Οι νευροτροφικές δράσεις ενδογενών και συνθετικών Νευροστεροειδών: μελέτες δομής-λειτουργίας των αλληλεπιδράσεων τους με υποδοχείς νευροτροφινών και ο βιολογικός τους ρόλος στη νευροπροστασία.

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Abstract

Neuronal cell fate is majorly governed by the actions of growth factors, such as neurotrophins. These specific ligands and their receptors activity are regulated by multiple cellular parameters. Indeed, we have recently shown that neurosteroid dehydroepiandrosterone (DHEA) prevents neuronal apoptosis through binding to NGF receptors, namely TrkA and $p75^{NTR}$. In this study we provide evidence that DHEA interacts with the other two mammalian neurotrophin receptors, i.e., the TrkB and TrkC, as well as their invertebrate counterparts (orthologs) in mollusks Lymnaea and Aplysia, and in cephalochordate fish Amphioxus, supporting the hypothesis that during evolution DHEA may have served as a primordial neurotrophic factor, promoting neuronal survival in species with less complex nervous systems. A large number of experimental and clinical studies suggest that endogenous neurotrophins are involved in the pathopysiology of many neurodegenerative diseases. However, despite their demonstrated beneficial effects on neuronal survival and protection, the therapeutic usefulness of neurotrophins is compromised by their polypeptide nature and their restricted penetrance to the blood-brain barrier (BBB). To overcome this limitation, small molecules –like DHEA- which could mimic the effects of neurotrophic factors would be therapeutically ideal. However, DHEA is metabolized *in vivo* to sex steroids, affecting the endocrine system. We have recently synthesized 17-spiro analogs of DHEA with anti-apoptotic, neuroprotective properties (IC_{50} at nanomolar levels), deprived of androgenic-estrogenic actions. In the present study, we report that synthetic DHEA derivative, BNN27, specifically interacts with both NGF receptors, TrkA and $p75^{NTR}$, at nanomolar concentrations. BNN27 induces TrkA tyrosine phosphorylation, affecting downstream signaling of Akt and MAPKs in sympathetic neurons and regulates TrkA internalization, however in a different time pattern than that of NGF. Moreover, BNN27 was shown to promote the interaction of $p75^{NTR}$ receptors with its effector factors RhoGDI, RIP2 and TRAF6. It also significantly reverses apoptosis of NGF-dependent embryonic sensory neurons of NGF null mice. BNN27 itself was not effective in to mimicking NGF in the induction of neurite elongation, perhaps due to its differential endosome's turn over. However, combination of BNN27 with NGF results in enhancement of neurites' length. BNN27 may serve as a lead molecule to develop BBB permeable, neurotrophin-like small molecules (*microneurotrophins*) with potential applications in the treatment of neurodegenerative diseases and brain disorders.

Περίληψη

Η οικογένεια των Νευροτροφινών αποτελείται στα θηλαστικά από 4 πολυπεπτιδικά μόρια, τα NGF (Nerve Growth Factor), BDNF (Brain Derived Growth Factor), NT-3 (Neurotrophin-3) και Neurotrophin-4/5 (Neurotrophin-4/5). Οι πρωτεϊνες αυτές ταυτοποιήθηκαν αρχικά σαν παράγοντες επιβίωσης των συμπαθητικών κι αισθητικών νευρώνων κι έκτοτε έχει δειχθεί πως ελέγχουν μια σειρά προ-επιβιωτικών λειτουργιών, ανάπτυξης και κυτταρικής λειτουργίας τόσο στον νευρικό ιστό όσο και σε άλλα συστήματα του οργανισμού.

Οι βιολογικές δράσεις κάθε νευροτροφικού παράγοντα διαμεσολαβούνται μέσω ειδικών κυτταρικών υποδοχέων που ανήκουν σε ένα ή περισσότερα μέλη της οικογένειας των κινασών τυροσίνης (Trk, Tropomyocin-related Kinase) TrkA, TrkB ή TrkC. Επιπροσθέτως, όλες οι νευροτροφίνες προσδένονται και ενεργοποιούν τον παν-υποδοχέα $p75^{NTR}$, που ανήκει στην υπεροικογένεια των υποδοχέων του TNF (Tumor Necrosis Factor). Εκτός των κλασσικών ενδογενών Νευροτροφινών, πρόσφατα δεδομένα κατέδειξαν για πρώτη φορά ότι και μια άλλη κατηγορία ενδογενών νευροπροστατευτικών μορίων, όπως το Νευροστεροειδές Διϋδροεπιανδροστερόνη (DHEA) διαθέτει την ικανότητα να αλληλεπιδρά άμεσα με τους υποδοχείς TrkA και $p75^{NTR}$, ενεργοποιώντας τα σηματοδοτικά μονοπάτια που εκκινούν από τους υποδοχείς αυτούς.

Το Νευροστεροειδές DHEA παράγεται στο κεντρικό νευρικό σύστημα, εκτός της βασικής θέσης παραγωγής τους στη περιφέρεια, το φλοιό των επινεφριδίων. Έχει προταθεί η πιθανή θεραπευτική χρησιμότητα του στην αντιμετώπιση των νευροεκφυλιστικών διαδικασιών, όπως οι νόσοι του Parkinson και Alzheimer αλλά και το εγκεφαλικό τραύμα και η ισχαιμία, και η Σκλήρυνση κατά Πλάκας, διαδικασίες στις οποίες παρατηρείται έντονη αποπτωτική απώλεια νευρώνων. Εν τούτοις η *in vivo* βιομετατροπή τους σε οιστρογόνα και ανδρογόνα καθιστά την κλινική τους χρήση προβληματική. Η ανάπτυξη συνθετικών αναλόγων της DHEA, που στερούνται ενδοκρινών δράσεων και διαπερνούν τον αιματοεγκεφαλικό φραγμό, παρέχει την δυνατότητα ανάπτυξης νέων μορίων ως μικρομοριακοί αγωνιστές των υποδοχέων των νευροτροφινών, και παρέχουν την δυνατότητα για την ανάπτυξη νέων θεραπευτικών ουσιών με νευροπροστατευτικές και πιθανές νευροαναγεννητικές ιδιότητες.

Η παρούσα εργασία στόχευσε κατ' αρχάς την μελέτη της ικανότητας του νευροστεροειδούς DHEA να αλληλεπιδρά και με τα υπόλοιπα μέλη της οικογένειας των υποδοχέων νευροτροφινών (TrkB και TrkC), αλλά και με τα αρχέγονα ανάλογά τους, Ltrk, ApTrk και AmphiTrk. Τα αποτελέσματα της παρούσας εργασίας ανέδειξαν την ικανότητα της DHEA να λειτουργεί ως ένας πιθανός αρχέγονος νευροτροφικος παράγοντας προάγοντας την νευρωνική επιβίωση σε είδη με λιγότερο σύνθετα νευρικά συστήματα.

Σημαντικός αριθμός πειραματικών και κλινικών μελετών υποστηρίζει την συμμετοχή των ενδογενών νευροτροφινών στην παθοφυσιολογία των νευροεκφυλιστικών νόσων και την πιθανή τους χρησιμότητα στην θεραπευτική τους. Εν τούτοις η κλινική θεραπευτική χρησιμότητα των νευροτροφινών περιορίεται από την πρωτεϊνική τους φύση και την αδυναμία τους να διέλθουν τον αιματεγκεφαικό φραγμό. Η προσπάθεια διεθνώς έχει εστιασθεί στην ανάπτυξη συνθετικών αναλόγων των Νευροτροφινών με νευροτροφικές, νευροπροστατευτικές δράσεις. Η ικανότητα της DHEA να συνδέεται και να ενεργοποιεί τους υποδοχείς των νευροτροφινών αποκτά ιδιαίτερη φαρμακολογικό ενδιαφέρον, ιδιαίτερα αυτή συνθετικών της αναλόγων, στα οποία απουσιάζουν οι ενδοκρινικές δράσεις.

Η παρούσα εργασία εστίασε κυρίως στην μελέτη της ικανότητας ενός πρότυπου συνθετικού αναλόγου της DHEA, του BNN27 να αλληλεπιδρά και να ενεργοποιεί εκλεκτικά τους υποδοχείς του Νευρικού Αυξητικού Παράγοντα (NGF). Αρχικά, μελετήθηκε η ικανότητα πρόσδεσης του BNN27 στους υποδοχείς των Νευροτροφινών (Trk και $p75^{NTR}$). Δεδομένα που προέκυψαν από πειράματα ανταγωνισμού σύνδεσης ([^{3}H]-DHEA displacement experiments) ανέδειξαν το BNN27 ως πιθανό αγωνιστή των υποδοχέων TrkA και $p75^{NTR}$ με Ki της τάξης του 1.86±0.4nM και 3.9±1.2nM για TrkA και $p75^{NTR}$ αντίστοιχα. Σε αντίθεση με την DHEA, το BNN27 δεν έδειξε χημική συγγένεια με τους υποδοχείς TrkB, TrkC, υποστηρίζοντας ότι η σύνδεση του BNN27 είναι εκλεκτική. Επιπλέον, μελετήθηκε η ικανότητα του BNN27 να επάγει συγκεκριμένα ενδοκυττάρια σηματοδοτικά μονοπάτια ευαίσθητα στον φυσιολογικό προσδέτη, τον NGF. Συγκεκριμένα, εξετάστηκε η ικανότητα του BNN27 να επάγει τη φωσφορυλίωση του υποδοχέα TrkA σε κύτταρα που τον εκφράζουν ενδογενώς (όπως οι συμπαθητικοί νευρώνες σε πρωτογενή καλλιέργεια ή η κυτταρική σειρά PC12) ή μετά από παροδική διαμόλυνση CHO κυττάρων με το cDNA του TrkA. Η επίδραση του BNN27 (100 nM) ήταν τόσο αποτελεσματική όσο και του NGF (100ng/ml) στην επαγωγή της φωσφορυλίωσης των TrkA υποδοχέων σε χρόνο 20 λεπτών, υποδηλώνοντας ότι η σύνδεση του BNN27 στους TrkA υποδοχείς είναι λειτουργική. Αντιθέτως, το BNN27 απέτυχε να επάγει τη φωσφορυλίωση του TrkB και TrkC υποδοχέα.

Επίσης, με μελέτες ανοσοκατακρήμνισης διαπιστώθηκε η ικανότητα του BNN27 να ενεργοποιεί σηματοδοτικά μονοπάτια που σχετίζονται με τον υποδοχέα $p75^{NTR}$. Μια από τις πρωτεΐνες που προσδένονται με τον $p75^{NTR}$ είναι η πρωτεϊνική κινάση RIP2, η οποία μπορεί να προσδένεται στην Death Domain (DD) του $p75^{NTR}$ μέσω της CARD περιοχής του. Μια επιπλέον πρωτεΐνη που έχει τη δυνατότητα να προσδένεται στην DD του $p75^{NTR}$ είναι ο αναστολέας της απόσπασης του GDP από την Rho (RhoGDP Dissociation Inhibitor, RhoGDI). Έχει δειχθεί ότι η αλληλεπίδραση του $p75^{NTR}$ με την RhoGDI επάγει την ενεργοποίηση της Rho, γεγονός που φαίνεται να συμβαίνει μέσω της απελευθέρωσης της Rho από την RhoGDI που οφείλεται στην αναδιαμόρφωση της στερεοδιάταξης της RhoGDI από την πρόσδεση στον $p75^{NTR}$. Πειράματα σε πρωτογενείς καλλιέργειες γλοιακών κυττάρων Schwann που εκφράζουν ενδογενώς τον $p75^{NTR}$ ή κύτταρα που δεν εκφράζουν τον $p75^{NTR}$ (MEF, HEK, CHO) παρά μόνο μετά από διαμόλυνση τους με το cDNA του $p75^{NTR}$ ανέδειξαν την ικανότητα του BNN27 (100nM) να στρατολογεί την RIP2 και τον TRAF6 στον $p75^{NTR}$ (20 λεπτά), ενώ διευκόλυνε την απομάκρυνση της RhoGDI από τον $p75^{NTR}$ (30 λεπτά).

Πειραματικά δεδομένα ανέδειξαν την ικανότητα του BNN27 να αναστρέψει στατιστικά σημαντικά την απόπτωση που προκαλείται λόγω στέρησης του νευροτροφικού παράγοντα (NGF) σε πρωτογενείς καλλιέργειες συμπαθητικών νευρώνων απομονωθέντων από τα ανώτερα θωρακικά συμπαθητικά γάγγλια (SCGs) νεογέννητων αρουραίων. Επίσης, χορήγηση του BNN27 κατά την διάρκεια τις κύησης οδήγησε σε μείωση τις αποπτωτικής απώλειας των TrkA θετικών αισθητικών νευρώνων των νωτιαίων γαγγλίων που παρατηρείται κατά την 14^η εμβρυική μέρα (E14) στους διαγονιδιάκους *ngf-/-* ποντικούς. Σε αντίθεση με τον NGF, το BNN27 δεν ήταν ικανό να προκαλέσει τη διαφοροποίηση των PC12 κυττάρων σε νευρώνες. Οι διαφορές στα ευρήματα που παρατηρήθηκαν ίσως να οφείλονται σε διαφορετική σενσχέση με τον NGF διαδικασία της ενδοκυττάρωσης του υποδοχέα TrkA.

Συμπερασματικά, η ανάπτυξη μικρομοριακών αναλόγων της DHEA, όπως του BNN27 που στερούνται ενδοκρινών δράσεων και διαπερνούν τον αιματοεγκεφαλικό φραγμό, παρέχει την δυνατότητα ανάπτυξης νέων συνθετικών μορίων (lead molecules) ως αγωνιστές των υποδοχέων των νευροτροφινών, στοχεύοντας στην ανάπτυξη νέων θεραπευτικών προσεγγίσεων των νευροεκφυλιστικών νόσων και του εγκεφαλικού τραύματος.

1. INTRODUCTION

1.1. Neurotrophins and their receptors

Polypeptidic neurotrophic growth factors (neurotrophins) are important regulators of neural survival, development, function and plasticity (*Eide et al., 1993; Segal & Greenberg 1996; McAllister et al., 1999; Sofroniew et al., 2001*). Four neurotrophins are characterized in mammals, namely Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4). All four neurotrophins are derived from a common ancestral gene and they have important homology and similarities in sequence and structure (*Hallbook, 1999*).

Neurotrophins show different distribution patterns throughout the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). In the CNS, NGF mRNA is present at high levels in the hippocampus, cerebral cortex, thalamus and hypothalamus, at medium levels in the striatum and brainstem, while lower levels were found in the cerebellum and spinal cord. Highest levels of BDNF mRNA and NT-3 mRNA in the adult mouse brain were found in the hippocampus, with 50 times more BDNF mRNA being present compared to this of NGF mRNA (*Hofer et al., 1990*). In the PNS, high levels of NGF mRNA were localised in sympathetic and sensory ganglia, in the sciatic nerve and lower levels in the trigeminal ganglion. Furthermore, NGF mRNA was also found in non-neuronal tissue such as the heart, submandibular gland and skin and lower levels in the adrenal gland, pituitary gland, testis, striated muscles and liver (*Goedert et al., 1986*). NGF and its receptors are expressed in a variety of cell types, such as neurons and glia cells including oligodendrocytes and Schwann cells, immune cells including lymphocytes, mast cells, macrophages and microglia, as well as by epithelial cells, fibroblasts and smooth muscle cells (*Lee et al., 1998*).

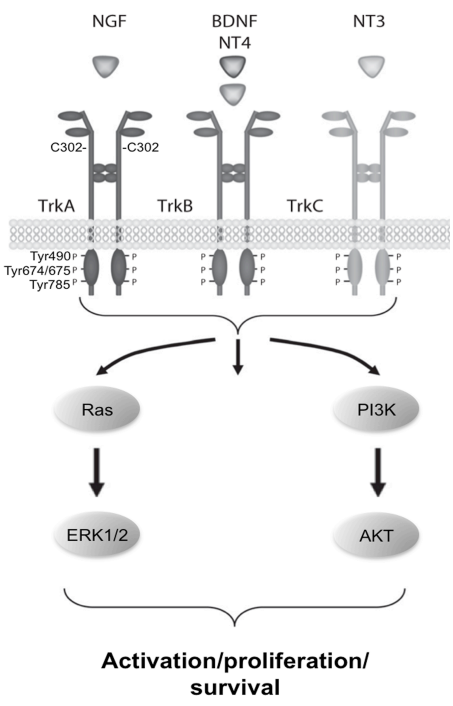
Although the production of NGF in many of these systems is mainly apparent during development, with their levels of expression been reduced or even absent during adulthood, NGF production is known to be homeostatically reactivated under pathological conditions, as a protective mechanism (*Lee et al., 1998*). BDNF expression is low in developing regions of the CNS and increases as these regions mature, whereas NGF expression varies during the development dependent upon the region. NT-3 is by far the most highly expressed in the immature CNS and its expression dramatically decreases with maturation (*Maisonpierre et al., 1990*).The mature forms of neurotrophins mediate their actions by interacting with two receptor types: the first one is the tyrosine kinase (Trk:Tropomyosin related kinase) family of receptors including TrkA, TrkB and TrkC which show high specificity for NGF, BDNF and NT3 respectively, and the second one is $p75^{NTR}$ which binds all neurotrophins with lower affinity (*Bothwell et al., 1995; Friedman et al., 1999; Lee et al., 2001*). In addition, unprocessed neurotrophins (pro-neurotrophins) have been found to display higher selectivity for $p75^{NTR}$ over Trk receptors (*Lee et al., 2001*).

