Hair-Cycle-Associated Remodeling of the Peptidergic Innervation of Murine Skin, and Hair Growth Modulation by Neuropeptides

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As the neuropeptide substance P can manipulate murine hair growth *in vivo*, we here further studied the role of sensory neuropeptides in hair follicle biology by determining the distribution and hair-cycle-dependent remodeling of the sensory innervation in C57BL/6 mouse back skin. Calcitonin-gene-related peptide, substance P, and peptide histidine methionine (employed as vasoactive intestinal peptide marker) were identified by immunohistochemistry. All of these markers immunolocalized to bundles of nerve fibers and to single nerve fibers, with distinct distribution patterns and major hair-cycle-associated changes. In the epidermis and around the distal hair follicle and the arrector pili muscle, only calcitonin-gene-related peptide immunoreactive nerve fibers were visualized, whereas substance P and peptide histidine methionine immunoreactive nerve fibers were largely restricted to the dermis and subcutis. Compared to telogen skin, the number of calcitonin-gene-related peptide, substance P, and peptide histidine methionine immunoreactive single nerve fibers increased significantly ($p < 0.01$) during anagen, including around the bulge region (the seat of epithelial stem cells). Substance P significantly accelerated anagen progression in murine skin organ culture, whereas calcitonin-gene-related peptide and a substance-P-inhibitory peptide inhibited anagen ($p < 0.05$). The inhibitory effect of calcitonin-gene-related peptide could be antagonized by coadministrating substance P. In contrast to substance P, calcitonin-gene-related peptide failed to induce anagen when released from subcutaneous implants. This might reflect a differential functional assignment of the neuropeptides calcitonin-gene-related peptide and substance P in hair growth control, and invites the use of neuropeptide receptor agonists and antagonists as novel pharmacologic tools for therapeutic hair growth manipulation. ***Key words: hair cycle/hair follicle/neuropeptide/calcitonin-gene-related peptide/peptide histidine methionine/substance P/vasoactive intestinal peptide. J Invest Dermatol 116:236–245, 2001***

Sensory nerve fibers contain neuropeptides synthesized in the cell body that are transported in vesicles to the peripheral nerve ending (Tausk *et al*, 1993). Neuropeptides exert a number of efferent functions, such as the modulation of blood flow, glandular activity, and inflammatory skin responses (Tausk *et al*, 1993; Maggi, 1995; Ansel *et al*, 1997).

Recently, there has been increasing interest in so-called ''trophic functions'' of skin innervation (Maggi, 1995; Paus *et al*, 1995, 1997), e.g., in wound healing (Tausk *et al*, 1993; Ansel *et al*, 1997) or in the maintenance of various epithelial structures such as epidermis, taste buds, and epidermal ridges (Lundberg *et al*, 1979; Morohunfola *et al*, 1992; Li *et al*, 1997; Botchkarev *et al*, 1999). In this context, several observations have suggested a role for sensory nerve fibers in hair growth control (Hordinsky and Ericson, 1996; Paus *et al*, 1997). As one of the most densely innervated organs of the mammalian body (**Fig 1**) (Rice and Munger, 1986; Winkelmann, 1988; Botchkarev *et al*, 1997b; Fundin *et al*, 1997), the hair follicle thus offers an intriguing model system for dissecting the piloneural interactions that are likely to underlie the ''trophic'' effects of neuropeptides (Paus *et al*, 1997).

In view of the complex and profound hair-cycle-dependent changes in the general architecture and composition of interfollicular and perifollicular innervation during the murine hair cycle (Botchkarev *et al*, 1997a; 1997b), it is interesting to ask whether the sensory innervation of mouse skin shows corresponding fluctuations. To this end, we have analyzed calcitonin-gene-related peptide (CGRP), a neuropeptide found primarily in small diameter sensory C-fibers (Karanth *et al*, 1991; Tausk *et al*, 1993); substance P (SP), which frequently coexists with CGRP (Hokfelt *et al*, 1989); and peptide histidine methionine (PHM), a neuropeptide that shares its precursor molecule with vasoactive intestinal polypeptide (VIP), is expressed in cholinergic and in sensory nerve fibers (Chery

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Abbreviations: CGRP, calcitonin-gene-related peptide; FNA, follicular (neural) network A; FNB, follicular (neural) network B; IR, immunoreactive; PHM, peptide histidine methionine; SP, substance P.

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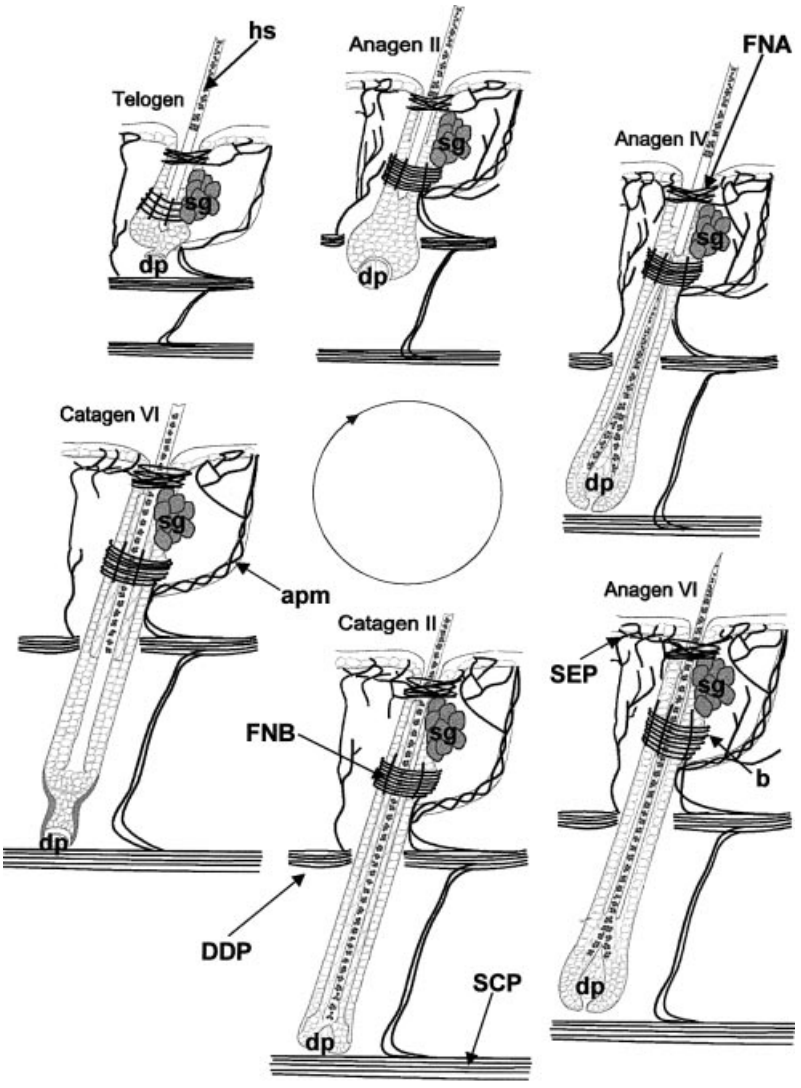

Figure 1. Schematic summary of CGRP-IR nerve fibers in murine pelage hair follicles during various stages of the induced murine hair cycle. This scheme qualitatively summarizes the most highly reproducible CGRP-IR patterns during the depilation-induced C57BL/6 hair cycle. For simplicity, the innervation of subcutaneous blood vessels has been excluded from the scheme. Abbreviations: apm, arrector pili muscle; b, bulge; DDP, deep dermal nervous plexus; dp, dermal papilla; e, epidermis; hs, hair shaft; SCP, subcutaneous nervous plexus; SEP, subepidermal nervous plexus; sg, sebaceous gland.

