Innervation of Melanocytes in Human Skin

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Summary

Communication between the nervous system and epidermal melanocytes has been suspected on the basis of their common embryologic origin and apparent parallel involvement in several disease processes, but never proven. In this study, confocal microscopic analysis of human skin sections stained with antibodies specific for melanocytes and nerve fibers showed intraepidermal nerve endings in contact with melanocytes. This intimate contact was confirmed by electron microscopy, which further demonstrated thickening of apposing plasma membranes between melanocytes and nerve fibers, similar to synaptic contacts seen in nervous tissue. Since many intraepidermal nerve fibers are afferent nerves that act in a "neurosecretory" fashion through their terminals, cultured human melanocytes were stimulated with calcitonin gene–related peptide (CGRP), substance P, or vasoactive intestinal peptide, neuropeptides known to be present in cutaneous nerves, to examine their possible functions in the epidermal melanin unit. CGRP increased DNA synthesis rate of melanocytes in a concentration- and time-dependent manner. Cell yields after 5 d were increased 25% compared with controls maintained in an otherwise optimized medium. Furthermore, stimulation by CGRP induced rapid and dose-dependent accumulation of intracellular cAMP, suggesting that the mitogenic effect is mediated by the cAMP pathway. These studies confirm and expand a single earlier report in an animal model of physical contact between melanocytes and cutaneous nerves and for the first time strongly suggest that the nervous system may exert a tonic effect on melanocytes in normal or diseased human skin (Mihara, K., K. Hashimoto, and M. Kumakiri. 1982. *J. Dermatol.* 19:1–43).

Interactions between the nervous system and cutaneous melanocytes have long been speculated on the basis of embryologic and phylogenic features of melanocytes as well as on several forms of clinical evidence. Embryologically, both melanocytes and peripheral nervous system neurons originate from the neural crest (1). In lower vertebrate species, movement of melanosomes within pigment cells leading to color change in the skin is directly regulated by autonomic nerves (2). In patients with tuberculoid leprosy, it is known that depigmentation and anesthesia coexist in the innervation area of the affected peripheral nerves (3). It has also been noted that skin color is normalized only after reinnervation of treated areas when hyperpigmented skin is dermabraded (4), a procedure expected to destroy many superficial nerve endings, and that degeneration of nerves occurs in segmental vitiligo lesions corresponding in location to the area of distribution of the local nerve, and regenerating nerves are subsequently observed during a course of therapy leading to repigmentation (5). Degenerative changes or increased subepidermal nerves have also been reported in other dyschromatic skin lesion (6).

Furthermore, increased numbers of nerve fibers have been found in benign melanocytic skin lesions such as acquired and congenital nevi (7). In contrast, in primary malignant melanoma lesions the number of nerve fibers is decreased in proportion to tumor thickness, suggesting that normal melanocytes produce neurite promoting factors. In addition, close association of melanocytes with nerve fibers containing calcitonin gene–related peptide (CGRP)[1], substance P (SP), and neuropeptide Y has been reported throughout all regions of the uvea (8–10), although their role in ophthalmic melanocyte biology is not known.

Finally, Mihara and co-workers (11) reported a direct contact, similar to synaptic contacts in the nervous system, between cutaneous nerve endings and epidermal melano-

M. Hara and M. Toyoda contributed equally to this paper. M. Toyoda performed the electron microscopic studies, and M. Hara performed all other experiments.

[1] *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CGRP, calcitonin gene–related peptide; NP, neuropeptide; SP, substance P; VIP, vasoactive intestinal polypeptide.

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cytes in guinea pig skin. However, this work was never confirmed and such contacts were never reported in human skin.

Skin is innervated primarily by sensory nerves and by postganglionic parasympathetic and sympathetic nerves. Sensory nerves have been shown to function not only as an afferent system to conduct stimuli from the skin to the central nervous system, but also as efferent system to stimulate target tissue by secreting several kinds of neuropeptides (NPs) through their branches (12). It is well known that some inflammatory skin disorders, including psoriasis and atopic dermatitis, characteristically worsen with anxiety (13, 14). Urticaria may also be triggered by tension (15). These interactions between psychic state and expression of skin disorders are possibly attributable to NP secretion from sensory nerves, in that many NPs are able to provoke inflammatory reactions (16). CGRP induces characteristic long-lasting erythema when injected intradermally (17). vasoactive intestinal polypeptide (VIP) and peptide-histidine isoleucine similarly stimulate vasodilatation (18); and VIP, SP, neurotensin, and somatostatin degranulate mast cells (19). Alternatively, NPs may modulate inflammatory conditions indirectly through regulating the immune system (20). SP enhances proliferation and function of T and B cells and stimulates IL-1 and IL-6 production by T cells (21, 22). VIP inhibits T cell proliferation and migration (23, 24). Furthermore, CGRP-containing nerves were recently reported to make close contact with epidermal Langerhans cells and, at least in vitro, CGRP was shown to inhibit their antigen-presenting function (25). In addition, the involvement of the nervous system in pathologic skin reactions strongly suggests that the nervous system may participate in maintenance of the physiological integrity of the skin. Indeed, SP-containing nerve fibers are regenerated during the early healing stage of burn wounds in guinea pig skin (26), and SP promotes proliferation of arterial smooth muscle cells and skin fibroblasts (27). VIP is mitogenic for human keratinocytes (28, 29), and CGRP induces proliferation of endothelial cells (30).

Neurotrophic effects of NPs are also reported in the nervous tissue. Some NPs, including CGRP, are normally made by both sensory nerves in dorsal root ganglia and by motor neurons; interestingly, CGRP synthesis is increased after nerve injury (31–33), suggesting that this peptide may play a role in nerve regeneration. Indeed, CGRP promotes Schwann cell proliferation through activating cAMP pathways (34). Because Schwann cells and melanocytes are both of neural crest origin, it is plausible that melanocyte behavior is also regulated by NPs.

