Hair Cycle-Dependent Changes in Adrenergic Skin Innervation, and Hair Growth Modulation by Adrenergic Drugs

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Skin nerves may exert ''trophic'' functions during hair follicle development, growth, and/or cycling. Here, we demonstrate hair cycle-related plasticity in the sympathetic innervation of skin and hair follicle in C57BL/6 mice. Compared with telogen skin, the number of nerve fibers containing norepinephrine or immunoreactive for tyrosine hydroxylase increased during the early growth phase of the hair cycle (anagen) in dermis and subcutis. The number of these fibers declined again during late anagen. β_2-adrenoreceptor-positive keratinocytes were transiently detectable in the noncycling hair follicle epithelium, especially in the isthmus and bulge region, but only during early anagen. In early anagen skin organ culture, the β_2-adrenoreceptor agonist isoproterenol promoted hair cycle progression from anagen III to anagen IV. The observed hair cycle-dependent changes in adrenergic skin innervation on the one hand, and hair growth modulation by isoproterenol, accompanied by changes in β_2-adrenoreceptor expression of selected regions of the hair follicle epithelium on the other, further support the concept that bi-directional interactions between the hair follicle and its innervation play a part in hair growth control. This invites one to systematically explore the neuropharmacologic manipulation of follicular neuroepithelial interactions as a novel therapeutic strategy for managing hair growth disorders. ***Key words: hair follicle/neuropeptide Y/neurotransmitter/norepinephrine/tyrosine hydroxylase. J Invest Dermatol 113:878–887, 1999***

There is increasing awareness of the role of neuroepithelial and neuromesenchymal interactions in the control of tissue homeostasis and remodeling (Jones and Munger, 1987; Abelli *et al*, 1993; Paus *et al*, 1994a, 1997; Hordinsky and Ericson, 1996; Ansel *et al*, 1997). The hair follicle (HF) offers an ideal model system for studying such interactions, as it represents one of the most densely innervated organs of the mammalian organism (Winkelmann, 1988; Hashimoto *et al*, 1990; Halata, 1993; Paus *et al*, 1997). Moreover, HF continuously and spontaneously undergo profound, highly organized, cyclic transformations involving massive epithelial cell proliferation, differentiation, and apoptosis (Paus, 1996; Stenn *et al*, 1998; Paus and Cotsarelis, 1999). Between resting (telogen), growth (anagen), and regression (catagen), neuroepithelial and neuromesenchymal interactions may significantly affect hair growth (for review, see Hordinsky and Ericson, 1996; Paus *et al*, 1997). General principles of epithelial and mesenchymal growth control by nerve- or glia-derived signals during development and tissue remodeling can be conveniently investigated in this readily accessible system under physiologic and pathologic conditions. Furthermore, novel neuropharmacologic strategies for the therapeutic manipulation of neuroepithelial and neuromesenchymal interactions, and for managing clinical hair growth disorders, can be explored in this system (Paus *et al*, 1997).

The highly synchronized depilation-induced murine hair cycle has proven to be a particular fertile testing ground for dissecting mesenchymal–neuroectodermal interactions (Paus *et al*, 1997). For example, the murine hair cycle is characterized by complex and profound hair cycle-dependent changes in the structure of extra- and perifollicular innervation. These are evidenced by significant fluctuations in the skin immunoreactivity (IR) of pan-neuronal markers (PGP 9.5, neurofilament 150), markers for growing nerve fibers (GAP-43, N-CAM) and Schwann cell markers (S-100, myelin basic protein) (Botchkarev *et al*, 1997a). In addition, mast cells, which are involved in the control of murine hair growth (Paus *et al*, 1994b; Maurer *et al*, 1997), and function as key elements of neuroimmune interaction (Williams *et al*, 1995), display abundant, and surprisingly hair cycle-dependent contacts with nerve fibers during the murine hair cycle (Botchkarev *et al*, 1997b).

In this study, we have followed-up these observations by focusing on the autonomic innervation, specifically on sympathetic nerves. Though the autonomic and sensory innervation of mammalian skin has been extensively characterized by numerous

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Abbreviations: APM, arrector pili muscle; β_2-AR, β_2-adrenoreceptor; DDP, deep dermal (nervous) plexus; FNA, follicular neural network A; FNB, follicular neural network B; HF, hair follicles; IR, immunoreactive/immunoreactivity; NA, norepinephrine; NGF, nerve growth factor; NP, neuropeptide; NPY, neuropeptide Y; SG, sebaceous gland; TH, tyrosine hydroxylase.

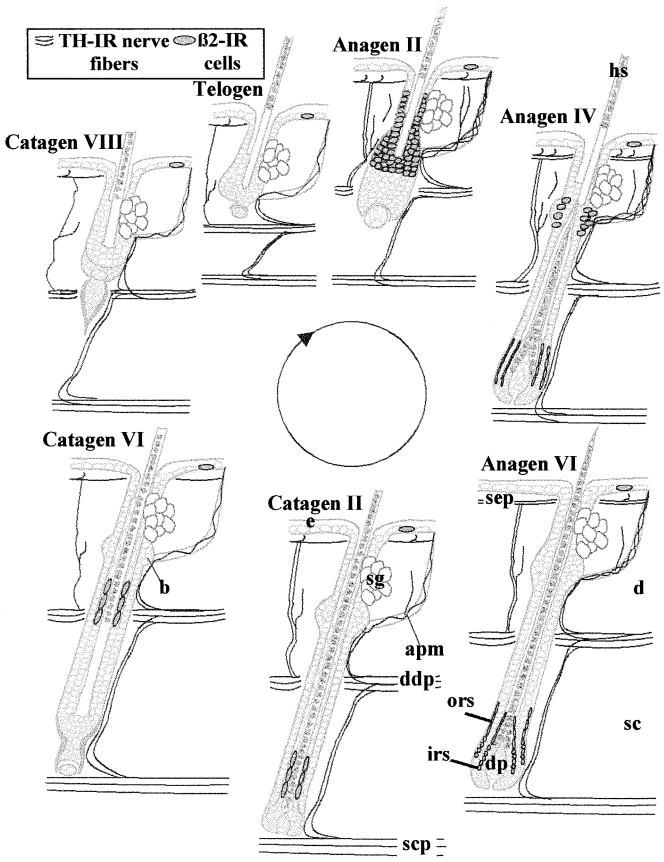


Figure 1. Schematic summary of TH-IR nerve fibers and β_2-AR-IR cells in murine pelage HF during various stages of the induced murine hair cycle. This computer-generated scheme summarizes the best reproducible TH- and β_2-AR expression patterns during the induced hair cycle of C57BL/6 mice (apm, arrector pili muscle; b, bulge; d, dermis; ddp, deep dermal (nervous) plexus; dp, dermal papilla; hs, hair shaft; sc, subcutis; scp, subcutaneous (nervous) plexus; sep, subepidermal (nervous) plexus; sg, sebaceous gland).

investigators over the last decades (e.g., Falck and Rorsman, 1963; Jenkinsson, 1970; Morishima, 1970; Wallengren *et al*, 1987; Alvarez *et al*, 1988; Dalsgaard *et al*, 1989; Karanth *et al*, 1991; Katoh *et al*, 1991; Rice *et al*, 1993, 1998; Rice *et al*, 1993; Tausk *et al*, 1993; Karanth, 1994; Hordinsky and Ericson, 1996; Fundin *et al*, 1997a, b), the autonomic innervation of murine dorsal skin and normal pelage HF is still fairly ill-defined (cf. Psenicka, 1965). In particular, the exact distribution of sympathetic nerve fibers in the HF-associated neural networks of murine skin awaits systematic characterization, and autonomic nerve fiber functions other than motor or vasodilatatory ones are underinvestigated.

