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Neurotrophins in healthy and diseased skin

Francesca Truzzi, Alessandra Marconi and Carlo Pincelli*

[1]Institute of Dermatology; School of Biosciences and Biotechnologies; University of Modena and Reggio Emilia; Modena, Italy

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Neurotrophins (NT) belong to a family of structurally and functionally related proteins that, depending on the tissue context and the receptors involved, promote either neuronal cell survival and differentiation or cell death. NT, and in particular NGF, were first identified as neurotrophic factors supporting the synthesis and development of sensory neurons in the central and peripheral nervous system. It is now widely accepted that NT also act as growth factors in non-neuronal cells, including the skin. In the skin, most cell types are able to secrete and/or to respond to stimulation by NT, creating a unique network of molecular signaling in the cutaneous microenvironment. Moreover, many skin diseases have been associated with an involvement of a number of neural factors including NT, but less attention has been given to the role of NT as growth factors in the development of skin pathologies. This review summarizes currently data on the expression and function of NT and their receptors in several cell types in the skin. Moreover it focuses on the role of the skin NT network in two cutaneous conditions, melanoma and psoriasis where NT are clearly involved.

Neurotrophins and Their Receptors

Neurotrophins (NT) belong to a group of functionally and structurally related proteins that were first identified as promoters for neuronal survival. Subsequently, it was demonstrated that they regulate many aspects of neuronal development and function, like synapse formation and synaptic plasticity.[1] Nerve growth factor (NGF) was the first NT to be discovered during a search for survival factors.[2] Brain-derived neurotrophic factor (BDNF) was the second NT to be characterized. It has been identified as a survival factor for several neuronal populations not responsive to NGF.[3] These two proteins revealed conserved features of the sequences, leading to isolation of clones encoding additional members of this family.

Nowadays, we know that four NT are expressed in mammals: NGF, BDNF, NT-3 NT-4. NT play a critical role in developmental neurobiology. They play an essential role in cellular interactions, in controlling cell survival and differentiation.[4] NGF is internalized by receptor-dependent mechanisms and transported along axons in membranous vesicles and energy-dependent processes.[5]

*Correspondence to: Carlo Pincelli; Email: carlo.pincelli@unimore.it
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The effects of neurotrophins are mediated by two classes of cell-surface receptors, a family of tyrosine kinase receptors called Trks (TrkA, TrkB and TrkC) and the p75 neurotrophin receptor (NTR). Whereas p75^{NTR} binds all neurotrophins with low affinity and specificity, Trk receptors bind neurotrophins with higher affinity and specificity. While TrkA binds NGF and NT-3, TrkB binds BDNF, NT-3, NT-4/5. TrkC only binds NT-3.

Trks are tyrosine kinase receptors, activated by ligand-induced formation of noncovalently associated receptor dimers. Several different mutation types in the trkA gene have been identified in congenital insensitivity to pain with anhydrosis (CIPA) patients.[6] The phenotypes associated with CIPA are believed to result in large part from loss of NGF-dependent neurons, including nociceptive sensory and sympathetic neurons, during embryogenesis.

A dominant mutation in TrkB (Y722C) that impairs TrkB kinase signalling has recently been described in a patient with severe hyperphagic obesity and severe impairments in nociception, learning and memory.[7]

NT dimerize the Trk receptors, resulting in activation through transphosphrylation of the kinases present in their cytoplasmic domains. NT interact with these receptors at the membrane-proximal immunoglobulin-like domain. The three-dimensional structures of this domain in each of the Trk receptors have been solved.[8] Expression of a specific Trk receptor confers responsiveness to the NT to which it binds. On the other hand the isoform of TrkA including an insert is also activated by NT-3 in addition to NGF,[9] while the similar isoform of TrkB is activated by NT-3 and NT-4 in addition to BDNF.[10] Thirty-six novel isoforms of TrkB proteins with unique properties have been described recently. This suggests high complexity in the synthesis, regulation and function of this important NT receptor, emphasizing the need for further study of these novel TrkB variants.[11]

