Growth Factors, Cytokines, Cell Cycle Molecules

Probing the Effects of Stress Mediators on the Human Hair Follicle

Substance P Holds Central Position

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Stress alters murine hair growth, depending on substance P-mediated neurogenic inflammation and nerve growth factor (NGF), a key modulator of hair growth termination (catagen induction). Whether this is of any relevance in human hair follicles (HFs) is completely unclear. Therefore, we have investigated the effects of substance P, the central cutaneous prototypic stress-associated neuropeptide, on normal, growing human scalp HFs in organ culture. We show that these prominently expressed substance P receptor (NK1) at the gene and protein level. Organ-cultured HFs responded to substance P by premature catagen development, down-regulation of NK1, and up-regulation of neutral endopeptidase (degrades substance P). This was accompanied by mast cell degranulation in the HF connective tissue sheath, indicating neurogenic inflammation. Substance P down-regulated immunoreactivity for the growth-promoting NGF receptor (TrkA), whereas it up-regulated NGF and its apoptosis- and catagen-promoting receptor (p75NTR). In addition, MHC class I and β 2-microglobulin immunoreactivity were up-regulated and detected ectopically, indicating collapse of the HF immune privilege. In conclusion, we present a simplistic, but instructive, organ culture assay to demonstrate sensitivity of the human HF to key skin

stress mediators. The data obtained therewith allow one to sketch the first evidence-based biological explanation for how stress may trigger or aggravate telogen effluvium and alopecia areata. (Am J Pathol 2007, 171:1872–1886; DOI: 10.2353/ajpath.2007.061206)

It has been hotly disputed for decades, whether or not stress can exert direct effects on human hair growth, for example in stress-induced telogen effluvium or alopecia areata.^{1–4} This controversial debate has recently been revived by our finding that a defined perceived stressor-inescapable noise as a model for psychoemotional stress indeed exerts multiple and significant hair growth-inhibitory effects in the mouse.^{4–12} However, epidemiological and clinical studies, so far, have failed to demonstrate a definite causal link between psychoemotional stressors and hair growth alterations in the human scalp.³

Stress inhibits murine hair follicle (HF) keratinocyte proliferation, prematurely induces intrafollicular keratinocyte apoptosis and HF regression (catagen), and triggers mast cell-dependent neurogenic inflammation.^{4,5,7,9} This critically depends on substance P, the prototypic stress-associated neuropeptide in skin and neurogenic inflammation.^{4,5,9,12–16} Substance P operates as a central mediator of intracutaneous neurogenic inflammation,^{13,17} and increased contacts between substance P-containing nerve fibers and mast cells with subsequent increased mast cell degranulation and cellular infiltration have been observed after stress exposure in mice.^{4,12}

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Alongside substance P, nerve growth factor (NGF) has recently been described as a stress-associated growth factor^{4,18} and is one of the key catagen-inducing factors involved in stress-mediated hair growth inhibition in the mouse.⁶ This neurotrophin does not only maintain and enhance substance P⁺ cutaneous innervation, but it also serves as a potent epitheliotrophin in murine and human keratinocytes and receives increasing attention as a promoter of neurogenic inflammation.^{4,18–21}

Premature catagen is a common feature of any damage to the HF.²² Inflammation-associated damage and premature HF regression are frequently accompanied by ectopic up-regulation of major histocompatibility complex (MHC) class I molecules in the HF epithelium, the break down of the so-called HF immune privilege, for example in alopecia areata^{27–31} or after interferon (IFN)- γ .^{32,33} The subsequent lack of protection from autoimmune attack likely plays a key role in disease pathogenesis thus linking stress and HF autoimmunity.^{23–26}

Therefore, to determine whether prototypic stress mediators exert stress effects on human HFs in other than a complex *in vivo* setting,^{34,35} we have taken to a simplistic and reductionistic but highly informative *ex vivo* approach to perform a pilot test on the effects of substance P directly.^{36,37} Elaborating on this approach, we now have addressed the following specific questions:

- Do human scalp HFs express receptors for the prototypic stress-associated neuropeptide substance P, specifically neurokinin-1 receptors (NK1)?
- Are human HFs responsive to signaling induced by the NK1 ligand, substance P? Specifically, does substance P affect human hair shaft growth, hair matrix keratinocyte proliferation/apoptosis, and HF cycling *in vitro*?
- Does substance P activate mast cells of the HF connective tissue sheath (as a first indication that this stress mediator can induce neurogenic inflammation within the pilosebaceous unit)?
- Are there any indications that these substance P effects involve the key catagen inducer and prototypic stress-associated growth factor, the neurotrophin NGF?
- Does substance P have any effect on MHC class I-based HF immune privilege?

Materials and Methods

Tissue and HF Sources

Temporal scalp skin was obtained from disposed excess skin samples derived from healthy patients undergoing elective plastic surgery (face lifting) after obtaining informed consent, following Declaration of Helsinki principles. A total of 20 different donor samples were obtained. After excision, tissue was maintained in Williams' E medium (Biochrom KG Seromed, Berlin, Germany) for transportation at 4°C for up to 24 hours. On arrival in the laboratory facility, the samples were divided: one part was immediately snap-frozen in

liquid nitrogen for immunohistochemistry and RNA extraction, and another part was processed for HF organ culture as described below. Immunohistochemistry was performed on each sample, and culture experiments were performed on at least five donor samples per group if not otherwise indicated in the figure legends.