Structural and functional role of TrK receptors

Evaluation of the distribution of TrkA protein and mRNA in the rat and human CNS shown that TrkA is expressed in basal forebrain cholinergic neurons (*Sobreviela et al., 1994; Steininger et al., 1993*) although there were some TrkA positive, $p75^{NTR}$-negative neurons in the striatum (*Allen et al., 1989; Dawbarn et al., 1988*). In contrast, TrkB and TrkC are more widely distributed (*Chao et al., 2002*). In the PNS, TrkA expression is confined in sympathetic and sensory

neurons. TrkB and TrkC receptors are not required for normal sympathetic or sensory development *in vivo*, while TrkA receptors are absolutely needed for sympathetic and sensory neuron survival during late embryogenesis and early postnatal development (*Fagan et al., 1996*).

Trk receptors are transmembrane glycoproteins of ~140 kDa, shares a common structural organization of their extra- and intracellular domains although they exhibit differences in their affinity toward the polypeptide neurotrophic ligands (*Bothwell et al., 1995*). Specifically, the extracellular region of TrkA is characterized by a number of distinct structural motifs (*Neilson et al., 1996*). The amino-terminal sequence consists of three tandem leucine repeats (LRM) flanked by two cysteine clusters. Following the cysteine-rich region, two immunoglobulin (Ig)-like C2 type domains are shown which contribute significantly to NGF binding



(Figure 1.1.a) (*Holden et al., 1997; Perez et al., 1995; Urfer et al., 1995*). It is well established that Trk receptors –as members of RTK receptors- are activated upon dimerization and intracellular phosphorylation at specific tyrosine residues. Activated Trk receptors initiate multiple cascades of phosphorylations, which activate specific pro-survival kinases. Tyrosine phosphorylation drives activation of signaling pathways like those of Ras-Raf-MAPKs, PI3K-Akt, and PLC-γ1 (*Kaplan et al., 2000; Foehr et al., 2000; Wooten et al., 1999*). The signaling pathways of Ras-Raf-MAPKs and PI3K-Akt are activated upon phosphorylation of Tyrosine 490 (Y490) of the Trk receptor, through subsequent

phosphorylation the Shc protein. Shc interacts directly with the receptor and mediates the phosphorylation of the Ras-Raf-MAPKs and PI3K-Akt pathways, controling finally several cellular events, such as the survival and differentiation of nerve cells. Another tyrosine residue at position 785 (Y785) is responsible for the activation of signaling cascades initiated by PLC-γ, and the production of diacylglycerol (DAG) and inositol-3-phosphate (IP3), which in their turn regulate the intracellular distribution of Ca^{+2}. Finally, receptors of tyrosine kinase have the ability to activate the synthesis of cAMP, which in turn participates in several signal pathways of survival, proliferation and cell differentiation. These signaling pathways of successive phosphorylation events culminate in the activation of transcription factors such as CREB and NF-κB and the subsequent regulation of gene expression and production of anti-apoptotic Bcl-2 proteins, affecting thus cellular survival (*Klein et al., 1991, Jing et al., 1992, Ip et al., 1993, Blum & Konnerth., 2005, Arevalo & Wu., 2006, Ibanez., 2002*).

TrkA mediates signaling events through its intracellular trafficking. Upon NGF binding, TrkA exits from membrane raft regions where it is initially located, undergoing a dynamin-dependent internalization process, probably *via* the coated pit pathway (*Zhang Y et al., 2000*). Once internalized into early endosomes, TrkA may either recycle to the plasma membrane through recycling endosomes or it might enter the degradation pathway by passing into late endosomes/multi-vesicular bodies (MVBs) to be finally degraded by lysosomes (*Jullien et al., 2002*). Internalized TrkA receptors signal activation of extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK), thereby regulating cell differentiation (J*ullien et al., 2003; Zhang Y et al., 2000*). Furthermore, the kinase activity of TrkA has been shown to play a major role in sorting of the internalized

TrkA from early to late endosomes/MVBs. Inhibition of TrkA kinase activity switches the sorting of TrkA from the degradative pathway to the recycling pathway in PC12 cells (*Saxena et al., 2005*).

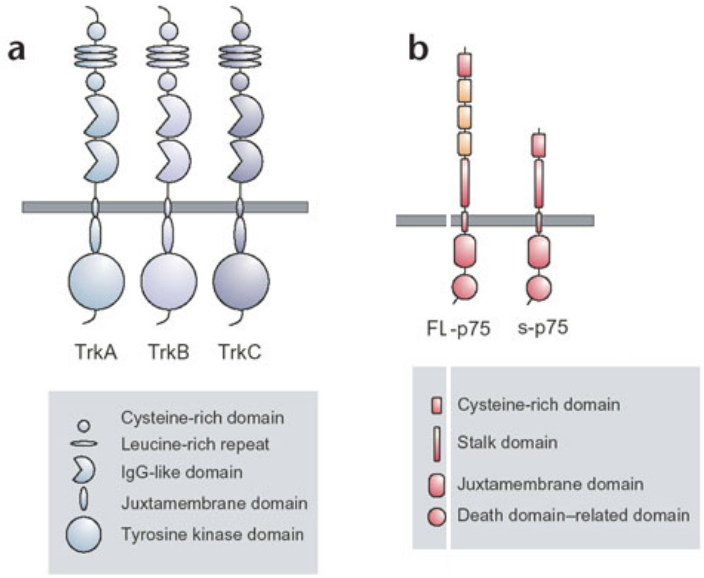


Figure 1.1. Schematic representation of the Trk (a) and $p75^{NTR}$ (b) receptors showing individual domains (*Dechant and Barde, 2002*)

Structure and functional role of $p75^{NTR}$ receptor

$p75^{NTR}$ is not widely expressed in the normal adult brain. Immunohistochemical and *in situ* analysis of the distribution of $p75^{NTR}$ in rat and primate brain (*Allen et al., 1989; Dawbarn et al., 1988; Kordower et al., 1988; Riopelle et al., 1987*) and co-localization of choline acetyltransferase (ChAT) and $p75^{NTR}$ immunoreactivity (*Dawbarn et al., 1988; Kordower et al., 1988*) show that $p75^{NTR}$ is almost exclusively located in basal forebrain cholinergic neurons. However, $p75^{NTR}$ is expressed in the nervous system during development or after injury (*Chao, 2003*) and it has been primarily studied as a regulator of survival and apoptosis in neurons and glia cells (*Dechant and Barde, 2002*).

$p75^{NTR}$ is a transmembrane protein, with its molecular weight (75 kDa) determined by glycosylation through both N- and O-linkages in the extracellular domain (*Grob et al., 1985; Johnson et al., 1986*). It consists of intracellular, transmembrane and extracellular domains (Figure 1.1.b), which are different from those in the other TNF receptors. First, the death of $p75^{NTR}$ is a type 2 molecule, which is different from type 1 molecules of other TNF receptors (*Liepinsh et al., 1997*) and, secondly, unlike other TNF receptors, $p75^{NTR}$ does not self-associate in solution (*Liepinsh et al., 1997*).

The crystal structure of the ligand-binding domain (CRDs 1 to 4) of $p75^{NTR}$ in complex with NGF had initially been explored by *Garcia and colleagues (He et al., 2004*). The complex was shown to be composed of an NGF homodimer asymmetrically bound to a single $p75^{NTR}$ molecule. $p75^{NTR}$ was proposed to bind along the homodimeric interface of NGF and to disable NGF's symmetry-related second binding site through an allosteric conformational change (*He et al., 2004*). Neurotrophins bind to the second and third CRD in $p75^{NTR}$ (*He et al., 2004; Welcher et al., 1991; Yan et al., 1991; Baldwin et al., 1995*).

In contrast to the aforementioned model of $p75^{NTR}$ interaction with its ligand, recent crystal structure studies of the complex of NGF with the $p75^{NTR}$, have shown that neurotrophins can interact with dimers of the p75 neurotrophin receptor. However, the exact mechanism of receptor activation remains elusive. Recently studies have shown that $p75^{NTR}$ forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys257 in its transmembrane domain (*Vilar et al., 2009*).

The intracellular domain of $p75^{NTR}$ interacts with a number of death-signaling binding partners (*Coulson et al., 2004; Frade, 2000; Roux & Barker,*

2002) and its C-terminal tail may bind to a PDZ domain containing proteins known for protein trafficking and receptor complex association.

p75^{NTR} can interact with tyrosine kinase receptors (TrkA, TrkB and TrkC constitute the high-affine receptors for NGF, BDNF and NT-3 respectively) to facilitate neuronal survival and differentiation in response to neurotrophins (*Hempstead, 2002*), or in the contrary, it can mediate an apoptotic response to pro-neurotrophins (the immature form of neurotrophins) by interacting with sortilin as a co-receptor (*Nykjaer et al., 2004; Teng et al., 2005; Volosin et al., 2006*). p75^{NTR} also binds non-neurotrophin ligands, which include the neurotoxic prion protein fragment PrP (*Della-Bianca et al., 2001*), and the Ab-peptide of the amyloid precursor protein (APP) (*Yaar et al., 2002*). In addition, p75^{NTR} is part of the Nogo receptor signaling complex which is activated by myelin proteins, including myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) to inhibit axonal growth (*Wang et al., 2002; Wong et al., 2002*). Therefore, p75^{NTR} may promote cell survival or death, and facilitate or inhibit axonal growth, depending on its receptor partners (Figure 1.2).

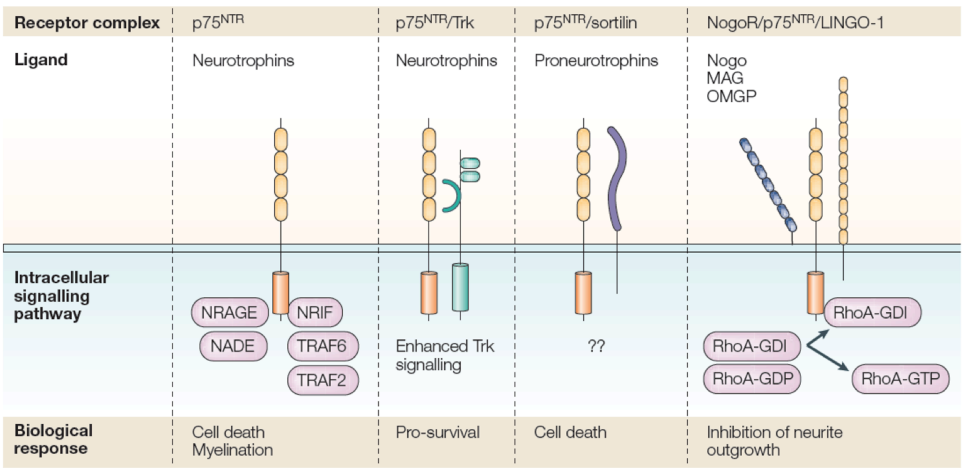
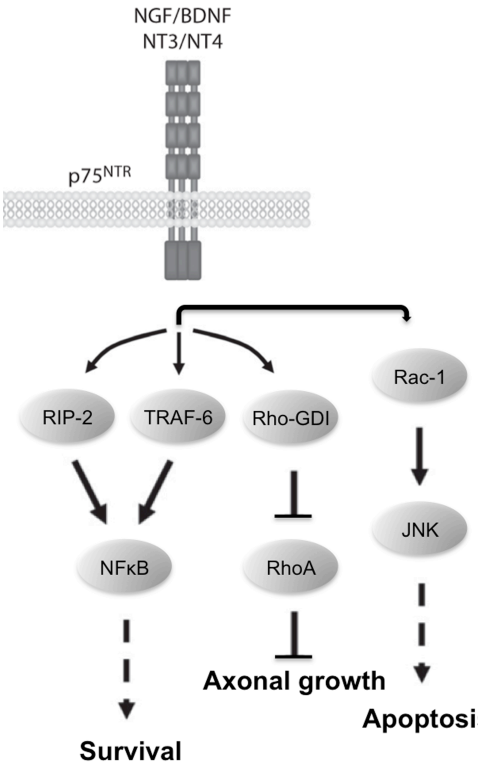


Figure 1.2. Multiple partners of p75^{NTR} (*Lu et al., 2005*).

p75^{NTR} has no kinase activity nor is linked to a G-protein-coupled pathway. Instead, p75^{NTR} interacts with different intracellular factors by recruiting intracellular binding proteins to mediate specific functions. Different binding proteins are recruited to the receptor in distinct cell types (*Barker, 2004; Yamashita et al., 2005; Arevalo & Wu, 2006; Blochl, 2007)*. The p75^{NTR}-mediated signaling pathways are only now becoming defined, in part due to the identification of proteins that bind the p75^{NTR} intracellular domain upon neurotrophin activation of p75^{NTR}. Many of the effectors identified have been implicated in cell cycle regulation, apoptosis, or both, suggesting pleiotropic roles for p75^{NTR} during development (*Dechant & Barde, 2002; Hempstead, 2002; Lopez-Sanchez & Frade; 2002; Roux & Barker, 2002*).



An important pro-survival signaling pathway activated by NGF, but not BDNF or NT-3, through p75^{NTR} is the NF-κB pathway (*Carter et al., 1996*). The activation of NF-κB requires several proteins, including TRAF6, p62, interleukin-1 receptor-associated kinase (IRAK), and receptor-interacting protein-2 (RIP-2) (*Kuruvilla et al., 2000; Yeiser et al., 2004; Casademunt et al., 1999; Mamidipudi et al., 2002; Khursigara et al., 2001*). Upon activation in response to neurotrophins, NF-κB translocates to the nucleus and triggers the expression of Hes1/5 to modulate dendritic growth (*Salama-Cohen et al., 2005*). This pro-survival pathway, unlike JNK activation, is not abolished by the expression of

TrkA and is more likely to be activated when the cells have been previously exposed to stress conditions, including TNF or serum deprivation (*Bhakar et al., 1999; Cosgaya et al., 2001*).

Among other functions, $p75^{NTR}$ can also modulate axonal growth with different outcomes depending on the molecule that binds to the receptor. Neurotrophin binding leads to axonal growth whereas myelin-derived growth inhibitors (MDGIs) evoke growth-cone collapse. These opposite effects are obtained by the regulation of the small GTPase RhoA, a member of the Rho family of proteins that have been shown to control the organization of the actin cytoskeleton in many cell types. In the absence of neurotrophins, a constitutive interaction between $p75^{NTR}$ and Rho-GDI (inhibitor of RhoA) maintains RhoA activation and inhibition of axonal growth. Neurotrophin binding to $p75^{NTR}$ causes dissociation of RhoGDI from the receptor, blocking RhoA activity and leading to axonal growth (*Yamashita et al., 1999, 2003*). Modulation of axonal growth through $p75^{NTR}$-RhoA pathways is not exclusive to neurotrophins. In the last few years several reports have implicated $p75^{NTR}$ as a part of a receptor complex with the Nogo receptor (NgR) that mediates the axonal outgrowth inhibitory signals of myelin derivatives, such as Nogo66, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (*Wang et al., 2002; Wong et al., 2002*). LINGO-1, a nervous system-specific transmembrane protein, also binds NgR1 and $p75^{NTR}$ and is a functional component of the NgR1/$p75^{NTR}$ signaling complex.

