Control of Human Hair Growth by Neurotrophins: Brain-Derived Neurotrophic Factor Inhibits Hair Shaft Elongation, Induces Catagen, and Stimulates Follicular Transforming Growth Factor β2 Expression

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Neurotrophins are important modulators of epithelial–mesenchymal interactions. Previously, we had shown that brain-derived neurotrophic factor (BDNF) and its high-affinity receptor tyrosine kinase B (TrkB) are prominently involved in the control of murine hair follicle cycling. We now show that BDNF and TrkB are also expressed in the human hair follicle in a manner that is both hair cycle dependent and suggestive of epithelial–mesenchymal cross-talk between BDNF-secreting dermal papilla fibroblasts of anagen hair follicles and subpopulations of TrkB+ hair follicle keratinocytes. As functional evidence for an involvement of BDNF/TrkB in human hair growth control, we show in organ-cultured human anagen hair follicles that 50 ng per mL BDNF significantly inhibit hair shaft elongation, induce premature catagen development, and inhibit keratinocyte proliferation. Quantitative real-time rtPCR analysis demonstrates upregulation of the potent catagen inducer, transforming growth factor β2 (TGFβ2) by BDNF, whereas catagen induction by BDNF was partially reversible through co-administration of TGFβ-neutralizing antibody. This suggests that TrkB-mediated signaling promotes the switch between anagen and catagen at least in part via upregulation of TGFβ2. Thus, human scalp hair follicles are both a source and target of bioregulation by BDNF, which invites to target TrkB-mediated signaling for therapeutic hair growth modulation.

Key words: brain-derived neurotrophic factor/hair cycle/human/neurotrophin/tumor growth factor β2/tyrosine kinase B


Since its discovery more than 20 y ago (Barde et al, 1982), the neurotrophin brain-derived neurotrophic factor (BDNF), the second member of the nerve growth factor family of growth factors, has become known to guide and sustain sensory and autonomic neuron development and their differentiation within peripheral neural networks (Barde et al, 1982; Botchkarev et al, 1999). BDNF is produced by peripheral inner-}

Abbreviations: BDNF, brain-derived neurotrophic factor; IR, immunoreactivity; rhBDNF, recombinant human BDNF; TGFβ2, transforming growth factor β2; TrkB, tyrosine kinase B; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labeling
vasculature (Mecklenburg et al., 2000), and profound changes of the skin architecture and immune function (Paus et al., 1999a). BDNF expression in the murine hair follicle fluctuates in a strikingly hair cycle-dependent manner and BDNF overexpression causes premature catagen transformation (Botchkarev et al., 1999a) and alters cutaneous innervation density (Botchkarev et al., 1999b).

Since nervous system and hair follicle epithelium share a common ectodermal origin, it is not surprising that TrkB is also expressed by cutaneous epithelial cells in mice and humans (Shibayama and Koizumi, 1996; Botchkarev et al., 1999a, b). In regressing hair follicles of C57BL/6 mice, TrkB is expressed on apoptotic keratinocytes during catagen and BDNF skin content is highest during anagen–catagen transformation (Botchkarev et al., 1999a). Simultaneous expression of BDNF by these cells suggests para- and autocrine growth-regulatory effects of BDNF on epithelial tissues (Shibayama and Koizumi, 1996; Botchkarev et al., 1999a). Although epidermal keratinocyte proliferation is stimulated by BDNF (Botchkarev et al., 1999b), a growth-inhibitory effect of BDNF on selected keratinocyte subpopulations was demonstrated with the report that BDNF induces premature catagen development in anagen hair follicles of C57BL/6 mice (Botchkarev et al., 1999a).

Given the very substantial inter-species differences that a given bioregulatory compound can exert, e.g. on murine versus human hair follicles (cf. Paus and Cotsarelis, 1999; Stenn and Paus, 2001) and the contrasting effects of BDNF on epidermal (stimulation of proliferation) (Botchkarev et al., 1999b) and hair follicle keratinocytes (promotion of apoptosis) (Botchkarev et al., 1999a) in mice, it remains totally unknown whether BDNF/TrkB signaling plays any role in human hair biology. With these limitations in mind, we have addressed the following specific questions:

1. Do human hair follicle keratinocytes and fibroblasts in normal scalp skin express BDNF and/or its high-affinity receptor TrkB mRNA and immunoreactivity (IR)?
2. Does BDNF promote catagen development in organ-cultured human anagen hair follicles, as it does in mice?
3. If so, does this include the recruitment of recently recognized common mechanisms of catagen induction in human hair follicles, such as downregulation of hair follicle keratinocyte proliferation, upregulation of keratinocyte apoptosis, and transforming growth factor β (TGFβ)-mediated catagen induction?
4. Can negative BDNF effects on human hair growth in vitro be antagonized?

These questions were addressed by analyzing the expression pattern of BDNF and TrkB in human scalp skin biopsies as well as in isolated anagen VI hair follicles by rtPCR analysis, in situ hybridization, and immunofluorescence. Analysis of human hair follicle cycling is difficult. Most available hair follicles for rtPCR, in situ analyses, or immunohistochemistry derive from scalp skin excised during elective plastic surgery. In such biopsies, 80%–90% of all hair follicles are in anagen VI, and the remainder are in telogen, with catagen or early anagen hair follicles being detectable only extremely rarely (Paus and Cotsarelis, 1999). Therefore, in situ expression analyses of growth factors and receptors largely are limited to studying anagen VI and telogen hair follicles. In addition, human anagen VI scalp skin hair follicles were organ cultured (Philpott et al., 1994; Philpott, 1999) to investigate the impact of recombinant human BDNF (rhBDNF) or BDNF plus TGFβ1-neutralizing antibodies on active hair shaft elongation and maintenance of the hair shaft-producing unit in the anagen hair bulb.