In this study, we show that melanocytes in human skin connect to intraepidermal nerve terminals via synapse like structures. Furthermore, CGRP, a neuropeptide known to exist in intraepidermal nerve terminals, upregulates DNA synthesis and proliferation of melanocytes through activating cAMP pathways. These results suggest an intimate relationship between the nervous system and epidermal melanocytes of probable relevance to normal and pathologic pigmentation in the skin.

Materials and Methods

Human Melanocyte Isolation and Culture. Neonatal foreskins obtained within 24 h of elective circumcision were used to culture human melanocytes as previously described (35). In brief, the epidermis was separated from the dermis after overnight incubation in 0.25% trypsin at 4°C. Primary cultures were then established in medium 199 (GIBCO BRL, Grand Island, NY) supplemented with 10 ng/ml epidermanl growth factor (Bethesda Research Laboratories, Gaithersburg, MD), 10^{-9} M triiodothyronine (Sigma Chemical Co., St. Louis, MO), 10 μg/ml transferrin (Sigma), 10 μg/ml insulin (Sigma), 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp., La Jolla, CA), 10^{-9} M cholera toxin (List Biological Laboratories, Inc., Campbell, CA),10 ng/ml basic fibroblast growth factor (bFGF) (Amgen Biologicals, Thousand Oaks, CA), and 5–10% fetal bovine serum. Post–primary cultures were maintained in a low calcium (0.03 mM) version of this defined melanocyte growth medium known to selectively support melanocyte growth (36). In experiments examining the effect of CGRP and bFGF on melanocyte yield, primary cultures were maintained in the same medium but, instead of cholera toxin, cells were supplemented with 10^{-4} M dibutyryl cAMP (dbcAMP) (Sigma) and 0.6 ug/ml bovine pituitary extract (Collaborative Biomedical Products, Lincoln Park, NJ). Post-primary cultures were maintained in low calcium (0.03 mM) version of this medium without dbcAMP.

Double Immunofluorescent Staining of Nerve Fibers and Melanocytes in Skin and Examination by Confocal Laser Scanning Microscopy. Fresh neonatal foreskin fragments were cut into small pieces; the specimens were immersed in 4% (wt/vol) paraformaldehyde, and 2% (wt/vol) picric acid in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C, then embedded in OCT compound. 10–20-μm cryosections were then processed in the following solutions: (*a*) 1% (vol/vol) Triton X-100 (Sigma) for 40 min; (*b*) mouse anti-TRP-1 antibody (MEL-5) (1:200) (Signet Laboratories, Inc., Dedham, MA) in PBS for 1 h; (*c*) FITC-conjugated anti–mouse IgG antibody (Sigma) (1:100) in PBS for 1 hr; (*d*) rabbit antineurofilament antibody (1:200) (Sigma) in PBS for 1 h; (*e*) rhodamine-conjugated anti–rabbit IgG antiserum (1:100) in PBS for 1 h. The specimens were washed two times gently with PBS between steps. Specimens were then mounted with antifade reagent (Molecular Probes, Inc., Eugene, OR) for observation.

Specimens were analyzed with a Leica confocal laser scanning microscope (Leica, Inc., Deerfield, IL) equipped with an argon ion laser with an output power of 2–50 mW. The continuously variable detection pin hole was set at the minimal size for optimal signal (37). The specimens were scanned first through the red channel (rhodamine) to follow the course of intraepidermal nerve fibers. Then the same field of vision was scanned through the green channel (fluorescein). Finally, images of the two channels were superimposed to create one image. Each image of the optical section was taken at 0.5–1 μm intervals in the 512 × 512 pixel format.

Electron-Microscopic Observation. Biopsy specimens obtained from facial skin of 25 donors were fixed with Trump's solution, cut into several small pieces, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections were made on an Ultracut S (Reichert-Jung, Vienna, Austria) and placed on Pelco 100 mesh grids (Ted Pella, Inc., Redding, CA) with 0.6% piloform (Ted Pella, Inc.) supporting films. The sections were stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (300; Philips, Holland). Between 296 and 348 sections per donor were analyzed for a total of more than 8,000 sections. At least 80 melanocytes were examined in each specimen.

