Inhibition of human hair follicle growth by endo- and exocannabinoids

Andrea Telek,[*,†,1] Tamás Bíró,[*,†,1,2] Enikő Bodó,[‡] Balázs I. Tóth,[*,2] István Borbíró,[*] George Kunos,[§] and Ralf Paus[‡]

[*]Department of Physiology and [†]Cell Physiology Research Group of the Hungarian Academy of Sciences, University of Debrecen, Medical and Health Science Center, Research Center for Molecular Medicine, Debrecen, Hungary; [‡]Department of Dermatology, University Hospital Schleswig-Holstein, Campus Lübeck, University of Lübeck, Lübeck, Germany; and [§]National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT **Recent studies strongly suggest that the cannabinoid system is a key player in cell growth control. Since the organ-culture of human hair follicles (HF) offers an excellent, clinically relevant model for complex tissue interaction systems, we have asked whether the cannabinoid system plays a role in hair growth control. Here, we show that human scalp HF, intriguingly, are both targets and sources of endocannabinoids. Namely, the endocannabinoid N-arachidonoylethanolamide (anandamide, AEA) as well as the exocannabinnoid Δ (9)-tetrahydrocannabinol dose-dependently inhibited hair shaft elongation and the proliferation of hair matrix keratinocytes, and induced intraepithelial apoptosis and premature HF regression (catagen). These effects were inhibited by a selective antagonist of cannabinoid receptor-1 (CB1). In contrast to CB2, CB1 was expressed in a hair cycle-dependent manner in the human HF epithelium. Since we successfully identified the presence of endocannabinoids in human HF, our data strongly suggest that human HF exploit a CB1-mediated endocannabinoid signaling system for negatively regulating their own growth. Clinically, CB1 agonists may therefore help to manage unwanted hair growth, while CB1 antagonists might counteract hair loss. Finally, human HF organ culture offers an instructive, physiologically relevant new research tool for dissecting "nonclassical" effects of endocannabinoids and their receptor-mediated signaling in general.—Telek, A., Bíró, T., Bodó, E., Tóth, B. I., Borbíró, I., Kunos, G., Paus, R. Inhibition of human hair follicle growth by endo- and exocannabinoids. *FASEB J.* 21, 3534–3541 (2007)**

Key Words: cannabinoid receptor · proliferation · apoptosis · hair cycle

Δ[9]-TETRAHYDROCANNABINOL (THC), the psychoactive component of marijuana, mimics the effects of numerous endogenous substances (collectively referred to as endocannabinoids) by binding to cannabinoid (CB) receptors (1–5). Centrally, these endogenous molecules are involved in regulating, *e.g.*, behavior and learning (1–3, 6–8), while their peripheral effects include the modulation of immune and cardiovascular functions (1–3, 9, 10) and the control of growth normal and transformed cells as well as cell death and survival (11–17). CB receptors reportedly are also found on human epidermal keratinocytes *in vitro*, with conflicting data as to which types (CB1, CB2) are actually expressed (18–20). Although activation of CB receptors may suppress growth, murine skin tumors (18) and human melanomas (16) and, furthermore, cannabinoids were suggested to modify *in vitro* proliferation and differentiation of transformed keratinocytes (19, 21), it is unclear whether CB receptors are functionally important in normal human skin physiology.

The organ culture of human scalp hair follicles (HF) in the growth stage of the hair cycle (anagen VI), which continue to grow rapidly after microdissection and produce hair shafts *in vitro* at almost the *in vivo*-speed seen on the human scalp (22), is ideally suited to follow-up the above reports of growth-modulatory effects of CB receptor ligands in the human system. Employing this assay, we had already shown, *e.g.*, that vanilloid receptor-1 (TRPV1) agonists (such as capsaicin) operate as potent inhibitors of human hair growth (23). Arguing, furthermore, that the HF is exquisitely sensitive to the effects of psychoemotional stress (24, 25); that THC is prominently incorporated into human hair shafts (26, 27); and that several psychotropic hormones have recently been recognized to modulate human hair growth (24, 28–32), we now have asked whether the endocannabinoid system is also involved in the control of human hair growth.

Since the cycling HF represents a prototypic, constantly remodeled epithelial-mesenchymal interaction system that switches between states of rapid epithelial proliferation (anagen), apoptosis-driven organ involution (catagen), and relative quiescence (telogen), the organ culture of human HF, which continues to un-

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Physiology, University of Debrecen, Nagyerdei krt. 98. PO Box 22, 4032 Debrecen, Hungary. E-mail: biro@phys.dote.hu

doi: 10.1096/fj.06-7689com

0892-6638/07/0021-3534 © FASEB

dergo the anagen-catagen transformation *in vitro*, offers a highly instructive, easily accessible model for probing the effects of test agents on complex human tissue interaction systems (33, 34). Therefore, as an integral part of the ongoing exploration of the intriguing "nonclassical" neuro-endocrine role of the skin both under physiological and pathological conditions (35–39), the human HF organ culture promised to offer an ideal, physiologically and clinically relevant general model system for dissecting the as-yet-unclear functions of cannabinoids in the control of human cell growth and death *in situ*.

MATERIALS AND METHODS

Materials

AEA, 2-AG, AM-251, THC, and interferon-γ (IFNγ) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Isolation and maintenance of hair follicles

The study was approved by the Institutional Research Ethics Committees and adhered to Declaration of Helsinki guidelines. Human anagen HF (n=18–24 per group) were isolated from skin obtained from females undergoing face-lift surgery (23, 31). Isolated HF were maintained in Williams E medium (Biochrom, Cambridge, UK) supplemented with 2 mM L-glutamine (Invitrogen, Paisley, UK), 10 ng/ml hydrocortisone, 10 μg/ml insulin, and antibiotics (all from Sigma). Medium was changed every other day, whereas treatment with various cannabinoids was performed daily.

Measurement of hair shaft elongation

Length measurements were daily performed on individual HF using a light microscope with an eyepiece measuring graticule (23, 31).

Histology, histochemistry, quantitative histomorphometry

Cryostat sections (8 μm thick) of cultured HF were fixed in acetone, air-dried, and stained with hematoxylin-eosin (Sigma). Hair cycle stage (anagen, catagen) of each HF was assessed according to defined morphological criteria whereas melanin pigment was visualized by the Masson-Fontana histochemistry (23, 34).

