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The neuropeptide galanin is a novel inhibitor of human hair growth

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none declared

What´s already known about this topic?

- The long-lasting research on the influence of neuropeptides on hair physiology developed a long list of hair follicle-influencing neuropeptides.

- Previous immunohistochemical studies on galanin in the skin suggest a potential role of galanin in hair physiology.

What does this study add?

- Human HFs not only show galanin expression, but also constitute a target of galanin, as galanin significantly inhibits hair shaft elongation and influences the hair cycle.

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Summary

Background Galanin is a trophic factor of the central and peripheral nervous system that shows widespread distribution in human skin. However, the exact localization and the role of galanin in the hair follicle (HF) remain to be clarified.

Objectives Therefore, we have characterized galanin expression in human scalp HFs and have examined the effects of galanin on normal human scalp HF growth in organ culture.

Methods Immunohistochemistry was performed on cryosections of human female scalp skin. Anagen HFs were microdissected and cultured up to 9 days and treated with 100 nM galanin. Staining for Ki-67, TUNEL and Masson-Fontana were used to analyze proliferation, apoptosis and hair cycle staging of the HFs. Functional galanin effects were tested in serum-free HF organ culture.

Results Galanin-like immunoreactivity (-LI) was detected in the outer root sheath (ORS) and inner root sheath. Additionally, galanin mRNA was detected in ORS keratinocytes and all HF samples tested. Galanin receptor transcripts (GalR2, GalR3) were also detected in selected samples. Galanin reduced proliferation of hair matrix keratinocytes *in situ* compared to vehicle-treated controls, shortened the hair growth phase (anagen) *in vitro* and reduced hair shaft elongation. This was accompanied by the premature development of a catagen-like morphology of galanin-treated HFs.

Conclusions Thus, we present the first evidence that human HFs are both, a source and a functionally relevant target of galanin. Due to its hair growth-inhibitory properties *in vitro*, galanin application deserves further exploration as a potential new treatment strategy for unwanted hair growth (hirsutism, hypertrichosis).

Introduction

In normal human skin presence of many neuropeptides including substance P (SP), neurokinin A, somatostatin, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), neurotensin, neuropeptide Y (NPY), galanin, and α- and γ-melanocyte stimulating hormone has been reported.[1-5] All of these peptides are moderately expressed in nerve fibers and almost all in hair follicles (HFs).[4,5]

The neuropeptide galanin is a 30 amino acid, C-terminally amidated peptide initially isolated from porcine intestine[6] but later found in many other species. Galanin has been shown to have a widespread distribution in the central and peripheral nervous systems of many mammalian species and a diversity of biological effects.[7-13]

In human skin, besides its localization on afferent sensory neurons and specialized cutaneous sensory structures such as Merkel cells and Meissner´s corpuscles,[3,14-18] galanin-like immunoreactivity (-LI) was detected in nerves beneath the epidermis and sweat glands.[16,19,20] Furthermore, galanin has been found in non-neuronal cells in the skin, such as in smooth muscle cells of dermal blood vessels, ductal cells of eccrine sweat glands, epidermal keratinocytes of the rat molar gingiva and rat skin as well as human follicular and interfollicular epidermal keratinocytes.[19-23] Additionally, secretion of galanin peptide has been observed in cultures of human primary keratinocytes.[21] Galanin can stimulate the expression of interleukins in cultured human keratinocytes.[24]

Three galanin receptor subtypes (GalR1-3), which belong to the superfamily of G-protein coupled transmembrane receptors, have been cloned and pharmacologically characterized to date.[25] Galanin binding sites have been detected in the basal zone of the epidermis as well as in inflamed dermis of the rat hind paw.[20] In human skin, galanin binding sites have been found in the vicinity of dermal arteries and arterioles and around sweat glands.[21] Furthermore, Dallos et al. detected GalR2-immunoreactivity around hair follicles in the dermis.[10]

The human HF is an unique mini-organ, capable of life-long cycles of massive growth (anagen), regression (catagen), and resting (telogen). Regulation of proliferation of follicular keratinocytes is an important feature of HF morphogenesis.[26] The HF is not only a very productive source of pigmented hair shafts (keratins and melanin) but also of many growth-, pigment-, and immunomodulators. It can synthesize or

metabolize an enormous number of hormones, neurotransmitters, neuropeptides and growth factors.[27] Due to their keratinocyte proliferation-inducing capacities, neuropeptides are suspected to be important players in epithelial tissue homeostasis and remodeling of cutaneous tissue and skin appendages.[28] The first description of influence of neuropeptides on hair growth was the study from Paus et al. in 1994, which describes the induction of hair growth by substance P (SP).[29]