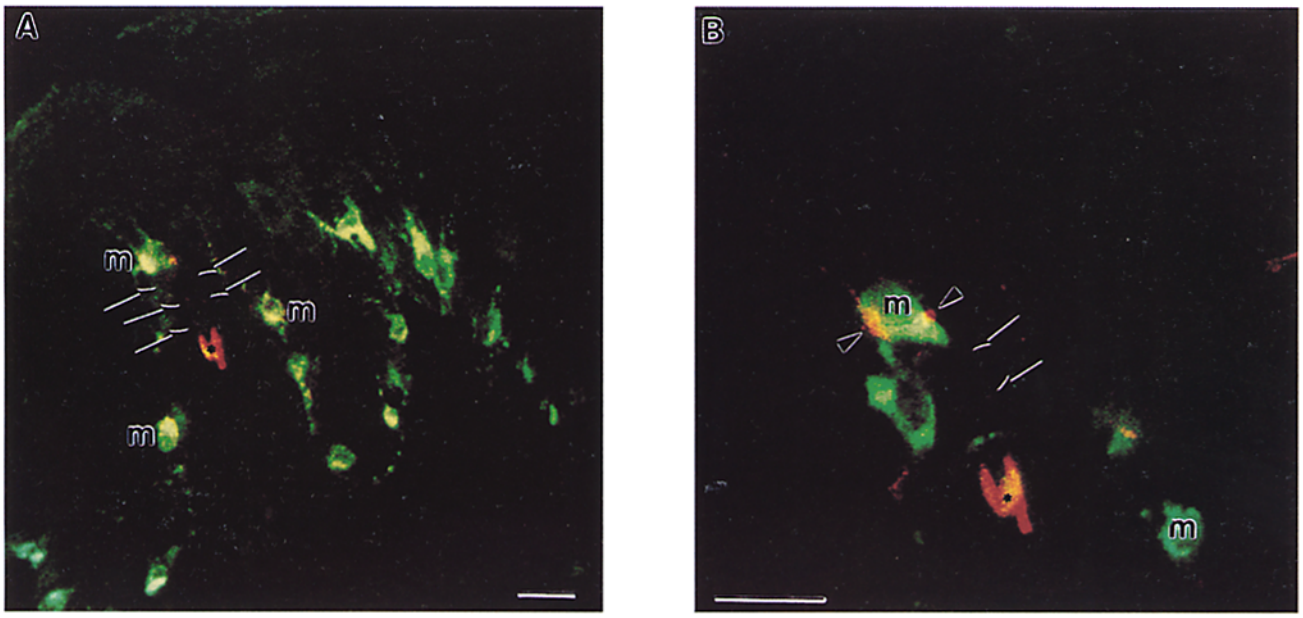

Figure 1. Confocal laser scanning microscopy of TRP-1–positive melanocytes (green fluorescence) and neurofilament-positive nerve fibers (red fluorescence) in human skin. (*A*) Low magnification of a representative normal skin cross-section. Melanocytes (*m*) located in the epidermal basal layer are stained in green. A branching axon stained in red is present in the dermal papilla (*asterisk*). Fine fibers with bead-like appearance (*arrows*) arise from it and ascend toward one of the melanocytes. (*B*) Higher magnification of the same field shown in (*A*). Orange color spots (*arrowheads*) present at the lateral sides of the melanocyte (*m*) presumably represent sites of axon terminals attached to the melanocyte cell body and are computer-generated when both fluorescent wavelengths are perceived in the same pixel of the 512 × 512 grid. Fine filamentous nerve fibers repeatedly entering and leaving the plane of section, are marked by arrows. Bar, 50 μm.

Cell Counts. Equal numbers of melanocytes (5–8 × 10^5/dish) were plated in paired 35-mm dishes with defined melanocyte medium. After overnight incubation culture dishes were supplemented with 10, 100, and 1,000 nM CGRP, SP, or VIP. In other experiments to compare the mitogenic effect of CGRP with the known primary melanocyte mitogen bFGF (38), cultures were maintained in defined medium lacking cholera toxin in the presence of 100 nM CGRP or 10 ng/ml bFGF shown to be bFGF optimal dose (39, 40). 5 d later, cells were detached by incubation for 3–5 min in 0.25% trypsin and cell number determined in an aliquot with a counter (Model ZM; Coulter Electronics Inc., Hialeah, FL). The number per dish is expressed as a percentage of the number seeded initially to accommodate the different seeding densities used among experiments.

[^{3}H]Thymidine Uptake. Melanocytes were plated in 24-well trays (Becton Dickinson and Co., Cockeysville, MD) at a density of 5–10 × 10^4 cells /ml. After overnight incubation, cells were stimulated with CGRP, SP, or VIP at a final concentration of 10–1,000 nM. After 24–72 h exposure to these neuropeptides, cells were labeled for 5 h with 1.0 μCi/ml [^{3}H]thymidine. Cells were washed three times with cold PBS, lysed with 2 N NaOH, and then neutralized with the same volume of 2 N HCl. Acid-insoluble materials were precipitated with 4 vol of 10% TCA, collected on filters, washed three times with 10% TCA and once with 100% ethanol, and dried. The radioactivity on the filters was determined using a liquid scintillation counter (Wallac Inc., Gaithersburg, MD) (41).

Tyrosinase Activity. Tyrosinase activity was measured according to Park et al. (42). In brief, 5 × 10^5 cells were briefly sonicated in 80 mM PO_4^{2-}, pH 6.8, containing 1% Triton X-100, and tyrosinase was extracted for 60 min at 4°C. 10–50 μg of cellular proteins was incubated with 250 nM L-tyrosine, 25 nM L-deoxyphenylalanine, 12.5 μg chloramphenicol, and 5 μCi L-[3,5-^{3}H]tyrosine for 30–60 min at 37°C. The reaction was stopped by addition of 500 μl of 10% TCA containing 0.2% BSA. TCA-soluble material was reacted with Norit A (Sigma), and released ^3H_2O was measured using a scintillation counter. The activity was expressed as counts per minute ^3H_2O release per microgram protein per hour minus the nonspecific incorporation of radioactivity, determined by using lysate boiled for 30 min (background). Background was generally <10% of the sample.

Measurements of Intracellular cAMP Accumulation. 1–2 × 10^6 melanocytes suspended in 1 ml melanocyte growth medium were supplemented with 10^{-10}–10^{-6} M CGRP. The cells were pretreated with or without 100 μM 3-isobutyl-L-methyl-xanthine (IBMX), reported to enhance CGRP's effect on cAMP (43), for 24 h before the experiment. After 5 min incubation at 37°C, cells were spun down and medium was replaced and resuspended with 0.05 M Tris-EDTA buffer/4 mM EDTA, pH 7.5. Samples were then boiled for 5 min and centrifuged; supernatants were measured for cAMP content with a radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA).

Results

Confocal Laser Scanning Microscopic and Electron Microscopic Observations of Melanocytes and Nerve Fibers in Human Skin. Double immunofluorescence staining was done on normal human skin using antibodies to TRP-1 and neurofilaments to identify melanocytes and nerve fibers, respectively. As expected, melanocytes, stained with green fluorescence,