TrkC has several characteristics of a tumor suppressor, and its expression in tumors has often been associated with good prognosis. TrkC was recently demonstrated to be a dependence receptor, transducing different positive signals in the presence of ligand but inducing apoptosis in the absence of ligand. Indeed, the TrkC ligand NT-3 (NT-3) is upregulated in a large fraction of aggressive human neuroblastomas (NB) and it blocks TrkC-induced apoptosis of human NB cell lines.[12,13]

p75^{NTR} was the first NT receptor to be identified as a low-affinity receptor for NGF, but it was subsequently shown to bind each of the neurotrophins with a similar affinity.[14,15] p75^{NTR} is a member of the tumour necrosis factor receptor superfamily with an extra-cellular domain containing four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a 'death' domain, similar to those present in other

members of this family.[16] Although this receptor does not contain a catalytic motif, it interacts with several protein that transmit important signals for regulating neuronal survival and differentiation as well as synaptic plasticity. The three-dimensional structure of the extracellular domain of $p75^{NTR}$ in association with an NGF dimer indicates that each of the four cysteine-rich repeats participates in binding to NGF.[16] The binding of NGF to $p75^{NTR}$ may result in dissociation of $p75^{NTR}$ multimers and is compatible with the possibility that Trk and $p75^{NTR}$ monomers simultaneously bind the same neurotrophin dimer. A gene related to $p75^{NTR}$, named NRH-2, has recently been identified. The product of this gene lacks the extracellular cysteine-rich repeats present in $p75^{NTR}$ and fails to bind NGF, but it is able to interact and influence the ligand-binding properties of TrkA.[17]

The role of $p75^{NTR}$ is still controversial: in the presence of Trk, $p75^{NTR}$ increases high-affinity NT binding, thereby enhancing the ability of Trk to promote survival.[18] By contrast, in the absence of Trk, $p75^{NTR}$ can induce apoptosis, via its own signal transduction, by interacting with a mounting number of downstream molecules.[19] Recently, it has been shown that the proform of NT proNGF binds $p75^{NTR}$, in association with its co-receptor sortilin, but not Trk.[20] More specifically, sortilin, a member of the vps-10 protein family, binds the "pro" region of NGF, whereas $p75^{NTR}$ binds mature NGF. The $p75^{NTR}$-sortilin complex couples with proNGF to induce apoptosis.[21]

Neurotrophins and the Skin

NT and in particular NGF were first identified as neurotrophic factors supporting the synthesis and development of sensory neurons, that in turn extend their dendrites into the skin, thus playing an essential role in skin innervation. Indeed, null mutations of genes from the NGF family of NT and their receptors (NTRs) lead to loss/reduction of specific neurons in sensory ganglia; conversely, cutaneous overexpression of NT results in skin hyperinnervation and increase in the number of sensory neurons innervating the skin.[22] In addition, NGF synthesized in the epidermis, is retrogradelly transported to the ganglia to stimulate the release of neuropeptides in the skin, thus favoring cutaneous neurogenic inflammation.[23] Subsequently, it became clear that NGF possesses a number of biological effects also on non-neuronal cells. In particular, Pincelli and co-workers first demonstrated that normal human keratinocytes synthesize and release NGF that can act as a growth factor for these cells.[24] Later, expression and function of NGF and other NT was shown in other skin cells, definitely indicating that NT play very important functions other than supporting skin innervation. In this review, we focus on NT as growth factors in most cutaneous cells and report the concept of a rich NT network in the skin.

Keratinocytes. Human keratinocytes synthesize and secrete biologically active NGF,[25,26] BDNF, NT-3 and NT-4.[27,28] NGF is secreted at highest levels as compared to the other NT, whereas NT-3 and NGF upregulate each other's secretion in human keratinocytes. NT-3 release is augmented by UVA, whereas NGF expression is downregulated by UVB irradiation.[29,30] In human skin, NGF is released in increasing amounts by proliferating keratinocytes, whereas secretion ends in more differentiated cells.[24] NGF is able to induce proliferation in human keratinocytes,[31] and it can either stimulate or inhibit murine epidermal and hair follicle keratinocyte proliferation in situ.[32] Human keratinocytes express the high affinity receptors TrkA and TrkC, but not the functional form of the TrkB.[28] Inhibiting TrkA phosphorylation with the natural alkaloid k252a, in absence of exogenous NGF, reduces keratinocyte proliferation.[33] This indicates that endogenous NGF autocrinally sustains keratinocyte proliferation. Keratinocytes transfected with NGF proliferate to a greater extent as compared to mock cells,[29] while increasing amounts of NT-3 antibody inhibits keratinocyte proliferation.[28] NT together with their receptors TrkA and TrkB are able to stimulate mouse keratinocyte proliferation in ex-vivo cultured skin explants.[27,32] NT also modulate susceptibility to apoptosis in the epidermis. While UVB downregulates both NGF and TrkA and induce apoptosis in human keratinocytes,[29] autocrine NGF protects these cells from UVB-induced apoptosis.[29] Because normal human keratinocytes typically lack TrkB expression, NT may exert different functions in this system, by binding $p75^{NTR}$ alone. In this context, $p75^{NTR}$ acts as a proapoptotic receptor. This is exemplified by BDNF and NT4, which induce a higher rate of apoptosis in normal human keratinocytes overexpressing $p75^{NTR}$, as compared to mock-transfected cells. On the other hand, $p75^{NTR}$ siRNA-transfected keratinocytes fail to undergo cell death after administration of NT4 (Truzzi F, et al. unpublished data).

