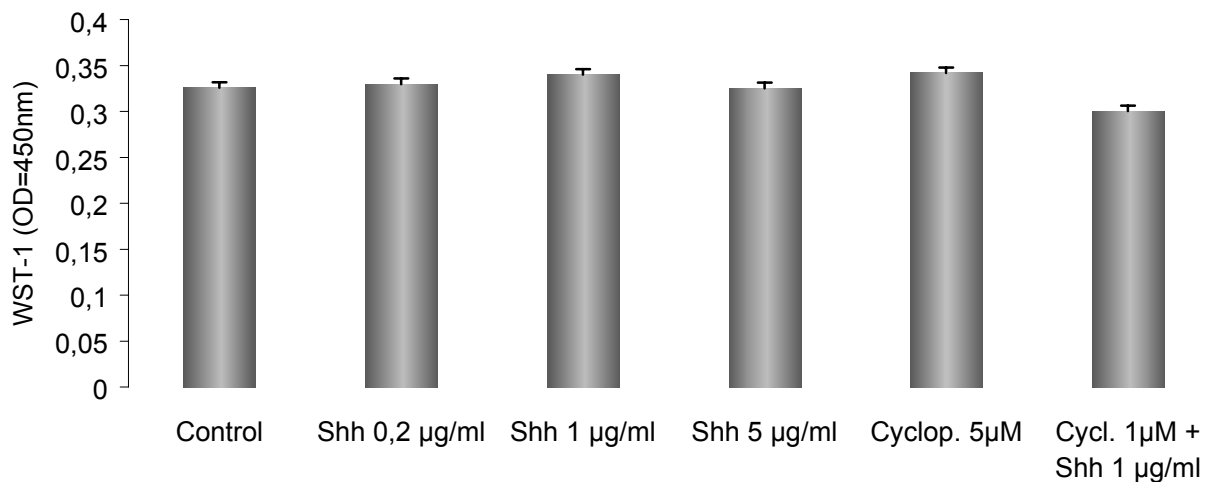
Figure 30.

Shh has no effect on GH3 cell proliferation

GH3 cells were seeded in 96-well plates, putting 2000 cells per well in 10% FCS medium. The next day, they were serum deprived for 24 hours and afterwards stimulated with different doses of Shh, Cyclopamine and the combinaiton of Shh and cyclopamine, diluted in stimulation medium containing 2% FCS. The experiment was run in quadruplicates, using as stimulation control cells in medium only and medium only without cells. Due to the degradation at 37°C, the stimulants were added after 48 hours. After 4 days in stimulation medium, WST-1 was added and 60 minutes afterwards, the results were measured in the Elisa reader, using a wave-length of 450 nm. The OD values were corrected after substracting the values of medium without cells.

5.   Discussion

Advances in defining the biological cascades behind pituitary gland development have shed light on the importance of the cross-talk between signaling molecules and differentiating factors as well as on cell-to-cell communication (reviewed in 4). Recent studies have revealed that differentiating factors needed for normal pituitary development like retinoic acid and BMP4 continue to have a very important role in pituitary physiology and tumorigenesis (18, 19). The present thesis demonstrates that another differentiating factor: Shh, also plays an important role in the cross-talk mechanisms that regulate the adult pituitary.

In the last decades, it has been shown that the pituitary gland, in addition to the classical hormones, also produces numerous polypeptide growth factors, cytokines, and neuropeptides. Expression of the corresponding receptors on pituitary cells enables these factors to influence growth and function of the pituitary by auto- or paracrine mechanisms. This evidence reveals that the intrinsic intercellular communication network seems to be involved in the control of pituitary homeostasis, in addition to the classical endocrine feed-back regulation (reviewed in 64 and 65). Auto-/paracrine mechanisms have an impact on the normal pituitary function and their disturbance may also play a crucial role in pituitary tumor progression.

Shh is absolutely required for early stages of normal pituitary development, but its role in the pituitary has not been taken further.
This work studied the expression and function of the Shh pathway in the adult pituitary and pituitary adenomas. Shh, its receptors Ptc1 and Ptc2 and the transcription factor of the Shh pathway Gli1 are all expressed in the human pituitary. Shh is produced in the corticotrophs, while each of other types of pituitary hormone secreting cells possesses one of the receptors and the transcription factor Gli1. At the same time, acting as an auto- or paracrine factor, Shh induces ACTH and GH hormone production. Especially the effect on ACTH is quantitatively very important and seems to be induced through signal transduction mechanisms that cross-react with the CRH pathway.