The $p75^{NTR}$ is expressed additionally to neurons in a variety of other cell types like glia. Among glial cells, Schwann cells have been the most extensively studied for $p75^{NTR}$ signaling. Schwann cells express high levels of $p75^{NTR}$ during

development and regeneratation of peripheral nerves, although the specific role of $p75^{NTR}$ in mediating myelination is unclear. *Cosgaya and his colleagues* demonstrated that the enhancement of myelin formation in co-cultures of dorsal root ganglia neurons and Schwann cells by endogenous BDNF is mediated by $p75^{NTR}$ receptors (*Cosgaya et al., 2002*). Oligodendrocytes were among the earliest cell populations shown to undergo $p75^{NTR}$-mediated apoptosis *in vitro* (*Casaccia et al., 1996*), mediated by Rac GTPase activity, JNK phosphorylation (*Harrington et al., 2002*) and caspase activation (*Gu et al., 1999*).

Numerous studies have demonstrated that astrocytes express neurotrophin receptors in primary culture (*Hutton et al., 1992*) while NGF treatment upregulates $p75^{NTR}$ mRNA levels (*Hutton et al., 1992*). There is uncertainty about the role of $p75^{NTR}$ in pathological conditions associated with astrocytes, although one possibility may be that $p75^{NTR}$ regulates the proliferation of astrocytes to restrict glial scar formation after CNS injury.

1.2. Neurotrophins and neurodegenerative diseases

Neurotrophins have potent neuroprotective and neurogenic effects in various neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS) and peripheral neuropathy. However they have not truly fulfilled their therapeutic potential in clinical trials because of the difficulties of protein delivery and pharmacokinetics in the nervous system. NGF promotes the growth, differentiation and survival of cholinergic neurons in the basal forebrain and is thus ideally suited as a neurotherapeutic (*Winkler et al., 1998*). Exogenous NGF rescues cholinergic

neurons in the basal forebrain and improves cognitive function in impaired, aged or cholinergically-lesioned rats (*Fischer, 1994*). Transplantation of fibroblasts genetically modified to secrete NGF led to a slowing of cognitive decline in a phase 1 clinical trial performed on patients suffering from mild AD (*Tuszynski et al., 2005*). NGF deprivation causes amyloid-beta (Aβ) production and apoptotic cell death in both differentiated PC12 cells (*Matrone et al., 2008*) and hippocampal neurons in primary cultures (*Matrone et al., 2008*). Furthermore, in these studies addition of an antibody against Aβ resulted in the reversal of apoptosis due to NGF withdrawal but had no effect on the levels of apoptosis due to serum deprivation. These results show that the production of Aβ is responsible for the induction of apoptosis due to NGF withdrawal. In addition to the link between NGF and AD there is some evidence to implicate changes in BDNF and TrkB in the disease process. Firstly, BDNF mRNA levels decreased in AD hippocampal samples, estimated by *in situ* hybridization (*Phillips et al., 1991*), and by immunocytochemical techniques; BDNF protein levels decrease in neurons of the hippocampus and temporal cortex (*Connor et al., 1997*). In the basal forebrain, immunostaining demonstrated a large reduction in TrkA, TrkB and TrkC expression in AD (*Salehi et al., 1996*).

NGF overexpressing fibroblasts transplanted into the rat brain enhanced survival and neurite outgrowth of both grafted fetal basal forebrain cholinergic neurons and resident cholinergic circuits in the cerebellar cortex in a model of AD (*Ernfors et al., 1989*). Injection of NGF overexpressing monocytes in organotypic brain slices protected cholinergic neurons of the basal forebrain from NMDA-induced neurotoxicity (*Zassler and Humpel, 2006*).

Parkinson's disease is a slow, progressive disease involving rigidity and

tremor of the limbs, postural instability and bradykinesia of the limbs and body, with increasing prevalence in elderly population. Although for the initiation of the disease many factors both genetic and environmental may play a role, the final outcome is apoptotic loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) that leads to extensive loss of striatal dopamine concentrations, the projection area of the SNc neurons (*Lewis and Barker, 2009*). Both NGF and TrkA are expressed in a large number of dopaminergic neurons in the substantia nigra in humans (*Nishio et al., 1998*). Significant neuroprotective effects of neurotrophins have been demonstrated in animal models of PD. Intrathecal infusion of NGF suppressed Parkinsonian signs in monkeys one week after treatment with the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys dopaminergic neurons and is widely used to model PD pathology (*Tsukahara et al., 1995*). Intraventricular administration of NGF increased the levels of dopamine and homovanillic acid in the striatum of MPTP treated mice but had no effect in vesicle treated control mice (*Garcia et al., 1992*).

ALS is a condition in which motor neurons in the spinal cord, brain stem and motor cortex progressively die. Alterations in neurotrophins and their receptors have been found in ALS. In one study in ALS muscle biopsies at *post-mortem* (*Stuerenburg et al., 1998*), BDNF was strongly up-regulated in the early stage of the disease, whereas levels of NGF, NT-3 and NT-4/5 gradually increased during the course of the disorder. In a study of ALS spinal cords TrkB mRNA was up-regulated but the receptor was found to be much less phosphorylated on tyrosine residues than was that of controls (*Mutoh et al., 2000*).

Peripheral neuropathy encompasses a range of neurological disorders resulting from damage to the peripheral nerves. The most common cause is diabetes, in which symptoms are predominantly related to the degeneration of sensory fibers. Serum levels of NGF in patients with diabetic neuropathy were found to be lower than in controls and the reduction in NGF levels was correlated with the decrease of motor nerve conduction velocity (*Faradji et al., 1990*). In rodent models of diabetes, deficits in NGF and TrkA were reported, presumably resulting in decreased support of NGF-dependent sensory neurons (*Tomlinson et al., 1996*). Treatment with exogenous NGF normalized these deficits (*Tomlinson et al., 1997*).

1.3. Neurosteroids

The term “neurosteroids” appeared in the early 1980s by Étienne-Émile Baulieu (*Baulieu et al., 2001*). Neurosteroids are produced in the brain after local synthesis or by conversion of peripherally-derived adrenal steroids or gonadal steroids. (*Baulieu and Robel, 1990*) The major neurosteroids are dehydroepiandrosterone (DHEA) and pregnenolone (PREG), their sulfated derivatives DHEAS and PREGS, progesterone (PROG), and allopregnanolone (Allo) *(Maninger et al., 2009).* Since neurosteroids are highly lipophilic and can readily cross the blood-brain barrier, neurosteroids synthesized in peripheral tissues accumulate in the brain and can influence brain function (*Schumacher et al., 1996*).

1.4. Dehydroepiandrosterone (DHEA)

Biosynthesis and metabolism of DHEA

DHEA is an endogenous steroid hormone. It is the most abundant circulating steroid in humans, in whom it is produced in the adrenal glands, the gonads, and the brain (*Robel and Baulieu, 1995*). The biosynthesis products of steroids and their biosynthetic enzymes have been well studied (Figure 1.3).

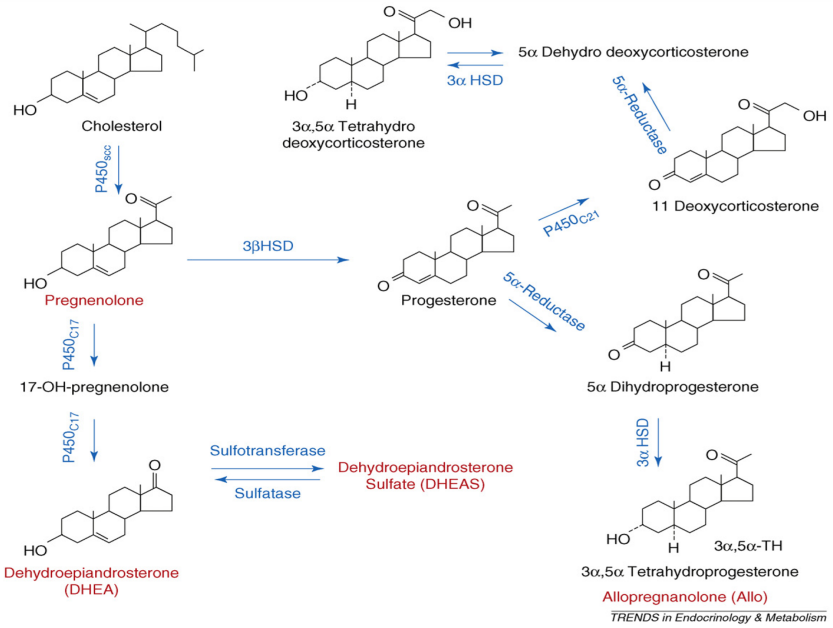


Figure 1.3. Schematic representation of major steroidogenic pathway catalyzed by cytochrome P450 (CYP) and HSD enzymes (*Maninger et al., 2009*).

Typically, there are three cytochrome P450 enzymes (CYP) such as P450 side chain cleavage (CYP11A), P450c17 (CYP17), and P450 aromatase (CYP19) and two types of hydroxysteroid dehydrogenases (HSDs) such as 3β-HSD and 17β-HSD. In particular, DHEA is synthesized from Pregnenolone (PREG) that is produced with the cleavage of cholesterol side chain by cytochrome CYP11A. PREG is metabolized to DHEA by the 17α-hydroxylase /

c17 and 20-lyse activity of cytochrome CYP17. Further on DHEA can be bi-directionally converted in to each sulfated derivative DHEAS by hydroxysteroid sulfotransferase and back to DHEA with a sulphatase (*Mellon, 2001*).

Neurons and glia express the necessary enzymes for the synthesis of DHEA

DHEA and its sulfated derivative (DHEAS) are the major neurosteroids in the brain (*Baulieu, 1997*). From the time that the local synthesis of DHEA in the brain was reported (*Corpechot et al., 1981*) there is considerable effort in the identification and localization of the enzymes required for its local synthesis in the nervous system.

During development in the mouse, cytochrome P450c17 protein expression starts as early as E10.5 in neural crest cells and shortly after is present in most of the spinal cord neural crest derived tissues, including peripheral nervous system (*Compagnone et al., 1995*). In the brain of rats cytochrome P450c17 is expressed during neonatal development and adulthood. The mRNA of P450c17 was found in the mesencephalon, cerebrum, diencephalon and cerebellum with the expression levels being higher in the mesencephalon. High levels of P450c17, in association with lower levels of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), the enzyme that transforms DHEA to androstenedione, in the mesencephalon shows that DHEA and not each metabolite might be the main neurosteroid in this area. (*Kohchi et al., 1998*). Furthermore, P450c17 has been identified in both neurons and astrocytes in hypothalamic and cortical cultures (Figure 1.4) (*Zwain and Yen, 1999*[1]*; Zwain and Yen, 1999*[2]) and in neurons, astrocytes and oligodendrocytes.

In the spinal cord of adult rats (*Kibaly et al., 2005*). P450c17 has been identified both in endoplasmic reticulum and in the presynaptic and postsynaptic regions of pyramidal neurons in CA1-CA3 regions and of granule neurons in the dentate gyrus, showing that DHEA may have fast neuromodulatory actions at the level of synapse (*Mukai et al., 2006; Hojo et al., 2004*). The presence of P450c17 in neurons and glial cells has been also described in the brain and pituitary of frog (*Rego et al., 2007*).

Finally, there is also evidence for an alternative pathway producing DHEA independently from P450c17 by Fe^{2+}. This Fe^{2+}-dependent alternative pathway has been shown to take place in both neurons and glial cells (*Cascio et al., 2000*).

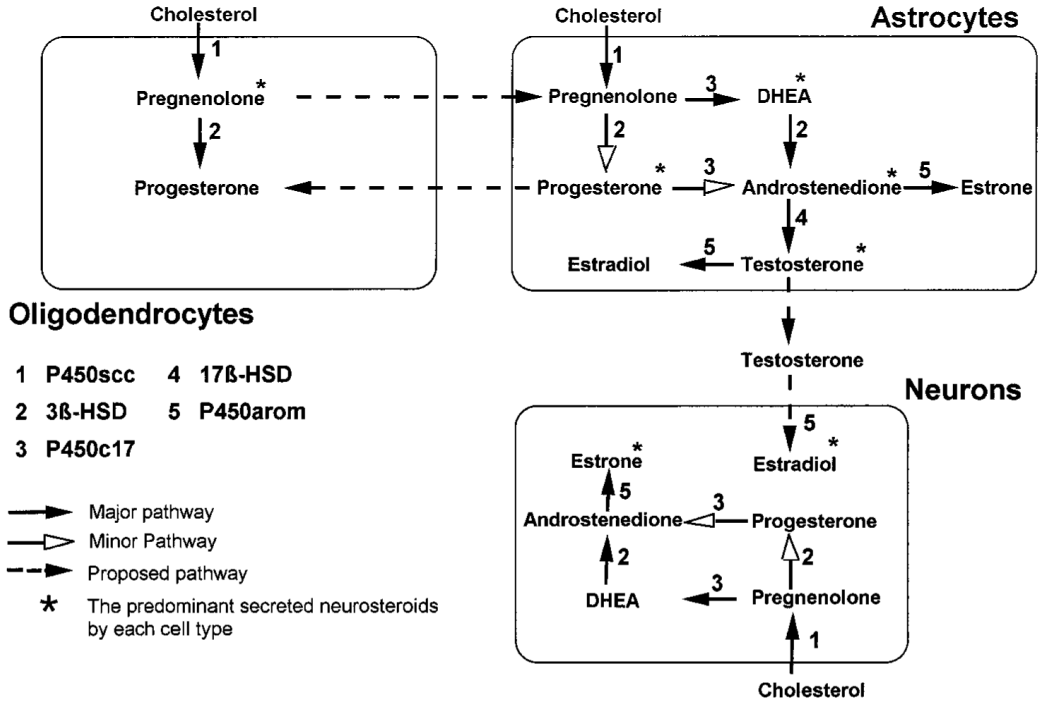


Figure 1.4. A schematic view of the neurosteroidogenic pathway in neurons and glia cells (*Zwain et al., 1999*).

Fluctuation of DHEA synthesis during development or aging

DHEA levels change profoundly throughout lifespan and fluctuations in the concentration of DHEA(S) during life coincides with important biological phenomena. This coincidence makes DHEA(S) a possible candidate as a regulatory molecule of these phenomena.

During development there are tempo-spatial fluctuations in each concentration, within the developing nervous system (Figure 1.5), showing that DHEA(S) may play an important role in the architecture of nervous system by controlling neural survival and apoptosis, and self renewal and differentiation of neural progenitors. Early in neonatal life the concentration of DHEA(S) starts to rise and remains at high levels throughout adulthood. These high levels may protect the brain from endogenous and exogenous neurotoxins and promote neurogenesis. During aging there is a progressive decline in the concentration of DHEA(S) (*Belanger et al., 1994, Migeon et al., 1957, Vermeulen et al., 1982*) and this decline have been associated with neural lose and neurodegenerative diseases (*Charalampopoulos et al., 2008*). Furthermore DHEA(S) may play a role in the shaping of neural responses acting as a neuromodulator by its interaction with neurotransmitter receptors (*Compagnone and Mellon, 2000*).

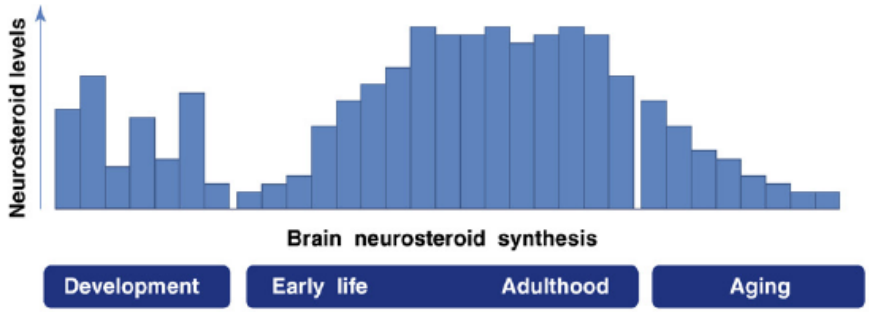


Figure 1.5. Changes in neurosteroidogenesis throughout lifespan (*Charalampopoulos et al., 2008)*.

1.5. Neurosteroid DHEA interacts with NGF receptors, preventing neuronal apoptosis

DHEA protects neuronal cells against apoptosis, activating pro-survival kinases PI3K/Akt and MEK1/2/ERK1/2, the transcription factors NF-κB and CREB, resulting in the expression of anti-apoptotic Bcl-2 proteins and the post-translational de-activation of pro-apoptotic Bad protein (*Charalampopoulos et al., 2004*). Similarities in the signal transduction pathways activated by DHEA and NGF suggested a role for NGF receptors in the anti-apoptotic actions of DHEA.