Interestingly, NGF is mostly expressed and released by stem keratinocytes (KSC),[28] and blocking TrkA inhibits the proliferation of this keratinocyte subpopulation.[28] This seems to indicate that a NGF-TrkA autocrine loop, by acting as both a mitogenic and survival factor, contributes to the maintenance of the so-called "stemness" in keratinocytes. On the other hand, $p75^{NTR}$ is almost exclusively expressed in the more differentiated transit amplifying (TA) cells, and it is barely detected in KSC. TA cells have been shown to be more susceptible than KSC to apoptosis.[34] Therefore, $p75^{NTR}$ being predominantly expressed in TA cells seems to be consistent with the pro-apoptotic role of this receptor in human keratinocytes (Truzzi F, et al. submitted). Taken together, these results point to a critical role of NT and their receptors in epidermal homeostasis.

Melanocytes. In human skin, melanocytes reside at the dermo-epidermal junction and in the hair matrix. During skin development, melanoblasts, which derive from neural crest, migrate into the skin and differentiate into melanocytes. Together with other paracrine signalling molecules, NT are important for melanocytes migration, viability and differentiation.[35,36] Normal human melanocytes seem to be a target of the NT skin network, because they express all the NT receptors both in vitro and in vivo.[37] Normal human melanocytes also express $p75^{NTR}$, which is upregulated by different stimuli, like UV irradiation.[38] NT influence melanocytes in paracrine fashion: for example, in vitro NGF is chemotactic for melanocytes and stimulate their dendrite formation.[26] NGF is implicated in melanocyte survival,[39] migration and dendricity,[26] and its synthesis and secretion are enhanced by UV irradiation.[40] Moreover, when melanocytes are irradiated with UV, NGF reduces apoptosis through upregulation of

the anti-apoptotic Bcl-2 protein in vivo.[30] From these data it is possibile to conclude that NT are important for protection of UV-induced oxidative stress and apoptosis in melanocytes. Melanocytes produce all NT, while when these factors are added to the cultures, they fail to stimulate cell proliferation.[37] When melanocytes are maintained in growth factor-depleted medium, NGF and NT-3 promote melanocyte survival.[41] Both NT3 and NT4 secretion promotes the synthesis of tyrosinase and tyrosinase-related peptide (TRP)-1, critical enzyme of melanin biosynthesis.[37] NT-3, NT-4 or NGF significantly increases the melanin content when stimulated with endotelin-1.[37]

Human melanocytes express Trk receptors.[37,41] Interestingly, phorbol-12-tetradecantate-13-acetate, a strong activator of protein kinase C, induces the expression of TrkA[41] and decrease the expression of TrkC, suggesting that NGF and NT3 mediate different signals through their specific high affinity receptors. Melanocytes expess also the NT low affinity receptor $p75^{NTR}$, which expression is upregulated after TPA treatment.[41]

