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Human scalp skin and hair follicles express neurotrophin-3 and its high-affinity receptor tyrosine kinase C, and show hair cycle-dependent alterations in expression

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Summary

Background Neurotrophin (NT)-3 and its high-affinity receptor tyrosine kinase C (Trk C) are essential for nervous system development. These members of the NT family are also involved in murine hair morphogenesis and cycling. However, their role in human hair follicle (HF) biology remains to be elucidated.

Objectives To explore the role of NTs in human skin and HF biology.

Methods The immunoreactivity (IR) of NT-3 and Trk C was studied in human scalp skin and HFs by immunofluorescent and light microscopic immunohistology. Skin biopsies were obtained from normal human scalp containing mainly anagen VI HFs from women (age 53–57 years) undergoing elective plastic surgery.

Results Both NT-3 and Trk C showed prominent, yet distinct, IR patterns in human scalp anagen HFs (anagen VI), whereas they were weakly expressed in catagen and increased again in telogen HFs. Within HF compartments, NT-3 IR was prominent in the outer root sheath, inner root sheath, dermal papilla and connective tissue sheath. Trk C IR was prominent in all HF epithelial and mesenchymal compartments. Outside the HF, both NT-3 and Trk C showed prominent IR in the epidermis, sebaceous glands and sweat glands.

Conclusions These observations provide the first indication that NT-3 and Trk C are expressed in human scalp skin and HFs, and suggest that Trk C-mediated signalling is involved not only in murine but also in human HF biology. They may be useful in determining therapeutic strategies for the treatment of hair cycle and skin-related disorders.

Neurotrophins (NTs) are a family of structurally and functionally related polypeptides which show about 50% amino acid sequence homology.[1] They include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4. NTs are essential for the development of the nervous system. They exert their biological effects via interaction with specific high-affinity receptors, tyrosine kinases A–C (Trk A for NGF, Trk B for BDNF and NT-4 and Trk C for NT-3) as well as the low-affinity receptor p75.[2]

Outside the nervous system, NTs can regulate tissue morphogenesis, proliferation and apoptosis.[3,4] NTs also control the development of kidney, teeth, muscles and heart.[5] NTs were recently found to be both expressed and involved in the development of the murine hair follicle (HF) as well as in the control of hair cycling.[6–8] Our studies with NGF and its receptor Trk A revealed that they are prominently expressed in human scalp anagen HFs (anagen VI), whereas they were weakly expressed in catagen and telogen HFs. Within HF compartments, NGF expression was prominent in the outer root sheath (ORS), dermal papilla (DP), connective tissue sheath (CTS) and inner root sheath (IRS). Trk A expression was prominent only in the ORS of the anagen HF. Outside the HF, both NGF and Trk A showed prominent expression in the epidermis, sebaceous and sweat glands (manuscript under review). In this study, we examined the role of another important member of the NTs, NT-3 and its receptor Trk C, in human skin and the HF cycle.

NT-3 can exert antiapoptotic actions in the developing nervous system and supports the survival of sensory neurones[9] and proprioceptive and cutaneous afferent nerve endings.[10] It also stimulates HF morphogenesis in mice[6] and controls catagen development during hair cycling.[7] In this context, it is

expected that NT-3/Trk C-mediated signalling is also involved in human skin and HF biology. The current study aims therefore at exploring: (i) the expression of NT-3 and Trk C in human scalp skin and HFs, and (ii) whether there are hair cycle-related alterations in expression.

Materials and methods

Skin samples

Skin biopsies from normal human scalp containing mainly anagen VI HFs were obtained after informed consent from women (age 53–57 years) undergoing elective plastic (cosmetic) surgery. After surgery, samples were maintained in Williams E Medium (Biochrom KG Seromed, Berlin, Germany) for transportation at 4 °C for up to 24 h. Skin specimens used for cryosections were frozen abruptly in liquid nitrogen and stored at −80 °C until use. Before immunostaining, samples were embedded and processed for longitudinal cryosections (8 μm). Sections were dried, fixed in cold acetone (−20 °C) and stored at −20 °C until used for immunohistochemistry.