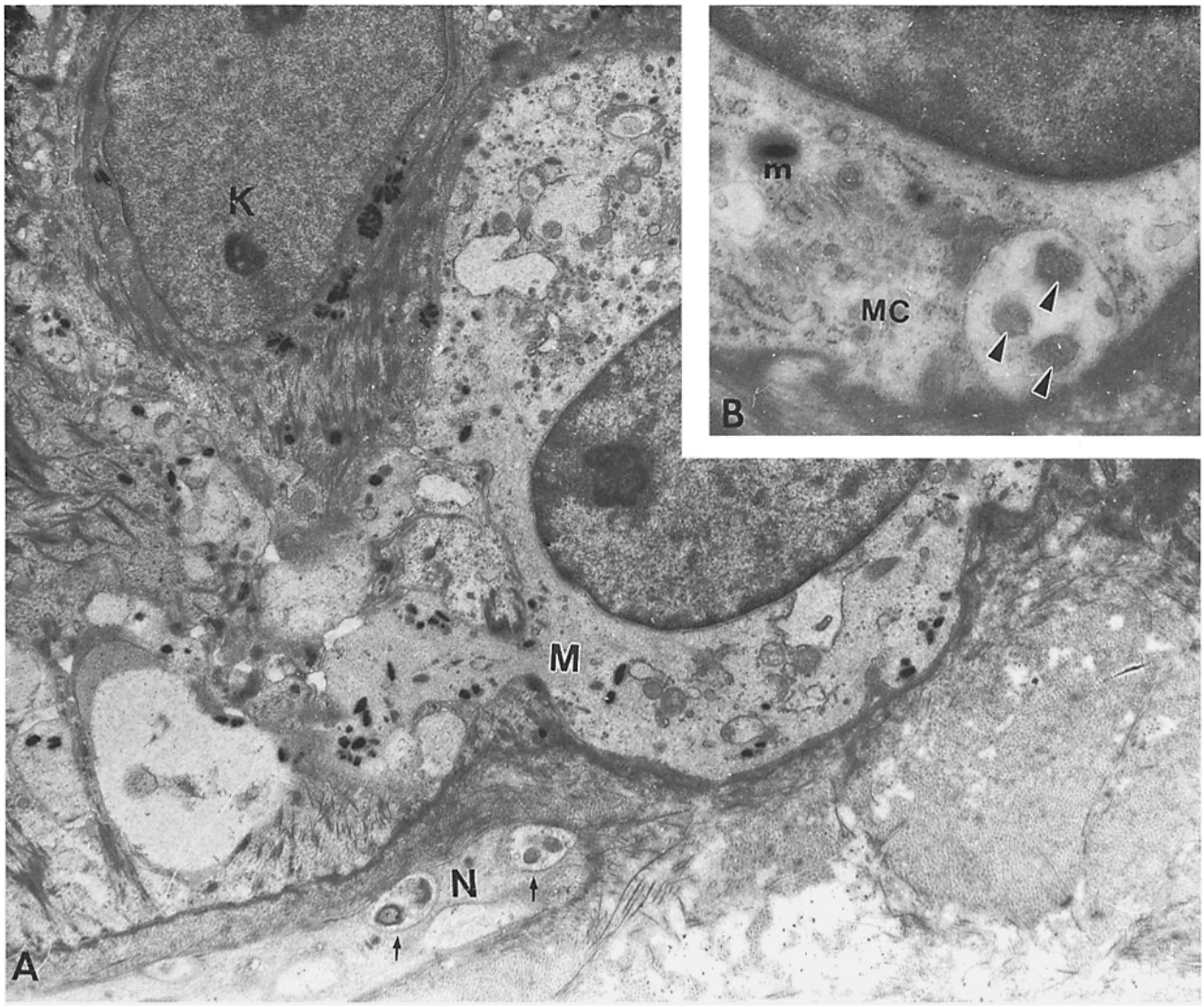

Figure 2. Melanocyte innervation in skin. (*A*) An electron micrograph at a low magnification shows a nerve fiber (*N*) containing axons (*arrows*) coursing toward a melanocyte (*M*) at the dermo–epidermal junction. (*B*) A step section of the same axon in *A* displayed in a higher magnification. The naked axon appears inside the melanocyte cytoplasm (*MC*). The axons contain electron-dense synaptic vesicles of 120–150 nm in diameter (*arrowheads*). *K*, keratinocyte; *m*, melanosome. Magnifications: (*A*) ×6,800; (*B*) ×15,800.

were located in the basal layer at intervals (Fig. 1). Many transverse or longitudinal profiles of nerve fibers stained with red fluorescence in a beaded pattern were observed in the dermal papillae. Some showed a Y-shaped bifurcation and coursed upward to the epidermis and made contact with one of the melanocytes in the basal layer. The contact site of nerve terminal and melanocyte was visualized as orange-yellow spots in the computer-generated color images when single-channel readings of green and red were superimposed (Fig. 1).

This intimate contact between melanocytes and nerves was confirmed by electron microscopic examination (Fig. 2–4). Melanocytes were identified by the presence of melanosomes in varying stages of maturity in the cytoplasm, and by lack of tonofilaments or desmosomes, well-known ultrastructural features of normal melanocytes in the basal layer that permit them to be easily distinguished from keratinocytes (44). As well, profiles of nerve fibers in the superficial dermis were identified by their characteristic features of unmyelinated axonal complexes, i.e., membrane-bound cylinders of electron-lucent cytoplasm with many microtubules centrally, corresponding to axons, enveloped by cell extensions of darker cytoplasm, corresponding to Schwann cell processes (45). The intraepidermal nerve profiles were confirmed to be axons by seeking in consecutive sections a proximal dermal part with characteristics of axonal complexes as described above. Unexpectedly, many axonal profiles were encountered in the epidermis, particularly in the basal layer. Occasionally, axons made synapselike close contact with the cell body of melanocytes in which plasma membranes of the axon terminal and melanocyte faced each other without intervening basal lamina (Fig. 4 *C*).