The most abundant neuropeptides in HFs are adrenocorticotropic hormone (ACTH),[30] α-melanocyte-stimulatin hormone (α-MSH),[31] corticotropin-releasing hormone (CRH),[30,32] prolactin,[33,34] SP,[35] and thyrotropin-releasing hormone (TRH).[36] Some of these stimulate HF growth and/or pigmentation and are also are recognized key mediators in systemic responses to psychoemotional stress. It is expected that many additional neuromediators are produced by the HF and may impact on HF growth and pigmentation.[37]

Although expression of galanin has been described in follicular and interfollicullar keratinocytes,[29] the exact localization and the role of galanin in human HFs has not yet been investigated. Therefore, we have characterized the expression of galanin in human HFs, and have studied the functional effects of galanin on normal, organ-cultured human scalp HFs.

We show for the first time that galanin mRNA and protein are expressed in human microdissected organ-cultured HFs. In addition, we show that galanin inhibits HF growth and induces premature catagen in cultured anagen VI scalp HFs.

Material and methods

Galanin immunohistochemistry

Sections of normal scalp skin were used to detect galanin-LI in the HF. Cryosections (6 µm) were air dried, fixed in acetone at –20°C, washed with PBS, blocked with 10% normal goat serum and incubated with rabbit anti-galanin primary antibody (T-4326 1:10000; Bachem, Bubendorf, Switzerland) overnight at 4°C. The detection system used was the LSAB method (Level 2 Ultra Streptavidin Detection System, Signet Laboratories Inc., Dedham, MA) with HistoGreen (Linaris, Wertheim-Bettingen, Germany) as peroxidase substrate.

A pre-absorption of the primary antibody was performed with synthetic human galanin peptide (Phoenix Pharmaceuticals, Burlingame, CA). The antibody was incubated with 5 µM peptide for 2 – 3 hours at 37°C. After centrifugation at 13 000 rpm for 10 minutes the supernatant was used for immunohistochemistry.

Human hair follicle culture

The study was approved by the Institutional Research Ethics Committee and adhered to Declaration of Helsinki guidelines.

Temporal scalp skin of female patients (mean age: 59·7 ± 9 years) was obtained from routine face-lift surgery after obtaining informed consent.

Serum-free organ culture of microdissected human scalp HFs was performed as previously described in detail.[38,56] After 24 h preincubation, HFs were treated with 100 nM galanin (Phoenix Pharmaceuticals, Inc., Burlingame, CA). Follicles were maintained in an incubator at 37°C in 95% air and 5% CO_2 atmosphere. Medium was changed every other day. Control wells received fresh medium only. On day 0, 1, 3, 5, 7 and 9 hair shaft lengths were measured using an inverted binocular microscope (Stemi 2000-C; Carl Zeiss, Oberkochen, Germany) with an eyepiece measuring graticule, and statistically analysed with GraphPad Prism software (version 4).

Masson-Fontana histochemistry and hair cycle staging

For Masson Fontana staining, cryosections were air dried and fixed in ethanol-acetic acid. The sections were washed in tris-buffered saline (TBS) and distilled water several times. Cryosections were treated with ammoniacal silver solution (Fluka, Seelze, Germany) for 40 min at 56°C in the dark. After washing in distilled water, the sections were treated with 5% aqueous sodium thiosulphate (Merck, Darmstadt, Germany) for 1 min. Next, the sections were washed in distilled water for 3 min and counterstained with Mayer´s hematoxylin (Merck). After washing under running tap water, sections were dehydrated and mounted in Eukitt (O. Kindler, Freiburg, Germany). Staging of HFs was performed after histomorphological criteria, following published guidelines.[39,40]

Isolation of total RNA and reverse transcription – polymerase chain reaction

Human eyebrow hairs of volunteers were plucked and outer root sheath keratinocytes (ORSK) were isolated as previously described in detail.[41-43]
Isolation of the RNA of 15-40 HFs and ORSK, respectively, was carried out by using Tri-Reagent (Molecular Research Centre, Cincinnati, OH, U.S.A.) according to the manufacturer´s protocol. DNAse I treatment (Ambion Inc., Austin, TX, U.S.A.) was performed and 2 µg of total HF RNA were used for cDNA synthesis (Superscript II reverse transcriptase; Invitrogen, Paisley, UK), and 1 µg of total ORSK RNA for cDNA synthesis with High Capacity cDNA kit (Applied Biosystems, Carlsbad, CA). PCR was performed on a Biometra T personal thermocycler (Biometra, Goettingen, Germany) using PerfeCTa SYBR Green Low Rox Mix (Quanta Biosciences, Gaithersburg, MD) and 200 nM primers (galanin forward: 5´-AGGTCATTCAGCGACAAGAAT-3´, reverse: 5´-CCGAAGATTATCCATTATCTT-3´, product size: 290 bp; GalR1 forward: 5´-CACTTGCATAAAAAGTTGAAG-3´, reverse: 5´-TTATCACACATGAGTACAATTGG-3´, product size: 378 bp; GalR2 forward: 5´-CTGCTCTTCGCGCTCATCTTCCTC-3´, reverse: 5´-AGGTTGGCCAGCTGCGACTG-3´, product size: 428 bp; GalR3 forward: 5´-GTTCATCCTCAACCTGGCG-3´, reverse: 5´-GTAGCTGAGGTAGGGCGC-3´, product size: 305 bp) (Eurofins MWG, Ebersberg, Germany) and the following temperature parameters: an initial denaturation step at 95°C for 3 min was followed