Immunohistochemistry

Cryosections of normal human scalp skin were immunostained using polyclonal rabbit antihuman NT-3 IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and polyclonal rabbit antihuman Trk C IgG antibody (Santa Cruz Biotechnology). Two labelling techniques were performed to visualize antigen–antibody complexes: avidin–biotin complex (ABC) labelling (Vector Laboratories, Burlingame, CA, U.S.A.) and the highly sensitive immunofluorescent tyramide signal amplification (TSA) labelling (Perkin Elmer Life Science, Boston, MA, U.S.A.). For the ABC labelling method, cryosections of normal human scalp skin were washed in Tris-buffered saline (TBS, 0·05 mol L^{-1}, pH 7·6) and preincubated with avidin–biotin blocking kit solution (Vector Laboratories) followed by incubation with protein blocking agent (Immunotech, Krefeld, Germany) to prevent nonspecific binding. Sections were then incubated with the primary antibodies diluted in TBS (1 : 100 for NT-3, 1 : 50 for Trk C) containing 2% goat serum for 1 h at room temperature or overnight at 4 °C. Thereafter, sections were incubated with biotinylated secondary antibody, goat antirabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) diluted 1 : 200 in TBS containing 2% goat serum for 30 min at room temperature. Next, sections were incubated with avidin–biotin alkaline phosphatase complex (Vecta-Stain Kits; Vector Laboratories) diluted in TBS (1 : 100) for 30 min at room temperature. The alkaline phosphatase colour reaction was developed by applying a staining protocol described before,[11–13] using fast red tablets (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Finally, sections were counterstained with Meyer's haematoxylin, covered with Kaiser glycerol (Dako, Glostrup, Denmark) and stored at 4 °C for microscopic examination and analysis.

For the TSA labelling technique, cryosections were washed in Tris–acid–Tween buffer (TNT, pH 7·5), followed by washing in 3% hydrogen peroxide. Sections were then incubated with lower concentrations of primary antibodies diluted in Tris–acid-blocking buffer (TNB, pH 7·2; 1 : 1000 for NT-3, 1 : 500 for Trk C) overnight at 4 °C. Next, sections were washed in TNT and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated $F(ab)_2$ fragments of goat antirabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories) diluted in TNB (1 : 200) for 30 min at room temperature. Thereafter, sections were incubated with streptavidin horseradish peroxidase (1 : 50 in TNB) for 30 min at room temperature. Finally, TRITC–tyramide amplification reagent was applied (1 : 50 in amplification diluent provided with the kit) for 30 min at room temperature, followed by counterstaining with 4′,6′-diamidino-2-phenylindole and mounting in levamisole (Dako, Carpenteria, CA, U.S.A.). The TSA signals were visualized under a fluorescence microscope (Zeiss, Jena, Germany). Negative controls were obtained by omission of primary antibodies, whereas postive controls were obtained by incubation of mouse brain cryosections with primary antibodies specific for both antigens to be detected.

Results

Neurotrophin-3 and tyrosine kinase C are expressed in human scalp hair follicles and their expressions peak during anagen, but decline during catagen and increase again in telogen