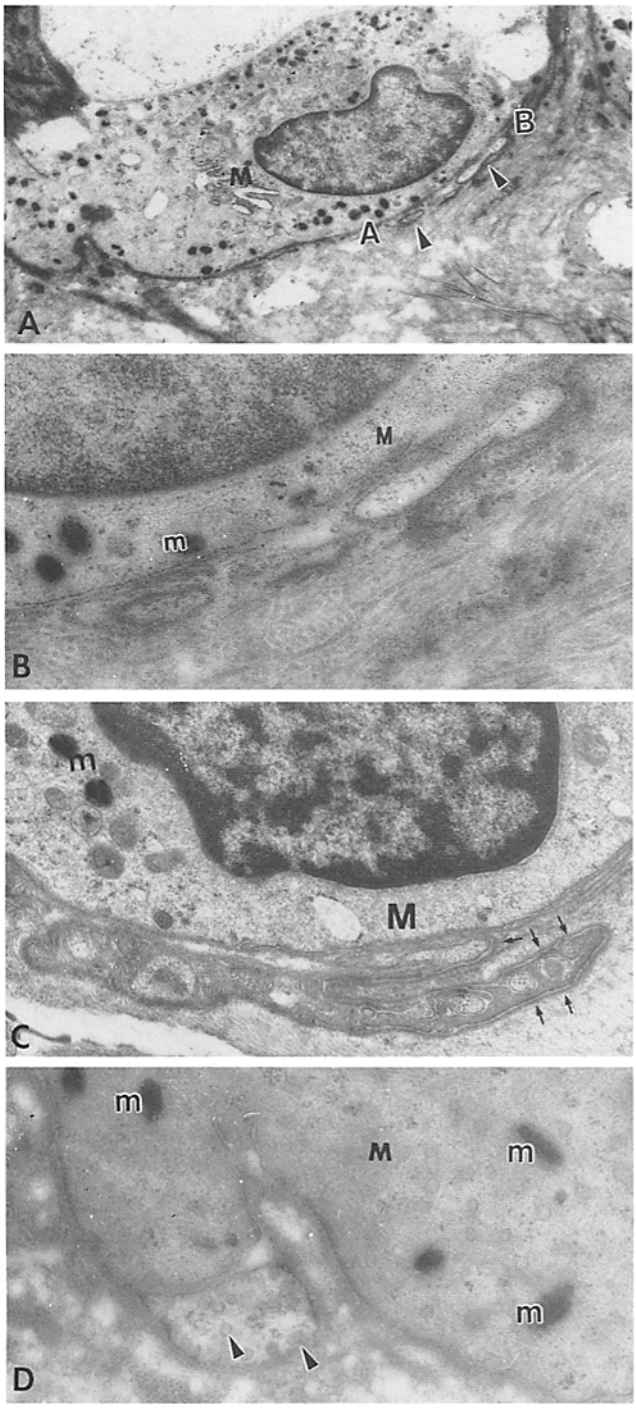

Figure 3. Contact between an epidermal melanocyte and an axon terminal. (*A*) A longitudinally sectioned axon (*arrowheads*) is situated directly beneath the melanocyte (*M*) and displays a broad attachment site with the melanocyte between positions A and B. (*B*) A higher magnification of the area between positions A and B shows that the axon terminal is in close contact with the melanocyte (*M*). (*C*) A step section of *A* shows that the distal part of the axon is capped by a Schwann cell sheath (*arrows*) and is in close apposition to the melanocyte (*M*). (*D*) A naked axon containing many clear small synaptic vesicles (*arrowheads*) forms a close contact with a melanocyte (*M*) that contains many stage III–IV melanosomes. *m*, melanosome. Magnifications: (*A*) ×2,800; (*B*) ×13,500; (*C*) ×11,500; (*D*) ×14,500.

Furthermore, some axons terminated in invaginations in the melanocyte body (Fig. 4 *B*). The plasma membrane of such melanocytes adjacent to the axon terminal was slightly thickened, closely resembling postsynaptic membrane specializations in nervous tissue (Fig. 4 *A*).

Effect of Neuropeptides on Melanocyte Proliferation. Since many intraepidermal nerve fibers are reported to contain several neuropeptides (reviewed in reference 16), including CGRP, SP, and VIP, and to modulate various skin functions through releasing these peptides (16), we supplemented melanocyte cultures with neuropeptides to examine the possible function of nerve–melanocyte interaction in skin.

The effect of neuropeptides on melanocyte yield was determined at three different doses (10, 100, and 1,000 nM). SP and VIP had no effect on cell number in any of the tested doses. After 5 d of culture in melanocyte growth medium in the presence of 100 nM of CGRP, SP, or VIP, melanocytes were counted. CGRP consistently increased melanocyte cell number ~25% compared to control (Fig. 5 *A*). Assuming that under our culture conditions melanocyte attachment is 50%, as previously determined (46), control cells underwent 0.96 ± 0.51 population doublings by day 5, while CGRP-treated cells underwent 1.43 ± 0.58 population doublings, a nearly 50% increase in actual growth rate. Modulation of cell number by SP and VIP was minimal in this experiment (Fig. 5 *A*).

Because CGRP is reported to exert its effect by upregulating intracellular cAMP, CGRP's effect on melanocyte proliferation was examined in cultures lacking cholera toxin, a known enhancer of cAMP levels in cells (47, 48). Duplicate cultures were stimulated with bFGF, to compare the mitogenic effect of CGRP to that of an acknowledged major melanocyte mitogen (38). Melanocyte yields increased within 5 d by ~70% in the presence of CGRP and by ~60% in the presence of bFGF.

Cell yields may be affected both by changes in mitotic rate and changes in survival rate, as illustrated for example by nerve growth factor, which appears primarily to modulate the extent of apoptotic cell death in cultured neurons and melanocytes (49, 51). To determine if CGRP stimulates DNA synthesis, thymidine incorporation rate was measured in melanocytes that were exposed to 100 nM of each of the three neuropeptides for 24 h before the assay. Significant increase of thymidine incorporation was observed in cultures stimulated with CGRP, roughly corresponding to the increase in cell yields (Fig. 6). Neither SP nor VIP induced significant changes in thymidine incorporation (Fig. 6).

We further examined the effect of CGRP on melanocyte DNA synthesis by examining its time course and concentration dependence (Fig. 7). DNA synthesis was upregulated within 24 h after stimulation by CGRP and the increase was sustained through 48 h with a return toward baseline levels by 72 h after a single supplementation (Fig. 7 *A*). CGRP induced statistically significant increases in DNA synthesis at 1-, 10-, and 100-nM concentrations (Fig. 7 *B*).