Fibroblasts. NT and their receptors are expressed both in dermal fibroblasts and in the more differentiated myofibroblasts. $p75^{NTR}$ and TrkB are expressed at higher levels in myofibroblasts than in fibroblasts, which in contrast express higher levels of TrkA. Dermal fibroblast and myofibroblasts secrete NGF and NT3 at higher levels than NT4 and BDNF. Exogenous NT per se fail to stimulate dermal fibroblast proliferation, possibly because endogenous production of NT is sufficient to normal cell activity and survival. Interestingly, NT also promote fibroblast differentiation into myofibroblasts, by inducing α-SMA expression, indicating that NT could have a functional role in the fibro-myofibroblast system (Palazzo E, et al. manuscript in preparation). It has been shown that NGF induce fibroblast-like keratinocyte differentiation into myofibroblasts,[42] their contraction in 3D collagen matrix[42] and the expression of MMP-9 (Metalloprotease-9) in keratoconjunctivitis-derived fibroblasts,[43] and different works show the applicative possibility of this NGF function.[44-46] It has been demonstrated that all NT promote fibroblast migration while NGF and BDNF promote their contractile activity. Therefore NGF and BDNF, produced by dermal and epidermal cells, could be key regulators of the biomechanical properties in the dermis.

Other skin cells. Skin mast cells express functional TrkA and produce NGF.[47] They also express $p75^{NTR}$ mRNA and protein.[48] NGF has been shown to play a collaborative action on mediator release from residential tissue mast cells in rat skin by in vivo extravasation assay. This result implies that the activation pathway in mast cells may occur in the pathophysiological condition. Activation of mast cells induces the release of histamine, leukotrienes and produces inflammatory cytokines, resulting in the recruitment and activation of circulating leukocytes to the area of allergic and non allergic inflammation.[49]

Merkel cells are sensory cells of neural crest origin. NT have been investigated during development in mice finding that neither NT-3 nor TrkC and $p75^{NTR}$ are expressed by Merkel cells in the murine whisker. At the time of birth, however, Merkel cells are immunoreactive for NT-3, TrkC and $p75^{NTR}$. In TrkC null and NT-3 null mice, Merkel cells differentiate initially, but undergo apoptosis perinatally. These results show that NT-3 signaling is not required for the differentiation of Merkel cells, but that it is essential for their postnatal survival.[50] Merkel cell of whisker follicles of NT3 null newborns exhibited decreased immunoreactivity for cytokeratin 8 and contained apoptotic bodies that were positive for cleaved caspase-3. It was suggested that perinatal apoptosis is responsible for the loss of Merkel cells lacking innervation in NT3 null mice. TrkB was shown to be important for Merkel cells development, as mice lacking TrkB lost 32% of neurons and this neuronal loss was associated with the absence of Meissner corpuscles and reduction of hair follicle-associated sensory nerve endings and Merkel cells.[51] Using mice lacking $p75^{NTR}$, indicates that Merkel cells require $p75^{NTR}$ during the late postnatal development.[52]

Neurotrophins in Diseased Skin

Many skin diseases have been associated with an involvement of a number of neural factors including NT. There is a huge body of literature on the increased nerve density in several dermatoses which has lead many investigators to conclude that augmented sprouting of nerves and upregulation of neuropeptide release are involved in the pathogenesis of certain cutaneous conditions. It remains to be defined whether hyperinnervation and the release of neural substances associated with neurogenic inflammation are primary phenomena or just amplification events in skin diseases.

By contrast, less attention has been given to the role of NT as growth factors in the development of skin pathologies. Here, we will review two cutaneous conditions, melanoma and psoriasis where NT are clearly involved.

Melanoma. Morphologically, melanomas can be subdivided in several subtypes, such as epithelioid, pleomorphic spindle cell and desmoplastic melanoma. Iwamoto et al. found that there was no detectable $p75^{NTR}$ immunostaining in melanocytes of the normal epidermis, whereas 13 of 14 benign nevi showed detectable $p75^{NTR}$. This was primarily within the spindled nevocytic structures within the dermis.[53] Interestingly, $p75^{NTR}$ is highly expressed in desmoplastic melanomas and spindle cell melanomas and weakly expressed in the epithelioid melanomas.[54] In a study by Huttenbach et al. only 33% of their desmoplastic melanomas stained positive with $p75^{NTR}$,[55] while more recently Lazova[56] showed $p75^{NTR}$ staining to be more diffuse and intense as compared with S100, as reported previously by Kanik et al.

