

Publications directly related to the subject of the dissertation

**THE ROLE OF NEUROPEPTIDES IN THE PHYSIOLOGY OF THE  
HUMAN SKIN**

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## Publications directly related to the subject of the dissertation

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- II. Dallos A, Kiss M, Polyánka H, Dobozy A, Kemény L, Husz S. 2006. Effects of the neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide and galanin on the production of nerve growth factor and inflammatory cytokines in cultured human keratinocytes. *Neuropeptides* (accepted for publication)
- III. Dallos Attila, Kiss Mária, Polyánka Hilda, Dobozy Attila, Kemény Lajos, Husz Sándor. 2006. Galanin receptorok azonosítása humán keratinocitákon és humán bőrben. *Bőrgyógy Vener Szle* (accepted for publication)

## Table of contents

<b>PUBLICATIONS DIRECTLY RELATED TO THE SUBJECT OF THE DISSERTATION .....</b>	<b>2</b>
<b>TABLE OF CONTENTS.....</b>	<b>3</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>5</b>
<b>1. INTRODUCTION.....</b>	<b>6</b>
1.1 Keratinocytes as cellular components of the skin immune system .....	7
1.2 Neuropeptides in the skin .....	8
1.3 Galanin as a less-known neuropeptid in the skin .....	9
1.3.1 <i>Galanin receptors</i> .....	9
1.4 The Nerve Growth Factor .....	11
<b>2. AIMS .....</b>	<b>13</b>
<b>3. MATERIALS AND METHODS.....</b>	<b>14</b>
3.1 Cell cultures.....	14
3.2 Neuropeptides treatment of the cell cultures.....	14
3.3 Reverse transcription (RT) – PCR .....	15
3.4 Quantitative reverse transcription (RT)-PCR.....	16
3.5 Immunocytochemistry and immunohistochemistry .....	17
3.6 Western blotting .....	17
3.7 ELISA technique.....	18
3.8 Measurement of intracellular $[Ca^{2+}]_i$ .....	19
3.9 Statistical analyses.....	19
<b>4. RESULTS.....</b>	<b>20</b>
<b>CHAPTER I. KERATINOCYTES CAN EXPRESS GALR2 BOTH mRNA AND PROTEIN LEVEL.....</b>	<b>20</b>
4.1 Identification of subtypes of GALR mRNAs in HaCaT cells and cultured human keratinocytes.....	20
4.2 GALR2 protein expression detected by Western blotting in human keratinocyte lysates .....	21
4.3 GALR2 is demonstrated by immunocytochemistry in HaCaT cells and cultured human keratinocytes.....	21
4.4 In situ expression of GALR2 in human skin.....	23
4.5 An increased level of intracellular $Ca^{2+}$ , $[Ca^{2+}]_i$ , was demonstrated in GAL-treated keratinocytes.....	24
<b>CHAPTER II. CUTANEOUS NEUROPEPTIDES UPREGULATE THE PRODUCTION OF INFLAMMATORY CYTOKINES AND NGF IN HUMAN KERATINOCYTE CULTURES .....</b>	<b>25</b>
4.6 Effects of SP on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes .....	25
4.7 Effects of CGRP on the production of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes .....	27
4.8 Effects of VIP on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes .....	29
4.9 Effects of GAL on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8	

and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes .....	31
4.10 Cultured human keratinocytes can secrete proNGF.....	33
<b>5. DISCUSSION .....</b>	<b>34</b>
5.1 GALR2 is expressed by human keratinocytes both at the level of mRNA and protein.....	34
5.2 The neuropeptides SP, CGRP, VIP and GAL upregulate the inflammatory cytokine expression in cultured human keratinocytes.....	36
5.3 Normal human keratinocytes secrete proNGF and the neuropeptides SP, CGRP, VIP and GAL can increase both proNGF production and NGF secretion in cultured human keratinocytes.....	38
<b>6. SUMMARY .....</b>	<b>40</b>
<b>7. REFERENCES.....</b>	<b>42</b>
<b>ACKNOWLEDGEMENTS.....</b>	<b>50</b>



## List of abbreviations

ANOVA	analysis of variance
CGRP	calcitonin gene-related peptide
ELISA	enzyme-linked immunosorbent assay
GAL	galanin
GALRs	galanin receptors
IL-1	interleukin-1
IL-8	interleukin-8
JNK	JUN N-terminal kinase
MAP kinase	mitogen-activated protein kinase
NGF	nerve growth factor
p75 <sup>NTR</sup>	75 kDa receptor
proNGF	nerve growth factor precursor
Q-RT-PCR	quantitative reverse transcription- polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	substance P
TBS	TRIS-buffered saline
TNF	tumor-necrosis factor
TrkA	tyrosine kinase receptor A
VIP	vasoactive intestinal polypeptide

## 1. Introduction

The human skin covers an area of average  $1.6 \text{ m}^2$  in adult. Function of the skin is complex. As the largest organ of the human body contribute to the structural and functional integrity of it. The human skin takes part in the establishment of endogenous homeostasis by protecting the body from exsiccation, controlling the normal body temperature, synthesizing of the important molecules and so on. The skin serves as an essential barrier to external harmful effects e.g. infections, UV-radiation, mechanical injury. In addition to mentioned above the skin functions as the most peripheral component of the immune system and initiates a primary immune response to external antigens. This primary immune reaction involves the resident cell types of the skin. This network is called Skin Immune System (SIS) consisting of Langerhans cells (antigen-presenting cell) which circulate between the skin and lymph nodes, keratinocytes, endothelial cells and fibroblasts, which produce a wide diversity of cytokines, chemokines and growth factors. Lymphocytes and macrophages extravasate from the blood vessels into the skin. These components cooperate with one another to give the best response against detrimental stimuli from outside (1,2,3,4,5,6).

The skin has considerable role in the perception of the sensory stimuli that stem from outside world. In this process the participant nerve terminals are components of the somatosensory system. The nerve terminals in the skin can be grouped on the basis of their morphology: free nerve endings (nociceptors) and encapsulated nerve endings (e.g. Meissner's corpuscles, Pacinian corpuscles). From point of view of our investigations the most interesting nerve terminals belong to a group of the free nerve endings, namely polymodal nociceptors, which can reach up as far as the epidermis and can be activated by chemical, mechanical stimuli and even heat.

Axons of these polymodal nociceptors make up the majority of very slowly conducting, unmyelinated C fibers (7). Up to now more and more experimental data have accumulated that the nervous system can modulate various immunological responses, including certain inflammatory events in the skin. Neuropeptides released from the cutaneous sensory free nerve endings have neurotransmitter and immunoregulatory roles,

they exert mitogenic actions and they can influence the functions of different cell types in the skin (8).

Moreover, cells of the skin are able to produce certain neuropeptides, growth factors (9, 10). Specific receptors and binding sites for neuropeptides have been described in different cell types in the skin (keratinocytes, Langerhans cells and fibroblasts) (11).

### *1.1 Keratinocytes as cellular components of the skin immune system*

Keratinocytes comprise about 90% of the total number of cells found in the epidermis. In the past they were highlighted for their production of keratins and lipids, which contributed to the structural integrity and barrier formation of the skin. For now it is obvious that these cells are active participants of the skin innate immune mechanisms. They are able to respond to nonspecific external stimuli with the production of wide range of inflammatory cytokines, chemokines, growth factors and receptors. Of primary inflammatory cytokines they can release interleukin-1 (IL-1 $\alpha$ , IL-1 $\beta$ ), interleukin-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) which can activate certain cellular signaling pathways including MAP kinase/JNK signaling system and the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway that regulates the genes for chemokines, cytokines and even interferons (3,5,12,13,14). IL-1 is present in keratinocytes constitutively, but can be upregulated by stimulating with lipopolysaccharide (LPS), physical, chemical or thermal injury, ultraviolet irradiation or a variety of cytokines and growth factors (i.e. granulocyte/macrophage colony stimulating factor (GM-CSF), TNF- $\alpha$ , IL-6, and IL-1 itself) (15).

Keratinocytes have also been identified as source of diverse range of the chemokines (both C-X-C chemokines [IL-8] and C-C chemokines) which are of crucial importance in the later phase of inflammation (16).

In addition the keratinocytes can produce and secrete several growth factors such as macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF) and GM-CSF to sustain and activate the Langerhans cell population (17). Transforming growth factor alpha (TGF- $\alpha$ ) and nerve growth factor (NGF) are able to influence the proliferation of the various cells types (18).

## 1.2 Neuropeptides in the skin

To date the presence of more than 20 neuropeptides and neurohormones has been verified in human skin by means of immunohistochemistry and radioimmunology (19,20,21). Of all the neuropeptides, the tachykinins, and especially the ubiquitous substance P (SP), have been best characterized (8,22). SP is involved in neurogenic inflammation. Outside the central nervous system, sensory neurons release SP from afferent termini directly into the peripheral tissues (23,24), where the neuropeptide can initiate numerous inflammatory reactions (25,26). Keratinocytes express specific receptors for SP (16), and SP is capable of upregulating the production of the proinflammatory cytokines IL-1 $\alpha$  and IL-1 $\beta$  and the chemokine IL-8 in murine and human keratinocytes (27). Further, SP is able to upregulate the expression of the NGF in human and murine keratinocytes (28).

SP is often co-localized with calcitonin gene-related peptide (CGRP), a neuropeptide present in considerable amount in the skin (29). The most important effects of CGRP are its *in vivo* vasodilatory actions on small and large vessels (30). It can enhance endothelial cell and keratinocyte proliferation (31). In addition to regulating the vascular function, CGRP can also influence immune responses (11,23,32,33,34).

Vasoactive intestinal polypeptide (VIP) belongs to the family of secretion peptides. In the epidermal layer, VIP immunoreactivity is low (35,36). Binding sites for VIP have been detected on keratinocytes (31) and immune cells (37). VIP can stimulate the proliferation and migration of human keratinocytes, which may play a role in both wound healing and hyperproliferation diseases of the skin, such as psoriasis (38,39,40). VIP exerts numerous considerable effects on the action of the immune system (41,42,43,44,45,46,47).