Croze *et al*, 1988; Ebara *et al*, 1992), and is here employed as a VIP marker. In addition, we have tested the functional relevance of these neuropeptides in hair growth control by testing the hair growth modulatory effects of CGRP, SP, PHM, and VIP in murine skin organ culture (Li *et al*, 1992; Botchkarev *et al*, 1999). As we had previously shown that subcutaneous implantation of SP-releasing microcapsules induces anagen (Paus *et al*, 1994a), we have also probed the hair cycle modulatory effect of CGRP-releasing subcutaneous implants *in vivo*.

MATERIALS AND METHODS

Mice Six to nine week old, syngeneic, 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 under 12 h light periods at the Charité/Virchow animal facilities, Berlin.

Hair cycle induction Anagen was induced in the back skin of mice by depilation as described previously (Paus *et al*, 1990), which induces the highly synchronized development of anagen follicles that are morphologically indistinguishable from spontaneous anagen follicles.

Neuropeptide administration in anagen skin organ culture Four millimeter punch biopsies were prepared under sterile conditions from C57BL/6 mouse back skin containing a homogeneous population of early, depilation-induced anagen hair follicles (i.e., day 3 after depilation, containing largely anagen II follicles) (Chase, 1954; Paus *et al*, 1994a), following previously described skin organ culture protocols ("histoculture", Li *et al*, 1992; Botchkarev *et al*, 1999). Six to eleven randomized skin fragments from two different mice were placed dermis down on prehydrated gelatin sponges (Gelfoam, Upjohn, Kalamazoo, MI) in 35 mm Petri dishes containing 5 ml Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 50 μg per ml L-glutamine, and an antibiotic/antimycotic mixture (Gibco, Grand Island, NY). After addition of 10^{-6} or 10^{-8} M of either CGRP, SP, SP antagonist N-acetyl-I-tryptophan 3,5-bi(trifluoromethyl)benzyl ester, PHM, or VIP, or a mixture of SP 10^{-6} M and CGRP 10^{-8} M (all obtained from Sigma, St. Louis, MO), organ cultures were incubated at the air–liquid interface for 72 h at 37°C, in 5% CO_2 and 100% humidity. The doses studied were selected because they are routinely used in neuropeptide studies (cf. Haegerstrand *et al*, 1989; Takahashi *et al*, 1993; Paus *et al*, 1994a, 1995). All skin fragments were fixed in 4% formalin and embedded in paraffin for routine histology.

CGRP administration *in vivo*: subcutaneous implantation of retarded-release poly(D,L-lactid-coglycolid) capsules Poly(D,L-lactid-coglycolid)/CGRP implants with a defined, retarded-release profile were kindly generated by Dr. K. Fechner at the Research Institute of Neuropharmacology, Berlin, following previously published protocols (cf. Paus *et al*, 1994a). The implants contained 0.214% rat CGRP, the CGRP-antagonistic CGRP fragment 8–37 (both obtained from Sigma), or no peptide. Twenty to twenty-four hours after carefully shaving the back skin of telogen mice without injuring the skin, implantation was performed with a trocar as described previously (Paus *et al*, 1994a). A total of 16 mice per group were treated in three independent experiments with CGRP or implants without peptide. Two mice out of each group were harvested the next day, five were harvested on day 20, and the rest were harvested 29 d after implantation. Five mice were treated with CGRP fragment 8–37 and harvested 29 d after implantation.

