View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Thyrotropin-Releasing Hormone Selectively Stimulates Human Hair Follicle Pigmentation

Erzsébet Gáspár[1,5,6], Kim T. Nguyen-Thi[1,5], Celine Hardenbicker[1], Stephan Tiede[1], Christian Plate[1], Enikő Bodó[1,7], Jana Knuever[1], Wolfgang Funk[2], Tamás Bíró[3] and Ralf Paus[1,4]

In amphibians, thyrotropin-releasing hormone (TRH) stimulates skin melanophores by inducing secretion of α-melanocyte-stimulating hormone in the pituitary gland. However, it is unknown whether this tripeptide neurohormone exerts any direct effects on pigment cells, namely, on human melanocytes, under physiological conditions. Therefore, we have investigated whether TRH stimulates pigment production in organ-cultured human hair follicles (HFs), the epithelium of which expresses both TRH and its receptor, and/or in full-thickness human skin *in situ*. TRH stimulated melanin synthesis, tyrosinase transcription and activity, melanosome formation, melanocyte dendricity, gp100 immunoreactivity, and microphthalmia-associated transcription factor expression in human HFs in a pituitary gland-independent manner. TRH also stimulated proliferation, gp100 expression, tyrosinase activity, and dendricity of isolated human HF melanocytes. However, intraepidermal melanogenesis was unaffected. As TRH upregulated the intrafollicular production of “pituitary” neurohormones (proopiomelanocortin transcription and ACTH immunoreactivity) and as agouti-signaling protein counteracted TRH-induced HF pigmentation, these pigmentary TRH effects may be mediated in part by locally generated melanocortins and/or by MC-1 signaling. Our study introduces TRH as a novel, potent, selective, and evolutionarily highly conserved neuroendocrine factor controlling human pigmentation *in situ*. This physiologically relevant and melanocyte sub-population-specific neuroendocrine control of human pigmentation deserves clinical exploration, e.g., for preventing or reversing hair graying.

JID JOURNAL CLUB ARTICLE: For questions, answers, and open discussion about this article please go to http://www.nature.com/jid/journalclub

Journal of Investigative Dermatology (2011) **131,** 2368–2377; doi:10.1038/jid.2011.221; published online 29 September 2011

INTRODUCTION

Thyrotropin-releasing hormone (TRH; synonyms: thyroliberin and thyrotropin-releasing factor) is a neurohormone that is best known as the upstream hypothalamic neuroendocrine stimulus that controls thyroid hormone production. TRH reaches the anterior pituitary gland through the hypophyseal portal system to induce the release of thyroid-stimulating hormone by pituitary ‘‘thyrotrope’’ cells. As the most proximal regulatory element of the hypothalamic–pituitary–thyroid axis, TRH is initially synthesized in medial neurons of the paraventricular nucleus of the hypothalamus as a 242 amino-acid precursor, which is proteolytically processed to the mature TRH tripeptide (Lechan and Hollenberg, 2003; Chiamolera and Wondisford, 2009).

However, the brain is not the only site of TRH expression and synthesis. For example, the gastrointestinal system and pancreatic islet cells have been reported to constitute extra-hypothalamic sites of TRH production (Martino *et al.*, 1978; Leduque *et al.*, 1989). This has greatly raised interest in the full range of *non-classical* TRH functions—for example, in ‘‘cytokine-induced sickness behavior’’ (Kamath *et al.*, 2009), the control of energy homeostasis (not only through TRH-controlled thyroid function but also through central effects of TRH on feeding behavior, thermogenesis, locomotor activation, and autonomic nervous regulation; Lechan and Fekete, 2006), regulation of cardiovascular functions (García and Pirola, 2005), and as a multifunctional hypophysiotropic modulator (Galas *et al.*, 2009).

On this background, it deserves emphasis that amphibian skin has long provided intriguing, yet insufficiently followed up, pointers to ancestral functions of TRH (Jackson and Reichlin, 1977; Vaudry *et al.*, 1999; Vazquez-Martinez

[1]Department of Dermatology, University of Lübeck, Lübeck, Germany; [2]Klinik Dr Koslowski, Munich, Germany; [3]Department of Physiology, Research Center for Molecular Medicine, University of Debrecen, Debrecen, Hungary and [4]School of Translational Medicine, University of Manchester, Manchester, UK

[5]These authors contributed equally to this work.

[6]Current address: Department of Radiology and Nuclear Medicine, University of Lübeck, Lübeck, Germany

[7]Current address: Agricultural and Molecular Research Institute, College of Nyíregyháza, Nyíregyháza, Hungary

Correspondence: Ralf Paus, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. E-mail: ralf.paus@uksh.de

Abbreviations: α-MSH, melanocyte-stimulating hormone; ASP, agouti-signaling protein; HF, hair follicle; HFPU, HF pigmentary unit; MC-1R, melanocortin-1 receptor; MITF, microphthalmia-associated transcription factor; POMC, proopiomelanocortin; TRH, thyrotropin-releasing hormone

Received 28 March 2011; revised 31 May 2011; accepted 13 June 2011; published online 29 September 2011

© 2011 The Society for Investigative Dermatology

et al., 2003; Jenks *et al.*, 2007, 2010; Galas *et al.*, 2009). Although these have largely been ignored in human biology, TRH may still have an important role in human tissue physiology beyond the well-recognized thyrotropic function of TRH within the hypothalamic–pituitary–thyroid axis. Specifically, we hypothesized that TRH may exert regulatory functions in the human pigmentary system. This hypothesis was based on the following considerations:

Frog skin contains surprisingly high amounts of TRH (Jackson and Reichlin, 1977; Bolaffi and Jackson, 1979; Vaudry *et al.*, 1999; Vazquez-Martinez *et al.*, 2003) and skin-derived TRH can exert hypophysiotropic functions (e.g., it stimulates pituitary thyroid-stimulating hormone, prolactin, and growth hormone release in adult frogs) that may extend to other vertebrates (Tonon *et al.*, 1980; Vaudry *et al.*, 1999; Galas *et al.*, 2009; Jenks *et al.*, 2010). TRH is also recognized as a potent stimulator of pituitary proopiomelanocortin (POMC) expression and α-melanocyte-stimulating hormone (α-MSH) secretion in frogs (Vaudry *et al.*, 1999; Jenks *et al.*, 2007, 2010). TRH derived from either the hypothalamus or the skin (Jackson and Reichlin, 1977; Vaudry *et al.*, 1999; Galas *et al.*, 2009) is thought to induce amphibian skin darkening *indirectly* by stimulating α-MSH release by the pituitary gland, inducing rapid skin darkening through melanophore stimulation (Jackson and Reichlin, 1977; Tonon *et al.*, 1980; Vaudry *et al.*, 1999; Vazquez-Martinez *et al.*, 2003; Jenks *et al.*, 2007, 2010).

However, it is not yet known whether TRH has direct pigmentation-modulatory effects on amphibian skin melanophores and/or on normal melanocytes of mammalian species in the absence of a pituitary gland. Intriguingly, it is not only amphibian skin that is a major source of TRH peptides (Jackson and Reichlin, 1977; Bolaffi and Jackson, 1979; Vaudry *et al.*, 1999) but even human skin and its cultured resident cell populations transcribe hypothalamic–pituitary–thyroid axis-related genes, such as *TRH*, *thyrotropin* (*TSH*), and their receptors (*TRH-R*, *TSH-R*) (Slominski *et al.*, 2002; Bodó *et al.*, 2009; Gáspár *et al.*, 2010; Paus, 2010). TRH-evoked α-MSH secretion in amphibians is mediated through TRH-R3, a receptor not detected in mammals (Bidaud *et al.*, 2004). Isolated neonatal human epidermal keratinocytes, dermal fibroblasts, and hair follicle (HF) papilla fibroblasts reportedly express *TRH* mRNA *in vitro* (Slominski *et al.*, 2002). Most recently, we have identified normal human scalp HFs as yet another extra-hypothalamic source of TRH production. TRH is not only expressed constitutively by human HFs on the gene and protein level but also stimulates hair shaft production and HF keratinocyte proliferation in organ-cultured human scalp HFs (Gáspár *et al.*, 2010). Following the above leads from frog skin to potential pigmentary activities of TRH in human skin, we have asked whether TRH can directly alter pigmentation in human epidermis and/or HFs in the absence of a pituitary gland.