Human HF Organ Culture

Anagen VI HFs were isolated by microdissection and cultured in Williams' E medium (Biocrom AG) supplemented with insulin (10 μ g/ml; Sigma, Taufkirchen, Germany), hydrocortisone (10 ng/ml) (Sigma), penicillin (Sigma), streptomycin (Sigma), and L-glutamine (2 mmol/L) (Sigma) using 24-well culture plates as published before.^{36,38,39} Each well contained 500 μ l of Williams' E medium and vehicle as well as test substances, which were added 100 times concentrated in a total volume of 5 μ l. Control HFs received only vehicle. Test groups were treated with 10^{-6} , 10^{-8} , and 10^{-10} mol/L substance P (Sigma) for a period of 3 days starting with day 1 (after dissection). The doses studied were selected because they are routinely used in neuropeptide studies with good effect on mast cells and keratinocytes.^{40–43,64} Higher concentrations yielded toxic effects in our culture model (not shown).

On days 0, 1, and 3, each well was photodocumented, and the total length of each HF was measured using an inverted microscope fitted with an eyepiece measuring graticule. Follicle length was defined as the distance between the tip of the protruding hair shaft and the base of the HF. Medium was replaced and fresh supplements added on day 1, and HFs were harvested for immunohistochemistry or RNA extraction on day 3 after isolation. To this effect HFs were washed in three rinses of sterile isotonic phosphate-buffered saline (PBS) and either embedded in Histogel embedding medium (Vector Laboratories, Peterborough, UK) or left without embedding medium before rapid snap-freezing in liquid nitrogen as described previously.²¹ Samples were then stored at -80°C until cryosectioning. For histochemical and immunohistochemical processing, 8- μ m-thick longitudinal cryosections through human scalp skin HFs were mounted on glass slides, fixed in ice-cold acetone for 10 minutes, and left for 1 hour to air-dry before staining procedures.

Only organ culture experiments, in which the control HFs showed at least the expected minimal growth activity (ie, $\sim 40\%$ hair shaft elongation during the culture period), were included in the current study. HF cultures with substandard HF growth in the control group (ie, 20% or less elongation during the culture period, indicating suboptimal HF preservation during surgery, transport, and/or microdissection) were excluded from analysis.

Hair Cycle Staging

Cultured HFs were staged after 3 days in culture by Ki-67-terminal dUTP nick-end labeling (TUNEL) double assay as described below, following previously pub-

Table 1. Antihuman Primary Antibodies

Antigen	Species	Source	Dilution	Technique
NK1	Rabbit, polyclonal	Novus, Littleton, CO	1:400	EnVision
NGF	Rabbit, polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	1:4000 1:50	TSA Standard immunofluorescence
p75	Mouse	Sigma, Taufkirchen, Germany	1:4000	TSA
TrkA	Rabbit, polyclonal	Santa Cruz Biotechnology	1:100	Standard immunofluorescence
c-Kit	Mouse, monoclonal	DAKO, Hamburg, Germany	1:100	TSA
MHC class I (HLA-ABC)	Mouse, monoclonal	DAKO	1:100 1:1000	EnVision TSA
β_2 Microglobulin	Mouse, monoclonal	BD Biosciences San Jose, CA	1:1000	EnVision
Ki-67	Mouse, monoclonal	DAKO	1:20	Standard immunofluorescence

lished morphological characteristics of the murine and human hair cycle⁴⁴ adapted for cultured human anagen HF as described previously.^{21,45,46} Briefly, HF were classified as anagen VI, early catagen, mid catagen, or late catagen. For statistical analysis (hair cycle score), HF in anagen VI were arbitrarily attributed a score of 0, in early catagen a score of 1, in mid catagen of 2, and in late catagen of 3. The sum of scores per group was then divided by the number of investigated HF. The mean value of these scores is a reliable quantitative indicator of the mean HF stage that has been reached on average by a larger population of HF after culture.

Routine Histochemistry

Sections prepared as described above were processed for Giemsa (Merck, Darmstadt, Germany) staining for the detection of mast cells in the connective tissue sheath after 3 days in culture as described elsewhere.⁴⁷ In brief, Giemsa was applied at a 1:10 dilution with 2% sodium borate solution for 45 minutes at room temperature. Differentiation was achieved using 0.02% acetic acid under microscopic control, and slides were dehydrated and mounted. Mast cells were classified as degranulated when eight or more granules could be found outside the cell membrane, as published before.^{4,5,6,7,9,12,47}

Standard Immunofluorescence

Sections prepared as described above were used to detect NGF, TrkA, and Ki-67 expression. After blocking nonspecific binding with 10% normal goat serum (DAKO, Hamburg, Germany) in Tris-buffered saline or PBS, pH 7.4, primary antibodies (Table 1) were applied in PBS with 2% normal goat serum overnight at 4°C. After washing, fluorescein isothiocyanate- or tetramethyl-rhodamine isothiocyanate-labeled goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA) was added for 1 hour at 37°C in PBS with 2% normal goat serum. All sections were counterstained with 4',6-diamidino-2'-phenylindol-dihydrochlorid (DAPI; Boehringer Mannheim, Mannheim, Germany) for identification of cell nuclei. All immunohistochemical staining steps

were performed in light-protected humidity chambers and interspersed by washing steps in Tris-buffered saline or PBS, respectively.