Results

Human anagen hair follicles express BDNF and TrkB mRNA rtPCR revealed that micro-dissected human scalp skin anagen hair bulbs, which include the hair follicle epithelium proximal to the isthmus and bulge region as well as the dermal papilla and the proximal connective tissue sheath of the hair follicle, contained transcripts of the BDNF and TrkB genes (Fig 1). This offered an indication, that, just like their murine counterparts, human anagen hair follicles are both sources and targets of BDNF/TrkB-mediated signalling.

BDNF mRNA is expressed both in the mesenchyme and epithelium of human anagen VI hair follicles, TrkB only in the epithelium (inner and outer root sheath) BDNF and TrkB transcription in human scalp anagen VI hair follicles was confirmed and localized by in situ hybridization using fluorescein-labeled oligonucleotide probe (Fig 2). All negative and positive controls (see Materials and Methods) confirmed the specificity and sensitivity of the used in situ stainings. Staining intensity for BDNF transcripts was strongest in the proximal inner root sheath and distal outer root sheath (Fig 2), and BDNF transcript-related signals were also detected in the dermal papilla. TrkB transcripts were present in the proximal inner and distal outer root sheath, but not in the dermal papilla (Fig 2).

Immunohistochemistry confirms translation of BDNF and TrkB mRNA in human anagen VI hair follicles All BDNF and TrkB translation in human scalp anagen VI hair follicles was confirmed and localized by immunohistochemistry (Fig 2). All positive and negative controls (see Materials and Methods) confirmed the specificity and sensitivity of the used immunohistochemistry (Fig 2). All BDNF and TrkB were detected in the dermal papilla. TrkB was also detected in the dermal papilla and the proximal connective tissue sheath of the hair follicle, contained transcripts of the BDNF and TrkB genes (Fig 1). This offered an indication, that, just like their murine counterparts, human anagen hair follicles are both sources and targets of BDNF/TrkB-mediated signalling.

Figure 1 Brain-derived neurotrophic factor (BDNF) and tyrosine kinase B (TrkB) are expressed in the human anagen hair follicle by rtPCR. rtPCR analysis was performed on isolated human hair follicles using primers for BDNF and TrkB. As positive control, RNA from full-thickness temporal human scalp skin was used and water was used as negative control.
negative and positive controls confirmed the specificity and sensitivity of the used immunohistochemical stainings: specific staining patterns were absent in negative controls without primary antibody, or were strongly diminished after preincubation with specific blocking peptide. Positive controls showed the expected IR patterns in human pancreatic epithelia (Miknyoczki et al., 1999). All extrafollicular IR patterns were not analyzed in detail, since they are the subject of a separate, ongoing study, and the schemes that summarize BDNF and TrkB IR have only been sketched in preliminary form (Fig 2).

Semiquantitative analysis of BDNF IR demonstrated strongest protein expression in more differentiated keratinocyte cell populations such as the proximal inner root sheath and in the mesenchymal compartment, e.g. the dermal papilla. TrkB IR, in contrast, was strongest in more undifferentiated keratinocyte cell populations such as the basal layer of the hair follicle ostium. TrkB IR was also detected in the hair matrix and proximal outer root sheath but not in the dermal papilla (Table I).

Low BDNF and TrkB is present in the telogen hair follicle Because of their low incidence in human scalp skin (10%–20% of all hair follicles), telogen hair follicles were detected only rarely in normal human scalp skin sections. In 13 (BDNF) and nine (TrkB) different donor samples (which contained more than 40 anagen hair follicles), only four telogen hair follicles, each of a different donor, were found per investigated antigen. Hair follicles in catagen or early anagen were not detected at all. Weakly positive BDNF staining (IR and in situ) was detected in the outer root sheath and secondary hair germ epithelium of all telogen hair follicles (Fig 2). BDNF staining was pronounced in the outer root sheath basal layer. TrkB staining was weak in the hair follicle epithelium (Fig 2). Interestingly and in contrast to our findings in anagen skin, in the telogen stage of the hair cycle the dermal papilla-stained negative for both antigens (Fig 2).

BDNF concentration dependently inhibits hair shaft elongation in cultured human anagen hair follicles In order to assess the functional relevance of BDNF/TrkB signaling in the anagen hair follicle, microdisected lower anagen hair follicles from normal human scalp skin were cultured in the presence of 1, 5, 25, 50, and 150 ng per mL rhBDNF. These experiments revealed a significant reduction of hair growth in hair follicles cultured with 50 and 150 ng per mL BDNF, whereby hair follicles treated with 150 ng per mL exhibited dystrophic features such as pigment incontinence as a sign of toxification. Therefore 50 ng per mL was chosen for the treatment of a larger number of samples (Fig 3).

Figure 2
Brain-derived neurotrophic factor (BDNF)- and tyrosine kinase B (TrkB)-expression patterns in human hair follicles. The schematic summaries display the most highly reproducible BDNF and TrkB IR patterns revealed in human anagen scalp skin hair follicles. Cryosections (immunohistochemistry [IHC]) or paraffin sections (in situ hybridization) from human scalp skin samples containing 80%–90% anagen hair follicles were stained with antisera against BDNF and TrkB (Santa Cruz Biotechnology) or were labeled with oligonucleotide probes to BDNF or TrkB. All images demonstrate representative IR patterns in anagen hair follicles and telogen hair follicles. If not otherwise labeled, arrows point at strongly stained structures. Boxes indicate anatomical location of the presented micrographs. The bottom panel gives examples of positive and negative controls. For the IHC-negative control, the primary antibody was omitted and the secondary antibody was applied (identical for BDNF and TrkB). The arrow points at the negative inner root sheath, which would stain strongly with primary antibody. Pancreatic ductal adenocarcinoma served as positive controls for IHC. Arrows point at strongly stained structures. Boxes indicate anatomical location of the presented micrographs. The bottom panel gives examples of positive and negative controls. For the IHC-negative control, the primary antibody was omitted and the secondary antibody was applied (identical for BDNF and TrkB). The arrow points at the negative inner root sheath, which would stain strongly with primary antibody. Pancreatic ductal adenocarcinoma served as positive controls for IHC. Arrows point at strongly stained structures. Boxes indicate anatomical location of the presented micrographs.
BDNF induces premature catagen development in vitro