### *1.3 Galanin as a less-known neuropeptid in the skin*

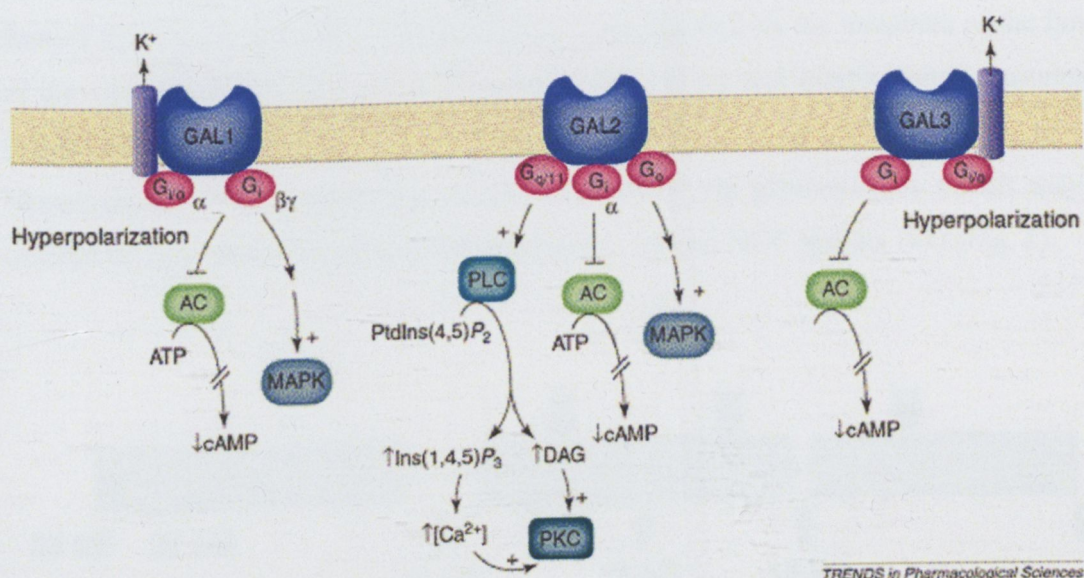
Galanin (GAL) is a conserved neuroendocrine peptide, consisting of 29 or 30 (in human) amino acids. The peptide was isolated first from the porcine intestine (48). The widespread distribution and diverse functions of GAL, together with its highly conserved sequence among species, suggest that GAL is an important messenger for inter- and intracellular communications. GAL has been found to be widely distributed and abundantly expressed in both the central nervous system (CNS) and the peripheral tissues, and this has led to studies demonstrating a variety of actions of GAL, including neurotransmitter and hormone release, spinal reflexes and nociception (49,50). Peripheral effects of GAL have been described both in the autonomic ganglia and in the endocrine-gastrointestinal axis, and it participates in the somato-sensory functions, especially pain signaling (51,52). Recent evidence suggests that GAL also plays mitogenic and neurotrophic roles of importance for the recovery from neuronal injury (53).

In human skin, GAL-like immunoreactivity was first reported in nerve terminals and fibers of the dermis (54). Later, GAL-like immunoreactivity was detected in the follicular and interfollicular epidermis and in the ductal cells of the eccrine sweat glands. Additionally, GAL-binding sites have been demonstrated around the eccrine sweat glands (55) and in the basal zone of the epidermis (56).

#### *1.3.1 Galanin receptors*

Biological effects of GAL on different cell types are mediated via three different GAL receptor subtypes (GALR1, GALR2 and GALR3) (57,58). The GALRs exhibit high interspecies homology and moderate homology to each other. They display a distinct tissue distribution. GALR1 is expressed mainly in the CNS (59), GALR2 is expressed in considerable amounts both in the CNS and in the peripheral tissues, and GALR3 is expressed in noteworthy quantities in the peripheral tissue and in lower amounts in the CNS (60,61). The signal transduction mechanisms of the three GALRs are complex. All three are able to inhibit adenylate cyclase (62). There is evidence that both GALR1 and GALR2 stimulate the MAP kinase in neuronal cells, which may lead to enhanced

proliferation (63,64). In contrast with GALR1, GALR2, via phospholipase C (PLC), can activate protein kinase C (PKC) (65,66) and increase inositol triphosphate (IP3) (63) which leads to mobilization of the  $\text{Ca}^{2+}$  from the intracellular stores in neuronal cells (67) (Fig. 1). The effects of GAL via its GALRs on non-neuronal cells may differ. GAL has been reported to be able to inhibit proliferation via GALR1 in oral squamous cell carcinoma (68) and via GALR1 and GALR3 in immature rat thymocytes (69). Moreover, GAL may play a role in the modulation of cutaneous inflammatory processes (70,71). GAL can inhibit cutaneous vasodilatation in the pigeon (72,73). Although GALRs are the key to the functional significance of the action of GAL, their expressions in cultured human keratinocytes have not yet been investigated.



**Figure 1. Galanin receptors subtypes and their various signal transduction mechanisms (51).**

receptor of 140 kD (TrkA) (89). Whereas the mature NGF is a high-affinity ligand for TrkA and low-affinity ligand for  $p75^{\text{NTR}}$  the proNGF can bind to  $p75^{\text{NTR}}$  with a higher-affinity than mature NGF (90,91) and thus it is an active participant in the initiation of the apoptosis mediated by  $p75^{\text{NTR}}$ . Accordingly, it can play a role in several pathophysiological conditions (92,93,94,95).

## 2. Aims

The aims of the study were:

- to investigate the mRNA and protein expressions of the different GALRs in the HaCaT immortalized keratinocyte cell line, in cultured human keratinocytes and in normal human skin.
- to verify that GALR2 is a functional receptor by measuring the alteration in cytosolic  $\text{Ca}^{2+}$  in cultured human keratinocytes under effect of the GALR2-ligand binding.
- to investigate systematically the effects of the neuropeptides SP, CGRP, VIP and GAL on the inflammatory mediator production (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) of the keratinocytes.
- to assess the role of the neuropeptides SP, CGRP, VIP, GAL in the regulation of the production of proNGF/NGF and their secretion from cultured human keratinocytes.



### 3. Materials and Methods

#### 3.1 Cell cultures

HaCaT is a spontaneous immortalized non-tumorigenic human keratinocyte-derived cell line (kindly provided by Dr N.E. Fusening, Heidelberg, Germany). HaCaT cells were cultured in a high-glucose Dulbecco's modified Eagle' medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal bovine serum, L-glutamine, antibiotics (penicillin and streptomycin) and an antimycotic (amphotericin B).

Adult epidermal keratinocytes were isolated from breast skin specimens from healthy donors (females; age group 20-40 years) undergoing plastic surgery. These skin specimens were first washed in an isotonic solution supplemented with antibiotic-antimycotic solution (Sigma, St. Louis, Missouri USA). To separate the dermis from the epidermis, an overnight incubation in dispase solution (Grade II, Roche Molecular Biochemicals) was carried out at 4 °C. The epidermis was then incubated in 0.25% trypsin-EDTA (Sigma, St. Louis, Missouri USA) for 20 min at 37 °C. Following trypsinization, the cell suspension was filtered through a 100 µm nylon mesh (BD, Falcon) and centrifuged at 1000 rpm for 10 min at 4 °C. The cell concentration and the percentage of viable cells were determined by means of trypan blue staining, using a hemocytometer. The cells were seeded in keratinocyte-serum free medium (Gibco BRL, Eggenstein, Germany) on 25 cm<sup>2</sup> tissue culture plastic flasks. The medium was changed every 2 or 3 days. Subconfluent primary cultures were harvested by trypsinization and were divided into equal two parts. Both cell types were maintained at 37 °C, with 5% CO<sub>2</sub> in the humidified atmosphere.

#### 3.2 Neuropeptides treatment of the cell cultures

On reaching approximately 80% confluence (after the third passage), the cells were treated with different neuropeptides, such as SP, CGRP, VIP and GAL at 10<sup>-8</sup> M for 30 min. Following incubation in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C, the keratinocytes were washed with neuropeptide-free medium and fed with fresh medium. For control purposes, cells were handled as mentioned above, but without neuropeptide

induction. Total RNA was isolated 3, 6 12 and 24 h after neuropeptide treatment. Cell lysates and keratinocyte culture supernatants were collected 10, 24, 48 and 72 h after neuropeptide treatment.

### 3.3 Reverse transcription (RT) – PCR

Total RNA was isolated from cultures by using the TRIzol reagent method (Invitrogen, Carlsbad, USA), carried out as described by the manufacturer. One microgram of total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After RT, amplification was carried out with an iCycler machine (iCycler IQ Real Time PCR, BioRad Laboratories, Hercules, CA, USA). Specific primers of GALR1 and GALR2 are listed in Table 1. For detection of the presence of GALR3 mRNA, Human GALR3 TaqMan® Gene Expression Assay (reference number: Hs00358572\_m1) (Applera Hungary Ltd., Budapest, Hungary) was used. The TaqMan probe was Fam dye-labeled. After the amplification, 15 µl of PCR products was run on 1.5% agarose gel in the cases of GALR1 and GALR2, or on 3% agarose gel for the GALR3 product, stained with ethidium bromide, photographed and evaluated with Kodak ID Digital Science software (Scientific Imaging Systems, New Haven, CT, USA). For the DNA sequence analysis, the PCR products were excised from the agarose gel under ultraviolet light and purified by using the DNA Gel Extraction Kit (V-gene Biotechnology Limited, Hangzhou, China) according to the manufacturer's instructions. The sequence of the purified PCR products was then determined by direct DNA sequencing.

gene	sequence	conc.
GALR1	sense: 5'-GTCATCTGCTTCTGCTATG-3'	450 nM
	anti-sense: 5'-GCAGCCAGGAGATTCCAA-3'	450 nM
GALR2	sense: 5'-TTCCTCATCTTCCTCACCATG-3'	450 nM
	anti-sense: 5'-ATGGCACACGGTCAGGTT-3'	450 nM

**Table 1.** Primer pairs and primer concentrations used to detect the presence of GALR1 and GALR2 subtypes

### 3.4 Quantitative reverse transcription (RT)-PCR

For quantitative real time RT-PCR, 1 µg purified total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After RT, amplification reactions were carried out with an iCycler machine (iCycler IQ Real Time PCR, BioRad Laboratories, Hercules, CA, USA). To detect 18S rRNA (as endogenous control), NGF and IL-8, we used sequence-specific TaqMan probes. The primers, probes and their concentrations are listed in Table 2. The specificities of the IL-1 $\alpha$  and TNF- $\alpha$  primers were determined by melting curve analysis of the amplicon. For the negative control, we omitted the cDNA template and replaced it with water. Each of the PCR reactions was carried out in duplicate. The PCR product was detected by measuring the increase in fluorescence. The amount of cDNA was calculated on the basis of the threshold cycle ( $C_T$ ) value, and was standardized by the amount of housekeeping gene (18S rRNA) by means of the  $2^{-\Delta\Delta C_T}$  method (96):

$$\Delta\Delta C_T = (C_{T, \text{target}} - C_{T, 18S \text{ rRNA}}) - (C_{T, \text{target}} - C_{T, 18S \text{ rRNA}})_{(\text{reference})}$$

where “target” means the gene of interest, and “reference” means the untreated keratinocytes. PCR results were expressed as fold increases over the control values.

gene	sequence	conc.
IL-1 $\alpha$	sense: 5'-AA TTG TAT GTG ACT GCC CAA G- 3'	200 nM
	antisense: 5'-TA GTT CTT AGT GCC GTG AGT T - 3'	200 nM
IL-8	sense: 5'-CA CAC TGC GCC AAC A-3'	300 nM
	antisense: 5'-CA TCT TCA CTG ATT CTT GGA T-3'	300 nM
	probe: 5'-/56-FAM/ CTG GGT GCA GAG GGT TGT GG /3BHQ/-3'	150 nM
TNF- $\alpha$	sense: 5'-CT CCT TCC TGA TCG TGG C-3'	300 nM
	antisense: 5'-GT TCA GCC ACT GGA GCT-3'	300 nM
NGF	sense: 5'-AG TGC CGG GAC CCA AAT-3'	200 nM
	antisense: 5'-GA GTT CCA GTG CTT TGA GTG AA-3'	200 nM
	probe: 5' - /56-FAM/ CCG TTG ACA GCG GGT GCC G /3BHQ/-3'	50 nM
18SrRNA	sense: 5'-GG CTA CCA CAT CCA AGG AA-3'	300 nM
	antisense: 5'-CT GGA ATT ACC GCG GCT-3'	300 nM
	probe: 5'-/TexasRed/ TGC TGG CAC CAG ACT TGC CCT C /3BHQ/-3'	50 nM

**Table 2.** Primer pairs and primer concentrations used to detect the presence of IL-1 $\alpha$ , IL-8, TNF- $\alpha$  and NGF mRNAs.