Tyramide-Substrate Amplification (TSA) Immunofluorescence

For the detection of NK1, p75, c-Kit (CD117), and MHC class I molecules (HLA-ABC) (Table 1) in human full thickness skin biopsies and in isolated HF after 3 days in culture, the TSA method was applied to sections prepared as described above, following the manufacturer's instructions [TSA plus tetramethylrhodamine (TMR) system; Perkin-Elmer, Boston, MA]. Briefly, after blocking of endogenous peroxidase activity, sections were first incubated with the respective primary antibody overnight at room temperature followed by incubation with biotinylated goat anti-rabbit (1:200; Jackson ImmunoResearch) or anti-mouse antibody (1:200; Beckman Coulter, Marseille, France) for 1 hour at 37°C. Finally biotinylated secondary antibodies were labeled with streptavidin-horseradish peroxidase (TSA), and the reaction was amplified by fluorescein or tetramethylrhodamine isothiocyanate-tyramide amplification reagent (TSA) at room temperature. DAPI counterstaining and washing steps were performed as described above.

Ki-67-TUNEL Double Assay

Sections were fixed in 1% paraformaldehyde and post-fixed in 2:1 ethanol to acetic acid at -20°C. TUNEL staining was performed as instructed by the manufacturer (ApopTag Plus fluorescein *in situ* apoptosis detection kit; Chemicon International, Hampshire, UK) and combined with the above-described standard immunofluorescence staining of Ki-67.

Alkaline Phosphatase-Based EnVision Technique for Light Microscopy

For the staining of NK1, MHC class I molecules (HLA-ABC), and β_2 -microglobulin, we used the alkaline phos-

phatase-based EnVision technique developed by DAKO as described before.^{25,48}

Controls for Immunohistochemistry

For negative controls the primary antibody was omitted and the secondary antibody applied without prior primary antibody incubation. For positive controls, well established staining patterns were determined: immunoreactivity in dermal and subcutaneous nerve fiber bundles for NK1, immunoreactivity in subcutaneous nerve fiber bundles for NGF, strong immunoreactivity of the basal layer of the epidermis for TrkA, immunoreactivity in single nerve fibers and small nerve fiber bundles in the subepidermal dermis for p75, immunoreactivity in dermal and subcutaneous mast cells and in epidermal melanocytes for c-Kit, immunoreactivity in all nucleated skin cells except for the proximal anagen hair bulb for MCH class I and β_2 -microglobulin, and basal layer epidermal and hair matrix keratinocytes for Ki-67. For TUNEL staining, murine spleen cryosections were used as positive control.

Photodocumentation and Histomorphometry

The sections were examined under an Axiophot microscope with a fluorescence device (Zeiss, Jena, Germany), and photodocumentation was performed with the help of a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan). All histomorphometric analyses were performed on photodocumented staining patterns. Mean fluorescence intensity was measured by the NIH-image system (Sun Microsystems, Santa Clara, CA) in two previously defined reference areas as indicated in the figures. Data were pooled per group, and statistically significant differences from control were determined by the Mann-Whitney *U*-test for unpaired samples. All results are expressed as means $\pm$ SEM.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (0.8 μ g) was reverse-transcribed using the First Strand cDNA synthesis kit (Roche, Mannheim, Germany) in an oligo-d(T)15-primed 20- μ l reaction. One μ l of cDNA was amplified by PCR using the Core PCR kit (Qiagen). The following primers were used: 5'-CTGCTGGATAAACTTCTTCAGGTAG-3' and 5'-AGGACAGTGACGAACATTTCTGG-3' to amplify NK1 mRNA, 5'-GCAATTCTGTGTTTCATCCGTTTG-3' and 5'-ATGTAGCCCAGCCAGTCAAC-3' to amplify a neutral endopeptidase (NEP) mRNA, 5'-GGCAGTATAATCCAAAGATGGTCAA-3' and 5'-GTCTGGCTTATATCCAACACTTCGT to amplify huPRT-R, 5'-CCAAGGGAGCAGCTTTCTATCC-3' and 5'-GGACATTGCTCTCTGAGTGTGGT-3' to amplify NGF mRNA, 5'-TGGACAACCCTTTCGAGTTC-3' and 5'-AGCCACCGAGACCCAA-3' to amplify TrkA mRNA and 5'-CCTACGGCTACTACCAGGATGA-

G-3' and 5'-TGGCCTCGTCGGAATACG to amplify p75NTR mRNA. The reactions were cycled for 5 minutes at 94°C, then 30 seconds at 94°C, 1 minute at 58°C, 1 minute at 72°C for 35 cycles, and then 10 minutes at 72°C. For NK1 and NEP, gut RNA served as positive control; for p75, NGF, and TrkA, full-thickness skin served as positive control.

