

# New effects of caffeine on corticotropin-releasing hormone (CRH)-induced stress along the intrafollicular classical hypothalamic–pituitary–adrenal (HPA) axis (CRH-R1/2, IP<sub>3</sub>-R, ACTH, MC-R2) and the neurogenic non-HPA axis (substance P, p75<sup>NTR</sup> and TrkA) in *ex vivo* human male androgenetic scalp hair follicles

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## Summary

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### Conflicts of interest

This study was supported in part by Dr. Kurt Wolff GmbH, and both T.W.F. and R.P. have served in a consultancy function for this company.

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**Background** Human hair is highly responsive to stress, and human scalp hair follicles (HFs) contain a peripheral neuroendocrine equivalent of the systemic hypothalamic–pituitary–adrenal (HPA) stress axis. Androgenetic alopecia (AGA) is supposed to be aggravated by stress. We used corticotropin-releasing hormone (CRH), which triggers the HPA axis, to induce a stress response in human *ex vivo* male AGA HFs. Caffeine is known to reverse testosterone-mediated hair growth inhibition in the same hair organ culture model.

**Objectives** To investigate whether caffeine would antagonize CRH-mediated stress in these HFs.

**Methods** HFs from balding vertex area scalp biopsies of men affected by AGA were incubated with CRH ( $10^{-7}$  mol L<sup>-1</sup>) with or without caffeine (0.001% or 0.005%). **Results** Compared to controls, CRH significantly enhanced the expression of catagen-inducing transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) ( $P < 0.001$ ), CRH receptors 1 and 2 (CRH-R1/2) ( $P < 0.01$ ), adrenocorticotrophic hormone (ACTH) ( $P < 0.001$ ) and melanocortin receptor 2 (MC-R2) ( $P < 0.001$ ), and additional stress-associated parameters, substance P and p75 neurotrophin receptor (p75<sup>NTR</sup>). CRH inhibited matrix keratinocyte proliferation and expression of anagen-promoting insulin-like growth factor-1 (IGF-1) and the pro-proliferative nerve growth factor receptor NGF-tyrosine kinase receptor A (TrkA). Caffeine significantly counteracted all described stress effects and additionally enhanced inositol trisphosphate receptor (IP<sub>3</sub>-R), for the first time detected in human HFs.

**Conclusions** These findings provide the first evidence in *ex vivo* human AGA HFs that the stress mediator CRH induces not only a complex intrafollicular HPA response, but also a non-HPA-related stress response. Moreover, we show that these effects can be effectively antagonized by caffeine. Thus, these data strongly support the hypothesis that stress can impair human hair physiology and induce hair loss,

and that caffeine may effectively counteract stress-induced hair damage and possibly prevent stress-induced hair loss.

### What is already known about this topic?

- Caffeine stimulates hair growth in male and female human hair follicles (HFs) *in vitro*.

### What does this study add?

- For the first time, corticotropin-releasing hormone induction of the hypothalamic–pituitary–adrenal (HPA) stress axis is documented in male human HFs from biopsies (balding vertex area) of men with androgenetic alopecia.
- First time, the non-HPA neurogenic stress axis is shown in the same male human HFs.
- Caffeine counteracts both stress axes.
- Inositol trisphosphate receptor was newly identified in human HFs.

### What is the translational message?

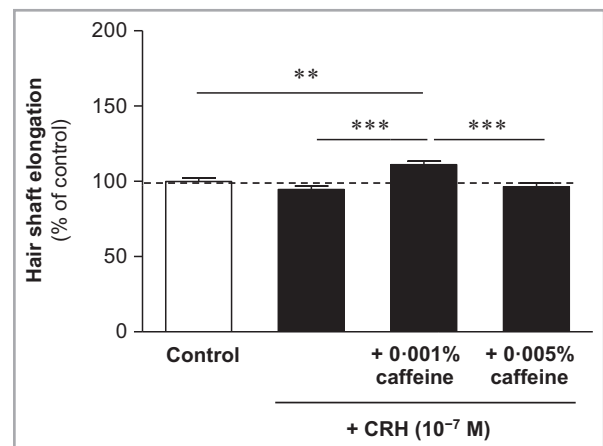
- Stress can impair human hair physiology and induce hair loss.
- Caffeine may effectively counteract stress-induced hair damage and possibly prevent stress-induced hair loss.

Hair loss is deeply stressful for affected patients,<sup>1–5</sup> while perceived stress might aggravate hair loss such as male and female pattern androgenetic alopecia (AGA) or diffuse alopecia.<sup>6–8</sup> Animal models have shown that stress impairs hair growth by catagen induction mediated by substance P (SP), mast cell activation and nerve growth factor (NGF),<sup>9–13</sup> as confirmed also in human hair follicles (HFs) *ex vivo*.<sup>14,15</sup>

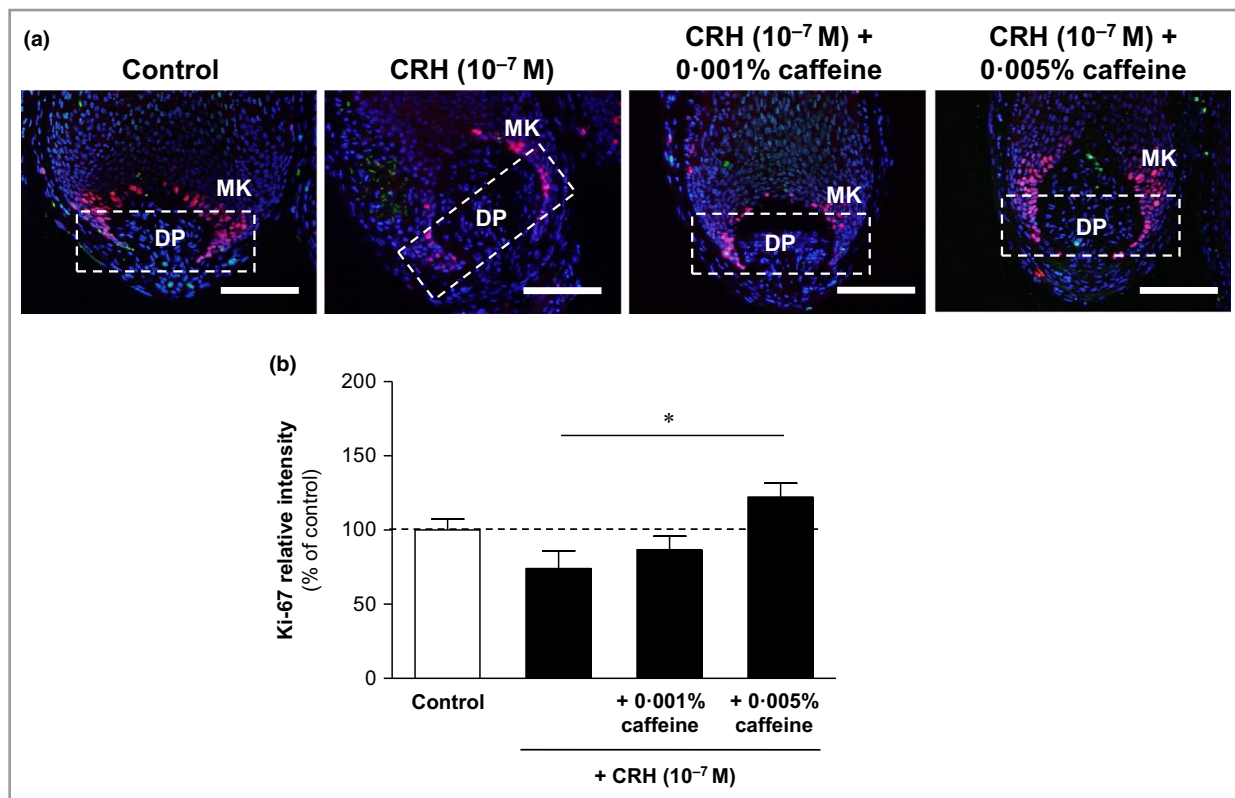
Stress can be mediated by oxidative stress or stress hormones.<sup>16–18</sup> The main actor of hormonal stress is corticotropin-releasing hormone (CRH).<sup>19</sup> CRH inhibits growth and induces premature entry into the catagen phase in human scalp HFs *ex vivo*,<sup>20</sup> which is consistent with the wide CRH expression and signalling in human skin,<sup>21,22</sup> including direct proinflammatory activities.<sup>23,24</sup> However, an established human hair stress model and related treatment principle are lacking so far.