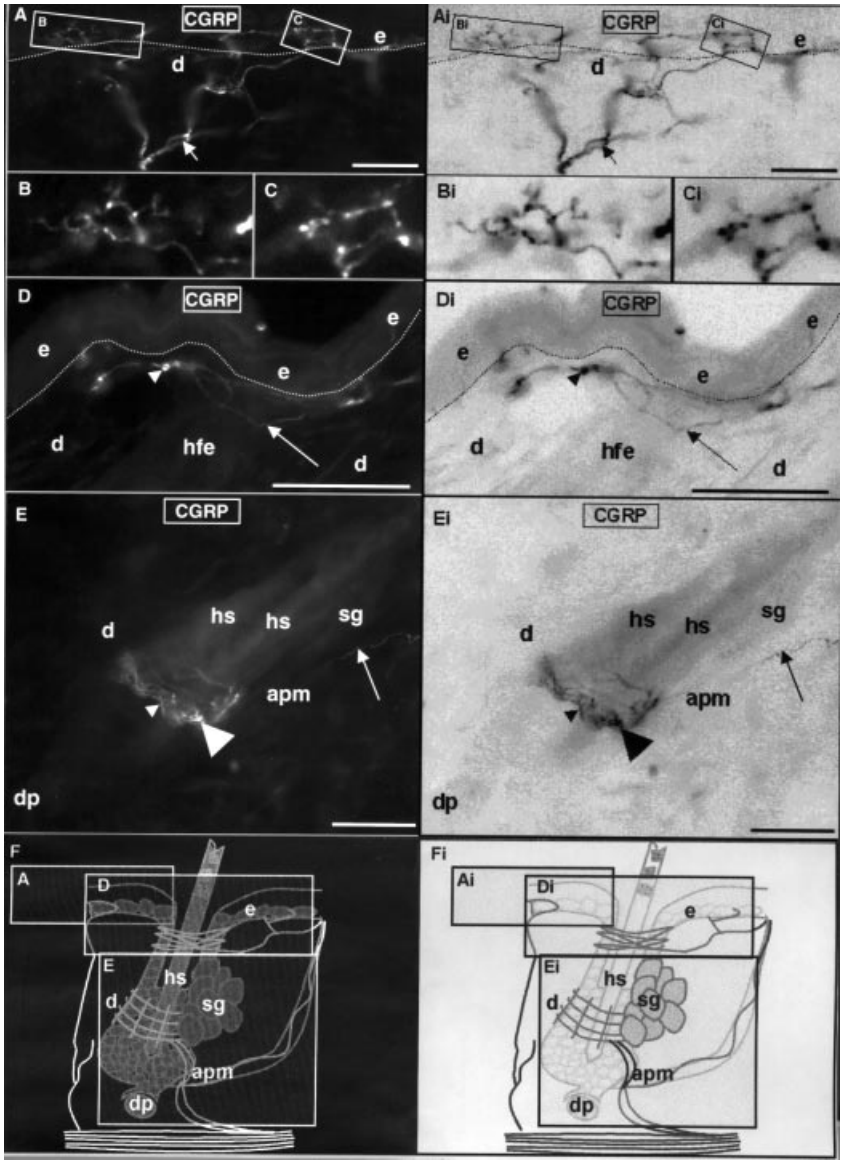

Figure 2. CGRP immunoreactivity in murine telogen skin. The dotted lines indicate basal lamina. (*A*)–(*E*) give immunofluorescent pictures. (*Ai*)–(*Ei*) give computer-generated inverted pictures corresponding to (*A*)–(*E*). Abbreviations (*A–Fi*): apm, arrector pili muscle; d, dermis; dp, dermal papilla; e, epidermis; hfe, hair follicle epithelium; hs, hair shaft; sg, sebaceous gland. *Scale bars*: 50 μm. (*A*), (*Ai*) CGRP-IR nerve fibers penetrate the basal lamina to terminate in the epidermis. Note that some of these nerve fibers appear to circle entire basal keratinocytes (*boxes*). (*B*)–(*Ci*) Magnifications of boxed areas in (*A*), (*Ai*). (*D*), (*Di*) CGRP-IR nerve fibers in the FNA (*arrow*). Note the small subepidermal bundle of nerve fibers belonging to the subepidermal nervous plexus (*arrowhead*) giving rise to the nerve fibers innervating the FNA. (*E*), (*Ei*) CGRP-IR circular (*small arrowhead*) nerve fibers in the FNB are rising from a nearby bundle of deep dermal nerve fibers belonging to the deep dermal nervous plexus (*large arrowhead*); they are surrounding the entire hair follicle, which is seen here to extend from the epidermis (cut off at the upper right-hand corner) to the dermal papilla (lower left-hand corner). Note the small nerve fiber innervating the arrector pili muscle (*arrow*). (*F*), (*Fi*) Schematic representations of CGRP immunoreactivity staining patterns as demonstrated in (*A*)–(*Ei*). Boxes indicate localization of where the above images were taken.

The repeat experiments gave highly comparable results to the initial one and so all data were pooled.

Immunohistochemistry Skin was perfusion-fixed and cryopreserved as described at defined days after depilation (unmanipulated telogen skin, day 0; anagen II, day 3; anagen IV, day 5; anagen VI, day 12; catagen, day 19) (Botchkarev *et al*, 1997a). For simplicity, skin with all follicles in telogen is referred to as ''telogen skin'' and the other stages as ''anagen/catagen skin''. For comparison, 10–12-wk-old mice were investigated with all back skin follicles in spontaneously developed anagen II-IV, or in anagen VI.

Cryostat sections (15 μm) were incubated overnight at room temperature with the primary antisera to CGRP (1:500, rabbit, polyclonal; Biogenesis, Poole, U.K.), SP (1:100, rat, monoclonal; Serva), VIP-PHI/PHM precursor (variable concentrations without result, rabbit antisynthetic sequence 111–122 of human prepro-VIP: Val-Ser-Ser-Asn-Ile-Ser-Glu-Asp-Pro-Val-Pro-Val; Genosys Biotechnologies, Cambridge, U.K.), VIP (variable concentrations without result, guineapig, polyclonal, Euro-Diagnostica, Malmö, Sweden; or rabbit, polyclonal, Paesel and Lorei, Frankfurt, Germany), and PHM (1:100, rabbit-antihuman, polyclonal; Paesel and Lorei). This was followed by an incubation of 1 h at 37°C with tetramethylrhodamine-isothiocyanate (TRITC) conjugated $F(ab)_2$ fragments of goat antirabbit or goat antirat IgG (Jackson ImmunoResearch).

For double staining of CGRP and SP, sections were first incubated with antiserum to SP, followed by TRITC-conjugated $F(ab)_2$ fragments of goat antirat IgG, and then with antiserum to CGRP, followed by fluorescein-isothiocyanate (FITC) conjugated $F(ab)_2$ fragments of goat antirabbit IgG as described before (Botchkarev *et al*, 1997b). For the analysis of hair follicle innervation, thick (120 μm) free-floating sections were incubated with the primary antiserum to CGRP for 72 h at room temperature, and then incubated with TRITC-conjugated $F(ab)_2$ fragment of a goat antirabbit IgG overnight at room temperature as described previously for PGP 9.5 (Botchkarev *et al*, 1997a). Four types of negative controls were run: (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 antisera (Sigma; 10–20 μg per ml, 37°C, 1 h); (iii) slides were incubated with rabbit/rat IgG as primary antibody; (iv) skin samples from surgically denervated skin were used for the standard staining procedure as described previously (Maurer *et al*, 1998). As positive controls, whole mount mouse fetuses were used. These controls confirmed the specificity and sensitivity of the immunohistologic techniques employed.

Qualitative and quantitative histomorphometry of skin and hair follicle innervation Sections were examined at 400× magnification under a Zeiss Axioscope fluorescence microscope. The number of single nerve fiber profiles in the dermis, including the arrector pili muscle, and in the subcutis, including blood vessels, was quantified by histomorphometry, as described previously (Botchkarev *et al*, 1997b). Hair follicle neural networks (Botchkarev *et al*, 1997a; Paus *et al*, 1997) were evaluated separately on 120 μm thick sections (Botchkarev *et al*, 1997a). For each stage of hair follicle cycling, at least 10 microscopic fields each of three to

five different mice, containing a minimum of 30 different hair follicles per mouse, were studied. The immunoreactivity (IR) patterns were also recorded qualitatively in computer-generated recording schemata.

Hair cycle staging and statistical analysis For the evaluation of the pharmacologic experiments, histomorphometric hair cycle staging was performed either on 5 μm paraffin sections (skin organ culture experiments) or on 10 μm cryosections (implantation experiments) as previously described (Botchkarev *et al*, 1999). Per investigated compound and time point the data were pooled from all samples, and the means ± SEM were calculated. p-values were determined by Student's *t* test for parametric samples or by the Mann–Whitney *U* test for nonparametric samples (Botchkarev *et al*, 1997a).

RESULTS

Interfollicular innervation: CGRP immunoreactivity is prominent in intraepidermal nerve endings, SP and CGRP immunoreactivity in dermal and subcutaneous nerve endings All three horizontal nervous plexuses in murine skin (Botchkarev *et al*, 1997a) displayed CGRP-IR, SP-IR, and PHM-IR nerve fibers. Within the innervated skin compartments, however, CGRP-IR, SP-IR, and PHM-IR were expressed differentially.

The subepidermal nervous plexus (**Fig 1**, **Fig 2*A***, ***Ai***) gave rise to nerve fibers in the epidermis (**Fig 1**, **Fig 2*A***, ***Ai***, ***B***, ***Bi***, ***C***, ***Ci***, **Fig 3*A***, ***Ai***), the follicular (neural) network A (FNA) (**Fig 1**, **Fig 2*D***, ***Di***, **Fig 3*B***, ***Bi***), and the subepidermal dermis. Freely terminating CGRP-IR fibers predominated in the epidermis (**Fig 2*A***, ***Ai***), some of which appeared to encircle entire, isolated cells in the basal layer (**Fig 2*B***, ***Bi***, ***C***, ***Ci***). SP-IR nerve fibers were only occasionally seen in cluster-like structures (**Fig 3*B***, ***Bi***) close to extra large hair follicles with two sebaceous glands (putative tylotrich hair follicles). PHM-IR nerve fibers could never be detected within the epidermis. All three neuropeptides were present, however, in the subepidermal dermis.

Bundles of nerve fibers forming the deep dermal nervous plexus (**Fig 1**, **Fig 2*E***, ***Ei***) were frequently seen close to the bulge region of hair follicles, where the arrector pili muscle inserts into the outer root sheath. Here they gave rise to nerve fibers in the follicular (neural) network B (FNB) and the arrector pili muscle (**Fig 1**, **Fig 2*E-Fi***), as well as to CGRP-IR, SP-IR, or PHM-IR nerve fibers that terminated freely in the dermis. Approximately 25%–30% of CGRP-IR nerve fibers were found to be SP-IR in the dermis, whereas essentially all SP-IR nerve fibers were also CGRP-IR (**Fig 4*A-Bii***). CGRP-IR nerve fibers that ran parallel to, and penetrated into, the arrector pili muscle were never SP-IR (not shown). Also the arrector pili muscle did not receive PHM-IR innervation.