Effect of Neuropeptides on Melanogenesis. One of the major functions of melanocytes is to synthesize melanin. Be-

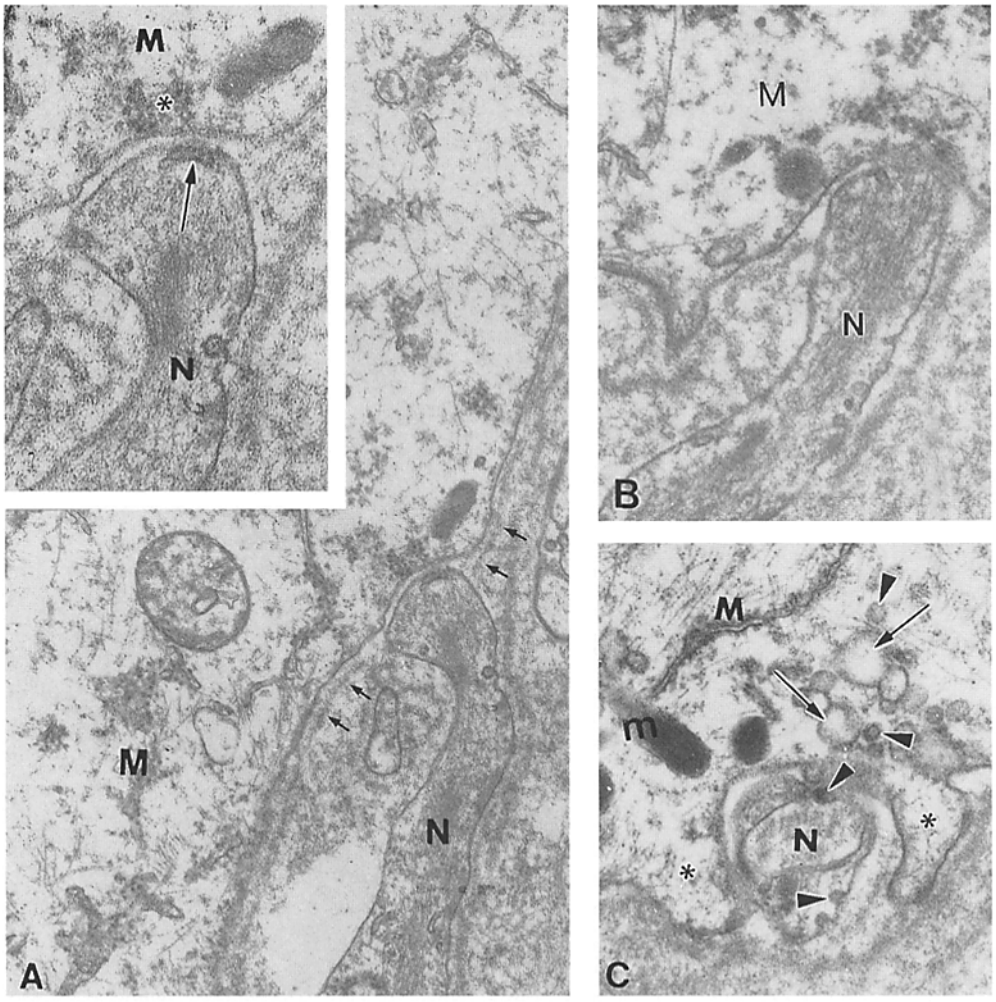

Figure 4. Step sections of a single axon innervating an epidermal melanocyte. A series of step sections of a single axon terminal are shown in *A–C*. (*A*) A naked nerve axon (*N*) forms a direct contact with a melanocyte (*M*). The lamina densa of the dermo–epidermal basement membrane, which is clearly seen below the melanocyte (*arrows*), is absent at the contact site between the melanocyte and the axon. (*Inset*) Higher magnification shows the axon terminal in close apposition to the melanocyte which is devoid of basement membrane at the area of contact with the axon. Note that, similar to other chemical synapses that are known to transmit signals from a neuron to its target cell (96), the presynaptic ending of the axon is enlarged to form a bouton. Futhermore, note the thickening of the plasma membrane of the bouton (*arrow*) and the subplasmalemmal specialization of the target melanocyte (*asterisk*), two specialized structures reported to be present in neural synapses (97). (*B*) A step section of the same axon shown in *A*. Note that the axon ends in a narrow invaginated groove in the melanocyte (*M*). (*C*) In an additional step section the axon terminal is enveloped by melanocyte cytoplasmic projections (*asterisks*). Many clear vesicles (*arrows*) ranging in diameter from 140 to 220 nm are aggregating in the melanocyte cytoplasm at the apposition site. As well, many synaptic vesicles (*arrowheads*) of ~50 nm in diameter, presumptively of axonal origin, are present in the cytoplasm of the melanocyte and in the space between the melanocyte and the nerve fiber. Magnifications: (*A*) ×11,600; (*inset*) ×17,500; (*B*) ×12,800; (*C*) ×17,000.

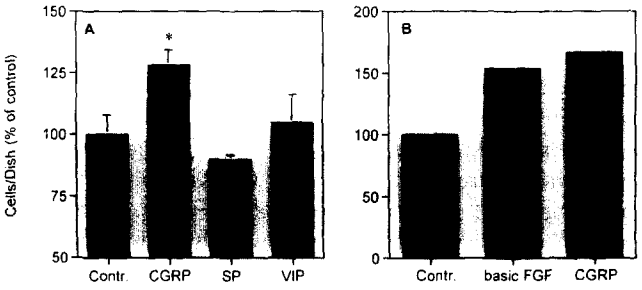

Figure 5. Effects of neuropeptides and bFGF on melanocyte proliferation. (*A*) Melanocyte cell yield was determined 5 d after supplementation with 100 nM CGRP, SP, VIP, or diluent alone. Neuropeptide effect on cell number is expressed as percentage of cell yield for diluent-treated control cultures (100%). Only CGRP supplementation showed statistically significant ($^*P < 0.05$, nonpaired *t* test) increases of melanocyte yield. Graph represents mean ± SD of three experiments. (*B*) Melanocytes were maintained for 5 d in medium lacking cholera toxin, in the presence of 100 nM CGRP or diluent, or in the presence of both CGRP and 10 ng/ml bFGF or diluent. bFGF increased melanocyte yield by ~60% and CGRP increased cell yield by ~70% above control (*Contr.*), suggesting comparable effects of CGRP and bFGF on melanocyte proliferation under these culture conditions. CGRP or bFGF effect on cell number is expressed as percentage of cell yield for control cultures (100%). Cell yield of two different culture dishes were averaged.