Hair cycle staging of hair follicles treated with 50 ng per mL rhBDNF after 48 h in culture did not show catagen-like development. First catagen-like hair follicles could be detected after 96 h, whereas none were seen in control hair follicles (not shown). This difference became highly significant after 10 d in culture with 50 ng per mL rhBDNF (Fig 4).

Table I. Summary of BDNF and TrkB staining intensities in various compartments of anagen VI scalp skin hair follicles

<table>
<thead>
<tr>
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<th>BDNF</th>
<th>TrkB</th>
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<tr>
<td></td>
<td>mRNA</td>
<td>Protein</td>
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<tr>
<td>Hair follicle ostium</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Inner root sheath</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Outer root sheath (including the isthmus and bulge region)</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Proximal hair follicle bulb</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hair matrix</td>
<td>(+)</td>
<td>(+)</td>
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<tr>
<td>Cuticle of the hair shaft</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Dermal papilla</td>
<td>++</td>
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The table lists the mean staining intensities from nine (TrkB) or 13 (BDNF) different human scalp skin samples. From each sample ten microscopic fields or six hair follicles were evaluated and assigned arbitrary scores (+, weak; ++, obvious staining; ++++, strong staining).

BDNF, brain-derived neurotrophic factor; TrkB, tyrosine kinase B.

Figure 3

Brain-derived neurotrophic factor (BDNF) reduces hair shaft elongation in organ culture. On the left, photodocumentation panels over a culture period of 10 d of one characteristic hair follicle per group are given. On the right, data pooled from six highly comparable, independent experiments (nine hair follicles per experiment, only pigmented hair follicles included) are shown. Growth rates are expressed as percent difference over day 0. Significances always refer to the growth rate of control hair follicles on the same day (p-values by Mann–Whitney U: +++ < 0.001, ++ < 0.01, + < 0.05).

Figure 4

Fifty nanograms per milliliter recombinant human brain-derived neurotrophic factor (rhBDNF)-induced catagen after 10 d in culture. This figure shows characteristic examples of photodocumented hair follicles cultured over 10 d and corresponding H&E-stained cryosections. Note the wide anagen hair bulb of the control hair follicle (left). In comparison, the hair bulb of a hair follicle treated with 50 ng per mL rhBDNF appears narrow, the dermal papilla has rounded up, and the hair follicle epithelium is not enclosing it. The graph represents data pooled from four highly comparable independent experiments (nine hair follicles per experiment). Data are represented as percentage hair follicles in anagen VI, early or late catagen, respectively, or as hair cycle score. Each anagen VI hair follicle was ascribed an arbitrary value of 100, early catagen-like of 200, mid-catagen-like of 300, and late catagen-like of 400 to calculate the hair cycle score. Values were added per group and divided by the number of staged follicles. The score thus represents the mean hair cycle stage of all hair follicles per group. Significances refer to the hair cycle score of control hair follicles on the same day (p-values by Mann–Whitney U: +++ < 0.001, ++ < 0.01, + < 0.05). c, club; dp, dermal papilla; hs, hair shaft; irs, inner root sheath, ors, outer root sheath; pu, pigmentary unit.

BDNF upregulates apoptosis and inhibits proliferation of human hair follicle keratinocytes in situ

Quantitative histomorphometry of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labeling (TUNEL) or Ki67 IR nuclei in organ-cultured human hair bulbs revealed significantly less Ki67 +, proliferating keratinocytes in hair follicles treated for 10 d with 50 ng per mL rhBDNF when compared with controls (Fig 5). Complementary hair follicles showed significantly more TUNEL + cells after 10 d of culture with 50 ng per mL rhBDNF (Fig 5). Quantitative TUNEL/Ki67 histomorphometry of hair follicles harvested after only 2 or 4 d of culture did not show significant differences (not shown).

BDNF upregulates TGFβ2 gene transcription in human anagen hair follicles

To investigate possible signaling loops recruited by BDNF in the control of catagen induc-
tion, we performed quantitative real-time rtPCR analysis of TGFβ2 steady-state mRNA levels in hair bulbs, since TGFβ2 is now appreciated as a key catagen-promoting growth factor in the human system (Soma et al., 2002; Tsuji et al., 2003; Hibino and Nishiyama, 2004). This showed substantial upregulation of TGFβ2 mRNA in hair bulbs that were cultured in the presence of 50 ng per mL rhBDNF over 48 h (Fig 6).

TGFβ-neutralizing antibodies abrogate BDNF-induced inhibition of hair shaft elongation Simultaneous treatment of cultured human anagen hair follicles with 50 ng per mL BDNF and 10 (not shown) or 25 μg per mL TGFβ-neutralizing antibodies completely abrogated the catagen-inductive and anti-proliferative effects of 50 ng per mL rhBDNF, e.g. hair shaft elongation rates did not differ from controls after 4 d in culture, when rhBDNF inhibition of hair shaft elongation became significantly different from control (Fig 7). Likewise, hair cycle staging, apoptosis (TUNEL), and proliferation analysis (Ki67) on hair follicles cultured over 4 or 10 d revealed anagen to early catagen hair bulbs with high numbers of Ki67+ proliferating and low numbers of TUNEL+ apoptotic keratinocytes in the control as well as the groups treated with BDNF together with TGFβ-neutralizing antibody (not shown). Hair follicles that were treated with 50 ng per mL rhBDNF showed striking upregulation of TGFβ2 expression in the hair bulb by immunohistochemistry especially in the hair follicle matrix, the inner root sheath, and the outer root sheath (Fig 7), whereas TGFβ2 expression was restricted to the most differentiated layer of the inner root sheath in control hair follicles and hair follicles treated with 50 ng per mL BDNF and 25 μg per mL TGFβ-neutralizing antibodies.