Immunostaining of cryosections of human scalp skin containing mainly anagen HFs besides a few HFs undergoing catagen and telogen revealed that the human HF expresses NT-3 (Fig. 1) and Trk C (Fig. 2) and that their expressions peak during anagen, decline during catagen and increase again in telogen. In human anagen VI HFs, prominent immunoreactivity (IR) of NT-3 and Trk C was indicated in both epithelial and mesenchymal compartments and was greater than in either catagen or telogen HFs. As shown in Figure 1, NT-3 is expressed in the ORS, IRS, CTS and DP, but it is absolutely absent from hair matrix cells. In the ORS, NT-3 immunostaining intensity varied considerably in the different regions of the follicle (Fig. 1Q,U). In the proximal region including the bulbar and lower suprabulbar ORS, NT-3 had a weak to moderate expression (Fig. 1F,G,M,N,U). Its expression increased in the central region, with greatest intensity in the innermost (companion) layer and lower intensity in the basal layer (Fig. 1E,L,U). In the distal region and the bulge, NT-3 showed a strong expression with increasing intensity towards the basal layer (Fig. 1D,K,U). In the isthmus and infundibulum, NT-3 IR was high in the basal layer and decreased gradually towards the inner layers (Fig. 1B,C,I,J,U). In the IRS, NT-3 IR was prominent in the proximal suprabulbar and central regions, but was absent from the bulbar and distal regions (Fig. 1D–G,K–N). Trk C showed strong expression in all follicle compartments, including hair matrix cells, with approximately identical intensities (Fig. 2A–D,F–H,K).