The classical effector nerves supplying the skin are postganglionic fibers of the paravertebral chain ganglia. Most of these fibers are immunoreactive for tyrosine hydroxylase (TH-IR), a rate-limiting key enzyme in catecholamine biosynthesis, and thus represent the sympathetic division of the autonomic nervous system (Tausk *et al*, 1993; Lundberg, 1996; Wallengren, 1997). To hairy skin, sympathetic fibers travel together with sensory fibers in order to innervate blood vessels, sweat glands, and HF (Jenkinsson, 1970; Karanth *et al*, 1991; Tausk *et al*, 1993; Hordinsky *et al*, 1995; Fundin *et al*, 1997a; Rice *et al*, 1998). Sympathetic nerve fibers also form neuromuscular junctions with the arrector pili and vascular smooth muscles, where they are partially deprived of their Schwann cell coat (Fuxe and Nilsson, 1965; Waris, 1978; Schotzinger and Landis, 1990; Roth and Kummer, 1994; Morris, 1995) and appear as single nerve fibers in the dermis (Jenkinsson, 1970; Karanth *et al*, 1991). Sebaceous glands (SG) are thought to be not innervated (Thody and Shuster, 1989; Tausk *et al*, 1993).

Some autonomic fibers also contain neuropeptides such as neuropeptide Y (NPY), which is often coexpressed with TH in adrenergic fibers (Johansson, 1986; Gibbins, 1990; Tausk *et al*, 1993; Walsh *et al*, 1994; Lundberg, 1996). Neuropeptides that are coexpressed in the skin with neurotransmitters may modulate transmitter functions, and may have growth-modulatory properties (Tanaka *et al*, 1988; Haegerstrand *et al*, 1989; Rozengurt, 1991; Panconesi *et al*, 1994; Paus *et al*, 1994a, 1995, 1997) as keratinocytes, fibroblasts, and mast cells all express receptors for adrenergic transmitters (Masini *et al*, 1982; Schallreuter *et al*, 1992, 1995; Williams *et al*, 1995; Steinkraus *et al*, 1996; Schallreuter, 1997). Sympathetic transmitters and neuropeptides are involved not only in the regulation of blood supply and thermoregulation, but probably also in the control of cell proliferation, differentiation, and migration as well as in collagen metabolism (Kishimoto *et al*, 1982; Donaldson and Mahan, 1984; Koizumi *et al*, 1991; Takahashi *et al*, 1993; Schallreuter *et al*, 1995; Ansel *et al*, 1997). In addition, they can exert immunomodulatory properties (cf. Blalock, 1994).

As a basic step towards exploring possible sympathetic mechanisms of hair growth control (Paus *et al*, 1997), we have defined changes in the skin content and distribution of norepinephrine (NA), TH, and NPY associated with adrenergic fibers (Gibbins, 1990) as well as the skin expression of β_2-AR (the most abundant adrenoreceptor in skin; Steinkraus *et al*, 1992; Schallreuter *et al*, 1993). The paraformaldehyde-condensation method (Falck *et al*, 1962) was used to visualize the sympathetic neurotransmitter NA, and immunofluorescence microscopy was employed to demonstrate TH, NPY, and β_2-AR. In order to manipulate putative piloneural interactions *in situ*, the β-AR agonist isoproterenol or the β_2-AR antagonist propranolol (Flaxman and Harper, 1975) were administered to early anagen murine skin organ culture in order to study whether this would affect anagen development *in vitro* in HF that had already been induced to enter into the growth stage of the hair cycle *in vivo*.

MATERIALS AND METHODS

Mice Six to 9 wk old, syngenic, female C57BL/6 mice in the telogen stage of the hair cycle were purchased from Charles River (Sulzfeld, Germany). The mice were housed in community cages with 12-h light periods at animal facilities in the Humboldt Universität Berlin, Virchow Klinikum, and were fed water and mouse chow *ad libitum*. All animal experiments were carried out in accordance with the protocols of the animal care committee of the Senate of Berlin.

Hair cycle induction Anagen was induced in the back skin of mice in the telogen phase of the hair cycle (identified by their homogeneously pink back skin color) by depilation, applying a 1:1 melted wax/rosin mixture under anesthesia, as previously described (Paus *et al*, 1990). After hardening, the wax/rosin mixture was peeled off the skin, plucking out all telogen hair shafts, which induces the predictable and highly synchronized development of anagen follicles that are morphologically indistinguishable from spontaneous anagen follicles (Chase, 1954; Paus *et al*, 1990).

Skin harvesting Skin was harvested at defined days after depilation (unmanipulated telogen skin = day 0, anagen II = day 3, anagen IV = day 5, anagen VI = day 12, early catagen = day 17, late catagen = day 19; **Fig 1**, cf. Paus *et al*, 1994c; Botchkarev *et al*, 1997a, b). For comparison, 10–12 wk old mice were investigated with all back skin follicles in spontaneously developed anagens II–IV or anagen VI. Mice were either anesthetized by i.p. injection of a lethal overdose of ketanest and perfusion-fixed with a mixture of 4% paraformaldehyde and 14% saturated picric acid as described (Ljungberg and Johansson, 1993; Botchkarev *et al*, 1997a, b) or killed by cervical dislocation. Back skin from the neck region was harvested perpendicular to the paravertebral line in order to obtain longitudinal HF sections. The samples were immediately frozen in liquid nitrogen as described before (Paus *et al*, 1994c).

Fluorescent histochemistry and cytospectrofluorometry For visualization of NA-containing nerve fibers, we used the conventional paraformaldehyde condensation method (Falck *et al*, 1962). The staining was confirmed by employing the method based on the glyoxylic acid-induced fluorescence of NA (Lindvall *et al*, 1980). For the

cytospectrofluorometric determination of NA content, the paraformaldehyde method, which produces less diffusion of the NA fluorophore into surrounding tissue, in our hands, gave more reliable results and was, therefore chosen for this study. Slides covered with unfixed, quick-frozen 15 μm cryostat sections were heated to 80°C in a closed chamber, containing paraformaldehyde (Aldrich-Sigma, St Louis, MO) 40–50% humidity for 1 h. Then, slides were dried at room temperature and mounted in a polystyrol/xylene mixture. For controls, sections were incubated with sodium borohydride ($NaBH_4$, Fluka AG) to reduce the specific signal (Fuxe and Johnson, 1973). Neuronal NA content was measured by cytospectrofluorometry (Dahlstrom *et al*, 1966; Dolezel *et al*, 1984), using a LUMAM-I3 fluorescence microscope (wavelength of the excitation light 410 ηm), equipped with a specially designed fluorometric block, FMAL-1A (LOMO, St Petersburg, Russia). Maximal fluorescence intensity was determined at the wavelength 480 nm, using an interference filter N6, and was documented in arbitrary units on a voltmeter scale.

Immunofluorescence For visualization of nerve fibers, perfusion-fixed (15 μm) or, for the visualization of β_{1+2}-AR, acetone-fixed cryostat sections (10 μm) were incubated overnight at room temperature in a humidity chamber with the primary anti-sera to TH (1:500, Biogenesis, Poole, U.K., Botchkarev *et al*, 1997b), NPY (1:100, Biogenesis, Poole, U.K.;Gibbins, 1990), β_1-AR (variable dilutions without result, Santa Cruz Biotechnology, Santa Cruz, CA, Inglese *et al*, 1994) or β_2-AR (1:100, Santa Cruz Biotechnology). All anti-sera used were rabbit polyclonal. This was followed by an incubation of 1 h at 37°C with tetrametylrhodamine-isothiocyanate-conjugated $F(ab)_2$ fragments of goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:200. All anti-sera contained 0.3% Triton X-100 and 2% normal goat serum. After incubation, sections were rinsed in 0.1 M Tris-buffered saline, pH 7.4, three times, 5 min each. After washing in Tris-buffered saline, all sections were mounted in Immunomount medium (Shandon, Pittsburgh, PA) and stored at –20°C until analyzed.

