Chapter 4

# Neurotrophin and Neurotrophin Receptor Involvement in Human Neuroblastoma

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## 1. Introduction

Neuroblastoma (NB) is an embryonic tumour that originates from cells of the neural crest (NC) arrested in their differentiation at different stages along the sympatho-adrenal lineage and, less frequently, from precursors of sensory neurons [1, 2]. As a consequence, NB can occur throughout the sympathetic chain from thoracic, abdominal and pelvic sites to the adrenal medulla, which accounts for the majority of NBs. Consistent with this, NBs exhibit a high degree of genetic heterogeneity and biological variability, including differences in catecholamine expression, according to their differentiation state along the sympathoadrenal lineage, with a small number of primitive midline and spinal NBs that do not secrete catecholamines considered to be of dorsal root sensory origin [1, 2].

Sympathetic nervous system development is orchestrated by neurotrophins (NT) and their respective neurotrophin receptors (NTR), which exhibit subtle temporal and spatial changes in expression that are critical for the delamination, migration, proliferation, survival, differentiation and apoptotic programs of NC lineages that form the fully differentiated and functional sympathetic nervous system. Not surprisingly NBs, consistent with their origin and particular differentiation state at the time of transformation, exhibit a variety of different patterns of NT and NTR expression. A great deal of research has focussed on characterising and exploiting these different patterns of expression for potential prognostic and therapeutic benefit. Recent studies have led to exciting new developments in understanding how blockages in sympathetic differentiation promote NB and how NBs utilise different patterns of NT

and NTR expression to select a more malignant, stress-resistant, invasive, genetically unstable, stem cell-like phenotype. Furthermore, they have also identified novel potential therapeutic targets and characterised patterns of NT/NTR expression of value in prognosis and therapeutic choice. In this chapter therefore, we will review the origins of NB during neural crest migation and sympathetic nervous system development, introduce NTs and NTRs and describe their roles NC and sympathetic nervous system development, examine patterns of NT/NTR expression in NB, review their potential roles in regulating spontaneous NB regression and metastatic NB progression, and discuss potential therapeutic ways to target the NT/NTR system in NB.

## 2. Formation of the neural crest, neural crest cell delamination and migration

NBs originate from NC cells (NCC) during sympathetic nervous system development. In this section therefore, we will briefly describe the natural history of neural crest, sensory dorsal root and sympathetic nervous system development, focussing attention on the sympatho-adrenal neuroblast lineage, which is responsible for generating neuroendocrine chromaffin tissues, SIF and ganglion cells, and in particular the adrenal medulla within which the majority (40-50%) of NBs develop [2, 3].

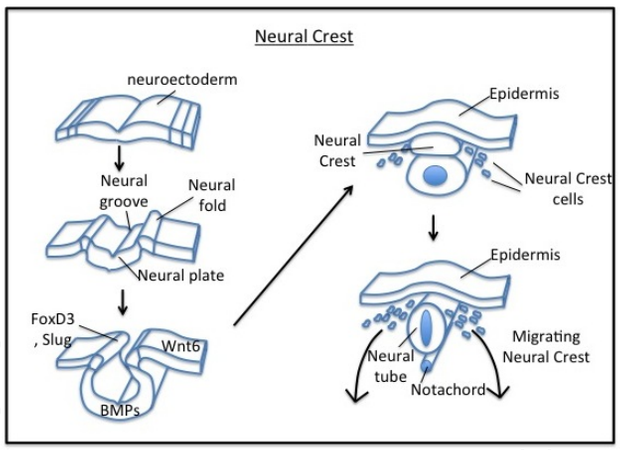


**Figure 1.** Formation of the Neural Crest and Neural Crest cell migration

During the 3rd week of human embryonic development the intra-embryonic mesoderm differentiates into paraxial, intermediate and lateral plate portions. The paraxial mesoderm organises into primitive segmented somites and the lateral plate mesoderm splits into somatic (parietal) and splanchnic (visceral) layers. This event occurs in a BMP-induced Notch-dependent "clock" and Wnt-dependent "wave" manner in a rostral to caudal gradient of FGF [2, 4-6] and results in the simultaneous formation of Somite pairs either side of the forming neural tube, in a head to tail direction along the entire length of the embryo, with

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each new somite forming on the caudal side of an existing somite. Somites further differentiate into dermomyotome and sclerotome structures that will eventually provide the cells for skin, muscle and skeletal formation. Contemporarily, the embryonic neuroectoderm undergoes progressive indentation to form the neural groove, neural folds and neural plate. This neurulation process causes the fusion of opposing neural folds at the future upper cervical level, which progresses in both rostral and caudal directions, eventually resulting in continuity between neural and squamous surface ectoderm. This event separates the presumptive epidermis from the neural plate, which in turn forms the distinct and separate columnar cellular structure of the Neural Tube. Interaction between the neural plate and presumptive epidermis is regulated by Wnts, BMPs and FGFs and results in mesenchymal transformation of the epithelial cells that line the margins of the neural fold. These cells organise between the epidermis and neural tube to form the transient Neural Crest (NC) embryonic structure [2, 6] (Fig. 1).

NC cells (NCC) delaminate from the NC and migrate initially in a ventrolateral manner and later in a dorsolateral direction, relative to the somites. Ventrolateral NCC migration occurs in chain-like manner [7] between the somites and neural tube and the rostral half of each somite [8]. NCC initially migrate through the inter-somitic boundary before switching to a sclerotome pathway controlled by semaphorin and its receptor neuropilin, with the entire dermomytome repulsing neuropilin positive trunk NCC [9] (Fig. 2).

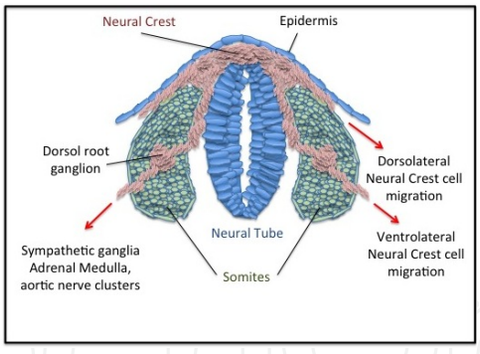


**Figure 2.** Neural Crest cell ventrolateral and dorsolateral migration

Dorsolateral NCC migration occurs between the developing dermis and the dorsal dermomyotome boundary [8, 10]. During NCC migration cells receive signals from adjacent structures that initiate a series of differentiation processes that will eventually lead to differentiation-commitment and specific cell fates at different locations. This process provides a wide variety of differentiated tissues, including: epidermal pigment cells (melanocytes); dorsal root, sympathetic and parasympathetic ganglia, neurons and plexuses; neuroglial and Schwann cells; endocrine/paracrine cells of the adrenal medulla, carotid body and organ of Zuckerland; cartilage and bones of the facial and ventral skull; corneal endothelium and stroma; tooth papillae; dermis, smooth muscle and adipose tissue of the head and neck;

connective tissue of the salivary, lachrymal, thymus, thyroid and pituitary glands and connective tissue and smooth muscle in arteries of aortic origin (Fig. 3).

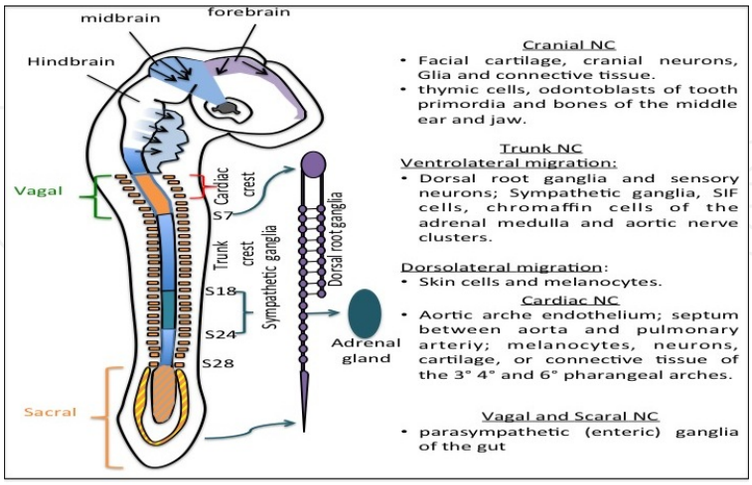


**Figure 3.** Neural crest cell destinations during embryonic development

## 3. The sympathetic nervous system

The vertebrate nervous system is composed of the central (CNS) and peripheral (PNS) nervous systems, the former comprised of the brain and spinal cord and the latter comprised of ganglia and associated plexuses that innervate and connect visceral organs and other tissues to the CNS.

The PNS is divided into the somatic and autonomic nervous systems, the former responsible for skeletal muscle function and the latter for innervation of visceral organs [11, 12]. The autonomic nervous system is further subdivided into sympathetic (SNS) and parasympathetic (PSNS) nervous systems, which are often antagonistic. Motor outflow from both systems is formed by serially connected neurons that initiate with pre-ganglionic neurons of the brain stem or spinal chord, which synapse with ganglia and post ganglion neurones outside the CNS. Parasympathetic ganglia lie close to or within the organs they innervate, whereas sympathetic ganglia lie at some distance from their target organ. Both have sensory fibres that feedback information concerning organ function to the central nervous system [11, 13].

The NC is fundamental for SNS formation. Pluripotent migratory NCC progenitors delaminate from the NC and migrate in a vetrolateral direction through the rostral half of each somite. NCC remaining within somites coalesce to form paraspinal dorsal root ganglia, which contain the nerve bodies of afferent spinal nerves responsible for relaying sensory information into the CNS. NCC that exit somites ventrolaterally initially lose segmental organisation, mix adjacent to the dorsal aorta then re-segregate to form sympathetic ganglia, helping to explain sympathetic ganglia heterogeneity [7]. At this point cells initiate differentiation that is responsible for the eventual formation of sympathetic ganglia, associated sympathetic neurones and

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Schwann cells, small intensely fluorescent (SIF) cells and chromaffin cells of the adrenal medulla and extra adrenal paraganglia (Fig. 4). Together, these components form the neuroendocrine SNS, which consists of preganglionic neurones that exit from spinal chord ventral routes of the 12 thoracic and 3 lumbar spinal segments that synapse with neurons of the sympathetic ganglia or specialised chromaffin cells of adrenal medulla and paraganglia. Sympathetic ganglia include paravertebral and prevertebral ganglia, with pairs of paravertebral ganglia each side of the vertebra interconnected to form the sympathetic chain. Normally, there are 21 to 22 pairs of paravertebral sympathetic ganglia, 3 cervical, 10-11 thoracic, 4 lumbar, 4 sacral and a single ganglion impar in front of the coccyx. Cervical superior, middle and stellar ganglia innervate viscera of the head and neck, thoracic ganglia innervate viscera of the trunk, and lumbar/sacral ganglia innervate the pelvic floor and lower limbs. Sympathetic ganglia also innervate blood vessels, muscle, skin, erector pilli and sweat glands [11, 13].