Recently Boiko et al. showed that $p75^{NTR}$ (CD271), which is considered a neural crest stem cell marker, allows the identification and isolation of melanoma cancer stem cells. Indeed, tumors derived from transplanted human $CD271^{+}$ melanoma cells were capable of metastasizing in vivo.[58] Marchetti et al. using melanoma cell lines, observed that NGF/$p75^{NTR}$ signaling promotes the survival of melanoma cells.[59] They also observed the presence of NGF and NT-3 in tumor adjacent tissues at the invasive front of melanoma brain metastases, which might indicate a paracrine activation of $p75^{NTR}$ and TrkC in melanoma cells by NGF and NT-3 produced by nearby glial cells. Besides promoting melanoma cell survival, neurotrophins also induce the expression in melanoma cells of heparanase, an important enzyme for local

invasion and metastasis, that cleave heparan sulfate chains of proteoglycans, thus modifying the extracellular matrix of tumor cells.[60] Moreover, Shonukan et al. established that neurotrophins are chemotactic for melanoma cells, triggered by $p75^{NTR}$-mediated dephosphorylation of the actin-bundling protein fascin.[61] In addition, TrkA is expressed by primary and metastatic melanomas and is associated with poor clinical outcome.[62]

Melanoma originates from melanocytes. Whereas melanocytes seem to benefit from the cutaneous neurotrophic network mainly as paracrine targets, once malignant transformation occurs, melanoma cells acquire self-renewal capabilities mediated also by autocrine NT loops. Indeed all NT, particularly NT-3 and NT-4, have been detected in conditioned medium of different melanoma cell lines.[63] Recent results show that melanoma cells proliferate through autocrine NT stimulation. K252a significantly reduces melanoma cell proliferation by inhibiting Trk phosphorylation. Proliferation is significantly reduced when endogenous NT are removed from the culture medium by soluble Trk/Fc receptors. NT appeared to be important for melanoma cell migration in vitro, with special respect for metastatic cell lines. The migratory phenotype is necessarily dependent on the presence of both the high- and low-affinity NT receptors. Cells treated with $p75^{NTR}$ small interfering RNA ($p75^{NTR}$ siRNA) fail to respond to NT stimulation. Similarly, the administration of K252a blocks melanoma cell migration, confirming that NT stimulate melanoma cell migration and invasion cooperation of the low- and high-affinity receptors.[63]

Psoriasis. Psoriasis is a chronic-relapsing inflammatory disease characterized by keratinocyte hyperproliferation and reduced apoptosis, leading to an increased epidermal turnover. Interestingly, NGF that is both a mitogen and a survival factor for keratinocytes, is overexpressed in psoriatic lesions as well as in psoriatic keratinocytes,[64,65] and its high-affinity receptor TrkA, that is located only in basal keratinocytes in healthy skin, is expressed throughout all epidermal layers in psoriasis.[66] On the other hand, $p75^{NTR}$ that plays a proapoptotic role in keratinocytes, is absent in psoriatic keratinocytes. The rate of apoptosis in psoriatic TA cells is significantly lower as compared to TA cells from normal epidermis. Interestingly, TA cells from psoriatic skin express lower levels of $p75^{NTR}$ as compared to TA from normal skin. Thus, absence of $p75^{NTR}$ in TA cells could account for the low keratinocyte apoptosis in psoriasis. In fact, infection of psoriatic TA with $p75^{NTR}$ restores apoptosis (Truzzi F, et al. submitted). Taken together, these results suggest that, under normal conditions, there is a balance between Trk and $p75^{NTR}$ functions, that allows epidermal homeostasis. On the contrary, in psoriasis, NGF and Trk upregulation associated with reduced $p75^{NTR}$ expression result in increased keratinocyte proliferation and reduced apoptosis, thus favoring epidermal thickness, a typical feature of this dermatosis. Interestingly, k252a, that has been shown to improve psoriasis in vivo in the SCID mouse model,[67] is now in Phase II clinical trial as a novel topical treatment for psoriasis.

In conclusion, NTs influence various cellular functions in normal and diseased skin. In the last decade, substantial progress has been made in the understanding of the molecular mechanisms involved in NT signaling. Additional studies are necessary to evaluate such molecular pathways in cells from healthy and pathologic skin. A translation of the relevant data from the field of cutaneous neurobiology into clinically-oriented research will unravel novel therapeutic strategies for the treatment of skin diseases, by targeting the skin NT network.

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