Figure 6 Brain-derived neurotrophic factor (BDNF) increases tumor growth factor [β2 (TGFβ2) transcription. Nine human hair follicles were cultured with or without 50 ng per mL BDNF over 48 h. Proximal (lower) portions were collected with surgical scalpels after the culture. The expression of TGFβ2 was measured by quantitative real-time PCR (TaqMan PCR) and normalized with GAPDH expression. Each PCR reaction was performed in triplicate. Graphs represent pooled data from these triplicates. Significance refers to control levels (p-values by Mann–Whitney U: **p < 0.01, *p < 0.05). Please note the numerous TUNEL IR terminally differentiating cell nuclei in the hair shaft. cs, connective tissue sheath; dp, dermal papilla; hs, hair shaft. Arrowheads point at TUNEL+ nuclei.

Figure 5 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labeling (TUNEL)/Ki67 double staining analysis on hair follicles cultured over 10 d. Per experiment harvested on day 10 after onset of culture, one to four of nine hair follicles allowed quantification of TUNEL (green) or Ki67 IR (red) nuclei. Only analysis of apoptosis and proliferation in keratinocytes yielded significant differences. Significances always refer to control levels (p-values by Mann–Whitney U: **p < 0.001, *p < 0.01, p < 0.05). Please note the numerous TUNEL IR terminally differentiating cell nuclei in the hair shaft. cs, connective tissue sheath; dp, dermal papilla; hs, hair shaft. Arrowheads point at TUNEL+ nuclei.

Discussion
Since it was first shown in mice that mammalian skin and especially keratinocytes may be a source for neurotrophins (Tron et al., 1990), a body of data has accumulated, in support of the concept that the skin epithelium is also an important target of neurotrophin signaling (Paus et al., 1994b; Pincelli and Yaar, 1997; Botchkarev et al., 1999a, b; Lambiase et al., 2000). More recently, our own studies have highlighted that neurotrophins are prominently involved in the control of murine hair follicle development and cycling and that the murine hair follicle is actually a prominent source of neurotrophins (Botchkarev et al., 1998a, c, 1999a, b, 2000). In this investigation on BDNF signaling in human hair growth control, we now show that selected neurotrophins act as hair growth regulators in the human system as well, e.g. via high-affinity tyrosinekinase receptors such as TrkB, and that the human hair follicle is both a source and target of neurotrophins. This underscores their importance in...
cutaneous biology and further supports that neurotrophins are important hair growth-modulatory agents in hair growth control across species barriers:

1. The distribution of BDNF and TrkB in human scalp skin hair follicles suggests auto- and/or paracrine BDNF activities within the human hair follicle:
   a. paracrine activities and mesenchymal–epithelial cross-talk between BDNF-expressing fibroblasts in the anagen dermal papilla on the one hand, and TrkB+ hair follicle keratinocytes on the other.
   b. para- and/or autocrine-signaling activities of BDNF within the hair follicle epithelium expressing BDNF and TrkB on gene and protein expression level.
   c. suggestive involvement of BDNF in the autonomous, intrafollicular control of follicle cycling because of the hair cycle-dependent changes in BDNF expression in the mesenchymal “command center” of the hair follicle, i.e. the dermal papilla, between anagen VI (present) and telogen (absent) (Paus et al, 1999b; Stenn and Paus, 2001).

2. This is supported by the observation that BDNF inhibits hair shaft elongation and promotes catagen development in organ-cultured human anagen VI hair follicles, and suggests that BDNF functions rather as a terminally differentiation-promoting factor than as a growth factor in human scalp hair follicles.

3. In line with this concept, higher doses of BDNF inhibit hair follicle keratinocyte proliferation and stimulate expression of the catagen-promoting and keratinocyte proliferation-inhibiting factor TGFβ2.

4. That BDNF effects on human hair growth in vitro can be antagonized by pan-TGFβ-neutralizing antibodies suggests that TGFβ-mediated pathways along BDNF/TrkB signaling serve at least as one important cellular “second messenger” system in the regressing human hair follicle.

The expression pattern of BDNF and TrkB in human scalp skin hair follicles in anagen VI and telogen shown here is similar to the expression patterns described in mice and rats with low expression during the resting phase (telogen) and high expression in the high growth phase (anagen VI) (Botchkarev et al, 1998b, 1999b, 2000; Bergman et al, 2000), suggesting a parallel function of BDNF in murine and human hair growth regulation. Here, we can confirm this hypothesis by showing that exogenous BDNF does indeed cause premature catagen development of organ-cultured human anagen hair follicles as does BDNF overexpression in mice (Botchkarev et al, 1999a).
Catagen hair follicles are only rarely detected in human scalp skin samples; we therefore cannot provide data on Trk expression of apoptotic cells in the regressing hair follicle as are seen in the mouse. In the mouse, the high-affinity BDNF-receptor TrkB is upregulated during catagen and co-localizes with markers of apoptotic cell death in the epithelium of the regressing hair bulb (Botchkarev et al., 2000). The promoting effect of BDNF on apoptosis-driven catagen development in the human anagen hair follicle may thus occur via BDNF secretion of the dermal papilla and/or more differentiated keratinocytes in the hair follicle, acting on TrkB + hair follicle keratinocytes initially in the inner and outer sheath and upon catagen onset in the upregulating hair bulb.