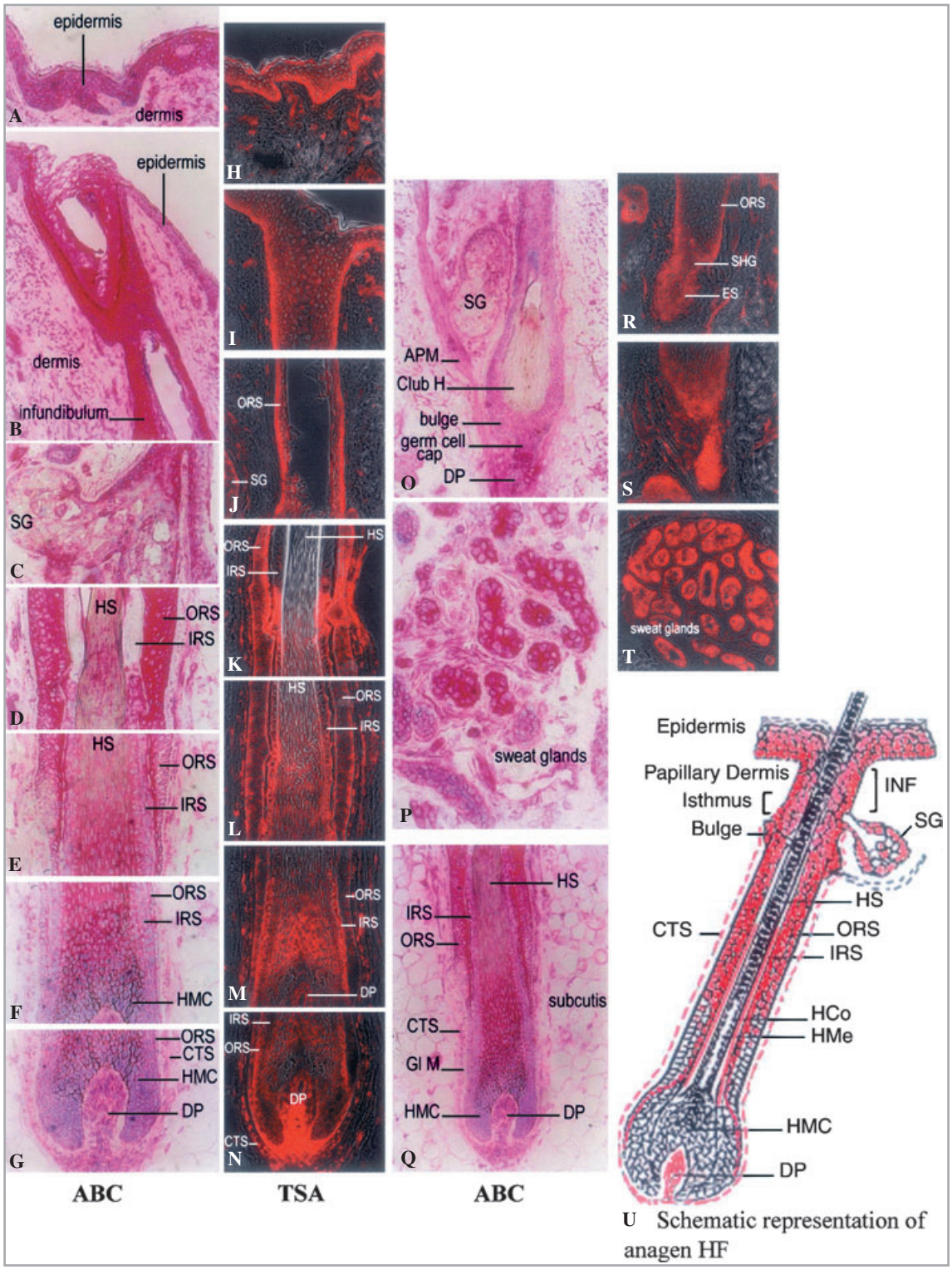

Fig 1. Immunoreactivity (IR) of neurotrophin-3 (NT-3) in human scalp skin and hair follicle (HF), shown in red with the avidin–biotin complex (ABC) and tyramide signal amplification (TSA) labelling techniques. (A) and (H) show the epidermis. (B–G) (panel 1) and (I–N) (panel 2) show IR in the anagen HF. (C) and (J) also show IR in the sebaceous gland (SG). (P) and (T) show IR in the sweat gland. (P) and (Q) show IR in some fibroblasts in the dermis and adipocytes in the subcutis. (O) and (S) show IR in telogen HF. (R) shows IR in catagen HF. (U) is a schematic representation of the anagen HF showing NT-3 IR in red. HS, Hair shaft; ORS, outer root sheath; IRS, inner root sheath; HMC, hair matrix cells; CTS, connective tissue sheath; DP, dermal papilla; APM, arrector pili muscle; Club H, club hair; SHG, secondary hair germ; ES, epithelial strand; INF, infundibulum, HCo, hair cortex; HMe, hair medulla.