To dissect the as yet obscure role of TRH in mammalian pigmentation, we performed organ culture of the extremely hormone-sensitive HF as an excellent, clinically relevant model for unraveling non-classical functions of neuropeptide hormones (Ito *et al.*, 2005; Kauser *et al.*, 2005, 2006; van Beek *et al.*, 2008; Bodó *et al.*, 2009; Schneider *et al.*, 2009; Gáspár *et al.*, 2010; Ramot *et al.*, 2010). Given the well-documented sensitivity of human HF pigmentation to neuroendocrine stimuli such as corticotropin-releasing hormone and the POMC products α-MSH, ACTH, and β-endorphin (Ito *et al.*, 2005; Kauser *et al.*, 2005, 2006; Slominski *et al.*, 2005a), we reasoned that the human HF should be particularly well suited to reveal clinically relevant pigmentation-modulatory action of TRH. TRH pigmentary effects observed in organ-cultured human HFs were compared with those seen in full-thickness human scalp skin organ culture (Lu *et al.*, 2007), to directly compare the TRH response of normal adult human epidermal with human HF melanocytes *in situ*. We focused on dissecting the impact of TRH on the human HF pigmentary unit (HFPU), the complex neuroendocrine regulation of which remains incompletely understood (Slominski *et al.*, 2004; Kauser *et al.*, 2005, 2006; Lu *et al.*, 2007; Schneider *et al.*, 2009; Paus, 2011; Tobin, 2011).

RESULTS

TRH stimulates melanin synthesis in normal human scalp HFs in the absence of a pituitary gland

First, we asked whether TRH, which is expressed by human scalp HFs on the gene and protein level (Gáspár *et al.*, 2010), can stimulate the pigmentation of microdissected and organ-cultured adult human scalp HFs. Only healthy HFs in the active growth stage of the hair cycle (anagen VI), during which the HF engages in maximal melanin production (Slominski *et al.*, 2005a; Schneider *et al.*, 2009; Tobin, 2011), were used. After 6 days of incubation with TRH (3 nM), qualitative and quantitative Masson–Fontana histochemistry revealed a significantly upregulated amount of histochemically detectable melanin granules in intact anagen hair bulbs compared with vehicle controls (Figure 1a–c).

TRH stimulates intrafollicular tyrosinase mRNA expression and enzyme activity

The fact that this reflects the *de novo* synthesis of melanin was suggested by *in situ* enzyme histochemistry for tyrosinase activity, the rate-limiting enzyme of melanogenesis (Slominski *et al.*, 2005a): Tyrosinase activity-associated immunofluorescence was significantly upregulated in HFs that had been cultured for 6 days in the presence of 30 nM and 300 nM TRH (Figure 1d–f). Moreover, compared with vehicle controls, TRH also stimulated *tyrosinase* gene transcription *in situ*, as demonstrated by quantitative RT-PCR from extracts of intact human anagen HFs (Figure 1g). This shows that TRH impacts on the enzymatic key control of melanogenesis by stimulating both gene transcription and enzyme activity.

Thus, in apparent contrast to amphibian skin melanophores, which are thought to require pituitary-derived signals for stimulation by TRH (Vaudry *et al.*, 1999; Vazquez-Martinez *et al.*, 2003; Jenks *et al.*, 2007, 2010), human HF melanocytes *in situ* are sensitive to TRH even in the absence of pituitary gland-derived melanocortins such as α-MSH. As human hair bulb epithelium expresses TRH on the gene and protein level *in situ* (Gáspár *et al.*, 2010), it is conceivable

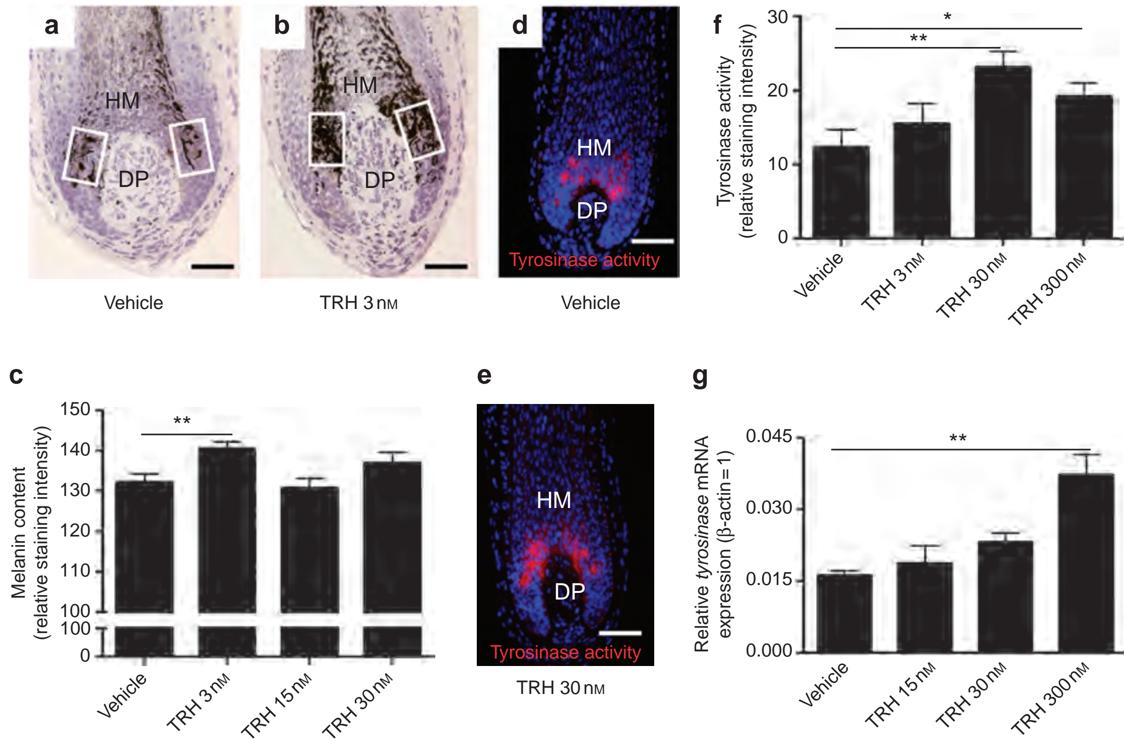

Figure 1. Thyrotropin-releasing hormone (TRH) stimulates melanin synthesis, tyrosinase mRNA expression, and tyrosinase activity in human scalp hair follicles (HFs) *in situ*. Histochemical staining for melanin in the hair bulb demonstrates elevated pigment production after 6 days in organ-cultured, normal human HFs incubated with TRH, compared with the vehicle-treated control (**a**, **b**). The increase of the melanin content—measured in the reference area (rectangles as indicated in **a**, **b**)—reached significance with 3 nM TRH (**c**). Red fluorescence staining represents tyrosinase enzyme activity in the pigmentary unit of TRH-treated HFs (**d**, **e**). Quantitative analysis of the tyrosinase staining intensity demonstrates significant increase at 30 nM and 300 nM TRH (**f**). Significant elevation of *tyrosinase* mRNA expression was observed after 300 nM TRH (**g**). Columns represent mean ± SEM of three independent experiments ($n = 15$–18 HFs per TRH concentration per each experiment), *$P < 0.05$ and **$P < 0.01$ versus vehicle (TRH 0 nM; bar = 50 μm). DP, dermal papilla; HM, hair matrix.

that keratinocyte-derived TRH has paracrine effects on melanocytes within the human HF.