The subcutaneous nervous plexus (**Fig 1**, **Fig 3*C***, ***Ci***, ***D***, ***Di***), located just above the panniculus carnosus, provided CGRP-IR, SP-IR, as well as PHM-IR nerve fibers for subcutaneous blood vessels (**Fig 3*C***, ***Ci***, ***D***, ***Di***).

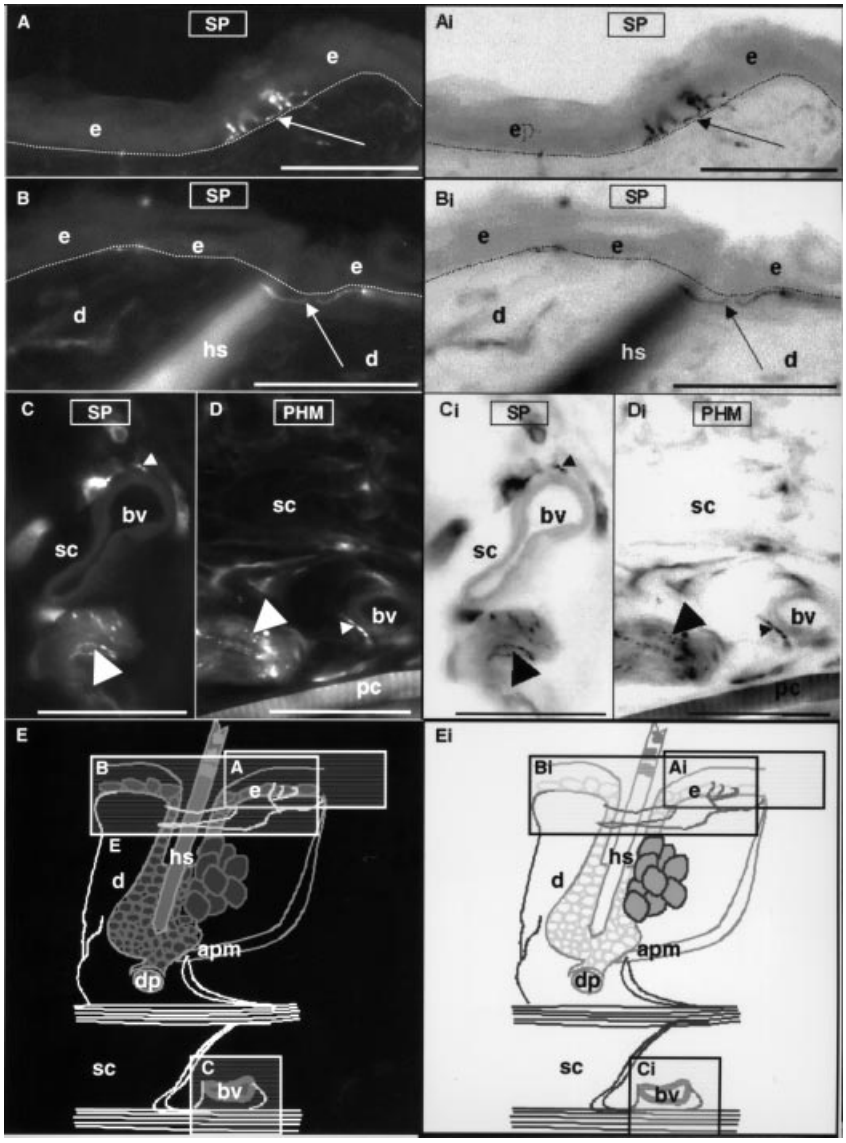

Figure 3. SP and PHM immunoreactivity in murine telogen skin. As secondary antibodies either FITC-conjugated $F(ab)_2$ fragments (*A–Ci*) or TRITC-conjugated $F(ab)_2$ fragments (*D*, *Di*) were used. The dotted lines indicate basal lamina. (*A*)–(*D*) give imunofluorescent pictures. (*Ai*)–(*Di*) give computer-generated inverted pictures corresponding to (*A*)–(*D*). Abbreviations (*A–Ei*): apm, arrector pili muscle; bv, blood vessel; d, dermis; dp, dermal papilla; e, epidermis; hs, hair shaft; pc, panniculus carnosus muscle; sc, subcutis. *Scale bars*: 50 μm. (*A*), (*Ai*) Rare example of a cluster of SP-IR nerve fibers (*arrow*) in the epidermis. (*B*), (*Bi*) SP-IR nerve fibers in the FNA (*arrow*) surrounding the distal outer root sheath of the hair follicle. Note the telogen hair shaft autofluorescence. (*C*), (*Ci*) SP-IR nerve fibers innervating a blood vessel (*small arrowhead*) in the subcutis. Note the SP-IR nerve fibers in the subcutaneous nervous plexus (*large arrowhead*). (*D*), (*Di*) PHM-IR nerve fibers innervating a blood vessel (*small arrowhead*) in the subcutis. Note the PHM-IR nerve fibers in the subcutaneous nervous plexus (*large arrowhead*). (*E*), (*Ei*) Schematic representations of SP-IR staining patterns as demonstrated in (*A*)–(*Ci*). Boxes indicate localization of where the above images were taken. Note that the localizations of pictures (*D*) and (*Di*) correspond to (*C*) and (*Ci*).