Four types of negative controls were done: (i) slides were incubated with the secondary antibody alone; (ii) slides were incubated with the primary antibody after preincubation with control peptides for the specific anti-sera (all obtained from Sigma; 10–20 μg per ml, 37°C, 1 h); (iii) slides were incubated with rabbit or rat IgG as primary antibody; and (iv) skin samples from surgically denervated skin were used for the standard staining procedure. Briefly, for surgical denervation the technique for denervation of rat back skin was employed (Doucette and Diamond, 1987). As positive controls, cryostat sections of whole mount mouse fetuses were used containing brain, spinal cord, heart, and gut. Negative controls incubated with the secondary antibody alone or with nonspecific IgG did not reveal specific IR comparable with any of the applied antibodies but only background staining similar to the background of the test slides was visible. In skin samples from surgically denervated mice no TH- or NPY-IR NF could be detected with the applied antibodies and specific staining was blocked completely when the antibody was incubated together with the specific control peptide.

For each stage of HF cycling, at least 10 microscopic fields per mouse of three to five different mice were studied, containing a minimum of 30 different HF (i.e., more than 200 microscopic fields were studied for each tested antibody and time point).

Skin organ culture To investigate a potential influence of the increased noradrenergic innervation occurring in early anagen, 4 mm punch biopsies were taken under sterile conditions from C57BL/6 murine back skin in early anagen (day 4 after depilation, when strong β_2-AR-IR was present in the HF bulge region), on autoclaved paper humidified with culture medium as described before (Li *et al*, 1992; Paus *et al*, 1994d). Five to 10 randomized skin punches per experimental group were placed dermis down on a gelatin sponge in a 35 mm Petri dish containing 5 ml Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 50 mg per ml L-glutamine, and antibiotic/anti-mycotic mixture (Gibco, Grand Island, NY). After the addition of 10^{-6}–10^{-8} M isoproterenol (Sigma), 10^{-6} M propranolol (Sigma), or 10^{-7} M isoproterenol together with 10^{-6} M propranolol, organ cultures were incubated for 48 h at 37°C, in 5% CO_2 and 100% humidity. Isoproterenol was chosen as β-AR agonist because of its greater stability and long half-life compared with NA, and because it binds with higher affinity and high specificity to keratinocyte β_2-AR (Gazith *et al*, 1983; Steinkraus *et al*, 1996). Subsequently, all fragments were washed repeatedly in phosphate-buffered saline buffer at 4°C, and quickly frozen in liquid nitrogen as described above. For the precise identification of the defined stages of HF cycling, histochemical detection of endogenous alkaline phosphatase activity was used, as this allows to visualize the morphology of the dermal papilla as a useful morphologic marker for staging HF development and cycling (Handjiski *et al*, 1994). The percentage of HF in defined anagen stages was calculated in five to 10 biopsies per group at magnification × 400 under a Zeiss Axioscope microscope, following accepted morphologic criteria (Chase, 1954; Paus, 1996), and statistical analysis was performed as described below.

Morphometry and statistical analysis Sections were examined at × 400 magnification under a Zeiss Axioscope fluorescence microscope and photodocumented with the aid of an image analysis system (ISIS, Metasystems, Altlussheim, Germany) and the entire individual image was further processed to reduce background signal by 8–11%, to achieve gamma correction, and to enhance sharpness (Paint Shop Pro, Jasc, Minnetonka, CA). The appearance of nerve fibers in circumscribed, previously defined nerve fiber networks [subepidermal (nervous) plexus, deep dermal (nervous) plexus (DDP), subcutaneous (nervous) plexus] or innervation targets [epidermis dermis including the arrector pili muscle (APM), subcutis including blood vessels, follicular network A (FNA) around the HF suprainfundibular outer root sheath (ORS), follicular neural network B (FNB) around the HF isthmus] were analyzed qualitatively. The number of single nerve fiber profiles in dermis and subcutis were then quantified by histomorphometry, as previously described (Botchkarev *et al*, 1997a). The results of the observed, representative IR patterns were also recorded in schematized, computer-generated recording protocols (as representatively demonstrated in **Fig 1**). For evaluation of the hair cycle progression in skin organ culture, histomorphometric hair cycle staging was performed on 10 μm sections of test or control skin and staging was performed as previously described (Handjiski *et al*, 1994; Paus *et al*, 1994c, e). The data were pooled from all biopsies or mice per investigated compound and time point, and the mean ± SEM calculated. p-values were determined by Student's t test for parametric samples or by Mann–Whitney U for nonparametric samples (cf. Botchkarev *et al*, 1997a, b).

RESULTS

Sympathetic innervation of murine skin and HF in telogen Here, we have investigated the expression and distribution of sympathetic innervation markers (NA, TH, NPY) in skin and HF-associated neural networks, as well as the β-AR distribution in murine skin epithelium. Previously, we have defined that the innervation of murine pelage HF can be structurally subdivided into two distinct perifollicular neural networks, FNA and FNB, which arise from the subepidermal and deep dermal (nervous) plexus (DDP), respectively (Botchkarev *et al*, 1997a; Paus *et al*, 1997).

FNA is a meshwork of very low caliber, PGP 9.5-IR unmyelinated nerve fibers, that extend from the perifollicular epidermis to the level of the SG and enwrap the suprainfundibular ORS of the HF (Botchkarev *et al*, 1997a). The FNA of telogen HF failed to display NA containing nerve fibers or IR for TH or NPY. The FNB is arranged around the isthmus part of the HF, just below the SG and above the insertion of the APM, and arises from the DDP (Botchkarev *et al*, 1997a). It consists of longitudinal PGP 9.5-IR fibers that are regularly spaced (often described as palisade-like) alongside and close to the HF ORS, together with circular fibers that encircle these longitudinal fibers in a centrifugal fashion. This piloneural nerve fiber complex has previously been described as being supplied by sensory nerve fibers (Bjorklund *et al*, 1986; Winkelmann, 1988; Rice *et al*, 1993; Fundin *et al*, 1997a, b). The FNB, however, also contains fibers reactive for the parasympathetic marker cholinesterase in a number of species (Jenkinson, 1970; Moustafa, 1989), and the sympathetic markers monoamine-oxidase, NA, and/or TH in rats, cats, and guinea-pigs (Jenkinsson, 1970; Roberts and Levitt, 1982; Roth and Kummer, 1994). Yet, in the C57BL/6 mouse, neither longitudinal nor circular fibers in the FNB of telogen HF displayed specific IR for TH in murine pelage HF (**Fig 2*A***). The FNB also stained negative for NA, using the paraformaldehyde method (not shown).