### *3.5 Immunocytochemistry and immunohistochemistry*

Cultured cells were harvested by trypsinization. After centrifugation, the cell pellet was resuspended in culture medium, and the cells were counted in a Burker chamber. Fifty thousand cells were seeded onto glass slides by cytocentrifuge. The slides were fixed in acetone for 6 min. Next, the slides were washed in TRIS-buffered saline (TBS, pH 7.2), and nonspecific binding sites were blocked with skimmed milk (5% in TBS) for 20 min. After washing in TBS, the slides were incubated in goat polyclonal antibody against human GALR2 (K-20) (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:10 overnight at 4 °C. Next day, after extensive washing in TBS, the slides were incubated with peroxidase-conjugated anti-goat secondary antibody (DakoCytomation, Glostrup, Denmark) at a dilution of 1:200 at room temperature for 30 min. Finally, peroxidase activity was detected with diaminobenzidine as substrate.

For immunohistochemical investigations, human skin samples were obtained from people undergoing plastic surgery. Samples were frozen in order to make sections. After fixing in acetone, the immunohistochemical staining procedure was essentially the same for the immunocytochemistry described above. In both cases, nuclei were counterstained with hematoxylin. For negative controls, the primary antibody was replaced in both cases with normal goat IgG. Images were acquired with an RT KE camera and SPOT RT v4.0 software (Diagnostic Instruments, Livingstone, Scotland) through an Axioplan microscope (Opton, Carl Zeiss AG, Oberkochen, Germany).

### *3.6 Western blotting*

Cultured human keratinocytes and human brain tissue (obtained from Department of Pathology) were lysed in 1 ml lysis buffer containing 62.5 mM TRIS (pH 6.8), 5 mM EDTA, 1.5% SDS, 5%  $\beta$ -mercaptoethanol and 0.2% proteinase inhibitor (Sigma, St. Louis, Missouri USA). Cell lysates were centrifuged for 15 min at 14000g. After separation by electrophoresis on 10% SDS-PAGE proteins were transferred to blotting membrane (BioRad Laboratories, Hercules, CA, USA), which was then washed in 2% skim milk in TBS (pH 7.4) for 1 h. To detect the presence of the GALR2, goat polyclonal antibody against human GALR2 (K-20) (Santa Cruz Biotechnology, California, USA)



was used at a dilution of 1:20 whereas to measure the amounts of the proNGF we used rabbit polyclonal antibody against human proNGF (Sigma, St. Louis, MO, USA) at a dilution of 1:100, both of them in 2% skim milk in TBS. Next day, the membrane was washed in TBS containing 0.5% Tween 20 (TTBS) and incubated with anti-goat IgG-conjugated alkaline phosphatase (Sigma, St. Louis, Missouri USA) at a dilution of 1:3000, anti-rabbit IgG-conjugated alkaline phosphatase (Sigma, St. Louis, MO, USA) at a dilution of 1:3800 for 75 min, respectively. After washing in TTBS and in TBS, the alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma, St. Louis, Missouri USA) as substrate.

The specificity of the polyclonal antibody used against GALR2 was verified by preabsorbing the antibody with the blocking peptide (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:500 for 4 h prior to the immunoblotting.

In case of measuring of the amount of the proNGF blots were developed with a 1:100 dilution of the rabbit polyclonal anti-actin antibody (Sigma, St. Louis, MO, USA) in order to check on the equal loading of the total protein in all lanes. Otherwise, the conditions were as described in case of proNGF.

The results of the Western blotting were filmed with a Kodak DC290 Zoom Digital Camera (Scientific Imaging System, New Haven, CT, USA) and the amount of the proNGF were quantitated densitometrically with Kodak 1D Digital Science software.

### *3.7 ELISA technique*

The protein levels of human IL-1 $\alpha$ , IL-8 and TNF- $\alpha$  in the supernatant of the third-passage epidermal keratinocytes were measured by using commercially available Quantikine human immunoassay kits, according to the manufacture's protocol (Quantikine R&D Systems, Minneapolis, MN, USA). To measure the protein level of NGF in the supernatant of the human keratinocyte culture, we used a NGF E<sub>max</sub> ImmunoAssay System (Promega, Madison, WI, USA), following the manufacturer's instruction.

### 3.8 Measurement of intracellular $[Ca^{2+}]_i$

After the second passage, cultured human keratinocytes were seeded onto glass slides in order for their culturing to continue until a confluence of approximately 90% was reached. The glass slides were then washed twice with a modified Hank's solution consisting of the following components: 127 mM NaCl, 0.33 mM  $MgSO_4$ , 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 0.33 mM  $Na_2HPO_4$ , 0.44 mM  $KH_2PO_4$ , and 10 mM HEPES. After washing, the cells were loaded with Fura-2/AM (Sigma, St. Louis, Missouri USA), a  $Ca^{2+}$ -sensitive fluorescent dye, at 3.5  $\mu M$  final concentration, followed by incubation at 37 °C for 45 min. Finally, the glass slides were subjected to a further two washings in order to eliminate the unnecessary dye. Before measurement of the fluorescence, the cells were placed into a dish filled with Hank's solution containing GAL (Sigma, St. Louis, Missouri USA) at  $10^{-8}$  M for 15 s. The fluorescence of the cultured human keratinocytes was measured with a Hitachi F-2500 spectrofluorometer. Fluorescence was excited at 340 and 380 nm and emission was measured at 505 nm. Maximal and minimal fluorescence values were obtained following the addition of Triton X-100 (final concentration: 0.1%) or EGTA (final concentration: 4 nM), respectively. Data were calculated according to the method of Grynkiewicz, et al (97).

### 3.9 Statistical analyses

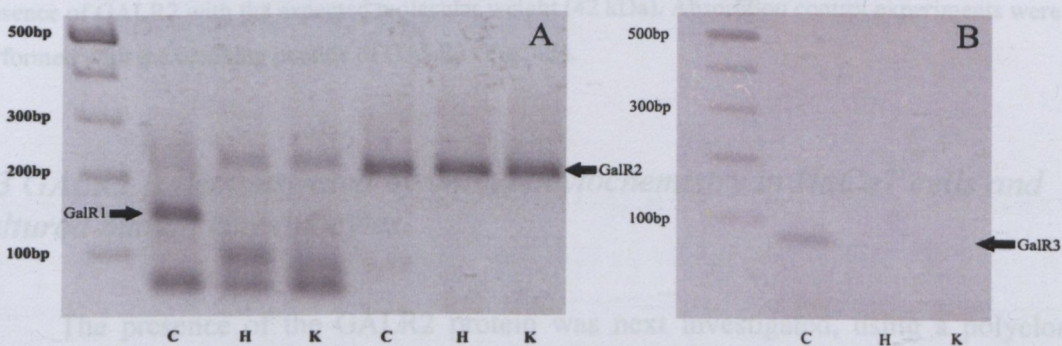
The data were collected from at least three independent experiments. Quantitative data are expressed as means  $\pm$  SD. Statistical significance was determined by using one-way ANOVA followed by Dunnett's and Tukey's *post hoc* tests (SPSS, Chicago, IL, USA). Differences were considered to be statistically significant when  $p < 0.05$ .

## 4. Results

### Chapter I. Keratinocytes can express GALR2 both mRNA and protein level

#### 4.1 Identification of subtypes of GALR mRNAs in HaCaT cells and cultured human keratinocytes

In the first step, the presence of different GALRs was investigated at the level of mRNA. GALR1 and GALR2 were detected by using human small intestine as positive control and positive control for GALR3 was human brain cDNA. In the case of GALR1, an amplified product with the expected molecular weight was detected on use of the human small intestine cDNA as template, but when the cDNAs of the HaCaT cells and keratinocytes were used, no amplified product with specific molecular weight could be demonstrated (Fig. 3A). On investigation of the expression of the GALR2 mRNA, we found a sharp band with the expected molecular weight in the gel when either human small intestine or cultured keratinocyte cDNAs were applied as templates for the PCR reactions (Fig. 3A). The amplified product was retrieved from the bands and subjected to DNA sequence analysis. This proved that the HaCaT cells and keratinocytes expressed GALR2 at the mRNA level. In the case of GALR3, we detected a weak band for the human brain cDNA, corresponding to the expected molecular weight, but neither the HaCaT nor the keratinocyte cDNA gave a signal (Fig. 3B).

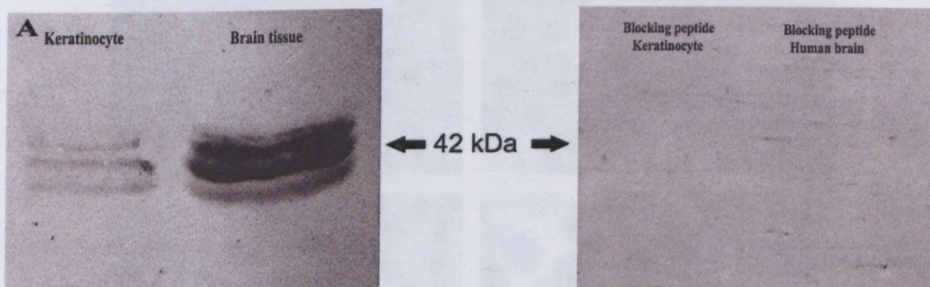


**Figure 3. Expression of the mRNA of GALRs in HaCaT cells and cultured human keratinocytes.** The PCR product of GALR1 was 143 bp, and that of GALR2 was 222 bp (Fig. 3A). The PCR product of GALR3 was 92 bp (Fig. 3B). (C = positive control, H = HaCaT cells, K = human keratinocytes)



#### 4.2 GALR2 protein expression detected by Western blotting in human keratinocyte lysates

We carried out Western blotting assays on cultured human keratinocyte extracts to show the presence of the GALR2 protein. The positive control was a human brain protein extract. By using both keratinocyte and brain extracts, we succeeded in demonstrating the presence of the GALR2 protein with the expected molecular weight, 42 kDa (Fig. 4A). We performed Western blotting experiments using the blocking peptide to verify the specificity of the goat polyclonal antibody used against human GALR2. In this case, in accordance with our expectations, we did not find a signal either for the keratinocyte extract or for the human brain extract (Fig. 4B).