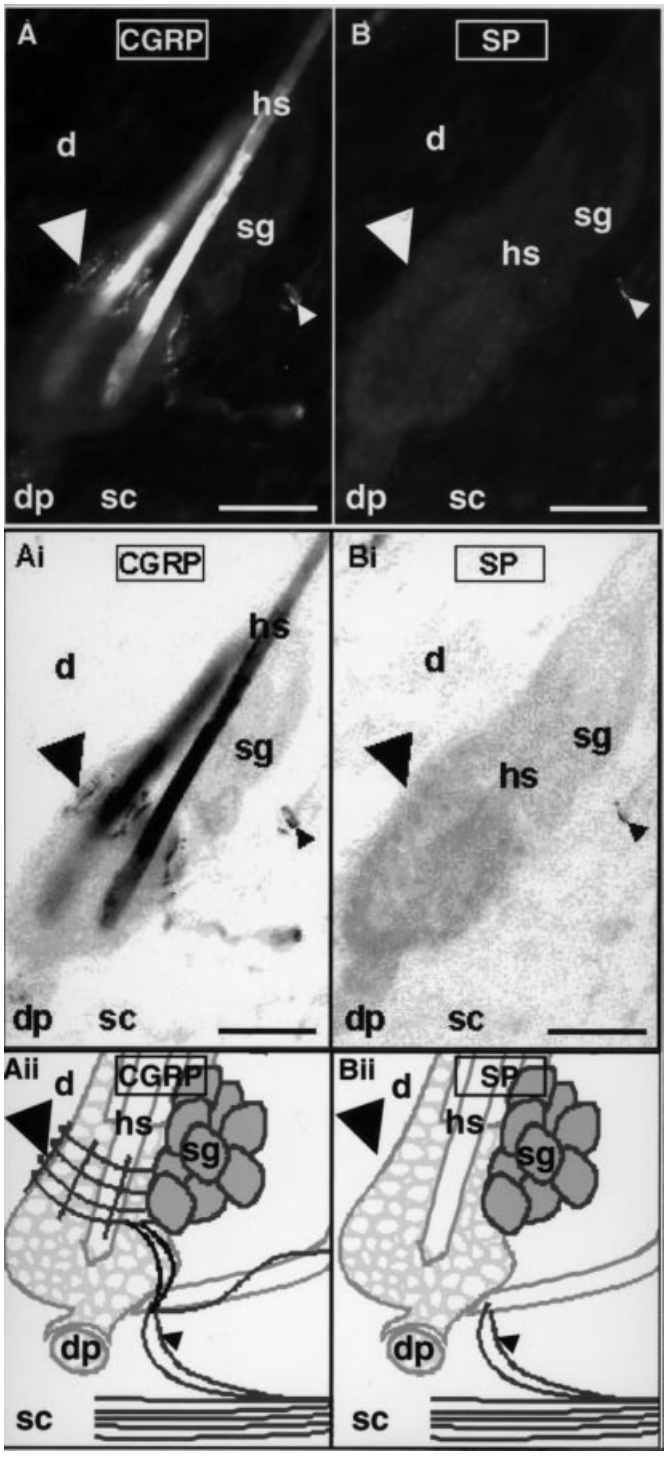

Figure 4. CGRP and SP immunoreactivity double labeling in murine telogen skin. As secondary antibodies either FITC-conjugated $F(ab)_2$ fragments (*A*, *Ai*) or TRITC-conjugated $F(ab)_2$ fragments (*B*, *Bi*) were used. (*A*), (*B*) give immunofluorescent pictures. (*Ai*), (*Bi*) give computer-generated inverted pictures corresponding to (*A*), (*B*); (*Aii*), (*Bii*) contain schematic drawings of (*A*), (*B*). Abbreviations (*A*–*Bii*): d, dermis; dp, dermal papilla; hs, hair shaft; sc, subcutis; sg, sebaceous gland. *Scale bars*: 50 μm. (*A*)–(*Bi*) Pictures of the same area were photographed with the respective filters: (*A*), (*Ai*) show fluorescein-labeled CGRP-IR in the FNB (*large arrowhead*) and in the dermis (*small arrowhead*); (*B*), (*Bi*) show rhodamine-labeled SP-IR in the dermis (*small arrowhead*), colocalized with CGRP-IR (compare *small arrowhead* in *A*, *Ai*). Note the absence of SP-IR in the FNB (*large arrowhead*). (*Aii*), (*Bii*) Schematic representations of CGRP-IR (*Aii*) and SP-IR (*Bii*) staining patterns as demonstrated in (*A*)–(*Bi*).

Follicular innervation: the FNA in telogen follicles contains CGRP and SP, whereas FNB contains only CGRP Nerve fibers forming the FNA (Botchkarev *et al*, 1997a; Paus *et al*, 1997) extended from the perifollicular epidermis to the infundibulum of the sebaceous gland, enwrapping the supra-infundibular outer root sheath of the hair follicle (Botchkarev *et al*, 1997a; Fundin *et al*, 1997). It contained some CGRP (**Fig 1**, **Fig 2*D***, ***Di***) and a few SP-IR nerve fibers (**Fig 3*B***, ***Bi***), but no PHM-IR nerve fibers were found in the FNA (not shown) and no prominent neuropeptide-IR connections between the FNA and the FNB were seen (**Fig 2*D*–*Ei***, **Fig 3*B***, ***Bi***, **Fig 4*A*–*Bi***).

The FNB consisted of two types of solely CGRP-IR nerve fibers (Botchkarev *et al*, 1997a; Fundin *et al*, 1997) arranged around the isthmus and bulge region of the outer root sheath, just below the infundibulum of the sebaceous gland and above the insertion of the arrector pili muscle (**Fig 1**, **Fig 2*E*–*Fi***). Longitudinal nerve fibers were regularly spaced alongside and close to the outer root sheath in a palisade-like manner (**Fig 2*E*–*Fi***), representing rapidly adapting C-type mechanoreceptors, which are frequently termed ''lanceolate nerve endings'' (Halata, 1993; Fundin *et al*, 1997). Additional nerve fibers encircled this region, representing slow adapting mechanoreceptors, which are also termed ruffini corpuscles when associated with a dense arrangement of circular collagen fibrils (Fundin *et al*, 1997) (**Fig 1**, **Fig 2*E*–*Fi***). None of the other tested antibodies yielded specific immunoreactivity in the FNB (**Fig 3*E***, ***Ei***). Also, no CGRP-IR, SP-IR, or PHM-IR nerve fibers were seen to innervate the connective tissue capsule of the sebaceous gland or sebocytes directly (**Fig 2*E*–*Fi***). With the methodology employed here, the entire cycling proximal part of hair follicles, including hair bulb and dermal papilla, did not show signs of peptidergic innervation.

Alongside hair cycle progression, substantial changes occur in the peptidergic innervation of various skin compartments and the hair follicle During synchronized anagen II-IV (**Fig 6**, compare **Figs 2**, **3**, **4** with **Fig 5**), the number of single CGRP-IR, SP-IR, or PHM-IR nerve fibers in the dermis increased significantly. This was followed by a significant decrease during late anagen (**Fig 6**). Finally, in catagen skin, the number of CGRP-IR and SP-IR single nerve fibers in dermis and subcutis returned to telogen levels (**Fig 6**).

Hair-cycle-dependent neuronal plasticity was also evident within the follicular neural networks. The peptidergic innervation of the FNA appeared denser in anagen hair follicles compared with telogen, and the FNB in anagen II-IV skin displayed substantially increased numbers of CGRP-IR circular nerve fibers (**Figs 7**, **8**). These numbers remained high during late anagen and catagen (**Fig 8**). There were only a few longitudinal CGRP-IR nerve fibers. There was a discrete but significant increase in the number of these fibers too, however, their number declined again during late anagen and catagen (**Fig 8**).

In more than 1200 individually analyzed hair follicles from all hair cycle stages, the proximal hair bulb and the dermal papilla did not show any peptidergic nerve fibers with any of the examined markers throughout the entire murine hair cycle. Also, there was no statistically significant difference between the data obtained from depilation-induced anagen and from spontaneously developed anagen (data not shown).

SP promotes and CGRP inhibits anagen progression in murine skin organ culture Quantitative histomorphometric analysis of early anagen skin cultured in the presence of CGRP, SP, PHM, or VIP revealed that SP (10^{-6} M) significantly promoted anagen progression from early anagen (I-II) to mid anagen (III), whereas SP antagonist and CGRP (10^{-6} and 10^{-8} M) had the opposite effect and blocked anagen progression compared with controls (**Fig 9*A*–*E***). Notably, when biopsies were cultured in the presence of CGRP plus SP (10^{-8} M and 10^{-6} M, respectively), no difference from control was seen (**Fig 9*A***, ***B***). Neither PHM nor