by 50 cycles of 95°C for 10 sec, 62°C for 10 sec and 72°C for 10 sec and a final extension of 1 min at 72°C.

Ki-67 and TUNEL staining

To compare proliferation and apoptosis of HFs in different hair cycle stages double immunolabelling of mouse anti-Ki-67 antiserum (DAKO, Glostrup, Denmark) and TUNEL (ApopTag Fluorescein In Situ Apoptosis detection kit; Millipore, Berlin, Germany) was performed as described previously.[36,44,45]

Results

Human hair follicles express galanin *in vivo* and *in vitro*

Previously, diffuse galanin-LI has been reported in the interfollicular and follicular epidermis, dermal arteries and innervating nerves and ductal cells of sweat glands, while its exact expression pattern in human HFs remains to be determined.[10,19,21] Therefore, we first characterized the exact distribution of galanin-LI in human HFs. In agreement with the literature[17], we were able to detect galanin-LI in the interfollicular epidermis of female skalp skin (Fig. 1a). Galanin-LI was abolished with 5 µM synthetic galanin peptide (Fig. 1b), which indicates the specificity of the antibody used.

In human scalp skin, galanin-LI was detected in the outer root sheath (Fig. 1c) of the HF, in smooth muscle cells of the arrector pili muscle (APM) (Fig. 1d) in the HF bulge region (i.e. the seat of epithelial and mesenchymal HF stem cells) [46] and in the inner root sheath of the examined HFs (Fig. 1e). Interestingly, keratinocytes of the hair bulb (around the dermal papilla) (Fig. 1f) and the mesoderm-derived connective tissue sheath showed no galanin-LI.

In agreement with our immunohistochemical analysis galanin mRNA was detected in ORSK as well as in all microdissected HF samples analysed (Fig. 2a).

No GalR1 mRNA was found in either in ORSK or in HF samples (Fig. 2b). GalR2 mRNA was found in both ORSK samples and in 2 out of 4 of the HF samples (Fig. 2c). Also GalR3 mRNA was detected in the ORSK samples, but only in 1 out of 4 of the HF samples (Fig. 2d). The primers used for PCR are located on different exons, and the bp fragment can therefore not be amplified from contaminating genomic DNA. Galanin receptor expression could not be followed-up on the protein level, because no reliable galanin receptor antibodies are available so far.[47]

Taken together, these data show that human scalp HFs are a prominent source of galanin expression on the gene and peptide level. The presence of galanin receptor transcripts (GalR2, GalR3) in several HF RNA extract samples suggests the possibility of auto- and/or paracrine signalling interactions of intrafollicularly produced galanin with locally expressed cognate receptors.

Galanin inhibits hair shaft elongation in human organ-cultured hair follicles

Therefore, we next investigated whether galanin directly exerts any growth-modulating effects on human HFs, which would suggest that human scalp HF express fully functional galanin receptors. Galanin (100 nM) was added to the microdissected hair bulbs of organ-cultured human anagen VI HFs (n=3). Microdissected human HFs from female temporal scalp skin were stimulated in serum-free organ culture with galanin or vehicle (control) for up to 9 days. The elongation of untreated and galanin-treated HFs was measured on day 0, 1, 3, 5, 7 and 9.

Human HF cultures (n=3, Supplementary Fig.) treated with 100 nM galanin every other day overall showed a significantly reduced hair shaft elongation rate compared to untreated controls ($p<0{\cdot}05$ after 7 and 9 days; Fig. 3a). In the control group, there was an increase in hair shaft length of 70.5% ± 32.7 after 9 days in contrast to 59·5% ± 25·4 in the galanin-treated group (Fig. 3a).

Galanin prematurely induces a catagen-like stage in organ-cultured human hair follicles

Using melanin histochemistry, a sensitive indicator of the hair cycle stage.[39,40], quantitative hair cycle histomorphometry revealed that galanin was able to accelerate spontaneous catagen development in human anagen HFs *in vitro*. Fig. 3b shows the staging after 9 days culture. A trend to more advanced catagen-stages is found in galanin-treated HFs and more anagen-stages are found in untreated HFs, respectively. Calculation of the hair cycle score (HCS), which allows to summarily assess and compare the full range of catagen stages between experimental groups, [48,49] showed a higher value for the group treated with 100 nM galanin (Fig. 3c). Furthermore, a lower HCS value for control HFs indicated that catagen development was advanced in galanin-treated HFs compared to untreated HFs.