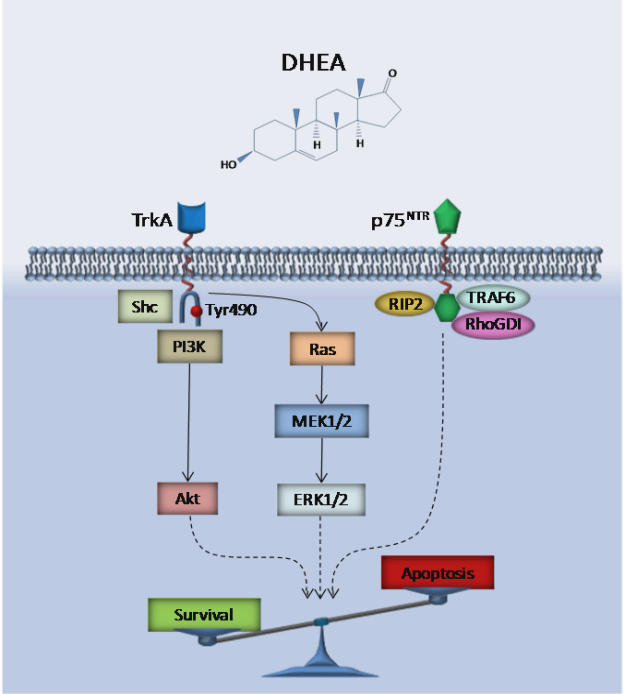


Figure 1.6. DHEA exerts its neurotrophic effects by directly interacting with the TrkA and p75NTR membrane receptors of nerve growth factor (NGF), and efficiently activates their downstream signaling pathways (*Lazaridis et al., 2011*).

We have recently reported that DHEA protects neuronal cells against apoptosis, interacting with TrkA (K_D at nanomolar levels), the high affinity prosurvival receptor of the neurotrophin, nerve growth factor. Specifically, binding of DHEA to TrkA results in TrkA autophosphorylation and the initiation of a downstream cascade of prosurvival kinases, such as the Shc-PI3KAkt and Src-

MEK-ERK signaling axes. In addition, DHEA also interacts with high affinity to the $p75^{NTR}$ receptors (K_D at nanomolar levels). Binding of DHEA to the pro-death receptor $p75^{NTR}$ affects the association of $p75^{NTR}$ with its effectors TRAF6, RIP2, and RhoGDI. The relative abundance of prosurvival TrkA and prodeath $p75^{NTR}$ receptors present in neuronal cells is thought to determine whether their fate is apoptosis or survival (*Lazaridis et al., 2011,* Figure 1.6).

1.6. Aims of the study

A) Based on our previous studies showing that DHEA is a ligand for NGF receptors, in the present study we explored its ability to bind to the other neurotrophin receptors, as well.

B) An effort was undertaken to develop synthetic analogs of DHEA, deprived of its endocrine properties but selectively interacting with NGF receptors, to propose them as lead compounds for the development of new neuroprotective and neurogenic agents in the therapeutics of neurodegenerative conditions.

A) Does DHEA binds to the other neurotrophin receptors?

It has been speculated that an alternative form of neurotrophic signaling should be present in invertebrates, possibly including several Trk related receptors like the mollusk *Lymnaea stagnalis* (Ltrk) (*Lee et al., 2001*), the marine mollusk *Aplysia californica* (ApTrk) (*Ormond et al., 2004*), and the cephalochordate amphioxus *Branhiostoma floridae* (AmphiTrk) (*Benito-Gutierrez*

et al., 2005). However, to date, researchers have been unable to identify neurotrophin homologs outside the vertebrate lineage (*Chao et al., 2000; Barde et al., 1994; Jaaro et al., 2001*), suggesting the presence of other types of molecules acting as potential activators of neurotrophin receptors. The only known example of such an invertebrate ligand, binding to fully conserved Trk receptor (ApTrk), is the ApNT, which is expressed exclusively in the central nervous system (CNS) of the marine mollusk *Aplysia californica*, playing a central role in learning related synaptic plasticity (*Ormond et al., 2004*). ApTrk is the only invertebrate Trk receptor with the structural and signaling features of its vertebrate Trk counterparts (*Kassabov et al., 2013*). In addition to Trk invertebrate ligands, the molluscan neurotrophic factor cysteine-rich neurotrophic factor (CRNF) has been found to interact with the mammalian pan-neurotrophin receptor $p75^{NTR}$ (*Fainzilber et al., 1996*), however invertebrate homologs of $p75^{NTR}$ have not been identified.

DHEA and the enzyme CYP17, which is required for DHEA biosynthesis, appeared early in evolutionary history (*Mizuta et al., 2007*). DHEA is a very "sticky" molecule, binding to many neutotransmitter and steroid hormone receptors, including the *N*-methyl-D-aspartate (NMDA), γ-aminobutyric acid type A ($GABA_A$), and σ1 receptors; the estrogen receptors α and β (ERα and ERβ); and androgen receptors (*Majewska et al., 1990; Johansson et al., 2005; Maurice et al., 1996; Kuiper et al., 1997*). Intrigued by its pleiotropic effects in the nervous system of a variety of species, in the present study we investigated the ability of DHEA to interact with the other two mammalian neurotrophin receptors, i.e., TrkB and TrkC, as well as their invertebrate counterparts (orthologs) in mollusks Lymnaea and Aplysia and in cephalochordate fish Amphioxus. Through these

studies we explored the hypothesis that, during evolution, DHEA might have served as a primordial neurotrophic factor, promoting neuronal survival in the less ancient complex nervous systems (*Pediaditakis et al., 2015*).

B) Developing synthetic DHEA analogs with binding affinity for NGF receptors (microneurotrophins).

Despite the demonstrated beneficial effect, the therapeutic usefulness of endogenous neurotrophins is compromised by their polypeptidic nature and their large size. Thus, enormous international interest has built up lately to develop small molecules, agonists of neurotrophin receptors, with therapeutic applications in neurodegeneration and brain trauma. Synthetic Blood Brain Barrier (BBB)-permeable small molecules that act as NGF receptor agonists with potent neuroprotective properties may represent a new therapeutic approach for NGF-dependent neurodegenerative diseases.

The neurosteroid DHEA was previously referred to protect various neuronal cell types by binding to neurotrophin receptors and inhibiting the apoptotic machinery (*Lazaridis et al., 2011*). This property of DHEA makes it a pharmacological candidate for its potential use in the treatment of neurodegenerative conditions. However, DHEA is metabolized in vivo to estrogens, androgens, and related metabolites, affecting the endocrine system, and altering the hormonal microenvironment in the brain. Therefore, the long-term use of DHEA as a potential treatment or chemio-prophylaxis of neurodegeneration is problematic, particularly in patients with genetic predisposition to hormone-dependent tumors (breast, endometrium, ovaries,

prostate). To overcome these unwanted effects of DHEA, we have previously synthesized DHEA analogs with modifications at positions C3 and C17 and evaluated these compounds for neuroprotective activity (*Calogeropoulou et al., 2009*). While these derivatives maintain strong neuroprotective, anti-apoptotic properties (EC_{50} at nanomolar levels), they are incapable of being metabolized towards estrogens and androgens and have no affinity for steroid hormone receptors, therefore do not cause endocrine effects (*Calogeropoulou et al., 2009*). The present study focused on the small, DHEA-derivative molecule, BNN27, which has been shown to effectively protect PC12 cells from serum-deprivation induced apoptosis (*Calogeropoulou et al., 2009*). Since, its prototype molecule (DHEA) binds to both NGF receptors (*Lazaridis et al., 2011*), we explored the ability of this newly synthesized molecule (BNN27) to bind and activate NGF receptors. Specifically, we assessed membrane binding of BNN27 in HEK293 cells transfected with the TrkA or $p75^{NTR}$ plasmid cDNAs, using binding assays. To investigate the domains of NGF receptors responsible for binding to BNN27, we performed binding assays in HEK293 cells transfected with mutated forms of both TrkA and $p75^{NTR}$ receptors at specific functional amino-acids. The direct physical interaction of BNN27 to NGF receptors was assessed by pull-down assays and NMR studies. Following our results on BNN27 ability to bind both NGF receptors, we to explored its efficacy -compared to NGF- in the activation of TrkA and $p75^{NTR}$ receptors. We examined TrkA phosphorylation using specific phospho-antibodies for all three (490, 675 and 785) known tyrosine residues known to be phosphorylated upon NGF treatment. The effectiveness of BNN27 to promote the interaction of $p75^{NTR}$ receptor with its effector proteins RhoGDI, RIP2 and TRAF6 was also examined. In order to determine whether the

anti-apoptotic effects of BNN27 are mediated through TrkA and $p75^{NTR}$ we used shRNAs against each one of the two receptors in PC12 cells using flow cytometry detection of caspase-3 activation. Furthermore, we examined the ability of BNN27 to rescue from apoptosis NGF receptor-sensitive dorsal root ganglia sensory neurons of NGF null mice, and NGF deprived rat superior cervical ganglia sympathetic neurons in culture. We investigated the possible role of BNN27 to potentiate the effects of NGF in axonal outgrowth in cultured DRG explants: co-incubation of BNN27 with NGF enhances the effect of the latter in neurite elongation. To evaluate the kinetics of the effect of BNN27 in TrkA internalization, we performed immunoprecipitation studies of TrkA with endosomal markers.

Our findings provide evidence that BNN27 exerts strong anti-apoptotic, neuroprotective actions via NGF receptors, differentially activating prosurvival signaling. BNN27 may serve as a lead molecule to develop BBB permeable, neurotrophin-like small molecules (microneurotrophins) with potential applications in the treatment of neurodegenerative diseases.

2. MATERIALS AND METHODS

Plasmids, Antibodies and Proteins

Plasmids of TrkA, $p75^{NTR}$, RIP2, RhoGDI and TRAF6 were previously described by *Lazaridis et al., 2011*. ERβ plasmid was obtained from Dr E.R. Levin (Irvine VA, Long Beach), as described in *Pettersson et al., 1997*. $TrkA^{\Delta ECD}$, $p75^{\Delta ECD}$ and $p75^{C257A}$ constructs were previously described by *Jung et al., 2003*, *Arevalo et al., 2000 and Vilar et al., 2009*, respectively. Normal expression of all constructs was verified by immunoblotting and FACS. PC12 cells were transfected with specific shRNAs for attenuating the expression of TrkA or $p75^{NTR}$ receptors (*Lazaridis et al., 2011*).

AmphiTrk plasmid was kindly provided by Dr. C.F Ibáñez (Karolinska Institutet, Sweden), TrkB and TrkC plasmids by Dr. Yves-Alain Barde (University of Cardiff, UK), ApTrk and ApTrk-GFP plasmids by Dr. Eric R. Kandel (Columbia University, New York) and the expression plasmid for Ltrk by Dr. Mike Fainzilber (Weizmann Institute, Israel).

The origin of antibodies was as follows: phospho-TrkA 490 (Cat. No. 9141; Cell Signaling), phospho-TrkA 490 (Cat. No. AP00221PU-N; Acris, ICC), phospho-TrkA 674/675 (Cat. No. 4621; Cell Signaling), phospho-TrkA 785 (Cat. No. 4168; Cell Signaling), TrkA (Cat. No. 06-574; Millipore), TrkA (Cat. No. sc-7268; Santa Cruz, ICC), pan-Trk (Cat. No. 4609; Cell Signaling), TrkB (Cat. No. ab33655; Abcam), TrkC (Cat. No. 3376; Cell Signaling), phospho-ERK1/2 (Cat. No. 4376; Cell Signaling), Erk1/2 (Cat. No. 4695; Cell Signaling), phospho-Akt (Ser473) (Cat. No. 4060; Cell Signaling), Akt (Cat. No. 4691; Cell Signaling), phospho-JNK (Cat. No. 4668; Cell Signaling), JNK (Cat. No. 9252; Cell Signaling), Rab5 (Cat. No. 610282; BD Transduction Laboratories), Rab7 (Cat. No. R8779; Sigma), Rab11 (Cat. No. 610657; BD Transduction Laboratories), c-

myc (Cat. No. 9E10, sc-40; Santa Cruz BiotechNology Inc.), RIP2 (Cat. No. ADI-AAP-460; Enzo Life Sciences Farmingdale), RhoGDI (Cat. No. R3025; Sigma), $p75^{NTR}$ for blotting [IB] and immunostaining (Cat. No. G3231; Promega), MC192 anti-$p75^{NTR}$ for immunoprecipitation [IP] (Cat. No. MAB365R; Millipore), GAPDH (Cat. No. 2118; Cell Signaling), and b-actin (Cat. No. A4700; Sigma). Secondary antibodies: horseradish peroxidase-conjugated anti-rabbit IgG (Cat. No. 65-6120; Invitrogen) and horseradish peroxidase-conjugated antimouse IgG (Cat. No. AP-124P; Millipore). Anti-rabbit-R-phycoerythrin conjugated (Cat. No. P9537; Sigma), anti-mouse-fluorescein conjugated (Cat. No. AP124F; Millipore), anti-rabbit Alexa Fluor 488 (Cat. No. A21206; Invitrogen), anti-rabbit Alexa Fluor 546 (Cat No. A10040; Invitrogen), GFP (Cat. No. ab290; Abcam). NGF (Cat. No. 01-125) was purchased from Millipore. BDNF (Cat. No. P3595) and NT3 (Cat. No. P4433) were purchased from Novus. TrkA inhibitor was purchased from Calbiochem (Cat. No. 648450).

For the pull-down assays and STD-NMR experiments, the recombinant Rat TrkA/Fc chimera and the recombinant mouse NGF R/TNFRSF16 Fc chimera (also named p75 neurotrophin receptor) were purchased by R&D Systems, Inc.

Tissue Culture and Cell Transfection

PC12, HEK293 and CHO cells were obtained from LGC Promochem. PC12nnr5 cells were kindly provided by Dr. Marçal Vilar (Instituto de Salud Carlos III-ISCII, Madrid, Spain). HEK293 and CHO cells were grown in DMEM medium containing 10% fetal bovine serum (charcoal-stripped for removing endogenous steroids), 100 units/ml penicillin, and 0.1 mg/ml streptomycin, at 5% CO_2 and 37^{o}C. PC12 and PC12nnr5 were grown in RPMI 1640 containing 2 mM

L-glutamine, 15mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% horse serum, 5% fetal calf serum (both charcoal-stripped for removing endogenous steroids) at 5% CO^2 and 37°C. MEFs were kindly provided from Dr C.F. Ibáñez (Karolinska Institutet) and cultured under standard conditions. Cells were transfected with TurboFect (Fermentas) or Lipofectamin 2000 (InVitrogen) according to manufacturers' instructions. Transfected cells were typically used on the 2nd day after transfection.

Schwann cells were dissected from P1 rat sciatic nerve and incubated in HBSS containing 0.25% trypsin (Sigma, St. Louis, MO) and 0.25% collagenase (Sigma) for 30 min. The cells were triturated and plated on poly-D-lysine-coated six-well plates cultured in DMEM containing 10% fetal calf serum and 2 μM forskolin (Sigma).

[^{3}H]-DHEA Binding Assays

HEK293 cells transfected with the cDNA expression plasmids coding for TrkA, TrkA$^{\Delta ECD}$, p75^{NTR} or p75$^{\Delta ECD}$ (FACS analysis and Western Blot inserts show the efficacy of transfection) were cultured, collected by scraping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7.4. After a centrifugation at 1200 rpm, cells were homogenized by sonication (3x30sec, at 4°C) in a 50 mM Tris-HCl buffer pH 7.4 (at 4°C) containing freshly added protease inhibitors (1 mM PMSF and 1 μg/ml apoprotin). Crude membrane fractions were isolated by differential centrifugation at 2,500g (10 min at 4^{o}C, to remove unbroken cells and nuclei) and 100,000g (1 h, at 4^{o}C). Membranes were washed once with ice-cold 50 mM Tris–HCl buffer, pH 7.4, and re-suspended in the same buffer. Membranes were then briefly acidified with 50 mM glycine pH 3

for 3 min on ice to dissociate any ligand bound to receptors, washed once, re-suspended in PBS (pH 7.4) with protease inhibitors, at a concentration of 2 mg/ml, and used immediately. A constant concentration of [^{3}H]-DHEA (5 nM) was incubated with increasing concentrations of BNN27 (from 10^{-12} to 10^{-6} M) or ADIOL (from 10^{-12} to 10^{-6} M) in a final volume of 100 µl. Membranes were then incubated overnight at 4^{o}C on a rotating plate; then they were collected on GF/B filters and pre-wetted in 0.5% PEI solution at 4^{o}C. Filters were washed five times with ice-cold PBS, dried and counted in scintillation fluid (SigmaFluor, Sigma) in a scintillation counter (Perkin Elmer, Foster City, CA) with 60% efficiency for Tritium.