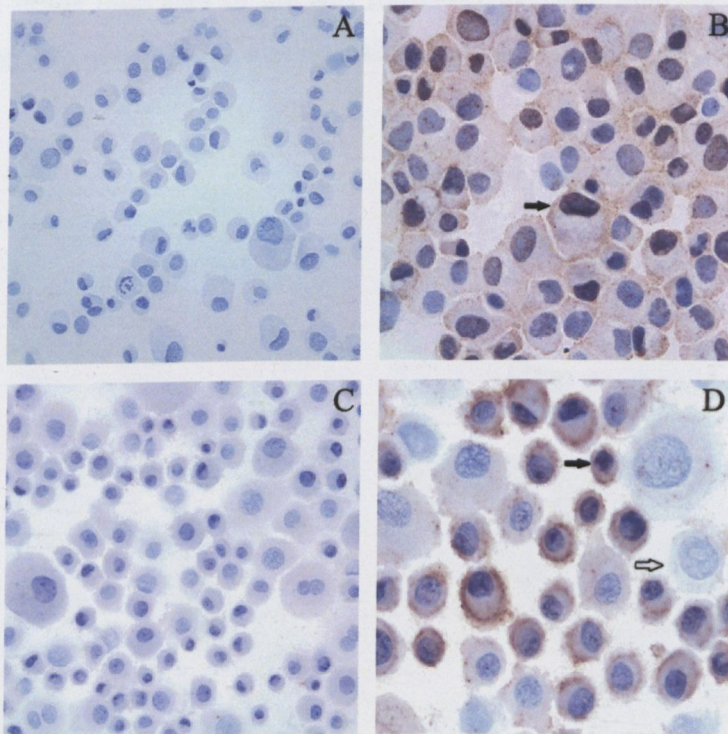
**Figure 4. Detection of the presence of GALR2 by Western blotting in protein extracts from cultured human keratinocytes and human brain.** Fig. 4A shows the presence of GALR2 with the expected molecular weight (42 kDa). Absorption control experiments were performed with the blocking peptide of GALR2 (Fig. 4B).

#### 4.3 GALR2 is demonstrated by immunocytochemistry in HaCaT cells and cultured human keratinocytes

The presence of the GALR2 protein was next investigated, using a polyclonal antibody against human GALR2. Immunocytochemical investigations were carried out with cultured HaCaT cells and human keratinocytes. In both cases, we made a negative control to enhance the reliability of the experiments. On negative control of the HaCaT cells (Fig. 5A), immune staining was not found, whereas with the polyclonal anti-GALR2



antibody (Fig. 5B) relatively uniformly distributed strong staining was observed on the total surface of the cells. On negative control of the cultured human keratinocytes (Fig. 5C), there was no specific staining, but a strong specific immune reaction was detected on the surface of the keratinocytes when the polyclonal anti-GALR2 antibody was used (Fig. 5D). These findings proved the presence of GALR2 on the surface of the human keratinocytes. Interestingly, the more differentiated cells appeared to be less intensively stained.

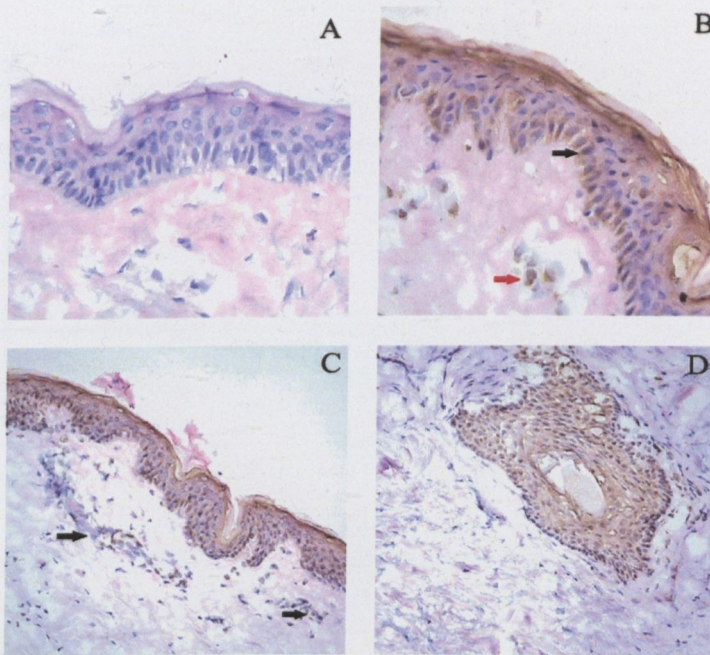


**Figure 5. Immunocytochemical analysis of GALR2 in HaCaT cells and cultured human keratinocytes.** Negative controls were made for both the HaCaT cells (Fig. 5A) and the keratinocytes (Fig. 5C). HaCaT cells (Fig. 5B, the black arrow shows the surface of a HaCaT cell) and cultured normal human keratinocytes (derived from adult female breast skin) (Fig. 5D) were subjected to immunocytochemical studies in order to investigate the expression of GALR2 (the black arrow shows the surface of the keratinocyte; the white arrow shows a differentiated cell). Nuclei were counterstained with hematoxylin.



#### 4.4 *In situ* expression of GALR2 in human skin

We carried out immunohistochemical investigations on skin samples from 5 persons undergoing plastic surgery in order to identify the layer of the human skin in which GALR2 is expressed and how intense this expression is. With the anti-GALR2 antibody, intense staining was demonstrated in the basal layer of the epidermis and around blood vessels in the dermis (Fig. 6B and 6C). Interestingly, we also found very strong staining in the bulb area of the hair follicles (Fig. 6D). This was in contrast with the negative controls, where there was no staining at all (Fig. 6A).

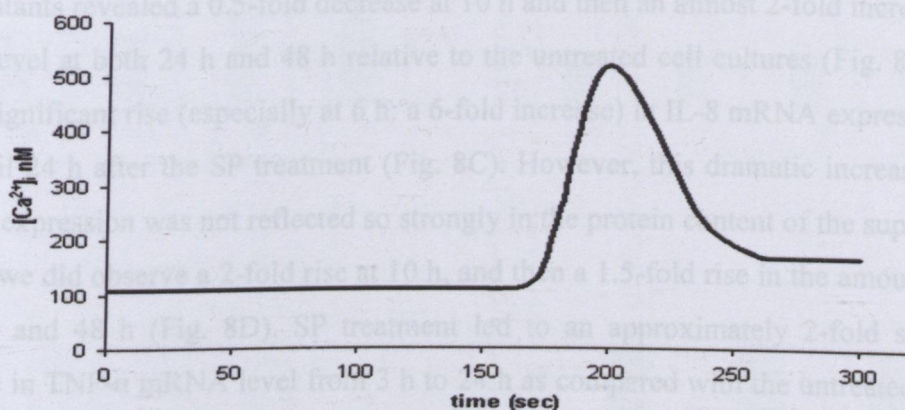


**Figure 6. Localization of GALR2 by immunohistochemistry in the different layers of normal human skin.** Figure shows the expression of GALR2 in normal human skin. A negative control was performed with normal goat IgG instead of primary antibody (Fig. 6A). Fig. 6B shows the more intensive coloration of the basal keratinocytes (black arrow) (orange arrow shows vessel endothelial cells). Positive blood vessel cells (black arrows) can be seen in Fig. 6C, while Fig. 6D presents a cross-section of a bulb of a hair follicle. Nuclei were counterstained with hematoxylin. Sections thickness: ~ 4  $\mu\text{m}$ .



#### 4.5 An increased level of intracellular $\text{Ca}^{2+}$ , $[\text{Ca}^{2+}]_i$ , was demonstrated in GAL-treated keratinocytes

The effect of GAL on the mobilization of intracellular  $\text{Ca}^{2+}$  was investigated in cultured human keratinocytes. The glass-cultured cells were placed in Hank's solution containing  $10^{-8}$  M GAL for 15 s. From the beginning of the measurement up to approximately 170 s, no change in  $[\text{Ca}^{2+}]_i$  was noted. A fairly sharp increase was then recorded, with a peak at approximately 200 s. The value of  $[\text{Ca}^{2+}]_i$  at the peak was increased roughly 5-fold as compared with the starting level.  $[\text{Ca}^{2+}]_i$  subsequently decreased until it reached 1.5 times the initial  $[\text{Ca}^{2+}]_i$  (Fig. 7).