Immunohistochemistry of CB receptors

For the detection of CB receptors on isolated HF, two complementary techniques, the tyramide-substrate amplification (TSA) and the alkaline phosphatase (AP) activity-based methods were used (23, 31). For the TSA technique, sections were first incubated by primary antibodies (1:400) against the N terminus of CB1 (H-150, sc-20754, Santa Cruz, Santa Cruz, CA, USA) or CB2 (Cat. No. 101550–1, Cayman Chemical, Ann Arbor, MI, USA). Samples were then labeled with biotinylated multilink swine anti-goat/mouse/rabbit IgG (1:200, DAKO, Glostrup, Denmark) and finally with streptavidine-horseradish peroxidase (TSA kit, Perkin Elmer, Boston, MA, USA) followed by an application of fluorescein-tyramide (1:50, TSA kit). Sections were counterstained by DAPI (1 μg/ml, Boehringer Mannheim, Mannheim, Germany). For the AP-based method, after staining with the appropriate CB-specific antibodies (1:40) and the biotinylated multilink swine anti-goat/mouse/rabbit IgG (1:200), sections were labeled by a streptavidin-AP conjugate (1% reagent mixture, Vector Laboratories, Burlingame, CA, USA). Immunoreactions were finally visualized using Fast Red (Sigma) and the sections were counterstained by hematoxylin (Sigma).

In both staining procedures, to further assess specificity of the immunostaining, primary labeling was also performed using goat C-terminus-specific antibodies: anti-CB1 (K-15, sc-10068, Santa Cruz) and anti-CB-2 (C-15, sc-10073, Santa Cruz). The application of these latter primary antibodies resulted in identical staining patterns (not shown). As negative controls, the appropriate primary antibodies were either omitted from the procedure or were preabsorbed with synthetic blocking peptides (purchased from Santa Cruz or Cayman). In addition, the specificity of CB receptor staining was also measured on tissues recognized to be CB1 (brain) or CB2 (spleen) positive (not show).

Image analysis

The intensity of fluorescence CB1-immunoreactivity in each section was measured at 5–10 previously defined reference regions of interest (ROI) of either the layers of distal ORS or the matrix keratinocytes at a 0–255 U/pixel intensity range using the Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Spring, MD, USA), and the average of the CB1-specific immunosignal (mean±SE) was calculated (23). A similar approach was employed to define the melanin content of the bulb regions of individual HF, labeled by Masson-Fontana histochemistry.

Double immunolabeling of proliferating and apoptotic cells

To evaluate apoptotic cells in colocalization with a proliferation marker Ki-67, a Ki-67/TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) double-staining method was employed (23, 31). Cryostat sections were fixed in formalin/ethanol/acetic acid and labeled with a digoxigenin-deoxyUTP (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Intergen, Purchase, NY, USA) in presence of terminal deoxynucleotidyl transferase (TdT), followed by incubation with a mouse anti-Ki-67 antiserum (DAKO). TUNEL+ cells were visualized by an antidigoxigenin FITC-conjugated antibody (ApopTag kit), whereas Ki-67 was detected by a rhodamine-labeled goat anti-mouse antibody (Jackson Immuno Research, West Grove, PA, USA). Finally, sections were counterstained by DAPI (1 μg/ml, Boehringer Mannheim). Negative controls were performed by omitting TdT and the Ki-67 antibody. The number of cells positive for Ki-67 and TUNEL immunoreactivity was counted per hair bulb and was normalized to the number of total (DAPI+) cells.

Quantitative "real-time" PCR (Q-PCR)

Q-PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the 5′ nuclease assay (23, 31). Total RNA was isolated from pools of freshly dissected HFs (n=100–200) using TRIzol (Invitrogen) and 3 μg of total RNA were reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (Promega, Madison, WI, USA) and 0.025 μg/μl random primers (Promega). PCR amplification was carried out by using the TaqMan primers and probes (Assay ID: Hs00275634_m1 for human CB1, Assay ID: Hs00361490_m1 for human CB2) using the TaqMan Universal PCR Master

Mix Protocol (Applied Biosystems). As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined (Assay ID: Hs99999905_m1 for human GAPDH). The amount of CB receptor-specific transcripts was normalized to those of GAPDH using the ΔΔCT method (23).

Determination of endocannabinoid levels

Freshly isolated HFs weighing ~50 mg were homogenized in 0.5 ml of an ice-cold solution of methanol: Tris buffer (50 mM, pH 8) 1:1 containing 7 ng of $^2H_4$-anandamide ($^2H_4$-AEA), synthesized as described in (40, 41). To each homogenate 2 ml of ice-cold chloroform:methanol 1:1 and 0.5 ml of 50 mM Tris buffer, pH 8, was added. The homogenate was centrifuged at 4°C (500 *g* for 2 min), and the chloroform phase was recovered and transferred to a borosilicate tube, and the water phase extracted two more times with ice-cold chloroform. The combined extract was evaporated to dryness at 32°C under a stream of nitrogen. The dried residue was reconstituted in 110 μl of chloroform, and 2 ml of ice-cold acetone was added. The precipitated proteins were removed by centrifugation (1800 *g*, 10 min) and the clear supernatant was removed and evaporated to dryness. The dry residues were reconstituted in 50 μl of ice-cold methanol, of which 35 μl was used for analysis by liquid chromatography/in line mass spectrometry, using an Agilent 1100 series LC-MSD, equipped with a thermostated autosampler and column compartment. Liquid chromatographic separation of endocannabinoids was achieved using a guard column (Discovery HS C18, 2 cm × 4.0 mm, 3 μm, 120A) and analytical column (Discovery HS C18, 7.5 cm × 4.6 mm, 3 μm) at 32°C with a mobile phase of methanol:water:acetic acid 85:15:0.1 (v/v/v) at a flow of 1 ml/min for 12 min followed by 8 min of methanol:acetic acid 100:0.1 (v/v). The MSD (model LS) was set for atmospheric pressure chemical ionization (APCI), positive polarity, and selected-ion-monitoring (SIM) to monitor ions m/z 348 for AEA, 352 for $^2H_4$-AEA, and 379 for 2-AG. The spray chamber settings: vaporizer 400°C, gas temperature 350°C, drying gas 5.0 l/min, and nitrogen, used as the nebulizing gas with a pressure of 60 psig. Calibration curves were produced using synthetic AEA and 2-AG (Cayman). The amounts of AEA and 2-AG in the samples were determined using inverse linear regression of standard curves. Values are expressed as fmol or pmol per mg wet tissue.

Statistical analysis

Statistical analysis was performed using a Mann-Whitney *U* test for unpaired samples (n=18–24 HFs per group) (23, 31).