[^{3}H]-17β-Estradiol Binding Assays

HEK293 cells transfected with the cDNA expression plasmids coding for ERβ (Western Blot inserts show the efficacy of transfection), were cultured, collected by scraping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7.4. After a centrifugation at 1200 rpm, cells were homogenized by sonication (3 x 30 sec, at 4°C) in a 50 mM Tris-HCl buffer pH 7.4 (at 4°C) containing freshly added protease inhibitors (1 mM PMSF and 1 µg/ml apoprotin). Cytoplasmic (supernatant) fractions were obtained by centrifugation at 100,000g (1 h, at 4^{o}C). For the competitive binding assay, increasing concentrations of BNN27 (from 10^{-12} to 10^{-6} M) or ADIOL (from 10^{-12} to 10^{-6} M) or Estradiol (from 10^{-12} to 10^{-6} M) were incubated with cytosol (~250µg protein) in the presence of 4 nm of [^{3}H] 17β- Estradiol for 18 h at 4^{o}C. Bound and free estradiol was separated using an equal volume of DCC (Dextran-coated charcoal) for 20 min at 4^{o}C followed by centrifugation at 3200g for 20 min at 4^{o}C. Bound [^{3}H] estradiol was

measured in scintillation fluid (SigmaFluor, Sigma) in a scintillation counter (Perkin Elmer, Foster City, CA) with 60% efficiency for Tritium.

.**Immunoprecipitation and Immunoblotting**

After 48 h transfection, cells were starved from serum for a few hours and stimulated with BNN27 or the appropriate neurotrophins for 15–30 min as indicated. Cells were suspended in lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4) supplemented with protease inhibitors (1 mM PMSF and 1 g/ml aprotinin). Lysates were pre-cleared for 1 h with protein G-plus Agarose beads (Cat No. sc-2002; Santa Cruz) and immunoprecipitated with the appropriate antibody overnight at 4^{o}C. Protein G-plus agarose beads were incubated with the lysates for 4 h at 4^{o}C with gentle shaking. Beads were collected by centrifugation, washed four times with lysis buffer and re-suspended in SDS loading buffer. Immunoblots were developed using the ECL Western Blotting Kit (Thermo Scientific) and exposed to Kodak X-Omat AR films. Image analysis and quantification of band intensities were done with ImageQuant (GE Healthcare).

Immunofluoresence

For immunofluoresent staining, cells were fixed in a solution of 4% PFA for 15 min on ice, washed with PBS and incubated with 5% BSA in PBS-T 0,3%. After blocking cells were incubated with primary antibodies in PBS-T 0,3% overnight at 4°C followed by washes with PBS and incubation with secondary fluorescent antibodies for 1 h in RT.

Flow Cytometry

The efficacy of transfection and expression of TrkA, TrkB, TrkC, $p75^{NTR}$, Ltrk, ApTrk, or AmphiTrk plasmids in HEK293 cells were analyzed in a Beckton-Dickinson FACSArray apparatus and the CELL-Quest software, using the relevant antibodies.

PC12 cells were cultured in 12-well plates, and 24 h later they were transfected with the si/shRNAs for TrkA or $p75^{NTR}$ receptors. 24h later the medium was aspirated and replaced either with complete medium (serum supplemented) or serum free medium in the absence or the presence of BNN27 at 100 nM. PC12nnr5 cell were transfected with the plasmid cDNAs of Ltrk, invertebrate ApTrk, AmphiTrk receptors or empty vectors (mock) and cultured for 24 hours in serum-starved conditions in the absence or the presence of 100 nM DHEA, or 100 ng/ml of mammalian neurotrophins.

PC12 and PC12nnr5 cells at a concentration of $1x10^{-6}$ were fixed and permeabilized by re-suspending in 100 μL of Cytofix/Cytoperm (BD Biosciences) buffer on ice for 30 min, followed by the addition of 0.5 ml of BD Perm/Wash buffer (1x) (BD Biosciences). After washing and centrifuging (300×g for 5 min), the PC12 cells were stained using the Cleaved Caspase-3 antibody (Cat. No 9664; Cell Signaling) for 30 min on ice. Afterwards, the cells were washed, centrifuged (300×g for 5 min) and the secondary antibody [anti-rabbit Alexa Fluor 488 (Cat. No A21206; Invitrogen)] was added for 30 min on ice. PC12nnr5 cells were stained using TUNEL (Cat. No. 11684795910; Roche) for 1 h at 37 ^{o}C. Following washing and centrifuging, the cells were re-suspended in 0.5ml BD Perm/Wash buffer (1x) and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Surface biotinylation assays

PC12 cells were biotinylated as described in *Yu et al., 2011* and then treated with NGF (100 ng/mL) or BNN27 (100 nM) for 5, 15 and 30 min. Biotin from non-internalized proteins was removed using reducing conditions as described in *Yu et al., 2011*. Cell lysates were prepared, surface proteins were subjected to precipitation with neutroavidin and western blot analyses were performed with the corresponding antibodies.

Cholesterol depletion and lipid rafts fractionation

CHO cells were transfected with TrkA plasmid and pretreated with 10 mM methyl- β-cyclodextrin (MβCD) for 20 min. MβCD treatment is a common method of examining lipid raft function, efficiently removing cholesterol from the plasma membrane (*Christian et al., 1997*). Cells were washed four times with serum and MβCD free medium and then stimulated with 100 nM BNN27 for 5, 15 and 30 min and lysed in lysis buffer. TrkA phosphorylation was analyzed by Western blot as described above. Cellular viability was monitored in parallel using trypan blue exclusion. Fractionation of detergent resistant membranes by sucrose gradient was performed on HEK cells as previously described (*Nikoletopoulou et al, 2010*). HEK cells were exposed to NGF or BNN for 15 minutes prior to lysis, while untreated cells served as a control. Twenty fractions were collected, of which the two uppermost (fractions 1 and 2) were devoid of proteins and hence omitted from further analysis. Fractions 3-20 were analyzed by Western blot on 10% acrylamide gels and blotted on nitrocellulose membranes following standard procedures. Membranes were then analyzed with an antibody against flotillin-1, a marker of lipid raft microdomains, and an antibody against TrkA.

Superior Cervical Ganglia Neuronal Cultures

Superior cervical ganglia (SCG) were dissected from newborn (P0–P1) rat pups and dissociated in 0.25% trypsin (Cat. No 15090; Gibco) for 30 min at 37°C. After dissociation SCG neurons were re-suspended in RPMI culture medium 1640 containing 3% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 mM antimitotic agent FdU with uridine, and 50 or 100 ng/ml NGF (Cat. No 01-125; Millipore). Cells were plated on collagen coated 24-well plates and cultured for 7 d prior to use. For NGF withdrawal experiments, cells were washed twice with NGF free medium and culture for 48 h in medium lacking NGF and containing anti-NGF antibody at 1:500 dilution (Cat. No AB1526; Millipore). At the end of the treatment period neurons were immunostained against neurofilament protein using NF200kD antibody and TUNEL assay (Cat. No 11684795910; Roche) was conducted according to manufacturer's instructions in order to stain apoptotic neurons.

***In Vivo* Experiments with the NGF Null Mice**

ngf +/− mice (*Crowley et al., 1994*) were obtained from the Jackson Laboratory and maintained on C57BL/6 background. They were kept in the Animal House of the Institute of Molecular Biology and Biotechnology (IMBB-FORTH, Heraklion, Greece). Animals were housed in a temperature-controlled facility on a 12 h light/dark cycle, fed by standard chow diet and water ad libitum. Genotyping was performed on tail DNA using the following primers: NGFKOU2 (5′CCG TGA TAT TGC TGA AGA GC3′), NGFU6 (5′CAG AAC CGT ACA CAG ATA GC3′), and NGFD1 (5′TGT GTC TAT CCG GAT GAA CC3′).

Genomic PCR reactions containing the 3 primers were incubated for 32 cycles at 95°C (30 s) / 59°C (30 s) / 72°C (1 min).

Mice heterozygous for the NGF gene disruption were interbred to obtain mice homozygous for the disrupted gene. The pregnant mothers were treated daily with an intra-peritoneal injection of BNN27 (100 mg/kg/day) or vehicle (4.5% ethanol in 0.9% saline) starting from the third day after gestation until day E13.5 or E17.5. At the end of the treatment mothers were euthanized by cervical dislocation and embryos were collected and fixed overnight in a solution of 4% PFA, 15% Picric Acid, 0.05% GA in phosphate buffer 0.1 at 4°C. The next day embryos washed in 0.1 M phosphate buffer and cryo-protected by being immersed in a 30% sucrose solution in PB 0,1 M at 4°C until they sink. Finally, embryos were frozen in OCT in isopentane at −40°C. The samples were sectioned (16 µm) in a freezing microtome and mounted onto Superfrost plus slides (Menzel-Glaser J1800AMNZ). Slides were post fixed for 10 min in cold acetone and left to dry for 10 min at room temperature. They were then washed in PB 0.1 M, and incubated for 1 h at RT with 5% horse serum in TBS-T 0.1%. After this slides were incubated with primary antibodies (anti-NF200Kd at 1:400 and anti- activated Caspase-3 diluted 1:250 or anti-TrkA 1:100), in TBS-T 0.1% with 1% horse serum overnight at 4°C, followed by 3 washes with TBS and incubation with anti-rabbit secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546, 1:1000 in TBS-T 0.1%) for 1 h at RT in TBS-T 0.1%. Sections were washed with TBS again and coverslipped with antifade reagent (InVitrogen) and visualized using confocal microscopy.

In order to quantify apoptotic neurons in embryos, caspase-3 positive cells inside the area of the DRG were counted in 12-14 sections being 160 µM apart

from one another across most of the rostro-caudal extent of the spinal cord and the mean of apoptotic cells per sections was estimated.

Statistical Analysis

Statistical analysis of our data was performed using Analysis of Variance, post hoc comparison of means, followed by the Fisher's least significance difference test. For data expressed as percent changes we have used the nonparametric Kruskal-Wallis test for several independent samples.

3. RESULTS

3.1. DHEA interacted with both vertebrate and invertebrate Trk receptors

Intrigued by DHEA pleiotropic effects on the nervous system across a variety of species, and the early evolutionary presence of CYP17, the enzyme responsible for its biosynthesis (*Mizuta et al., 2007; Kirischian et al., 2011*), we examined, the ability of DHEA to bind to representative members of Trk receptors of various species and more specifically to the vertebrate TrkA, TrkB, TrkC receptors and to the invertebrate Trk orthologs, Ltrk and ApTrk (mollusks Lymnaea and Aplysia respectively), and AmphiTrk (cephalochordate fish Amphioxus). Competition binding assays showed that DHEA effectively displaced binding of [^{3}H]-DHEA to membranes isolated from HEK293 cells (not expressing endogenous Trk receptors) transfected with the cDNAs of TrkA, TrkB or TrkC receptors (Ki: 7.40±1.7 nM, 4.49±1.7 nM and 6.33±1.3 nM, N=3, respectively), suggesting that DHEA appears to interact with all forms of vertebrate Trk receptors (Figure 3.1.A), although with affinities approximately two orders of magnitudes lower compared to that of polypeptide neurotrophins (0.01– 0.1 nM) (*Bothwell et al., 1995*). Moreover, DHEA was proved equally effective in displacing binding of [^{3}H]- DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of Ltrk, ApTrk or AmphiTrk receptors (Ki: 3.6 ±0.4 nM, 1.14±0.11 nM, 0.47±0.18 nM, N=3, respectively) (Figure 3.1. B-D).

Membranes isolated from HEK293 cells transfected with the empty vector (mock) showed no specific binding of tritiated DHEA. We have previously shown that DHEA-S, the sulfated ester of DHEA, binds to TrkA with an affinity comparable to this of DHEA (*Lazaridis et al., 2011*). Our competition assays showed that DHEA-S also binds to all three invertebrate receptor types at nanomolar concentrations (Figure 3.1.E).

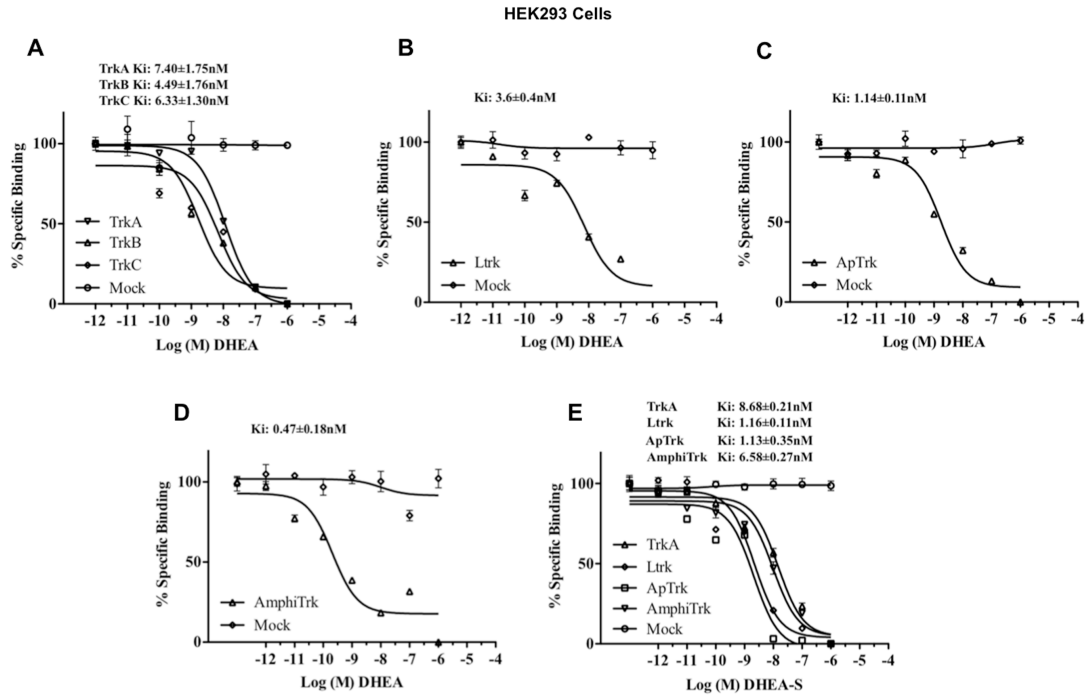


Figure 3.1. A-E. Competition binding assays of tritiated [^{3}H]-DHEA in the presence of increasing concentrations of non-labeled DHEA (A-D) or DHEAS (E) were performed using membranes isolated from HEK293 cells transfected with the plasmid cDNAs of mammalian TrkA, TrkB, TrkC receptors (A) or invertebrate Ltrk, ApTrk, AmphiTrk receptors (B-E). (KI represents the mean±SE of three experiments).

However, the affinity of DHEA-S for AmphiTrk was significantly lower compared to this of DHEA, suggesting that even small structural differences significantly affect binding of these steroids to neurotrophin receptors.

3.2. DHEA was able to activate all types of invertebrate Trk homologs

To examine if binding of DHEA to Trk receptors is functional, we assessed its effectiveness in inducing tyrosine phosphorylation of Trk receptors, the sine qua non proof in assessing activation of Trk receptors. We have found that DHEA induced tyrosine phosphorylation of TrkA and TrkC receptors in CHO cells transfected with the appropriate cDNAs, but did not affect the phosphorylation of

TrkB receptors (Figure 3.2.A). To our knowledge, DHEA is the only molecule that successfully binds to all three vertebrate Trk receptors, activating two of them. The invertebrate Ltrk receptor has a conserved transmembrane and intracellular tyrosine kinase domain but lacks the immunoglobulin-like domains at the extracellular part of the protein (*van Kesteren et al., 1998*). Ltrk has been shown to bind mammalian NT-3 (but not NGF or BDNF), however with no apparent signaling response to this neurotrophin (*van Kesteren et al., 1998*). It is interesting that DHEA was also able to induce tyrosine phosphorylation of the Ltrk receptors in CHO cells transfected with the Ltrk cDNA, an effect not seen in any polypeptide neurotrophin (Figure 3.2.B). In *Aplysia*, ApTrk is highly conserved compared to vertebrate Trk receptors possessing an identical domain architecture that includes two immunoglobulin G (IgG) domains in its ligand-binding extracellular region and the two main signaling sites. BDNF and Aplysia's neurotrophin ApNT were able to bind and activate ApTrk, while NGF and NT3/4 failed to activate ApTrk (*Kassabov et al., 2013*). DHEA also induced tyrosine phosphorylation of the ApTrk receptor in CHO cells transfected with the ApTrk cDNA (Figure 3.2.C). Amphioxus AmphiTrk receptors share the basic protein domain structure of the mammalian Trk receptors at both intracellular and extracellular level, interacting with mammalian neurotrophins NGF, BDNF, NT3 and NT4 (*Benito-Gutierrez et al., 2005*). DHEA also induced tyrosine phosphorylation of AmphiTrk receptors in CHO cells transfected with the AmphiTrk cDNA (Figure 3.2.D).