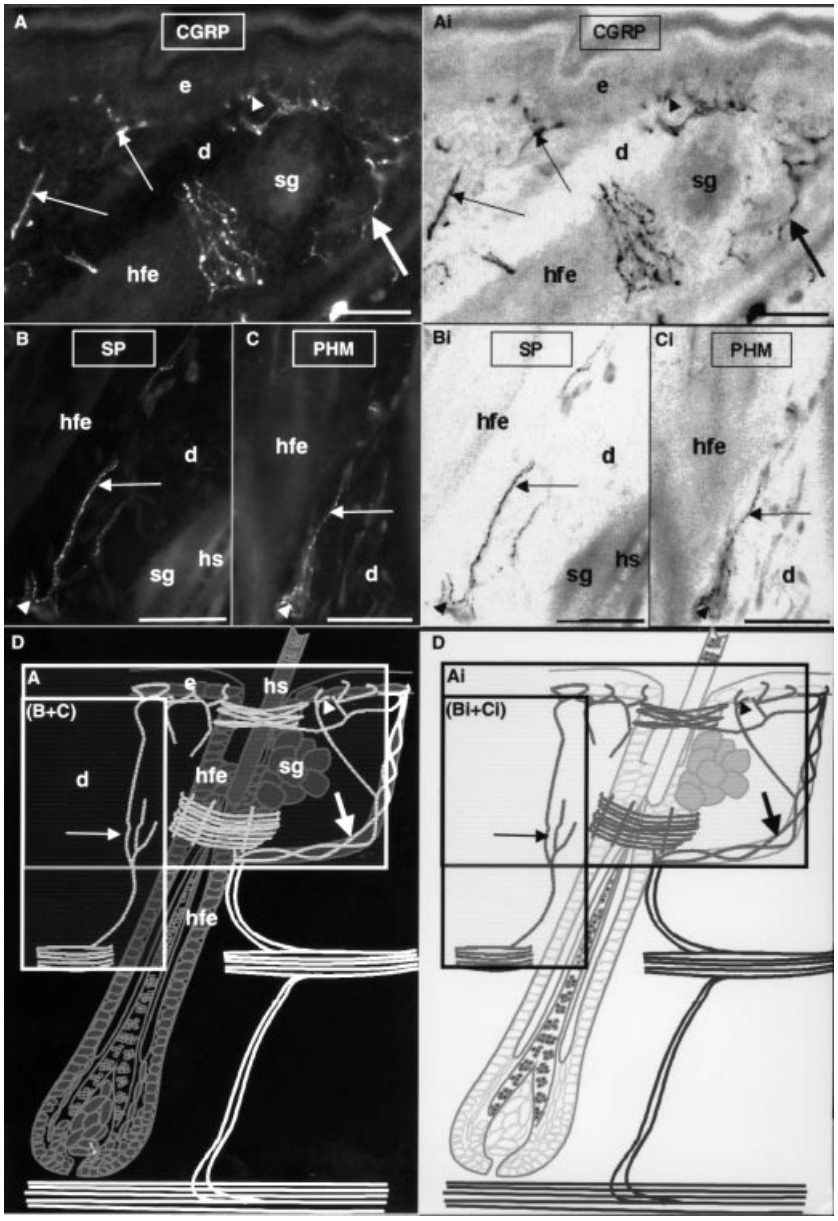

Figure 5. CGRP, SP, and PHM immunoreactivity in murine anagen skin. Cryostat sections of anagen IV skin (5 d after anagen induction by depilation) were stained. (*Ai*)–(*Ci*) give computer-generated inverted pictures corresponding to (*A*)–(*C*). Abbreviations (*A–C*): d, dermis; e, epidermis; hfe, hair follicle epithelium; sg, sebaceous gland. *Scale bars* (*A–C*): 50 μm. (*A*), (*Ai*) Strongly positive varicose dermal CGRP-IR nerve fibers in anagen IV skin innervate the arrector pili muscle (*large arrow*) or terminate freely in the dermis (*small arrows*). (*B*), (*Bi*) Strongly positive varicose dermal SP-IR nerve fibers in anagen IV skin (*arrows*) extend from a bundle of deep dermal nerve fibers (*arrowhead*) at the border between dermis and subcutis to terminate freely in the interfollicular dermis (*arrow*). (*C*), (*Ci*) Strongly positive varicose dermal PHM-IR nerve fibers in anagen IV skin (*arrows*) extend from a bundle of deep dermal nerve fibers (*arrowhead*) at the border between dermis and subcutis to terminate freely in the interfollicular dermis (*arrow*). (*D*), (*Di*) Schematic representations of CGRP-IR staining patterns as demonstrated in (*A*), (*Ai*). Boxes indicate localization of where the above images were taken. Note that the localizations of pictures (*B*)–(*Ci*) correspond to the indicated boxes.

VIP exerted any significant influence on hair cycle progression in this model (**Fig 9*A*, *B***).

CGRP fails to induce anagen *in vivo* CGRP or CGRP-antagonist-releasing subcutaneous implants did not have a macroscopic effect on anagen induction in murine telogen skin, such as skin graying or hair growth, over an observation time of 29 d. Release of CGRP was evident, however, from the long-lasting skin erythema observed in test but not control mice during the first 48–72 h after implantation (cf. Wallengren and Hakanson, 1987; data not shown). Comparative histomorphometric analysis of treated and control skin harvested 20 or 29 d after implantation likewise demonstrated skin containing exclusively telogen hair follicles in the vicinity of the implants (not shown).

DISCUSSION

Here we identify distinct and unique patterns of sensory neuropeptide distribution in the neural networks of murine back skin and hair follicles, completing our previous analyses made with pan-neuronal (Botchkarev *et al*, 1997a) and adrenergic markers (Botchkarev *et al*, 1999). The observed hair-cycle-associated plasticity in these peptidergic networks (**Figs 1–5**) underscores that hair follicle cycling is associated with substantial remodeling of hair follicle innervation, and is in line with our previous observation that the frequency of close physical contacts between mast cells and peptidergic nerve fibers in murine interfollicular skin shows a striking hair cycle dependence (Botchkarev *et al*, 1997b). Peptidergic hair follicle innervation is concentrated around and above the bulge region, where at least one major population of epithelial hair follicle stem cells resides (Cotsarelis *et al*, 1990). Interestingly, the number of CGRP-IR nerve fibers in the FNB of non-tylotrich murine pelage hair follicles was significantly lower than the total number of PGP 9.5-IR FNB nerve fibers (Botchkarev *et al*, 1997a). Also, the exclusive CGRP-IR of the FNB in non-tylotrich murine pelage hair follicles contrasts with the more complex peptidergic IR profile described for the FNB of various hair follicle types in other species, especially in facial skin (Hartschuh *et al*, 1984; Gibbins *et al*, 1987; Dalsgaard *et al*, 1989; Ebara *et al*, 1992; Fundin *et al*, 1995; Schulze *et al*, 1997). This indicates the presence of one or more additional CGRP-negative nerve fiber subset(s) in the FNB of murine pelage hair follicles, whose neuropeptide profile was not recognized by the antibodies used in our study. Indeed, in other follicle populations, additional VIP-IR and/or SP-IR nerve fibers have been described (e.g.,

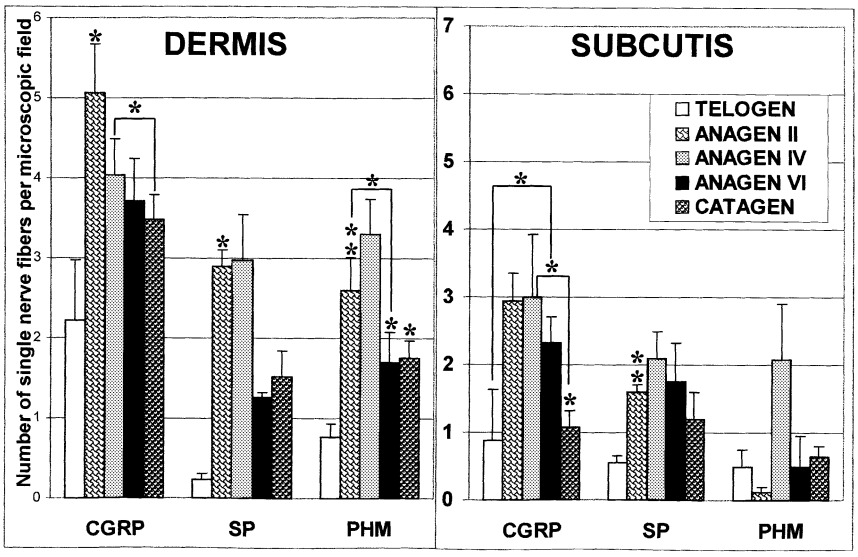

Figure 6. Hair-cycle-dependent numeric changes of peptidergic single nerve fibers in murine skin. The number of single CGRP-IR, SP-IR, or PHM-IR nerve fiber profiles in dermis or subcutis were counted per microscopic field in a minimum of 10 microscopic fields per mouse in three to five mice per time point shown. Numbers were pooled and statistics performed as indicated in *Materials and Methods*. p-values always refer to the previous hair cycle stage if not otherwise indicated (*p-value < 0.05; **p-value < 0.01).