RESULTS

AEA, unlike 2-AG, inhibits hair growth

First, microdissected, organ-cultured human scalp HF in the growth stage of the hair cycle (*i.e.*, anagen VI) (22, 23, 31) were stimulated with one of the best-characterized endocannabinoids, N-arachidonoylethanolamide (anandamide, AEA) (1–5, 42). AEA significantly (P<0.05) and dose-dependently inhibited hair shaft elongation (**Fig. 1*a***) and (as revealed by determining the number of Ki67 positive cells) hair matrix keratinocyte proliferation (Fig. 1*b*). In contrast, the endocannabinoid significantly (P<0.05) stimulated keratinocyte apoptosis in the epithelial hair bulb (as assessed by TUNEL labeling, Fig. 1*b*) as well as premature HF entry into apoptosis-driven organ involution (catagen) (Fig. 1*c*). It is worth noting, however, that AEA did not affect HF pigmentation (32, 33), since the melanin content of anagen VI HF remained unchanged (not shown).

We have also investigated the effect of the other main endocannabinoid, 2-arachidonoylglycerol (2-AG) (1–5, 42). Interestingly, 2-AG did not significantly alter human hair shaft elongation *in vitro* (Fig. 1*a*), HF proliferation, apoptosis, or catagen entry (not shown).

CB1, but not CB2, is expressed in the HF, and its level is regulated by the hair cycle

We then assessed whether HF express the molecular targets of cannabinoids (3, 5). By mutually complementary and confirmatory, independent immunohistochemical methods (**Fig. 2*a*, *b***), specific CB1 immunoreactivity (CB1-ir) was identified in the HF epithelium, primarily in outer root sheath (ORS) keratinocytes (but not on the fibroblasts of the HF dermal papilla). In addition, transcription of the CB1 gene in freshly isolated, microdissected human scalp HFs (more precisely: anagen VI hair bulbs) was demonstrated by quantitative RT-PCR (Fig. 2*d*). In contrast, of great importance, neither immunohistochemistry nor Q-PCR indicated the expression of CB2 in the HF (not shown).

Intriguingly, CB1 protein expression significantly increased on hair matrix (and, yet only marginally, on ORS) keratinocytes of cultured HF, which had been experimentally induced to undergo premature HF involution (catagen) phase by interferon-γ (IFNγ) treatment (23) (Fig. 2*b, c*). Moreover, we have also found that the intensity of CB1-ir was also up-regulated on AEA-treated catagen HF (not shown). These data show that normal human scalp HFs express CB1 (but not CB2) on the gene and protein level, and suggest that the intrafollicular CB1 expression is hair cycle-dependent.

Effects of AEA are mediated by CB1 but not by TRPV1

The above data also support the argument that the effects of AEA on the human scalp HF may be transmitted by CB1 receptors. Further in line with this hypothesis, we found that the specific CB1 antagonist AM-251 (1, 3, 5), which alone did not modify hair shaft elongation, completely abrogated the hair growth-inhibitory effect of AEA and normalized hair growth parameters to the vehicle control level (Fig. 1*a*). This finding corroborates the missing evidence of CB2 expression in human scalp HF on either the protein or gene level and suggests that the potent hair growth-inhibitory actions of the endocannabinoid AEA are most likely mediated by CB1.

However, previous reports have also documented that AEA may also activate TRPV1 and hence may act as

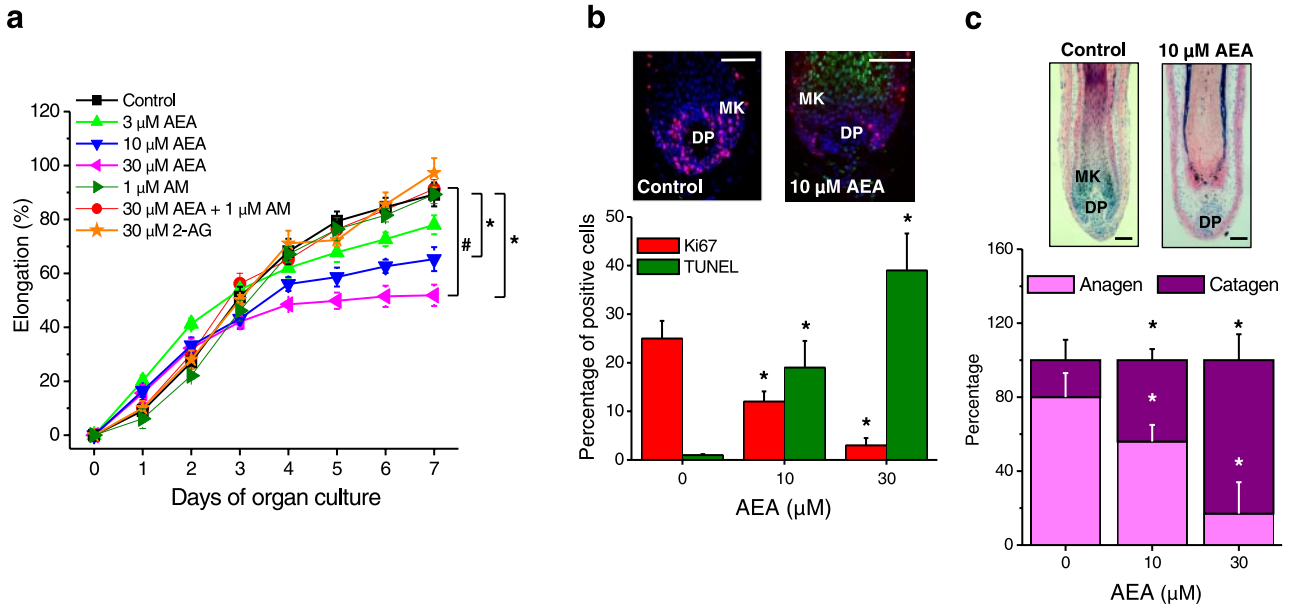

Figure 1. AEA inhibits hair shaft elongation and proliferation whereas it stimulates apoptosis of cultured human HF. *a)* Hair shaft elongation curves (mean±SE, 18–24 HF per group). $^{*}P < 0.05$ when compared to control; $^{\#}P < 0.05$ when values of the 30 μM AEA-treated group were compared to those of the 30 μM AEA + 1 μM AM-251 (AM) group. Note the lack of effect of 2-AG. *b)* Coimmunolabeling of proliferating (Ki-67+, red fluorescence) and apoptotic (TUNEL+, green fluorescence) cells, along with nuclei (DAPI, blue fluorescence). Statistical analysis of number of Ki-67+ and TUNEL+ cells as compared to the number of DAPI+ cells (mean±SE). *c)* Quantitative hair cycle histomorphometry on hematoxylin-eosin-stained sections. Percentage of HFs in anagen or catagen state (23, 33, 34) was determined (mean±SE). *b, c)* DP, dermal papilla, MK, matrix keratinocytes. Scale bars, 10 μm. $^{*}P < 0.05$ when compared to control.

an "endovanilloid" substance (3, 43, 44). In addition, we have previously shown that the human HF epithelium also expresses TRPV1 and that the specific activation of the TRPV1-coupled signaling by the exovanilloid capsaicin (a pungent ingredient of hot chili peppers) inhibits hair shaft elongation and proliferation, and induces apoptosis-driven catagen regression (23), very similar to the above action of AEA. Therefore, we also measured the possible role of TRPV1 in mediating the effects of AEA.