HFs are skin appendages that survive and proliferate independently of the skin *in vitro*, for example in the human hair organ culture model.<sup>25,26</sup> They undergo lifelong repeating growth cycles;<sup>27</sup> synthesize, metabolize and process numerous (neuroendocrine) hormones;<sup>28–31</sup> and consequently represent an essential part of the skin neuroendocrine system.<sup>19,32,33</sup> Additionally, HFs (and skin) express a fully functional peripheral cutaneous equivalent of the central hypothalamic–pituitary–adrenal (HPA) stress axis [CRH → adrenocorticotrophic hormone (ACTH) → cortisol],<sup>19–21,34,35</sup> responsive even to ultraviolet B-induced stress.<sup>36</sup> Finally, HFs (and the skin) manage locally occurring stress<sup>20,33,37</sup> and therefore can serve excellently as a human *ex vivo* hair stress organ culture model. The

HPA axis is initiated by CRH,<sup>38</sup> which stimulates the pituitary CRH receptor type 1 (CRH-R1), leading to production and secretion of proopiomelanocortin (POMC)-derived peptides, including melanocyte-stimulating hormone and ACTH.<sup>39</sup>



**Figure 1** Effects of corticotropin-releasing hormone (CRH) and caffeine on hair shaft elongation. Hair follicles were incubated with the culture medium (control), CRH ( $10^{-7}$  mol L<sup>-1</sup>) alone or CRH in combination with caffeine (0.001% or 0.005%). CRH slightly reduced hair shaft elongation, while caffeine at the concentration of 0.001% significantly increased it compared with control at 120 h. Data are expressed as the pooled mean  $\pm$  SEM of 18 independent experiments. Values are expressed as the percentage of the control value. Statistically significant variations are indicated as \*\*\* $p$  < 0.01, \*\*\*\* $p$  < 0.001.



**Figure 2** Effects of corticotropin-releasing hormone (CRH) and caffeine on matrix keratinocyte proliferation (Ki-67). (a) Hair follicles were stained with immunofluorescence-labelled antibodies for the proliferation marker Ki-67 (red fluorescence) in matrix keratinocytes (MKs) localized above and around the dermal papilla (DP). Bars = 50  $\mu$ m. (b) Evaluation of the fluorescence intensity was conducted using Image J 1.38d software within the indicated white dashed rectangular areas. The data are expressed as the pooled mean  $\pm$  SEM. \* $p < 0.05$ .

The current study aimed now to establish a CRH-based hair stress model – for the first time in male *ex vivo* human HF derived from AGA patients and to investigate the effects of a possible counteracting substance, the well-known phosphodiesterase inhibitor caffeine.<sup>40–42</sup>

## Materials and methods

### Reagents

All of the reagents used are listed in Appendix S1 (see Supporting Information).

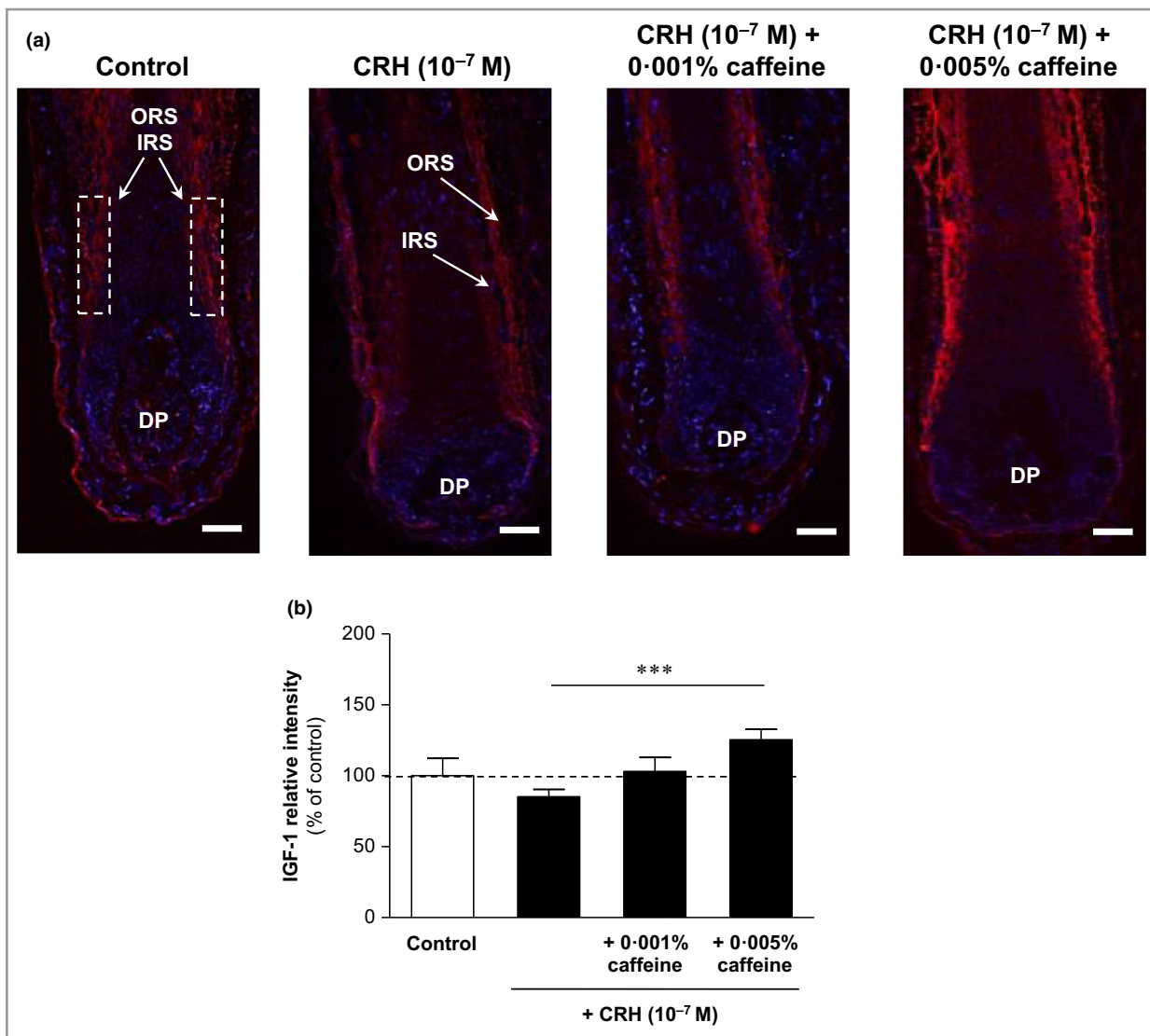
### Scalp biopsies

Whole human HF (anagen VI) were microdissected from elective biopsies (0.5  $\times$  1.5 cm) from the balding vertex region in the border area of the dense to the shedding area (androgen sensitive) of 18 men (age range 25–44 years, mean 35.4) with AGA in the moderate stage (Norwood–Hamilton stage III vertex and IV). The study was approved by the ethics committee of the University of Lübeck (reference 06-109) and written informed consent was obtained from the patients in accordance with the Declaration of Helsinki.

### Hair follicle microdissection and organ culture

Extracted HF were cultivated in a validated hair organ culture model as previously described (Appendix S2; see Supporting Information).<sup>25,26,41,43,44</sup>

Experimental treatment was started by adding fresh medium containing CRH at 10<sup>-7</sup> mol L<sup>-1</sup> or 10<sup>-8</sup> mol L<sup>-1</sup>, or CRH- and caffeine-free culture medium (control) to identify the most suppressive concentration of CRH on hair shaft elongation and matrix keratinocyte proliferation. CRH at 10<sup>-7</sup> mol L<sup>-1</sup> was most inhibitory, thus the following treatment conditions were established: 10<sup>-7</sup> mol L<sup>-1</sup> CRH, 10<sup>-7</sup> mol L<sup>-1</sup> CRH + 0.001% or 0.005% caffeine, or CRH- and caffeine-free culture medium (control). HF were cultivated for 120 h at 37 °C and 5% CO<sub>2</sub>. Control and treatment media were changed every other day. Hair shaft elongation was measured every 24 h using a scaled microscopic eyepiece. At the end of the 120-h cultivation, follicles were frozen at –80° C and cryosections of 6  $\mu$ m in thickness were performed for immunofluorescence. For reverse-transcriptase polymerase chain reaction (RT-PCR), 15 HF per treatment condition were collected, placed in Eppendorf tubes and suspended in 600  $\mu$ L TRIzol (Invitrogen, Carlsbad, CA, USA), then homogenized and frozen at –80° C until RT-PCR.



**Figure 3** Effects of corticotropin-releasing hormone (CRH) and caffeine on insulin-like growth factor (IGF)-1 in organ-cultured hair follicles. (a) Hair follicles were stained with immunofluorescence-labelled antibodies for IGF-1 (red fluorescence) in the inner root sheath (IRS) and outer root sheath (ORS). DP, dermal papilla. Bars = 50  $\mu$ m. (b) Evaluation of the fluorescence intensity was conducted using Image J 1.38d software within the indicated white dashed rectangular areas. The data are expressed as the pooled mean  $\pm$  SEM. \*\*\* $P$  < 0.001.