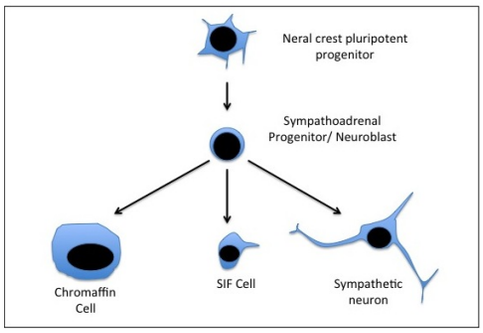


**Figure 4.** Cell types generated from differentiated sympathoadrenal neuroblast progentitors

In general, preganglionic neurons utilise acetylcholine as the major neurotransmitter, whereas post-ganglionic neurons are noradrenergic and utilise noradrenalin as the major neurotransmitter, combined with specific neuropeptide transmitters (e.g. neuropeptide Y, somatostatin, vasointestinal peptide and calcitonin related peptide), utilised in an organ-specific manner. Under normal conditions the sympathetic nervous system provides local adjustments (e.g. sweating) and relax adjustment to the cardiovascular system. Under conditions of stress, the entire SNS can activate to induce the "fight or flight" response, during which adrenalin released from the adrenal gland leads to rapid increases in heart rate, cardiac output, skeletal muscle vasodilation, cutaneous and gastrointestinal vasoconstriction, pupil dilation, bronchial dilation and pili-erection, in preparation for imminent danger [11, 13].

### 3.1. Sympatho-adrenal progenitors, SIF and Chromaffin cells of the neuroendocrine SNS

The vast majority (40-50%) of NBs arise from neuroblastic NCCs within the developing adrenal gland [2, 3]. Therefore, a description of normal adrenal gland development is also warranted at this point.

The fully developed functional adrenal gland is composed of cortex and medulla. The adrenal medulla is composed of neuroendocrine-differentiated chromaffin, SIF and ganglion cells, which are also present in extra-adrenal paraganglia of the carotid body and organ of Zucker-

land [14]. Chromaffin and SIF cells, characterised by their affinity from chromium salts, are closely related to sympathetic neurons and, like sympathetic neurons, synthesise, store, uptake and release catecholamines and express enzymes for noradrenalin synthesis including tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH). Unlike sympathetic neurons, chromaffin cells also synthesise, store and release adrenalin and retain their capacity proliferate but do not produce axons or dendrites [15]. Adrenal and extra-adrenal chromaffin tissues, like sympathetic ganglia, are innervated by pre-ganglionic neurones originating from the spinal chord [16]. Chromaffin, SIF and sympathetic neurons exemplify the wide spectrum of sympathoadrenal cell types that originate from NCC [17].

Chromaffin and SIF cells differentiate from pluripotent NCC progenitors that delaminate from the NC at the "adreno-medullary" somite level (somites 18-24 in avian development) [18]. These cells migrate ventrolaterally initially between Somite dermomyotome and sclerotome then through the rostral sclerotome mesenchyme to arrive at para-aortic sites [18-20]. At these sites, NCC mix, re-segregate and coalesce to form sympathetic ganglia. At the same time, NCC derived from the "adreo-medullar" somite region coalesce adjacent to the adrenal cortex anlage then invade the anlage in considerable numbers, initially in a nerve fibre-independent then nerve fibre-dependent manner [21], in a Sox transcription factor-dependent manner [22]. Once within the adrenal primordium, NCCs form rosettes, nests and nodules along nerve fibres, proliferate and initiate pheo-chromoblast differentiation. This process continues throughout foetal development and into the neonatal period, providing differentiated Chromaffin and SIF adrenal medulla cell populations. In humans, the gestational period between 17 and 20 weeks is critical for adrenal sympathetic component development, with neuroblastic NCC proliferation peaking during this period in terms of maximal nodule size and number, waning thereafter. Neuroblastic nodules tend to disappear during the third trimester and are usually absent at birth. However, nodules that continue to grow and persist into neonatal life are not infrequent and have been classified as *in situ* NB. A sizeable number of these NBs spontaneously regress and are likely, therefore, to represent delayed differentiation in addition to neoplastic transformation [23].

Chromaffin cells, SIF cells and sympathetic neurons develop from catecholaminergic sympathoadrenal (SA) progenitors [18, 24, 25] and their formation involves BMP signalling [18, 26-28]. However, the classical concept that a common SA lineage acquires neuronal and catecholaminergic traits prior to migration to secondary sympathetic ganglia and adrenal sites [24, 25] has now been discounted, as chromaffin cells undergo catecholaminergic differentiation within the adrenal anlage and not within primary sympathetic ganglia, and do not express neuronal markers at the onset or even following induction of TH expression [29, 30]. Therefore, sympathetic neuronal and chromaffin lineages must separate upstream prior to catecholaminergic differentiation, despite evidence of sympathoadrenal marker expression in some migrating cells [22, 24, 30], and enter the adrenal primordium as undifferentiated Sox10 expressing NCC [22, 30]. Indeed, chromaffin and sympathetic neurones originate at the same axial level from common NC progenitors but differ in the time of catecholaminergic differentiation [18]. Furthermore, NCC populations migrating to the adrenal anlage and within the

adrenal medulla exhibit heterogeneity, and consist of SOX10/Phox2B/p75NTR, SOX10/p75NTR and PHox2B/p75$^{NTR}$ sub-populations [22].

### 3.2. Transcriptional regulation of sympathoadrenal differentiation

Chromaffin and sympathetic neuron differentiation is regulated by BMP-induced transcription factors Phox2B, Mash-1, Insm1, Hand2 and Gata 2/3 [31]. Knockout technology has identified a fundamental role for Phox2B in chromaffin and sympathetic neuronal differentiation [18], with Phox2B knockout increasing neuron but not chromaffin precursor death. This not only relates to specific cell traits but also differences in environment and migration [32, 33], and confirms that adrenal anlage are colonised by undifferentiated NCC progenitors. Knockout technology has also characterised a role for Mash-1 as an accelerator of sympathetic neuronal and chromaffin differentiation [34, 35], a role for Insm-1 as a regulator of catecholamine synthesising enzyme expression and, therefore, endocrine differentiation [36, 37], a role for Hand2 in the induction and maintenance of noradrenergic differentiation [38, 39] and a role for Gata3 in the differentiation of both sympathetic ganglia and chromaffin cells [31, 40].

It has now been confirmed that adrenal cortex glucocorticoids are not responsible for chromaffin cell differentiation [41] but they do, however, regulate postnatal chromaffin cell survival and phenyl ethanolamine N-methyl transferase expression [30, 42]. The adrenal cortex is also dispensable for chromaffin differentiation, which is also found in extra-adrenal neuroendocrine tissue, but may regulate adrenal chromaffin cell numbers and associated vascularity [30, 43]. Within the adrenal gland, hypoxia has recently been shown to promote chromaffin/SIF cell differentiation from neuroblasts [44-46].

## 4. Neurotrophins and neurotrophin receptors in neural crest, sympathetic nervous system and adrenal development

Neurotrophins (NTs) and NT receptors (NTRs) are critical for the development and maintenance of the vertebral CNS and PNS [47-50], NTs and NTRs are also expressed by human NBs and have been implicated in both NB regression and malignant progression. In this section, therefore, we will introduce NTs and NTRs and describe their potential involvement in normal SNS and adrenal development.

### 4.1. Neurotrophins (NTs)

NTs are a family of growth, differentiation, survival and apoptosis-inducing factors that are involved in many aspects of nervous system development, maintenance and function. They comprise four structurally related basic 115-130 amino acid containing polypeptides, nerve growth factor (NGF), brain-derived growth factor (BDNF), and the neurotrophins 3 (NT-3) and 4/5. NGF was first NT to be described and purified from the mouse salivary gland [51]. This was followed by the discovery of BDNF, NT-3 and NT-4/5 some 30 years later [52-54].

NTs exhibit close structural homology, with the exception of NT4/5 that exhibits only 50% homology to the others NTs, and all contain six conserved cysteines that form structurally important disulphide bridges [55, 56]. NTs are expressed by both neuronal and non-neuronal cells as pre-NTs and are converted to pro-NTs upon signal peptide removal. This can occur within the endoplasmic reticulum (ER), in which NTs are converted to mature-NTs by furins. Alternatively, NTs are transported to the cell surface and released following signal peptide removal as pro-NTs. Secreted pro-NTs, which also exhibit biological activity, are converted to mature NTs by enzymes including plasmin and the matrix metalloproteinases MMP-7 and MMP-9 [56-58]. Within the extracellular environment, pro- and mature NTs form homo-dimers and bind specific receptors to induce an array of biological activities, including cell migration, proliferation, survival, differentiation, apoptosis and neuronal synapse/junction plasticity, depending upon the cell population, receptor expression and activation status [57, 60]. The human *NGF* gene localises to chromosome 1p13.1 [61], the human *BDNF* gene localises to chromosome 11p13 [62], the human *NT-3* gene localises to chromosome 12p13 [62] and the human *NT4/5* gene localises to chromosome 19q13.3 [63]. Since the discovery of NTs, their respective receptors have been identified and many of their roles in nervous system development and function have been elucidated.

### 4.2. NT receptors

#### *4.2.1. Tropomyosin-related kinases TrkA, TrkB and TrkC*

The family of NT receptors includes the tropomyosin-related tyrosine kinases TrkA, TrkB and TrkC [64]. TrkA is the preferred receptor of mature NGF but also binds the mature neurotrophin NT-3 [64, 65]. Identified following the discovery of the first tumour-associated TrkA oncogene [64, 66, 67], the 25kb human *TrkA* gene maps to chromosome 1q21-22 and is organised into 17 exons [68-70]. TrkA proteins are expressed either as the fully spliced gp140kDa TrkAII receptor, alternatively spliced TrkA L0 and L1 variants that exhibit differential exon 2-4 use [71], the TrkAI variant that exhibits exon 9 skipping [72] or the TrkAIII variant, which exhibits in-frame skipping of exons 6 and 7 combined with exon 9 omission [73]. TrkA L0 (exons 2, 3 and 4 alternatively spliced) and TrkA L1 (exons 2 and 3 alternatively spliced) are expressed during rat development [71] as truncated receptors with in-frame deletions of leucine-rich sequences encoded within exons 2-4 [68]. Since, TrkA leucine rich sequences may modulate ligand binding [74], these variants may exhibit altered ligand-binding activity similar to analogous alternative TrkB splice variants [75]. TrkAI (exon 9 exclusion) and TrkAII (exon 9 inclusion) splice variants [72] are expressed as cell surface transmembrane receptors and exon 9 omission does not result in ligand-independent receptor activation. TrkAI and TrkAII variants bind NGF and NT3 [72, 76] but TrkAII exhibits higher levels of NT-3-mediated activation when co-expressed with the low affinity neurotrophin receptor CD271/p75$^{NTR}$ [76]. TrkAII is predominantly expressed within the nervous system, whereas TrkAI expression predominates in the thymus [72].