Combined Ki-67 and TUNEL immunohistomorphometry was performed to assess the percentage of proliferating and apoptotic hair matrix cells compared to the total amount of cells (DAPI+) in this area. Though these data did not reach the level of significance when only anagen VI HFs were compared with each other in test and control groups, they showed a trend towards reduced proliferation (i.e. a lower

number of Ki-67-positive cells) in the hair matrix of galanin-treated HFs than in vehicle controls; no differences were seen with respect to the percentage of apoptotic hair matrix keratinocytes of test versus control hair bulbs (data not shown).

Discussion

Here, we demonstrate that the outer and inner root sheaths (ORS, IRS) of human scalp HFs express galanin peptide and that HF and/or cultured ORSK transcribe GalR2 and GalR3 mRNA. Therefore, the human HF is both a target and source of galanin. That galanin shortened anagen, induced premature catagen development, and inhibited hair shaft elongation in HF organ culture suggests that endogenously produced galanin serves as a novel molecular “brake” on human HF cycling.

Since HFs represent a major potential port of microbial entry into the skin, yet rarely show clinical signs of infection,[50] the presence of local, efficient, anti-infection defense systems in the distal HF epithelium is increasingly being appreciated.[51,52] One function of the galanin gene and peptide expression in human HF keratinocytes might therefore be the production and secretion of galanin message-associated peptide (GMAP) as an antimicrobial peptide: For example, GMAP is encoded on the galanin precursor peptide and inhibits growth of *Candida* species and the transition of *Candida albicans* from the budded yeast growth form to the invasive hyphal growth form.[53,54] The current study underscores that some of these antimicrobial peptides,[50] such as galanin, apparently double as hair growth modulators. This makes their systematic exploration in investigative and clinical dermatology even more pertinent, and raises the intriguing question which function developed first during evolution: its growth-modulatory or its antimicrobial activity?

While at least some human HF and/or ORSK RNA extracts contain GalR2 and GalR3 transcripts, unfortunately, our attempts to clarify the galanin receptor subtype that is chiefly responsible for the observed hair growth-inhibitory effects by co-administration of selective receptor subtype-specific agonists and antagonists gave inconclusive results and could not be continued due to the extreme difficulty to obtain sufficient human HF specimen. The great interindividual variability of human HF organ culture responses to galanin stimulation (e.g. a significant decrease of HF proliferation by

galanin administration was only observed in the HF organ cultures from two out of three distinct individuals (Supplementary Fig.1)) may in part reflect interindividual differences in galanin receptor expression or receptor polymorphisms.
The expression of GalR2 mRNA detected in the present study is in agreement with the observation of Dallos et al., that GalR2 is present around HFs.[10]

The hair growth-inhibitory effects of galanin place it into the ever-growing rank of neuropeptides and peptide neurohormones recognized to modulate human hair growth, such as SP,[35] TRH,[36] prolactin,[33] calcitonin gene-related peptide (CGRP),[55] and melanocortins such as α-MSH or ACTH.[26] Interestingly, several studies have already shown that the human hair growth inhibitors, galanin and SP, which are co-expressed around HFs,[19] interact along various pathways, mainly in skin inflammation. For example, galanin inhibits cutaneous plasma extravasation induced by SP.[13,56] Furthermore, galanin has been suggested to modulate neurogenic edema formation via a presynaptic mechanism by inhibiting release of SP.[57]
SP is also involved in hair cycling and anagen/catagen transition by progression toward catagen after injection of SP in mice, mimicking the effect of stress exposure.[58]

All these neuromediators and the relevant signal transduction systems remain to be systematically targeted for therapeutic purposes so as to develop urgently needed novel classes of human hair growth inhibitors or stimulators. The current data suggest that galanin can serve as a new lead compound in the search for novel agents that reduce or prevent unwanted hair growth. On the basis of the current findings, GalR2 and GalR3 receptor agonists deserve further systematic exploration as candidate anti-hirsutism and anti-hypertrichosis agents, while cognate antagonists might be recruited for hair growth-stimulation purposes.