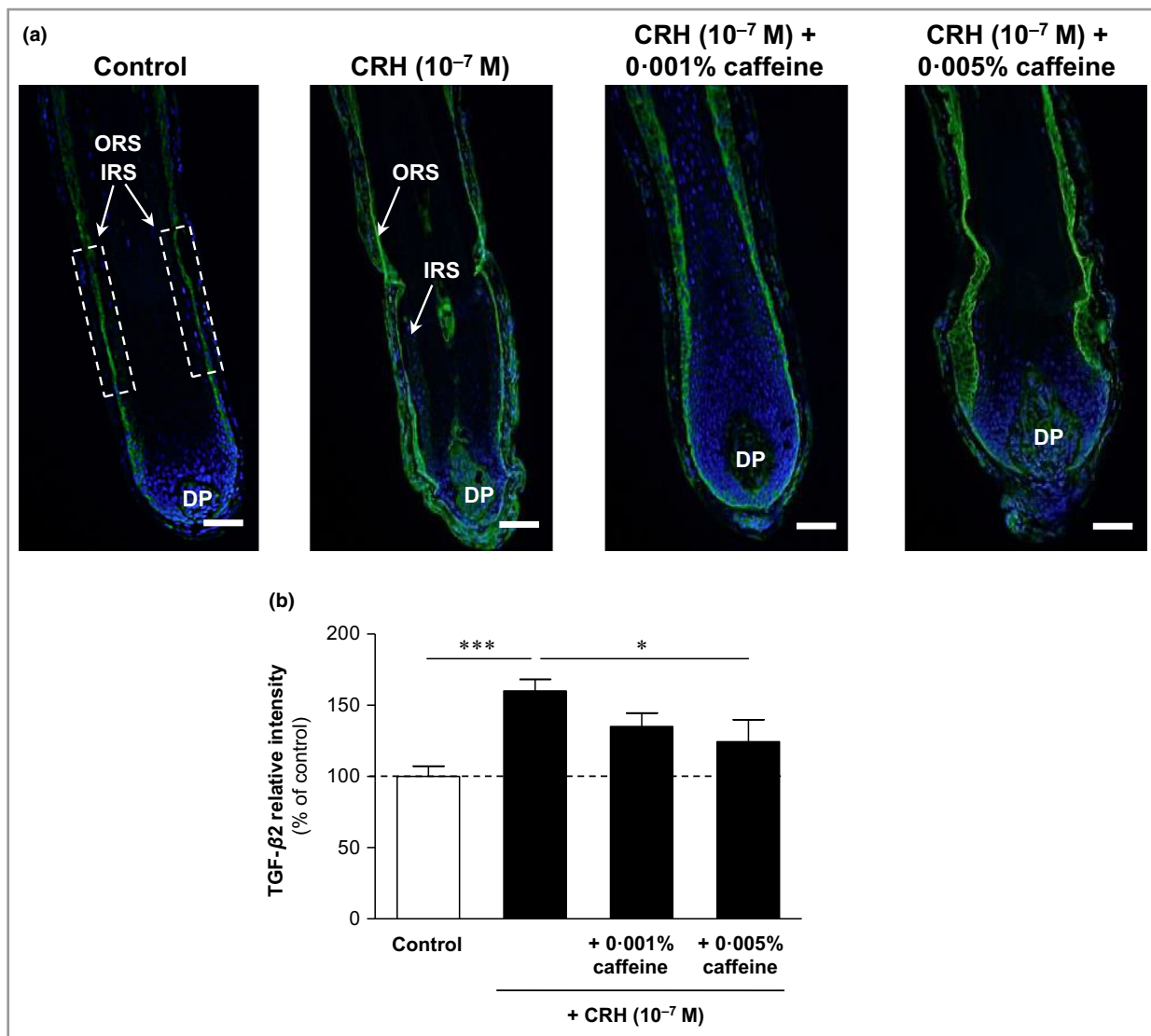
### Real-time reverse-transcriptase polymerase chain reaction assay

Total RNA from HFs was extracted using TRIzol, the RNA concentration was quantified (NanoDrop 3300; Fisher Scientific, Wilmington, DE, USA) and 1  $\mu$ g of total RNA was reverse transcribed with the SuperScript First-Strand Synthesis System (Applied Biosystems, Foster City, CA, USA). Primers for real-time PCR amplification were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) according to earlier published PCR detection of the CRH gene (Table S1; see Supporting Information).<sup>45</sup> The reaction was performed in triplicate with the SYBR Green I Master Mix (Roche, Mannheim, Germany) on a Light Cycler 480 (Roche). The amount of amplified product for each gene was

compared with that of  $\beta$ -actin using a comparative  $\Delta\Delta$ CT method  $\pm$  SD, and the data are presented as the fold change.

### Immunofluorescence labelling

For detection and visualization of protein expression in HFs, tyramide signal amplification (TSA) was applied for CRH-R1/2, insulin-like growth factor (IGF)-1, inositol triphosphate receptor (IP<sub>3</sub>-R), melanocortin receptor 2 (MC-R2), p75 neurotrophin receptor (p75<sup>NTR</sup>) and transforming growth factor (TGF)- $\beta$ 2, and the standard immunofluorescence procedure for ACTH, SP, NGF-tyrosine kinase receptor A (TrkA) and Ki-67. A detailed description of the procedures is provided in Appendix S3 and Table S2 (see Supporting Information).



**Figure 4** Effects of corticotropin-releasing hormone (CRH) and caffeine on the catagen-inducing factor transforming growth factor (TGF)- $\beta$ 2 in organ-cultured hair follicles. (a) Hair follicles were stained with immunofluorescence-labelled antibodies for TGF- $\beta$ 2 immunoreactivity (green fluorescence), which was detected in the outer root sheath (ORS) and partly in the inner root sheath (IRS). DP, dermal papilla. Bars = 50  $\mu$ m. (b) Evaluation of the fluorescence intensity was conducted using Image J 1.38d software within the indicated white dashed rectangular areas. The data are expressed as the pooled mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

### Statistical analysis

A description of the statistical analyses is provided in Appendix S4 (see Supporting Information).

## Results

### Identification of growth and proliferation inhibitory concentrations of corticotropin-releasing hormone in male human androgenetic alopecia hair follicles *in vitro*

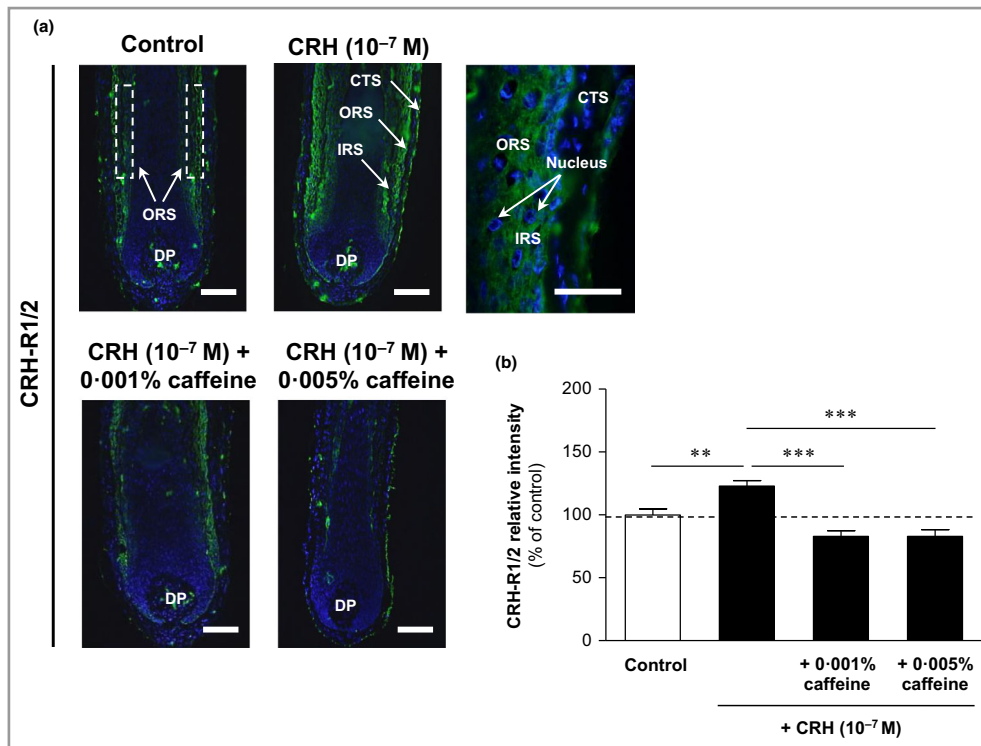
Incubation of male human AGA HF in culture with CRH at  $10^{-7}$  mol  $L^{-1}$  and  $10^{-8}$  mol  $L^{-1}$  or with CRH-free medium (control) identified  $10^{-7}$  mol  $L^{-1}$  as the most

suppressive concentration regarding hair shaft elongation and matrix keratinocyte proliferation (Ki67 positivity; data not shown), corresponding to results in female human HF.<sup>20</sup>