TRH stimulates melanosome formation and melanocyte dendricity in the human HFPU

To corroborate these observations by independent, complementary pigment biological parameters, we investigated the expression of the pre-melanosomal protein gp100 (silver/Pmel17; essential for transition of stage I (pre-) melanosomes to stage II melanosomes (Singh *et al.*, 2008)). Immunodetection of this pigmentation-specific protein provides a very sensitive tool for visualizing both melanosome transfer from melanocytes to keratinocytes and non-pigmented (amelanotic) melanocytes in the HF (Singh *et al.*, 2008; Kloepper *et al.*, 2010). As assessed by quantitative immunohistomorphometry, TRH significantly increased both the total gp100-related immunoreactivity detectable in human anagen hair bulbs (Figure 2a–c) and the dendrite formation of human HF melanocytes *in situ* (Figure 2a, b, and d). TRH treatment also increased slightly, but not significantly, the total number of human anagen hair bulb melanocytes that were identifiable *in situ* by gp100 immunofluorescence (data not shown).

To induce hair shaft pigmentation, mature melanosomes are transferred through melanocyte dendrites within the HFPU to neighboring keratinocytes of the precortical hair matrix (Slominski *et al.*, 2005a; Kauser *et al.*, 2006; Singh *et al.*, 2008; Tobin, 2011). Therefore, the stimulation of gp100 production and the enhancement of HF melanocyte dendricity by TRH suggest considerably more complex and profound modulatory activities of TRH on human HF melanocyte biology *in situ* than the mere stimulation of enzymatic melanogenesis (Slominski *et al.*, 2005a). Thus, our gp100 data independently confirm the above histochemical and enzymatic pigmentation data (Figure 1) and show that TRH actually stimulates melanin biosynthesis and melanosome transfer to recipient keratinocytes within the precortical hair matrix of normal human scalp HFs.

TRH also stimulates melanogenesis in isolated, cultured human HF-derived melanocytes

Next, we asked whether TRH also stimulates pigmentation in primary cultured human HF melanocytes isolated from the outer root sheath of normal adult human scalp HFs; i.e., in the absence of the normal epithelial and mesenchymal signaling environment of HF melanocytes. As expected, these normally amelanotic cells switched on melanin production *in vitro* (Kauser *et al.*, 2005). As shown before *in situ*, TRH significantly also stimulated dendricity (Figure 2e–g), gp100 expression (Figure 3a–c), and tyrosinase activity (Figure 3d–f) in cultured human HF melanocytes. Intriguingly, even the proliferation of human HF melanocytes was upregulated by TRH *in vitro*, as shown by quantitative Ki67/gp100 double

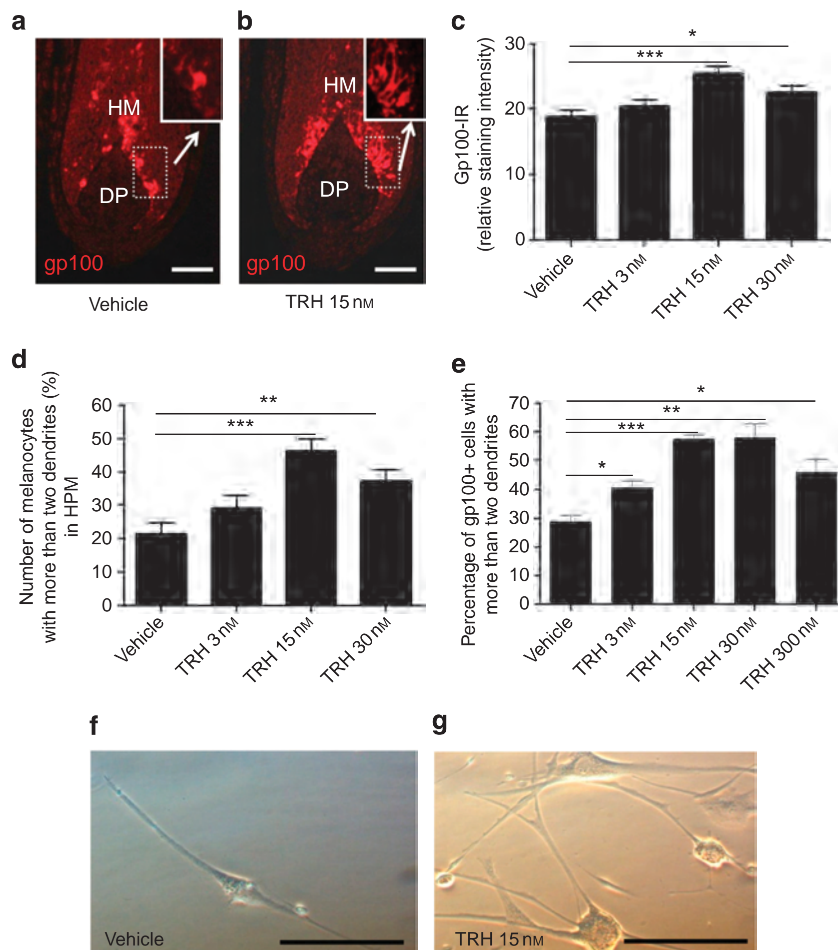

Figure 2. Thyrotropin-releasing hormone (TRH) stimulates melanosome formation and dendricity in human melanocytes *in situ* and *in vitro*. TRH administration *in vitro* (3–300 nM). TRH significantly increased the pre-melanosome marker gp100 expression (red) in human anagen hair bulbs (**a**, **b**, **c**) and the dendrite formation of human hair follicle (HF) melanocytes *in situ* (**d**). TRH administration *in vitro* (15–300 nM) significantly stimulated the number of gp100 + melanocytes (**e**, **f**, **g**). Columns represent mean ± SEM of three independent experiments (n = 15–18 HFs per TRH concentration per each experiment), *P<0.05, **P<0.01, and ***P<0.001 versus vehicle (TRH 0 nM; bar = 50 μm). DP, dermal papilla; HM, hair matrix; HPM, hair follicle pigmentary unit; IR, immunoreactivity.

immunofluorescence (Figure 3g–i). These TRH-induced effects were seen even in cultured human HF melanocytes not showing TRH-R immunoreactivity (data not shown); indeed, the latter reportedly do not express *TRH-R* mRNA (Slominski *et al.*, 2002).

TRH does not stimulate human epidermal melanogenesis *in situ*

We asked whether TRH is a general pigmentation stimulator in human skin, or whether this effect is specific to the HF. As shown in Figure 4a, TRH did not significantly upregulate the histochemically determined melanin content of organ-cultured human epidermis. Moreover, TRH failed to significantly upregulate epidermal gp100 immunoreactivity and tyrosinase activity *in situ* (Figure 4b and c). Thus, under physiological conditions, TRH selectively stimulates human HF melanogenesis, but not intraepidermal melanogenesis.