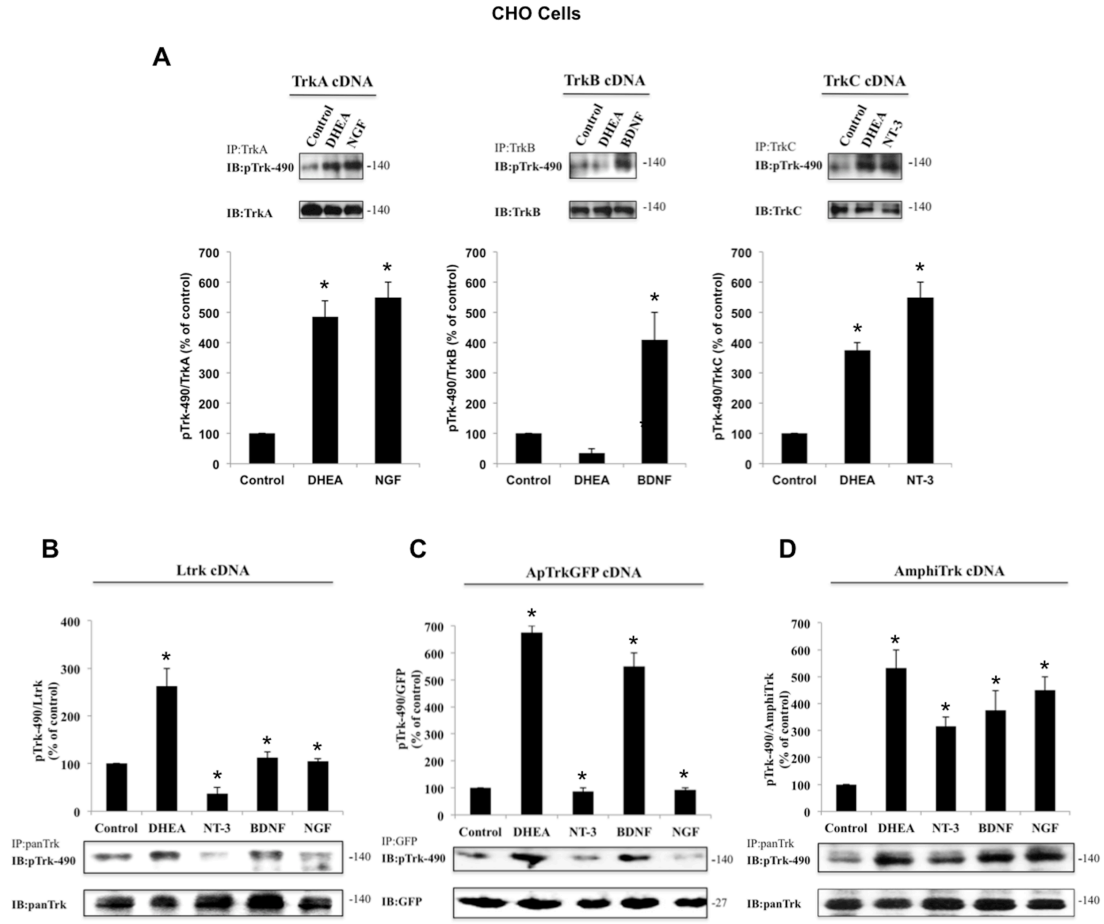


Figure 3.2. **A.** CHO cells were transfected with the plasmid cDNAs of mammalian TrkA, TrkB or TrkC receptors. Transfectants were then exposed for 15 minutes to 100 ng/ml of mammalian neurotrophins NGF (left panel), BDNF (middle panel), NT-3 (right panel) or to 100 nM of DHEA. Cell lysates were immunoprecipitated with specific antibodies against TrkA, TrkB, or TrkC, then immunoblotted with pY490 antibodies. Total lysates were analyzed for TrkA, TrkB or TrkC expression by immunoblotting. **B-D.** CHO cells were transfected with the plasmid cDNAs of invertebrate Ltrk, ApTrk or AmphiTrk receptors and then exposed for 15 minutes to DHEA or to mammalian neurotrophins, as above. Cell lysates were immunoprecipitated with panTrk or GFP antibodies, and then immunoblotted with pY490 antibodies. Total lysates were analyzed for Trk expression by immunoblotting. (mean±SEM of three independent experiments, **P <0.05* relative to control, serum free condition).

3.3. DHEA induced the activation of Erk1/2 and Akt kinases via all types of invertebrates Trk receptors

To evaluate whether activation of invertebrate Trk receptors by DHEA do

activate downstream signaling pathways involving the pro-survival Erk1/2 and Akt kinases, we used PC12nnr5 cells, a clone of PC12 cells not expressing endogenous Trk receptors, but expressing the pan-neurotrophin receptor $p75^{NTR}$ (*Loeb et al., 1993*). DHEA effectively induced phosphorylation of both Erk1/2 and Akt kinases in PC12nnr5 cells transfected with the cDNAs of Ltrk, ApTrk or AmphiTrk receptors (Figure 3.3. A-C). It should be stressed however that in the same transfectants, DHEA failed to induce phosphorylation of PLC-γ (data not shown), a protein that is implicated in neuronal differentiation and synaptic plasticity (*Benito-Gutierrez et al., 2005; Obermeier et al., 1994*)

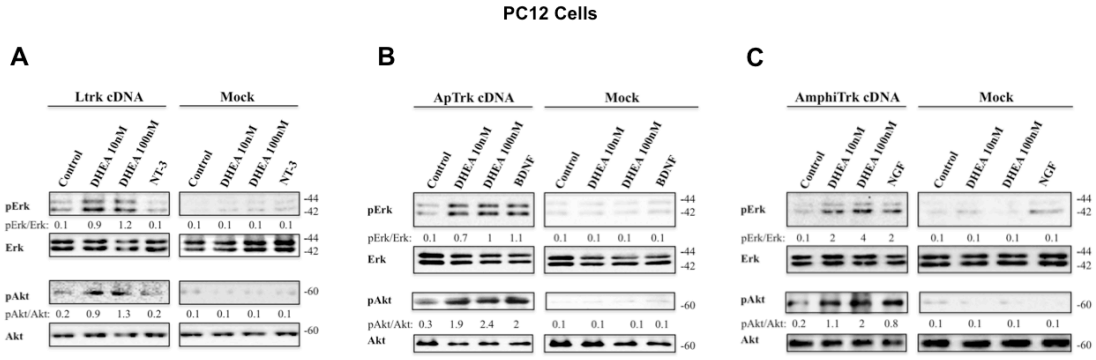


Figure 3.3. A-C. PC12nnr5 cell were transfected with the plasmid cDNAs of Ltrk, ApTrk, AmphiTrk receptors and control plasmid (mock), treated for 15 minutes with DHEA at various concentrations or 100 ng/ml NT-3, 100 ng/ml BDNF, 100 ng/ml NGF respectively, and then lysed and analyzed by western blotting with the indicated antibodies. Mean±SEM of three independent experiments.

3.4. The invertebrate Trk receptors mediated the anti-apoptotic effects of DHEA

In this set of experiments, we have found that DHEA effectively reversed the serum deprivation-induced apoptosis in Ltrk-, or ApTrk-transfected PC12nnr5 cells (from 0.31±0.03 in serum-free conditions to 0.163±0.008 and 0.146±0.003 respectively, N=3, *$*P<0.05$*) or AmphiTrk-transfected PC12nnr5 cells (from 0.253

±0.008 in serum-free conditions to 0.183±0.016, N=3, **P<0.05*) (Figure 3.4. A-C).

Mammalian neurotrophins NGF or BDNF exerted similar anti-apoptotic actions in ApTrk- or AmphiTrk-transfected PC12nnr5 cells. However, mammalian NT-3 increased further the apoptosis induced by serum-deprivation in Ltrk-transfected PC12nnr5 cells, possibly due to inability of NT-3 to activate Ltrk in one hand, and on the other, it can activate the pro-apoptotic $p75^{NTR}$ receptor (which is also expressed) and thus increasing apoptosis. Finally, DHEA was unable to induce neurite outgrowth in Ltrk-, ApTtrk- or AmpiTrk-transfected PC12nnr5 cells, confirming previously published observations in mammalian, endogenously TrkA expressing PC12 cells (*Lazaridis et al., 2011*).

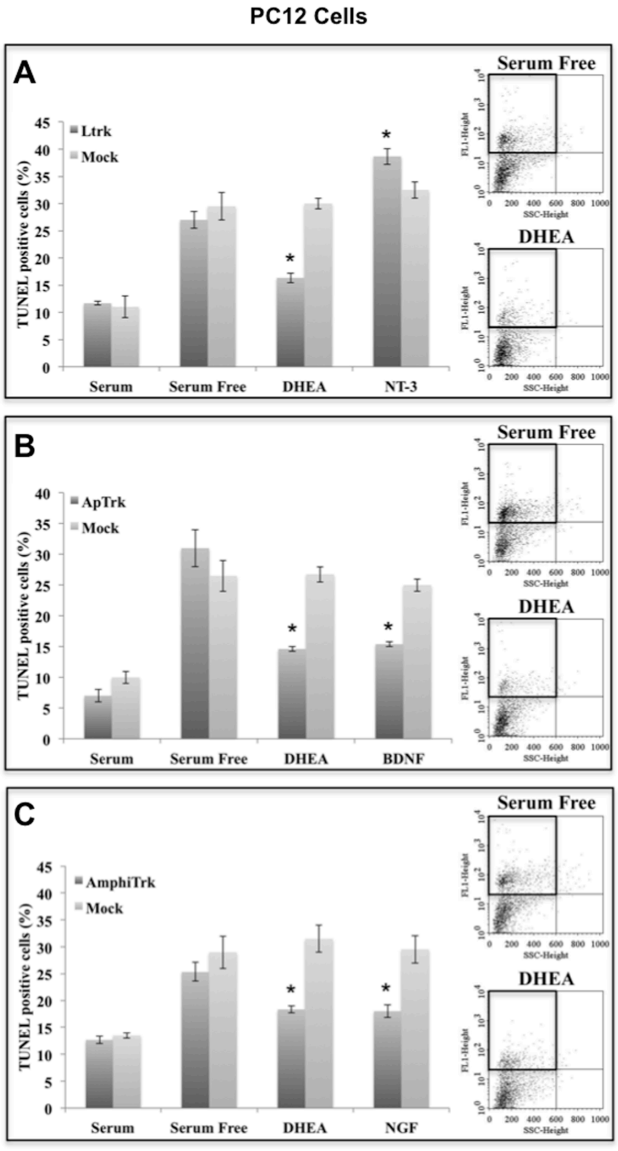


Figure 3.4. A-C. PC12nnr5 cell were transfected with the plasmid cDNAs of Ltrk, invertebrate ApTrk, AmphiTrk receptors or empty vectors (mock) and cultured for 24h in serum-starved conditions in the absence or the presence of 100 nM DHEA, or 100 ng/ml of mammalian neurotrophins. FACS analysis shows the percentage of TUNEL positive cells compared with Serum Free condition, measuring 10.000 cells. (mean±SEM of three independent experiments, **P <0.05*, FACS diagrams on the right panel show only the results from transfected cells).

3.5. BNN27 specifically interacts with TrkA and p75^{NTR} receptors

Competition assays using [^{3}H]-DHEA, showed that BNN27 effectively displaces binding of [^{3}H]-DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and p75^{NTR} receptors (Ki: 1.86±0.4nM and 3.9±1.2nM respectively) (Figure 3.5.A, B, Table 1). In contrast, BNN27 had no affinity for TrkB, TrkC (Figure 3.5.C).

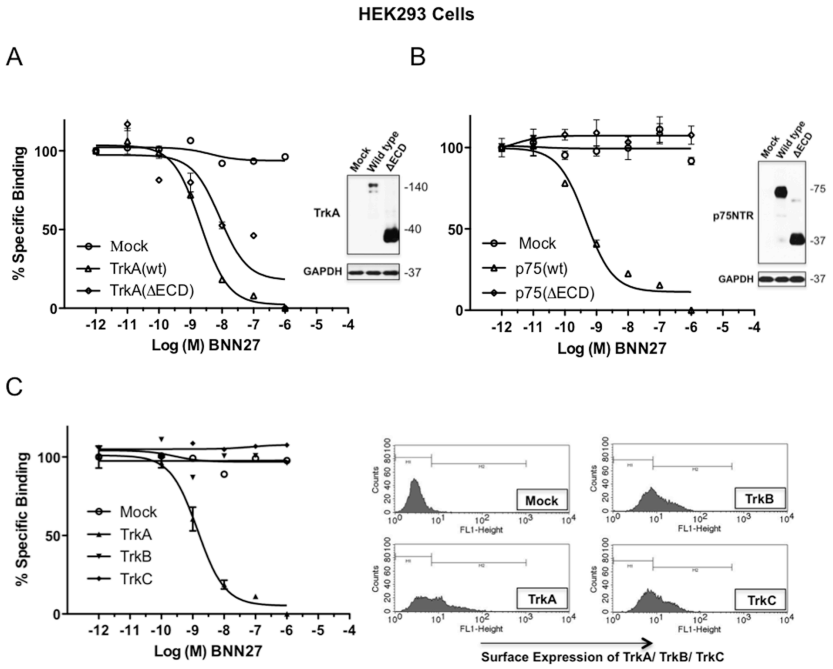


Table 1

Neurotrophin Receptors	BNN27 Ki (nM)
TrkA	1.31±0.6
TrkA (ΔECD)	6.3±0.9
p75NTR	3.7±1.3

Figure 3.5. A. Competition binding assays of [^{3}H]-DHEA in the presence of increasing concentrations of BNN27 using membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or TrkAΔECD receptors. **B.** Competition binding assays of [^{3}H]-DHEA in the presence of increasing concentrations of BNN27 using membranes isolated from HEK293 cells transfected with the cDNAs of p75^{NTR} or p75^{NTR}ΔECD receptors. **C.** BNN27 showed no affinity for TrkB or TrkC receptors. Competition binding assays of [^{3}H]-DHEA in the presence of increasing concentrations of BNN27 using membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or TrkB or TrkC receptors. (Ki represents the mean±SE of five (5) experiments).

Following the ability of BNN27 to bind to NGF receptors, and in order to specify the domains of the receptors that are necessary to afford the binding of BNN27, we performed competition studies using HEK293 cells expressing TrkA or $p75^{NTR}$ mutants that lack the entire Extracellular Domain (ECD), necessary for full receptor activity after binding of its native ligand (*Jung et al., 2003, Arevalo et al., 2000 and Vilar et al., 2009*). Our binding experiments clearly showed that the extracellular domain was not required for the interaction of BNN27 to TrkA receptor, which was not the case for its binding to the extracellular domain of $p75^{NTR}$ receptor. Indeed, BNN27 showed a similar binding ability to $TrkA^{\Delta ECD}$ (with a small increase of Ki, Table 1) (Figure 3.5.A), while no binding was detected in membranes isolated, from $p75NTR^{\Delta ECD}$-transfected cells (Figure 3.5.B).

DHEA and its metabolite ADIOL (5-androsten-3 beta, 17 beta -diol), were shown to bind, the former with low and the latter with high affinity to Estrogen Receptor beta (ERβ) (Ki: 200 nM and 1 nM respectively) (*Kuiper et al, 1997*). BNN27 was unable to compete binding of [3H]-Estradiol to cytosolic preparations isolated from HEK293 cells, transfected with the cDNA plasmid of ERβ receptors (Figure 3.5.D), supporting the selective binding profile of this compound. In addition, ADIOL was unable to reverse binding of [3H]-DHEA to membranes isolated from $HEK293^{TrkA}$ transfectants, while effectively competing its binding to membranes from $HEK293^{p75NTR}$ transfectants (Ki: 1.2±0.1nM) (Figure 3.5.E).