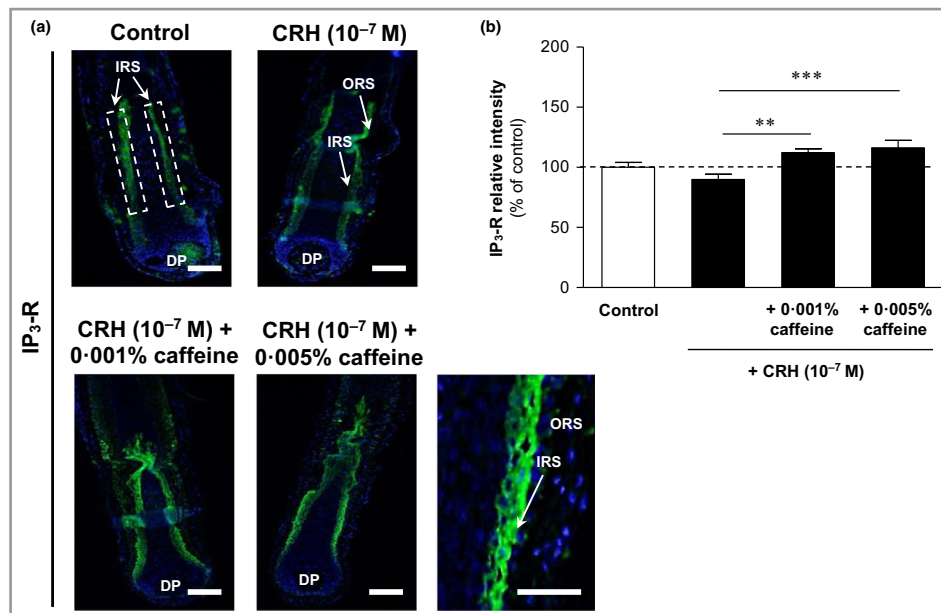
### Corticotropin-releasing hormone suppressed, whereas caffeine significantly increased hair shaft elongation

CRH ( $10^{-7}$  mol  $L^{-1}$ ) slightly decreased hair shaft elongation by 5% after 120 h in culture compared with control (Figure 1). Caffeine 0.001% in co-culture with CRH significantly enhanced hair shaft elongation by 11% ( $P < 0.01$ ) compared to control, and by 17% ( $P < 0.001$ ) compared to CRH alone.

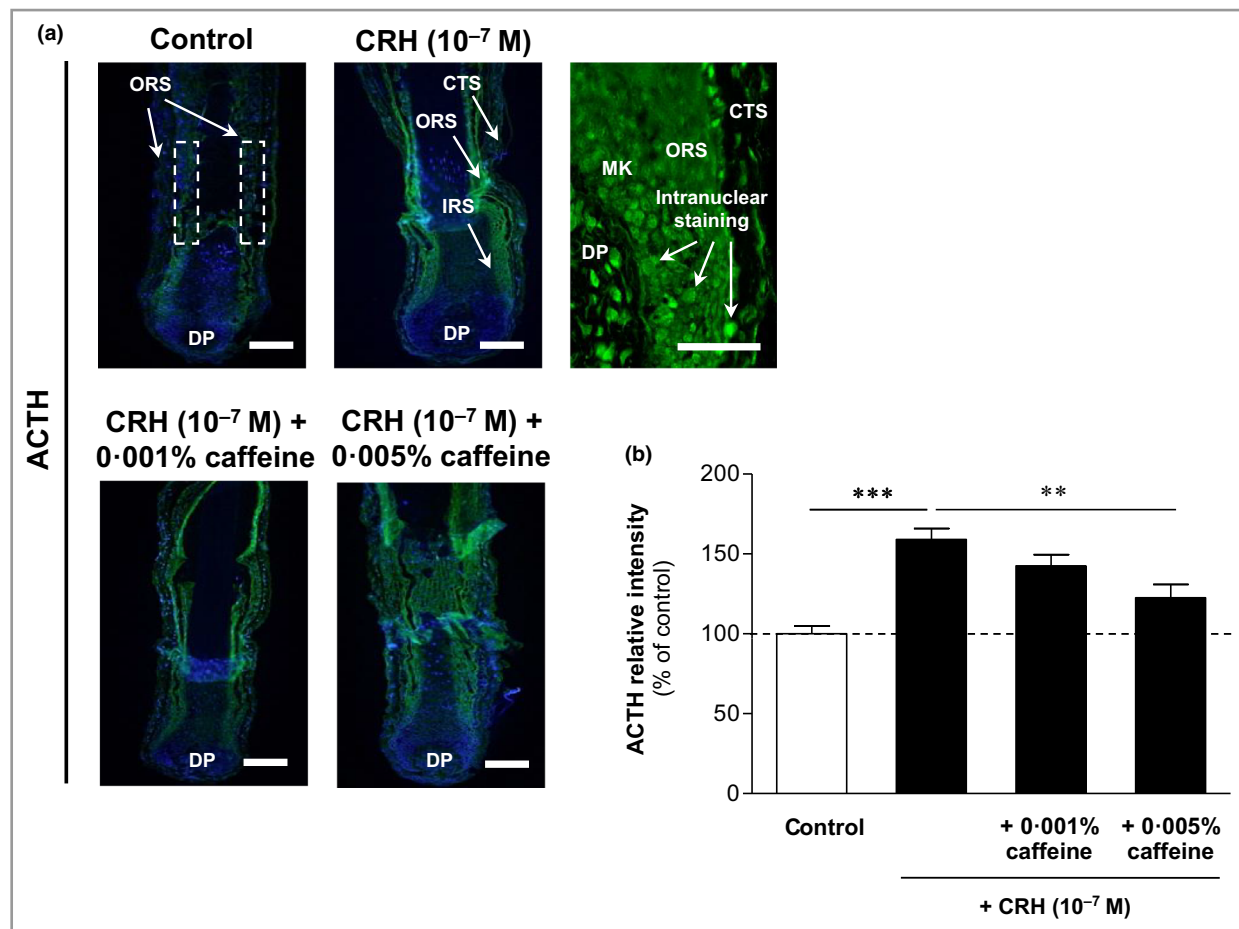




**Figure 5** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal axis-dependent stress parameter CRH receptors (CRH1/2) in human hair follicles. (a) CRH-R1/2 immunoreactivity (green fluorescence) was detected in the outer root sheath (ORS) and inner root sheath (IRS) (white dashed rectangular areas), with membrane-bound and cytosolic detection (magnification in upper right image). Single fibroblasts in the dermal papilla (DP) were also CRH-R1/2 positive. CTS, connective tissue sheath. Bars = 50  $\mu$ m. (b) Relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the IRS and ORS. Data are presented as the mean  $\pm$  SEM. \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.



**Figure 6** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal axis-dependent stress parameter inositol triphosphate receptor (IP<sub>3</sub>-R) in human hair follicles. (a) IP<sub>3</sub>-R immunoreactivity (green fluorescence) was detected in the inner root sheath (IRS) (white dashed rectangular areas) with cytosolic localization (magnification in lower right panel). DP, dermal papilla. Bars = 50  $\mu$ m. (b) The relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the inner root sheath (IRS). Data are presented as the mean  $\pm$  SEM. \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.



**Figure 7** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal axis-dependent stress parameter adrenocorticotrophic hormone (ACTH) in human hair follicles. (a) ACTH immunoreactivity (green fluorescence) was observed in the outer root sheath (ORS) and inner root sheath (IRS) (white dashed rectangular areas), as well as in matrix keratinocytes (MKs) and in fibroblasts in the dermal papilla (DP), with mainly intranuclear localization (magnification in upper right panel). Weak ACTH immunoreactivity was also detected in the connective tissue sheath (CTS) (magnification in upper right panel). Bars = 50  $\mu$ m. (b) The relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the ORS. Data are presented as the mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### Corticotropin-releasing hormone reduced matrix keratinocyte proliferation (Ki-67) and insulin-like growth factor 1, while caffeine significantly counteracted these effects

CRH reduced matrix keratinocyte proliferation (Ki-67) (Figure 2) and protein expression of IGF-1 detected in the inner and outer root sheaths (Figure 3). Caffeine at 0.005% significantly enhanced the number of Ki-67-positive matrix keratinocytes by 65% ( $P < 0.05$ ), and the IGF-1 positivity in the inner and outer root sheath by 47% ( $P < 0.001$ ) compared to HFs incubated with CRH alone.

#### Corticotropin-releasing hormone significantly increased the catagen inducer transforming growth factor- $\beta$ 2, while caffeine significantly reduced it

The protein expression of the key catagen inducer TGF- $\beta$ 2 was significantly enhanced by CRH by 60%

( $P < 0.001$ ) compared to control (Figure 4). Immunoreactivity was detected in the outer root sheath, and partly, but more weakly, in the inner root sheath. Caffeine 0.005% in co-incubation with CRH led to a significant reduction of CRH-induced TGF- $\beta$ 2 immunoreactivity, by 22% ( $P < 0.05$ ).

#### Corticotropin-releasing hormone and caffeine modulated the hypothalamic–pituitary–adrenal axis-dependent stress parameters

Corticotropin-releasing hormone (CRH) receptor 1/2 was significantly upregulated by CRH but downregulated by caffeine

Cultivation of extracted HFs with CRH ( $10^{-7}$  mol L $^{-1}$ ) and in combination with caffeine at 0.001% and 0.005% showed a strong impact on HPA axis-dependent stress parameters. Firstly, the expression of CRH-R1/2 was detected in the outer and inner root sheaths, with predominance in the outer root

sheath, and was mainly membrane bound. Single fibroblasts in the dermal papilla were also CRH-R1/2 positive. CRH-R1/2 immunoreactivity was significantly enhanced by 23% ( $P < 0.01$ ) compared with control after incubation with CRH  $10^{-7}$  mol L $^{-1}$ . Co-incubation with caffeine 0.001% and 0.005% led to a distinct decrease of this effect by 33% ( $P < 0.001$ ) (Figure 5).