The APM showed dense sympathetic innervation. TH-IR fibers enwrapped and penetrated the muscle in a meshwork-like manner (**Fig 2*A***). Frequently, nerve fibers in the DDP (cf. Botchkarev *et al*, 1997a; **Fig 2*B***) were seen very close to the insertion of the APM into the follicular connective tissue sheath that separates the bulge region of the ORS from the APM (**Fig 2*A***). These TH-IR DDP

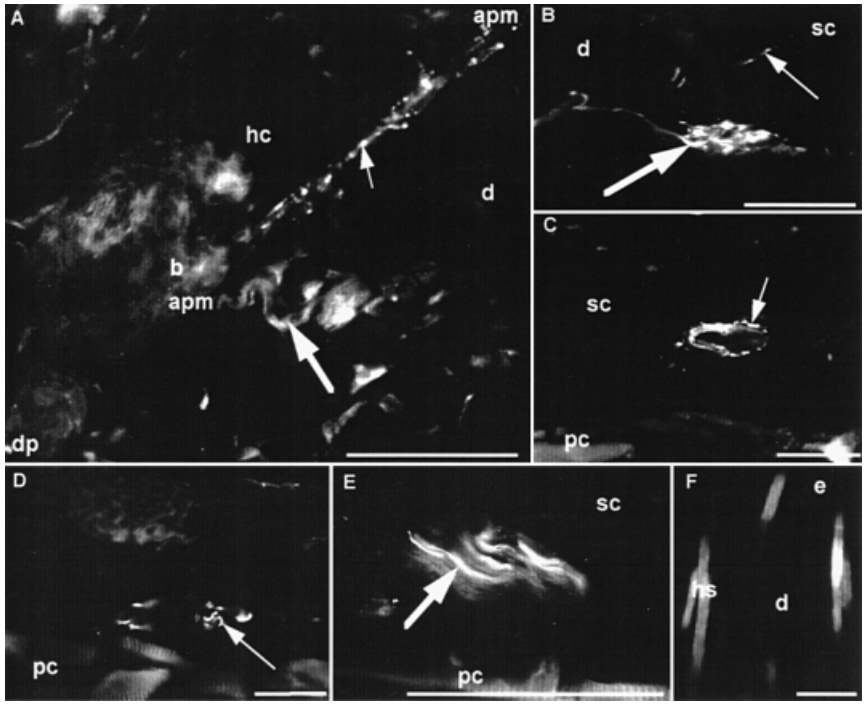


Figure 2. TH- and NPY-IR in murine telogen skin. Back skin cryostat sections (15 μm) of adolescent C57BL/6 mice with all HF in a resting state of the hair cycle (telogen) were stained with antiserum against TH (*A*, *B*, *D*) or NPY (*C*, *E*). apm, arrector pili muscle; b, bulge; d, dermis; dp, dermal papilla; e, epidermis; hc, hair canal; hs; hair shaft; pc, panniculus carnosus muscle; sc, subcutis. *Scale bars*: 50 μm. (*A*) TH-IR nerve fibers innervate the APM of a pelage HF (*small arrow*). Note the anatomical closeness of the insertion point of the APM to the HF bulge region. *Large arrow* indicate TH-IR nerve fibers in the DDP. (*B*) TH-IR nerve fibers within the deep dermal (nervous) plexus (*large arrow*) at the border between dermis and subcutis. Note the fine fiber extending into the dermis (*small arrow*). (*C*) NPY-IR nerve fibers innervate a subcutaneous blood vessel (*arrow*) in the subcutis, close to the panniculus carnosus. Note the varicose appearance of the nerve fibers. (*D*) TH-IR nerve fibers innervate a subcutaneous blood vessel (*arrow*) in the subcutis, close to the panniculus carnosus. Note the varicose appearance of the nerve fibers. (*E*) NPY-IR nerve fibers within the subcutaneous nerve fiber (nervous) plexus (*large arrow*) close to the panniculus carnosus. (*F*) Negative control from surgically denervated skin, stained for TH-IR. Note the strong autofluorescence of the hair shafts. No TH-IR nerve fibers are seen in the dermis.

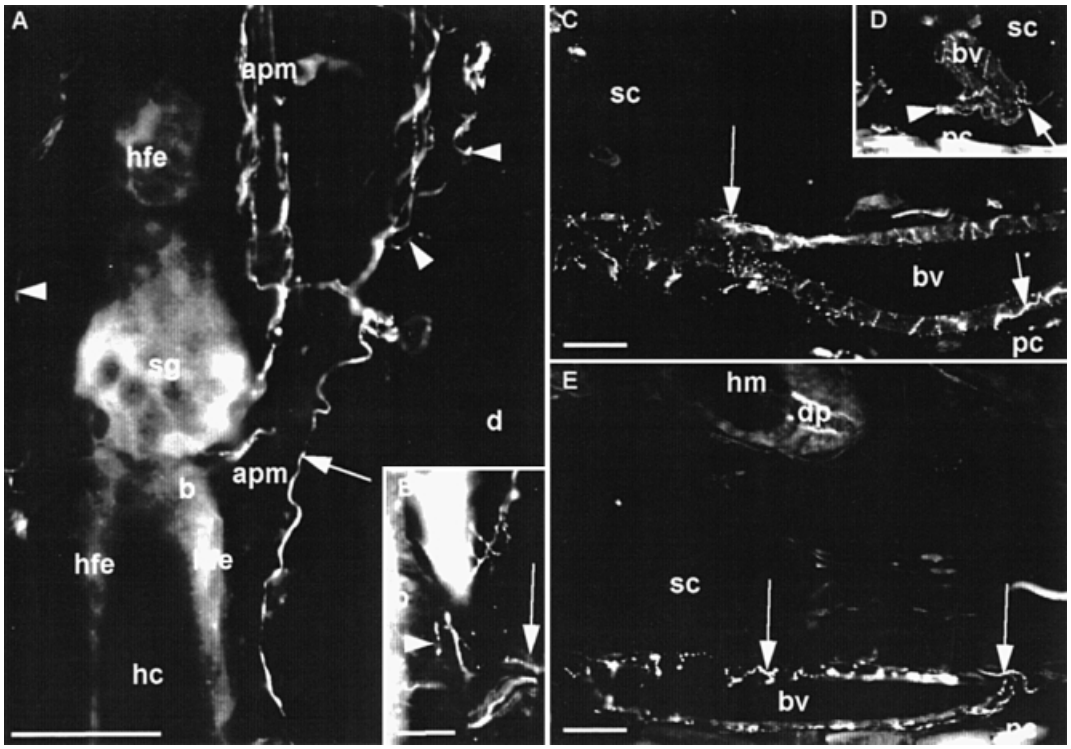
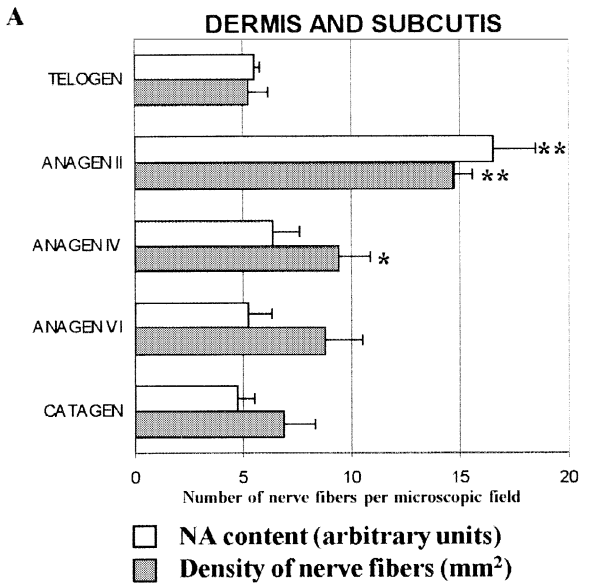