TrkAIII was identified as an unexpected RT-PCR product in primary human NBs [73]. This variant exhibits exon 6 and 7 skipping plus exon 9 omission, resulting in the in-frame deletion

of amino acids 192-284 that encode the D4 extracellular immunoglobulin-like domain, several functional N-glycosylation sites and introduces a valine substitution at the novel exon 5/8 splice junction [73]. In addition to being expressed by primary human NBs, TrkAIII is also developmentally regulated and is detected from stages E13-E18 of mouse embryonic development and is also expressed by immature thymocytes within the developing thymus [73, 77]. Unlike fully spliced TrkA receptors, TrkAIII is not expressed at the cell surface but is retained within intracellular membranes of the endoplasmic reticulum (ER), GN and ER/GN intermediate compartment (ERGIC) [73, 78, 79], within which it exhibits interphase-restricted spontaneous ligand-independent activation [73, 78, 79].

TrkB is the preferred receptor of BDNF but also binds NT-4/5 [65, 80-82]. The 590kb human *TrkB* gene maps to chromosome 9q22 and contains 24 exons [70, 83]. In addition to fully spliced gp145kDa TrkB, eight TrkB variant isoforms have been described, including a gp95kDa C-terminal truncated receptor that lacks the tyrosine kinase and Shc binding domains; a C-terminal truncated receptor that lacks the tyrosine kinase domain but retains the Shc binding site; a C-terminal truncated receptor that lacks exons 23 and 24 but retains tyrosine kinase activity and four N-terminal truncated receptors that exclude combinations of exons 1-5 and upstream signal sequence [75, 83-85]. The *TrkB* gene has also been reported to encode up to 100 different transcripts ranging from 0.7-9kb, at least 36 of which can be translated into functional TrkB proteins [85-87]. Both full length and C-terminal truncated TrkB receptors are expressed in the brain and share 100% extracellular domain homology, consisting of 5 highly glycosylated extracellular binding domains (D1-5) [75, 85, 86].

TrkC binds NT-3 and no other NT [88]. The 387kb human *TrkC* gene maps to chromosome 15q25 and is organised into 18 exons [70] and six TrkC isoforms have been described. In addition to the fully spliced gp145kDa receptor, these isoforms include C14/K2, C25/K3 and C39 variants which contain 14, 25 and 39 additional amino acid insertions between kinase subdomains VII and VIII, downstream of the TDYYR motif of the putative Trk receptor family autophosphorylation site [89] and NC1/T1 and NC-2/T2 non-catalytic variants truncated in the tyrosine kinase domain by short C-terminal sequences [90-92]. Full-length TrkC receptors are expressed during development, whereas truncated receptors predominate in later life in post mitotic cerebellar granule neurons and young stem cell-derived differentiated neurons but not in proliferating neural stem cells. TrkC NC1/T1 and NC2/T2 variants do not support NT-induced neuritogenesis, suggesting that TrkC variants could exert different roles during nervous system development [90, 93].

#### *4.2.2. CD271/p75 neurotrophin receptor*

The p75 neurotrophin receptor (CD271/p75$^{NTR}$) is a member of the tumour necrosis factor (TNFR)/FAS receptor superfamily and binds all NTs in pro-form with high affinity and mature NGF with low affinity [94, 95]. The 3.4kb *CD271/p75$^{NTR}$* gene is organised into 5 exons and maps to chromosome 17q21-q22 [96]. In addition to the fully spliced 75kDa CD271/p75$^{NTR}$ receptor, a truncated alternative s-p75$^{NTR}$ splice variant has been described that is devoid of exon III. S-p75$^{NTR}$ lacks the NT binding domain, does not bind NTs and is expressed by several neural tissues [97]. The fully spliced CD271/p75$^{NTR}$ extracellular-domain contains four 40-

amino acid repeats with 6 cysteine residues at conserved positions that are required for NT binding, a serine/threonine-rich region, a single transmembrane domain and a 155-amino acid cytoplasmic domain, which does not exhibit catalytic activity. CD271/p75$^{NTR}$ acts either as an independent NT receptor, a NT receptor complex with Sortilin or a co-receptor for TrkA, and is involved in regulating death, differentiation or survival signals [94, 98]. The CD271/P75$^{NTR}$ receptor is devoid of intrinsic catalytic activity, indicating that signalling from this receptor must depend upon intracellular interactors [99, 100].

*4.2.3. Sortilin*

Sortilin is a member of the Vps10p domain-containing transmembrane proteins that binds both mature NGF and the neurotrophins NGF, BDNF and NT-3 in pro-form [98, 101, 102]. The 7kb human *Sortilin* gene localises to chromosome 1p13.3 and is expressed as a gp95-100kda glycoprotein [103, 104]. Sortilin co-expression with CD271/p75$^{NTR}$ results in the formation of a co-receptor complex that augments affinity for proNGF and acts principally as an inducer of apoptosis [105].

### 4.3. NT receptor structure and ligand binding

All three Trk receptors share significant sequence homology and a conserved domain organization. This organization comprises from N-terminus to C-terminus of five extracellular domains, a transmembrane region and the intracellular kinase domain.

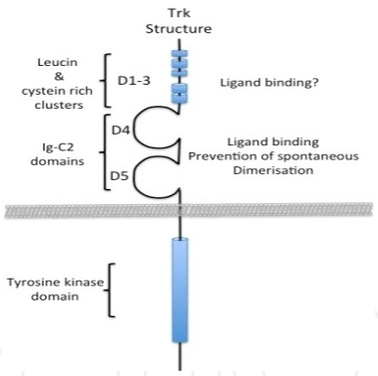


**Figure 5.** NT receptor structure and ligand binding domains

The first three extracellular domains consist of a leucine-rich region (D-2) flanked by two cysteine-rich regions (D-1 and D-3), and domains 4 and 5 are immunoglobulin-like domains. Studies on TrkB and TrkC have shown that D-5 is sufficient for the binding of ligands and is responsible for binding specificity [106-109], although the D-4 domain, leucines and cysteine clusters may regulate ligand binding [55, 73]. Receptor transmembrane and juxta-membrane regions are critical for signal internalisation and transduction. The intracellular tyrosine-rich carboxyl terminal cytoplasmic domain exhibits tyrosine kinase activity upon ligand-mediated activation and is responsible for propagating post-receptor signal transduction [74, 107, 110-114]. The immunoglobulin-like D4 and D5 domains stabilise receptors in monomeric form

and prevent spontaneous receptor oligomerisation and activation. Deletions, chimeric receptors and point mutations that disrupt the structure of the first (D4) and second (D5) immunoglobulin-like domains result in ligand-independent spontaneous receptor activation and the acquisition of oncogenic activity [73, 110, 115] (Fig. 5).

CD271/p75$^{NTR}$ receptors modulate the affinity and enhance the specificity of TrkA for NGF, and TrkB for BDNF, with optimal affinity reflecting the ratio of Trk to CD271/p75$^{NTR}$ receptors [116-118]. In contrast, CD271/p75$^{NTR}$ reduces TrkAI activity in response to NT-3 and TrkB activity in response to NT-3 and NT-4/5 [76, 119, 120]. The CD271/p75$^{NTR}$ receptor analogue neurotrophin-related homolog-2 (NRH2) that is expressed by neural cells, also interacts with TrkA to promote high affinity NGF binding [121].

### 4.4. NT receptor signalling

In the absence of ligand, Trk receptors are maintained as inactive oligomers [120], concentrated within caveolin and cholesterol-containing cell membrane caveolae invaginations, which also contain components of the Ras signalling pathway [122]. Receptor oligomers are maintained in an inactive state by mature extracellular domain N-glycosylation, intact D4 and D5 domains and by receptor-associated protein tyrosine phosphatases (PTPases) [110, 123-126]. Upon ligand binding, oligomeric Trk receptors dimerize, alter their conformation and acquire tyrosine kinase activity, facilitated by temporary inactivation of receptor-associated PTPases, which results in auto- and trans-phosphorylation of receptor tyrosine residues Y490, Y670, Y674/675, Y751 and Y785, in TrkA and their equivalents in TrkB and TrkC. These tyrosines act as phosphorylation-dependent binding sites for a variety of signalling proteins, including the adapters Shc and FRS-2; Grb-2 and SOS; the IP3K subunit p85$\alpha$ and PLC$\gamma$. These interactions, which are modulated by CD271/p75$^{NTR,}$ provide avenues for signal transduction through RAS/MAPK, IP3K/Akt/NF-κB and PKC pathways that mediate NT effects upon migration, proliferation, survival, differentiation and apoptosis [73, 111, 127-141]. Cell surface Trk localisation and NT-mediated Trk activation also involves interaction with the heat shock protein chaperone Hsp90 [78].

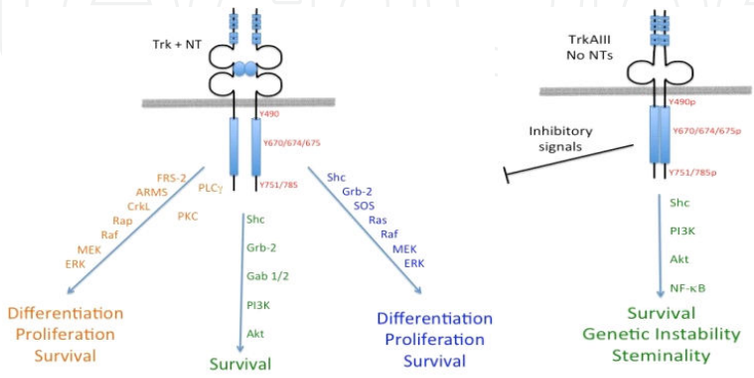


**Figure 6.** Trk receptor signalling and outcome

Trk receptors activated by NTs use two main pathways to activate MAPKs. The first pathway involves Shc, Grb-2, SOS, Ras and Raf, and the second pathway involves CrkL, Rap and Raf [142, 143]. Trk activation of MAPK is now considered to depend not only upon the phosphorylated Trk Y490 tyrosine residue [144, 145] but also the ankyrin repeat-rich membrane spanning protein ARMS, acting through CrkL [146, 147]. MAPKs activate CREB transcription factors to promote differentiation and survival [148-150]. Trk activation of PI3K/Akt signalling occurs through Shc/Grb-2 and Gab-1 and induces pro-survival signals [73, 151, 152], resulting from the phosphorylation of Bad and activates the pro-survival transcription factor NF-κB [73, 153, 154]. PLCγ is activated as a consequence of being recruited to the phosphorylated Trk tyrosine Y785 [73, 132] and provides additional differentiation and survival signals that involve MAPK [155] (Fig. 6).

The alterative TrkAIII splice variant, in contrast to other Trk receptors (see above), is not expressed at the cell surface but accumulates within intracellular membranes. Intracellular TrkAIII does not bind extracellular NTs and is prone to spontaneous ligand-independent intracellular activation [73, 78, 79]. In contrast to ligand activated cell surface TrkA signalling, spontaneously active TrkAIII signals through PI3K/Akt/NF-κB but not Ras/MAPK, resulting in increased survival and the induction/maintenance of a stem cell-like undifferentiated phenotype [73, 78, 79, 156] (Fig. 6).