Results

Normal Human Scalp Skin Anagen HF Express Prominent Immunoreactivity for the Substance P Receptor, NK1

In healthy human scalp skin biopsies, prominent NK1 receptor-like immunoreactivity was found on the epidermal keratinocytes of the stratum granulosum (Figure 1), whereas expression in the anagen VI HF was localized to the distal, suprainfundibular outer root sheath (ORS), the inner root sheath (most prominently in the proximal HF)

Figure 1. Native human scalp skin and native human anagen VI HF express immunoreactivity for the NK1 receptor. The schematic summaries display the most highly reproducible NK1 immunoreactivity patterns observed in human anagen scalp skin HF. Cryosections (immunohistochemistry, IHC) from human scalp skin samples containing 80 to 90% anagen HF were stained with antisera against NK1 (for details see Materials and Methods). All images demonstrate representative immunoreactivity patterns generated with the TSA technique in anagen HF. Boxes indicate anatomical location of the presented micrographs. Color in schematic drawings and bright red immunolabeling in photomicrographs indicate localization of positive immunoreactivity. Original magnifications, $\times 200$.

Figure 2. Substance P promotes development of a catagen-like hair bulb morphology in cultured human anagen scalp skin HF. Human anagen scalp skin HF were cultured for 3 days in the presence of substance P. Hair shaft elongation was measured on days 0 and 3, and HF were harvested for immunohistochemistry and hair cycle staging on day 3. cts, connective tissue sheath; dp, dermal papilla; hs, hair shaft; irs, inner root sheath; m, matrix; ors, outer root sheath; pu, pigmentary unit. **A:** Hair shaft elongation is decreased in HF treated with substance P after 3 days in culture. The effect is dose-dependent, and the differences to control are significant by Mann-Whitney *U*-test for unpaired samples ($^{**}P < 0.01$, $^{***}P < 0.001$). Each group contains $n = 44$ to 78 HF derived from four different donors, which yielded highly reproducible results. **B, i–iii:** For hair cycle staging, labeling of proliferating cells by Ki-67-immunoreactivity (**i**) against a background of DAPI⁺ cell nuclei (**ii**) and apoptotic cells by TUNEL labeling (**iii**) was performed. We observed classical morphological changes in the form and size of the dermal papilla in relation to hair bulb and matrix diameter. For example, high numbers of proliferating cells can be observed in a control hair bulb with several layers of proliferating cells in the matrix all around the early catagen-like mildly dilated dermal papilla. No TUNEL⁺ cells can be detected. In HF treated with substance P, the HF epithelium containing proliferating cell nuclei is decreased in area and less Ki-67⁺ cells can be detected around the rounded up mid catagen-like dermal papilla. Note the TUNEL⁺ cells within the HF epithelium (**arrows**) and in the connective tissue sheath. **C:** Statistical analysis of control versus HF treated with 10^{-8} , 10^{-10} , or 10^{-12} mol/L substance P for 3 days shows an increase in hair cycle score, assessed as described in Materials and Methods, in substance P-treated HF indicating a high number of anagen VI HF in the control group and a high number of late catagen HF in the substance P groups. The differences to control are significant by Mann-Whitney *U*-test for unpaired samples ($^{*}P < 0.05$, $^{***}P < 0.001$). Each group contains $n = 10$ to 23 HF derived from four different donors, that yielded highly reproducible results. Original magnifications, $\times 200$.

Figure 3. NK1 and NEP expression in human hair bulbs is altered after 3 days in culture with substance P. Twenty human anagen scalp skin HF s derived from two different donors were cultured per group for 3 days in the presence of substance P as described in the Materials and Methods section. **A, i and ii:** Immunohistochemistry of human anagen scalp skin HF s after 3 days in culture against DAPI staining of nuclei (**i**) reveals persistence of the NK1 expression in the inner root sheath and matrix of a control HF (**ii**). Treatment with substance P leads to a gradual decrease in NK1 expression with the strongest effect observable after 10^{-12} mol/L substance P. Please note the individual NK1⁺ cells that light up in the connective tissue sheath after 10^{-8} mol/L substance P (arrows). Basement membranes between dermal papilla and HF epithelia are indicated by white dotted lines. **B:** RT-PCR reveals a strong NK1 expression in the gut and in isolated control HF s. HF s treated with substance P show decreased NK1 expression. **C:** RT-PCR reveals a weak NEP expression in the gut and in isolated control HF s. HF s treated with substance P show increased NEP expression. Original magnifications, $\times 200$.

(Figure 1), and the nucleated hair shaft (Figure 1). With exception of distal ORS keratinocytes, all other NK1⁺ cell populations were characterized by lack of proliferation and ongoing terminal differentiation, leading to complete keratinization.

Weak NK1 immunoreactivity was observed throughout the basal layers of the epidermis, the HF matrix, and the ORS (Figure 1). These compartments show high proliferation and low differentiation. Outside of epidermis and HF epithelium, strong NK1 immunoreactivity was observed in the sebaceous gland (Figure 1) and nerve fibers (not shown), and somewhat weaker expression was observed in dermal microvascular cells and sweat glands (not shown).