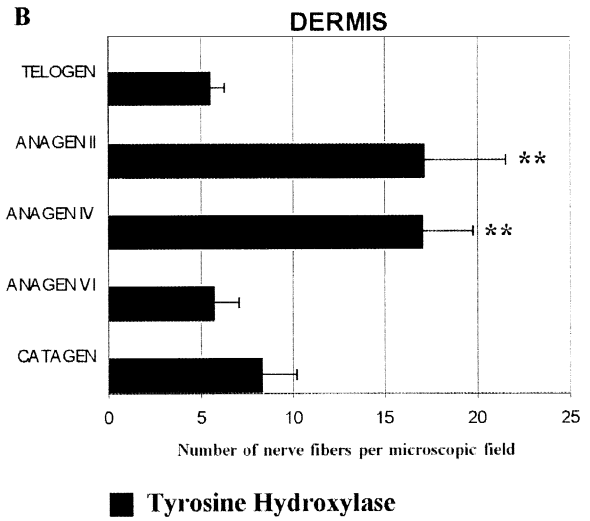
Figure 3. TH- and NPY-IR in murine anagen skin. Back skin cryostat sections (15 μm thickness) of adolescent C57BL/6 mice with all HF in defined stages of anagen (*A*, *B*: anagen II = 3 d after anagen induction by depilation; *C–E*: anagen VI = 8–12 d after anagen induction) were stained with an antiserum against TH or NPY. apm, arrector pili muscle; b, bulge; bv, blood vessel; d, dermis; hc, hair canal; hfe, hair follicle epithelium; hm, hair matrix; pc, panniculus carnosus muscle; sc, subcutis; sg, sebaceous gland). *Scale bars*: 50 μm. (*A*) Dermal TH-IR nerve fibers in anagen II skin: strongly positive, varicose nerve fibers (*arrow*) innervate the APM, or terminate freely in the dermis (*small arrowheads*). The bulge region, located just underneath the SG where the APM inserts, appears close to the nerve fibers innervating the APM. (*B*) TH-IR nerve fibers in the DDP (*arrow*) at the border between the dermis and subcutis giving rise to other TH-IR nerve fibers (*arrowhead*) which enter into the APM where it inserts into the ORS, close to the bulge region (*b*). (*C*) Numerous TH-IR nerve fibers (*arrows*) innervating a longitudinally sectioned large subcutaneous blood vessels close to the panniculus carnosus, as frequently seen in anagen skin. (*D*) TH-IR nerve fibers (*arrow*) leave the subcutaneous (nervous) plexus (*arrowhead*) to innervate a cross-sectioned blood vessel close to the panniculus carnosus. (*E*) Numerous NPY-IR nerve fibers (*arrows*) innervating a longitudinally sectioned large subcutaneous blood vessel close to the panniculus carnosus. Note the anagen hair bulb in the upper part of the micrograph.

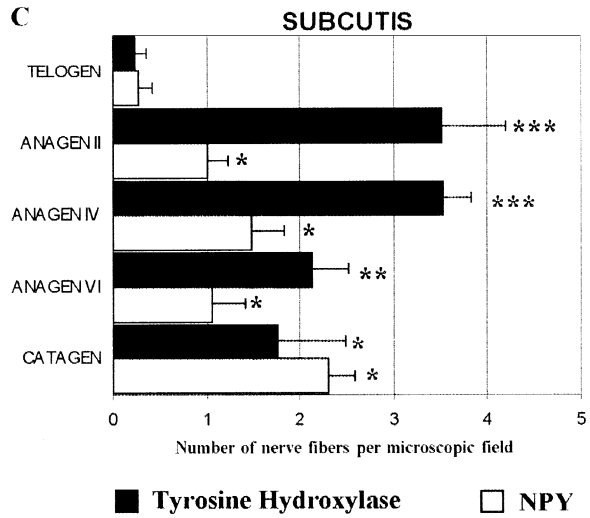
fibers appeared to give rise to the sympathetic innervation of the APM. No TH-IR nerve fibers were seen to innervate the connective tissue capsule of the SG or sebocytes directly.

NPY-IR was only seen in the innervation of subcutaneous blood vessels (**Fig 2*C***), where TH-IR nerve fibers were also observed (**Fig 2*D***). In contrast to previous observations made in murine vibrissae (Ebara *et al*, 1992), no NPY-IR fibers were noted in FNA or FNB. TH-IR single nerve fibers and TH-IR fibers innervating the APM or subcutaneous blood vessels as well as NPY-IR nerve fibers innervating subcutaneous blood vessels, displayed a varicose appearance (**Fig 2*A***, ***C***, ***D***). This suggests the presence of numerous sites for neurotransmitter and/or NPY release (cf. Tausk *et al*, 1993). All three distinct (nervous) plexus of the murine skin [subepidermal (nervous) plexus, DDP (**Fig 2*A***, ***B***), and subcutaneous (nervous) plexus, **Fig 2*E***] contained fibers stainable for TH, but only the subcutaneous (nervous) plexus also contained NPY-IR nerve fibers (**Fig 2*E***).

None of the specific staining patterns mentioned above was observed in any of the control stains (e.g., **Fig 2*F***). Likewise, no other cells in murine skin showed comparably strong NA content or TH and NPY-IR.







Hair cycle dependent changes in the noradrenergic innervation of murine skin Depilation of murine back skin with all follicles in the resting stage (telogen) results in the immediate onset of a new, highly synchronized anagen wave with practically all HF in the same stage of anagen development when harvested at defined time points after depilation (Chase, 1954; Paus *et al*, 1990; Paus, 1996). For simplicity, back skin with all follicles in a defined hair cycle stage will therefore be referred to as "telogen" or "anagen/catagen skin". Besides spontaneously developed telogen and anagen II–VI skin from untreated animals, depilation-induced anagen I, II, IV, and VI (days 1, 3, 5, and 12 postdepilation, respectively) as well as catagen skin (day 19 postdepilation) were studied (note that catagen develops spontaneously in depilation-induced anagen follicles). These follicles are morphologically and functionally indistinguishable from spontaneously developed hair cycle stages (Paus *et al*, 1990, 1994e; Slominski *et al*, 1994).

With the applied methods, NA-containing or TH- and NPY-IR nerve fibers remained undetectable within the FNA and FNB of nontylotrich pelage HF throughout the entire hair cycle. In more than 1500 analyzed HF from all hair cycle stages the proximal hair bulb and the dermal papilla never revealed nerve fibers positive for any of the investigated adrenergic markers (NA, TH, NPY).

In contrast, substantial changes were observed during the depilation-induced hair cycle in the innervation of the APM, blood vessels, and various skin compartments (**Fig 1**). The innervation of the APM gained complexity during anagen development. It exhibited numerous interconnected NA- and TH-IR nerve fibers, which left the DDP close to the HF bulge region, and gave rise to small single nerve fibers terminating in the dermis (**Fig 3*A***, ***B***). NA-, TH-, or NPY-IR nerve fibers could now readily be seen to innervate different caliber blood vessels in

Figure 4. Dynamics in TH- and NPY-IR as well as NA-containing nerve fiber number and intraneuroneal NA content throughout the depilation induced hair cycle. (*A*) The number of NA-containing single nerve fibers in the dermis and subcutis increase in early anagen, and decreases significantly when the hair cycle proceeds towards late anagen. In catagen skin, the number of NA-containing single nerve fibers returns to telogen levels. Parallel to this development, intraneuroneal NA content peaks in early anagen, and returns almost to telogen levels in catagen. (*B, C*) TH-IR single nerve fibers in the dermis and subcutis increase significantly during anagen II–IV. NPY-IR nerve fibers remain restricted to subcutaneous blood vessels throughout the entire hair cycle, but their number also increases significantly in early anagen. TH-IR nerve fiber number decrease significantly ($p < 0.05$) during the later anagen stages. In catagen skin, the number of TH-IR in the dermis and subcutis returns to the telogen levels, whereas the number of NPY-IR nerve fibers in the subcutis seems to fall towards late anagen, and to rise again in catagen, although these changes are not significant. For each stage of HF cycling, at least 30 microscopic fields derived from three to five different mice were studied (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; all p values listed are in comparison with telogen). Error bars represent mean ± SEM, n = 3–5.