An additional feature of TrkA receptors is retrograde transport signalling within the cell. This depends upon receptor/ligand interaction, internalisation and retrograde transport of activated receptors, resulting in signal transduction within the cell body. Sympathetic neurons most dramatically illustrate this activity, with retrograde transport of NGF-activated TrkA occurring along the axonal length to the neuronal cell body. This phenomenon involves ubiquitin mediated receptor internalisation through interaction with CD271/$p75^{NTR}$ and TRAF6, receptor endocytosis within clatherin-coated vesicles and receptor endocytosis facilitated by the endocytosis inducing protein EHD4/Pincher [157-159]. In addition, immature Trk receptors also localise to intracellular membranes of the Golgi Network (GN) and can be trans-activated by agonists of the G-protein linked $A_{2A}$ adenosine receptors, potentially through the non-receptor tyrosine kinase Src [160, 161], providing evidence for intracellular neurotrophin-independent Trk activation. Post receptor signal transduction from GN-associated TrkA differs from cell surface-activated TrkA, by signalling through IP3K/Akt but not RAS/MAPK, which results in NF-κB transcription factor activation, inducing a more stress-resistant phenotype, not dissimilar to that induced by the intracellular alternative TrkAIII splice variant [73, 124, 160]. TrkA localisation to the GN may not only reflect transient passage of de-novo synthesised receptors but also alterations in receptor extracellular domain N-glycosylation and folding.

CD271/$p75^{NTR}$ receptors regulate cell survival, apoptosis, differentiation and proliferation. CD271/$p75^{NTR}$ is a positive modulator of Trk-mediated survival, and within this context, it is likely that CD271/$p75^{NTR}$ does not directly bind NTs in competition with Trks [162] but acts as a co-receptor, interacting with Trk dimers ligated to active NTs, refining receptor specificity (e.g. increasing specificity for NGF, while restricting NT-3 binding) [163]. This may be responsible for shifting NT dependence during development coincident with CD271/$p75^{NTR}$

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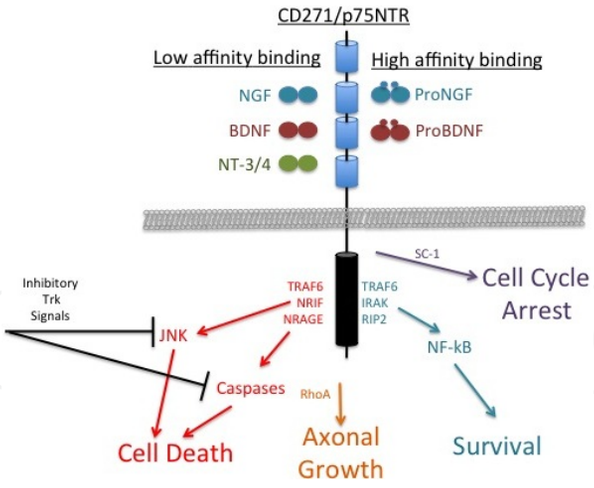


**Figure 7.** CD271/p75$^{NTR}$ receptor signalling and outcome

expression, which is exemplified by the shift from NT-3/TrkC to NGF/TrkA dependence observed during SNS development [164]. CD271/p75$^{NTR}$ may also influence Trk signalling by binding of the Shc adapter, which also binds to activated Trk, to augment or inhibit Trk signalling [165, 166], and in Trk-complexed form may result in different signalling to that from Trk dimers alone [147], resulting in differences in capacity to complete differentiation programs [167] (Fig. 7).

As a pro-apoptotic receptor CD271/p75$^{NTR}$ also exhibits Trk-independent activity. The cytoplasmic tail of CD271/p75$^{NTR}$ contains death domains and its role in apoptosis has been clearly demonstrated in CD271/p75$^{NTR}$ exon 3 knockout mice [168]. CD271/p75$^{NTR}$ exon 3 knockout mice combined with TrkA knockout mice have highlighted the dual function for CD271/p75$^{NTR}$ in refining innervation and eliminating neuronal excess during early development and later in neuronal survival [169, 170]. Apoptosis induced by CD271/p75$^{NTR}$ involves JNK, phosphorylated c-jun, p53, Bad, Bim and activated caspases [168, 169, 171-174]. Apoptosis induced by CD271/p75$^{NTR}$ may also involve β-secretase-mediated release of the intra-cytoplasmic domain, its subsequent nuclear transport and potential involvement in transcriptional regulation, together with TRAF6, NRAGE, NADE, NRIF and SC-1. TRAF6 interaction with NRIF has been implicated in the generation of death signals through the activation of JNK [169, 175]. NRAGE interaction with CD271/p75$^{NTR}$ is involved in inducing cell death through JNK and caspase activation, and is blocked by TrkA [176]. A role for NADE in CD271/p75$^{NTR}$–mediated apoptosis, involving NGF but not BDNF or NT-3, has been reported [177], whereas CD271/p75$^{NTR}$ interaction with SC-1 has been implicated in cell cycle arrest via transcriptional repression of cyclins [178] (Fig. 7). Further advances in the understanding of this effect have come with the observation that inactive pro-form NT precursors bind CD271/p75$^{NTR}$ receptors with high affinity and trigger apoptosis at far lower concentrations than active counterparts, which bind with low affinity (Lee et al., 2001). Up to 60% of NTs released by cells are proform [56]. Indeed proNGF induces death in CD271/p75$^{NTR}$ expressing cells, highlighting an opposite effect to activated NGF in cells, including sympathetic neurones [56]. The capacity of proNGF to activate CD271/p75$^{NTR}$ but not TrkA is now known to depend upon Sortilin, a 95kDa member

of the Vps10p-domain receptor family [98, 101]. In this interaction, complexes between CD271/p75$^{NTR}$ and Sortilin are augmented by proNGF, which simultaneously binds both receptors to induce apoptosis. Thus Sortilin acts as an essential co-receptor capable of switching cells that co-express TrkA and CD271/p75$^{NTR}$ from survival to apoptosis.

CD271/p75$^{NTR}$ receptors, therefore, promote either survival or death in response to NTs, depending upon NT status and the cellular context. Survival through CD271/p75$^{NTR}$ receptors involves NF-κB activated through TRFA6, p62, Interleukin-1 receptor-associated kinase IRAK and receptor interacting protein RIP2 [179]. CD271/p75$^{NTR}$ promotion of axon growth involves neurotrophin-mediated dissociation of axonal growth inhibitory complexes between CD271/p75$^{NTR}$ and the G-protein Rho [180]. Furthermore, the proteolytic shedding of cell surface CD271/p75$^{NTR}$ releases an intracellular domain that moves to the nucleus and may act as a transcription factor [181].

### 4.5. Trks A and C are dependence receptors

A classical concept is that NT activation of Trk receptors inhibits default apoptotic programs to promote NT-dependent survival [134]. This concept is considered to involve PI3K/Akt/NF-κB signalling and induction of Bcl-2 inhibitor of mitochondrial apoptosis. In this mechanism, NT depletion results in the turning-off of PI3K/Akt signalling, which reduces Bad phosphorylation and releases it from the chaperone 14-3-3. This results in Bcl-2 and Bcl-XL sequestration [182, 183], reduces FOX03A phosphorylation resulting in nuclear translocation, induces pro-apoptotic FAS, Trail, Puma and BIM transcription [184-188], abrogates CREB and NF-κB survival signals [189, 190] and activates pro-apoptotic JNK, inducing BIM expression [188], which together trigger apoptosis (Fig. 8).

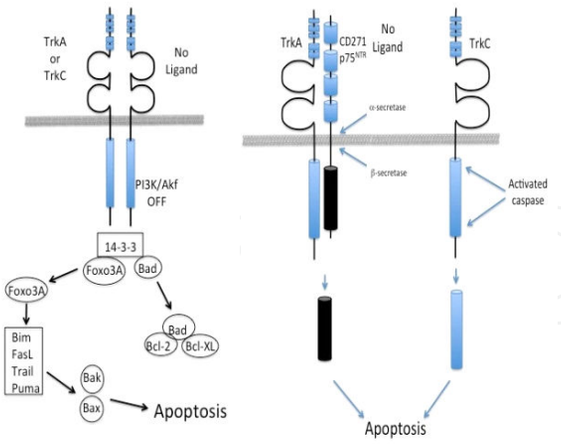


**Figure 8.** TrkA and TrkC receptors and Apoptosis

Recently, however, both TrkA and TrkC have also been characterised as true dependence receptors. In one study, TrkC but not TrkA or TrkB triggered apoptosis in the absence of NT-3 in a variety of cell lines by an activated caspase-dependent cleavage mechanism, releasing a pro-apoptotic intracellular TrkC domain capable of inducing caspase-9 dependent death [191].

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In a separate study, TrkA and TrkC but not TrkB induced apoptosis in neurons differentiated from stable transfected embryonic stem cells and promoted loss of all TrkA and TrkC but not TrkB transfected cells with associated loss of nervous system at E13.5 during mouse embryonic development, through a CD271/p75$^{NTR}$ -mediated mechanism, in which CD271/p75$^{NTR}$ is recruited as a "hired killer" [192]. Therefore in the absence of ligand, TrkC acts directly as a death receptor and TrkA death receptor activity appears to depend upon CD271/p75$^{NTR}$, whereas TrkB does not exhibit death receptor activity (Fig. 8)

## 5. NTs and NTRs in sympathetic nervous system development

### 5.1. TrkC and NT–3

TrkC is the only NT receptor expressed during early embryogenesis. During avian development TrkC expression coincides with neurulation and is detected in both neural tube and neural plate anlage [193, 194]. TrkC is also expressed in hindbrain rhombomeres 3 and 5. This, however, does not associate with lateral NCC migration, suggesting that either TrkC positive NCC cells die prior to NCC migration or that they migrate away from these regions. NT-3 expression is low at this time and the recent characterisation of TrkC as pro-apoptotic dependency receptor, supports the former hypothesis [191, 192]. Neither TrkC nor NT-3 knockout prevent neurulation but do result in neuronal loss from sympathetic ganglia [195-197], indicating that TrkC/NT-3 interactions are not required for neurulation but are required for later stages of SNS development. Consistent with this, the NT-3 protein is detected at later developmental stages. There have been no reports concerning the expression of alternative TrkC isoforms during early development.