Substance P Inhibits Hair Shaft Elongation and Promotes Development of a Catagen-Like Morphology in Organ-Cultured Human Anagen HFs

As early as 3 days after microdissection, we observed a significant reduction in hair shaft elongation in HFs treated with 10⁻⁸ and 10⁻¹⁰ mol/L substance P (Figure 2A). Reduced hair shaft elongation was accompanied by development of a catagen-like morphology in hair bulbs of HFs treated with various concentrations of substance P, as evidenced by a rounded dermal papilla, a decreased area containing Ki-67⁺-proliferating keratinocytes in the hair bulb epithelium, and the occurrence of TUNEL⁺ apoptotic cell nuclei in the HF epithelium (Figure 2B). This shift toward a catagen-like morphology was significant as evidenced by quantitative hair cycle histomorphometry (Figure 2C).

Substance P Reduces NK1 Expression in the Hair Bulb and Up-Regulates NEP

After 3 days in culture with various concentrations of substance P, we observed a striking down-regulation of NK1 expression in the HF matrix and ORS (Figure 3A). This immunohistomorphometric observation was confirmed by decreased levels of NK1 mRNA products (Figure 3B). Complementarily, the down-regulation of the most prominent cutaneous substance P receptor was accompanied by an up-regulation of the substance P-

degrading enzyme NEP, as determined by RT-PCR (Figure 3C).

Substance P Increases Mast Cell Degranulation and Decreases the Total Mast Cell Number in the Connective Tissue Sheath

Human anagen VI scalp skin HFs cultured for 3 days with 10⁻¹⁰ mol/L and 10⁻¹² mol/L substance P showed increased numbers of degranulated mast cells in their connective tissue sheath close to the ORS (not shown). In HFs treated with 10⁻¹⁰ mol/L, virtually all histochemically detectable mast cells were degranulated as evidenced by Giemsa staining (Figure 4A) and histomorphometric analysis (Figure 4B). Interestingly, after treatment with the highest concentration of substance P, 10⁻⁸ mol/L, no mast cells were detectable by Giemsa labeling (not shown), which only labels mast cells if granules are present.

To determine whether mast cells were still present in the connective tissue sheath of HFs treated with 10⁻⁸ mol/L substance P, we performed c-Kit staining to determine total mast cell numbers (Figure 4C). This staining labels both mature, granulated mast cells and immature, ungranulated mast cells alike, all of which express c-Kit on their cell surface.⁴⁹⁻⁵¹ We observed a dose-dependent effect of substance P on c-Kit⁺ connective tissue mast cell numbers. The strongest decrease was observed after 10⁻⁸ mol/L substance P, but there was still a significantly lower number of c-Kit⁺ CTS mast cells after stimulation with 10⁻¹² mol/L substance P (Figure 4D).

Substance P Up-Regulates Intrafollicular NGF and p75NTR Protein Expression and Down-Regulates TrkA Immunoreactivity

Furthermore, treatment of cultured human anagen HFs with substance P up-regulated intrafollicular immunoreactivity for NGF (Figure 5, A and D), the catagen-promoting neurotrophin,²¹ which is also appreciated as the prototypic neurotrophin up-regulated during systemic responses to perceived stress.⁶ At the same time, the low-affinity pan-neurotrophin receptor p75, which plays a central role in NGF-driven apoptosis

Figure 4. Human connective tissue sheath mast cells in culture respond to substance P treatment by degranulation and maturation *in situ*. Thirty human anagen scalp skin HFs derived from three different donors (Giemsa) and 18 human anagen scalp skin HFs derived from two different donors (c-Kit) were cultured per group for 3 days in the presence of substance P as described in the Materials and Methods section. **A:** HFs were labeled by Giemsa staining. On the **left** two examples of connective tissue mast cells in control HFs are shown. **Arrows** point at densely stained, compact mast cells. No individual mast cell granules can be distinguished within or detected outside the cell membrane. On the **right** two examples of degranulated mast cells in HFs treated with an intermediate concentration of substance P (10⁻¹⁰ mol/L) are shown. **Arrowheads** point to enlarged cells with sparse granulation. Metachromatic mast cell granules can clearly be distinguished, whereas the cell membrane containing them is hard to identify. **B:** Statistical analysis of control versus HFs treated with 10⁻¹⁰ mol/L substance P for 3 days shows a low percentage of degranulated mast cells in the connective tissue sheath of control HFs as compared to 100% degranulated mast cells as defined in the Materials and Methods section in HFs treated with 10⁻¹⁰ mol/L substance P. The differences to control are significant by Mann Whitney *U*-test for unpaired samples (***P* < 0.01). **C:** c-Kit immunoreactivity labels primarily melanocytes in the pigmentary unit (pu), blood vessels in the dermal papilla (dp), and mature as well as immature connective tissue sheath (cts) mast cells (**arrowheads**). Expression in all compartments is here shown to be high in a control HF. Three days of treatment with 10⁻⁸ mol/L substance P dramatically decreases the number of c-Kit⁺ mast cells in the connective tissue sheath but also the c-Kit expression in the pigmentary unit and in blood vessel endothelia. The same effect is present, although less pronounced, after treatment with 10⁻¹⁰ mol/L and 10⁻¹² mol/L substance P. **D:** Statistical analysis of control versus HFs treated with 10⁻⁸, 10⁻¹⁰, or 10⁻¹² mol/L substance P for 3 days shows a high number of c-Kit immunoreactive mast cells in the connective tissue sheath of control HFs as compared to substance P-treated HFs. The effect is dose-dependent, and the differences to control are significant by Mann-Whitney *U*-test for unpaired samples (***P* < 0.01). Original magnifications: ×560 (**A**); ×200 (**C**).