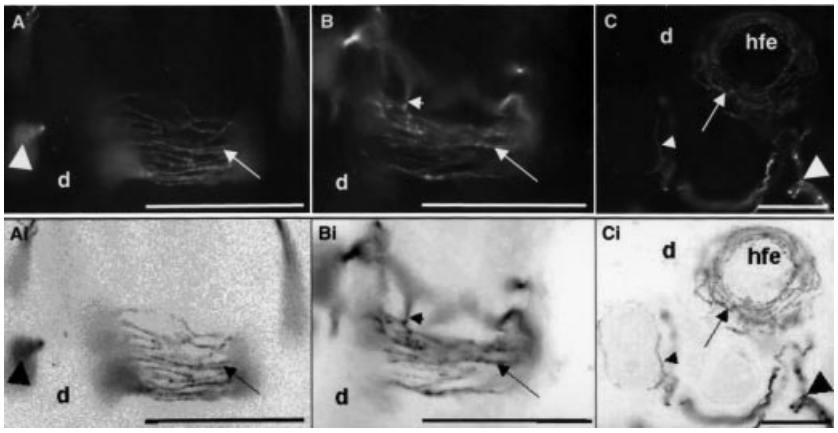

Figure 7. CGRP-IR nerve fibers in FNB of anagen IV hair follicles in murine skin. Cryostat sections of anagen IV skin (5 d after anagen induction by depilation) were stained. (*Ai*)-(*Ci*) give computer-generated inverted pictures corresponding to (*A*)-(*C*). Abbreviations (*A-C*): d, dermis; hfe, hair follicle epithelium. *Scale bars* (*A-C*): 50 μm. (*A*), (*Ai*), (*B*), (*Bi*) Multiple circular nerve fibers in FNB (*arrow*, compare with **Fig 2*E*, *Ei***) in anagen IV. Pictures show the identical hair follicle photographed at different focuses. Note the few longitudinal nerve fibers in FNB (*small arrowhead*) and the nearby bundle of nerve fibers (*large arrowhead*). (*C*) Transversal cut through a large tylotrich hair follicle in anagen IV at the level of the FNB showing multiple circular nerve fibers (*arrow*) surrounding the hair follicle epithelium. Note the small nontylotrich pelage hair follicles nearby also surrounded by circular nerve fibers (*small arrowhead*) and the bundle of nerve fibers (*large arrowhead*) giving rise to the nerve fibers innervating the FNB.

Hartschuh *et al*, 1984; Dalsgaard *et al*, 1989; Ebara *et al*, 1992; Fundin *et al*, 1995; Schulze *et al*, 1997).

No specific VIP immunoreactivity signal was obtained with the methodology employed here, despite a variation of applied antibodies and staining protocols. VIP and PHM share a common precursor peptide, however, and have been shown to consistently coexist (Hauser Kronberger *et al*, 1992; Takahashi *et al*, 1993). Also, except for perifollicular nerve fibers, PHM-IR nerve fibers in our study showed a distribution pattern similar to that described earlier for VIP/PHM double labelings (Itoh *et al*, 1983; Kirkeby *et al*, 1992). Thus, VIP may well be present in both PHM-IR and even PHM-negative nerve fibers (Lindh *et al*, 1989), but may have been below the detection limit of our technique, or subject to rapid degradation.

Peptidergic nerve fibers have frequently been found in close contact with keratinocytes (Ishida Yamamoto *et al*, 1989; Karanth *et al*, 1991), fibroblasts (Forster *et al*, 1975), mast cells (Botchkarev *et al*, 1997b), dendritic cells (Asahina *et al*, 1995), macrophages (Forster *et al*, 1975), melanocytes (Hara *et al*, 1996), endothelial cells (Fundin *et al*, 1997), and Schwann cells (Botchkarev *et al*, 1997b), and may modulate the functional activities of these cells (Asahina *et al*, 1995; Ansel *et al*, 1997). This is particularly true for CGRP and SP that stimulate, for example, mast cell degranulation (Paus *et al*, 1995), which in turn modulates hair growth in mice (Paus *et al*, 1994b; Maurer *et al*, 1997). Also, the increase of CGRP-IR nerve fibers in early anagen skin (**Figs 5–8**) may play a role in the striking, anagen-associated downregulation of contact hypersensitivity in murine skin (Hofmann *et al*, 1996), as CGRP inhibits antigen presentation by Langerhans cells (Asahina *et al*, 1995; Ansel *et al*, 1997).

It is difficult to explain conclusively why SP, but not CGRP, exerts a dose-dependent effect on anagen development in murine skin organ culture. It is conceivable that the anagen-promoting effect of SP reflects the net result of multiple, distinct functional activities of SP (e.g., on keratinocyte differentiation, fibroblast proliferation and migration, growth factor release by mast cells and other immunocytes). These statistics may affect various cell populations involved in hair growth control differentially, in a manner that differs from that of CGRP.

SP has been used in cell and tissue culture experiments at various concentrations ranging from 10^{-12} M to 10^{-4} M (e.g., Paus *et al*, 1995; Furutani *et al*, 1999). Picomolar concentrations of SP

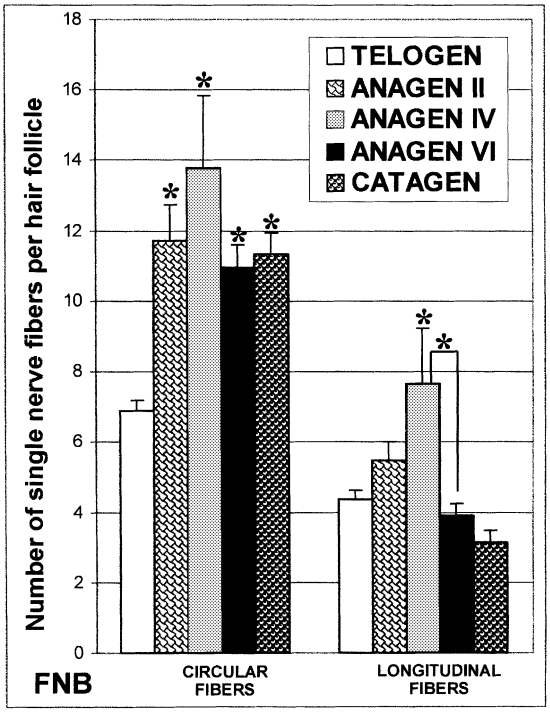

Figure 8. Hair-cycle-associated number changes in CGRP-IR nerve fiber in FNB. The number of CGRP-IR circular or longitudinal nerve fiber profiles in completely visible FNB (120 μm sections) of a minimum of 10 hair follicles per mouse were counted in three to five mice per time point shown. Numbers were pooled and statistics performed as indicated in *Materials and Methods*. p-values always refer to telogen if not otherwise indicated (*p-value < 0.05).

potently promote proliferation and/or migration of keratinocytes or fibroblasts (Ziche *et al*, 1990; Paus *et al*, 1995). Instead, (sub)micromolar concentrations of SP ($> 10^{-7}$) reportedly do not affect proliferation and growth (Pincelli *et al*, 1992), but increase intracellular calcium in cultured human keratinocytes (McGovern *et al*, 1995). They also stimulate mast cell degranulation, vasodilatation, and various immunologic responses (Tomoe *et al*, 1992; Paus *et al*, 1995). Therefore, it is conceivable that, under *in situ* conditions, only high local SP concentrations generate net effects that culminate in anagen promotion.