#### Expression of inositol triphosphate receptor (IP<sub>3</sub>-R) was significantly enhanced by caffeine, but not by corticotropin-releasing hormone

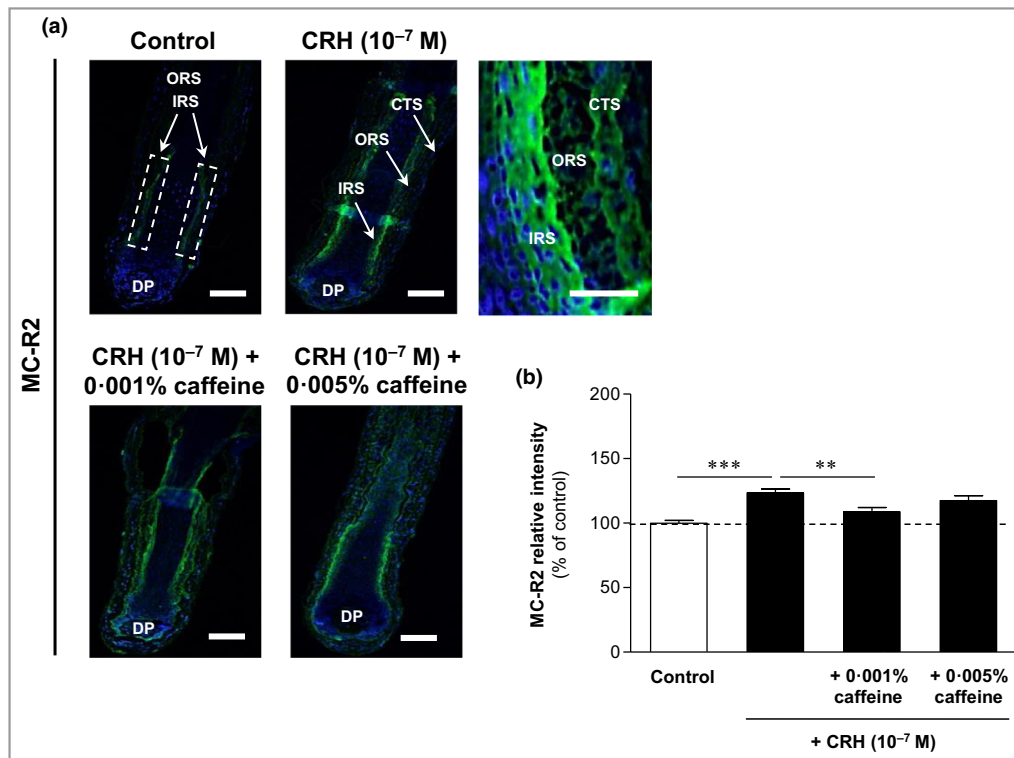
The immunoreactivity of the intracellular second messenger receptor IP<sub>3</sub>-R was detected in the inner root sheath with cytosolic localization (Figure 6a), and was slightly reduced by CRH  $10^{-7}$  mol L $^{-1}$  compared with control (not significant) (Figure 6a, upper right image). Co-cultivation with caffeine at 0.001% and 0.005% led to a strong and significant enhancement of IP<sub>3</sub>-R immunoreactivity along the inner root sheath (Figure 6a, lower image panel). The enhancements compared to CRH-treated HF<sub>s</sub> were +24% ( $P < 0.01$ ) and +29% ( $P < 0.001$ ), respectively (Figure 6b).

#### Adrenocorticotrophic hormone (ACTH) was significantly upregulated by corticotropin-releasing hormone and downregulated by caffeine co-administration

ACTH immunoreactivity was observed in the outer and inner root sheaths, as well as in matrix keratinocytes and in dermal papilla fibroblasts, with mainly intranuclear localization (Figure 7a, upper middle image). Weak ACTH immunoreactivity was also detected in the connective tissue sheath (Figure 7a, upper right image). ACTH in the outer and inner root sheaths was significantly increased by CRH  $10^{-7}$  mol L $^{-1}$ , by 59% compared to control ( $P < 0.001$ ) (Figure 7b), while co-cultivation with caffeine 0.005% led to a significant ACTH decrease by 23% compared to ACTH in HF<sub>s</sub> incubated with CRH  $10^{-7}$  mol L $^{-1}$  alone ( $P < 0.01$ ) (Figure 7b).

#### Melanocortin receptor 2 (MC-R2) was significantly upregulated by corticotropin-releasing hormone and downregulated by caffeine

MC-R2, the key receptor for ACTH, was detected membrane bound in the outer and inner root sheaths, with predominance in the outer parts of the inner root sheath (Figure 8a, upper panel). Weaker MC-R2 immunoreactivity was found in



**Figure 8** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal axis-dependent stress parameter melanocortin receptor 2 (MC-R2) in human hair follicles. (a) MC-R2 immunoreactivity (green fluorescence) was detected in the outer root sheath (ORS) and inner root sheath (IRS) (white dashed rectangular areas) with membrane-bound localization (magnification in upper right panel). Weaker MC-R2 immunoreactivity was found in the connective tissue sheath (CTS) (magnification in upper right panel). DP, dermal papilla. Bars = 50  $\mu$ m. (b) The relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the IRS. Data are presented as the mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



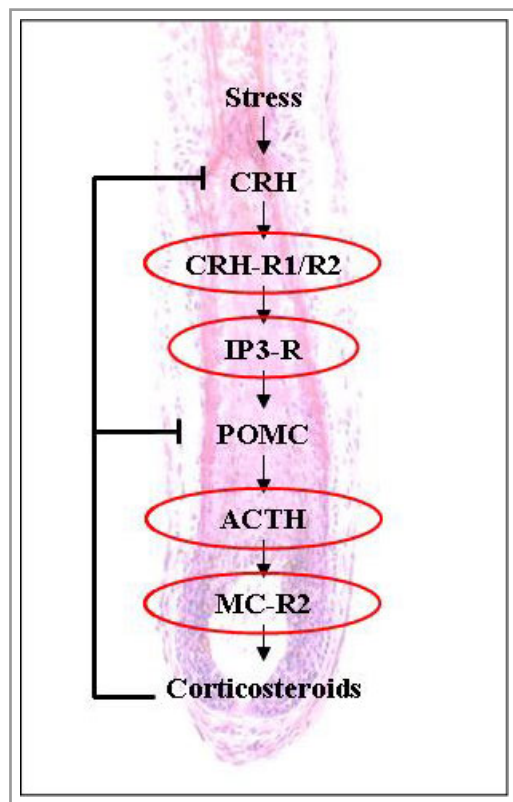
the connective tissue sheath (Figure 8a, upper right image). While MC-R2 was seen rather weakly in control HF, it was significantly enhanced by CRH  $10^{-7}$  mol L $^{-1}$ , by 23% compared with the control follicles ( $P < 0.001$ ) (Figure 8b). Co-incubation with caffeine 0.001% significantly decreased MC-R2 by 11% compared to CRH alone ( $P < 0.01$ ) (Figure 8b).

The investigated stress parameters dependent on the HPA axis and their downstream positions along the HPA axis are presented in Figure 9.

### Corticotropin-releasing hormone and caffeine regulated the non-hypothalamic–pituitary–adrenal axis stress parameters substance P, p75 neurotrophin receptor and NGF-tyrosine kinase receptor A

Substance P was significantly enhanced by corticotropin-releasing hormone but reduced by caffeine

CRH and caffeine were identified to influence non-HPA-axis dependent stress parameters such as SP, p75<sup>NTR</sup> and TrkA. SP was detected in the Henle layer of the inner root sheath (Figure 10a, upper image panel). CRH ( $10^{-7}$  mol L $^{-1}$ )



**Figure 9** A representation of the downstream positions of the hypothalamic–pituitary–adrenal axis (HPA)-dependent stress parameters along the HPA axis. The red coloured ellipses indicate the stress parameters investigated in this study. ACTH, adrenocorticotrophic hormone; CRH, corticotropin-releasing hormone; CRH-R, CRH receptor; IP<sub>3</sub>-R, inositol triphosphate receptor; MC-R2, melanocortin receptor 2; POMC, proopiomelanocortin.

significantly increased SP in the Henle layer by 17% ( $P < 0.05$ ) (Figure 10a, upper right panel, Figure 10b). Co-incubation with caffeine at 0.001% and 0.005% significantly decreased SP by 21% ( $P < 0.001$ ) and 17% ( $P < 0.01$ ), respectively (Figure 10).

### Proapoptotic p75<sup>NTR</sup> was significantly induced by corticotropin-releasing hormone but reduced by caffeine

Immunoreactivity of the pro-apoptotic neurotrophin receptor p75<sup>NTR</sup> was detected in the outer root sheath with membrane-bound localization (Figure 11a, upper panel). It was significantly increased by CRH  $10^{-7}$  mol L $^{-1}$  by 27% ( $P < 0.01$ ), while it was significantly reduced by co-incubation with caffeine 0.001%, by 24% ( $P < 0.01$ ) (Figure 11b).