Figure 5. Substance P alters immunoreactivity for NGF and its receptors (TrkA, p75NTR) in organ-cultured human hair bulbs. Twenty-one human anagen scalp skin HF s derived from three different donors were cultured per group for 3 days in the presence of substance P as described in the Materials and Methods section. Basement membranes between dermal papilla and HF epithelia are indicated by white dotted lines. **A:** NGF expression in a control hair bulb is present throughout the HF epithelium. In HF s treated with substance P this expression is enhanced, and additional weak expression can be observed in the dermal papilla. Note the nuclear pronouncement of the staining pattern. **B:** p75 pan-neurotrophin receptor expression is present in the ORS as well as the connective tissue sheath (dotted arrow) of a control HF. After treatment with substance P, the expression of p75 is greatly enhanced in the ORS and connective tissue sheath (dotted arrows), and additional weak staining can be observed in the proximal matrix (arrows) and individual cells of the ORS (arrowheads). **C:** Expression of the high-affinity NGF receptor TrkA locates to the ORS in a control HF. After substance P treatment this expression is virtually absent and only a weak expression can be detected in a HF treated with 10^{-12} mol/L substance P. **D:** The mean fluorescence intensity was measured at two previously defined reference areas as indicated in A–C. The observed effects are dose-dependent, and the differences to controls are significant by Mann-Whitney *U*-test for unpaired samples ($*P < 0.05$). **E:** NGF, p75NTR, and TrkA mRNA levels in cultured human HF s after 3 days of culture with or without SP. TaqMan RT-PCR data pooled from three different donors donating 3 to 12 HF s each are displayed. Each extraction, containing three HF s, was measured in duplicate, and produced highly reproducible results. Amount of mRNA is presented in percentage of control levels, when control levels equal 100. Original magnifications, $\times 200$.

promotion and HF regression,^{20,52} was up-regulated in the proximal ORS. Additional weak staining became visible in the proximal HF matrix after substance P treatment (Figure 5, B and D) (the same phenomenon is characteristically observed after catagen induction.^{20,52} In contrast, immunoreactivity for the high af-

finity, growth- and survival-promoting NGF receptor (TrkA)^{20,52} virtually disappeared from the ORS of HF s treated with 10^{-8} and 10^{-10} mol/L substance P and was greatly diminished in HF treated with 10^{-12} mol/L substance P (Figure 5, C and D). Data obtained by quantitative RT-PCR revealed a decrease in both

p75NTR and NGF transcription, whereas TrkA was below detection limit at the time of catagen-induction.

Substance P Up-Regulates Expression of MHC Class I and Associated Molecules and Induces Ectopic MHC Class I Expression in the Anagen Hair Bulb

Using two highly sensitive MHC class Ia immunostaining methods established for light (EnVision) and fluorescence (TSA) microscopy,²⁵ virtually no trace of MHC class I molecule immunoreactivity was found in the matrix, inner root sheath, and proximal ORS of vehicle-treated, organ-cultured human anagen VI HF (Figure 6A), whereas MHC class I was expressed within the fibroblastic compartments (the dermal papilla and connective tissue sheath) (Figure 6A), as expected from our previous studies.^{25,53}

In contrast, we observed an up-regulation of MHC class I expression in the connective tissue sheath and the dermal papilla of organ-cultured anagen scalp skin HF by 10^{-8} , 10^{-10} , and 10^{-12} mol/L substance P (Figure 6A). More importantly, there was also aberrant, ectopic MHC class I immunoreactivity in hair matrix keratinocytes (Figure 6A), and in some cases in the inner root sheath and ORS of the HF (not shown). After substance P treatment, NIH image analysis revealed that the up-regulation of MHC class I expression in the dermal papilla and HF matrix was significant in all three treatment groups (Figure 6B). The effect was strongest in HF keratinocytes of follicles treated with 10^{-8} mol/L substance P and appeared to be dose-dependent (Figure 6B).

As independent confirmation of these results, β_2 -microglobulin expression was seen to mirror the follicular expression of MHC class Ia: β_2 -microglobulin immunoreactivity was very low to absent in matrix keratinocytes and weakly present in the dermal papilla and the connective tissue sheath of control HF (Figure 6C), whereas treatment with substance P strongly up-regulated β_2 -microglobulin in the connective tissue sheath (Figure 6C). In addition, aberrant β_2 -microglobulin expression became detectable in the HF matrix with the strongest up-regulation and additional expression in the ORS after 10^{-8} mol/L substance P (Figure 6C).