Interestingly, preliminary observations with adult frog skin organ culture in our laboratory suggest that high doses of TRH can stimulate melanosome dispersion in intracutaneous *Xenopus tropicalis* melanophores *in situ* in the absence of a pituitary gland (D Pattwell and R Paus, unpublished observation). These frog skin organ-culture observations suggest that, contrary to conventional wisdom (Jackson and Reichlin, 1977; Vaudry *et al.*, 1999), even in amphibian skin TRH can exert direct pituitary gland-independent pigmentation-stimulatory effects. Although these intracutaneous pigmentation-stimulatory effects of TRH appear to have been lost in human epidermal melanocytes *in situ*, we demonstrate here that they have been preserved in the human HFPU. Moreover, this underscores the validity of the concept that epidermal and hair melanocytes represent phenotypically and functionally rather distinct sub-populations, which underlie differential controls (Slominski *et al.*, 2004; Plonka *et al.*, 2009; van Raamsdonk *et al.*, 2009; Tobin, 2011).

The HFPU does not seem to express TRH-R protein

A major challenge remains to identify the mechanism(s) of action by which TRH may exert its complex pigmentary-stimulatory function on human HF melanocytes *in situ* and *in vitro*. We had already shown that *TRH-R* mRNA is

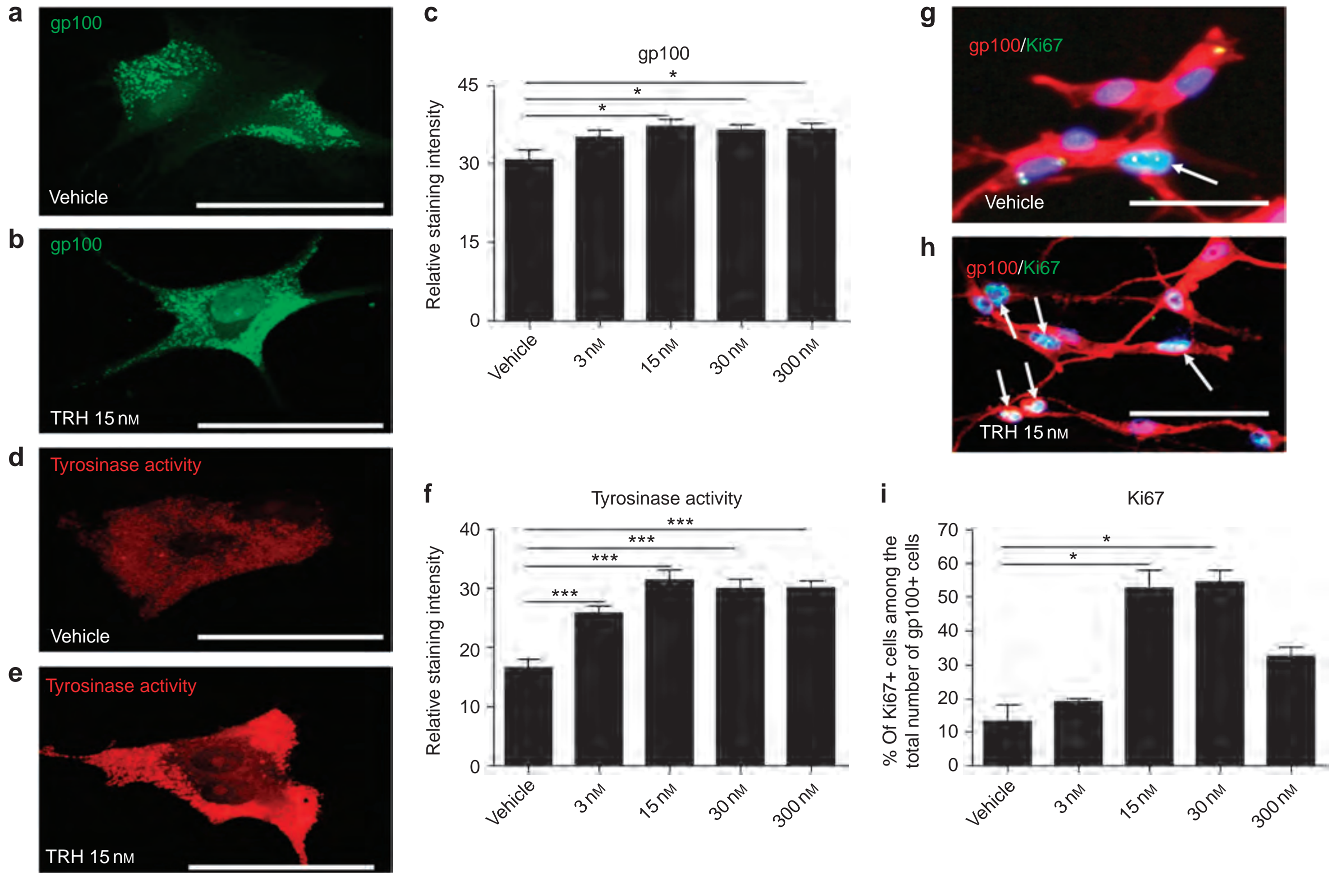

Figure 3. Thyrotropin-releasing hormone (TRH) increases melanogenesis and proliferation of human hair follicle (HF) melanocytes *in vitro*. Green fluorescence shows normal, HF melanocytes *in vitro* stained for the pre-melanosome marker gp100 (**a**, **b**). TRH administration (15–300 nM) significantly stimulated the gp100 expression (green fluorescence) of cultured HF melanocytes (**c**). Red fluorescence staining represents tyrosinase activity of cultured HF melanocytes (**d**, **e**). The tyrosinase activity was also significantly upregulated after TRH (3–300 nM) treatment (**f**). Ki67 (green)/gp100 (red) double immunofluorescence staining (arrows; **g**, **h**) shows significantly increased proliferation of cultured HF melanocytes after TRH (15–30 nM) treatment (**i**). Columns represent mean ± SEM of three independent experiments ($n = 15$–18 HFs per TRH concentration per experiment), $^{*}P < 0.05$ and $^{***}P < 0.001$ versus vehicle (TRH 0 nM; bar = 50 μm).

detectable in human HF extracts, although the expression of TRH-R protein *in situ* appears largely limited to the inner root sheath of the HF (Gáspár *et al.*, 2010). Also, isolated human HF melanocytes do not express TRH-R mRNA *in vitro* (Slominski *et al.*, 2002). This made it difficult to understand how TRH exerts its HF pigmentary effects on the HFPU.

Therefore, we double checked this earlier observation with a highly sensitive light-microscopic immunohistology technique (Envision-ABC), so as to more accurately define the anatomical localization of the TRH-R protein expression pattern, using optimal positive and negative immunoreactivity controls. However, this method independently confirmed the TRH-R negativity of the human HFPU (Supplementary Figure S1 online). If melanogenically active human HF melanocytes express any functional TRH-R protein at all *in situ*, this was below our limit of detection. Even isolated, cultured human HF melanocytes from the outer root sheath showed no evidence of specific TRH-R immunoreactivity (data not shown). This suggests that the intrafollicular pigmentary effects of TRH are unlikely to be mediated by direct stimulation of TRH-Rs expressed by melanocytes of the human HFPU.

POMC-dependent signaling may mediate the pigmentary effects of TRH

Therefore, we next asked whether an indirect mode of action may explain the profound pigmentary effects of TRH. Our HF organ culture data had clearly shown that hypothalamus- and/or pituitary gland-derived signals are dispensable for the observed pigmentary effects of TRH. However, as human scalp HFs express as an astounding variety of ''hypothalamic'' and ''pituitary'' neurohormones (Slominski *et al.*, 2000, 2002; Kauser *et al.*, 2004, 2005, 2006; Ito *et al.*, 2005; Paus, 2010, 2011), TRH may well have upregulated intrafollicular POMC expression and α-MSH and/or ACTH production from this prohormone. In fact, the epithelium of human scalp HFs has established a fully functional peripheral equivalent system of the hypothalamic–pituitary–adrenal axis (Ito *et al.*, 2005), and human HFs are now appreciated to prominently transcribe and translate POMC, from which ACTH and α-MSH are processed (Slominski *et al.*, 2000; Ito *et al.*, 2005; Kauser *et al.*, 2005, 2006).