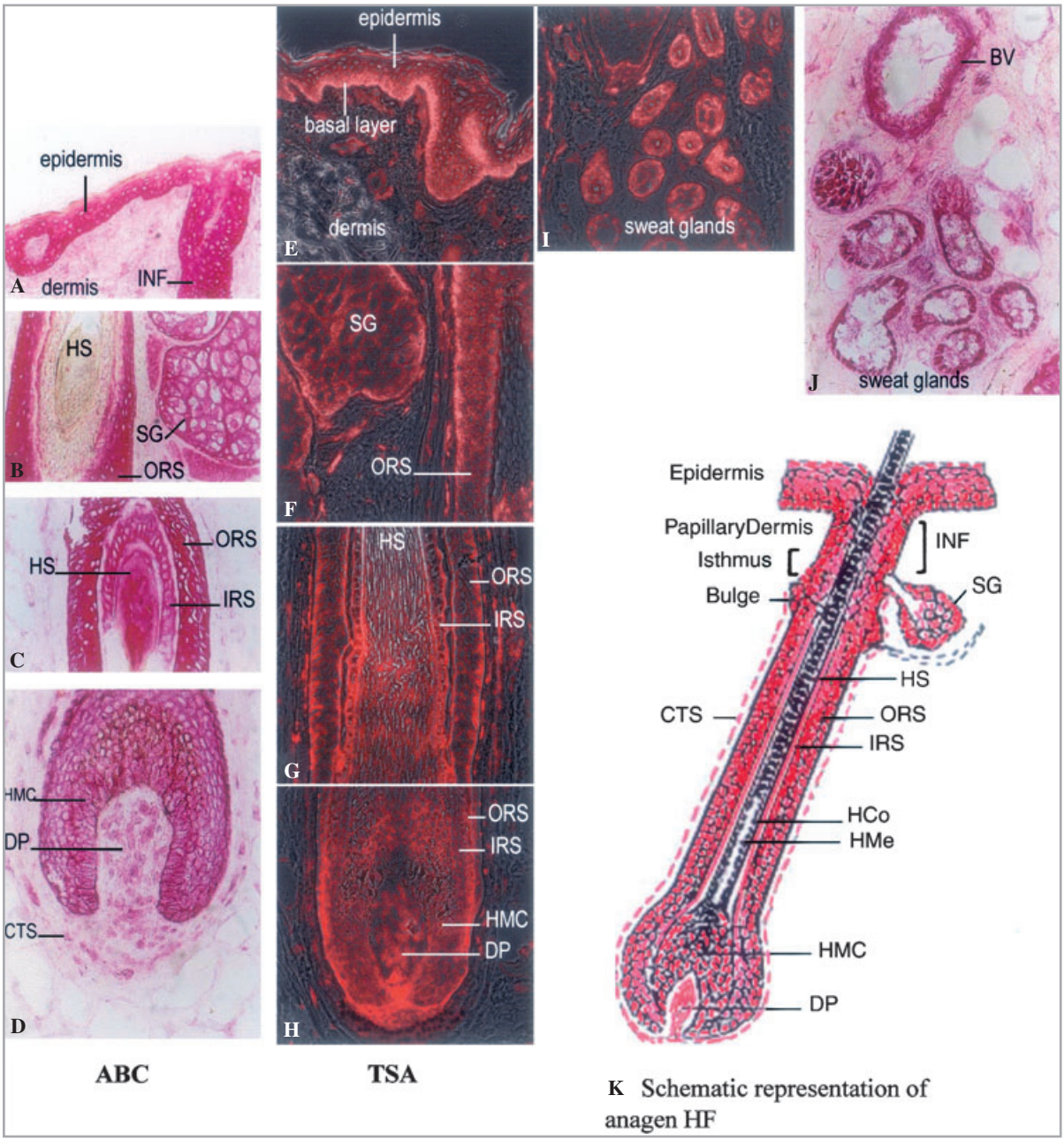

Fig 2. Immunoreactivity (IR) of tyrosine kinase C (Trk C) in human scalp skin and hair follicle (HF), shown in red with the avidin–biotin complex (ABC) and tyramide signal amplification (TSA) labelling techniques. (A) and (E) show the epidermis. (B–D) (panel 1) and (F–H) (panel 2) show IR in the anagen HF. (B) and (F) also show IR in the sebaceous gland (SG). (I) and (J) show IR in the sweat gland. (K) is a schematic representation of the anagen HF showing Trk C IR in red. INF, Infundibulum; HS, hair shaft; ORS, outer root sheath; IRS, inner root sheath; HMC, hair matrix cells; DP, dermal papilla; CTS, connective tissue sheath; BV, blood vessel; HCo, hair cortex; HMe, hair medulla.

In catagen HFs, NT-3 and Trk C IR was greatly decreased and became confined to the basal and few outer suprabasal layers of the ORS, as indicated in the central region of catagen VI HFs (Fig. 1R). Strong NT-3 expression was, however, found in the secondary hair germ and regressing epithelial strand, indicating its role also in catagen (Fig. 1R).

In telogen, NT-3 and Trk C IR increased prominently. NT-3 expression extended to include all layers of the ORS in the bulge and isthmus regions (Fig. 1O,S). Strong NT-3 expression was also seen in the DP and germ cell cap (Fig. 1O).

Neurotrophin-3 and tyrosine kinase C show prominent expression in human scalp skin and extrafollicular structures

Both NT-3 and Trk C had distinct expression in the skin and certain extrafollicular structures, clearly in the sebaceous

gland, sweat glands, arrector pili muscle, dermal fibroblasts and subcutaneous fat cells. Strong NT-3 IR was indicated in scalp skin, with increasing positivity in the basal layer and stratum corneum (Fig. 1A,H). Moderate to strong NT-3 IR was also seen in the sebaceous gland (Fig. 1C,J), sweat glands (Fig. 1P,T), arrector pili muscle (Fig. 1O), dermal fibroblasts (Fig. 1B,I) and subcutaneous fat cells (Fig. 1Q). Trk C IR was indicated in the skin with increasing strength towards the basal layer (Fig. 2A,E). Strong Trk C IR was found in the sebaceous gland (Fig. 2B,F), sweat glands (Fig. 2I,J) and blood vessel walls (Fig. 2J). Weak Trk C IR was also shown in some dermal fibroblasts (Fig. 2A,E) and subcutaneous fat cells (Fig. 2D).