During PNS formation, TrkC is expressed by neurogenic pre-migratory and migrating NCC subsets [194, 198, 199] and TrkC/NT-3 interactions are required prior to NCC arrival at destination [200]. Indeed, NT-3 acts as a NCC survival factor and promotes NCC proliferation in the presence of somites [201]. Furthermore, somites express NT-3 during this period [198, 202], sympathetic neuroblasts and neurons also express TrkC and NT-3, NT-3 is expressed by non-neuronal sympathetic cells [194, 199, 203], and NT-3 and TrkC expression during this time is stimulated by neuroregulin, PDGF and CNTF [204]. NT-3 in sympathetic tissues increases mature neuron numbers by promoting the survival of proliferating neuroblast and their subsequent differentiation, without directly effecting proliferation [205]. This temporary effect subsequently declines with a switch to NGF-dependence [206], associated with reduced TrkC expression and the induction of TrkA and later CD271/p75$^{NTR}$ expression [204, 207]. NT-3 continues to be expressed by both sympathetic neural and non-neural cells [198, 199, 204], by adult non-neural cells [208] and TrkA expression is regulated in part by NT-3 [207]. Therefore, NT-3 acts as both a survival and differentiating factor through TrkA, eventually rendering differentiating post-mitotic neurons dependent upon NGF produced by effector tissues. NT-3, at this stage, acts as an autocrine interim and not peripherally derived paracrine factor, corroborated by the lack of target innervation at this time [207]. In support of this, NT-3 knockout mice exhibit sympathectomy [106, 197, 209-212] caused principally by neuroblast

apoptosis, which is partially rescued by exogenous NGF [212]. In the adult, NT-3 continues to be expressed by a wide range of tissues [202, 203, 213, 214] and, together with NGF, continues to be important for post-natal sympathetic neuron survival [214, 215]. Consistent with this, exogenous NT-3 promotes target organ sympathetic innervation in NT-3 knockout animals [211, 212], suggesting that the switch from NT-3 to NGF dependence observed in sympathetic neurons *in vitro* [216] does not actually occur *in vivo*. This may relate to environmental differences, corroborated by the mitogenic effect of NT-3 on neuroblasts *in vitro* [201] but not *in vivo* [217], the susceptibility of TrkC transcription to environmental factors and also by the capacity of NT-3 to bind and activate TrkA receptors, and in particular TrkAII [76]. This helps to explain how NT-3 rescues NGF-dependent neurons from NGF depletion and *vice versa* and is consistent with the characterisation of TrkA as a functional NT-3 receptor *in vivo*. However, one difference between these two NTs is that exogenous NGF but not NT-3 induces sympathetic ganglia hyperplasia [218].

NT-3 released from effector tissues and acting through TrkA also promotes sympathetic innervation of target organs [202, 213, 214, 219, 220]. In support of this, effector tissue elimination induces the death of innervating neurons, which cannot be completely reversed by exogenous NGF alone, and adult sympathetic neurons expressing TrkA are immunoreactive for both NGF and NT-3 [49, 221, 222]. Therefore, NT-3 plays an important role throughout sympathetic neuron life-cycle from neuroblast to neuron, acting initially through the TrkC receptor as an autocrine/paracrine factor stimulator of migration and survival in proliferating sympathetic neuroblasts and later as a paracrine promoter of sympathetic neuron differentiation, survival and target organ innervation acting through the TrkA receptor. CD271/p75$^{NTR}$ is also required for optimal neurotrophin sensitivity since CD271/p75$^{NTR}$ deficient dorsal root and sympathetic neurons exhibit reduced sensitivity to NGF [223].

During sympathoadrenal development, progenitors switch from being dependent upon NT3 and TrkC to dependence upon NGF and TrkA, through an intermediate stage of combined TrkA and TrkC expression. In murine thoracic sympathetic ganglia TrkC expression alone is detected at E14-15, whereas both TrkA and TrkC expression are detected at E16.5-17 and only TrkA at E19.5 [224]. Interestingly, sympathetic chromaffin tissues of the adrenal medulla and paraganglia, which form in parallel to sympathetic ganglia, exhibit differences in NT receptor expression consistent with upstream progenitor separation. This difference is characterised by the expression of TrkC but not TrkA by NCCs migrating into the adrenal anlage, at times when TrkC expression is lost in associated with the induction of TrkA expression by NCCs within sympathetic ganglia [225].

### 5.2. TrkA and NGF

Unlike TrkC, TrkA is not expressed during neurulation, NC development or NCC dorsolateral or vetrolateral migration. In rodent development, TrkA is detected at E12.5 within sensory cranial and spinal dorsal root ganglia and subsequently in the paravertebral sympathetic ganglia [226]. NGF is expressed during the mid-stage of development initiating within CNS structures then within PNS structures at later stages of development [227, 228]. Within the developing adrenal gland NGF exhibits a brief period of post-natal expression, whereas NT-3

is expressed by both the developing and adult adrenal gland [229]. This suggests that TrkA/NGF interactions are of transient importance in adrenal gland development. Consistent with this, both TrkA and NGF knockout mice exhibit a relatively normal adrenal medulla chromaffin cell content, although cholinergic innervation of pre-ganglionic origin is lost in TrkA knockout animals [230], and both chromaffin and SIF cells express TrkA but do not depend upon NGF for survival. Normal NCC progenitors entering the developing adrenal glands express TrkC and begin to express TrkA upon seeding under the influence of the adrenal environment. This event may depend upon NT-3 and/or NT4/5 expressed by the adrenal cortex anlage, with subsequent chromaffin/SIF differentiation and survival regulated by these NTs. In contrast, sympathetic neurones in paravertebral sympathetic ganglia, despite their common origin, express TrkA and require NGF for their development, differentiation and survival [229-231]. In support of this, NGF neutralising antibodies do not delay adrenal development nor induce chromaffin cell degeneration [232]. Differences between human and rodent adrenal development include observations that the adult rat adrenal cortex but not medulla express TrkB or TrkC [229, 230], whereas TrkA immunoreactivity is restricted to the adrenal cortex and TrkC immunoreactivity to the adrenal medulla with no TrkB immunoreactivity detected in the human adult adrenal glands [233]. Interestingly, stress induces a massive release of NGF from salivary glands, which targets adrenal chromaffin cells inducing marked adrenal medullary hyperplasia and catecholamine synthesis through enhanced TH and BDH expression [234-236]. In chromaffin tissues, sympathoadrenal cells of the carotid body express NGF and TrkA, providing an autocrine/paracrine mechanism [237]. Pre-natal and post-natal differentiating and differentiated chromaffin cells express TrkA mRNA within the adrenal medulla [238], which increases with development, at times when NGF expression is all but absent [229]. TrkA knockout eliminates the acetylcholine positive component but does not influence chromaffin content of the adrenal medulla [230], indicating that chromaffin cells, unlike their sympathetic neuronal cousins, do not depend upon NGF/TrkA interactions for survival [232, 239]. Chromaffin cells do, however, respond to NGF with acute hyperplasia [235] and eventual neuronal differentiation [240, 241]. In rodents, immature sympathoblasts within sympathetic ganglia cells express TrkA from E14 onwards and express CD271/$p75^{NTR}$ from E16 to birth, in association with acquisition of NGF-responsiveness [242]. Differentiated neurons within sympathetic ganglia express TrkA but not NGF [208].

### 5.3. TrkB, BDNF and NT4/5

TrkB, like TrkA, is also not expressed during neurulation but is expressed by motor progenitors in hindbrain rhombomere 2 at later stages 9-10 and 12, during avian development, either side of the floor plate in the caudal midbrain, extending through the hindbrain and into the spinal chord [193]. Alternative TrkB splice variant expression has not been assessed during early development.

Following neurulation, TrkB expression is detected within motor neuron progenitors of the ventral neural tube and corresponds to BDNF expression by elements within dorsal neural tube, which coordinate motor neuron development [243]. Consistent with this, both TrkB and

BDNF knockout mice exhibit the loss of motor and sensory neurons from dorsal root, trigeminal, nodose/petrosal, vestibular, and geniculate ganglia [244].

During SNS development, TrkB exhibits expression restricted to sub-populations of pre-ganglionic cells [230, 245], and sympathoblasts within coalescing sympathetic ganglia, which exhibit transient TrkB expression prior to differentiating [225]. Sympathoblasts that express TrkB within coalescing sympathetic ganglia are non-proliferating but do proliferate in response to BDNF *in vitro*, suggesting that the concentration of BDNF within coalescing sympathetic ganglia is sub-threshold at this time [225]. Pre-ganglionic cells respond to BDNF expressed and release by effector tissues, resulting in pre-ganglionic innervation [230]. Within the adrenal gland, chromaffin cells express NT4/5 but not TrkB, which is weekly expressed by the adrenal cortex, providing a neurotrophic source for extra-adrenal TrkB expressing pre-ganglionic neurons located in spinal chord segments T7-T10. Thes cells use adrenal medullary NT-4/5 to project axons into the adrenal medulla in a TrkB-dependent manner [230]. BDNF, on the other hand, is expressed by sympathetic neurones and regulates sympathetic synaptic complexity [246].

The fact that NT4/5 but not TrkB is expressed within the developing adrenal medulla [230, 238, 247] has prompted hypotheses that medullary NT-4/5 may also ligate and activate TrkA receptors expressed by adrenal medullary neuroblasts and chromaffin cells [63, 80, 229, 230]. However, adrenal medullary chromaffin tissues do exhibit rapid stress-induced TrkB expression, which facilitates the adrenal catecholamine response to stress-induced elevation of blood bourn BDNF [248].

### 5.4. CD271/P75$^{NTR}$

CD271/p75$^{NTR}$ is a neural crest marker that is expressed by NC crest stem cells during early development, by NC stem cells in peripheral neural tissues during late development after NCC migration has ceased, and by nerve associated post natal and adult NC stem cells [249]. CD271/p75$^{NTR}$ expressing adult NC stem cells have been identified as a potential origin for adult tumours of the PNS and NC, including adult NB [249, 250]. Within the human foetal adrenal medulla, CD271/p75$^{NTR}$ immunoreactivity is detected in nerve fibres and primitive neuroblast clusters, and in the adult adrenal medulla is detected in nerve fibres, ganglion cells and connective tissue cells of septi but not chromaffin cells [251, 252]. CD271/p75$^{NTR}$ is required for normal sympathetic neuronal death and the death of damaged neurons [253-255]. CD271/p75$^{NTR}$ knockout alters synapses within sympathetic ganglia and reduces sympathetic target organ innervation, consistent with its function in enhancing NT-responsiveness [223, 256].

## 6. Neurotrophins and neurotrophin receptors in human neuroblastoma

### 6.1. TrkA and NGF expression in NB

The cloning of the TrkA receptor in 1991 [113] initiated the study of TrkA expression in human NBs [257]. This initial report detected an inverse relationship between TrkA mRNA

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levels and N-myc amplification and expression, with low to no TrkA mRNA expression associated with poor prognosis. This salient study not only implicated Nmyc in the repression of TrkA expression but also reported moderate to high TrkA expression in non-Nmyc amplified disease. The inverse relationship between Nmyc amplification and expression, low TrkA expression and advanced stage disease has now been confirmed by many studies, and it is generally accepted that low TrkA expression combined with Nmyc amplification and expression characterises unfavourable NB and carries poor prognosis [156, 257-270]. In support of this, NBs that form in the root ganglia of Nmyc transgenic mice also exhibit reduced TrkA expression [271]. Nmyc amplified NBs, however, also exhibit heterogeneity [272] and a small number of these tumours exhibit high TrkA expression and favourable histology [270], suggesting that the relationship between NMYC and TrkA in NB is not always straightforward.