### Antiapoptotic NGF-tyrosine kinase receptor A (TrkA) was enhanced by caffeine compared to corticotropin-releasing hormone (CRH), while CRH alone did not influence TrkA

Immunoreactivity of the antiapoptotic nerve growth factor receptor TrkA was detected in the outer root sheath with membrane-bound localization (Figure 12a). CRH  $10^{-7}$  mol L $^{-1}$  only slightly reduced TrkA compared to control (not significant), but caffeine 0.001% significantly enhanced TrkA immunoreactivity by 20% compared to CRH-treated HF ( $P < 0.01$ ) (Figure 12b). An overview of CRH- and caffeine-mediated effects in all immunofluorescence parameters is presented in Table 1.

### Gene expression regulation of corticotropin-releasing hormone (CRH) receptors 1 and 2 in human hair follicles by CRH and caffeine

Co-cultivation of extracted HF with caffeine 0.001% and CRH  $10^{-7}$  mol L $^{-1}$  led to a significant 200-fold upregulation of CHR-R1 and an approximately 1000-fold upregulation of CHR-R2 gene expression in human HF compared with control HF (Figure 13). CRH  $10^{-7}$  mol L $^{-1}$  alone had no effect on CHR-R1, but led to a significant 15-fold upregulation of CHR-R2 gene expression (Figure 13).

## Discussion

While the original identification of the hair organ-specific local HPA-axis was shown earlier in female human hair follicles gained from routine face-lift surgery,<sup>20</sup> the present study provides the first evidence for a functional HPA axis in HF electively taken from the balding area of male human individuals with AGA, a condition found in every second man in European countries.<sup>46</sup> Additionally, a new player along the HPA axis – IP<sub>3</sub>-R – was identified for the first time in human HF.

While in the non-HPA-axis stress response of human organ cultured HF a substance P mediated up-regulation of NGF

and its apoptosis- and catagen-promoting receptor p75<sup>NTR</sup> and a down-regulation of the growth promoting, anti-apoptotic NGF receptor TrkA was shown earlier,<sup>15</sup> the present study shows for the first time that CRH regulates SP, p75<sup>NTR</sup> and TrkA in human male AGA HF.

Thus, the CRH-related human hair organ culture model has excellently proven to be a well-suited human hair stress model in a multifaceted way, and caffeine counteracted this stress response significantly. Caffeine was used at the same concentrations previously shown to be effective by our group in male or female human HF.<sup>41,42</sup>

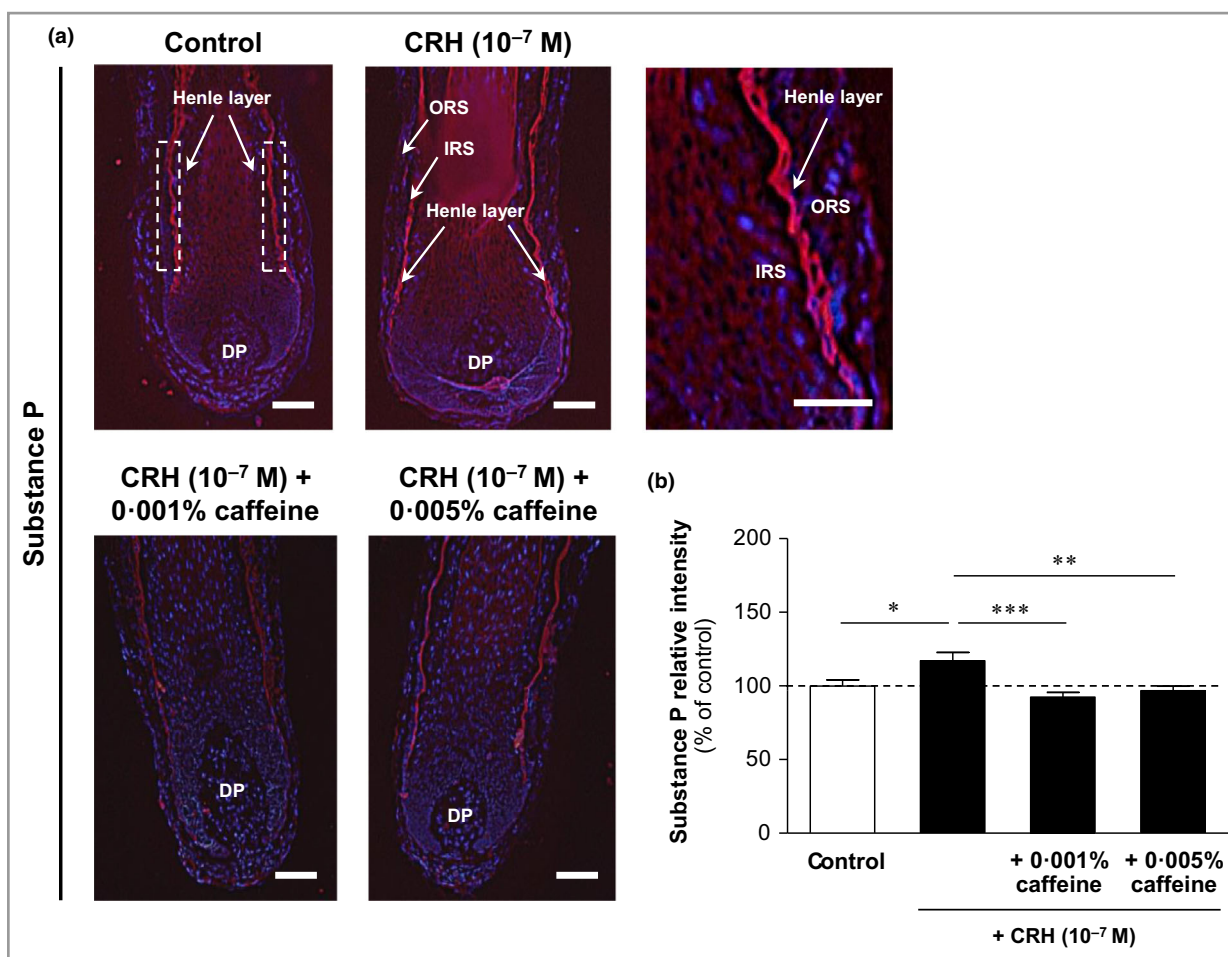
Consistent with our previous studies,<sup>41,42</sup> we used anagen stage VI (terminal) HF and not intermediate HF as suggested by Miranda *et al.*<sup>47</sup> The latter are suitable for investigation of hair growth stimulation, but are more sensible in stress induction (e.g. by CRH) or experimentally induced growth suppression (e.g. by testosterone<sup>41,42</sup> or ultraviolet irradiation)<sup>48</sup> as in the present and previous studies.

CRH induces 'growth suppression' in cell cultures of skin cells of different origin,<sup>49</sup> with the most reported suppressive concentrations in cell culture and female HF of  $10^{-7}$  mol L<sup>-1</sup> and  $10^{-8}$  mol L<sup>-1</sup>.<sup>20,49,50</sup> We confirmed the concentration of  $10^{-7}$  mol L<sup>-1</sup> as the most inhibitory. Concentrations higher than  $10^{-7}$  mol L<sup>-1</sup> were not tested by our group or others as they might be considered unphysiological.<sup>51</sup>