At the same time, comparative expression studies reveal a remarkable reduction of Shh signaling in pituitary tumors. Although present in normal corticotrophs, Shh is found in none of Cushing tumors studied. The absence of the Gli1 transcription factor also emphasizes that there is no Shh signaling.

In ACTH producing cell line models, Shh reduces cell proliferation by 50%. Therefore, Shh helps in maintaining corticotrophs in a non-proliferative state. In the absence of Shh, corticotrophs proliferate more and this results in ACTH-producing adenoma formation.

Expression of Shh pathway members in the pituitary gland

Shh is expressed in the adenohypophysis at the protein level. Double-staining methods reveal that it is localized in the pituitary corticotrophs. Being a secreted protein, Shh is able to diffuse and induce effects in other neighboring cells. So, the protein produced in corticotrophs, is theoretically available for other cells, where it may also have important functions.

The finding of both Patched receptors (Ptc1 and Ptc2) and of the transcription factor Gli1 in the pituitary gland, helps to define a hypothesis for the role of this pathway. Ptc1 is co-localized in gonadotrophs and in thyrotrophs, while Ptc2 is co-localized in corticotrophs, somatotrophs and to a lesser degree in lactotrophs. The Ptc1 and Ptc2 receptors keep also in the pituitary gland the general rule of not overlapping with each other: they are expressed in different cell types. The transcription factor Gli1 was also found in all types of hormone-producing pituitary cells. Our findings suggest an active Shh pathway in the adenohypophysis. It is clear that Shh coming from corticotrophs, binds to receptors Ptc1 and Ptc2 and induces effects in all types of hormone-secreting cells. Therefore, the Shh pathway could contribute to co-ordinate the regulation of different cell types in the pituitary gland.

Corticotrophs also possess the Patched-2 receptor and the transcription factor Gli1. Patched-2 is known to be co-expressed with Shh also in other tissues (31). The above results show that corticotrophs produce Shh and also respond to Shh at the same time. Apparently, Shh acts in an autocrine way to maintain an active signal transduction pathway in ACTH-secreting cells.

Corticotrophs originate directly from one branch of Rathke's pouch stem cells in the early stages of pituitary development. Other adenohypophysis cells derive from a second branch, having more gene markers in common with each other than with the corticotrophs (66). So one may speculate that corticotrophs are needed at early stages in the pituitary gland development. They produce Shh, which becomes later through paracrine mechanisms available to other pituitary hormone-producing cells, and maintains an active Shh signaling pathway in the normal pituitary gland.

Hormone secretion from the rat pituitary cells in primary culture

It is known that normal pituitary cells do not proliferate, their main physiological activity is specific hormone secretion in response to different stimuli. Therefore, after the first evidence for an active Shh pathway in the pituitary gland, the most important question raised was whether there was an impact on hormone production.

The stimulation experiments done in the rat pituitary primary culture show that Shh increases ACTH production even more than CRH. Given the fact that CRH is the most important physiological stimulus of increasing ACTH basal secreting levels, this finding is very important and intriguing. At the same time, CRH and Shh have synergistic effects on ACTH secretion. The combination of both of them is a very potent stimulus of ACTH secretion in the pituitary primary culture.

Shh was also found to increase over 2 fold the GH secretion in the pituitary primary culture. This effect does not seem as high as the ACTH increase in corticotrophs, but it seems proportionate to the amount of Gli1 presence found in the pituitary by IHC (or to the percentage of cells containing an active Shh pathway). The transcription factor Gli1 is expressed in over 50% of corticotrophs, but not in many somatotrophs and even in less lactotrophs. Shh induces an increase in prolactin secretion, but this effect is not statistically significant. So the Shh pathway seems to be more active in the corticotroph cells.

Nevertheless, Gli1 expression in at least part of other anterior pituitary epithelial cell types, indicates a possible Shh physiological effect. Even if this would not be direct hormone secretion, there could be synergy with other stimuli, release of other

paracrine factors, etc. This is why these results seem to open other possibilities and another line of research in the challenging pituitary research field.

Shh effects on ACTH secretion in the ATT-20 cell line

The AtT-20 murine corticotroph cell line (54) is one of the most studied pituitary cell lines, that has been used since decades as a model to understand the physiology and pathology of corticotrophs action. This cell line responds to CRH and is a classical model for studying the effects of stimulants that induce ACTH secretion above basal levels. The effects of classical ACTH secretagogues in this cell line are well known: CRH increases up to two-fold ACTH secretion (67), forskolin by activating adenylate cyclase activates also the CRH pathway, while vasopressin has a little significant effect only when stimulated in the absence of serum (68).