CGRP reportedly stimulates intracellular cAMP formation and proliferation in cultured keratinocytes (Takahashi *et al*, 1993). Yet, in our assays, CGRP proved unable to promote anagen *in vivo* or in skin organ culture (**Fig 9**). This contrasts with the hair-growth-promoting effects of SP, and calls to mind the similarly antagonistic effects of SP and CGRP observed in the ''weal and flare reaction'' (Wallengren and Wang, 1993; Maggi, 1995). On the basis of the currently available literature, it is quite difficult to explain conclusively why SP and CGRP operate as functional antagonists in the control of anagen progression. With the methodology employed here, however, it is not possible to distinguish whether the opposing effects result from a direct interaction between CGRP and SP or – more likely – from a combination of opposing functional effects that eventually neutralize each other's hair growth effects.

Though sensory nerves are nonessential for anagen development to occur (Maurer *et al*, 1998), our data suggest that they can still exert significant hair growth modulatory effects. Furthermore, the functionally antagonistic effects of SP and CGRP on anagen progression suggest that it is prudent to jointly study SP *and* CGRP in neuropharmacologic hair growth manipulation. In order to appreciate the functional role of SP and CGRP in hair biology more fully, however, it will be critical to study next the hair-cycle-dependent expression of the relevant high affinity receptors (Ansel *et al*, 1997; Holzer, 1998), hair growth abnormalities in corresponding receptor knockout mice, and the hair cycle modulatory effects of selective, synthetic receptor ligands *in vivo*.

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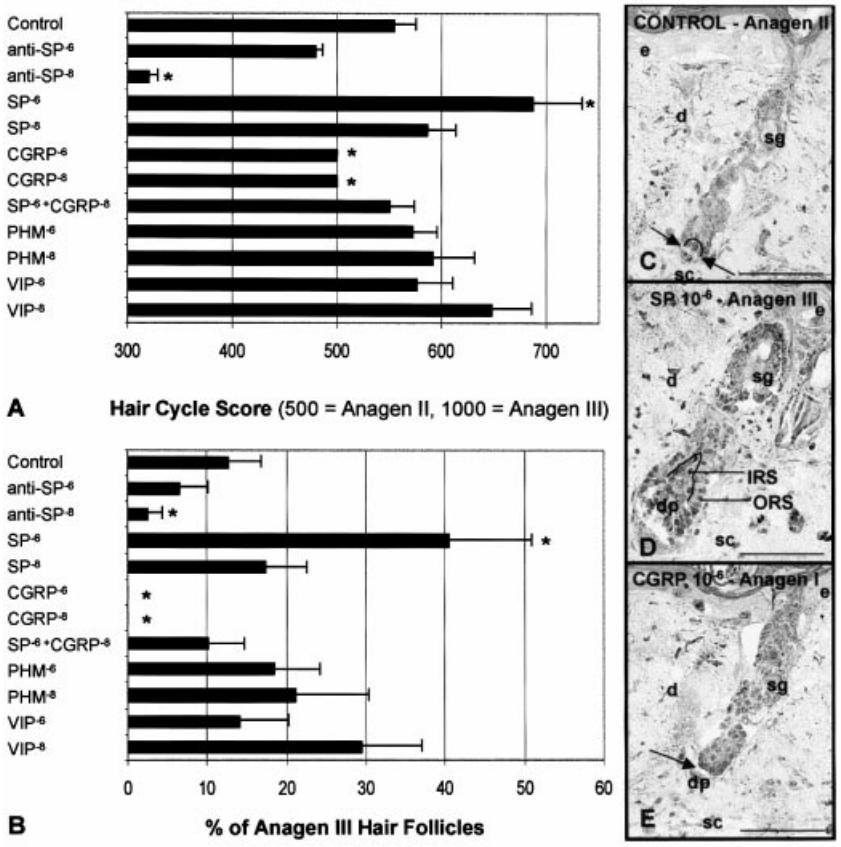

Figure 9. Altered hair cycle progression by CGRP and SP in murine skin organ culture. Four millimeter punch biopsies of C57BL/6 mouse skin, taken 3 d after anagen induction by depilation so that they contained only hair follicles in early anagen stages I-III, were cultured for 72 h in the presence of either SP (10^{-6} or 10^{-8} M), SP-antagonist (10^{-6} or 10^{-8} M), CGRP (10^{-6} or 10^{-8} M), PHM (10^{-6} or 10^{-8} M), VIP (10^{-6} or 10^{-8} M), or a combination of SP (10^{-6} M) and CGRP (10^{-8} M). Quantitative histomorphometric analysis was performed blindly on a minimum of 10 randomly chosen hair follicles per tested biopsy by two different investigators and the number of follicles in telogen, anagen I, anagen II, and so forth was documented. The hair cycle score (HCS) was calculated assigning a score of 0 to each anagen I hair follicle, 500 to anagen II, or 1000 to anagen III. HSC were added per sample and divided by the number of evaluated hair follicles, mean HCS per follicle were then added, and mean and ± SEM determined. Also, the percentage of hair follicles in the most progressed hair cycle stage was calculated per sample. Abbreviations (*C-E*): d, dermis; e, epidermis; dp, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; sc, subcutis; sg, sebaceous gland. *Scale bars* (*C-E*): 50 μm. (*A*) CGRP significantly retarded anagen progression from early anagen to mid anagen and maintained almost all follicles in anagen I-II. SP on the other hand accelerated this progression significantly in a dose-dependent manner, and when skin fragments were cultured in the presence of both CGRP and SP the effect of either was abolished and no significant difference could be seen between test and control. Neither PHM nor VIP produced significant differences from controls. (*B*) CGRP significantly abolished the development of anagen III hair follicles. SP significantly increased the percentage of anagen III hair follicles compared with controls. (*C*) Anagen II hair follicle from a biopsy cultured control medium not containing any additional neuropeptides. Note the rounded and small dermal papilla not yet capped by keratinocytes forming an inner root sheath (basal membrane between hair follicle keratinocytes and dermal papilla is indicated by *arrows* and *black line*). (*D*) Anagen III hair follicle from a biopsy cultured with 10^{-6} M SP. Note the enlarged dermal papilla and the developing inner root sheath (border between inner and outer root sheath is indicated by *black line*). (*E*) Anagen I hair follicle derived from a biopsy cultured with 10^{-6} M CGRP. Note the flat and small dermal papilla only capped by keratinocytes (basal membrane between hair follicle keratinocytes and dermal papilla is indicated by *arrow*).

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