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Figure legends

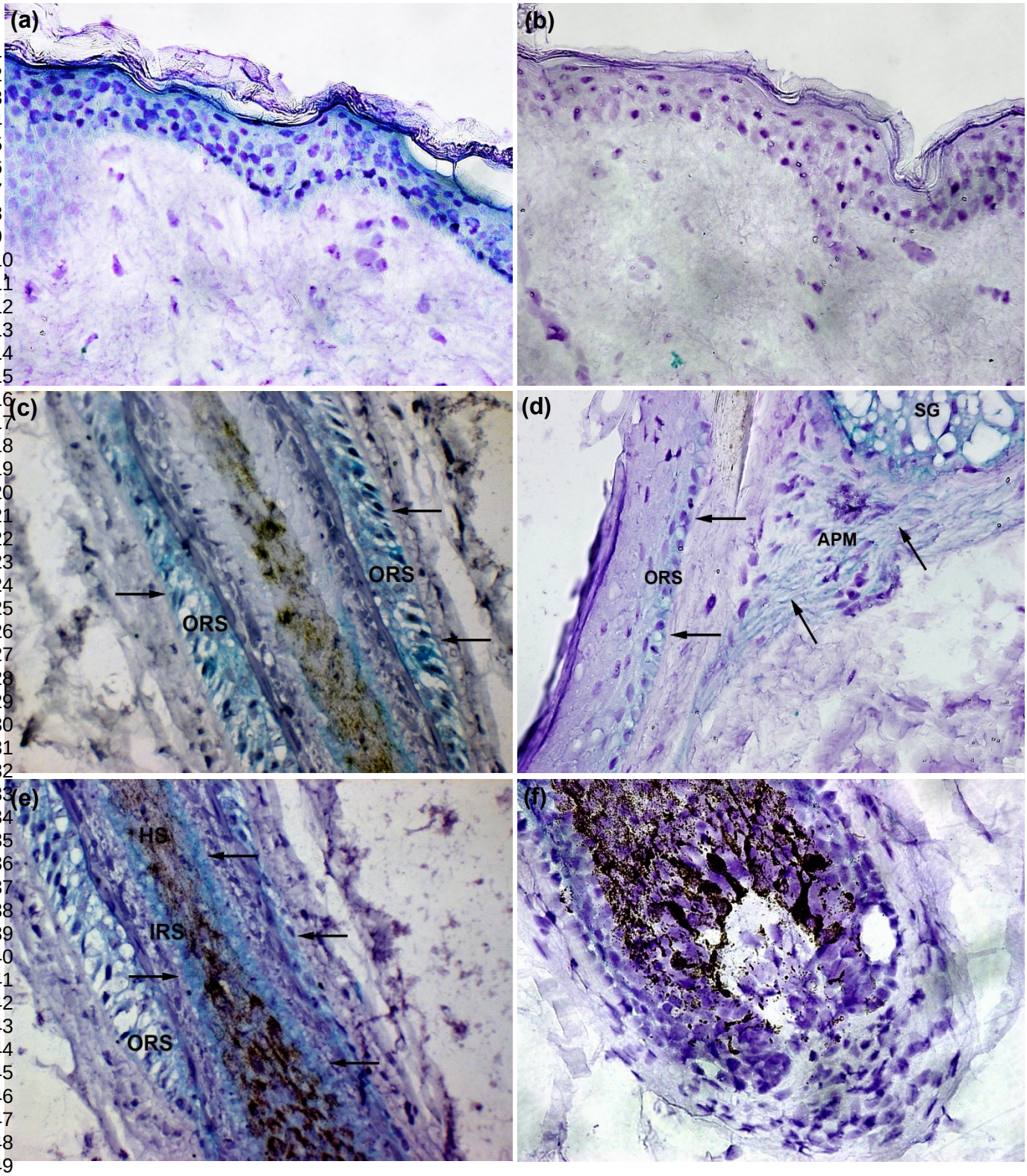
Fig 1. Galanin-LI (green-blue colour) in the epidermis of human scalp skin (a). Pre-absorption with synthetic peptide (5 µM) (b). Galanin-LI was found in the outer root sheath of hair follicles (c), in the bulge region and smooth muscle cells of the arector pili muscle (d), and the inner root sheath (e). Green staining is indicated by black arrows. No galanin-LI was found in the hair bulb (f). APM, arrector pili muscle; HS, hair shaft; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland.