The effect of Shh in the AtT-20 cell line is even higher than the one in the pituitary primary culture (expected, as the cell line contains 100% corticotrophs. A 4-5 times increase in ACTH secretion and also a marked synergy with CRH and Forskolin was observed. While CRH increases ACTH secretion less than 2 times, the combination Shh plus CRH induces an 11 fold increase. This is one of the most potent stimuli in increasing ACTH secretion in the AtT-20 cell line, which contains already persistently high ACTH basal levels.

ACTH increase caused by Shh is partially reversible by its specific antagonist Cyclopamine. This partial effect may be caused by the modest doses of Cyclopamine used (higher doses are toxic for the cells). Nevertheless, it is interesting that cyclopamine does not have any statistically significant effect on ACTH basal secretion. This suggests that Shh would not play a role in maintaining the basal ACTH secretion in this cell line.

Shh effects in the GH3 cell line

The mammosomatotroph GH3 cell line (55) is another accepted model for studying GH and Prolactin secretion. The effects of Shh here were quite similar to the ones in the pituitary primary culture: a more than 4-fold increase in GH production and no statistically significant effect on Prolactin secretion.

Shh increases CRH-R1 protein levels in synergy with CRH
CRH induces a major increase in Shh protein levels in the AtT-20 cell line

This work also aimed to find out the mechanism of the stimulatory effect of Shh on ACTH and its synergy with CRH.

CRH pathway becomes active in corticotrophs after the binding of CRH to its membrane receptor CRH receptor 1 (CRH-R1) (69). Shh increases CRH-R1 protein basal levels and this effect is synergic with CRH. So one may speculate that Shh is needed in corticotrophs to help in the response to CRH.

One interesting result is the finding that CRH induces a great increase in Shh protein levels. This makes Shh one of the few known target genes of CRH. There is a cross-talk between CRH and Shh pathways at the protein level. One could speculate that Shh helps to increase the response to CRH from the hypothalamus, while CRH itself is important in maintaining and increasing the Shh levels in the corticotrophs.

Knowing that corticotrophs are the only local source of Shh in the adenohypophysis, this result becomes more interesting. Knowing that Shh pathway is active and may have functions in other pituitary cells, it can be speculated that CRH may be an important factor not only for the maintenance of ACTH secretion, but also for the maintenance and coordination of other physiological parameters and functions of the anterior pituitary.

Transfection results in the AtT-20 cell-line
(Gli1 expression and reporter plasmid results)

Gli1 is the transcription factor that mediates the effects of the Shh pathway. It becomes activated in the cytoplasm and then translocates to the nucleus to induce the transcription of target genes. It is generally known that among the genes induced is also Gli1 itself. Our experiments in the AtT-20 cell line, verified that this rule is also present in the AtT-20 cell line.

Shh as well as the Gli1 expression plasmid increase Gli1 transcriptional activity. The increase caused by the Gli1 expression plasmid is over 100 fold. The conclusion is that Shh signals inducing the Shh pathway, activate Gli1, which by increasing its own transcription, induces a quantitatively much higher response of target genes. So, not much Shh is apparently required, as its signaling cascade amplifies itself. At the same time, Gli1 increases POMC promoter transcription about 6-times compared to the control.

It is known that CRH induces POMC promoter transcription mainly through the PKA pathway and the most important downstream transcription factors are Cre and AP-1. Trying to reveal possible mechanisms of Shh-CRH synergy, the effect of the CRH pathway on Gli1 transcription was studied. CRH and cAMP-CPT (the most potent stimulator of PKA) both increase the Gli1 transcriptional activity. The stimulation obtained shows that the CRH and PKA act upstream to Gli1.

At the same time was studied the effect of Gli1 on Cre and AP-1 reporter plasmids, revealing that Gli1 increases the transcription of both of them. So, Gli1 can act upstream to CREB and AP-1. However, CREB can be directly phosphorilated by PKA. Therefore, CREB and Gli1 can act in parallel.

It seems that there is a multiple cross-talk between Shh and CRH pathways at the transcriptional level. These pathways seem to regulate each other mutually at different levels, aiming in any case to obtain a well-controlled and high increase of ACTH secretion.