In this context, it is crucial to understand that neurotrophins utilize more than one mode of interaction with their receptors (Lopez-Sanchez and Frade, 2002): acting as high-affinity growth factors activated by low concentrations of neurotrophins (Yaar et al., 1994; Barker, 1998; Roux and Barker, 2002), activation of Trk receptors by high levels of neurotrophins has been shown to induce apoptosis depending on receptor availability and receptor density (cf. Roux and Barker, 2002). Apoptosis through the common low-affinity neurophin receptor p75, a member of the TNF-receptor family leading to rapid apoptosis of the expressing cells, is another possible pathway involved in BDNF-mediated hair follicle regression (Botchkarev et al., 2000) and is the subject of an ongoing study on neurotrophin signaling in human hair growth control through this receptor (Peters et al., 2005). Moreover, careful examination for morphological indicators of toxicity (see Materials and Methods) did not reveal any evidence that the hair growth-inhibitory effects of BDNF presented here were related to hair follicle toxicity.

In our model, BDNF was also able to upregulate the keratinocyte differentiation factor TGFβ2 (Min et al., 1999; Hashimoto, 2000; Amendt et al., 2002) in the hair bulb as early as 48 h after start of culture. The observed TGFβ2 IR pattern in hair follicles treated with 50 ng per mL BDNF is in good accordance with the expression pattern observed by others during progression of the hair cycle and induction of catagen (Soma et al., 2002). Moreover, in our study hair growth could be maintained by co-administration of BDNF with anti-TGFβ1, 2, and 3-neutralizing antibodies. These observations suggest that TGFβ2, known to be a potent catagen inducer in murine (Foitzik et al., 2000) and human hair follicles (Philpott et al., 1994; Soma et al., 1998; Inui et al., 2002; Soma et al., 2002; Tsuji et al., 2003; Hibino and Nishiyama, 2004), is at least one major player in BDNF-induced premature catagen development. As evidenced by our functional experiment utilizing a pan-TGFβ-neutralizing antibody, TGFβ1 or 3 may also contribute to the BDNF-induced premature catagen development observed in our experiments. But TGFβ2 was shown to be a more potent catagen inducer in human scalp hair follicles than TGFβ1 (Soma et al., 1998, 2002; Tsuji et al., 2003; Hibino and Nishiyama, 2004). Furthermore, to the best of our knowledge, no effect of BDNF has previously been reported on TGFβ3 expression, and TGFβ3 is widely considered to be of negligible importance in both human and murine hair growth control since there is no published evidence to support any such role (cf. Soma et al., 1998, 2002; Foitzik et al., 1999; Stenn and Paus, 2001).

In glial astrocytes, BDNF can induce cell cycle arrest proteins such as p53 (Jordon-Sciutto et al., 2001) and in corneal keratinocytes, BDNF promotes differentiation as shown by colony formation but not proliferation (You et al., 2000). Together with our findings of TGFβ1 upregulation in unison with catagen induction and reduced keratinocyte proliferation by BDNF presented here, these observations lead us to the hypothesis that BDNF/TrkB signaling in the context of the growing human hair follicle promotes terminal differentiation rather than proliferation directly and indirectly, e.g. by modulating other growth factor-mediated pathways.

Our findings support an important role for BDNF as a trophic factor outside neural networks in the human and suggest to further use the cultured human hair follicle as an easily accessible and all the while complex miniature organ-culture system for future analysis of neurotrophins in epithelial and mesenchymal growth control. Local BDNF has been applied, with few side effects to patients suffering from diabetic polyneuropathy (Wellmer et al., 2001). Therefore, the topical application of TrkB agonists or the upregulation of endogenous BDNF production in terminal anagen hair follicles may be useful future tools in the management of unwanted hair growth (hirsutism), whereas the local application of specific TrkB antagonists not yet available holds promise for the future management of telogen effluvium in human.

Materials and Methods

Tissue and hair follicle sources Temporal scalp skin containing mainly anagen VI hair follicles was obtained from disposed excess skin samples derived from patients undergoing elective plastic surgery after obtaining informed consent, following accepted ethic guidelines of the University of Hamburg, Germany. The study was conducted according to the Declaration of Helsinki Principles. After excision, tissue was maintained in Williams E Medium (Biochrom KG seromed, Berlin, Germany) for transportation at 4°C up to 24 h. Upon arrival, the samples were divided by three: one part was immediately snap-frozen in liquid nitrogen for immunohistochemical analysis, the second was processed for paraffin embedding to allow routine hair follicle staining and in situ hybridization, and the third was processed for rPCR and quantitative real-time rPCR analysis and hair follicle culture as described below.

Conventional rPCR analysis Single human anagen VI hair follicles were microdissected from human skin biopsies. Microdissected hair follicles consisted of the hair bulb and the distal hair follicle, up to the level where the arrector pili muscle inserts into the bulge region (including the hair shaft, the inner and outer root sheath, the connective tissue sheath, and the dermal papilla). RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). 0.8 μg of total RNA was reverse transcribed using the first Strand cDNA Synthesis Kit for rPCR (Roche, Mannheim, Germany) in an oligo-d(T)15-primed 20 μL reaction. rPCR was carried out using the Core PCR Kit (Qiagen) in 25 μL reactions containing 1 μL cDNA, 2.5 mM MgCl2, 200 μM dNTP, 0.5 U Taq polymerase, and 0.5 pmol of each primer. The following oligonucleotide primers were designed to amplify target cDNA. 5'-TCCACCAGGGTGAAGAC-3'.