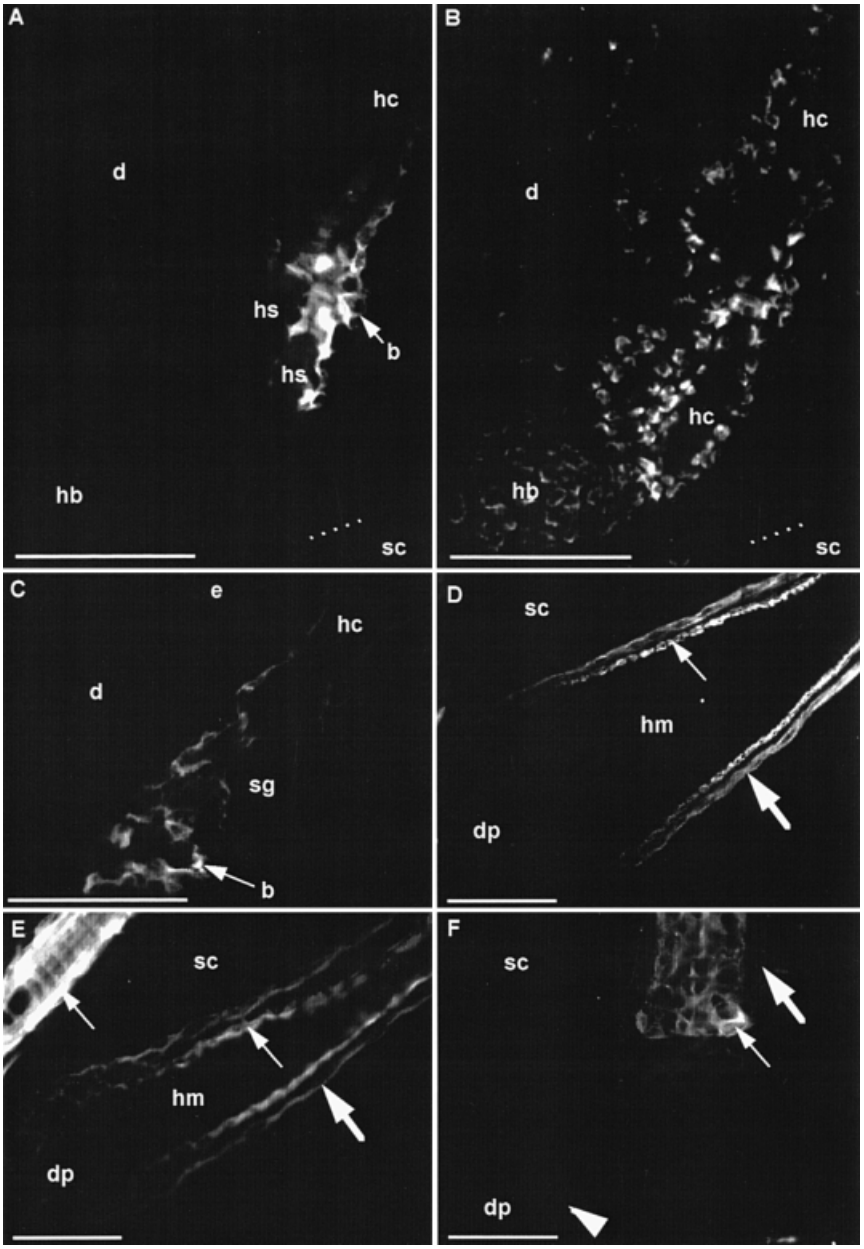


Figure 5. Adrenoreceptor-IR in different stages of the hair cycle. Back skin cryostat sections (15 μm thickness) of adolescent C57BL/6 mice with all HF in defined stages of the induced hair cycle were stained with an anti-serum against β_2-AR. (*A*, *B*) β_2-AR-IR cells in the HF, isthmus, and bulge region (b, *arrow*) of anagen II follicle (*A*). HF cells, largely reflecting ORS keratinocytes, are made visible with Hoechst-counterstaining, which demarcates the nuclei (*B*). The HF extends from the epidermis (cut off at the upper right-hand corner) towards the subcutis [the dotted line represents the border between dermis (d), and subcutis (sc)]. Note the weak autofluorescence of the hair shafts (hs). (hc, hair canal; left behind by the missing hair shaft; hb, hair bulb). (*C*) β_2-AR-IR in the bulge (b) keratinocytes (*arrow*) in anagen IV HF. Sebaceous gland (sg) is negative. (*D*) β_2-AR-IR cells within the inner (*small arrow*) and ORS (*large arrow*) in an anagen VI hair bulb, which extend into the subcutis. Note the negative dermal papilla (dp) and hair matrix (hm). (*E*) In catagen II HF only keratinocytes of proximal inner root sheath express β_2-AR-IR (*small arrows*), whereas no specific IR is seen in ORS (*large arrow*), hair matrix (hm), and dermal papilla (dp). (*F*) In catagen VI HF only single keratinocytes in the regressing inner root sheath express β_2-AR-IR (*small arrow*), whereas ORS (*large arrow*), epithelial strand (*arrowhead*), and dermal papilla (dp) are negative. d, dermis; e, epidermis; sc, subcutis. *Scale bars*: 50 μm.

the subcutis (**Fig 3*C–E***). Whether this striking increase of blood vessels associated TH- or NPY-IR nerve fibers during anagen development was due to an increased number of blood vessels, or reflected an increased adrenergic innervation of individual blood vessels, could not be distinguished in this study.

Synchronized early anagen exhibited a highly significant increase ($p < 0.05$) in the number of noradrenergic cutaneous nerve fibers. When stained for NA content, or for TH- or NPY-IR, the previously observed rise in PGP 9.5-IR nerve fibers during anagen II (Botchkarev *et al*, 1997a) was paralleled by a dramatic increase of NA-containing (**Fig 4*A***) or TH-IR single nerve fibers (**Fig 4*B***, ***C***) in the dermis and subcutis. We also observed a substantial increase of paraformaldehyde-induced fluorescence of NA in individual dermal nerve fibers during anagen II (**Fig 4*A***). NPY-IR nerve fibers remained restricted to subcutaneous blood vessels throughout the entire hair cycle, but their number also increased significantly in early anagen (**Fig 4*C***). TH-IR and NA-containing nerve fibers declined significantly in number during the later anagen stages of anagen development ($p < 0.05$). TH-IR nerve fiber numbers in spontaneously developed anagen II, IV, and VI skin corresponded well with these findings from the depilation-induced murine hair cycle (not shown). In catagen skin, the number of TH-IR (**Fig 4*B***, ***C***) or NA-containing single nerve fibers in dermis and subcutis returned to telogen levels (**Fig 4*A***). The number of NPY-IR nerve fibers in the subcutis appeared to decline slightly towards late anagen and to rise again in catagen; however, these changes were not statistically significant ($p > 0.05$, **Fig 4*C***).

Hair cycle-dependent follicular expression of β-AR immunoreactivity Next, we were interested in learning whether these hair cycle-dependent changes in the noradrenergic innervation of murine skin corresponded to any changes in the follicular expression of β-AR. As expected from the published distribution of β-AR in human skin (Steinkraus *et al*, 1992) β_1-AR-IR could not be detected at any time point during the hair cycle (not shown).