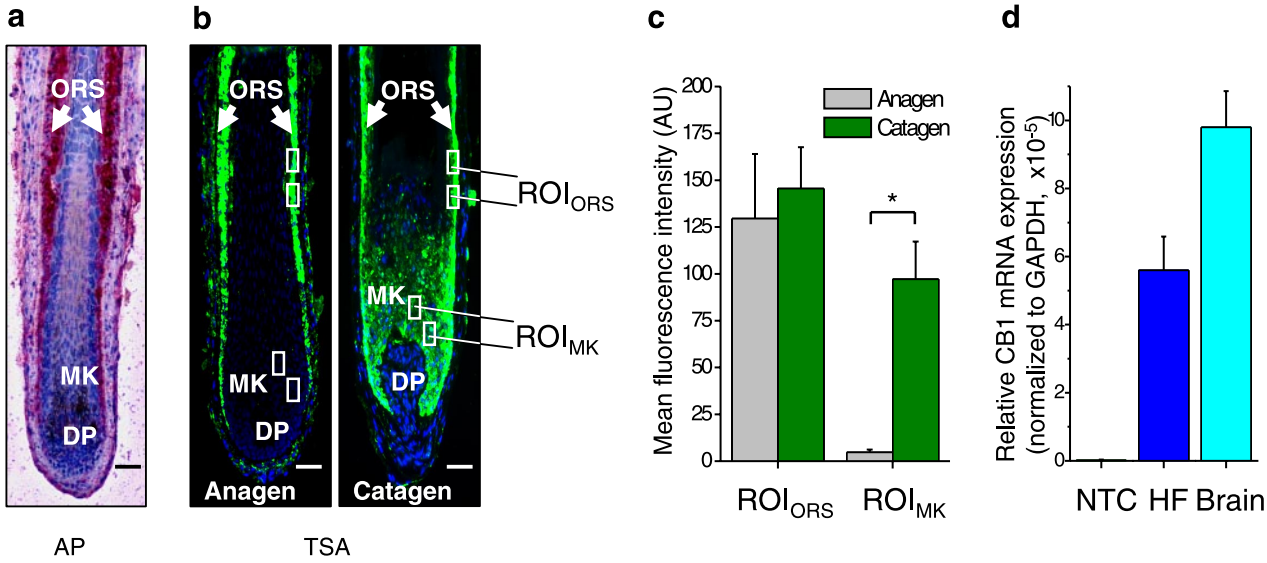

Figure 2. CB1 is expressed in the epithelium of the HF and is up-regulated in catagen. Immunodetection of CB1 using the alkaline phosphatase (AP) activity-based (*a*) and the tyramide-substrate amplification (TSA, *b*) methods. *b)* Expression of CB1 in anagen (no treatment) and catagen (1000 IU/ml IFNγ treatment for 5 days) HF. *c)* Statistical image analysis of CB1-specific fluorescence intensities in the outer root sheath (ORS) and matrix keratinocyte (MK) regions (mean±SE); 5–10 regions of interest (ROI, boxes in (*b*) per section, 18–24 HF per group. DP, dermal papilla. Scale bars, 10 μm. $^{*}P < 0.05$ when compared to control. *d)* Identification of CB1-specific mRNA transcripts in HF by quantitative RT-PCR. NTC, nontemplate control, Brain, positive control. Mean ± SE of quadruplicate determination.

As seen in **Fig. 3*a***, the TRPV1 antagonist iodoresiniferatoxin (I-RTX), which on its own did not modify "basal" hair growth (23), was unable to prevent the effect of AEA to inhibit hair shaft elongation suggesting the lack of involvement of TRPV1. Further corroborating this statement, we have also found that AEA and the TRPV1 agonist capsaicin exerted similar and, of great importance, additive effects to suppress hair growth (Fig. 3*a*), to inhibit the proliferation of HF matrix keratinocytes, and to induce intrafollicular apoptosis (Fig. 3*b*). Since the hair growth-inhibitory effect of capsaicin (confirming our previous results) (23) was fully abrogated by the TRPV1 antagonist I-RTX but not affected by the CB1 antagonist AM-251 (Fig. 3*a*), these findings strongly support the argument that the synergistic endocannabinoid and vanilloid systems operate independently to inhibit human hair growth and hence the effects of AEA are indeed exclusively mediated by CB1.

HF are sources of endocannabinoids

We were also interested in to define whether HF, besides responding to the action of cannabinoids and expressing CB1, also produce certain endocannabinoids. Therefore, in a pilot study, HF collected from two individuals, processed as described under Materials and Methods, and subjected to mass spectrometry to measure the presence of endocannabinoids. We showed for the first time that freshly dissected HF not only respond to but, intriguingly, also express such endocannabinoids as AEA and 2-AG. However, it was noteworthy to observe that whereas the level of AEA (6.6–11.2 fmol/mg tissue, range, $n=2$) was comparable to those of, *e.g.*, heart samples (~7.7 fmol/mg tissue) (9, 45), the level of 2-AG was much lower (0.2–0.3 pmol/mg tissue, range, $n=2$) than previously found in cardiac tissues (~3.5 pmol/mg tissue) (9, 45). Obviously, these initial, very preliminary data demand careful and systematic repetition using tissue extracts of many additional HF from several different individuals before definitive conclusions on the spectrum of endogenous cannibinoid receptor ligands can be drawn. In addition, these need to be integrated with information on the endocannabinoid content of healthy human skin and organ-cultured HF of various stages of HF cycling (*i.e.*, anagen, catagen).