GAGTG-3' and 5'-ACTTGACTCTGAGCCATCAC-3' to amplify a 319 bp fragment corresponding to nucleotides 277–595 of BDNF mRNA (Genbank: X60,201), and 5'-ATGCTGGTGGTATTGC-3' and 5'-CCTTCTATGCAAACATTGAGTCTG-3' to amplify a 97 bp fragment corresponding to nucleotides 1652–1748 of the TrkB mRNA (Genbank: U12,140 common to all known isoforms of TrkB). The reactions were run in a thermal cycler (PE Biosystems, Foster City, California) for 5 min at 95 °C, then 30 s at 95 °C, 30 s at 60 °C, 45 s at 72 °C for 35 cycles, and then 5 min at 72 °C. Products were run on a 1% agarose gel (Sigma, St Louis, Missouri). The identity of the products was confirmed by sequencing.

Quantitative real-time rPCR analysis Hair follicles cultured over 48 h in the presence of 50 ng per mL rhBDNF (for details, see Table II) were dissected into proximal region consisting of the hair bulb and distal region consisting of the hair shaft and inner and outer root sheaths with the adjacent connective tissue sheath with a surgical scalpel under a microscope. Snap-frozen proximal hair follicle regions were homogenized with a glass homogenizer (neolab, Heidelberg, Germany) and processed as described above. Amplification reactions were performed as 50 μL triplicates in a 96-well microplate format containing 1 × SYBR Green Master Mix (PE Biosystems), first strand cDNA, and each primer set. Primers were designed using the Perkin Express 1.0 Software (PE Biosystems) and each set of primers are situated in different exons as human TGFβ2 5'-AAAGTGGACGTAGGCAGCAATTA-3' forward primer, human TGFβ2 5'-GACCAACCGGCGGAAGA-3' reverse primer, human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) 5'-TGGGTGTGAACCATGAGAAG-3' reverse primer, and human GAPDH 5'-GCTAAGCAGTTGGTGGTGC-3' forward primer, respectively. The reaction mixture was subjected to the following thermal cycle conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Analysis of the reactions was carried out in an ABI Prism 7700 Sequence Detection System (PE Biosystems) monitoring after each cycle. The expression of TGFβ2 was normalized with that of GAPDH. Each TaqMan experiment was performed in triplicate.

In situ hybridization Oligonucleotide probes to TrkB (Genebank: AF410,899 5'-GAT TGC CCA CCA GGA TCA GTT CAG ACG TGT TTG AAC CTG CCT TG-3') and BDNF (Genebank: M61,176 5'-ATG ATG ATC ACA GTC TCG AAC CTG CGT TG-3'), labeled with fluorescein at both the 3' and 5' ends, were obtained from Biognostik (Göttingen, Germany; designed and used as recommended by vendor, Biognostik). Four micrometer paraffin sections of human skin were dewaxed and rehydrated before being treated with 50 μg per μL proteinase K (Roche) in proteinase K buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 10 mM NaCl) for 15 min at 37 °C. Sections were then post-fixed for 10 min with 1% paraformaldehyde in PBS (0.68 M NaCl, 26.82 mM KCl, 17.64 mM KH2PO4, 0.10 M Na2HPO4, pH 7.4) followed by washes in PBS and water for 10 min each. Sections were then prehybridized in hybridization buffer (2 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 mg yeast tRNA (Sigma, Taufkirchen, Germany), 150 mM NaCl, 50% formamide) for 2 h at 30 °C. Hybridization was carried out for 16 h at room temperature (25 °C) with 1.5 pmol probe in the hybridization buffer. Following hybridization, excess unbound probe was washed off by a brief rinse followed by two washes for 15 min each with 0.1 × SSC at 37 °C. Sections were then equilibrated in TN buffer (pH 7.5, 100 mM Tris, pH 7.5, 150 mM NaCl) for 5 min before being incubated with blocking solution (TN (pH 7.5) containing 0.1% Triton-X 100% and 1% normal goat serum) for 30 min at 37 °C. The blocking solution was then replaced with antibody (anti-fluorescein AP FAB fragments (Roche)) diluted 1:1000 in blocking solution, and the sections were incubated for another 1 h at 37 °C. The slides were then washed twice in TNT (100 mM Tris, pH 7.5, 200 mM NaCl, 0.1% Tween-20) for 15 min each time, and then equilibrated in TN (pH 9.5, 100 mM Tris, pH 9.5, 150 mM NaCl) for 5 min. Sections were then incubated in the dark with color solution (TNM (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl2) to which 20 μL NBT/BCIP mix (Roche) had been added directly before use) for 3 × 1 h, with sections rinsed in TN (pH 9.5) and a fresh color solution added each hour.

Immunohistochemistry Cryostat sections (8 μm) of human scalp skin, fixed in acetone (at −20 °C, 10 min), were preincubated with 10% normal goat serum (20 min, room temperature) and then incubated overnight at room temperature with the primary antisera to BDNF, TrkB, or TGFβ2 (BDNF; rabbit, polyclonal, raised against AA 128–147 mapping at C-terminus of h-BDNF; Santa Cruz Biotechnology, Santa Cruz, California; cf. Lommatzsch et al., 1999; Botchkarev et al., 1999a, b; diluted 1:50; n = 14. BDNF mouse, monoclonal, raised against rhBDNF; Sigma, St Louis, MI, USA; diluted 1:200; n = 5. TrkB; rabbit, polyclonal, raised against AA 794–808 mapping at the C-terminus of TrkB gp 145; Santa Cruz Biotechnology; cf. Lommatzsch et al., 1999; Botchkarev et al., 1999a, b; diluted 1:75; n = 9. TGFβ2; rabbit, polyclonal, raised against peptide mapping at the C-terminus of TGFβ2 of human origin; Santa Cruz Biotechnology; cf. Lyall et al., 2001; diluted 1:100; n = 4). For BDNF we found two working antibodies yielding comparable staining results, which mapped different epitopes of BDNF. For TrkB only antibodies mapping the same epitope were commercially available. Working BDNF and TrkB antibodies were available from Santa Cruz Biotechnology; we therefore chose these for immunofluorescence screening of all available skin sam-