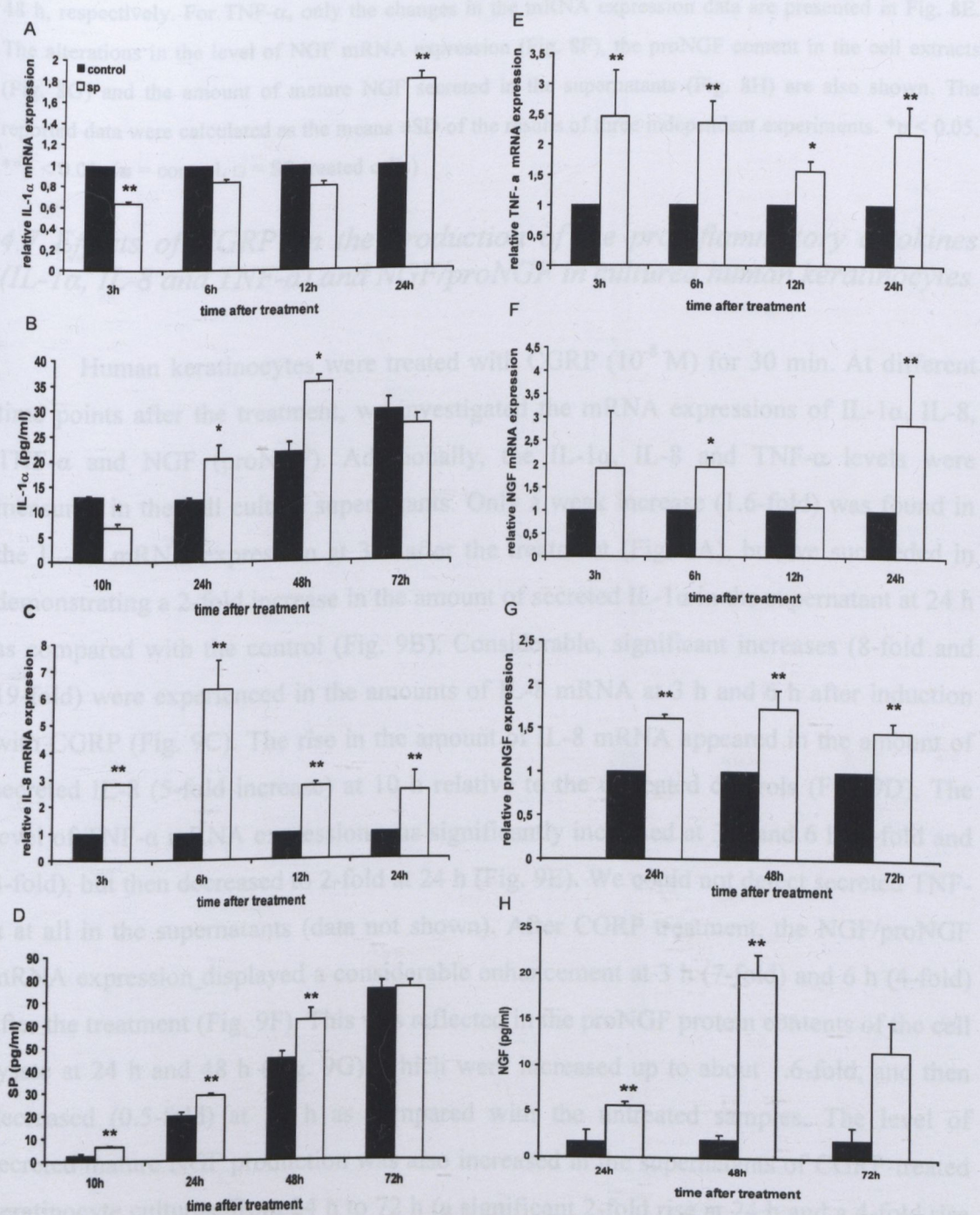
**Figure 7. Effect of GAL on  $[\text{Ca}^{2+}]_i$  in cultured human keratinocytes.** The change in  $[\text{Ca}^{2+}]_i$  caused by  $10^{-8}$  M GAL in cultured human keratinocytes.  $[\text{Ca}^{2+}]_i$  was calculated from three independent experiments.

## **Chapter II. Cutaneous neuropeptides upregulate the production of inflammatory cytokines and NGF in human keratinocyte cultures**

### *4.6 Effects of SP on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes*

After the induction of subconfluent cultured human keratinocytes with SP ( $10^{-8}$  M), a modest, but significant decrease was experienced in the amount of IL-1 $\alpha$  mRNA as compared with time-matched controls at 3 h after the treatment. Later, the IL-1 $\alpha$  mRNA levels gradually increased in the SP-treated cells, but the difference between the SP-treated cells and the controls after 24 h was not yet significant (Fig. 8A). Investigation of the supernatants revealed a 0.5-fold decrease at 10 h and then an almost 2-fold increase in the IL-1 $\alpha$  level at both 24 h and 48 h relative to the untreated cell cultures (Fig. 8B). There was a significant rise (especially at 6 h: a 6-fold increase) in IL-8 mRNA expression from 3 h until 24 h after the SP treatment (Fig. 8C). However, this dramatic increase in IL-8 mRNA expression was not reflected so strongly in the protein content of the supernatants, though we did observe a 2-fold rise at 10 h, and then a 1.5-fold rise in the amounts of IL-8 at 24 and 48 h (Fig. 8D). SP treatment led to an approximately 2-fold significant increase in TNF- $\alpha$  mRNA level from 3 h to 24 h as compared with the untreated samples (Fig. 8E), but we failed to demonstrate the presence of TNF- $\alpha$  in the keratinocyte supernatants by ELISA assay (data not shown). As concerns the impact of SP on the expression of NGF/proNGF, we experienced biphasic kinetics in the level of the NGF/proNGF mRNA. There were significant increases in the levels of NGF/proNGF mRNA at 6 h and 24 h after the treatment (Fig. 8F). The relative proNGF protein contents of the SP-treated keratinocyte lysates as compared with time-matched controls were evaluated by means of Western blotting. We found an approximately 1.6-fold increase in the level of proNGF as compared with the control from 24 h up to 72 h (Fig. 8G). The mature NGF concentrations of the cultured keratinocyte supernatants were also significantly increased after SP treatment, the rise being dramatic (10-fold) at 48 h (Fig. 8H).





**Figure 8. The effects of SP on the expressions of proinflammatory cytokines and the NGF/proNGF system in cultured human keratinocytes.**

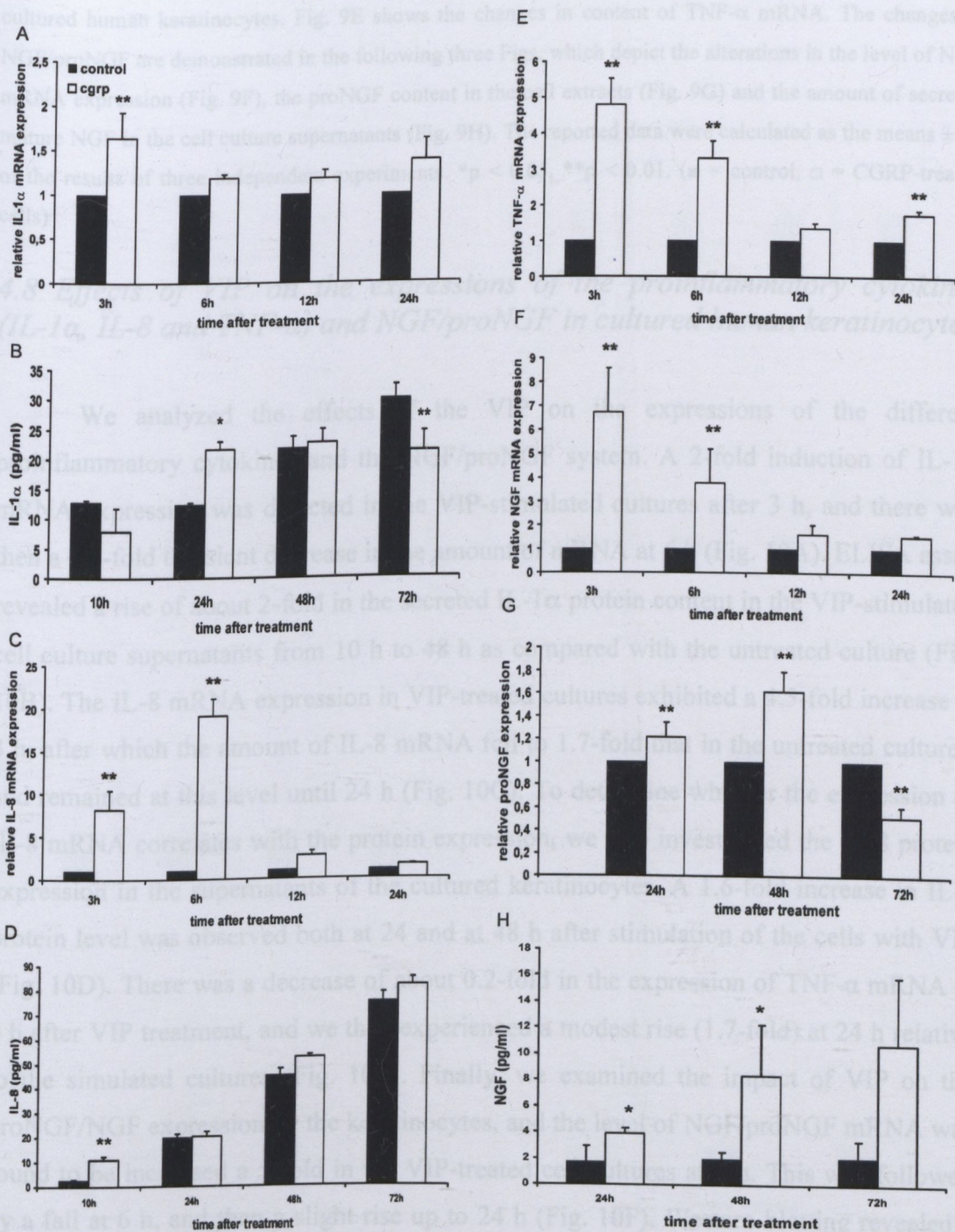
Fig. 8A depicts the changes in IL-1 $\alpha$  mRNA expression from 3 h to 24 h after SP treatment ( $10^{-8}$  M). The changes in the secreted IL-1 $\alpha$  protein concentration are demonstrated in Fig. 8B. Figures 8C and 8D present the changes in IL-8 mRNA expression and IL-8 protein secretion from 3 h to 24 h and from 10 h to

48 h, respectively. For TNF- $\alpha$ , only the changes in the mRNA expression data are presented in Fig. 8E. The alterations in the level of NGF mRNA expression (Fig. 8F), the proNGF content in the cell extracts (Fig. 8G) and the amount of mature NGF secreted in the supernatants (Fig. 8H) are also shown. The reported data were calculated as the means  $\pm$ SD of the results of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . (■ = control, □ = SP-treated cells)

#### *4.7 Effects of CGRP on the production of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes*

Human keratinocytes were treated with CGRP ( $10^{-8}$  M) for 30 min. At different time points after the treatment, we investigated the mRNA expressions of IL-1 $\alpha$ , IL-8, TNF- $\alpha$  and NGF (proNGF). Additionally, the IL-1 $\alpha$ , IL-8 and TNF- $\alpha$  levels were measured in the cell culture supernatants. Only a weak increase (1.6-fold) was found in the IL-1 $\alpha$  mRNA expression at 3 h after the treatment (Fig. 9A), but we succeeded in demonstrating a 2-fold increase in the amount of secreted IL-1 $\alpha$  in the supernatant at 24 h as compared with the control (Fig. 9B). Considerable, significant increases (8-fold and 19-fold) were experienced in the amounts of IL-8 mRNA at 3 h and 6 h after induction with CGRP (Fig. 9C). The rise in the amount of IL-8 mRNA appeared in the amount of secreted IL-8 (5-fold increase) at 10 h relative to the untreated controls (Fig. 9D). The level of TNF- $\alpha$  mRNA expression was significantly increased at 3 h and 6 h (5-fold and 3-fold), but then decreased to 2-fold at 24 h (Fig. 9E). We could not detect secreted TNF- $\alpha$  at all in the supernatants (data not shown). After CGRP treatment, the NGF/proNGF mRNA expression displayed a considerable enhancement at 3 h (7-fold) and 6 h (4-fold) after the treatment (Fig. 9F). This was reflected in the proNGF protein contents of the cell lysate at 24 h and 48 h (Fig. 9G), which were increased up to about 1.6-fold, and then decreased (0.5-fold) at 72 h as compared with the untreated samples. The level of secreted mature NGF production was also increased in the supernatants of CGRP-treated keratinocyte cultures, from 24 h to 72 h (a significant 2-fold rise at 24 h and a 4-fold rise at 48 h) (Fig. 9H).