Discussion

The current study revealed that NT-3 and Trk C are strongly expressed in both epithelial and mesenchymal compartments of human anagen VI HFs, whereas they are weakly expressed in HFs undergoing catagen. This suggests a role for NT-3 in anagen induction and/or prolongation. This task may be aided either by keratinocyte proliferation and differentiation in HF epithelium or by exerting an antiapoptotic effect against apoptosis-induced catagen. A role for NT-3 in stimulating proliferation of epidermal keratinocytes was found in murine skin.[14] Our data coincide with those of Botchkarev *et al.*,[6,7] who found expression and involvement of NT-3 and Trk C in murine HF morphogenesis and cycling. Our data showed that NT-3 and Trk C were more strongly expressed in anagen and telogen than in catagen, indicating a stronger role in anagen induction in human HFs. A role for NT-3 in stimulating keratinocyte proliferation was found also *in vitro*, either directly or indirectly via upregulation of NGF. The addition of NT-3 slightly upregulated the secretion of NGF, whereas NGF strongly augmented NT-3 release.[15] In the nervous system, NT-3-dependent increase in Bcl-2 levels is an important mechanism by which NT-3 can promote proliferation and prevent apoptosis in oligodendrocyte progenitor cells.[16] NT-3 is a differentiation and proliferative factor for cholinergic neurones as well as a link between NT and neurotransmitter plasticity.[17]

Our results are also consistent with findings showing that NT-3 is an antiapoptotic factor. For examples, Rohon Beard (RB) cells are embryonic primary sensory neurones that are removed by apoptosis during larval development in zebrafish. The RB neurones expressing Trk C1 can survive, whereas those that are Trk C1-negative undergo apoptosis. This is further supported by the finding that antibodies that depleted NT-3 induced apoptosis of RB, while exogenous application of NT-3 reduced RB cell death.[9] NT-3 can also promote the survival and prevent the apoptosis of both proprioceptive afferents and cutaneous afferents[10] and mediate enhancement of Merkel cell number.[18] The mechanisms for neuronal survival in the central nervous system (CNS) are complex, with possible autocrine and redundant neurotrophic support. A new report confirms that the novel catecholaminergic CNS cell line, CAD, is capable of autocrine survival and that this is mediated by NT-3.[19] NT-3 displayed an antiapoptotic effect on cultured cortical neurones through a mechanism involving the recruitment of the phosphoinositide-3 (PI-3) kinase/Akt signalling pathway.[20]

Nervous system and skin epithelium share a common ectodermal origin, and it is not surprising that some NTs can modulate keratinocyte proliferation and apoptosis.[15] The expression and function of NTs and NT receptors have been investigated in cultured human keratinocytes. These studies revealed that the keratinocytes synthesize NT-3, BDNF, NT-4/5 and Trk A–C. Alternatively, only the truncated extracellular isoform of Trk B, the high-affinity BDNF and NT-4/5 receptor, is detected in keratinocytes. Moreover, NT-3, BDNF and NT-4/5 proteins are secreted by human keratinocytes at low levels. Keratinocyte stem cells synthesize the highest amounts of NGF, while they secrete higher levels of NGF compared with transit amplifying cells.