cause it is well-established that melanin synthesis is largely regulated by tyrosinase, the rate-limiting enzyme of the pathway (52), to determine if neuropeptides affect melanogenesis, tyrosinase activity was measured 72 h after exposure to 100 nM CGRP, VIP, or SP. No significant effect was seen for any of the neuropeptides examined (data not shown).

Effect of CGRP on Intracellular Levels of cAMP. Since it was reported in other cell types that CGRP mediates its biological actions via cAMP-dependent pathways by activating adenylate cyclase (34), melanocytes were stimulated with CGRP and intracellular cAMP levels were measured. As shown in Fig. 8, CGRP increased cAMP accumulation

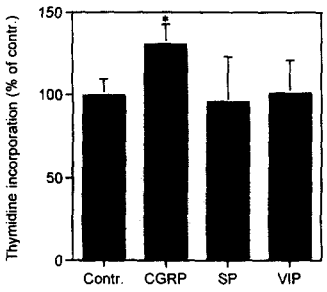

Figure 6. Effect of neuropeptides on DNA synthesis of melanocytes. 24 h after exposure to 100 nM CGRP, SP, or VIP, melanocytes were incubated with [^{3}H]thymidine for 5 h. Incorporated [^{3}H]thymidine was then counted. Melanocytes exposed to CGRP incorporated ~30% more [^{3}H]thymidine than diluent-treated controls (*P <0.05, nonpaired t test). Graph represents mean ± SD of two experiments.

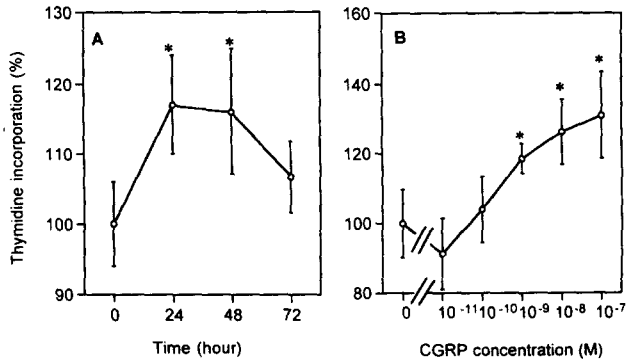

Figure 7. Time- and dose-dependency of CGRP's effect on melanocyte DNA synthesis. (*A*) Melanocytes were exposed up to 72 h to 100 nM CGRP and then were provided with [^{3}H]thymidine. [^{3}H]thymidine incorporation was determined as described in the legend to Fig. 6. Statistically significant increase of [^{3}H]thymidine uptake was observed at 24 and 48 h after CGRP supplementation (*P <0.05, nonpaired t test). (*B*) 24 h after exposure to increasing CGRP doses (10^{-11}–10^{-7} M) [^{3}H]thymidine incorporation was measured in melanocytes. Cultures stimulated with 10^{-9}–10^{-7} M CGRP had significantly (*P <0.05, nonpaired t test) higher [^{3}H]thymidine incorporation than diluent supplemented control cultures. Graph shows mean ± SD of two experiments.

in melanocytes in a dose-dependent manner. The maximal cAMP level, induced by 10^{-8} M CGRP, was approximately twice the basal level.

Discussion

An interaction between epidermal melanocytes and the nervous system has long been speculated but never accepted (1), despite one report of their physical proximity in murine skin (11). Here we show an intimate physical connection of intraepidermal axon terminals to melanocytes by immunohistochemical and electron microscopic means. It is notable that the sites of contact between nerve axons and melanocytes are characterized by synapselike specialization, including thickening of pre- or postsynaptic plasma membranes and submembraneous aggregation of vesicles, similar to synaptic structures in nervous system. Because intraepidermal free nerve endings contain neuropeptides and presumptively perform their afferent function through secretion of these neuropeptides (16), neuropeptides reported to be present in skin were examined in vitro for their effect on melanocytes. Of the three neuropeptides examined, CGRP induced significant increases of DNA synthesis rate and melanocyte number. The increased intracellular cAMP level in melanocytes after exposure to CGRP, known to mediate its effects through the cAMP pathway (34), further supports a direct action of CGRP on melanocytes. Effects of substance P, VIP, or indeed CGRP on melanocytes other than changes in growth rate and melanogenesis were not examined.

Pigmentary disorders of skin sometimes cause serious psychosocial problems for patients through their pronounced cosmetic disfigurement. Many are strongly associated with neurologic abnormalities and/or manifest characteristic dermatomal distribution patterns (53–57), suggesting regulation of melanocyte function by the nervous system. Although embryologic defects of the neural crest, the origin of both melanocytes and peripheral nerves, might account for pigmentary disorders in a dermatomal distribution pattern, much evidence suggests that postdevelopmental neurologic abnormalities are also involved in the pathogenesis of pigmentary disturbances. For example, in segmental vitiligo, in which skin areas devoid of melanocytes occurs in a dermatomal distribution, degenerative changes in nerves were reported (58). In some cases, segmental vitiligo appeared on neurologically compromised skin (59) after peripheral nerve injury (60) or in association with viral encephalitis (61). As well, leprosy, a mycobacterial disease primarily affecting the skin and peripheral nervous system, is often manifested by anesthetic hypomelanotic macules. Electron microscopic studies of such lesions show decreased numbers of epidermal melanocytes and degenerative changes of superficial nerve fibers (62). In other disorders, both hy-