**Figure 9. The effects of CGRP on the expressions of IL-1 $\alpha$ , IL-8 and TNF- $\alpha$  and the NGF/proNGF system in cultured human keratinocytes.**

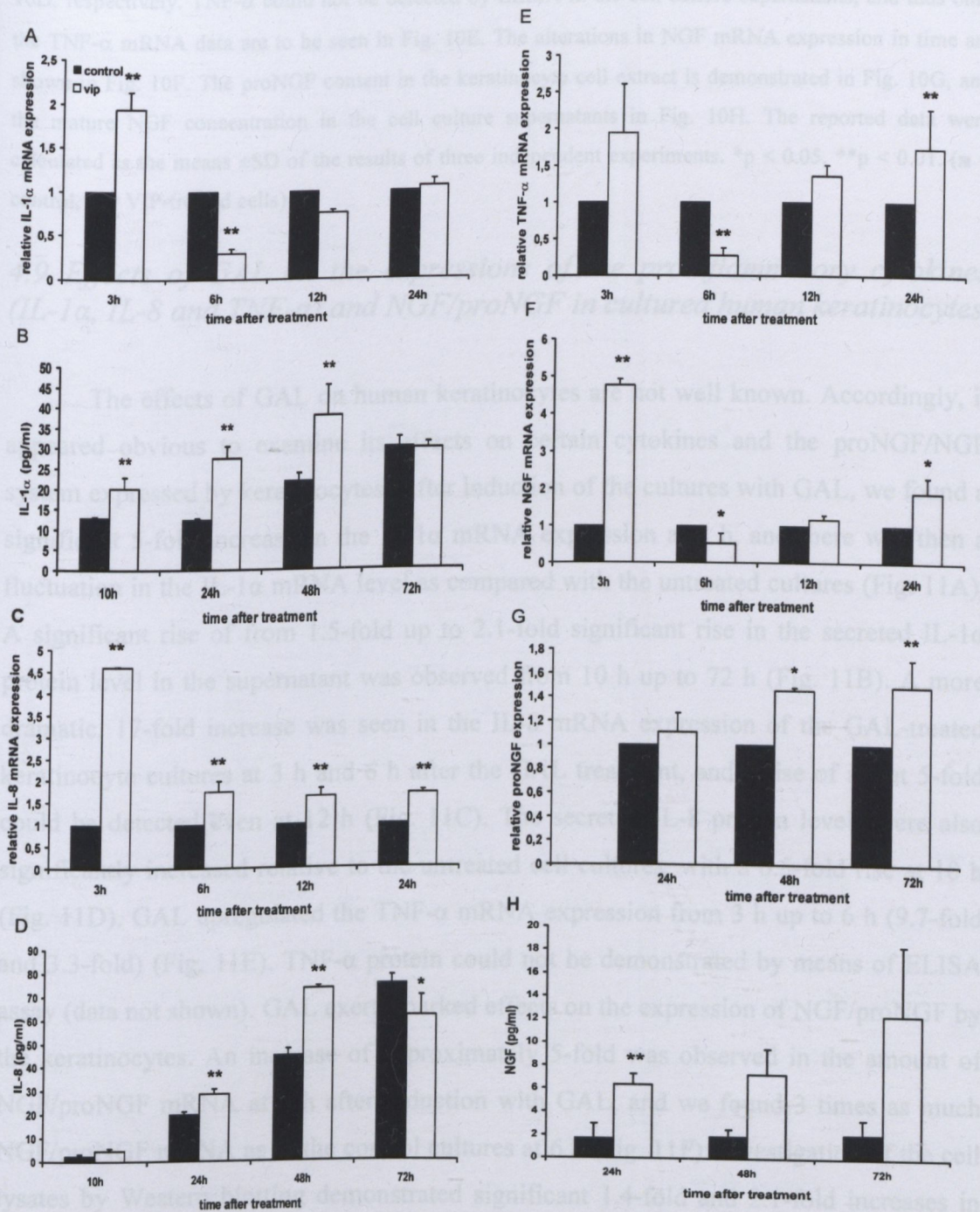
The changes in IL-1 $\alpha$  mRNA expression and secreted IL-1 $\alpha$  protein concentration in the CGRP-induced human keratinocytes are presented in Figs. 9A and 9B. Figs. 9C and 9D demonstrate the concentrations of IL-8 mRNA and secreted IL-8 protein in different time intervals after treatment with CGRP ( $10^{-8}$  M), of



cultured human keratinocytes. Fig. 9E shows the changes in content of TNF- $\alpha$  mRNA. The changes in NGF/proNGF are demonstrated in the following three Figs. which depict the alterations in the level of NGF mRNA expression (Fig. 9F), the proNGF content in the cell extracts (Fig. 9G) and the amount of secreted mature NGF in the cell culture supernatants (Fig. 9H). The reported data were calculated as the means  $\pm$ SD of the results of three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01. (■ = control, □ = CGRP-treated cells)

#### *4.8 Effects of VIP on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes*

We analyzed the effects of the VIP on the expressions of the different proinflammatory cytokines and the NGF/proNGF system. A 2-fold induction of IL-1 $\alpha$  mRNA expression was detected in the VIP-stimulated cultures after 3 h, and there was then a 0.3-fold transient decrease in the amount of mRNA at 6 h (Fig. 10A). ELISA assay revealed a rise of about 2-fold in the secreted IL-1 $\alpha$  protein content in the VIP-stimulated cell culture supernatants from 10 h to 48 h as compared with the untreated culture (Fig. 10B). The IL-8 mRNA expression in VIP-treated cultures exhibited a 4.5-fold increase at 3 h, after which the amount of IL-8 mRNA fell to 1.7-fold that in the untreated cultures, and remained at this level until 24 h (Fig. 10C). To determine whether the expression of IL-8 mRNA correlates with the protein expression, we also investigated the IL-8 protein expression in the supernatants of the cultured keratinocytes. A 1.6-fold increase in IL-8 protein level was observed both at 24 and at 48 h after stimulation of the cells with VIP (Fig. 10D). There was a decrease of about 0.2-fold in the expression of TNF- $\alpha$  mRNA at 6 h after VIP treatment, and we then experienced a modest rise (1.7-fold) at 24 h relative to the simulated cultures (Fig. 10E). Finally, we examined the impact of VIP on the proNGF/NGF expression by the keratinocytes, and the level of NGF/proNGF mRNA was found to be increased a 5-fold in the VIP-treated cell cultures at 3 h. This was followed by a fall at 6 h, and then a slight rise up to 24 h (Fig. 10F). Western blotting revealed a 1.5-fold increase in the level of proNGF in the keratinocyte lysates relative to the untreated cultures at 48 and 72 h (Fig. 10G). Although the proNGF content of the keratinocytes underwent only a modest change, the level of secreted mature NGF exhibited a significant increase at 24 h (3.7-fold) in the supernatants of the VIP-treated keratinocytes (Fig. 10H).



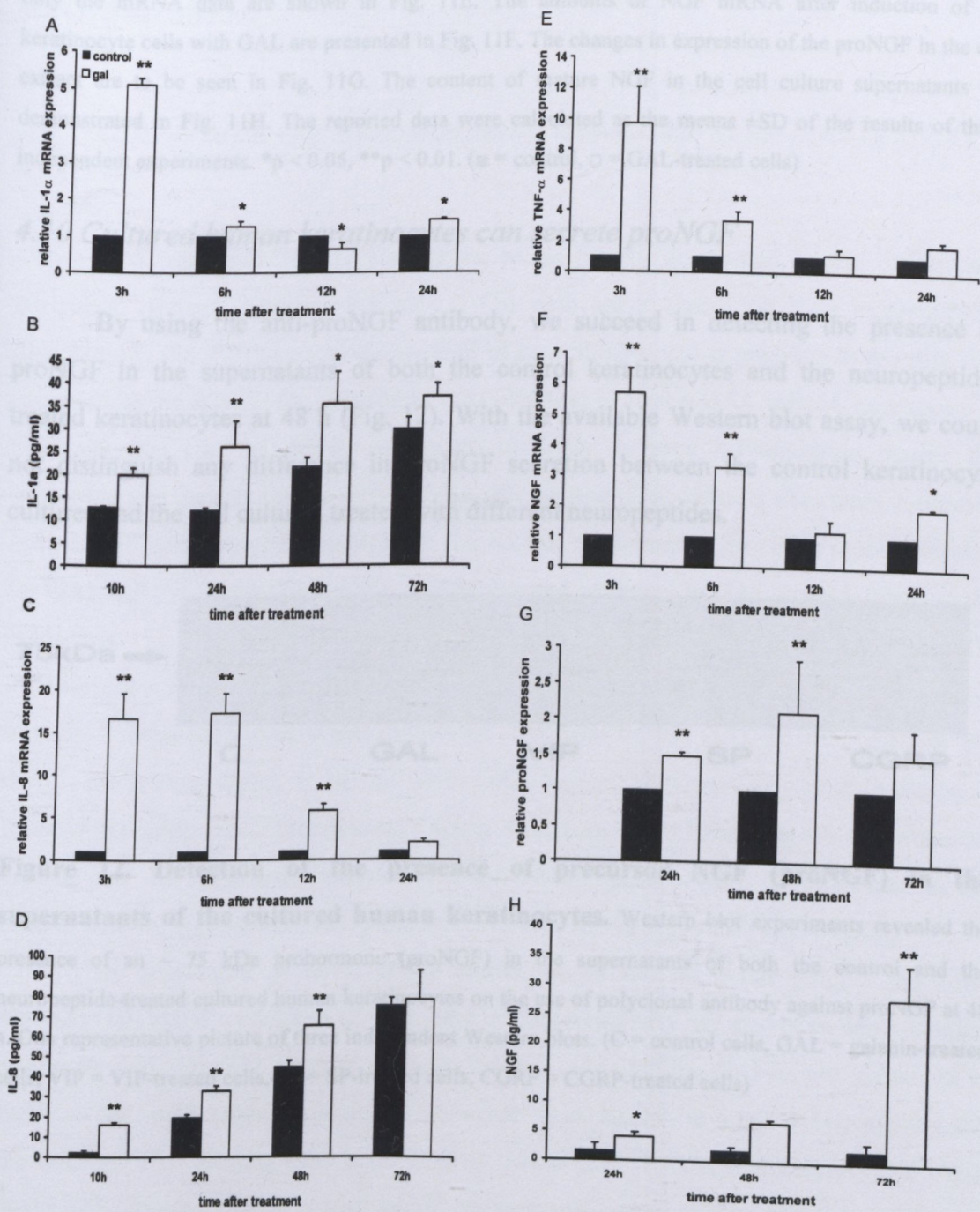
**Figure 10. The effects of VIP induction on the expressions of proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and the NGF/proNGF system in cultured human keratinocytes** Cultured human keratinocytes were treated with VIP at  $10^{-8}$  M for 30 min. The effects of the treatment on the levels of IL-1 $\alpha$  mRNA and IL-1 $\alpha$  protein are presented in the Figs. 10A and 10B, and the changes in the expression of IL-8 mRNA and the secreted IL-8 protein concentration in Figs. 10C and