Adding to the observation that moderate to high TrkA levels associate with non-Nmyc amplified NB [257, 258], Shimada and colleagues extended the clinical relationship between TrkA expression in NB to include outcome, prognostic significance, biological relevance and histopathological status. They reported that TrkA expression could not distinguish prognostic groups but could distinguish between Nmyc amplified (low TrkA) and non-Nmyc (high TrkA) amplified NB, between Nmyc amplified NB with favourable (high TrkA) and unfavourable (low TrkA) histology, but could not distinguish between non-Nmyc amplified NB with favourable histology (moderate to high TrkA) and unfavourable histology (moderate to high TrkA) [270]. This contrasts with some reports [257, 258, 264] but not others [263]. Adult NBs are aggressive non-Nmyc amplified tumours that express high TrkA levels and in such bear similarity to non-Nmyc amplified paediatric NBs [156, 268, 273].

Low TrkA expression by Nmyc amplified NBs may relate to an origin along the sympathoadrenal lineage within non-TrkA expressing NCC subpopulations that colonize coalescing sympathetic ganglia, paraganglia and adrenal medulla anlage during development [225]. Alternatively, reduced TrkA expression in Nmyc amplified NBs may occur post transformation, since Nmyc represses TrkA transcription by promoting TrkA promoter methylation and TrkA promoter methylation is detected in Nmyc amplified NBs [274, 275].

Moderate to high TrkA levels in non Nmyc-amplified NBs may also relate to cellular origin within undifferentiated TrkA expressing NCC subpopulations of the sympathetic chain and adrenal primordia [225, 276], or may also occur post-transformation, regulated by NTs, growth factors and/or cytokines [277-279].

Despite elevated TrkA expression in advanced stage non-Nmyc amplified and in a small subgroup of Nmyc amplified NBs with favourable histology [270], full length TrkA exhibits a tumour suppressor function in NB models, suggesting that defects in TrkA receptor signalling occur in NB [280]. Consistent with this, TrkA gene transfection in the absence of CD271/$p75^{NTR}$ restores NGF responsiveness to NB cells, inducing either neuronal differentiation, growth arrest and/or apoptosis in response to NGF [73, 281-284]. Differentiation induced by NGF in TrkA transfected NB cells involves insulin growth factor II [285], RET [286], c-Src [287], protein kinase C-ε [288] and Ras/MAPK/Erk signalling [289, 290], and associates with reduced angiogenic factor expression and angiogenesis resulting in reduced tumorigenic activity [291,

292]. Furthermore, full length TrkA does not promote genetic instability [73, 293] or invasive behaviour of NB cells [294]. Apoptosis induced by TrkA in NB cells is p53-dependent [295], involves the cerebral cavernous malformation 2 protein, CCM2 [296], ERK and caspase-7, and can also be augmented by NGF [297]. As stated above, TrkA may also acts as a true dependency receptor, recruiting CD271/p75$^{NTR}$ as a hired killer to promote apoptosis in the absence of NGF [192]. TrkA responsiveness and specificity for NTs is optimised by CD271/p75$^{NTR}$, which in its own right acts as a Fas-like apoptosis receptor in response to pro-NTs, supporting the hypothesis that NBs that coexpress TrkA and CD271/p75$^{NTR}$ are favourable tumours that carry good prognosis [298, 299]. It should be noted, however, that metastatic bone marrow NB infiltrates induced in SCID mice express TrkA [300] and human NB metastatic bone marrow infiltrates express CD271/p75$^{NTR}$ [301].

Although, TrkA gene rearrangements have not been described in NB, a c.1810 C>T TrkA polymorphism has been detected in approximately 9% of NB, with potential to predict disease relapse in non-Nmyc amplified NB [302].

### 6.2. The alternative TrkAIII splice variant in NB

Anomalies of TrkA expression that do not support an exclusively tumour suppressing role for TrkA in NB, include moderate to high TrkA expression reported in non-Nmyc amplified advanced stage, metastatic unfavourable NBs. These reports may be explained by TrkAIII expression [73], an increase in which was originally reported in advanced stage NB [73], and later confirmed [156, 303, 304]. Recently, TrkAIII expression in a cohort of 500 NBs was found to be significantly higher in high TrkA expressing unfavourable NBs compared to high TrkA expressing favourable NBs ($p<0.0001$) and to correlate with worse prognosis [156]. Furthermore in the latter study, TrkAIII promoted a cancer stem cell NB phenotype [156], helping to explain high TrkA levels in unfavourable non-Nmyc amplified NB, adult NB and a subset of relapsing NBs [73, 156, 270, 303, 304]. In support of this, gene-based outcome prediction studies focussed on exon-specific expression, have identified a TrkA splicing difference between stage I and stage IV non-Myc amplified NBs [305, 306], and an exon gene array analysis using TrkAI/II specific primers, excluding TrkAIII, reported to provide a significant prognostic and predictive statistical advantage, associating high TrkAI/II expression with better prognosis in NB [307].

TrkAIII represents a developmental and stress-regulated TrkA isoform [73, 77] that exhibits spontaneous ligand-independent activation and oncogenic activity in NB models [73, 78, 79] and promotes a nestin, CD117, CD133 and Sox2 positive NB stem cell phenotype [156]. In contrast to full length TrkA, TrkAIII does not restore NGF responsiveness to NB cells nor induce NB cell differentiation or apoptosis [73, 78, 79] but interfers with NGF/TrkA signalling through Ras/MAPK, augments genetic instability by promoting centrosome amplification [79] and promotes angiogenesis by altering the equilibrium between MMP-9, VEGF and thrombospondin, through IP3K/Atk. Together these phenomena promote NB cell xenograft primary [73] and metastatic tumorigenic activity [308]. Furthermore, TrkAIII increases NB cell resistance to stress, doxorubicin and geladanamycin-induced cytotoxicity [73, 78, 79].

TrkAIII expression in human NB cells is regulated by hypoxic stress [73] and by agents that promote stress within the endoplasmic reticulum (unpublished observations). TrkAIII signalling through IP3K/Akt but not Ras/MAPK, combined with interference in NGF/TrkA signalling, would permit tumours to override NT-dependence, whilst promoting survival and staminality to provide a selective advantage [73, 78, 79]. Therefore, TrkAIII expression in non-Nmyc amplified NBs may parallel the selective advantage provided by BDNF/TrkB in Nmyc amplified NBs [309-313], and NT-3/TrkC in a subgroup of advanced stage NBs [314].

It remains to be determined whether TrkAIII can counteract the pro-apoptotic effects of the Sortlin-CD271/p75$^{NTR}$ complex in the presence of pro-NTs or prevent CD271/p75$^{NTR}$-mediated apoptosis in the absence of NTs.

### 6.3. CD271/ p75$^{NTR}$ expression in NB

The CD271/p75$^{NTR}$ low affinity nerve growth factor receptor is a neural crest stem cell marker [249, 250, 315] and is expressed by neural crest-derived melanoma and NB cancer stem cells [250, 316, 317]. In a model of non-Nmyc amplified NB cancer staminality, self replicating CD133, CD271/p75$^{NTR}$ positive clonogenic stem cells produce both a non-malignant fibromuscular lineage and a malignant neuronal (N)-type cell lineage defective in terminal neuronal differentiation. Although Trk expression in this NB population remains to be determined, CD271/p75$^{NTR}$ positive self-replicating neural stem cells have been shown to express TrkA, TrkAIII, TrkB and TrkC [73, 318].

Consistent with a restricted pattern of CD271/p75$^{NTR}$ expression in NB, primary human NBs have been reported to not express CD271/p75$^{NTR}$ [252, 259, 319] or to express variable levels of CD271/p75$^{NTR}$ [251, 319], which either correlate [259] or don't correlate with TrkA expression [259, 264]. Indeed, differences in CD271/p75$^{NTR}$ co-expression with TrkA have been associated with survival, with the co-expression of CD271/p75$^{NTR}$ and TrkA in NB associated with a 100% survival probability, TrkA expression in the absence of CD271/p75$^{NTR}$ with a 62.3% (intermediate) survival probability and no TrkA or p75$^{NTR}$ expression with a 0% probability of survival [259]. Consistent with this, a lack of CD271/p75$^{NTR}$ expression has been reported in Nmyc amplified and undifferentiated NB [252, 319, 321] and high CD271/p75$^{NTR}$ expression reported in more favourable differentiating NBs, ganglioneuromas and ganglioneuroblastomas [251, 252, 299, 320]. However, despite the general concept that high level CD271/p75$^{NTR}$ expression associates with favourable NB behaviour and outcome [259, 264, 322], CD271/p75$^{NTR}$ expression characterises GD2 positive stage IV metastatic bone marrow NB infiltrates [301] and aggressive adult NBs [268, 323].

Consistent with a general association with favourable NB, CD271/p75$^{NTR}$ exhibits a tumour suppressor role in NB models, promoting differentiation, apoptosis and reducing tumorigenic activity [299, 324, 325]. Differentiation promoted by CD271/p75$^{NTR}$ depends upon the molecular context and may involve an IP3K-Akt-mediated BcL-X-dependent survival pathway [326, 327] or a TrkA-dependent pathway, in which CD271/p75$^{NTR}$ plays a subtle but critical role in optimising and prolonging NGF-mediated TrkA activation [328-331]. Indeed, mutation of CD271/p75$^{NTR}$ within a TrkA context results in proliferation and not differentiation in response to NGF [332]. Coexpression studies in NB cells have also indicated that, in response to NTs,

CD271/p75$^{NTR}$ alone induces mild differentiation, TrkA alone causes a more marked differentiation and coexpression an even more marked and rapid differentiation [333].

CD271/p75$^{NTR}$ can acts as either an anti-apoptotic or pro-apoptotic factor, depending upon the molecular context. At low TrkA to CD271/p75$^{NTR}$ ratios the anti-apoptotic activity of NGF requires binding to CD271/p75$^{NTR}$, whilst at higher TrkA to CD271/p75$^{NTR}$ ratios involves a mechanism independent of CD271/p75$^{NTR}$ binding [334]. Conversely, NGF also induces apoptosis in NB cells with a high CD271/p75$^{NTR}$ to TrkA ratios [335]. In the absence of NTs and TrkA, CD271/p75$^{NTR}$ induces apoptosis and inhibits NB tumorigenic activity [299, 336-338]. It has been reported that apoptosis, induced by non-ligated CD271/p75$^{NTR}$ is inhibited by non-ligated TrkA but this may reflect spontaneous activation of overexpressed TrkA [337]. Furthermore, agents such as prion proteins activate CD271/p75$^{NTR}$ to promote apoptosis in NB cells via NF-κB [339]. In the absence of spontaneous TrkA activation and NT expression, however, the coexpression of CD271/p75$^{NTR}$ and TrkA promotes more marked apoptosis [333]. In the presence of BDNF CD271/p75$^{NTR}$ interaction with TrkB promotes NB cell proliferation and survival, through RAS/MAPK and PI3K/AKT/NF-κB [322]. These reports suggests that CD271/p75$^{NTR}$ is a pivotal regulator of the disparate behaviour of TrkA and TrkB expressing NBs, exhibiting capacity to enhance differentiation and apoptotic responses in TrkA expressing NBs and enhance proliferation and survival responses in TrkB expressing NBs, by increasing receptor sensitivity to low NT concentrations and blocking responses to promiscuous NTs.