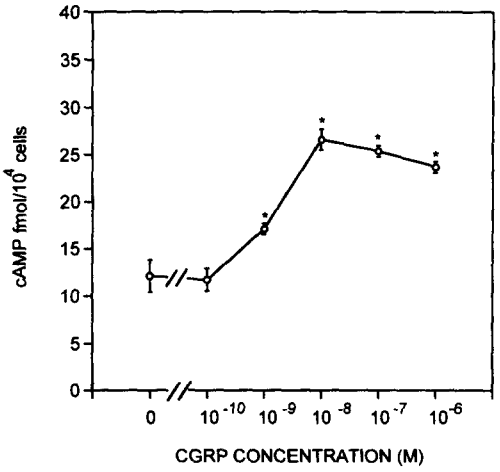

Figure 8. Effect of increasing CGRP concentrations on cAMP level in melanocytes. 10^6 melanocytes were suspended in 1 ml of melanocyte-defined medium and exposed to various concentrations of CGRP for 5 min. Cells were extracted, and cAMP level was measured as described in Materials and Methods. CGRP increased intracellular cAMP in a dose-dependent manner with maximal increase of approximately twice the basal level with 10^{-8} M CGRP. (*P <0.02, nonpaired t test.)

pomelanotic and dyschromic lesions are accompanied by degenerative changes of dermal nerve fibers (6).

Past studies of human skin have reported contact between the basal lamina of Schwann cells and the basal lamina of melanocytes (63, 64). However, these contacts were found only in skin involved in pigmentary disorders like vitiligo and incontinentia pigmenti achromians. One study of guinea pig's skin showed direct contact between melanocytes and nerve fibers and the presence of specialized structures such as postsynaptic thickening of plasma membrane and vesicle aggregation (11). However, such observations were never reported for human skin, leading to a lack of appreciation of a physical interaction between neurons and melanocytes in humans.

For many years it was thought that cutaneous nerves were restricted to the dermis, but sophisticated immunohistochemical studies have revealed free nerve fibers even in the upper part of the epidermis (65, 66). Ample evidence suggests that intraepidermal free nerves participate in epidermal homeostasis as well as in the pathogenesis of certain skin diseases through release of neuropeptides (67–69). So far, neuropeptides demonstrated in the epidermis include SP and CGRP (67). In addition to such well-described functions of SP as vasodilation (70, 71), mast cell degranulation (72), and immunomodulation (73), SP has shown to be mitogenic for fibroblasts and epithelial cells (74, 75). Under certain circumstances, SP also suppresses keratinocyte growth (29). CGRP has a mitogenic effect on endothelial cells (76) and Schwann cells (77) as well as its vasodilatory and immunomodulatory effects. In this study, we examined the melanocytic effect of these neuropeptides, as well as VIP, a peptide widely distributed in dermis, especially around blood vessels and sweat glands (78). CGRP consistently induced DNA synthesis and increased cell number. Although in an experimental medium containing cholera toxin the effect was less than that reported for the keratinocyte-derived melanocyte mitogens bFGF (79, 80) and endothelin (81), in medium lacking cholera toxin their effect was comparable. Cholera toxin is known to increase cAMP levels and to reduce the apparent effect in melanocytes of agents such as α-melanocyte-stimulating hormone (α-MSH) that exert their effects through the cAMP-dependent pathway (82). This phenomenon is particularly interesting in the present case in that the biological actions of CGRP in several cell types are mediated through the cAMP pathway (83). Rapid accumulation of intracellular cAMP in melanocytes after stimulation by CGRP is consistent with published data in other cell types (76, 77, 83); and the more modest increase measured in the present study (100% vs 400–600%) (83) is consistent with an artifactually increased basal level in those cultured human melanocytes.

CGRP's effect on melanocyte proliferation is particularly interesting in light of its known role during cutaneous wound healing. CGRP and other neuropeptides have a trophic effect in regeneration of UV-damaged skin (84). Thus, it is possible that neuropeptides play a role in melanocyte proliferation during cutaneous wound healing, as well. Another possible function of neuropeptides is to induce melanocyte dendricity. Neuropeptides have been reported to translocate adhesion molecules like P- and E-selectin to the cell membrane (85) and to affect phosphorylation of the focal adhesion protein paxillin (86). Hence, neuropeptides participate in the regulation of cell-substrate adhesion and ultimately cell motility and shape (87). Furthermore, in neural crest–derived astrocytes, neuropeptides regulate dendrite formation (88). It is possible that neuropeptides may similarly affect melanocyte dendricity and/or their adhesion to the basement membrane or surrounding keratinocytes.

Neuropeptides such as VIP have been reported to act as survival factors for certain neuronal populations (88, 89). Therefore, they may act as survival factors for cutaneous melanocytes. Interestingly, one neuropeptide, neuropeptide Y, was reported to regulate the secretory activity of melanin-containing cells in *Xenopus laevis* (90). It is possible that in cutaneous melanocytes neuropeptides may not regulate melanin synthesis per se but may affect melanosomal transfer to surrounding keratinocytes.

Finally, neuropeptides play an important role in regulating the synthesis and secretion of immune cytokines including IL-1, IL-2, IL-4, IL-6, and TNF-α (91–93). Melanocytes are known to express a variety of cytokines including, IL-1, IL-6, IL-8, and TNF-α (94). Furthermore, melanocytes are capable of phagocytosing antigens and as such are speculated to function as accessory immune cells within the skin (95). It is tempting to speculate that cutaneous neuropeptides may modulate melanocyte immune function, particularly during cutaneous inflammation.

In summary, this paper expands an earlier study and demonstrates that in human skin there are intimate synapselike connections of axon terminals to melanocytes. CGRP, one of the neuropeptides present in epidermal nerve endings, induces melanocyte proliferation. These data expand our understanding of normal and disordered epidermal pigmentation and the complex relationship of melanocytes to their epidermal environment.

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