10D, respectively. TNF- $\alpha$  could not be detected by ELISA in the cell culture supernatants, and thus only the TNF- $\alpha$  mRNA data are to be seen in Fig. 10E. The alterations in NGF mRNA expression in time are shown in Fig. 10F. The proNGF content in the keratinocyte cell extract is demonstrated in Fig. 10G, and the mature NGF concentration in the cell culture supernatants in Fig. 10H. The reported data were calculated as the means  $\pm$ SD of the results of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . (■ = control, □ = VIP-treated cells)

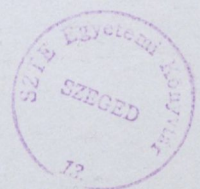
#### *4.9 Effects of GAL on the expressions of the proinflammatory cytokines (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) and NGF/proNGF in cultured human keratinocytes*

The effects of GAL on human keratinocytes are not well known. Accordingly, it appeared obvious to examine its effects on certain cytokines and the proNGF/NGF system expressed by keratinocytes. After induction of the cultures with GAL, we found a significant 5-fold increase in the IL-1 $\alpha$  mRNA expression at 3 h, and there was then a fluctuation in the IL-1 $\alpha$  mRNA level as compared with the untreated cultures (Fig. 11A). A significant rise of from 1.5-fold up to 2.1-fold significant rise in the secreted IL-1 $\alpha$  protein level in the supernatant was observed from 10 h up to 72 h (Fig. 11B). A more dramatic, 17-fold increase was seen in the IL-8 mRNA expression of the GAL-treated keratinocyte cultures at 3 h and 6 h after the GAL treatment, and a rise of about 5-fold could be detected even at 12 h (Fig. 11C). The secreted IL-8 protein levels were also significantly increased relative to the untreated cell cultures, with a 6.5-fold rise at 10 h (Fig. 11D). GAL upregulated the TNF- $\alpha$  mRNA expression from 3 h up to 6 h (9.7-fold and 3.3-fold) (Fig. 11E). TNF- $\alpha$  protein could not be demonstrated by means of ELISA assay (data not shown). GAL exerts marked effects on the expression of NGF/proNGF by the keratinocytes. An increase of approximately 5-fold was observed in the amount of NGF/proNGF mRNA at 3 h after induction with GAL, and we found 3 times as much NGF/proNGF mRNA as in the control cultures at 6 h (Fig. 11F). Investigation of the cell lysates by Western blotting demonstrated significant 1.4-fold and 2.1-fold increases in the proNGF level at 24 h and 48 h, respectively, as compared with the non-induced cultures. (Fig. 11G). Interestingly, the level of mature NGF secreted into the GAL-treated cell culture supernatants was highest (14-fold) at 72 h (Fig. 11H).





**Figure 11. The effects of GAL on the expressions of proinflammatory cytokines and the NGF/proNGF system by cultured human keratinocytes** The changes in time of the IL-1 $\alpha$  mRNA expression in the cultured human keratinocytes after GAL induction (10<sup>-8</sup> M) are illustrated in Fig. 11A, and the alterations in the secreted IL-1 $\alpha$  protein concentration in Fig. 11B. The changes in expression of IL-8 mRNA and IL-8 protein are depicted in Figs. 11C and 11D, respectively. For TNF- $\alpha$ ,

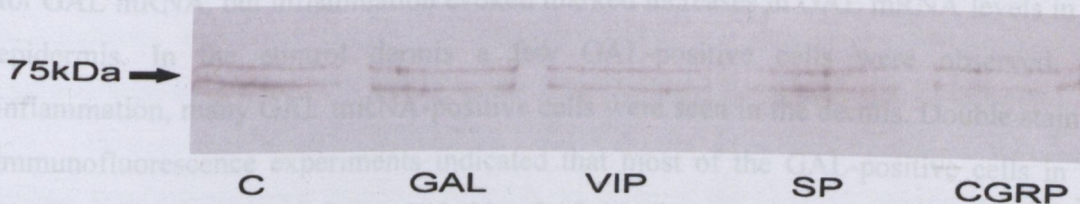




only the mRNA data are shown in Fig. 11E. The amounts of NGF mRNA after induction of the keratinocyte cells with GAL are presented in Fig. 11F. The changes in expression of the proNGF in the cell extract are to be seen in Fig. 11G. The content of mature NGF in the cell culture supernatants are demonstrated in Fig. 11H. The reported data were calculated as the means  $\pm$ SD of the results of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . (■ = control, □ = GAL-treated cells)

#### 4.10 Cultured human keratinocytes can secrete proNGF

By using the anti-proNGF antibody, we succeed in detecting the presence of proNGF in the supernatants of both the control keratinocytes and the neuropeptide-treated keratinocytes at 48 h (Fig. 12). With the available Western blot assay, we could not distinguish any difference in proNGF secretion between the control keratinocyte cultures and the cell cultures treated with different neuropeptides.



**Figure 12. Detection of the presence of precursor NGF (proNGF) in the supernatants of the cultured human keratinocytes.** Western blot experiments revealed the presence of an ~ 75 kDa prohormone (proNGF) in the supernatants of both the control and the neuropeptide-treated cultured human keratinocytes on the use of polyclonal antibody against proNGF at 48 h. One representative picture of three independent Western blots. (C = control cells, GAL = galanin-treated cells, VIP = VIP-treated cells, SP = SP-treated cells, CGRP = CGRP-treated cells)

## 5. Discussion

### *5.1 GALR2 is expressed by human keratinocytes both at the level of mRNA and protein*

GAL and the GALRs are known to be widely distributed in the central and peripheral nervous systems, but only a few data are available on their extraneuronal distributions (98,99). The first description of its extraneuronal expression in the skin was provided by Ji, et al. (56), who found GAL-binding sites and GAL mRNA expression in the epidermis and the dermis of the rat hindpaw. In their inflammation model, the epidermal and dermal expressions of GAL mRNA and GAL-binding sites were compared in inflamed versus normal rat skin. In the normal epidermis, a few cells labeled weakly for GAL mRNA, but inflammation evoked marked increases in GAL mRNA levels in the epidermis. In the control dermis a few GAL-positive cells were observed. On inflammation, many GAL mRNA-positive cells were seen in the dermis. Double staining immunofluorescence experiments indicated that most of the GAL-positive cells in the dermis are immunocytes. In normal skin, GAL-binding sites were observed in the basal layer of the epidermis. There was no distinct difference in GAL-binding sites between the normal and the inflamed epidermis. No GAL-binding sites were demonstrated in the normal dermis, whereas the inflammation evoked a strong signal of GAL binding.

The expression of GAL in the epithelium of the rat molar gingiva has been reported (10) and the presence of GAL-like immunoreactivity has also been demonstrated in human skin (55). The latter study revealed that GAL and the GALRs are expressed in neuronal and extraneuronal positions in human skin. Kofler, et al. (55) investigated the presence of GAL-binding sites in the skin by means of [<sup>125</sup>I]-GALR autoradiography and found GAL-binding sites around blood vessels and sweat glands, but they could not detect GAL-binding sites on the keratinocytes. In fact, there is a discrepancy between the lack of GAL-binding sites in the human skin as reported by Kofler, et al. and our own findings. We used a different methodology: we first identified mRNA for GALR2 in cultured human keratinocytes and then demonstrated GALR2 protein expression in cultured keratinocytes by Western blotting and by immunohistochemistry, using a

polyclonal antibody against human GALR2.

The functions of GAL are mediated by three distinct G-protein-coupled receptors, GALR1, GALR2 and GALR3. We investigated the mRNA expressions of all three GALR subtypes, using a PCR technique with GALR-specific primer pairs in HaCaT cells and cultured human keratinocytes. Human small intestine cDNA was used as control for GALR1 and GALR2 (100) and human brain cDNA for GALR3 (61). GALR1 was detected only when the small intestine cDNA was used as template; with the keratinocyte cDNA, we could not demonstrate any amplified product at the specific molecular weight. Interestingly, we detected an amplified product with around twice the molecular weight as compared with that found in the case of the human small intestine cDNA. The explanation for this might be dimerization of the amplified product, but additional investigations are required for a precise explanation. On the basis of the direct sequencing of the purified GALR2 PCR products, both the HaCaT cells and the cultured human keratinocytes exhibited GALR2 mRNA expression. We could not demonstrate the expression of GALR3 mRNA in either HaCaT cells or cultured human keratinocytes.

Besides the detection of GALR2 mRNA expression in HaCaT cells and cultured human keratinocytes, we demonstrated the presence of the GALR2 protein in both cell types. Both the HaCaT cells and the cultured human keratinocytes displayed specific immunohistochemical staining, with greater intensity along the cell surface of the keratinocytes. We used commercially available polyclonal antibody against human GALR2, directed against a specific sequence of the C-terminal end of the receptor. It has been reported (101) that it is difficult “to stain” 7-transmembrane, G-protein-coupled receptors because false-positive staining may occur. There are convincing data for the specificity of this antibody in the literature (70). Moreover, we have demonstrated the presence of GALR2 protein in the human brain and human keratinocyte protein extracts by using the same polyclonal antibody on Western blots. By performing absorption control experiments with the blocking peptide for the GALR2 antibody, we were able to completely block the GALR2-antibody binding in the Western blotting assay.

In our investigations of the biological relevance of the presence of GALR2 on keratinocytes, we observed a transient increase in the cytosolic  $[Ca^{2+}]_i$  during the GAL treatment of cultured human keratinocytes. This phenomenon is be apparently dependent



on GALR2 via the IP3 pathway, where the released  $\text{Ca}^{2+}$  may cause the activation of protein kinase C, starting the process of differentiation or proliferation of the keratinocytes (102).

Work with GAL knock-out and transgenic animals have indicated the possible trophic and developmental roles of GAL (53). In GAL knock-out mice, a subset of small neurons of dorsal root ganglia with small-diameter, unmyelinated axons (i.e. probably nociceptors) are missing and neuroregeneration in these animals is retarded *in vivo* (103). The nerve injury-induced dramatic upregulation of GAL in both the central and the peripheral systems is another indicator of the trophic effects of GAL (53,104). After axotomy, the GAL mRNA level is dramatically increased in the sensory neurons (105). Other microarray studies on sensory ganglia strongly suggest that similar mechanisms operate in both sensory and autonomic neurons (106).