| Table II. Hair follicle organ-culture treatment schedules |
|---------------------------------|----------------|----------------|
| Treatment | Duration of culture | Number of donors | Processed for |
| A | 5 or 50 ng per mL rhBDNF | 10 d | 11 different donors | Seven for hair shaft elongation; four for proliferation, staging analysis, TGFβ2 immunohistochemistry; eight for apoptosis analysis |
| Aa | 50 ng per mL rhBDNF | 96 h | One donor | Apoptosis/proliferation and staging analysis |
| Ab | 50 ng per mL rhBDNF | 48 h | Two different donors | One for apoptosis/proliferation and staging analysis, one for TGFβ2 RTPCR |
| B | 5, 10, 25, 50, 150 ng per mL rhBDNF | 10 d | Five different donors | Hair shaft elongation |
| C | 5 and 50 ng per mL anti-BDNF | 10 d | Eight different donors | Hair shaft elongation, two for apoptosis/proliferation and staging analysis |
| D | 5, 10 or 25 μg per mL anti-TGFβ | 250 ng per mL rhBDNF | 96 h | Three different donors | Apoptosis/proliferation and staging analysis, TGFβ2 immunohistochemistry |

Each donor also contributed to controls.
rhBDNF, recombinant human brain-derived neurotrophic factor; TGFβ2, transforming growth factor β2.
ples. All anti-sera were supplemented with 2% normal goat serum. This was followed by an incubation of 45 min at room temperature with tetramethylrhodamine-isothiocyanate-conjugated F(ab)2 fragments of goat anti-rabbit or goat anti-mouse IgG (Dianova, Hamburg, Germany) diluted 1:200 in Tris-buffered saline (TBS). Incubation steps were interspersed by three washes with TBS (5 min each). Then sections were stained with 4', 6-diamidino-2'-phenylindol-dihydrochlorid (DAPI, Boehringer Mannheim, Mannheim, Germany) with a concentration of 1 µg per mL (1 min, room temperature) for identification of cell nuclei. Two types of negative controls were run: (a) slides were incubated with the secondary antibody alone; (b) slides were incubated with a mixture (incubated overnight at 4 °C in tubes covered with 1% milk powder in TBS) of the primary antibody, and the control peptide for the specific anti-sera (Santa Cruz Biotechnology, 5 and 10 and 20 times higher concentrated than primary antibody, over-1% milk powder in TBS) of the primary antibody, and the control were isolated following the protocol published by Philpott et al. (Promega, Madison, Wisconsin) or rhBDNF with TGFβ1 isomer (Dianova, Hamburg, Germany) at a concentration of 1 ng per µL (1 min, room temperature) for identification of cell nuclei. As positive controls, cryopreserved samples from human pancreatic ductal adenocarcinoma (Miknyczoki et al, 1999) were kindly supplied by Prof. Kalthoff (Molecular Oncology, Department of Surgery, University of Kiel, Germany) and Dr Hosch (Department of Surgery, University Hospital Hamburg-Eppendorf, Germany).

**Histomorphometry** A minimum of six sections of nine (TrkB) or 14 (BDNF) scalp skin samples, each derived from a different donor, were examined at 250 magnification under a Zeiss Axioscope 2 fluorescence microscope using appropriate filters (Zeiss, Göttingen, Germany). IR was assessed by histomorphometry in defined compartments such as the hair follicle, hair follicle epithelium between the epidermis and entry of the sebaceous gland into the hair canal, inner root sheath, outer root sheath, cuticle, dermal papilla, matrix, and proximal hair bulb epithelium (below Auber's line. For details on these follicle compartments, see Müller-Röver et al, 2001). Intensity was recorded in a semi-quantitative manner by assigning arbitrary units to different staining intensities (0, negative; +, barely visible staining; +++, apparent staining; ++++, strong staining). For each IR pattern, follicular compartments were evaluated at least six different anagen hair follicles per sample and the extrafollicular compartments in a minimum of ten microscopic fields. The photodocumentation was carried out with the help of a modular imaging program (Openlab, ImproVision, Heidelberg, Germany). The results of the observed, representative IR patterns were also recorded qualitatively in schematized recording protocols.

**Hair follicle organ culture** Normal human scalp skin hair follicles in the anagen VI stage of the hair cycle (Müller-Röver et al, 2001) were isolated following the protocol published by Philpott et al (1994) and Philpott (1999) with slight modifications. Briefly, after separation of the epidermis and dermis from subcutaneous fat under a binocular dissecting microscope, the proximal two-thirds of anagen hair follicles located in the subcutaneous fat were isolated using watchmaker's forceps and subsequently collected in Petri dishes containing complete hair follicle culture medium (Williams E, Biochrom KG: seromed); 1% penicillin-streptomycin (Life Technologies, Eggenstein, Germany); 1% L-glutamine 200 mM (Life Technologies); 0.02% hydrocortisone (Sigma, Taufkirchen, Germany); and 0.1% insulin (Sigma). Three hair follicles per well were then randomly distributed and cultured in 24-well plates (Costar, Bremen, Germany) containing 500 µL of complete hair follicle culture medium per well. Per experiment, a minimum of three wells (containing three hair follicles each) was assigned to each test group, and was supplemented with different concentrations of rhBDNF (Promega, Madison, Wisconsin) or rhBDNF with TGFβ1-neutralizing antibody (monoclonal anti-TGFβ1-3 antibody (R&D Systems, Weisbaden, Germany)); binds to all three TGFβ isoform (Roberts et al, 1985; Cheifetz et al, 1990). An equal number of control follicles were cultured in complete hair follicle culture medium alone. Each experiment was repeated with hair follicles from different donors (for details see Table II). Every second day, each well was photodocumented, the total length of each hair follicle was measured, medium was replaced, and fresh supplements were added. After 2, 4, or 10 d hair follicles were snap-frozen in a drop of OCT Cryochrome (Shandon, Pittsburgh, Pennsylvania) and stored at –80°C until cryosectioning.