Therefore, we tested by RT-PCR whether *POMC* mRNA steady-state levels after TRH treatment were altered. After 8 h

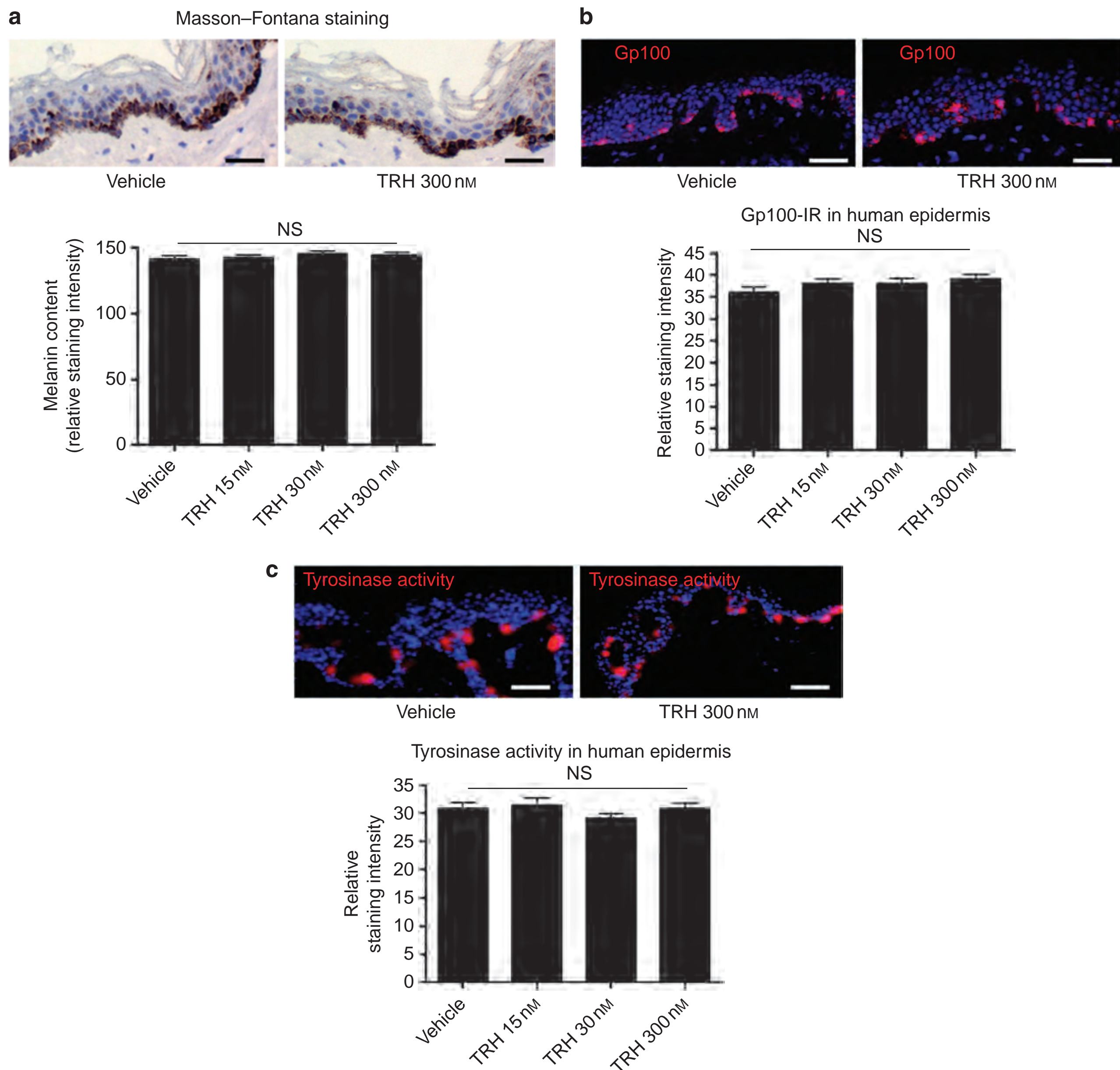

Figure 4. Thyrotropin-releasing hormone (TRH) does not affect human epidermal melanogenesis *in situ*. TRH did not stimulate melanin formation in normal human epidermal melanocytes (**a**). (**b**) Gp100 expression (red fluorescence staining) in epidermal melanocytes was not significantly increased after TRH treatment. (**c**) Tyrosinase activity (red fluorescence staining) of epidermal melanocytes remained unchanged after 6 days of TRH administration. IR, immunoreactivity; NS, not significant. Bar = 50 µm.

of incubating organ-cultured HFs with 15 nM TRH, intrafollicular *POMC* transcript levels were significantly increased (Figure 5a). On the protein level, quantitative immunohistomorphometry revealed that TRH significantly upregulated the intrafollicular immunoreactivity for ACTH *in situ* (Figure 5b; Supplementary Figure S2a online). Even though the immunoreactivity for α-MSH also showed a slight increase after TRH stimulation in the proximal epithelium of some HFs (as illustrated, e.g., in Supplementary Figure S2b online), overall this was not statistically significant (Supplementary Figure S2c online). It is conceivable that intrafollicularly generated TRH (Gáspár *et al.*, 2010) upregulates intrafollicular *POMC* expression and its processing to melanotropic, POMC-derived peptides (such as ACTH), and thereby stimulates human HF pigmentation indirectly.

TRH may stimulate HF pigmentation by binding to the melanocortin-1 receptor

Given that both ACTH and α-MSH stimulate human HF melanocytes and intrafollicular melanogenesis by binding to the melanocortin-1 receptor (MC-1R), we focused next on MC-1R. MC-1R is not only prominently expressed in the human anagen hair bulb (Slominski *et al.*, 2005a, b; Kauser *et al.*, 2006; Tobin, 2011), but TRH can also use this receptor