Cutaneous nerve injury and inflammation give rise to increased GAL production in the skin by both neuronal and extraneuronal routes. GAL may stimulate keratinocytes via GALR2 and a protein kinase C pathway to produce nerve-regenerating factors, i.e. GAL can cause an upregulation of NGF mRNA expression in cultured human keratinocytes.

## *5.2 The neuropeptides SP, CGRP, VIP and GAL upregulate the inflammatory cytokine expression in cultured human keratinocytes*

Keratinocytes, key cellular components as concerns both the homeostasis and pathophysiological processes of the skin, secrete a number of cytokines and are stimulated by various biological modifiers (i.e. neuropeptides). In our study, cultured human keratinocytes were stimulated with neuropeptides, namely SP, CGRP, VIP, GAL. Throughout the study we used a constant concentration of stimulating peptide,  $10^{-8}$  M. We took into considerations which have been described for each neuropeptide to occur physiologically in the serum:  $10^{-9}$  M for SP (107),  $10^{-11}$  M for CGRP (108) and  $10^{-11}$  M for VIP (109). Data could not find concerning GAL serum concentration. We included relatively higher concentrations of stimulating peptides since serum concentrations of growth factors, hormones and neuropeptides not necessarily reflect the concentrations in the direct pericellular microenvironment, which can exceed serum levels. Based on our

unpublished preliminary experiments and other's studies (31,51,110) finally we choosed  $10^{-8}$  M as the constant concentration of the stimulating peptide. We have demonstrated that SP, CGRP, VIP and GAL, which can be present in cutaneous epidermal nerve endings, upregulate the expression of the inflammatory cytokine IL-1 $\alpha$  mRNA and the amount of secreted IL-1 $\alpha$  in cultured human keratinocytes. CGRP, VIP and GAL caused a rapid transient increase in IL-1 $\alpha$  mRNA expression, observed 3 h after treatment, and the concentration of secreted IL-1 $\alpha$  protein in CGRP, VIP or GAL neuropeptide-treated cultures were 50-60% higher 24 h after the treatment than in their time- matched controls. In our study, SP was found to exert late inductive effects on IL-1 $\alpha$  mRNA production (at 24 h) and on IL-1 $\alpha$  secretion (at 48 h and 72 h). All of the investigated neuropeptides upregulated the IL-8 mRNA expression to a considerable extent way 3 h to 6 h after the treatment, but the enhancements in the amounts of secreted IL-8 protein were modest. Interestingly, the degree of upregulation of IL-1 $\alpha$  mRNA expression was much lower than that of IL-8 mRNA, but the amounts of secreted IL-1 $\alpha$  and IL-8 displayed a reverse pattern. The explanation of this may be that the preformed IL-1 $\alpha$  protein in the cells contributes to the increase in the amount of secreted IL-1 $\alpha$ , while IL-8 must have been produced *de novo* after the neuropeptide stimulus. SP, CGRP and GAL all caused significant increases in TNF- $\alpha$  mRNA expression, but no secreted TNF- $\alpha$  could be detected in the cell culture supernatants. It is noteworthy that other authors experienced similar difficulties with the detection of TNF- $\alpha$  in the supernatants of cultured human keratinocytes by the ELISA method (111). TNF- $\alpha$  is initially synthesized as proTNF- $\alpha$ , a type II integral membrane protein, and is then cleaved proteolytically by specific enzymes (112). Therefore, the preformed TNF- $\alpha$  mRNA is not translated into protein or the TNF- $\alpha$  protein production may be stored intracellularly and further microenvironmental stimuli are needed for its release. Overall, keratinocytes are capable of accepting information from the nervous system via the released neuropeptides. Nerve-ending injuries or stimuli from the central nervous system can provoke the release of neuropeptides, which can then induce the production of IL-1 $\alpha$  and the potent chemokine IL-8, a key cytokine in the recruitment of neutrophils. Our experimental findings furnish

further evidence that neuropeptides may play a direct role in the pathogenesis of inflammatory dermatoses (113,114).

### *5.3 Normal human keratinocytes secrete proNGF and the neuropeptides SP, CGRP, VIP and GAL can increase both proNGF production and NGF secretion in cultured human keratinocytes*

In the second part of our study, we examined the proNGF/NGF expression produced by cultured human keratinocytes. Alternative splicing of the whole NGF gene can produce precursors of 27 and 35 kDa (115), which are cleaved to produce mature NGF (87). ProNGF can be further post-translationally modified by glycosylation, forming distinct molecular weight species (116,117,118). Our Western blotting experiments demonstrated for the first time that proNGF is the predominant form detected in the lysates of cultured human keratinocytes, at molecular weights of 75 and 50 kDa. At the lower molecular weight, we failed to demonstrate any form of proNGF or mature NGF in the cell lysates on using either anti-proNGF or anti-NGF antibody. Yardley et al. (117) reported the presence of the 73 kDa NGF prohormone in extracts of ovine flank skin, but they failed to reveal the presence of the mature 13 kDa NGF in the same extracts. Hasan et al. (119) recently detected the secretion of proNGF forms into the cell culture media by sympathetic neurons. This result is in accordance with those of our Western blot studies, which demonstrated the presence of the 75 kDa proNGF in the supernatants of cultured human keratinocytes. We were also able to detect the presence of mature NGF in the supernatants of cultured human keratinocytes. As mature NGF was not found in the extract of the keratinocytes, but it was present in the supernatants of the keratinocytes, it is possible that the 75 kDa proNGF was secreted by the cells and that extracellular proteases can cleave mature NGF (86). Lee et al. (86) further revealed that proNGF has a high affinity for the  $p75^{\text{NTR}}$  receptor and the proNGF –  $p75^{\text{NTR}}$  receptor interaction can lead to neuronal apoptosis (92). Since both proNGF and mature NGF were found in the supernatants of the cells, and the keratinocytes possess both TrkA and  $p75^{\text{NTR}}$  (120), it may be supposed that the balance between cell survival and cell death may be determined by the ratio of the secreted proNGF and the formed mature NGF.

On induction of the keratinocyte cultures with the different neuropeptides, the amount of NGF was changed distinctly. SP treatment of the keratinocytes caused significant increases in both NGF mRNA production and the amount of secreted NGF. Our results are in accordance with the findings of Burbach et al. (28). The effects of CGRP, VIP and GAL on proNGF/NGF production have not yet been investigated. We demonstrated that all four neuropeptides can increase the NGF mRNA expression, though to different extents. In the cell lysate of neuropeptide-treated keratinocytes, only proNGF can be detected, in a concentration about 2-fold that in the time-matched controls. Both the control cultures and the neuropeptide-treated cultures can secrete proNGF and mature NGF, but the neuropeptide-treated cell cultures produce markedly higher (3-7-fold) amounts of NGF-like immunoreactive materials. The exact proNGF/NGF ratio in the supernatants of cultured human keratinocytes treated with different neuropeptides is not known at present. This question necessitates further investigations.

Our results support the important regulatory capacity of the neuropeptides released from cutaneous nerves after an injurious stimulus. SP, CGRP, VIP and GAL are able to induce an upregulation of inflammatory cytokine (IL-1 $\alpha$ ) and chemokine (IL-8) production, and they can influence proNGF/NGF expression and secretion from keratinocytes. The concept of the direct influence of cutaneous nerves on the regulation of keratinocyte proNGF/NGF makes a contribution to our understanding of neural effects on skin health and disease.

## 6. Summary

Neuropeptides released from the cutaneous sensory nerve endings have neurotransmitter and immunoregulatory roles; they exert mitogenic actions and can influence the functions of different cell types in the skin. The one of these neuropeptides is the galanin, the role of which is less-known in the skin. Galanin (GAL) is a biologically active neuropeptide that is widely distributed in the nervous system. GAL exerts diverse action via the GAL receptors (GALR1, GALR2 and GALR3), which belong in the superfamily of G-protein-coupled transmembrane receptors. Although the GALRs are essential for biological functions, the expressions of different GALR subtypes in cultured human keratinocytes have not yet been investigated. The aim of our study was to investigate the mRNA and protein expressions of the different GALRs in the HaCaT immortalized keratinocyte cell line and in cultured human keratinocytes. When reverse transcription PCR was used with different GALR-specific primers, only GALR2 mRNA was identified in cultured HaCaT cells and keratinocytes. Sequencing of the PCR products proved the presence of GALR2 mRNA in the keratinocytes. The presence of GALR2 protein was next investigated, using a polyclonal antibody against human GALR2. Both the HaCaT cells and the cultured keratinocytes displayed specific immunohistochemical staining, with higher intensity on the surface of the keratinocytes. Immunohistochemical investigations of normal human skin specimens revealed that GALR2 was expressed with high intensity in the basal layer of the epidermis and also around the hair follicles in the dermis. GAL treatment of the keratinocytes resulted in an increase in cytosolic  $\text{Ca}^{2+}$  concentration, suggesting that GALR2 is a functional receptor.

The further aims of our study were a systematic investigation of the effects of the neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and galanin (GAL) on the inflammatory cytokine production (IL-1 $\alpha$ , IL-8 and TNF- $\alpha$ ) of the keratinocytes, and a study of their role in the production and secretion of nerve growth factor (NGF) and its precursor molecule (proNGF). Cultures of normal human keratinocytes were treated with  $10^{-8}$  M SP, CGRP, VIP or GAL for 30 min. After different time intervals, cells were harvested for total RNA isolation; in addition, cell lysates and supernatants were collected. The effects of the

neuropeptides on the mRNA expressions of the different cytokines and NGF were investigated by Q-RT-PCR and the protein levels were studied by means of ELISA assays and Western blotting. Each of the four neuropeptides induced increases in the expressions of IL-1 $\alpha$ , IL-8 and TNF- $\alpha$  mRNA. Increases appeared in the amount of the IL-1 $\alpha$  protein in the supernatants of neuropeptide-treated cells, and the IL-8 secretion was mildly elevated, while secretion of TNF- $\alpha$  remained undetectable. The four neuropeptides increased the NGF mRNA expression to different extents. In the cell lysates of the keratinocytes, only proNGF could be detected, its concentration in the neuropeptide-treated cells being approximately twice that in the time-matched controls. Both control cultures and neuropeptide-treated cultures were found to secrete proNGF and mature NGF, but neuropeptide-treated cell cultures produced markedly higher (3-7-fold) amounts of NGF-like immunoreactive materials. The results demonstrated that neuropeptides released from cutaneous nerves after an injurious stimulus are able to induce an upregulation of IL-1 $\alpha$  and IL-8 production; they are additionally able to influence the expressions of proNGF/NGF and their secretion from the keratinocytes. These findings may contribute toward an understanding of the neural influence on skin health and disease.

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