**Hair cycle staging** Cultured hair follicles were staged after 2, 4, and 10 d (for detail see Table II) in culture, either by morphometric evaluation of entire hair follicles photodocumented by inversion light microscopy or by routine H&E histomorphometry, following previously published hair cycle staging guidelines for the murine hair cycle (Müller-Röver et al, 2001) and morphological characteristics of the human hair cycle (Kligman, 1959).

Microscopically, hair follicles were classified as anagen VI if the hair bulb was wider than the rest of the hair follicle, the dermal papilla was partially covered by melanin, and if the pigmented hair shaft was emerging directly from the hair follicle pigmented unit in the precortical hair matrix. Histologically, anagen VI hair follicles show a narrow, elongated, oval-shaped dermal papilla of loose consistency, which is fully enclosed by the hair bulb keratinocytes, residing in an onion-shaped, large hair bulb. Hair follicles were classified as “early catagen” if the hair bulb was more narrow, i.e. had essentially the same diameter as the rest of the hair follicle, if a slightly condensed, tear-drop-shaped dermal papilla was visible, if the pigmented zone of the hair shaft was beginning to retract in the distal direction from the pigmented unit, and/or if the number of visible melanin granules in the distal hair bulb was substantially decreased, compared with high anagen. Hair follicles were classified as being in “mid-catagen” when melanin granules were almost absent from the hair bulb and if, on longitudinal sections, the bulb appeared opened at its proximal end, with a compact, ball-shaped dermal papilla. Hair follicles were classified as being in “late catagen”, if the hair bulb had become narrower than the distal rest of the cultured hair follicle, if the dermal papilla was clearly visible in an unpigmented hair bulb, yet was further condensed compared with mid-catagen, and if the proximal part of the pigmented hair shaft was fully separated from the dermal papilla. Histologically, these late catagen hair follicles display an epithelial strand, a column of epithelial cells between the compact dermal papilla and the germ capsule (secondary hair germ) (Müller-Röver et al, 2001), the likely seat of epithelial stem cells from which the hair matrix is reconstructed during the subsequent anagen stage (Panteleyev et al, 2001). Furthermore, late catagen hair follicles have already developed the so-called club hair, the hair shaft of follicles in the “resting” stage (telogen), whose proximal end is unpigmented and appears separated from the dermal papilla. In addition to hair cycle staging, signs of hair follicle dystrophy such as extraepithelial pigmentation (melanin incontinence), ectopic localization of melanin granules in the hair bulb (Paus et al, 1994a; Tobin et al, 1998), or hair shaft kinking were also documented. For statistical analysis (hair cycle score [HCS]), anagen VI hair follicles were arbitrarily attributed a score of 100, hair follicles in early catagen a score of 200, in mid-catagen of 300, and in late catagen of 400. The sum of scores per group was than divided by the number of investigated hair follicles (Maurer et al, 1997; Peters et al, 1999). The mean value of these scores therefore is a reliable quantitative indicator of the mean hair follicle stage that had been reached on average by a larger population of hair follicles after culture (Maurer et al, 1997; Peters et al, 1999).

**Apoptosis/proliferation assay** Cryostat sections of cultured hair follicles were processed for TUNEL labeling (apoptotic cell nuclei) and Ki67 labeling (proliferating cell nuclei) after 2, 4, or 10 d (for detail, see Table II) according to our previously published protocol (Lindner et al, 1997) adapted for human antigens. The TUNEL assay detects DNA fragmentation by enzymatic labeling of free 3′-OH ends according to the manufacturer’s instructions (Apopdetect Fluorescein, QBiogene, Heidelberg, Germany). Subsequently, sec-
tions were incubated with mouse anti-human Ki67 antiserum (anti-human Ki67 antigen, monoclonal, mouse, DAKO, Hamburg, Germany), detected by a rhodamine-conjugated goat anti-mouse antibody. Cell nuclei were counterstained with DAPI as described above. For negative controls, the TdT-enzyme step was omitted.

Hair bulbs were photographed using light microscopy. The results between different experiments using samples from different donors were highly comparable so that the experiment. The results between different experiments using samples from different donors were highly comparable so that the experiment.

**Statistical analysis:** The HCS was assessed and calculated as described (Maurer et al, 1997; Peters et al, 1999). Scores, TUNEL, and Ki67 IR cell nuclei numbers were pooled per group for each experiment. The results between different experiments using samples from different donors were highly comparable so that the mean scores and numbers of TUNEL IR or Ki67 IR cell nuclei per group and per experiment from all samples were then pooled again and statistical differences between groups were determined by the Mann–Whitney U test for unpaired samples.

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**References**

Alieva E, Santucci D: Psychosocial versus "physical" stress situations in rodents and humans: Role of neurotrophins. Physiol Behav 73:313–320, 2001


Lopez-Sanchez N, Frade JM: Control of the cell cycle by neurotrophins: Lessons from the p75 neurotrophin receptor. Histol Histopathol 17:1227–1237, 2002