NT-3 enhances substantial separation of dermatomes postnatally and may exert its effects either by enhancing competition, or by direct effects on the stability and formation of sensory endings in the skin.[21] Indeed, NT-3 can also act as an 'epitheliotrophin' and may be intimately involved in the control of epidermal homeostasis;[14] this may subsequently have application in the treatment of skin diseases due to disorders in epidermal homeostasis. HF is both a source and a target of NT-3, and NT-3/Trk C signalling is functionally important in the control of HF regression. Therefore, Trk C agonists and antagonists deserve systematic exploration in the management of hair growth disorders that are related to premature catagen such as alopecia/effluvium or those that are related to retarded catagen such as hirsutism/hypertrichosis.[14]

Our results are further supported by the finding that the Trk family of receptors mediates NGF and NT-3 effects in melanocytes.[22] This in turn opens new ways in treatment of disorders of pigmentation, e.g. vitiligo. NT-3, through its receptor Trk C, plays a critical role in regulating the Th1/Th2 balance,[23] and this may represent a very important tool for the prevention and treatment of many related skin disorders such as allergic and autoimmune diseases. NT-3 was also shown to be involved in the maturation of mast cells (MCs), either by Trk C-mediated stimulation of MC differentiation from MC precursors and/or by enhancing the migration of circulating MC precursors into the skin,[24] and this subsequently has a vital role in MC stability and the prevention of MC instability-associated diseases. Moreover, it was revealed that ultraviolet (UV) B irradiation augments NT-3 and NT-4/5 protein levels, and UVA irradiation increases the level of NT-3. This work may delineate the mechanism by which UVB and UVA can treat many skin diseases and induce epidermal homeostasis, and implies that these effects are mediated by upregulation of NT-3 level.[14] Furthermore, it was found in human diabetic skin that epidermal Trk C expression is upregulated as a compensatory mechanism for the decreased autocrine NT action due to the reduction in NT-3 level in diabetes.[25] This is supported, on one hand, by the finding that insulin-like growth factor-I upregulates NT-3 expression.[26] On the other hand, this implies

that NT-3 can combat diabetic damage and the *in vivo* treatment of diabetes with insulin is mediated by NT-3, which could prevent the loss of mitochondrial membrane potential.[27] The mechanism of insulin- and NT-3-dependent modulation of mitochondrial membrane potential involves the activation of the PI-3 kinase pathway.[28] Downstream targets of PI-3 kinase, such as Akt and the transcription factor cAMP response element-binding protein, which regulate sensory neurone gene expression, are activated by insulin and NT-3. It has been shown that in adult sensory neurones, treatment with insulin can elevate the input of reducing equivalents into the mitochondrial electron transport chain, and this leads to greater mitochondrial membrane polarization and enhanced ATP synthesis.[27] Mitochondrial dysfunction in diabetic sensory neuropathy could be aborted by downregulation of inducible nitric oxide synthase, and thus subsequent reduction in nitric oxide level, via NT-3 treatment, and this, in turn, proves that NT-3 is antioxidant.[29] This explains the expected powerful roles of NT-3 in skin and hair disease treatment or prevention.

It has been shown also that adrenalectomy suppresses the NT-3 mRNA,[30] and this can address the question of whether corticosteroids exert their functions in the treatment of alopecias or allergic and autoimmune diseases via the upregulation of NT-3. A novel study showed that all-*trans* retinoic acid can also potentiate the survival and neurite outgrowth-promoting activities of NT-3.·[31] This, in turn, means that there is an interaction or a shared mechanism between NT-3 and retinoid efficacy. This may promise a new strategy of replacing retinoids in the treatment of skin and hair disorders with NT-3, as NT-3 will be expected not to produce the side-effects associated with use of retinoids. Iatrogenic neuropathy caused by administration of chemotherapeutic drugs represents an excellent target for a human trial to assess the potential of gene therapy neuropathy prevention[32] and this, in turn, suggests that NT-3 may have a role in the treatment of alopecia induced by cytotoxic drugs. It has been speculated that NT-3 and other NTs may only be effective in the treatment of disorders in which apoptosis, but not necrosis, is the major process,[33] and this, in turn, will strengthen our results in exploiting NT-3/Trk C-mediated signalling in the treatment of certain skin and HF disorders.

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