THC also inhibits hair growth

THC, the key active ingredient in hashish and marijuana, is one of the best-investigated exocannabinoid (1–5) and is deposited at high levels in the hair shafts of human cannabis consumers (26, 27), *e.g.*, after inhalation or ingestion and of tumor patients treated with THC as an antiemetic, psychotropic agent. Therefore, we finally wished to investigate the effects of this prototypic exocannabinoid, which binds to both CB1 and CB2 (1–5), on human HF growth in organ-culture.

Almost identical to the actions of AEA reported above, THC significantly inhibited hair shaft elongation in a dose-dependent fashion, suppressed proliferation of HF keratinocytes, and induced both hair matrix

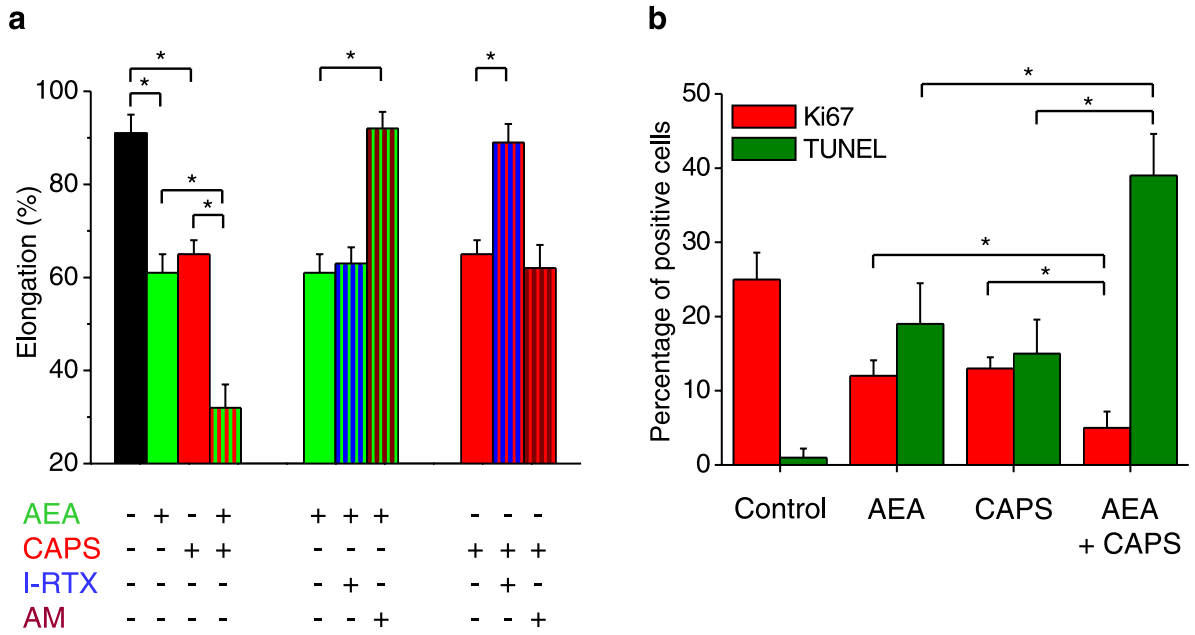

Figure 3. Effect of AEA to inhibit hair growth is mediated by CB1 but not by TRPV1. *a)* HF were treated with 10 μM AEA, 10 μM capsaicin (CAPS), 1 μM AM-251 (AM), and 50 nM I-RTX in the above combinations and hair shaft elongation was determined daily (mean±SE, 18–24 HF per group). Data represent elongation values at day 7 of organ-culture. *b)* HF (18–24 per group) were treated with 10 μM AEA, 10 μM CAPS, or their combination and, at day 7, coimmunolabeling of proliferating (Ki-67+) and apoptotic (TUNEL+) cells, along with nuclei (DAPI+), was performed. Data represent values of statistical analysis of number of Ki-67+ and TUNEL+ cells as compared to the number of DAPI+ cells (mean±SE). In both panels, asterisks mark significant ($P<0.05$) differences.

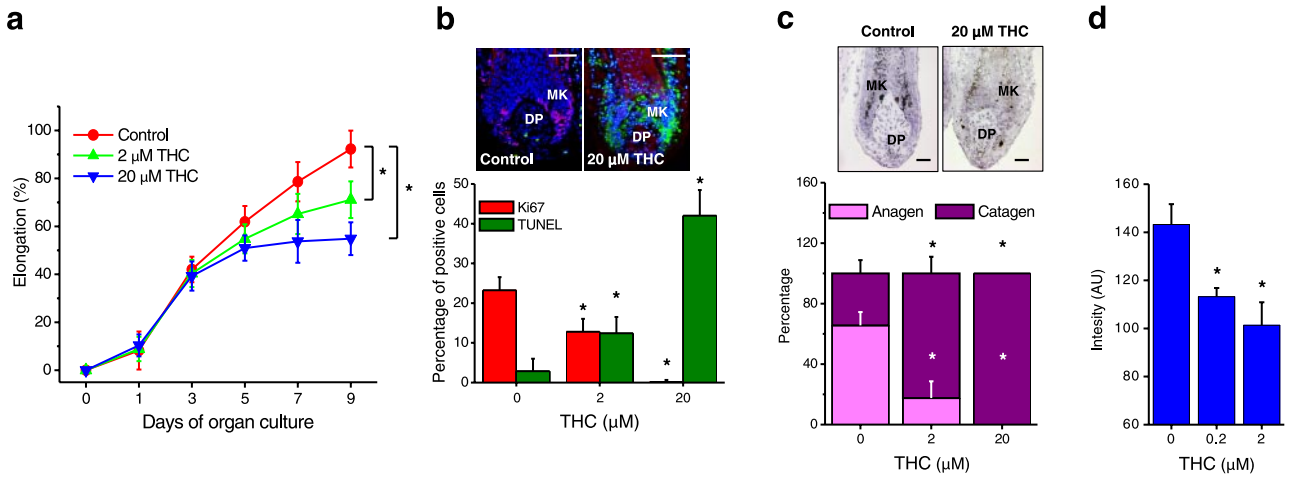

Figure 4. THC inhibits hair shaft elongation and proliferation, whereas it stimulates apoptosis of cultured human HF. *a)* Hair shaft elongation curves (mean±SE, 18–24 HFs per group). *$P < 0.05$ when compared to control (*b*) Coimmunolabeling of proliferating (Ki-67+, red fluorescence) and apoptotic (TUNEL+, green fluorescence) cells, along with nuclei (DAPI, blue fluorescence). Statistical analysis of number of Ki-67+ and TUNEL+ cells as compared with the number of DAPI+ cells (mean±SE). *c)* Quantitative hair cycle histomorphometry on hematoxylin-eosin-stained sections. Percentage of HF in anagen or catagen state (23, 33, 34) was determined (mean±SE). *b, c)* DP, dermal papilla, MK, matrix keratinocytes. Scale bars, 10 μm. *d)* Statistical image analysis of melanin content in the bulb region of those THC-treated HF which were not yet transformed to catagen (mean±SE); 5–10 regions of interest per section, 18–24 HF per group. *$P < 0.05$ when compared to control.

keratinocyte apoptosis and premature catagen development **(Fig. 4*a–c*)**. These data, therefore, suggest that exocannabinoids can mimic the hair growth-inhibitory effects of endocannabinoids.