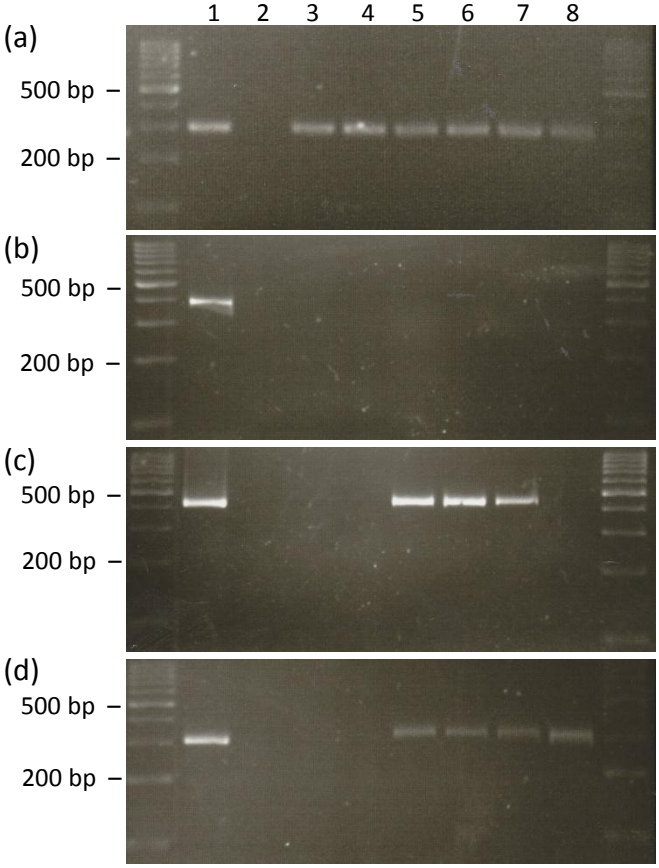
Fig 2. Expression of (a) galanin, (b) GalR1, (c) GalR2 and (d) GalR3 mRNA in two different outer root sheath keratinocytes (lane 5: male; lane 6: female), and four HF samples (female; lanes 3+4 and 7+8). M = 100 bp ladder. lane 1: positive control (galanin: cDNA from human keratinocytes from neonatal foreskin; GalR1: cDNA from SH-SY5Y-R1[59]; GalR2: cDNA from SH-SY5Y-R2[59]; GalR3: cDNA from HEK-R3[60]). lane 2: negative control; no cDNA added.

Fig 3. Hair shaft elongation of three independent cultures of untreated and galanin-treated cultures (n=62-135 per day and group), cultured up to 9 days. * indicates significant change compared to untreated controls, $p < 0{\cdot}05$. Error bars indicate standard error of the mean. (a) Percentage of HFs of two independent cultures (HF08-109, 08-120) at defined hair cycle stages after 9 days culture. (b) Calculation of the hair cycle score (HCS). All HFs of each group were staged and each stage of the hair cycle has been scored as follows: anagen VI = 100, early catagen = 200, mid catagen = 300, late catagen = 400. The HCS indicates the mean of the stages of all HFs per group. (c)

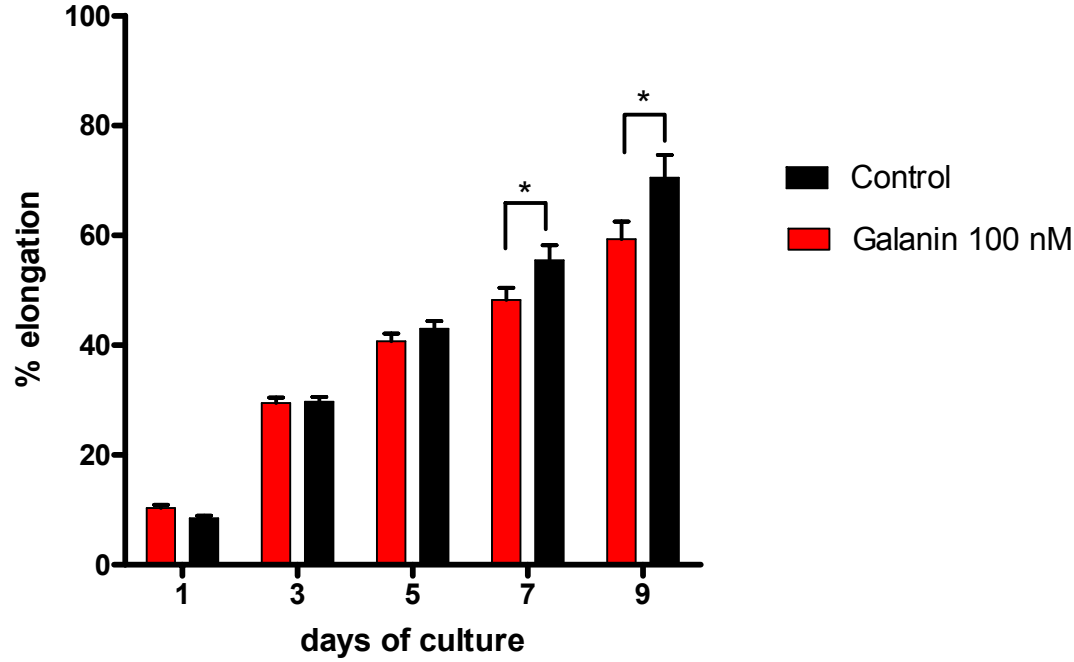
Supplementary Fig.