Discussion

In this instructive and clinically relevant organ culture assay for exploring the direct effects of stress mediators on a complex human miniorgan, we demonstrate sensitivity of the human HF to the key stress-associated neuropeptide, substance P. We show expression of cognate receptors (NK1) on the gene and protein level, hair growth inhibition and premature catagen induction by substance P, down-regulation of NK1, and up-regulation of substance P-degrading enzymes in the HF epithelium. Just as for the substance P-dependent stress response previously seen in and around the HF of mice *in vivo*,^{5-7,9,12,31,55} the *in vitro* response of human HF to stimulation with this key

stress-associated neuropeptide exhibited signs of neurogenic inflammation. In addition, substance P up-regulates the intrafollicular expression of NGF and its apoptosis- and catagen-promoting receptor p75NTR, whereas the hair growth-promoting NGF receptor TrkA is down-regulated. Finally, and perhaps most importantly, substance P up-regulates (ectopic) MHC class I and β_2 -microglobulin expression, suggestive for a stress-associated collapse of the MHC class I-based HF immune privilege.

These data allow one to sketch four plausible hypothetical avenues along which psychoemotional stress via substance P may inhibit human hair growth: 1) down-regulation of proliferative substance P-signaling in HF keratinocytes; 2) deleterious neurogenic inflammation induced by substance P activation of perifollicular mast cells; 3) activation of catagen-inductive growth factor cascades; and 4) enhanced immune attack on the no-longer-immune-privileged HF epithelium. This offers the first evidence-based biological explanations for how stress may trigger or aggravate telogen effluvium and alopecia areata in human individuals (Figure 7).

The current focus on substance P was not only motivated by the central role that we had previously identified for this neuropeptide in murine stress-induced hair growth inhibition (see above) but also by the implication of substance P in stress-sensitive clinical conditions, in which psychoemotional stress is thought to trigger hair loss.^{3,27,28,31,54} Moreover, human keratinocytes and scalp HF express NK1 receptors.⁵⁶ In culture, substance P stimulates proliferation of keratinocytes⁵⁷ and organ-cultured murine epidermis.⁵⁸

That substance P inhibited hair matrix keratinocyte proliferation in our assay, rather than stimulating it, underscores the very distinct, often underestimated, regulatory controls of HF versus epidermal keratinocytes.⁵⁹⁻⁶¹ Moreover, the growth-promoting effects of substance P on keratinocytes are concentration-dependent and may rely on the presence of additional growth modulators in the culture medium.^{62,63} In an *in situ* context, ie, in a physiological epithelial-mesenchymal interaction milieu containing degranulating dermal mast cells, inhibition of keratinocyte proliferation has been shown before at concentrations used in our study.^{42,55,64} Low levels of cortisol as observed under chronic stress or chronic inflammation may feed into SP skin signaling because it enhances SP production by keratinocytes.¹⁴ Also, substance P reportedly induces selective tumor necrosis factor- α release by mast cells,⁶⁵ a cytokine recognized for its keratinocyte-apoptotic and hair growth-inhibitory effects.⁶⁶⁻⁶⁸

The observed down-regulation of NK1 within the HF epithelium and the up-regulation of intrafollicular NEP immunoreactivity after substance P administration (Figure 3), both of which may reduce the sensitivity of the HF epithelium to direct substance P-induced HF effects,⁶⁹ further support the hypothesis that indirect mast cell-mediated effects of substance P release play an important part at least in stress-associated hair growth inhibition.

The observed decline in the number of both more and less differentiated mast cells suggests two mechanisms

Figure 7. Hypothetical scenario: human hair growth inhibition by substance P. Hypothetical scenario depicting direct and indirect pathways by which substance P may inhibit human hair growth in the context of psychoemotional stress and neurogenic inflammation. On the **left** an anagen VI hair bulb is depicted. Gray cells with black membrane show location of proliferating Ki-67⁺ cells in the hair matrix that also express weak NK1 and TrkA immunoreactivity. On the **right** a regressing (mid catagen) hair bulb is depicted. Light gray cells indicate rarified proliferating Ki-67⁺ cells that also express NGF and some of which express p75NTR and show ectopic MHC I expression (gray cells with black membrane). Close by a nerve fiber in contact with degranulated mast cells and some monocytes are shown. Note that NK1, TrkA, p75NTR, and MHCII immunoreactivity outside the proliferative compartment are not schematized for easier comprehensibility. MKC, matrix keratinocyte.

by which mast cells may contribute to deleterious substance P HF effects: substance P activates CTS mast cells, thereby causing a degranulation-induced decline in the number of histochemically detectable mast cells, and changes in growth factor availability and cytokine expression, as commonly observed during inflammation and catagen-like development, here by substance P, may lead to mast cell apoptosis. Mast cell apoptosis seems to be a common mechanism in inflamed tissue to terminate the acute inflammatory response and prevent perpetuation and potential chronification.^{70,71} Decreased c-Kit ligand stem cell factor,⁷² increased tumor growth factor- β 1,⁷³ and up-regulated IFN- γ ,²⁴ all of which can induce mast cell apoptosis,^{71,74,75} may play a role in this process. However, possibly because of the very low number of apoptotic events in the mast cell compartment, this escaped detection with the TUNEL methodology used here (data not shown).

It is important to note here that our previous findings in the murine system suggest that substance P effects on the HF are strictly hair cycle-dependent: substance P inhibits late anagen HF growth *in vivo*^{9,31,55} but stimulates the growth of resting (telogen) HFs *in vivo*⁷⁶ and of early anagen HFs *in vitro*.⁴³ Because human early anagen HFs cannot easily be microdissected and studied in organ culture, it is as yet unknown whether a similar Janus-faced effect of substance P holds true for human HF biology.