We also determined the effect of THC on the melanin content of the HF. During this measurement, to differentiate the effect of the exocannabinoids from the well-known catagen-associated "shut-down" of follicular melanogenesis (33, 34, 46–48), the melanin content of only those THC-treated HF were defined, which were not yet transformed to catagen. Interestingly, as opposed to findings with AEA, we found that THC significantly and dose-dependently suppressed the melanin content of the HF (Fig. 4*d*), suggesting THC may also exert inhibitory effects on follicular melanogenesis *in situ* (independent of the normal, catagen-associated suppression of the melanin production of the HF) (33, 34, 46–48).

DISCUSSION

Exploration of cannabinoid functions in skin biology and pathology is an important, integral part of the ongoing exploration of the skin as a "nonclassical" neuro-endocrine organ. As a part of this quest, in this paper, we provide the first evidence that the prototypic endocannabinoid, AEA (which may even be produced within human HF), and the—notoriously abused—exocannabinoid, THC, both inhibit human hair shaft elongation and induce apoptosis-driven HF involution (catagen) *in vitro*. We show that these effects are most likely mediated via CB1 receptor-mediated signaling mechanism. Furthermore, we show that intrafollicular expression of the "targeted" CB1 is hair cycle-dependent and is up-regulated during catagen. Given that these effects were generated with intact components of a normal human miniorgan and under assay conditions that preserve *in vivo*-like key functions of this organ during the test period, our findings are both physiologically and clinically relevant. Furthermore, these data support the concept that human HF are both targets and sources of endocannabinoids, and exploit a physiologically relevant paracrine-autocrine endocannabinoid system for negatively regulating their own growth.

Since previous reports have extensively documented that AEA might exert its cellular actions via CB1, CB2, and/or TRPV1-coupled signaling mechanisms (1–5, 42–44), a central core of the current study was to identify the molecular target(s) of this endocannabinoid. Our results that *i)* the hair growth-inhibitory actions of AEA was fully abrogated by the CB1-specific antagonist AM-251; *ii)* the effects of AEA was not modified by the TRPV1 antagonist I-RTX; and *iii)* CB1 was successfully identified in the HF (both at the protein and mRNA levels), whereas CB2 was not found; suggest that (although TRPV1 is also functionally expressed in the HF) (23) AEA may exclusively act on CB1 to inhibit human hair growth and to modulate the hair cycle.

Experimental data with the coadministration of AEA and the TRPV1 agonist capsaicin, by showing that the similar effects of the two agents were additive (at least indirectly) further strengthened the above argument. However, these results (along with our presentation that the effect of capsaicin was not modified by the CB1 antagonist AM-251) also propose that the otherwise very intimately related (and often "overlapping") endocannabinoid and (endo)vanilloid systems (3, 43) synergistically yet, of importance, independently function to regulate various biological processes (elongation, pro-

liferation, apoptosis, cycling) of the human HF. This may also be further strengthened by our recent report showing that TRPV1 knockout mice (which possess an essentially unaltered endocannabinoid system) (49) exhibit a significant delay in the onset of the first spontaneous catagen during the morphogenesis of the HF (50). (Our currently running investigation of the morphogenesis and hair cycle of CB1 knockout mice will hopefully explore this interaction "the other way around").

Our findings that catagen development, *per se* (at least in organ-cultured human scalp HF), is already associated with a marked up-regulation of CB1 expression, suggests that, once catagen has been induced by either AEA or cannabinoid-independent mechanisms (such as, *e.g.*, IFNγ or on TRPV1 activation) (23), the HF substantially increases its susceptibility to (additional) stimulation by endocannabinoids *via* this receptor. This may then further accelerate the speed of catagen development, depending on the availability of endogenous agonists.

Our exciting pilot mass spectrometry data (which, as detailed above, demand further careful and systematic repetition using tissue extracts of many additional HF from several different individuals), which demonstrate the intrafollicular presence of substantial AEA levels in microdissected, rigorously washed human scalp HF, suggest that endogenous CB agonists may even be produced locally, *i.e.*, within the anagen hair bulb. However, it was surprising to observe that, unlike in most tissues (1–3, 42), the level of 2-AG was very low in the HF. This may reflect, *e.g.*, high intrafollicular levels of fatty acid amide hydrolase and monoacylglycerol lipase, which participate in the degradation of 2-AG (1–3, 42). Although further studies are to be performed to quantitatively define the expression of these molecules in the HF, the above hypothesis may, at least in part, explain our results that of the two major endocannabinoids (produced by the HF) only AEA was able to inhibit hair growth and that HF were unresponsive to 2-AG stimulation.

In our hands, the CB1 antagonist AM-251 alone did not modify hair shaft elongation which, at the first glance, might suggest that the endogenous cannabinoid tone does not affect hair growth. However, it is well documented that during certain pathological conditions (*e.g.*, inflammatory and autoimmune diseases), the level of numerous endocannabinoids (including AEA and 2-AG) and the expression profile of CB receptors are markedly altered (1–3, 51). Since inflammation as well as alterations in the activity of the immune system was shown to markedly contribute to the pathogenesis of several hair loss disorders (such as alopecia areata, effluvium) (52, 53), it might be hypothesized (and to be definitely measured in the near future) that endocannabinoid expression may, *e.g.*, be increased in such diseases. Therefore, our demonstration that the CB1 antagonist effectively abrogated the hair growth-inhibitory effects of AEA may be interpreted as a first, tentative proof-of-principle for a novel, CB1 *antagonist*-based adjuvant treatment option in the clinical management of certain human hair loss disorders.