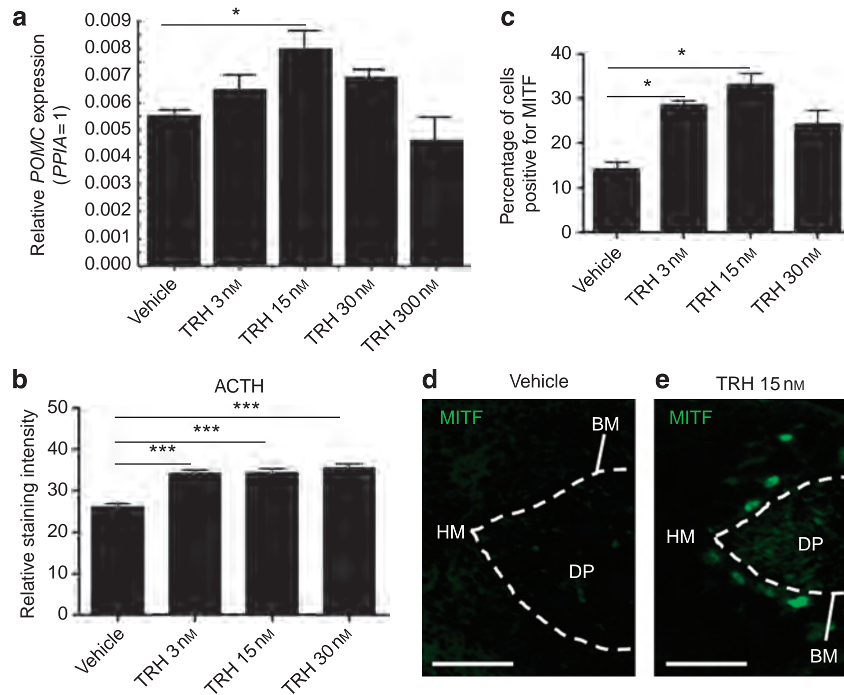

Figure 5. Thyrotropin-releasing hormone (TRH) stimulates *proopiomelanocortin* (*POMC*) mRNA expression and ACTH and micropathalmia-associated transcription factor (MITF) immunoreactivity in human scalp hair follicles (HFs) *in situ*. TRH 15 nM significantly upregulated *POMC* mRNA expression (**a**), and TRH 3–30 nM upregulated the ACTH immunoreactivity in organ-cultured human HFs *in situ* (**b**). TRH also significantly increased intrafollicular MITF protein expression (**c**). Green fluorescence shows the immunoreactivity for MITF in TRH-treated hair bulbs (**d**, **e**). Columns represent mean ± SEM of three independent experiments ($n = 15–18$ HFs per TRH concentration per each experiment), $*P < 0.05$ and $***P < 0.001$ versus vehicle (TRH 0 nM; bar = 50 µm). BM, basal membrane; DP, dermal papilla; HM, hair matrix.

as an alternative mode of signaling (Schiöth *et al*., 1999). As highly selective TRH-R antagonists are currently not available, we resorted to administering TRH (3 or 15 nM) in the presence of an excess amount of agouti-signaling protein (ASP; 100 nM), a recognized MC-1R antagonist (Lu *et al*., 1994; Ollmann *et al*., 1998).

Coadministration of TRH with ASP reduced both the melanin content and the tyrosinase enzyme activity *in situ* to the level of vehicle-treated control HFs or even below this level (data not shown). These preliminary results suggest that the stimulatory effects of TRH on human HF pigmentation could indeed be mediated, at least in part, by binding to the MC-1R. This would also resolve the quandary how TRH can stimulate apparently TRH-R-negative human HF melanocytes *in situ* and *in vitro*, as both prominently express MC-1R (Kauser *et al*., 2005, 2006; Slominski *et al*., 2005a, b; Tobin, 2011). In addition, this would explain the ability of follicular melanocytes of POMC knockout mice to still produce eumelanin even in the absence of POMC products (Slominski *et al*., 2005b).

TRH stimulates intrafollicular MITF protein expression

Finally, we assessed the impact of TRH on microphthalmia-associated transcription factor (MITF), the ''master regulator of pigmentation'' (Nishimura *et al*., 2005; Vachtenheim and Borovanskỳ, 2010). This transcription factor is not only activated by MC-1R stimulation (Rouzaud *et al*., 2006; Chou *et al*., 2010) but also controls more than 25 genes in pigment cells that affect melanogenesis, melanocyte development, proliferation, and survival, as well as intrafollicular melanocyte stem cell functions (Nishimura *et al*., 2005). Therefore, we asked whether TRH upregulated the intrafollicular expression of MITF protein. As seen in Figure 5c–e, immunoreactivity for MITF hair bulbs was significantly increased in TRH-treated HFs compared with vehicle controls. In addition, the percentage of MITF-positive cells increased under TRH administration (Figure 5c). Given that a MITF-dependent decline in the number of human HF melanocyte stem cells is considered by many investigators to be a chief cause of hair graying (Nishimura *et al*., 2005; Tobin, 2011), it is particularly intriguing to note that TRH can upregulate intrafollicular MITF expression.

DISCUSSION

Our study presents the first evidence that TRH (1) exerts any effects on normal mammalian melanocytes *in situ*, (2) stimulates melanin production in appendages of normal adult human skin, (3) stimulates human HF, but not human epidermal melanocytes, and (4) that these pigmentation-stimulatory effects are independent of the pituitary gland . Moreover, our study reveals a novel and apparently HF-specific and evolutionarily highly conserved neuroendocrine control system of human pigmentation (Paus, 2011) that may date back at least to the role that these controls attained already in amphibian skin biology.

This newly identified, physiologically relevant, and melanocyte sub-population-specific control of human pigmentation

deserves full exploration not only in melanocyte biology but also as a potential target to prevent or reverse hair graying (Tobin and Paus, 2001; Tobin 2011). As TRH is an unusually stable tripeptide, it is conceivable that high intrafollicular TRH doses could be achieved after topical application, e.g., by HF-targeting liposomes (Ciotti and Weiner, 2002; Chourasia and Jain, 2009). This could help to circumvent an undesired rise in systemic TRH levels that may induce excessive thyroid hormone secretion.

The proposed key role of MITF in graying of human hair (Nishimura *et al.*, 2005) and the stimulatory effect of TRH that we have observed here on the intrafollicular expression of this master regulator of pigmentation and of HF melanocyte stem cells (Nishimura *et al.*, 2005; Vachtenheim and Borovanskỳ, 2010; Tobin, 2011) encourage one to explore TRH and/or TRH-mimetic peptides as antigraying agents. The fact that TRH is already regularly used in clinical endocrinology (TRH stimulation test in thyroid medicine) should greatly facilitate the clinical testing of this tripeptide as a new candidate agent for halting or reversing human hair graying.

Although the full range of mechanisms through which TRH exerts its complex stimulatory effects on human HF pigmentation remains to be definitively clarified, our currently available data suggest that TRH stimulates intrafollicular *POMC* expression by HF keratinocytes. This then primarily upregulates the intrafollicular production of ACTH, which binds to MC-1R and MC-2R receptors in human HFs (Ito *et al.*, 2005; Kauser *et al.*, 2005). This mechanistic scenario is supported by clinical evidence: Intravenous administration of TRH to pregnant women, or to a subgroup of patients with Cushing's disease, reportedly induces increased serum ACTH levels (Pieters *et al.*, 1982a, b). TRH may exploit the intrafollicular production of classical ''pituitary'' melanotropic hormones to stimulate HF pigmentation indirectly. Moreover, TRH appears to non-classically stimulate intrafollicular MC1-R-mediated signaling.

Our data suggest that MC-1R is the key receptor in transducing the pigmentary effects of TRH. This is supported by several lines of argumentation: (1) The HFPU does not express immunohistochemically detectable TRH-Rs; (2) cultured HF melanocytes do not express detectable TRH-R protein or transcripts, but functional MC-1R (Abdel-Malek *et al.*, 1999; Slominski *et al.*, 2002; Kadekaro *et al.*, 2003; Kauser *et al.*, 2005), yet respond to TRH stimulation; (3) TRH can non-classically signal via the human MC1-R (Pieters *et al.*, 1982a); (4) the stimulatory effects of TRH on human hair pigmentation can be partially antagonized by the selective competitive MC-1R antagonist, ASP; (5) as proposed before, the unexpected production of eumelanin in POMC knockout mice on a C57BL/6 background indicates the existence of an alternative ligand for MC1-R (Slominski *et al.*, 2002); our findings raise the possibility that (e.g., intrafollicularly generated) TRH could be this alternative ligand.

However, additional potential mechanisms of action should be considered and explored in future studies. For example, TRH could also stimulate intrafollicular β-endorphin processing, as this POMC product can stimulate human HF pigmentation (Kauser *et al.*, 2004). Even unprocessed POMC itself may stimulate melanin production by human melanocytes *in vitro* (Rousseau *et al.*, 2007). As we show here that TRH upregulates intrafollicular *POMC* transcription, this additional mechanism of action deserves careful consideration. In theory, even receptor-independent signaling events might contribute to the pigmentary effects of this small tripeptide neurohormone *in situ* and *in vitro*. However, our finding that ASP partially antagonizes TRH pigmentary effects argues in favor of a predominantly MC1-R-dependent mechanism.