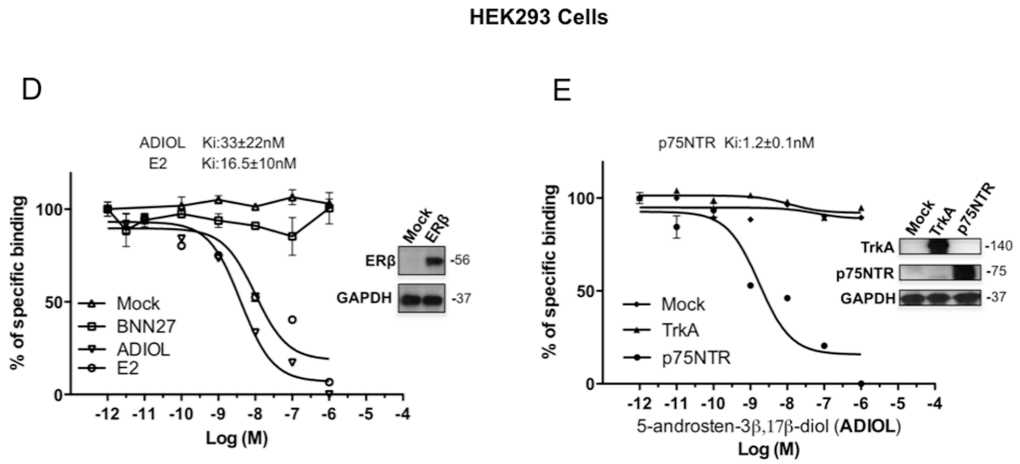


Figure 3.5. D. BNN27 showed no affinity for ERβ receptors. Competition binding assays of a single concentration of [^{3}H]-Estradiol in the presence of increasing concentrations of BNN27, ADIOL or Estradiol (E2) in cytosol isolated from HEK293 cells transfected with the cDNAs of ERβ receptor. Western blot inserts show the efficacy of transfection. **E.** DHEA metabolite ADIOL interacts with neurotrophin receptor p75^{NTR} but not with TrkA. Competition binding assays of a single concentration of [^{3}H]-DHEA in the presence of increasing concentrations of ADIOL on membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or p75^{NTR} receptors. Western blot inserts show the efficacy of transfection (Ki represents the mean±SE of four (4) experiments).

3.6. Pull-down Assays and STD NMR Spectroscopy of the interaction of BNN27 with NGF receptors

Pull-down assays and NMR studies confirmed the direct physical interaction of BNN27 with recombinant TrkA and p75^{NTR} receptors: polyethylene-BNN27 beads effectively pull-down recombinant TrkA and p75^{NTR} proteins (Figure 3.6. A, B). Furthermore, pre-incubation of the recombinant proteins with BNN27 or NGF in excess did not abolish the ability of BNN27-PEG to pull down both receptors, suggesting a synergistic conformational effect of these two molecules.

The application of saturation transfer difference (STD) NMR spectroscopy enabled the monitoring of the interactions between BNN27 and the extracellular domains (ECDs) of TrkA and $p75^{NTR}$ in the absence or presence of the neurotrophin NGF (Figure 3.6.C).

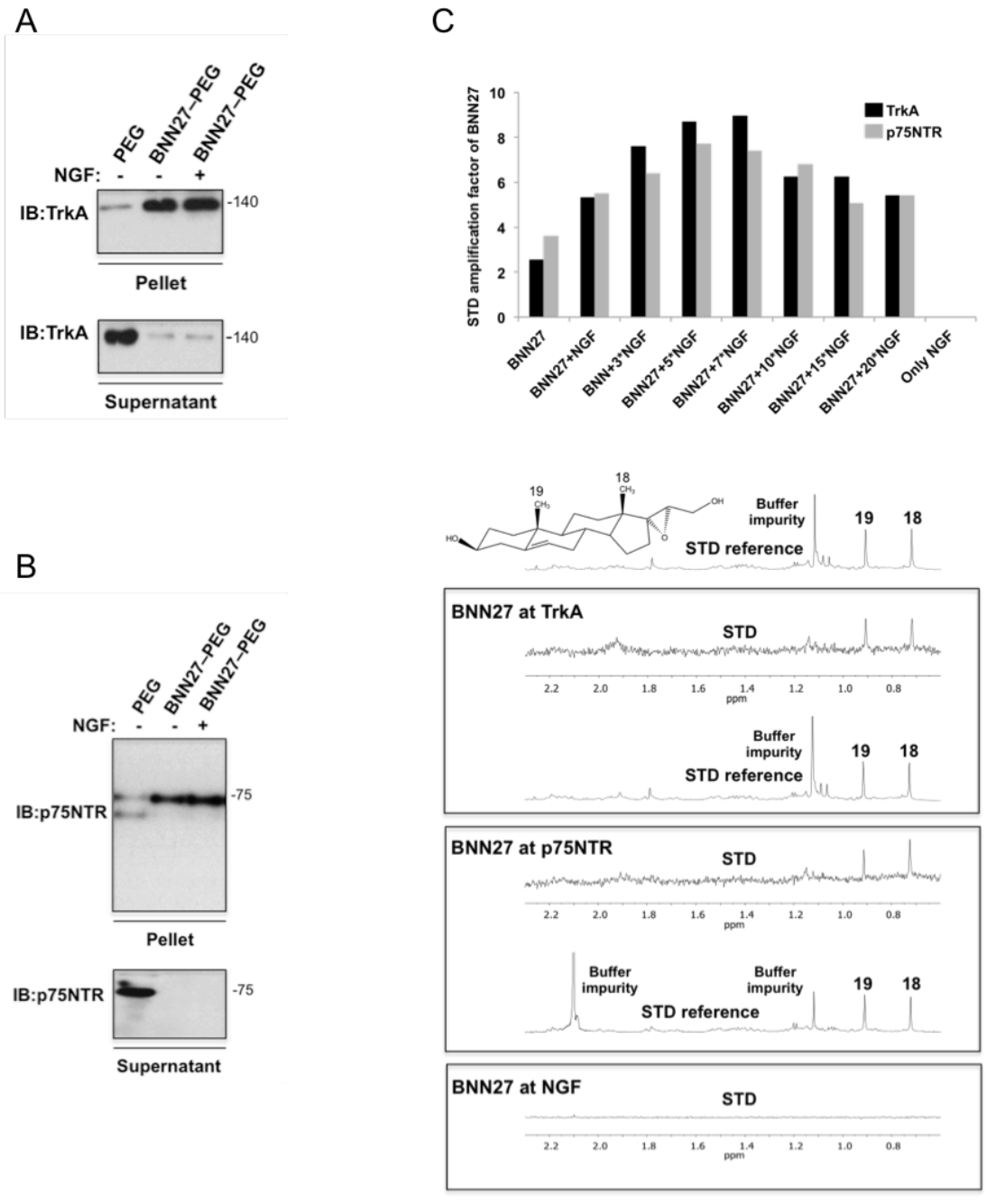


Figure 3.6. Polyethylene-BNN27 beads effectively pull-down recombinant TrkA **(A)** and $p75^{NTR}$ **(B)** proteins. **C.** NMR studies revealed a specific interaction of BNN27 to TrkA receptors, as well as with p75NTR receptors (Collaboration with Maria Zervou under the supervision of Dr. Calogeropoulou, Istitute of Biology, Medicinal Chemistry and Biotechnology (I.B.M.C.B.), Athens).

The concept of this method relies on the magnetization transfer from the effectively saturated protein to the bound ligand (*Mayer and Meyer, 1999; Meyer and Peters, 2003*). Briefly, a non-equilibrium magnetization is created on the receptor by applying selective rf-irradiation on a protein resonance band for a quite long saturation period. The spin diffusion mechanism accomplishes the quick spread of the saturation through the whole receptor proton network and eventually to the bound ligand protons. Upon dissociation, the ligand magnetization decays in solution providing resonance lines with a decreased intensity, due to the NOE effect modulation during the binding event. The line shapes of non-binders are not affected by the presence of protein.

Two distinct spectra are recorded, the on-resonance as described above and the off-resonance or reference spectrum (STD-REF) by applying rf-irradiation to a distal spectral region away from all resonance peaks. The binding event is monitored by their difference (STD-NMR), which exhibits solely the binder peaks while resonances of non binders are cancelled out. The fast exchange between the free and bound states of the ligand as well as the excess of the ligand concentration in respect to the target ([L]:[T] ~ 100:1) ensure that a maximum possible number of ligand molecules will occupy the binding site and subsequently transfer the magnetization into solution leading to an efficient build up of the NMR signal.

The STD amplification factor consists a measure of the ligand:protein interaction expressing the fractional STD effect multiplied by the ligand excess. It is calculated according to the formula: STD amplification factor = $(I_0-I_{sat})/I_0$ × ligand excess, where (I_0-I_{sat}) corresponds to the STD intensity of one resonance

signal and I_0 is the corresponding intensity obtained from the off resonance spectrum.

STD-NMR revealed the binding of BNN27 at the ECDs of the TrkA and $p75^{NTR}$ receptors as depicted by the presence of BNN27 resonance peaks in the respective spectra (Figure 3.6.C). On the other hand, the lack of the corresponding resonances in the NGF solution (Figure 3.6.C) indicates that BNN27 does not interact with the neurotrophin NGF in the absence of TrkA or $p75^{NTR}$ receptors.

The STD amplification factor was obtained by averaging the respective STD amplification factors for the C18 and C19 methyl group peaks of BNN27 in: (a) the TrkA and $p75^{NTR}$ solutions, (b) the respective solutions upon successive additions of NGF and, (c) a preparation only with NGF (Figure 3.6.C). The addition of NGF to the TrkA (or $p75^{NTR}$) solutions (1:1 moral ratio) induces an increase of the BNN27 STD amplification factor indicating an enhancement of BNN27 binding. STD amplification factor increases monotonically upon successive additions of NGF until a molar ratio of TrkA (or $p75^{NTR}$): NGF in the order of 1:(5-7), followed by a decay and a plateau formation for molar ratios above 1:15. Further additions of NGF to the solution TrkA/BNN27 up to a molar ratio of 1:30 (TrkA:NGF) confirmed this trend to a greater extend (data not shown).

3.7. BNN27 effectively activates both TrkA and $p75^{NTR}$ receptors in neurons and glial cells

Following our results on the ability of BNN27 to bind to both NGF receptors, we compared its efficacy to this of NGF to activate TrkA and $p75^{NTR}$

receptors. We firstly examined the phosphorylation of TrkA using specific phospho-antibodies for all three (490, 675 and 785) known tyrosine residues that have been shown to be phosphorylated upon NGF treatment. We used primary cultures of sympathetic neurons, (known to be NGF dependent), to test the ability of BNN27 to induce the phosphorylation of TrkA receptor. Sympathetic neurons were isolated and cultured for 7d in the presence of 100 ng/ml NGF, then were deprived of NGF for 12h and treated for 15 min with NGF or BNN27 in the presence of anti-NGF antibodies in order to block endogenously secreted NGF. As Figure 3.7.B (and Figure 3.7.A for CHO cells) depicts, BNN27 was able to activate TrkA receptor, inducing phosphorylation at tyrosine Y490 as well as the down-stream ERK1/2 and Akt kinases (Figure 3.7.B), potential effectors of its anti-apoptotic actions. Similar studies in CHO cells transfected with the TrkA plasmid showed that BNN27 effectively induced the phosphorylation of all three tyrosine residues at the intracellular domain of TrkA receptor (Figure 3.7.A). These findings were confirmed with immune-fluorescent experiments in PC12 cells. TrkA-positive PC12 cells were treated with NGF, BNN27, Estradiol (E2) or DHEA-derivative, ADIOL for 15 min (Figure 3.7.C). Then, the cells were fixed and stained for total or phospho-TrkA. This experiment also clearly indicates that BNN27 specifically activates TrkA phosphorylation, while the other two steroids had no effect on the activation of the receptor, effect which was also confirmed for ADIOL in CHO cells (Figure 3.7.D).

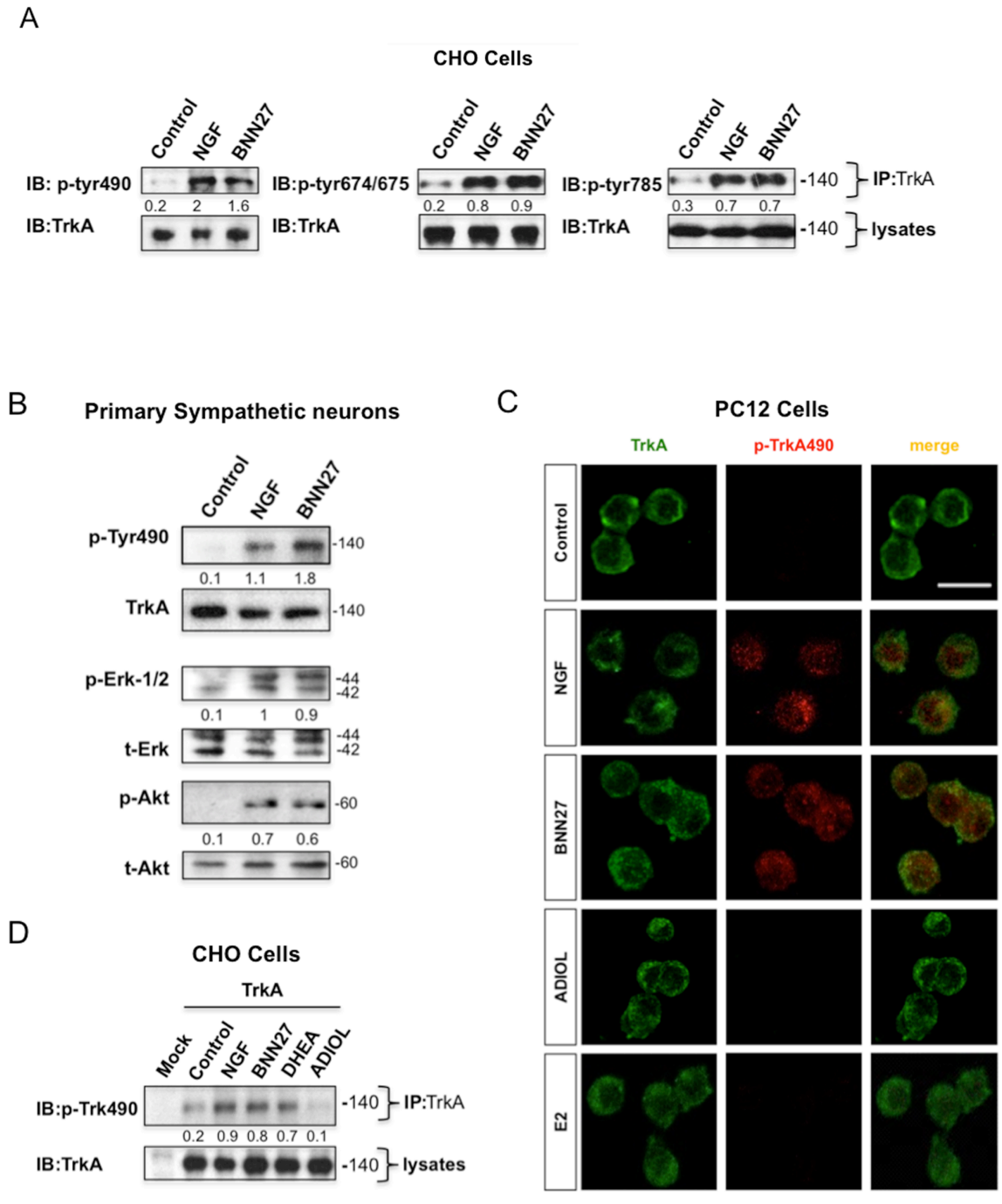


Figure 3.7. A. Phorphorylation of Y490, Y674/675 and Y785 in TrkA receptors, upon exposure to BNN27. CHO cells were transfected with the plasmid cDNA of TrkA. Transfectants were then exposed for 15min, to NGF (100ng/ml) or BNN27 (100nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with specific for pY490, pY674/675 and pY785 antibodies. Total lysates were analyzed for TrkA expression by immunobloting. Mean ±SEM of triplicate measurements. **B.** BNN27 induces TrkA tyrosine phosphorylation (p-Tyr490), affecting downstream signaling pathways MAPKs and Akt, in primary sympathetic neurons. Sympathetic neuron cultures were NGF-deprived overnight and then stimulated with

NGF(100ng/ml) or BNN27(100nM) for 20min. Lysates were subjected to SDS-PAGE and immunoblotted for the indicated proteins. Upper and lower blots represent different samples. Mean±SEM of triplicate measurements. **C.** BNN27 stimulates the tyrosine phosphorylation of TrkA in PC12 cells. Cells were grown in 24-well tissue culture plates on 12-mm circular glass slides coated with poly-D-lysine for 24h prior experimental treatment. Cells were treated with NGF (100 ng/ml) or BNN27 (100 nM) or ADIOL (100 nM) or E2 (100 nM) for 15 min. PC12 were then fixed, permealized and immunostained with the polyclonal rabbit p-Trk490 antibody and the moNoclonal mouse TrkA antibody followed by anti-rabbit Alexa Fluor 546 (red) and anti-mouse Alexa Fluor 488 (green), respectively. Cell staining was analyzed qualitatively using confocal fluorescence microscopy. The data are a representation of one out of three experiments showing similar results. **D.** CHO cells were transfected with the plasmid cDNA of TrkA. Transfectants were then exposed for 15 min to NGF (100 ng/ml) or BNN27 (100 nM) or DHEA (100 nM) or ADIOL (100 nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with p-Trk490. Total lysates were analysed for TrkA expression by immunobloting. Mean ±SEM of triplicate measurements.