β_2-AR-IR was also absent in telogen follicles, but became visible in the isthmus and bulge region of the ORS in early anagen (**Fig 5*A***, ***B***), where it disappeared again during late anagen (**Fig 1**). During anagen IV, single keratinocytes in the bulge and proximal

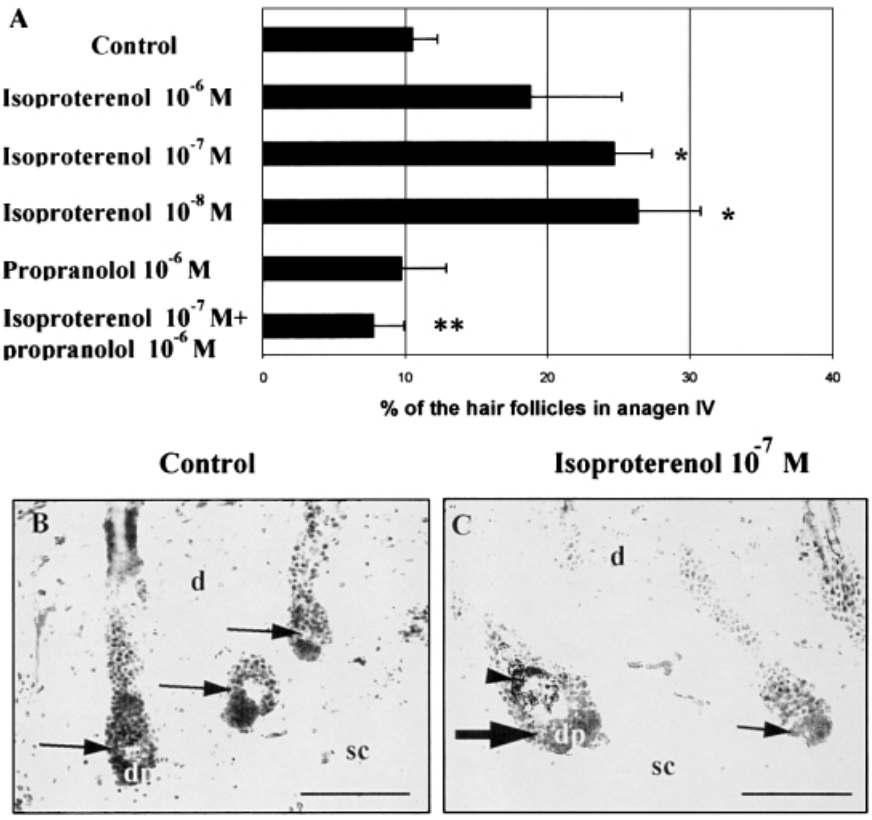


Figure 6. Isoproterenol accelerates hair cycle progression in murine early anagen skin organ culture. Four millimeter punch biopsies from C57BL/6 murine back skin containing mainly anagen III HF (day 4 after depilation), were incubated for 48 h without or after the addition of 10^{-6}–10^{-8} M isoproterenol, 10^{-6} propranolol, or 10^{-7} M isoproterenol together with 10^{-6} M propranolol. Cryostat sections were processed for histochemical detection of endogenous alkaline phosphatase activity so as to demarcate the dermal papilla shape as useful marker for hair cycle stage classification (Handjiski *et al*, 1994), and percentage of the anagen IV HF was evaluated. Error bars represent mean ± SEM, n = 5–10. (*A*) The β-AR selective NA analog isoproterenol was able to significantly promote the hair cycle progression from anagen III, as mainly present in control skin, to anagen IV. This effect was selectively and highly significantly inhibited by the addition of propranolol in a concentration sufficient to block completely isoproterenol activity. Surprisingly, low concentrations of isoproterenol (10^{-8} M) were more effective than higher concentrations (10^{-6} M) (*$p < 0.05$; **$p < 0.01$). (*B*) Histologic sample from control skin, showing HF in anagen III (*arrows*). Note, that the HF do not yet extend into the subcutis. (*C*) Histologic sample from skin treated with isoproterenol (10^{-8} M), showing an anagen III (*small arrow*), and an anagen IV (*large arrow*) HF. The anagen IV HF shows the characteristic melanin granules above the dermal papilla (*arrowhead*) and an enlarged proximal hair bulb. Note, that the HF do extend into the subcutis. d, dermis; dp, dermal papilla; sc, subcutis. *Scale bar*: 100 μm.

ORS showed β_2-AR-IR (**Fig 5*C***). Intriguingly, β_2-AR-IR also appeared in a single layer of cells within the proximal inner root sheath in late anagen (**Fig 5*D***). At the same time a layer of β_2-AR-IR cells was stainable in the proximal part of the ORS at the level of the β_2-AR-IR inner root sheath cells (**Fig 5*D***, **Fig 1**). During early catagen, β_2-AR-IR was present only in the proximal inner root sheath, whereas a substantial decrease of IR was seen in the proximal ORS (**Fig 5*E***). During late catagen, β_2-AR-IR was found only in single keratinocytes of the regressing inner root sheath (**Fig 5*F***). A few scattered cells in the epidermis were β_2-AR-IR during all hair cycle stages (**Fig 1**), yet we never saw the continuos layer of β_2-AR-IR cells observed in human epidermis by others (e.g., Steinkraus *et al*, 1996), neither could we detect β_2-AR-IR of fibroblast populations, including dermal papilla fibroblasts (**Fig 5*D***).

Isoproterenol accelerates hair cycle progression in skin organ culture Thus, we have identified a substantial increase of the noradrenergic innervation of murine skin (**Fig 4*A–C***), and of β_2-AR-IR expression, namely in the bulge region (**Fig 5*A***), during early and middle anagen. Therefore, we were interested in learning whether NA had any modulatory properties on hair cycle progression in skin organ culture (Li *et al*, 1992). We found that, in murine skin organ-cultured day 4 after depilation *in vivo*, when almost all HF are in anagen III, only 10% of all HF progressed into anagen IV within 48 h when cultured in control medium (**Fig 6*A***, ***B***). If the medium was supplemented with the β-AR-selective NA analog isoproterenol, however, this significantly promoted hair cycle progression with a maximal anagen IV promotion seen after addition of 10^{-7}–10^{-8} M isoproterenol (**Fig 6*A***, ***C***). This effect was inhibited, when the β-AR antagonist propranolol was added. Propranolol alone did not alter HF cycling in organ culture. Surprisingly, the lowest concentration of isoproterenol (10^{-8} M) was more effective than higher concentrations (10^{-6} M) (for discussion, see below; note that this skin organ culture assay does not distinguish between a true anagen acceleration, and an inhibitory effect of the test agent on the constitutive tendency of explanted HF to degenerate slowly in skin organ culture).

DISCUSSION

Here we demonstrate that the previously described hair cycle-dependent plasticity of skin and HF innervation in mice is not only evident from the general increase of PGP 9.5-IR nerve fibers during early anagen and their decline during catagen (Botchkarev *et al*, 1997a), but that these changes are largely paralleled by adrenergic nerves and β_2-AR expression. In addition, we provide evidence that suggests an involvement of the adrenergic innervation in the control of HF cycling. Specifically, we show here that synchronized anagen development is associated with an increase in adrenergic skin innervation, namely with a peak in the intraneuroneal NA content and in the number of NA- or TH-IR dermal nerve fibers during anagen II–IV (**Figs 3*A*** and **4*A***, ***B***).

The regulation and function of this relative adrenergic hyperinnervation during anagen remain, at present, unclear. Yet, several lines of argumentation suggest that the cycling HF itself plays a part in this rearrangement of sympathetic nerve fibers in murine anagen skin, and that β-AR ligands released from these fibers promote the completion of anagen development. Recent data from our laboratory suggest that the cycling HF is an important source of multiple neurotrophins (Paus *et al*, 1997; Botchkarev *et al*, 1998a, 1999).