The current study strongly encourages one to systematically follow up available pointers to evolutionarily ancient controls of pigmentation that may still be conserved in human skin. In this context, amphibian skin neuroendocrinology (Jackson and Reichlin, 1977; Bolaffi and Jackson, 1979; Vaudry *et al.*, 1999; Vazquez-Martinez *et al.*, 2003; Jenks *et al.*, 2007, 2010; Slominski 2007; Slominski *et al.*, 2008; Galas *et al.*, 2009) promises valuable lessons for the mammalian system that may bear direct relevance for the management of human pigmentary disorders. We show that human HF organ culture and the highly hormone-sensitive HF melanocyte populations provide excellent discovery tools for elucidating ancestral, evolutionarily conserved, and clinically relevant non-classical neurohormone functions in human biology.

Our study also underscores the value of dissecting the physiological controls of human melanocyte biology in organ culture, rather than in non-physiological cell culture systems where melanocytes operate in the absence of crucial epithelial and mesenchymal cues. As human HF organ culture facilitates the study of human melanocyte functions within their natural tissue habitat and under full preservation of normal keratinocyte–fibroblast–melanocyte interactions (Slominski *et al.*, 2005a; Plonka *et al.*, 2009), it offers an instructive, accessible, and clinically relevant test system for characterizing the neuroendocrine controls of melanogenesis and human melanocyte biology *in situ* (Ito *et al.*, 2005; Kauser *et al.*, 2005, 2006; Schneider *et al.*, 2009; Paus, 2011).

MATERIALS AND METHODS

HF organ culture

The study adhered to the Declaration of Helsinki Principles, was approved by the Institutional Research Ethics Committee of the University of Lübeck, and patients gave their written informed consent. Anagen VI HFs were microdissected from normal human scalp skin obtained from three healthy adult females undergoing routine face-lift cosmetic surgery (Kloepper *et al.*, 2010). Serum-free HF organ culture was performed in the presence of vehicle, TRH (3–300 nM; Bachem, St Helens, UK), and/or ASP (100 nM; Phoenix Pharmaceuticals, Burlingame, CA; see Supplementary Materials and Methods online).

Human scalp skin organ culture

Full-thickness human scalp skin organ culture was performed for 6 days under serum-free conditions in the presence or absence of 3–300 nM TRH as described before (Lu *et al.*, 2007; see Supplementary Materials and Methods online).

Human HF melanocyte culture

The isolation and culture of melanocytes from human anagen VI scalp HFs can be found in the Supplementary Materials and Methods online.

Quantitative melanin histomorphometry

Masson–Fontana histochemistry was used to visualize the melanin content in cryosections (demarcated as black dots). Melanin staining intensity was analyzed in precisely defined, selected reference areas of the HFPU by using the ImageJ software (http://rsbweb.nih.gov/ij/download.html; see Supplementary Materials and Methods online).

Immunohistochemistry or immunofluorescence

ACTH, gp100, MITF, α-MSH, and TRH-R immunohistochemistry was performed on 7 μm cryostat sections of HFs fixed in acetone at −20 °C (10 min). Quantitative immunohistomorphometry in precisely defined reference areas was performed by using Image J software (see Supplementary Materials and Methods online).

Immunocytochemical analysis of cultured HF melanocytes

Human HF melanocytes grown on coverslips were incubated with Nki/beteb (Monosan, Uden, The Netherlands; 1:30 in phosphate-buffered saline), anti-Ki-67 (1:100 in phosphate-buffered saline, Zymed Laboratories, South San Francisco, CA), or a polyclonal rabbit anti-TRH-receptor antibody, which recognizes the human TRH receptor (homolog to murine TRH-R1; 1:100 in TBS + 2% NGS; Acris, Hiddenhausen, Germany) overnight at 4 °C (see Supplementary Materials and Methods online).

In situ tyrosinase enzyme activity

Tyrosinase enzyme activity was measured by tyramide-based tyrosinase immunohistochemistry (Han *et al.*, 2002). The fluorescence staining intensity, which reflects tyrosinase activity *in situ*, was quantified with ImageJ software. This assay was also used to measure tyrosinase activity of cultured human outer root sheath melanocytes.

Quantitative PCR for *tyrosinase* and *POMC* mRNA

Normal human scalp skin HFs were microdissected and cultured in the absence or presence of TRH (15–300 nM) for 8 h. Total RNA was isolated from 20 HFs per experimental group (see Supplementary Materials and Methods online).

Statistical analysis

Student's *t*-test for unpaired samples was calculated with GraphPad Prism 4.00 program (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Drs Björn E Wenzel, Andrzej Slominski, Robert Opitz, and Markus Böhm for expert advice; István Borbíró for performing Q-PCR; and G Scheel for excellent technical assistance. This work was supported in part by a grant from the Manchester Academic Health Sciences Center to RP.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abdel-Malek Z, Suzuki I, Tada A *et al.* (1999) The melanocortin-1 receptor and human pigmentation. *Ann NY Acad Sci* 20:117–33

Bidaud I, Galas L, Bulant M *et al.* (2004) Distribution of the mRNAs encoding the thyrotropin- releasing hormone (TRH) precursor and three TRH receptors in the brain and pituitary of *Xenopus laevis*: effect of background color adaptation on TRH and TRH receptor gene expression. *J Comp Neurol* 477:11–28

Bodó E, Kromminga A, Bíró T *et al.* (2009) Human female hair follicles are a direct, nonclassical target for thyroid-stimulating hormone. *J Invest Dermatol* 129:1126–39

Bolaffi JL, Jackson IM (1979) Immunohistochemical localization of thyrotropin-releasing hormone (TRH) in skin of *Rana pipiens*. *Cell Tissue Res* 202:505–8

Chiamolera MI, Wondisford FE (2009) Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism. *Endocrinology* 150:1091–6

Chou TH, Ding HY, Hung WJ *et al.* (2010) Antioxidative characteristics and inhibition of alpha- melanocyte-stimulating hormone-stimulated melanogenesis of vanillin and vanillic acid from *Origanum vulgare*. *Exp Dermatol* 19:742–50

Chourasia R, Jain SK (2009) Drug targeting through pilosebaceous route. *Curr Drug Targets* 10:950–67

Ciotti SN, Weiner N (2002) Follicular liposomal delivery systems. *J Liposome Res* 12:143–8

Galas L, Raoult E, Tonon MC *et al.* (2009) TRH acts as a multifunctional hypophysiotropic factor in vertebrates. *Gen Comp Endocrinol* 164:40–50

García SI, Pirola CJ (2005) Thyrotropin-releasing hormone in cardiovascular pathophysiology. *Regul Pept* 128:239–46

Gáspár E, Hardenbicker C, Bodó E *et al.* (2010) Thyrotropin releasing hormone (TRH): a new player in human hair-growth control. *FASEB J* 24:393–403

Han R, Baden HP, Brissette JL *et al.* (2002) Redefining the skin's pigmentary system with a novel tyrosinase assay. *Pigment Cell Res* 15:290–7

Ito N, Ito T, Kromminga A *et al.* (2005) Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J* 19:1332–4

Jackson IM, Reichlin S (1977) Thyrotropin-releasing hormone: abundance in the skin of the frog, *Rana pipiens*. *Science* 198:414–5

Jenks BG, Kidane AH, Scheenen WJ *et al.* (2007) Plasticity in the melanotrope neuroendocrine interface of *Xenopus laevis*. *Neuroendocrinology* 85:177–85