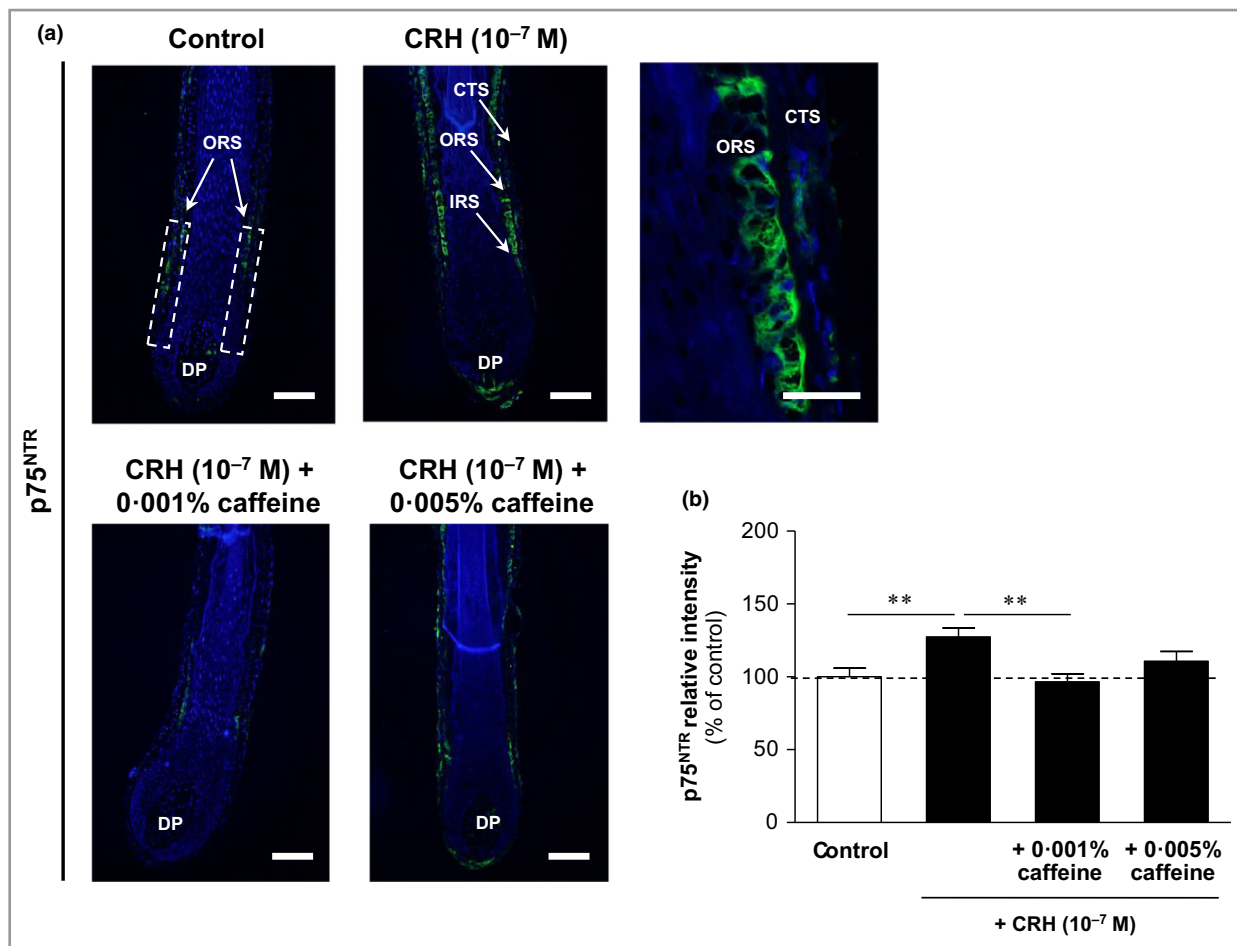
CRH plays an important role in skin and hair biology, as shown by hair-cycle-dependent expression of CRH-R1 and CRH-R2 in mice,<sup>52,53</sup> and by a CRH-induced functional cascade along the HPA axis in human normal epidermal melanocytes and dermal fibroblasts.<sup>54,55</sup>

The CRH stress-mediated decrease of IGF-1 and increase of TGF- $\beta$ 2 and their counter-regulation by caffeine are in agreement with our own previous results in testosterone-treated AGA HF.<sup>42</sup>

CRH  $10^{-7}$  mol L<sup>-1</sup> activated its own receptor CRH-R1/2 in the HF, corresponding to previous observations in human



**Figure 10** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal (HPA) axis-independent stress parameter substance P in human hair follicles. (a) Substance P immunoreactivity (red fluorescence) was detected precisely in the Henle layer of the inner root sheath (IRS) (white dashed rectangular areas in the upper left image, and magnification in the upper right panel). DP, dermal papilla. Bars = 50  $\mu$ m. (b) The relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the Henle layer of the inner root sheath (IRS). The data are presented as the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 11** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal (HPA) axis-independent stress parameter p75 neurotrophin receptor (p75<sup>NTR</sup>) in human hair follicles. The immunoreactivity of p75<sup>NTR</sup> (green fluorescence) was detected in the outer root sheath (ORS) with membrane-bound localization (white dashed rectangular areas in the upper left image, and magnification in the upper right image). CTS, connective tissue sheath; DP, dermal papilla. Bars = 50  $\mu$ m. (b) The relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the outer root sheath (ORS). The data are presented as the mean  $\pm$  SEM. \*\* $P < 0.01$ .

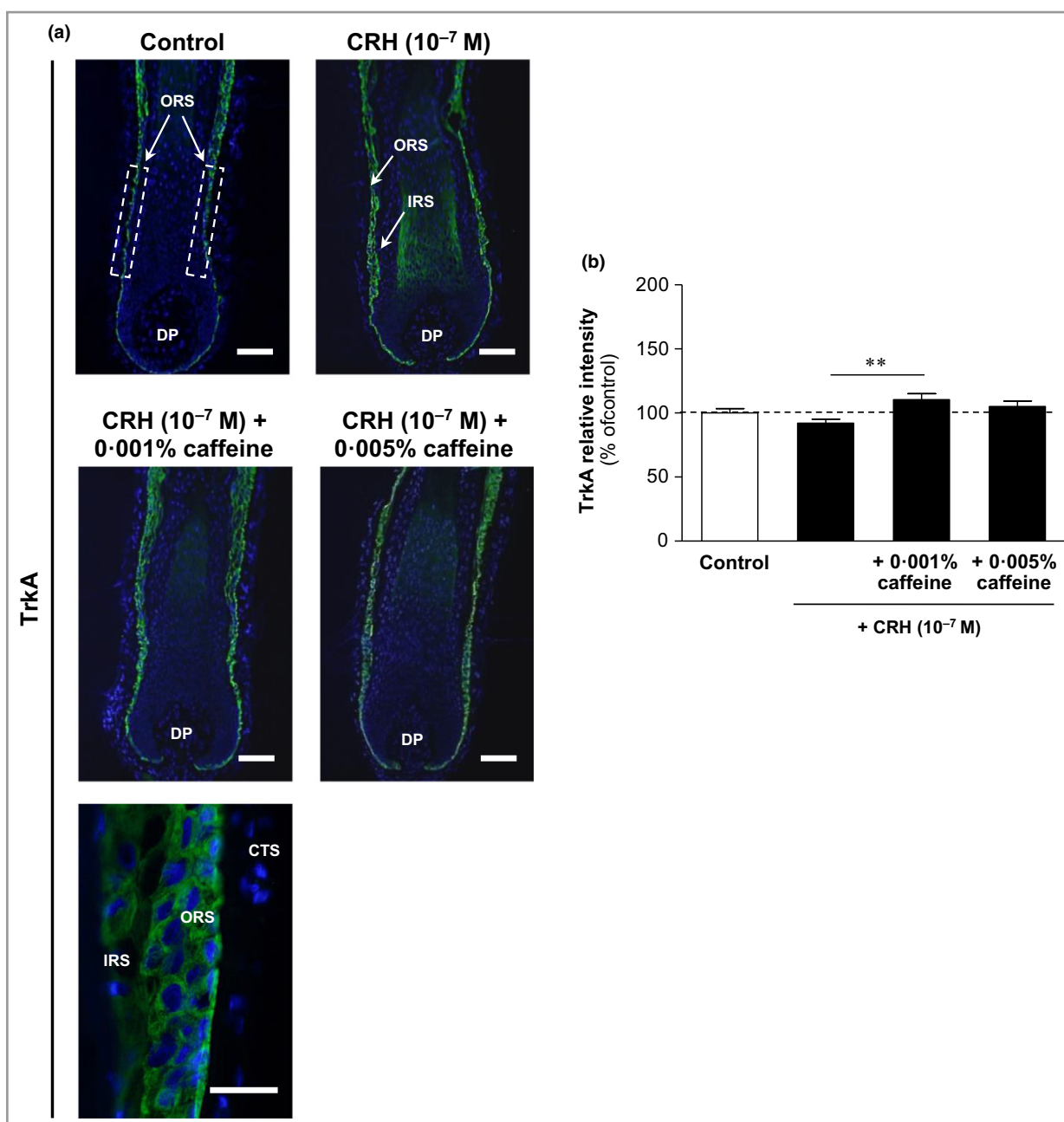
organ-cultured HFs as well as in *in situ* models.<sup>20,21,35</sup> The normalization of CRH-R1/2 expression by caffeine might be explained by caffeine-mediated inhibitory gene regulation of CRH-R1/2 synthesis; however, we observed the opposite: caffeine lead to over-upregulation of the CRH-R1 and CRH-R2 genes. This might be explained by the assumption that caffeine would directly reduce CRH-R1/2 expression leading to reduced receptor density on the cell membrane, or, alternatively, caffeine might directly and competitively inhibit CRH binding at its receptor. The first hypothesis is more likely, as a reduced availability of free CRH receptors would reactively upregulate its gene expression, as shown by our results.