CD271/p75$^{NTR}$ also interacts with Sortilin and other proteins, complicating potential responses to both pro- and active NTs. The CD271/p75$^{NTR}$-Sortilin co-receptor complex augments affinity for proNGF and induces apoptosis [105, 340]. Furthermore, CD271/p75$^{NTR}$ also interacts with NRIF, TRAF, NRAGE and MAGE proteins to promote apoptosis [340-342].

With respect to the regulation of CD271/p75$^{NTR}$ expression in NB, Nmyc acts as a transcriptional repressor of CD271/p75$^{NTR}$ expression by promoting promoter methylation [274]. This effect can be reversed by HDAC inhibitors, resulting in the resoration of NGF-mediated apoptosis [274]. This novel pathway, detected in Nmyc amplified NB, may help to explain the inverse relationship between CD271/p75$^{NTR}$ and Nmyc expression detected in human Nmyc amplified NBs and in root ganglia NBs in Nmyc transgenic mice [271, 343]. The histone methyltransferase EZH2A has also been reported to repress CD271/p75$^{NTR}$ providing an additional Nmyc-independent CD271/p75$^{NTR}$ transcriptional repressing mechanism that may contribute to the genesis and maintenance of undifferentiated CD271/p75$^{NTR}$ negative NBs [344].

At the therapeutic level, CD271/p75$^{NTR}$ protects NCC and NB cells from apoptosis induced by antimitotic agents [345], and histone deacetylase inhibitors induce NB cell apoptosis and restore CD271/p75$^{NTR}$ and TrkA expression [274, 346].

### 6.4. TrkB and BDNF in NB

Fully spliced TrkB is expressed by a subpopulation of Nmyc amplified NBs [311, 347-349]. Despite observations that Nmyc alone is insufficient to induce TrkB expression [348], TrkB

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expression in NB exhibits a positive correlation with Nmyc amplification and expression [309, 311, 347, 348, 350]. TrkB expression is stimulated by activated c-erbA in NB cells, unveiling a potential oncogenic receptor tyrosine kinase-mediated mechanism for promoting TrkB expression [351]. Aggressive unfavourable Nmyc amplified NBs also express BDNF, which when coexpressed with TrkB provides an autocrine/paracrine survival mechanism in tissues that do not express NTs [309-313]. Recently, BDNF variants encoding exons 4, 6 and 9 have been associated with unfavourable NB outcome [312, 313].

TrkB expression by sympathoblasts subpopulations during SNS development provides a potential origin for TrkB expressing NBs. However, this population proliferates in response to BDNF *in vitro* but does express BDNF *in vivo* [225, 245], suggesting that BDNF expression may be acquired at a later stage. TrkB transcription in NB cells is also up regulated by hypoxia inducible factor-1, providing a potential epigenetic mechanism through which tumour-associated hypoxia could augment TrkB expression [352].

In contrast to signals from NGF-activated TrkA, which induces NB cell differentiation and growth arrest [73, 324, 353, 354], BDNF activation of TrkB induces partial differentiation in the absence of growth arrest, through Ret tyrosine kinase [354-357]. BDNF activation of TrkB increases NB cell survival [358], resistance to chemotherapeutic agents [358-363], augments invasive capacity [294] in cooperation with c-Met [364] and galectin-1 [365], promotes angiogenesis and angiogenic factor expression [292, 350, 366], augments genetic instability [293] (Schulte et al., 2008) and increases metastatic behaviour by inhibiting anoikis [367]. In contrast, NB cells expressing truncated TrkB lacking the tyrosine kinase domain, display a more differentiated phenotype [311] and this receptor is more frequently detected in ganglioneuroblastomas and ganglioneuromas. Consistent with this, truncated TrkB overexpression in NB cells promotes differentiation suggesting that this receptor variant promotes a more benign phenotype [368]. Oxidative stress up-regulates the expression of full length TrkB relative to the truncated isoform, providing an additional epigenetic mechanism for regulating TrkB involvement in NB [369].

### 6.5. TrkC and NT3 in NB

TrkC is expressed by migrating NCC progenitors, sympathoblasts and sympathetic neurons [194, 201, 203], providing many potential origins for TrkC expressing NBs. High level TrkC expression in low stage NBs is associated with favourable outcome (309, 310, 349, 353, 370-372], and is often accompanied by TrkA expression [257, 258, 309]. Recently, however, a subset of advanced stage IV NBs has been identified that exhibit high level NT-3 and TrkC co-expression, providing an autocrine/paracrine survival and proliferation mechanism for selecting these NBs in tissues that do not express NT-3 [314]. This expression pattern bears close similarity to migrating, proliferating NCC sympathoblasts prior to sympathetic neuronal differentiation, which also coexpress NT-3 and TrkC [194, 201, 203], identifying a potential origin for this NB subset. TrkC expression in NB, like that of TrkA, inversely correlates with Nmyc amplification and expression, and Nmyc amplified NBs either do not express TrkC at all, or express truncated TrkC [371, 372]. With the exception of NBs that coexpress NT3 and TrkC [314], the co-expression of TrkC, TrkA and CD271/$p75^{NTR}$ in NB carries the best prognosis and associates

more frequently with spontaneous regression, differentiation and chemo-responsiveness [100, 258, 259, 333, 370, 371]. The *TrkC* gene, however, encodes multiple NT-3 receptors with distinct biological properties and substrate specificities [89, 373] and, although *TrkC* gene rearrangements in NB have not been reported, the effect of differential TrkC isoform expression in NB remains to be elucidated.

Association between high TrkC expression and favourable NB outcome, in the absence of NT-3 [333, 371], is consistent with pro-apoptotic TrkC dependency receptor function, which promotes apoptosis in the absence of CD271/p75$^{NTR}$ and NT expression [191, 192]. Furthermore, NT-3 activation of TrkC induces NB cell differentiation [374] and the co-expression of TrkC with CD271/p75$^{NTR}$ lowers tumorigenic potential and tumour growth [375] but may protect NB cells from doxorubicin and cisplatin cytotoxicity [375].

With respect to the transcriptional regulation of TrkC, Nmyc silencing increases TrkC expression in human NB cells [376], corroborating the inverse relationship reported for TrkC expression and Nmyc amplification [371,372]. TrkC expression, furthermore, is abrogated by the activation of c-erbA, providing a potential oncogenic tyrosine kinase-mediated mechanism for repressing TrkC expression in NB [351]. Retinoic acid induces TrkC expression in human NB cells, restoring NT-3-dependent differentiation [152]. Retinoids also induce the expression of microRNAs-9, 125a and 125b that repress truncated kinase domain-deleted TrkC, resulting in altered growth and highlighting a role for the truncated TrkC receptor in the regulation of NB growth and differentiation [377]. MiR-151-3p represses full length TrkC expression, whereas miRs-128, 485-3p, 765 and 768-5p repress truncated TrkC expression in NB cells [378], indicating that full length and truncated TrkC receptors are regulated by different miRs, linking NT-mediated processes to miR expression in NB.

### 6.6. General considerations on NT and NTR expression patterns in NB

The concept that different NT and NTR receptor expression profiles characterise NB subsets and that these differences are involved in divergent NB behaviour and therapeutic susceptibility, continues to evolve with potential to improve prognosis and therapeutic choice, whilst identifying novel potential therapeutic targets.

The hypothesis that high TrkA, high TrkC and/or high CD271/p75$^{NTR}$ expression always associate with low disease stage and better prognosis in NB is clearly not the case. Moderate to high levels of TrkA, TrkC and/or CD271/p75$^{NTR}$ can also characterise advanced stage and relapsing non-Nmyc amplified NBs and a subset of Nmyc amplified NB with favourable histology (see section 6.1). However, high TrkB expression appears to distinguish advanced stage Nmyc amplified from non-Nmyc amplified NB and carries poor prognosis associated with potential therapeutic resistance (see section 6.4). It is also now apparent that NTRs can be expressed as different isoforms with altered biological activity and can interact with one other and with a variety of ancillary proteins to modulate function (see section 6.3), complicating prognosis and potential therapeutic outcome, as outlined below (Fig. 9).

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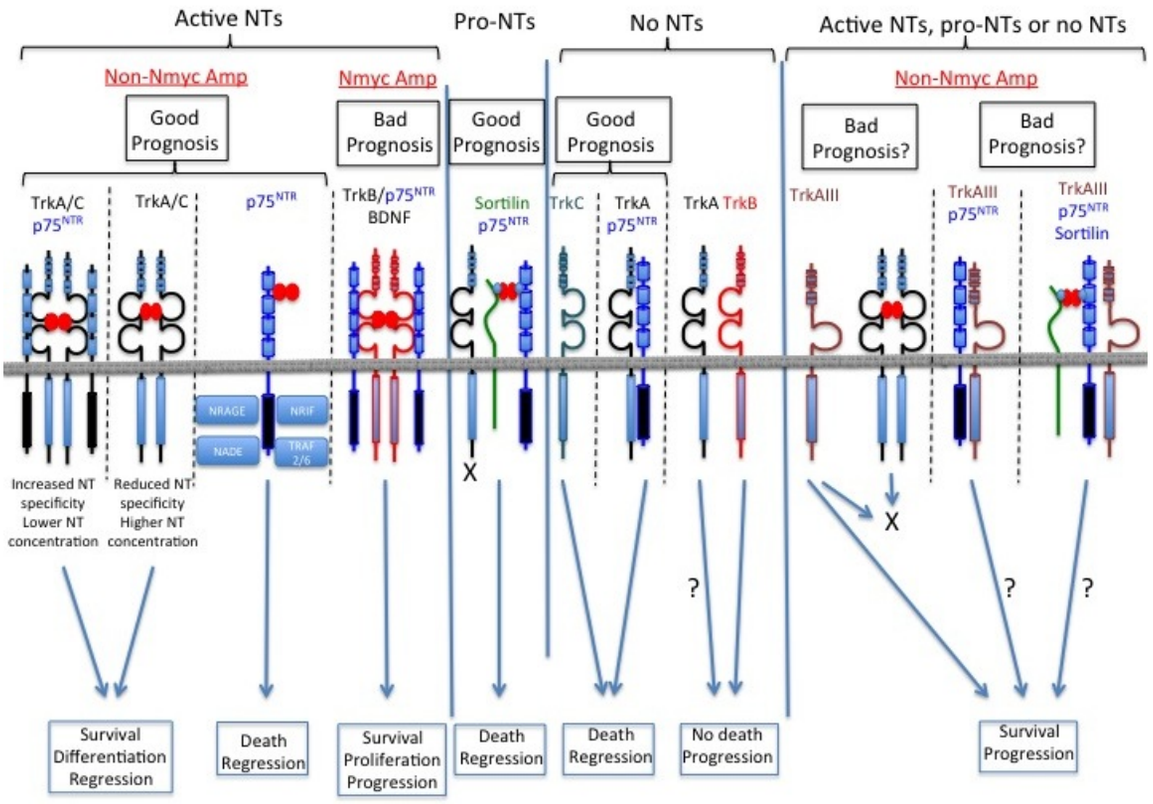


**Figure 9.** Different combinations, potential outcomes and prognosis of NTs and NTRs in NB

NTR expression in low stage non-Nmyc amplified NB characterised by the coexpression of TrkA and CD271/p75$^{NTR}$ may carry the best prognosis. These tumours may terminally differentiate in response to NGF (TrkAI) or NT-3 (TrkAII), or undergo TrkA and/or CD271/p75$^{NTR}$–mediated apoptosis in the absence of NTs, depending upon the CD271/p75$^{NTR}$ to TrkA expression ratio. Furthermore, the coexpression of Sortilin in these tumours would extend apoptotic potential to include pro-NTs (see Sections 4.2.1-4.2.4). NBs that express TrkA but not CD271/p75$^{NTR}$ may have a worse prognosis, as they require higher NT concentrations for TrkA activation and signalling and would also respond to promiscuous NTs potentially with a response of proliferation, survival and/or partial differentiation. In the absence of CD271/p75$^{NTR}$, these NBs would neither exhibit TrkA dependency receptor-mediated apoptosis nor Sortilin-CD271/p75$^{NTR}$ complex-mediated apoptosis in response to pro-NTs.