Jenks BG, Galas L, Kuribara M *et al.* (2010) Analysis of the melanotrope cell neuroendocrine interface in two amphibian species, *Rana ridibunda* and *Xenopus laevis*: a celebration of 35 years of collaborative research. *Gen Comp Endocrinol* 170:57–67

Kadekaro AL, Kanto H, Kavanagh R *et al.* (2003) Significance of the melanocortin 1 receptor in regulating human melanocyte pigmentation, proliferation, and survival. *Ann NY Acad Sci* 994:359–65

Kamath J, Yarbrough GG, Prange AJ Jr *et al.* (2009) The thyrotropin-releasing hormone (TRH)-immune system homeostatic hypothesis. *Pharmacol Ther* 121:20–8

Kauser S, Thody AJ, Schallreuter KU *et al.* (2004) beta-Endorphin as a regulator of human hair follicle melanocyte biology. *J Invest Dermatol* 123:184–95

Kauser S, Thody AJ, Schallreuter KU *et al.* (2005) A fully functional proopiomelanocortin/melanocortin-1 receptor system regulates the differentiation of human scalp hair follicle melanocyte. *Endocrinology* 146:532–43

Kauser S, Slominski A, Wei ET *et al.* (2006) Modulation of the human hair follicle pigmentary unit by corticotropin-releasing hormone and urocortin peptides. *FASEB J* 20:882–95

Kloepper JE, Sugawara K, Al-Nuaimi Y *et al.* (2010) Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* 19:305–12

Lechan RM, Hollenberg AN (2003) Thyrotropin-releasing hormone (TRH). In: Henry HL and Norman AW (eds). *Encyclopedia of Hormones*. New York: Elsevier Science, pp 510–24

Lechan RM, Fekete C (2006) The TRH neuron: a hypothalamic integrator of energy metabolism. *Prog Brain Res* 153:209–35

Leduque P, Bulant M, Dubois PM *et al.* (1989) Processing of thyrotropin-releasing hormone prohormone (pro-TRH) in the adult rat pancreas: identification and localization of pro-TRH- related peptides in beta-cells of pancreatic islets. *Endocrinology* 125:1492–7

Lu D, Willard D, Patel IR *et al.* (1994) Agouti protein is an antagonist of the melanocyte-stimulating- hormone receptor. *Nature* 371:799–802

Lu Z, Hasse S, Bodo E *et al.* (2007) Towards the development of a simplified long-term organ culture method for human scalp skin and its appendages under serum-free conditions. *Exp Dermatol* 16:37–44

Martino E, Lernmark A, Seo H *et al.* (1978) High concentration of thyrotropin-releasing hormone in pancreatic islets. *Proc Natl Acad Sci USA* 75:4265–7

Nishimura EK, Granter SR, Fisher DE (2005) Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. *Science* 307:720–4

Ollmann MM, Lamoreux ML, Wilson BD *et al.* (1998) Interaction of Agouti protein with the melanocortin 1 receptor *in vitro* and *in vivo*. *Genes Dev* 12:316–30

Paus R (2010) Exploring the ''thyroid-skin connection'': concepts, questions, and clinical relevance. *J Invest Dermatol* 130:7–10

Paus R (2011) A neuroendocrinological perspective on human hair follicle pigmentation. *Pigment Cell Melanoma Res* 24:89–106

Pieters GF, Smals AG, Goverde HJ *et al.* (1982a) Paradoxical responsiveness of adrenocorticotropin and cortisol to thyrotropin releasing hormone (TRH) in pregnant women. Evidence for intermediate lobe activity? *J Clin Endocrinol Metab* 55:387–9

Pieters GF, Smals AG, Goverde HJ *et al.* (1982b) Adrenocorticotropin and cortisol responsiveness to thyrotropin-releasing hormone and luteinizing hormone-releasing hormone discloses two subsets of patients with Cushing's disease. *J Clin Endocrinol Metab* 55:1188–97

Plonka PM, Passeron T, Brenner M *et al.* (2009) What are melanocytes really doing all day long…? *Exp Dermatol* 18:799–819

Ramot Y, Bíró T, Tiede S *et al.* (2010) Prolactin—a novel neuroendocrine regulator of human keratin expression *in situ*. *FASEB J* 24:1768–79

Rousseau K, Kauser S, Pritchard LE *et al.* (2007) Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis. *FASEB J* 21:1844–56

Rouzaud F, Costin GE, Yamaguchi Y *et al.* (2006) Regulation of constitutive and UVR-induced skin pigmentation by melanocortin 1 receptor isoforms. *FASEB J* 20:1927–9

Schiöth HB, Prusis P, Muceniece R *et al.* (1999) Thyrotropin releasing hormone (TRH) selectively binds and activates the melanocortin 1 receptor. *Peptides* 20:395–400

Schneider MR, Schmidt-Ullrich R, Paus R (2009) The hair follicle as a dynamic miniorgan. *Curr Biol* 19:132–42

Singh SK, Nizard C, Kurfurst R *et al.* (2008) The silver locus product (Silv/gp100/Pmel17) as a new tool for the analysis of melanosome transfer in human melanocyte-keratinocyte co-culture. *Exp Dermatol* 17:418–26

Slominski A, Wortsman J, Luger T *et al.* (2000) Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol Rev* 80:979–1020

Slominski A, Wortsman J, Kohn L *et al.* (2002) Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. *J Invest Dermatol* 119:1449–55

Slominski A, Tobin DJ, Shibahara S *et al.* (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 84: 1155–228

Slominski A, Wortsman J, Plonka PM *et al.* (2005a) Hair follicle pigmentation. *J Invest Dermatol* 124:13–21

Slominski A, Plonka PM, Pisarchik A *et al.* (2005b) Preservation of eumelanin hair pigmentation in proopiomelanocortin-deficient mice on a nonagouti (a/a) genetic background. *Endocrinology* 146:1245–53

Slominski A (2007) A nervous breakdown in the skin: stress and the epidermal barrier. *J Clin Invest* 117:3166–9

Slominski A, Wortsman J, Paus R *et al.* (2008) Skin as an endocrine organ: implications for its function. *Drug Discov Today Dis Mech* 5:137–44

Tobin DJ, Paus R (2001) Graying: gerontobiology of the hair follicle pigmentary unit. *Exp Gerontol* 36:29–54

Tobin DJ (2011) The cell biology of human hair follicle pigmentation. *Pigment Cell Melanoma Res* 24:75–88

Tonon MC, Leroux P, Leboulenger F *et al.* (1980) Thyrotropin-releasing hormone stimulates the release of melanotropin from frog neurointermediate lobes *in vitro*. *Life Sci* 26:869–75

Vachtenheim J, Borovanskỳ J (2010) ''Transcription physiology'' of pigment formation in melanocytes: central role of MITF. *Exp Dermatol* 19: 617–27

van Beek N, Bodó E, Kromminga A *et al.* (2008) Thyroid hormones directly alter human hair follicle functions: anagen prolongation and stimulation of both hair matrix keratinocyte proliferation and hair pigmentation. *J Clin Endocrinol Metab* 93:4381–8

van Raamsdonk CD, Barsh GS, Wakamatsu K *et al.* (2009) Independent regulation of hair and skin color by two G protein-coupled pathways. *Pigment Cell Melanoma Res* 22:819–26

Vaudry H, Chartrel N, Desrues L *et al.* (1999) The pituitary-skin connection in amphibians. Reciprocal regulation of melanotrope cells and dermal melanocytes. *Ann NY Acad Sci* 885:41–56

Vazquez-Martinez R, Castaño JP, Tonon MC *et al.* (2003) Melanotrope secretory cycle is regulated by physiological inputs via the hypothalamus. *Am J Physiol Endocrinol Meta* 285:E1039–46