IP<sub>3</sub>-R is an intracellular cation channel that is located primarily at the membrane of the endoplasmic reticulum in a large range of cell types.<sup>56</sup> Inositol-1,4,5-trisphosphate (IP<sub>3</sub>) is the most important ligand and leads after receptor binding to a fast release of Ca<sup>2+</sup> ions, inducing a complex local and global Ca<sup>2+</sup> signal response. IP<sub>3</sub>-R has additional activating

ligands such as Ca<sup>2+</sup> itself, ATP and cAMP.<sup>57</sup> Via CRH-R1, CRH stimulates IP<sub>3</sub> production, shown in human keratinocytes treated with CRH at 10<sup>-7</sup> mol L<sup>-1</sup>.<sup>58</sup> A similar effect with Ca<sup>2+</sup> release might prove relevant also in HF keratinocytes. The slight CRH-induced reduction of its receptor, IP<sub>3</sub>-R, might be considered a negative feedback mechanism, observed also for example with the insulin receptor.<sup>59</sup> Caffeine was reported to inhibit IP<sub>3</sub>-mediated cellular responses.<sup>60,61</sup>

However, as a phosphodiesterase inhibitor, caffeine increases cAMP,<sup>62</sup> opens Ca<sup>2+</sup> channels and increases intracellular Ca<sup>2+</sup> levels.<sup>63</sup> Thus, caffeine shows paradoxical effects on the intracellular Ca<sup>2+</sup> balance, which might be dependent on the caffeine concentration. In our study, the increase of IP<sub>3</sub>-R by caffeine is most likely due to a decrease of intracellular Ca<sup>2+</sup> and a consecutive upregulation of IP<sub>3</sub>-R as a positive feedback mechanism.

ACTH showed an immunoreactivity pattern in our HFs corresponding to previous reports,<sup>20,48</sup> as well as its



**Figure 12** Effects of corticotropin-releasing hormone (CRH) and caffeine on the hypothalamic–pituitary–adrenal (HPA) axis-independent stress parameter NGF-tyrosine kinase receptor A (TrkA) in human hair follicles. (a) TrkA immunoreactivity (green fluorescence) was detected in the outer root sheath (ORS) with membrane-bound localization (white dashed rectangular areas in the upper left image, and magnification in the lower image). CTS, connective tissue sheath; DP, dermal papilla. Bars = 50  $\mu$ m. (b) Relative fluorescence intensity was measured with Image J 1.38d software within the dashed rectangular areas along the outer root sheath (ORS). The data are presented as the mean  $\pm$  SEM. \*\* $p < 0.01$ .

enhancement by CRH  $10^{-7}$  mol L $^{-1}$ .<sup>20</sup> The downregulating effect of caffeine might be mediated by phosphodiesterase inhibition because the activation of POMC gene expression and ACTH processing is mediated by the cAMP signal pathway.<sup>37,54,55</sup>

Also, the observed expression pattern of MC-R2 corresponded to the study of Ito *et al.*<sup>20</sup> CRH might directly regulate MC-R2; however, an indirect stimulatory effect via ACTH was also

described by Chakraborty and Pawelek.<sup>64</sup> Caffeine showed a significant decrease of MC-R2 expression down to an almost normal level, thus confirming the potent stress inhibitory effects of caffeine at multiple points along the HPA axis.

SP immunoreactivity was significantly enhanced in the Henle layer by CRH. So far, it has been shown that SP can only be built in the dermal nerve fibres that surround the HF,<sup>65</sup> seen also in alopecia areata scalp skin biopsies.<sup>66</sup> Our



**Table 1** Overview of the effects of corticotropin-releasing hormone (CRH)  $10^{-7}$  mol L $^{-1}$  plus caffeine 0.001% or 0.005% on the investigated parameters assessed by immunofluorescence

Parameter	CRH vs. control	CRH + caffeine 0.001% vs. CRH	CRH + caffeine 0.005% vs. CRH
HSE	↓	↑ ***	—
Ki-67	↓	↓	↑ *
IGF-1	↓	↑	↑ ***
TGF- $\beta$ 2	↑ ***	↓	↓ *
CRH-R1/2	↑ **	↓ ***	↓ ***
IP $_3$ -R	↓	↑ **	↑ ***
ACTH	↑ ***	↓	↓ **
MC-R2	↑ ***	↓ **	↓
Substance P	↑ *	↓ ***	↓ **
p75 <sup>NTR</sup>	↑ **	↓ **	↓
TrkA	↓	↑ **	↑

CRH was used at  $10^{-7}$  mol L $^{-1}$  in all comparisons. Differences indicated with up or down arrows are  $\geq 5\%$  increases or decreases, respectively. Differences indicated with '—' are no differences or differences  $< 5\%$ . Significant differences are indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ACTH, adrenocorticotrophic hormone; CRH-R, CRH receptor; HSE, hair shaft elongation; IGF, insulin-like growth factor; IP $_3$ -R, inositol triphosphate receptor; MC-R2, melanocortin receptor 2; TGF, transforming growth factor; TrkA, NGF-tyrosine kinase receptor A.

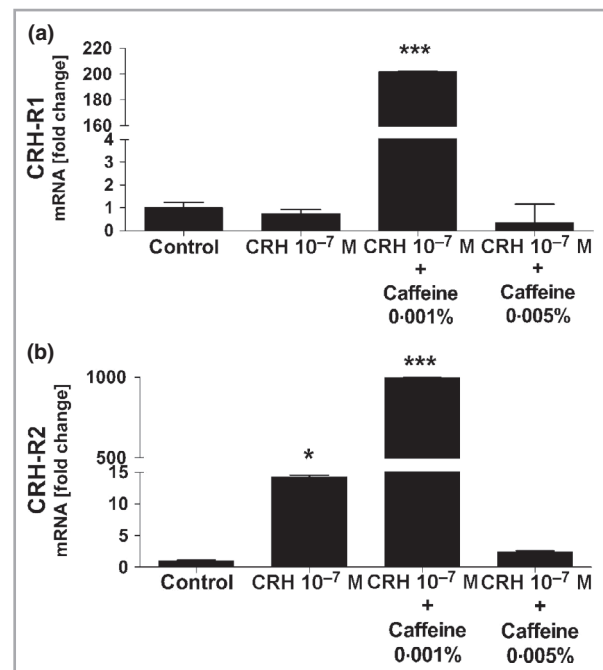
extracted HF's were lacking the surrounding tissue and nerves, and therefore SP had to be derived from other sources. Such a source might be mast cells and human keratinocytes from the inner and outer root sheaths, which are known to liberate SP,<sup>67,68</sup> or the connective tissue sheath, which is known to be an HF-associated reservoir of SP.<sup>15</sup> Ito *et al.* reported mast cell activation and degranulation in human HF's by CRH  $10^{-7}$  mol L $^{-1}$ ,<sup>69</sup> confirming our observation. To the best of our knowledge, this is the first report of a supposedly autocrine liberation of SP by the HF itself. The caffeine-induced reduction of SP might be mediated by its stimulatory effect on SP-degrading enzymes (e.g. endopeptidase and angiotensin-converting enzyme).<sup>70</sup>

The immunoreactivity localization of p75<sup>NTR</sup> in our study corresponds to that in previous studies.<sup>15,71</sup> p75<sup>NTR</sup> mediates apoptotic signals<sup>72,73</sup> and is strongly expressed in catagen and telogen.<sup>71</sup>

Expression of the transmembrane antiapoptotic high-affinity NGF tyrosine kinase receptor TrkA was found predominantly in the outer root sheath in the hair shaft area, as well as in the bulb region, which is also consistent with previous reports.<sup>15,71,74,75</sup>

The central role of the neurogenic non-HPA axis stress mediators SP, p75<sup>NTR</sup> and TrkA is described here for the first time in human male AGA HF's and confirms results from stressed mice developing hair loss.<sup>3,11,76</sup> Also, the influence of caffeine on the neurogenic non-HPA axis stress response is a new observation.

In conclusion, our study shows for the first time a parallel CRH-induced stress response along the peripheral intrafollicular HPA axis and the neurogenic SP-mediated non-HPA axis in



**Figure 13** Gene expression of CRH-R1 and CRH-R2 in human hair follicles. Whole human hair follicles were lysed, then RNA was extracted and reverse transcribed for real-time polymerase chain reaction amplification for detection of the expression of (a) CRH-R1 and (b) CRH-R2. (a) CRH-R1 gene expression was 200-fold and significantly (\*\* $P < 0.001$ ) upregulated in human hair follicles co-cultivated with caffeine 0.001% and corticotropin-releasing hormone (CRH)  $10^{-7}$  mol L $^{-1}$  compared to hair follicles incubated with control or only CRH  $10^{-7}$  mol L $^{-1}$ . (b) CRH-R2 gene expression was 15-fold and significantly (\* $P < 0.05$ ) upregulated in human hair follicles incubated with CRH  $10^{-7}$  mol L $^{-1}$  alone compared to controls, while it was approximately 1000-fold and significantly (\*\* $P < 0.001$ ) upregulated in human hair follicles co-cultivated with caffeine 0.001% and CRH  $10^{-7}$  mol L $^{-1}$  compared to control or only CRH  $10^{-7}$  mol L $^{-1}$ .

human HF's from men with AGA. Both stress responses are inhibited by caffeine, suggesting its potential for the clinical treatment of AGA or stress-induced hair loss.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Appendix S1** Reagents.

**Appendix S2** Hair follicle microdissection and organ culture.

**Appendix S3** Immunofluorescence labeling, tyramide signal amplification staining and standard immunofluorescence.

**Appendix S4** Statistical analysis.

**Table S1** List of quantitative real-time polymerase chain reaction forward and reverse primers used for gene-expression analysis of CRH-R1 and CRH-R2.

**Table S2** Antibodies and staining techniques used for fluorescence labelling of the investigated proteins.