Hair shaft elongation of untreated and galanin-treated hair follicles of three independent cultures (n=62-135 HFs per day and group), cultured up to 9 days. HF08-109, 08-120 and 10-047 are lab-internal culture numbers. * indicates significant change compared to untreated controls, $p < 0{\cdot}05$

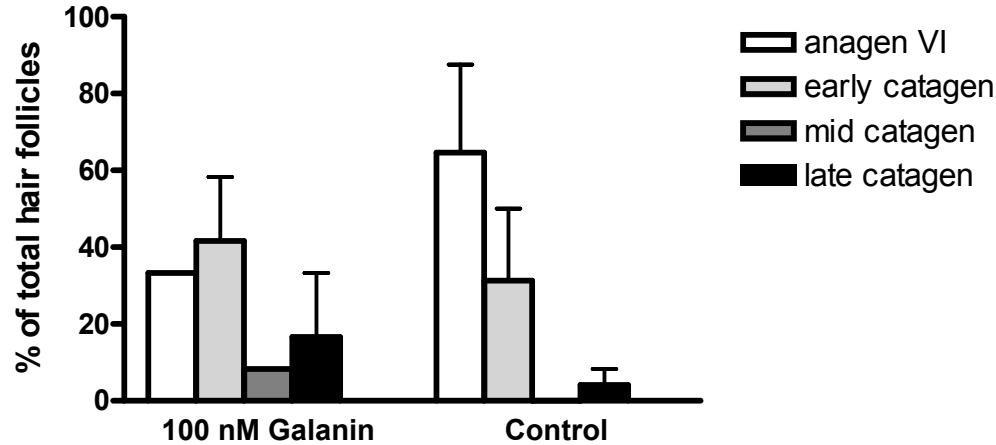

(a)
(b)
(c)
ORS
ORS
(d)
SG
APM
ORS
(e)
HS
IRS
ORS
(f)


M
+
-
HF
ORSK
HF
M
1
2
3
4
5
6
7
8
(a)
500 bp
200 bp
(b)
500 bp
200 bp
(c)
500 bp
200 bp
(d)
500 bp
200 bp

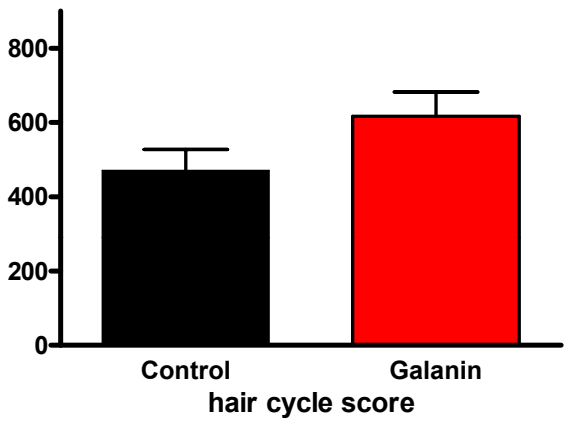
(a)


100
80
60
40
20
0
% elongation
*
*
1
3
5
7
9
days of culture
Control
Galanin 100 nM

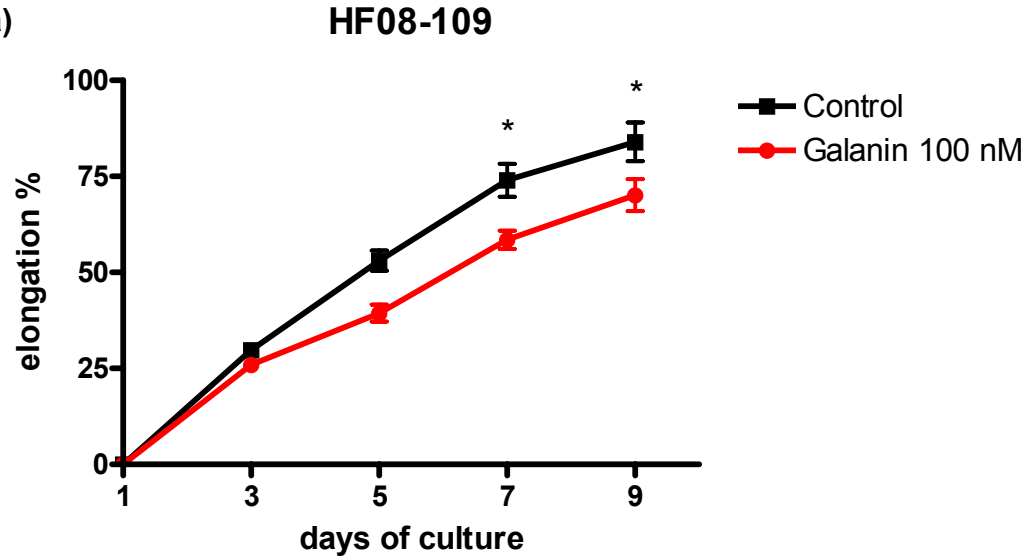
(b)