For example, HF cycling in mice is associated with profound changes in the extrafollicular and intrafollicular expression of nerve growth factor (NGF), neurotropin-3, brain-derived neurotropic factor, and neurotropin-4 (Paus *et al*, 1997; Botchkarev *et al*, 1998a, 1999),[1] which may be related to these hair cycle associated changes

in adrenergic skin innervation. In particular, the maximal steady-state levels of NGF protein that are detectable in early anagen skin (Paus *et al*, 1997),[1] may explain the early anagen-associated peak in adrenergic skin innervation observed here, as sympathetic ganglia, which supply adrenergic fibers to skin, are highly NGF-dependent (Lewin and Barde, 1996). The peak of cutaneous neurotropin-3, brain-derived neurotropic factor, and neurotropin-4 gene expression during late anagen and catagen in murine skin (Botchkarev *et al*, 1998a, 1999) may underlie the maximal number of NPY-IR nerve fibers observed in the subcutis during catagen (**Fig 4*C***), as sympathetic ganglia also depend on target-derived neurotropin-3 and brain-derived neurotropic factor (Lewin and Barde, 1996; Botchkarev *et al*, 1998b). Overexpression of NGF and neurotropin-3 in the HF epithelium induces profound alterations in skin innervation (Albers *et al*, 1994, 1996). Therefore, it is likely that hair cycle-dependent fluctuations in the cutaneous neurotrophin milieu, much of which seem to arise from the neurotrophin-secretory activity of the HF epithelium itself (Botchkarev *et al*, 1998a, 1999), underlie the observed changes in adrenergic skin innervation. It now remains to be dissected whether HF which have entered into anagen development begin to secrete neurotrophins, that induce an increase in adrenergic innervation, which in turn promotes further anagen development.

That the increase in sympathetic innervation can indeed stimulate hair growth has already been observed in rabbit skin (Crowe *et al*, 1993), and it is conceivable that the common clinical observation of localized hypertrichosis after thoracotomy (hemitrichosis) in humans and dogs (Kobayashi *et al*, 1958) is the consequence of sympathetic hyperinnervation. Vice versa, sympathetic nerve fibers degenerate in lesional skin of patients with alopecia areata (Thies, 1960). β_2-AR blockers such as propranolol can cause reversible telogen-effluvium (i.e., hair loss by premature termination of anagen; Martin *et al*, 1973; Dawber, 1997). Moreover, surgical or chemical sympathectomy initially inhibits hair growth in dogs, rabbits, and neonatal mice (Kobayashi *et al*, 1958; Crowe *et al*, 1993; Asada Kubota, 1995).

In murine back skin, NA- and TH-IR nerve fibers were mostly associated with the APM and with blood vessels, but were not detected in the follicular neural networks FNA or FNB of the nontylotrich pelage HF investigated in this study (**Figs 2** and **3**). This corresponds well to findings from rat facial skin, where TH-IR nerve fibers were not associated with HF nerve networks (Fundin *et al*, 1997a, b; Rice *et al*, 1998). TH expression, however, has clearly been observed around the distal ORS in NGF transgenic mice (Davis *et al*, 1997). This provides further support for the existence of adrenergic nerve fibers around the HF infundibulum (FNA) even though they do not contain immunochemically detectable levels of TH under normal conditions.

How might adrenergic signals affect hair growth? One possibility is that adrenergic nerve fibers may also influence HF cycling via alterations of skin blood supply, as TH- and NPY-IR nerve fibers innervating blood vessels are intimately involved in the control of skin perfusion (Schotzinger and Landis, 1990; Braverman, 1997), and hair cycle-associated increase of subcutaneous innervation, mainly representing blood vessels, was also detected in our study (**Figs 3*C***, ***D*** and **4*C***).

Another, probably even more important, conceivable mechanism is the modulation of β-AR expression and function by the HF epithelium via the release of appropriate neurotransmitters from sympathetic nerve fibers. Of the known adrenoreceptors, β_2-AR are the most abundant ones in human skin (Steinkraus *et al*, 1992; Steinkraus *et al*, 1996) and keratinocyte proliferation and differentiation is associated with a rise in β-AR activity (Koizumi *et al*, 1991; Schallreuter *et al*, 1992, , 1995). In the light of the strong β_2-AR staining in the distal epithelium of early anagen HF, this may reflect an important role of catecholamines in modulating the extremely high level of keratinocyte proliferation associated with early anagen (cf. Paus *et al*, 1990). Interestingly, pharmacologic β-AR blockade inhibits keratinocyte proliferation (Orenberg and Wilkinson, 1982), and undifferentiated keratinocytes (Schallreuter *et al*, 1995) and keratinocytes in prophase (Clausen *et al*, 1982) seem to be the most susceptible to proliferative and differentiative β-AR stimulation. Therefore, it is reasonable to speculate that follicular β-AR stimulation by sympathetic neurotransmitters released from adrenergic skin nerves is an important element of hair growth control by adrenergic signals.

In this context, it is interesting to note that the TH-IR DDP nerve fibers and the TH-IR nerve fibers innervating the APM, are located very close to the HF bulge region (**Figs 2*A*** and **3*A***, ***B***), which contains at least one key epithelial stem cell population of the HF (Cotsarelis *et al*, 1990). Moreover, the immunohistology employed in our study revealed intense β_2-AR-IR keratinocytes in the HF bulge region only during anagen II–IV (**Fig 5*A***, ***C***), i.e., the time of most rapid keratinocyte proliferation in the HF (Hansen *et al*, 1984; Paus *et al*, 1990). Although evidence for direct axonal contacts between TH-IR nerve fibers and those bulge keratinocytes expressing β_2-AR remains to be generated, catecholamines diffuse more easily than neuropeptides and may thus reach even remote targets (Tausk *et al*, 1993). Taken together with the observation that the β-AR agonist isoproterenol stimulates HF anagen progression in murine skin organ culture (**Fig 6**), this suggests that adrenergic nerve fibers may indeed promote anagen development via interaction with follicular β_2-AR, in particular those expressed by bulge region keratinocytes.

Interestingly, it has recently been demonstrated that keratinocytes and melanocytes are capable of producing catecholamines (Schallreuter *et al*, 1995; Chang *et al*, 1997). Furthermore, $6BH_4$, the rate-limiting cofactor for TH activity, whose synthesis may actually be induced by NGF (Anastasiadis *et al*, 1996), is also found in high amounts in early anagen skin in the same mouse model that we have employed in this study (Schallreuter *et al*, 1998). Therefore, we cannot exclude an autocrine mechanism for β-AR activation of HF keratinocytes by intraepithelially generated catecholamines. Although we did not detect any TH-IR or NA in either the HF epithelium or the HF mesenchyme during anagen, the amount of catecholamines produced by the HF itself may easily be below the level of detectability of our methods employed here.

In summary, this study provides suggestive evidence for the existence of bi-directional HF–neuronal interactions, and for the concept that hair cycle-associated remodeling of adrenergic skin innervation, at least in mice, is functionally relevant to hair growth control. On the one hand, this invites one systematically to explore targeted pharmacologic manipulations of adrenergic signaling pathways in skin as means of modulating hair growth in health and disease (Paus, 1996; Paus and Cotsarelis, 1999). On the other hand, the murine hair cycle offers an excellent model for dissecting the as yet ill-understood controls of peripheral tissue innervation by sympathetic fibers in response to changing innervation demands of these tissues (cf. Paus *et al*, 1997).

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