A proposed mechanism explaining the synergic effect of CRH and Shh on ACTH secretion is given in Fig. 31.

Figure 31.

Cross-talk between Shh and CRH pathways
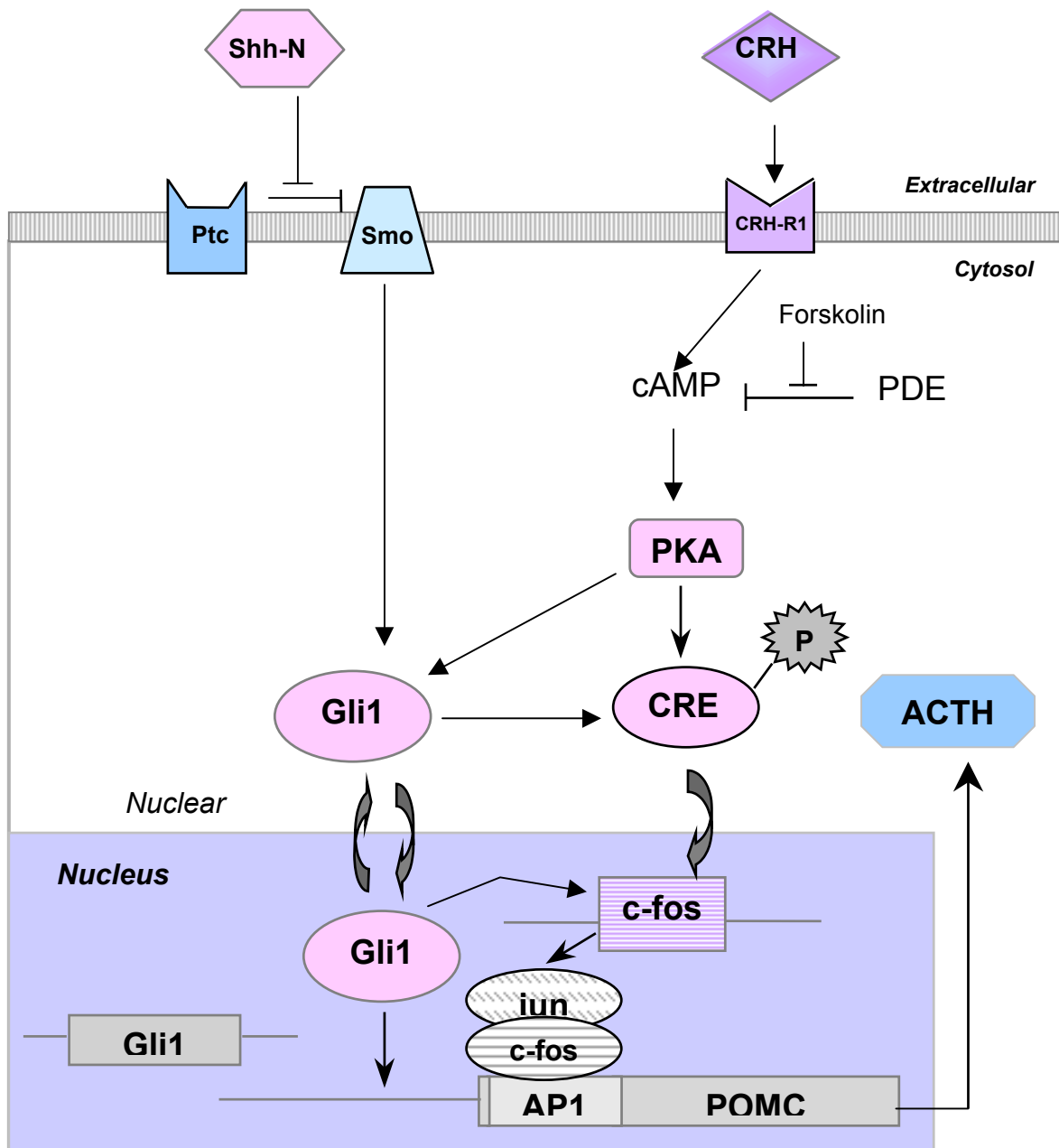
Shh binds to its receptor complex Ptc-Smo and then activates the transcription factor Gli1. CRH binds to the receptor CRH-R1 and acting through the transcription factors AP1 and CRE, increases POMC promoter transcription. The results of this thesis reveal that Gli1 is up-regulated by CRH and PKA and increases POMC promoter transcriptional activity, acting upstream to CREB and AP1.