In addition, substance P may inhibit human hair growth indirectly also by down-regulating TrkA expression and by up-regulating both p75NTR and NGF expression in the epithelium of anagen VI HFs.^{20,52,77} Like in the murine situation during late anagen-catagen transition, however, high NGF protein levels were accompanied by low NGF mRNA expression.⁷⁸ This seeming discrepancy may reflect a negative feedback loop installed toward the end of anagen-catagen transition to control the proapoptotic cascade boosted by NGF/p75NTR signaling during the short period of HF regression.

The neurotrophin signaling cascade has long been implicated in the termination of murine hair growth, especially via activation of the p75NTR.^{52,77,78} Moreover, we have shown that NGF is critically involved in stress-associated hair growth termination in mice^{4,6} and can terminate human anagen VI hair growth and induce premature catagen entry, using the same organ culture model as used here.²¹

Furthermore, the up-regulation of intrafollicular NGF expression by substance P demonstrated here must be expected to further aggravate inflammatory events, because NGF promotes, eg, inter- and perifollicular mast cell activation/proliferation.^{79–83} Experiments in the murine system show that stress-induced premature HF regression depends on NK1 expression and mast cells.⁷ It is therefore feasible to speculate that altered neurotrophin signaling is a result of neurogenic inflammation rather than of direct substance P-keratinocyte effects, especially because substance P⁺ nerve fibers appear close to HFs only after NGF expression has increased during cycling.⁸⁴ However, cell culture experiments demonstrate induction of NGF by substance P, whereas a direct effect on receptor expression remains to be shown.^{85,86}

Most strikingly, the up-regulation of MHC class I and β 2-microglobulin expression after substance P treatment indicates that substance P is capable of triggering a collapse of MHC class I-based immune privilege

Figure 6. Substance P treatment causes up-regulation and ectopic expression of MHC class I molecules and β 2 microglobulin in cultured human anagen VI HFs. Thirty human anagen scalp skin HFs derived from three different donors were cultured per group for 3 days in the presence of substance P as described in the Materials and Methods section. cts, connective tissue sheath; dp, dermal papilla; hs, hair shaft; irs, inner root sheath; m, matrix; ors, outer root sheath; pu, pigmentary unit; bv, blood vessel. **A:** Using EnVision and TSA technique weak MHC class I staining can be observed in the dermal papilla and connective tissue sheath of a control HF, whereas the HF epithelium is virtually free of immunoreactivity. HFs treated with 10^{-8} , 10^{-10} , or 10^{-12} mol/L substance P, as described in Materials and Methods, show increased MHC class I immunoreactivity in the fibroblastic compartments and additional immunoreactivity in the HF matrix generated with EnVision technique. Note the prominently stained connective tissue sheath blood vessels that are easily distinguishable in the sections stained with the TSA technique. This staining technique gives a more precise staining result but less anatomical detail. Reference areas used for determination of mean fluorescence intensity as shown in **B** are shown exemplarily in a HF treated with 10^{-8} mol/L substance P. Basement membranes between dermal papilla and HF epithelia are indicated by white dotted lines. **B:** The mean fluorescence intensity was measured at two previously defined reference areas in the matrix and dermal papilla, respectively, as indicated in **A** (number of investigated HFs per group = 20). The effect is dose-dependent, and the differences to controls are significant by Mann-Whitney *U*-test for unpaired samples (**P* < 0.05). **C:** β 2 Microglobulin is low in a control HF and mainly labels the connective tissue sheath and dermal papilla. In substance P-treated HFs, an up-regulation of β 2-microglobulin can be observed in dermal papilla and connective tissue sheath, whereas ectopic induction occurs in the proximal HF epithelium, especially in the HF matrix. Original magnifications, $\times 200$.

of human anagen HF. This may destroy the follicle's capacity to sequester anagen-associated autoantigens from immune recognition and renders affected HF's substantially more susceptible to autoaggressive inflammatory events in immunogenetically predisposed individuals with autoreactive T cells.^{23,24,26,87} To the best of our knowledge, substance P is here shown to be the first neuropeptide that can induce ectopic MHC class I expression on a normally immunoprivileged human tissue *in situ*. This activity places substance P alongside IFN- γ , which we had also shown to be capable of doing so, both in murine⁸⁸ and human HF's.²⁵ In this context, it is particularly intriguing to note that substance P can even stimulate IFN- γ expression in mononuclear cells.^{32,33} Given that other clinical autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and lupus erythematosus, have also long been felt to be aggravated by psychoemotional stress, as has already been demonstrated in corresponding animal models of these diseases,^{89–91} the current data encourage one to explore now the specific role of stress-associated neuropeptides such as substance P and their linkage to IFN- γ -induced autoaggressive inflammation in stress-induced triggering, progression, or relapse of other autoimmune diseases.

The leads found here with studying substance P complement our recent study with NGF (the key stress-associated growth factor) in this system^{77,91} and can now be followed up by studying the effects other major stress mediators, such as CRH, ACTH, cortisol (all of which are generated by human scalp HF's³⁷), and catecholamines, on a complex, exemplary human *in vitro* model system for physiologically relevant neuroectodermal-mesodermal interaction.⁹²

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