Irrespective of their potential clinical implications and further intriguing applications (*e.g.*, future exploitation of the growth-inhibitory effect of CB *agonists* in the putative management of unwanted hair growth such as hirsutism), our results also show that human HF organ culture offers a very simple, yet highly instructive new research tool for exploring and dissecting "non-classical", growth- and apoptosis-modulatory effects of endo- and exocannabinoids and of receptor-mediated signaling in general under *physiologically relevant* conditions. Using microarray techniques (*cf.* 23, 32), this prototypic tissue interaction system can now even be exploited to identify novel target genes of CB-mediated signaling in the human system *in situ*. Certainly, the intriguing concept that human HF (at least on the scalp) may always (or hair cycle-dependently) more or less "stoned", and the challenge to selectively get the HF (rather than the central nervous system. . .) "high" in a clinically desired manner will surely excite patients, investigators, industry, regulatory institutions, the lay press, and politicians alike. FJ

This work was supported in part by Hungarian research grants (OTKA T049231, OTKA K063153, NKFP 1A/008/04) to T.B. and by a DFG grant (Pa 345/11–2) to R.P. The authors declare no competing financial interests.

REFERENCES

1. Di Marzo, V., Bifulco, M., De Petrocellis, L. (2004) The endocannabinoid system and its therapeutic exploitation. *Nat. Rev. Drug Discov.* **3,** 7712–7784
2. Piomelli, D. (2005) The endocannabinoid system: a drug discovery perspective. *Curr. Opin. Investig. Drugs* **6,** 672–679
3. Pacher, P., Batkai, S., and Kunos, G. (2006) The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* **58,** 389–462
4. Di Marzo, V., and De Petrocellis, L. (2006) Plant, synthetic, and endogenous cannabinoids in medicine. *Annu. Rev. Med.* **57,** 553–574
5. Felder, C. C., Dickason-Chesterfield, A. K., and Moore, S. A. (2006) Cannabinoids biology: the search for new therapeutic targets. *Mol. Interv.* **6,** 149–161
6. De Vries, T. J., Shaham, Y., Homberg, J. R., Crombag, H., Schuurman, K., Dieben, J., Vanderschuren, L. J., and Schoffelmeer, A. N. (2001) A cannabinoid mechanism in relapse to cocaine seeking. *Nat. Med.* **7,** 1151–1154
7. Freund, T. F., Katona, I., and Piomelli, D. (2003) Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev.* **83,** 1017–1066
8. Jiang, W., Zhang, Y., Xiao, L., Van Cleemput, J., Ji, S. P., Bai, and G., Zhang, X. (2005) Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *J. Clin. Invest.* **115,** 3104–3416
9. Batkai, S., Jarai, Z., Wagner, J. A., Goparaju, S. K., Varga, K., Liu, J., Wang, L., Mirshahi, F., Khanolkar, A. D., Makriyannis, A., Urbaschek, R., Garcia, N. Jr., Sanyal, A. J., and Kunos, G. (2001) Endocannabinoids acting at vascular CB1 receptors mediate the vasodilated state in advanced liver cirrhosis. *Nat. Med.* **7,** 827–832
10. Croxford, J. L., Miller, S. D. (2003) Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J. Clin. Invest.* **111,** 1231–1240
11. Galve-Roperh, I., Sanchez, C., Cortes, M. L., del Pulgar, T. G., Izquierdo, M., Guzman, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation

and extracellular signal-regulated kinase activation. *Nat. Med.* **6,** 313–319
12. Bifulco, M., Di Marzo, V. (2002) Targeting the endocannabinoid system in cancer therapy: a call for further research. *Nat. Med.* **8,** 547–550
13. Blazquez, C., Casanova, M. L., Planas, A., Del Pulgar, T. G., Villanueva, C., Fernandez-Acenero, M. J., Aragones, J., Huffman, J. W., Jorcano, J. L., and Guzman, M. (2003) Inhibition of tumor angiogenesis by cannabinoids. *FASEB J.* **17,** 529–531
14. Portella, G., Laezza, C., Laccetti, P., De Petrocellis, L., Di Marzo, V., and Bifulco, M. (2003) Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis. *FASEB J.* **17,** 1771–1773
15. Bifulco, M., Laezza, C., Valenti, M., Ligresti, A., Portella, G., and DiMarzo, V. (2004) A new strategy to block tumor growth by inhibiting endocannabinoid inactivation. *FASEB J.* **18,** 1606–1608
16. Blazquez, C., Carracedo, A., Barrado, L., Real, P. J., Fernandez-Luna, J. L., Velasco, G., Malumbres, M., and Guzman, M. (2006) Cannabinoid receptors as novel targets for the treatment of melanoma. *FASEB J.* **20,** 2633–2635
17. Fernandez-Ruiz, J., Romero, J., Velasco, G., Tolon, R. M., Ramos, J. A., and Guzman, M. (2007) Cannabinoid CB(2) receptor: a new target for controlling neural cell survival? *Trends. Pharmacol. Sci.* **28,** 39–45
18. Casanova, M. L., Blazquez, C., Martinez-Palacio, J., Villanueva, C., Fernandez-Acenero, M. J., Huffman, J. W., Jorcano, J. L., and Guzman, M. (2003) Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. *J. Clin. Invest.* **111,** 43–50
19. Maccarrone, M., Di Rienzo, M., Battista, N., Gasperi, V., Guerrieri, P., Rossi, A., and Finazzi-Agro, A. (2003) The endocannabinoid system in human keratinocytes. Evidence that anandamide inhibits epidermal differentiation through CB1 receptor-dependent inhibition of protein kinase C, activation protein-1, and transglutaminase. *J. Biol. Chem.* **278,** 33896–33903
20. Ständer, S., Schmelz, M., Metze, D., Luger, T. A., and Rukwied, R. (2005) Distribution of cannabinoid receptor 1 (CB1) and 2 (CB2) on sensory nerve fibers and adnexal structures in human skin. *J. Dermatol. Sci.* **38,** 177–188
21. Wilkinson, J. D., and Williamson, E. M. (2007) Cannabinoids inhibit human keratinocyte proliferation through a non-CB1/CB2 mechanism and have a potential therapeutic value in the treatment of psoriasis. *J. Dermatol. Sci.* **45,** 87–92
22. Philpott, M. P., Green, M. R., and Kealey, T. (1990) Human hair growth in vitro. *J. Cell Sci.* **3,** 463–471
23. Bodó, E., Bíró, T., Telek, A., Czifra, G., Griger, Z., Tóth, I. B., Mescalchin, A., Ito, T., Bettermann, A., Kovács, L., and Paus, R. (2005) A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am. J. Pathol.* **166,** 985–998
24. Arck, P. C., Handjiski, B., Kuhlmei, A., Peters, E. M., Knackstedt, M., Peter, A., Hunt, S. P., Klapp, B. F., and Paus, R. (2005) Mast cell deficient and neurokinin-1 receptor knockout mice are protected from stress-induced hair growth inhibition. *J. Mol. Med.* **83,** 386–396
25. Peters, E. M., Arck, P. C., and Paus, R. (2006) Hair growth inhibition by psychoemotional stress: a mouse model for neural mechanisms in hair growth control. *Exp. Dermatol.* **15,** 1–13
26. Uhl, M., and Sachs, H. (2006) Cannabinoids in hair: strategy to prove marijuana/hashish consumption. *Forensic. Sci. Int.* **145,** 143–147
27. Skopp, G., Strohbeck-Kuehner, P., Mann, K., and Hermann, D. (2006) Deposition of cannabinoids in hair after long-term use of cannabis. *Forensic. Sci. Int.* [Epub ahead of print]
28. Paus, R., Theoharides, T. C., and Arck, P. C. (2006) Neuroimmunoendocrine circuitry of the 'brain-skin connection'. *Trends. Immunol.* **27,** 32–39
29. Botchkarev, V. A. (2003) Stress and the hair follicle: exploring the connections. *Am. J. Pathol.* **162,** 709–712
30. Arck, P. C., Handjiski, B., Peters, E. M., Peter, A. S., Hagen, E., Fischer, A., Klapp, B. F., and Paus, R. (2003) Stress inhibits hair growth in mice by induction of premature catagen development and deleterious perifollicular inflammatory events via neuropeptide substance P-dependent pathways. *Am. J. Pathol.* **162,** 803–814
31. Ito, N., Ito, T., Kromminga, A., Bettermann, A., Takigawa, M., Kees, F., Straub, R. H., and Paus, R. (2005) Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J.* **19,** 1332–1334
32. Foitzik, K., Krause, K., Conrad, F., Nakamura, M., Funk, W., and Paus, R. (2006) Human scalp hair follicles are both a target and a source of prolactin, which serves as an autocrine and/or paracrine promoter of apoptosis-driven hair follicle regression. *Am. J. Pathol.* **168,** 748–756
33. Paus, R., and Cotsarelis, G. (1999) The biology of hair follicles. *N. Engl. J. Med.* **341,** 491–497
34. Stenn, K. D., and Paus, R. (2001) Controls of hair follicle cycling. *Physiol. Rev.* **81,** 449–494
35. Slominski, A., and Wortsman, J. (2000) Neuroendocrinology of the skin. *Endocr. Rev.* **21,** 457–487
36. Slominski, A., Wortsman, J., Luger, T., Paus, R., and Solomon, S. (2000) Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol. Rev.* **80,** 979–1020
37. Paus, R., Schmelz, M., Bíró, T., and Steinhoff, M. (2006) Frontiers in pruritus research: scratching the brain for more effective itch therapy. *J. Clin. Invest.* **116,** 1174–1186
38. Arck, P. C., Slominski, A., Theoharides, T. C., Peters, E. M., and Paus, R. (2006) Neuroimmunology of stress: skin takes center stage. *J. Invest. Dermatol.* **126,** 1697–1704
39. Slominski, A., Wortsman, J., Tuckey, R. C., and Paus, R. (2007) Differential expression of HPA axis homolog in the skin. *Mol. Cell Endocrinol.* **265,** 143–149
40. Giuffrida, A., Rodriguez de Fonseca, F., and Piomelli, D. (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. *Anal. Biochem.* **280,** 87–93
41. Batkai, S., Osei-Hyiaman, D., Pan, H., El-Assal, O., Rajesh, M., Mukhopadhyay, P., Hong, F., Harvey-White, J., Jafri, A., Hasko, G., Huffman, J. W., Gao, B., Kunos, G., and Pacher. P. (2007) Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury. *FASEB J.* [Epub ahead of print]
42. Kogan, N. M., and Mechoulam, R. (2006) The chemistry of endocannabinoids. *J. Endocrinol. Invest.* **29,** 3–14
43. Di Marzo, V., Blumberg, P. M., and Szallasi, A. (2002) Endovanilloid signaling in pain. *Curr. Opin. Neurobiol.* **12,** 372–379
44. Van Der Stelt, M., and Di Marzo, V. (2004) Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. *Eur. J. Biochem.* **271,** 1827–1834
45. Pacher, P., Batkai, S., Osei-Hyiaman, D., Offertaler, L., Liu, J., Harvey-White, J., Brassai, A., Jarai, Z., Cravatt, B. F., and Kunos, G. (2005) Hemodynamic profile, responsiveness to anandamide, and baroreflex sensitivity of mice lacking fatty acid amide hydrolase. *Am. J. Physiol. Heart. Circ. Physiol.* **289,** H533–541
46. Slominski, A., Paus, R., Plonka, P., Chakraborty, A., Maurer, M., Pruski, D., and Lukiewicz, S. (1994) Melanogenesis during the anagen-catagen-telogen transformation of the murine hair cycle. *J. Invest. Dermatol.* **102,** 862–869
47. Slominski, A., Tobin, D. J., Shibahara, S., and Wortsman, J. (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol. Rev.* **84,** 1155–1228
48. Slominski, A., Wortsman, J., Plonka, P. M., Schallreuter, K. U., Paus, R., and Tobin, D. J. (2005) Hair follicle pigmentation. *J. Invest. Dermatol.* **124,** 13–21
49. Pacher, P., Batkai, S., and Kunos, G. (2004) Haemodynamic profile and responsiveness to anandamide of TRPV1 receptor knock-out mice. *J. Physiol.* **558,** 647–657
50. Bíró, T., Bodó, E., Telek, A., Géczy, T., Tychsen, B., Kovács, L., and Paus, R. (2006) Hair cycle control by vanilloid receptor-1 (TRPV1): Evidence from TRPV1 knockout mice. *J. Invest. Dermatol.* **126,** 1909–1912
51. Carrier, E. J., Patel, S., and Hillard, C. J. (2005) Endocannabinoids in neuroimmunology and stress. *Curr. Drug. Targets CNS Neurol. Disord.* **4,** 657–665
52. Paus, R., Ito, N., Takigawa, M., and Ito, T. (2003) The hair follicle and immune privilege. *J. Investig. Dermatol. Symp. Proc.* **8,** 188–194
53. Paus, R., Nickoloff, B. J., Ito, T. (2005) A 'hairy' privilege. *Trends Immunol.* **26,** 32–40

Received for publication March 6, 2007.
Accepted for publication May 10, 2007.