NBs that co-express TrkC and CD271/p75$^{NTR}$ but not TrkA or TrkB, may have better prognosis with potential to differentiate in response to NT-3 but alternatively could proliferate and survive in response to NT-3, complicating prognosis. NT-3 is rarely expressed in NBs, increasing the potential for TrkC dependency receptor-mediated apoptosis, in the presence or absence of CD271/p75$^{NTR}$ (see Sections 4.5 and 6.3). The coexpression of Sortilin with CD271/p75$^{NTR}$ in these NBs would increase apoptotic potential to include a response to pro-NTs (see Sections 4.2.3 and 6.3). Advanced stage NBs coexpressing TrkC and NT-3 would be expected to carry worse prognosis as a result of this autocrine survival and proliferation

mechanism that may also extend to NBs expressing TrkC but not NT-3 in tissues that express NT-3, and could be further optimised by co-expression of CD271/p75$^{NTR}$ (see Section 6.3).

High levels of BDNF and TrkB expression in Nmyc-amplified NBs, in the absence of TrkA, TrkC and CD271/p75$^{NTR}$, carries the worse prognosis as a result of autocrine/paracrine BDNF-mediated TrkB activation, which would be expected to promote proliferation, survival and metastatic capacity. Furthermore in the absence of BDNF, TrkB would not be expected to promote apoptosis, as TrkB does not act as a dependence receptor (see Sections 6.4). As with other Trk receptors, TrkB co-expression with CD271/p75$^{NTR}$ would be expected to optimise NT-specificity and responsiveness, which would be expected to further promote aggressive bahaviour in TrkB expressing NBs.

NBs that express CD271/p75$^{NTR}$ but not Trks may carry better prognosis, as they would be expected to respond to active NTs with an apoptotic response and if co-expressed with Sortilin in the absence of Trks, would also be expected to exhibit an apoptotic response to pro-NTs, which comprises up to 50% of secreted NTs (see Sections 4.2.2 and 6.3).

Non-Nmyc amplified NBs that express TrkAIII may carry worse prognosis, as spontaneous TrkAIII activation would override NT-dependency, provide a selective growth advantage in tissues including those that do not express NTs, promote NB cell stamilality, survival, angiogenesis and genetic instability, resulting in a more tumorigenic, metastatic and stress-resistant phenotype (see Sections 4.2.1 and 6.2). Although it remains to be elucidated whether TrkAIII may interfere with CD271/p75$^{NTR}$ –mediated apoptosis in the presence or absence of Sortilin, its expression in NB may represent the biological equivalent to BDNF/TrkB expression in Nmyc amplified NB and TrkC/NT3 expresssion in a subset of advanced stage NBs, as an indicator of poor prognosis.

## 7. Potential therapeutic approaches

### 7.1. Trk kinase inhibitors

Trk kinase inhibitors would be more suitable for use in advanced stage Nmyc amplified TrkB expressing NBs and advanced stage unfavourable non-Nmyc amplified NBs that express the TrkAIII oncogene but may also reduce survival in NBs expressing full length TrkA and TrkC and their corresponding NTs.

Therapeutic Trk kinase inhibitors include the selective Trk kinase inhibitors AZ-23 and AZ623, which inhibit Trk kinase activity at low nanomolar concentrations. AZ-23 has shown efficacy following oral administration in a TrkA-driven mouse allograft NB model [379], whereas AZ623 inhibits BDNF-mediated signalling and NB proliferation, and when combined with topotecan prolongs the inhibition of tumour regrowth and reduces chemo and radio therapeutic resistance [380, 381]. Lestaurtinib (CEP-701) is a small-molecule receptor tyrosine kinase inhibitor that competitively inhibits ATP binding to the Trk kinase domain at nanomolar concentrations. This compound not only inhibits the tyrosine kinase activities of full-length Trk receptors but also inhibits the kinase activity of the alternative TrkAIII splice variant [73,

78,79]. Lestaurtinib inhibits NB growth *in vitro* and *in vivo*, and substantially enhances the efficacy of conventional chemotherapy, such as 13-cis-retinoic acid, ferenteride and bevacizumab, presumably by inhibiting autocrine TrkB/BDNF [382-384] and/or spontaneous TrkAIII activity [73]. Lestaurtinib is an active metabolite of the Trk kinase inhibitor CEP-751, and is more suitable for clinical trials, as it can be administered orally [384, 385]. These Trk tyrosine kinase inhibitors not only target tumour promoting effects of Trk receptor activation but also Trk-mediated chemotherapeutic resistance, which has been attributed not only to TrkB [348, 358-363] but also to TrkC [375], fully spliced TrkA [375] and TrkAIII [73, 78, 79]. CEP-701 synergises with retinoids in the treatment of NB by inhibiting TrkB activity [386].

Nifutimox, a drug used for years to treat Chagas disease, is also currently in clinical trials for refractory or relapsed NB, and has been shown to suppress TrkB-mediated Akt activation and induce caspase-dependent apoptosis of NB cells *in vitro* and *in vivo* [387].

### 7.2. TrkAIII inhibitors

Tyrosine kinase inhibitors K252a and CEP-701 inhibit TrkAIII tyrosine kinase activity. TrkAIII activity is also inhibited by the Hsp90 inhibitor geladanamycin and its clinically relevant analogues 17-AAG and 17-DMAG, and by the ARF inhibitor Brefeldin A (BFA) [78, 79]. CEP701 inhibits TrkAIII activity and TrkAIII-induced centrosome amplification at nanomolar concentrations, whereas BFA reversibly inhibits spontaneous TrkAIII activation in association with disruption of the Golgi Network and the endoplasmic reticulum/Golgi Network intermediate compartment [78, 78]. Geldanamycin and its analogues reversibly inhibit TrkAIII tyrosine kinase activity and reduce proliferation of TrkAIII expressing NB cells *in vitro* [78]. Inhibitors of TrkAIII activity, however, do not inhibit TrkAIII expression nor promote TrkAIII elimination but cause retention within the endoplasmic reticulum, with potential to induce an ER stress response. This may help to explain the high level of resistance to GA-mediated cytotoxicity exhibited by TrkAIII but not TrkAI transfected NB cells, despite inhibition of TrkAIII activity [78, 79]. This suggests that, in addition to other off target effects, reversible TrkAIII tyrosine kinase inhibitors may increase stress-resistance by promoting TrkAIII-ER retention and inducing an ER stress response. Consistent with this, geldanamycin selects slow growing TrkAIII expressing NB cells from mixed populations, with TrkAIII re-activation post drug-removal, suggesting a mechanism for potential post therapeutic relapse [78]. To counter this, we have also developed a specific peptide nucleic acid (PNA) inhibitor of TrkAIII expression based upon the novel exon 5/8 splice junction (TrkAIII PNA conjugate $(KKAA)_4$-GGCCGGGA-CAC) [78, 79] for use in combination with with TrkAIII tyrosine kinase inhibitors, to maximise therapeutic efficacy.

### 7.3. Agents that conserve Trk tyrosine phosphorylation and facilitate signal transduction

TrkA activation and signal transduction is fundamental for NB differentiation and the loss of TrkA expression or defective activation and/or signalling probably contributes to NB pathogenesis. Agents that optimise TrkA activation and facilitate subsequent signal transduction may, therefore, overcome defective TrkA signalling and restore differentiation and/or apoptotic responses to NTs. In this context, a novel cyclophane compound CPPy, with low

toxicity, has been shown to facilitate NGF-induced TrkA signal transduction through RAS/MAPK and to induce NB differentiation [388]. Since, CD271/p75$^{NTR}$ optimizes TrkA responses to NTs and augments NT specificity, agents such as CPPy may be particularly useful in NBs that express TrkA but not CD271/p75$^{NTR}$.

### 7.4. DNA methylation and HDACs inhibitors

Recent reports have identified promoter methylation as an important mechanism in the transcriptional repression of TrkA and CD271/p75$^{NTR}$ in NB [274, 389, 390]. Therapies that reverse or inhibit DNA methylation may, therefore, be useful in malignant NBs to restore the expression of favourable NB genes. In support of this, the DNA methylation inhibitor 5-aza-2'-deoxycytidine and histone deacetylase inhibitors 4-phenylbutyrate, trichostatin A and Romidepsin, have been shown to restore TrkA and CD271/p75$^{NTR}$ expression in NB cells, decrease proliferation, reduce tumorigenicity and promote caspase-dependent apoptosis [291, 346, 390]. Romidepsin is presently in clinical trials [346].

### 7.5. Liposome targeting of TrkB expressing cells

Considering the importance of TrkB in advanced stage Nmyc amplified NB, a recent report has characterised liposomes that target TrkB expressing cells, providing the opportunity to deliver nanotherapeutic cargos to TrkB expressing cells within NBs [392].

## 8. Concluding remarks

The complex nature of NT and NTR expression during normal development of the sympathetic nervous system is reflected in the different patterns of NT and NTR expression exhibited by human NB, which is consistent with their NCC origin at different stages along the differentiating sympathoadrenal lineage. The different biological potentials of TrkA, TrkB, TrkC, CD271/p75$^{NTR}$ and Sortilin receptors expressed alone or in different combinations, range from promotion of proliferation and/or differentiation to survival and/or apoptosis and to chemotherapeutic resistance. This complexity is increased by the potential of each receptor to be expressed as a functionally altered alternative splice variant, the recent characterisation of TrkA and TrkC as true dependency receptors, and the pro-apoptotic behaviour of the CD271/p75$^{NTR}$ -Sortilin complex, providing an exciting array of new potential ways to restore and/or modulate Trks, CD271/p75$^{NTR}$ and Sortilin behaviour for therapeutic purposes, based upon accurate characterisation of NT and NTR expression profiles in individual tumours.

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