Shh pathway is downregulated in pituitary tumors

The screening of 55 pituitary tumors for the protein levels of Shh pathway members, shows a marked reduction of Shh, Ptc1 and Gli1 in all pituitary tumors.

Ptc1 is reduced to a lesser extent. Ptc1 is a negative transducer of Shh signaling, so it may be present in the cell even when the pathway is inactive. On the other hand, Ptc is a known target of Gli1, so it makes sense to have a reduction in Ptc1 signaling when the Gli1 levels are also low or negative.

The reduced Shh and Gli1 expression in Prolactinomas, Acromegaly, Non-functioning pituitary tumors and in TSH-secreting pituitary tumors may be compatible with the fact that these tumors do not contain corticotroph cells, essential for secreting Shh. A few rare cases where Shh or Gli1 may be present, may reflect a mixture with some normal pituitary tissue during tumor removing surgery. Nevertheless, the impact of this Shh pathway downregulation in all these tumors is not known. Whether there is any effect in tumor ethiopathology, maintenance and progression, it is difficult to judge.

The loss of Shh in Cushing tumors is very striking. There is no Shh or Gli1 expression at all in all Cushing tumors studied.

Shh inhibits AtT-20 cell proliferation in culture conditions, while the effect on the pituitary primary culture is impossible to test as these cells do not proliferate.

It might well be that Shh is necessary for the maintenance of a low/non proliferation state in the corticotrophs. This is also supported by the development data: Shh must be absolutely present in the embryo, but excluded from the Rathke's pouch in the first stages of pituitary organogenesis to allow proliferation. According to our hypothesis, this may allow pituitary stem cells to proliferate and differentiate, but the Shh signaling may be needed later on to stop the proliferation and help in the hormone secreting activity, at least in corticotrophs.

The results presented here support the hypothesis that in the absence of Shh, corticotroph cells proliferate more and this could lead to adenoma formation.

6.   Summary

This work investigates for the first time the expression and the role of Sonic hedgehog signaling pathway in adult pituitary and in pituitary tumors. Shh is a signaling protein, important in regulating patterning and proliferation in the embryo and the adult. It has a crucial role in pituitary development and Shh deficient mice do not even have a rudimentary Rathke's pouch (the development structure that gives rise to anterior pituitary).

This study reveals the presence of an active Shh pathway in the post-developmental pituitary gland, with major impacts on hormone secretion and cell proliferation.

After embryonic development, Shh continues to be expressed in the normal adult pituitary, being mainly co-localized in corticotrophs. These cells express also the receptor Ptc2 and the Shh induced transcription factor Gli1, being so Shh-producing and Shh-responsive cells. Shh acts in an autocrine way inside corticotrophs, inducing a major stimulation of ACTH secretion in the normal rat pituitary and in the AtT-20 cell line.

The Shh induced ACTH secretion effect is synergistic with CRH. Shh stimulation increases CRH-R1 levels, up-regulating so the response of corticotrophs to CRH. At the same time, Gli1 is not only activated by Shh, but also by CRH and PKA. Gli1 itself activates POMC-transcription and acts in parallel upstream to CREB and AP-1. A major increase in Shh protein levels is seen as a result of CRH stimulation. All these results put in evidence a multiple cross-talk between these two important pathways acting at different levels to insure the final ACTH stimulation.

Other types of hormone-secreting adenohypophysial cells possess one of Shh receptors (Ptc1 or Ptc2) and the transcription factor Gli1, so they have an active Shh pathway. Shh produced in the corticotrophs is a signaling protein, so it diffuses and acts also in distance. Shh increases GH secretion from the rat pituitary somatotrophs and from the GH3 cell line, while the effect on Prolactin is not statistically significant.

The Sonic hedgehog pathway is downregulated in pituitary adenomas. Screening of 55 pituitary tumors reveals that they have a significantly reduced expression of Shh and Gli1. Although Shh in the normal pituitary is secreted by corticotroph cells, all

the Cushing tumors screened had no Shh expression at all. Cell culture experiments performed in the AtT-20 corticotroph cell line *in vitro* show that Shh reduces cell proliferation by 50% and this effect is partially reducible by Cyclopamine. So Shh maintains the low proliferative capacity of corticotrophs in the normal pituitary gland and its loss may be one of the factors leading to tumor progression.