100
80
60
40
20
0
% of total hair follicles
100 nM Galanin
Control
anagen VI
early catagen
mid catagen
late catagen

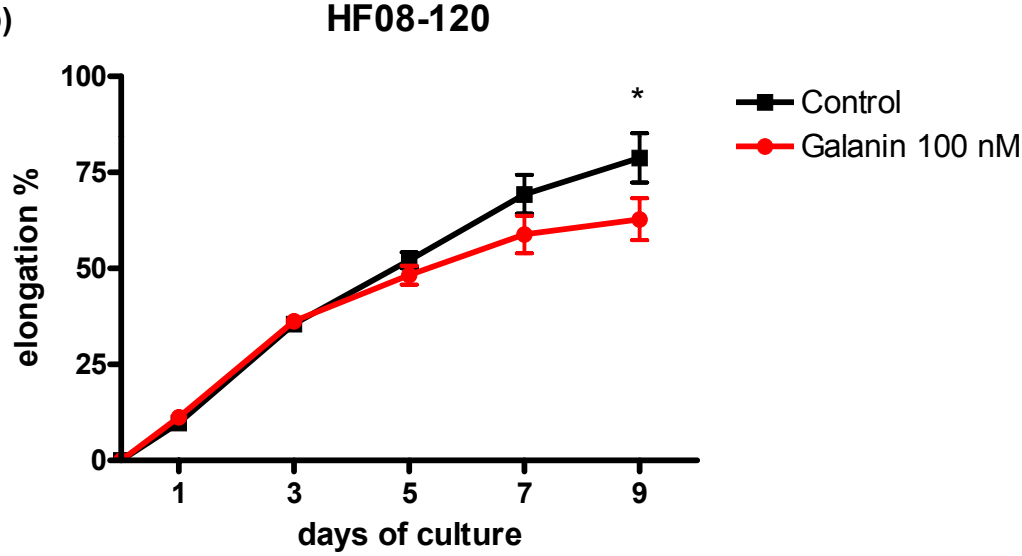
(c)


800
600
400
200
0
Control
Galanin
hair cycle score

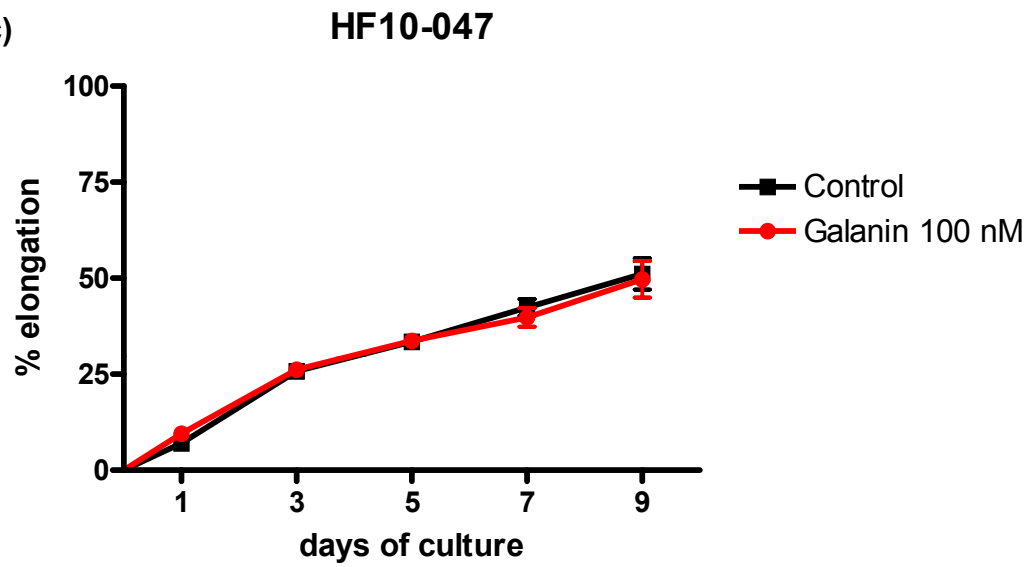
(a)


HF08-109
100
75
50
25
0
elongation %
1
3
5
7
9
days of culture
*
*
Control
Galanin 100 nM

(b)


HF08-120
100
75
50
25
0
elongation %
1
3
5
7
9
days of culture
*
Control
Galanin 100 nM

(c)


HF10-047
100
75
50
25
0
% elongation
1
3
5
7
9
days of culture
Control
Galanin 100 nM