It is concluded that Shh is produced in the anterior pituitary gland, is a major stimulant of ACTH and GH secretion, acts synergistically with CRH, opposes corticotroph cell proliferation and is downregulated in pituitary adenomas.

6.1   Zusammenfassung

In der vorliegenden Arbeit wurde erstmals die Expression und die Rolle des Sonic Hedgehog (Shh) Signalweges in adulten Hypophysen und Hypophysentumoren untersucht. Shh ist ein Signalprotein, das im Embryo und beim Erwachsenen wichtig für die Regulation der Zelldifferenzierung und –proliferation ist. Es ist für die Entwicklung der Hypophyse von herausragender Bedeutung, da Shh-defiziente Mäuse noch nicht einmal eine rudimentäre Rathkesche Tasche (aus der sich der Hypophysenvorderlappen entwickelt) bilden können.

Die hier präsentierte Untersuchung zeigt, daß es in der voll entwickelten Hypophyse einen aktiven Shh-Signaltransduktionsweg gibt, der von beträchtlicher Bedeutung für die Hormonsekretion und Zellproliferation ist.

Nach der Embryonalentwicklung wird Shh weiter in der normalen, erwachsenen Hypophyse exprimiert, wobei es vorwiegend in kortikotropen Zellen lokalisiert ist. Diese Zellen exprimieren auch den Shh-Rezeptor Ptc2 und den Shh-induzierten Transkriptionsfaktor Gli1 und sind somit sowohl Shh-produzierende als auch Shh-responsive Zellen. Shh wirkt auf autokrine Weise in den kortikotropen Zellen, indem es eine erhebliche Stimulation der ACTH Sekretion in der normalen Hypophyse und in der kortikotropen AtT20 Zelllinie induziert.

Shh wirkt synergistisch mit CRH auf die Induktion der ACTH Freisetzung. Die Stimulation mit Shh erhöht die Expression des CRH-Typ 1 Rezeptors (CRH-R1) und erhöht so die Empfindlichkeit kortikotroper Zellen auf CRH. Gleichzeitig wird Gli1 nicht nur durch Shh, sondern auch durch CRH und PKA erhöht. Gli1 selbst aktiviert die POMC Transkription parallel zu CREB und AP-1. Ein erheblicher Anstieg der Shh-Proteinspiegel wird nach CRH-Stimulation beobachtet. In ihrer Gesamtheit weisen diese Ergebnisse auf vielfältige Interaktonen auf unterschiedlichen Ebenen zwischen diesen zwei wichtigen Signalwegen hin, um letztlich die ACTH Stimulation zu gewährleisten.

Auch andere hormonsezernierende Hypophysenzellen besitzen einen der Shh-Rezeptoren (Ptc1 oder Ptc2) und den Transkriptionsfaktor Gli1 und haben daher ein aktives Shh-System. Shh, das in kortikotropen Zellen gebildet wird, ist ein Signalprotein, das nach Diffusion auch in einiger Entfernung in benachbarten Zellen wirksam ist. Shh steigert die GH Sekretion in somatotropen Zellen und in

laktosomatotropen GH3 Zellen, während sein Effekt auf Prolaktin statistisch nicht signifikant ist.

Der Shh-Signaltransduktionsweg ist in Hypophysenadenomen herunterreguliert. Die Untersuchung von 55 Hypophysenadenomen ergab, daß in den Tumoren die Expression von Shh und Gli1 signifikant reduziert ist. Obwohl in der normalen Hypophyse Shh von kortikotropen Zellen sezerniert wird, exprimierten alle untersuchten kortikotropen Adenome überhaupt kein Shh. Zellkulturexperimente mit AtT20 Zellen zeigten, daß Shh die Zellproliferation um 50 % reduzierte und daß dieser Effekt teilweise durch Cyclopamine, einem Inhibitor des Shh-Signaltransduktionsweges, aufgehoben werden kann. Das weist darauf hin, daß Shh für die geringe proliferative Kapazität von kortikotropen Zellen in der normalen Hypophyse verantwortlich ist, und daß sein Verlust einer der Gründe ist, die zur Progression von Hypophysentumoren führen.

Zusammenfassend kann man feststellen, daß Shh im Hypophysenvorderlappen produziert wird, daß es ein bedeutender Stimulator der ACTH und GH Sekretion ist, daß es syergistisch mit CRH wirkt, daß es der Proliferation von kortikotropen Zellen entgegenwirkt und daß es in Hypophysenadenomen herunterreguliert ist.

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