BNN27 induces TrkA phosphorylation in a dose- and time-dependent manner, with the most effective concentration to be at 100nM (Figure 3.8.A, B). The kinetics of BNN27-mediated phosphorylation of TrkA, differs from these of NGF: BNN27 induces phosphorylation from 15 min up to 6 h, (Figure 3.8.B). NGF induces phosphorylation within 15 min but its effect is less pronounced in 3 h and 6 h. The effect of BNN27 is specific to Trk receptors, since no effect was observed in TrkB or TrkC phosphorylation (Figure 3.8.C) in agreement with our bindings experiments in these receptors (Figure 3.5.C).

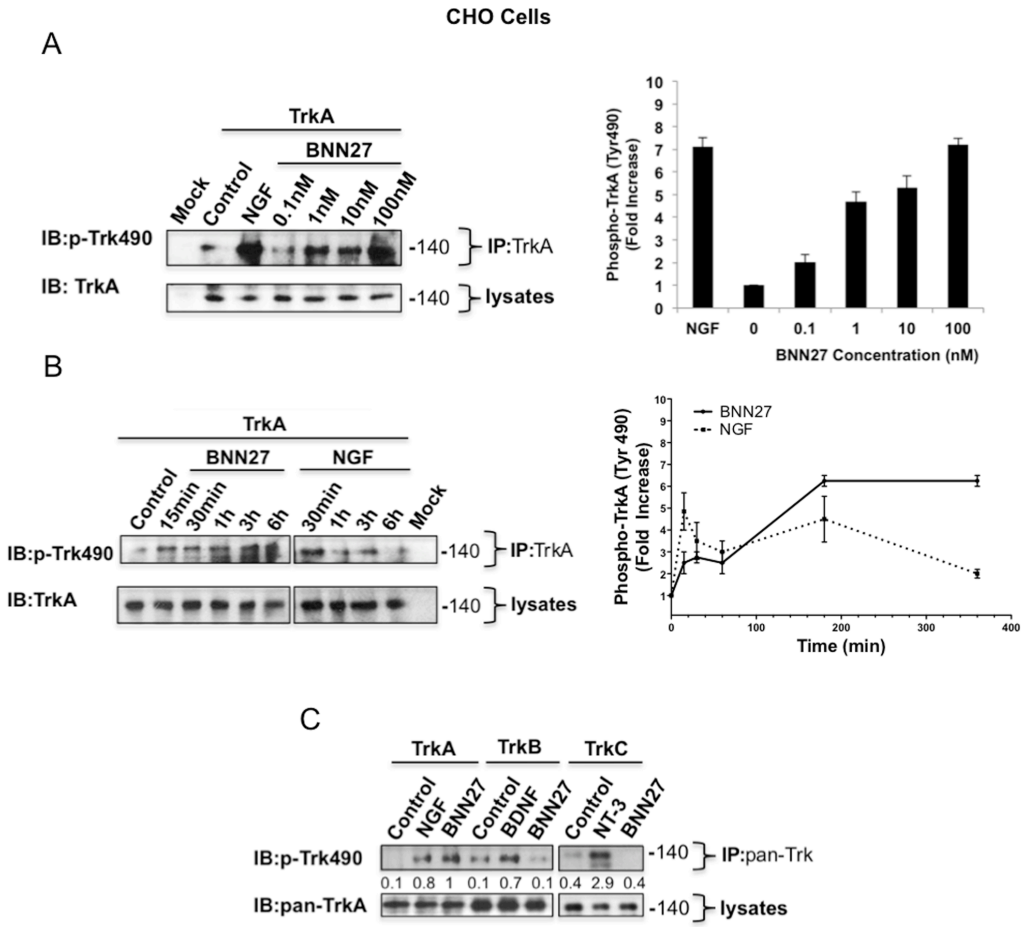


Figure 3.8. A. BNN27 dose-dependently induced TrkA tyrosine phosphorylation in CHO cells. Cells were transfected with the plasmid cDNA of TrkA. Transfectants were then exposed for 15min to NGF (100ng/ml) or BNN27 (0.1nM, 1nM, 10nM, 100nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with anti-pY490 antibodies. Total lysates were analysed for TrkA expression by immunobloting. Mean±SEM of triplicate measurements. **B.** BNN27 at 100 nM induced TrkA phosphorylation even 6h latter, compared to within 15 min short acting NGF. CHO cells were transfected with the plasmid cDNA of TrkA. Transfectants were then exposed for 15min, 30min, 1h, 3h, 6h to NGF (100 ng/ml) or BNN27 (100 nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with anti-pY490 antibodies. Total lysates were analysed for TrkA expression by immunobloting. Mean±SEM of triplicate measurements. **C.** BNN27 can induce tyrosine phosphorylation of TrkA but not of TrkB or TrkC. CHO cells were transfected with the plasmid cDNAs of TrkA, TrkB, TrkC. Transfectants were then exposed for 15 min, to NGF (100 ng/ml) or BNN27 (100 nM), and lysates were immunoprecipitated with pan-Trk antibody, and then immunoblotted with anti-pY490 antibodies. Total lysates were analysed for TrkA expression by immunobloting. Mean±SEM of triplicate measurements.

BNN27 also induced p75^{NTR} signaling, in cells, endogenously expressing this death receptor, such as mouse Schwann cells or Mouse Embryonic Fibroblasts (MEFs). Indeed, BNN27 induced the recruitment of RIP2 (Figure 3.10.A, B) and TRAF6 (Figure 3.10.C) in p75^{NTR}, and it increased the release of RhoGDI (Figure 3.10.D, E), mimicking the effects of NGF. A partial release of RhoGDI was also observed with DHEA metabolite ADIOL, indicating that this steroid may also activate p75^{NTR} receptor, in contrast to E2 (Figure 3.10.A). The ability of BNN27 to regulate RhoGDI through p75^{NTR} was further confirmed by measuring the activity of RhoA, a target protein of RhoGDI. Indeed, liberation of RhoGDI upon activation of the receptor reduces RhoA activity (*Yamashita et al., 1999, 2003*). BNN27 and ADIOL as well as NGF were effective in decreasing RhoA activity in MEFs cells transfected with the p75^{NTR} cDNA (Figure 3.10.F).

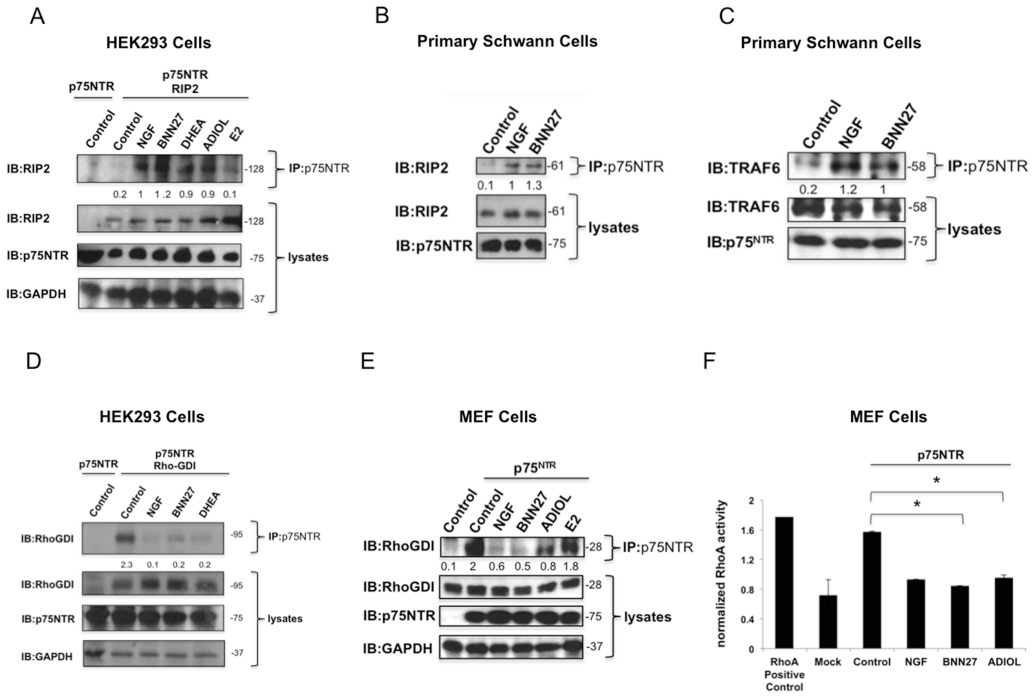


Figure 3.10. A. HEK293 cells were co-transfected with the plasmid cDNAs of p75^{NTR} and RIP2. Tranfectants were exposed for 20 min to NGF (100 ng/ml), BNN27 (100 nM), DHEA (100 nM), ADIOL (100 nM) or E2 (100 nM), and lysates were immunoprecipitated with p75^{NTR}-specific

antibodies and then immunoblotted with antibodies against RIP2. Total lysates were analysed for RIP2, $p75^{NTR}$ or GAPDH expression by immunobloting. Mean±SEM of triplicate measurements. **B, C.** BNN27 efficiently induced the association of $p75^{NTR}$ with effectors RIP2 and TRAF6 in Schwann cells. Schwann cells were isolated from P1 sciatic nerve, cultured for 6 days, and then treated with NGF (100 ng/ml) or BNN27 (100 nM) for 20 min. The cells were lysed, immunoprecipitated with $p75^{NTR}$-specific antibodies, and subsequently immunoblotted for either anti-RIP2 or anti-TRAF6. Endogenous $p75^{NTR}$ levels were confirmed in both panels. Mean±SEM of triplicate measurements. **D.** HEK293 cells were co-transfected with the plasmid cDNAs of $p75^{NTR}$ and RHOGDI. Transfectants were exposed for 30 min to NGF (100 ng/ml) or BNN27 (100 nM) or DHEA (100 nM), and lysates were immunoprecipitated with $p75^{NTR}$-specific antibodies and then immunoblotted with antibodies against RhoGDI. Total lysates were analysed for RhoGDI, $p75^{NTR}$ or GAPDH expression by immunobloting. Mean±SEM of triplicate measurements. **E.** BNN27 induces the release of RhoGDI from $p75^{NTR}$. MEF cells were transfected with the plasmid cDNA of $p75^{NTR}$. Transfectants were exposed for 30 min to NGF (100 ng/ml), BNN27 (100 nM), ADIOL (100 nM) or E2(100 nM), and lysates were immunoprecipitated with $p75^{NTR}$-specific antibodies, and then immunoblotted with antibodies against RhoGDI. Total lysates were analysed for $p75^{NTR}$ or GAPDH expression by immunobloting. Mean±SEM of triplicate measurements. **F.** Regulation of RhoA activity by BNN27 in MEF cells. Constitutively active RhoA protein was used as positive control. Results are expressed as mean of triplicate measurements ±SEM Normalized to un-stimulated $p75^{NTR}$ (Control). Similar results were obtained in three (3) independent experiments.

In addition, to further study the domains of TrkA receptors, affording activation by BNN27, we examined the efficacy of BNN27 to induce phosphorylation of the $TrkA^{\Delta ECD}$ and $TrkA^{C302S}$ mutants. $TrkA^{\Delta ECD}$ lacks the entire extracellular domain, and $TrkA^{C302S}$ has a point mutation at the cysteine residue 302, which completely abolishes NGF binding (*Arevalo et al., 2000*). BNN27 - in contrast to NGF- effectively induced phosphorylation of receptor at Y490 residues in cells transfected with the $TrkA^{C302S}$ mutant. Cells transfected with the $TrkA^{\Delta ECD}$ mutant showed constitutive activation of TrkA phosphorylation, independently of the presence of ligands (data not shown). These findings suggest that BNN27 does not need the extracellular domain to activate TrkA phosphorylation, in contrast to NGF (Figure 3.11.A). However, this was not the case for $p75^{NTR}$

receptors. We tested the efficacy of BNN27 to recruit the effector protein RIP2 on two $p75^{NTR}$ mutants, $p75NTR^{\Delta ECD}$ lacking the entire extracellular domain, and $p75NTR^{C257A}$ lacking the ability to mediate RIP2 recruitment due to its low dimerization capacity (*Vilar et al., 2009*), Our findings show that the extracellular domain of $p75^{NTR}$ and its dimerized conformation are necessary to afford the ability of BNN27 to facilitate recruitment of RIP2 on, $p75^{NTR}$ receptors (Figure 3.11.B), confirming our results from the binding assays (Figure 3.5.B).

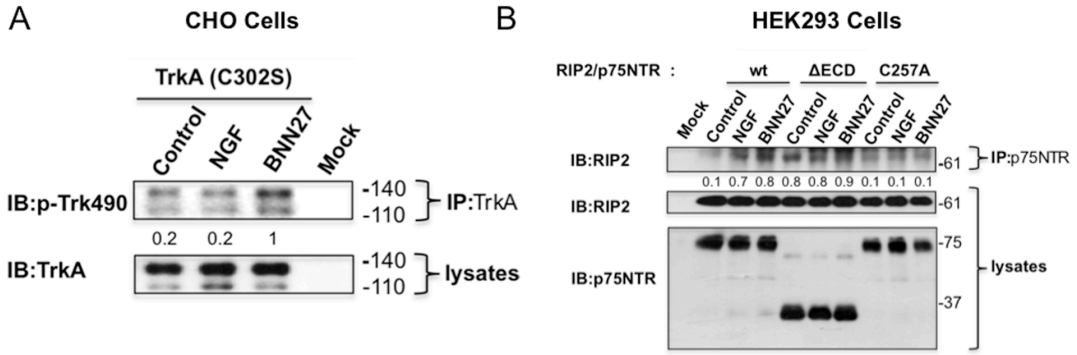


Figure 3.11. A. The effects of BNN27 to induce phosphorylation of TrkA were tested in CHO cells transfected with TrkA mutant receptor (C302S) lacking NGF binding. Transfectants were exposed for 15min to NGF (100ng/ml) or BNN27 (100nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with pY490. Total lysates were analyzed for TrkA expression by immunobloting. Mean±SEM of triplicate measurements. **B.** HEK293 cells were co-transfected with the plasmid cDNAs of $p75^{NTR}$ wild type (wt) or $p75^{NTR}$ mutants that lack the entire Extracellular Domain (ΔECD) or p75C257A and interactor RIP2. Tranfectants were exposed for 20min to NGF(100 ng/ml) or BNN27 (100nM), and lysates were immunoprecipitated with $p75^{NTR}$-specific antibodies, and then immunoblotted with antibodies against RIP2. Total lysates were analyzed for $p75^{NTR}$ or RIP2 expression by immunobloting. Mean±SEM of triplicate measurements.

3.8. BNN27 inhibits apoptosis of NGF-deprived sympathetic neurons in culture, via NGF receptors

Previous studies have shown that NGF as well as DHEA and BNN27 rescue PC12 pheochromocytoma cells from apoptotic cell death, occuring in cultures in serum free condition (*Greene et al., 1978; Charalampopoulos et al., 2004; Calogeropoulou et al., 2009*). In order to define whether the anti-apoptotic effects of BNN27 are mediated through TrkA and $p75^{NTR}$ receptors we used shRNAs against each one of the two receptors. The effectiveness of shRNAs manipulation was documeted by the remarkable decrease of the expression of both TrkA and $p75^{NTR}$ in PC12 cells, analysed with FACS (Figure 3.12.A).

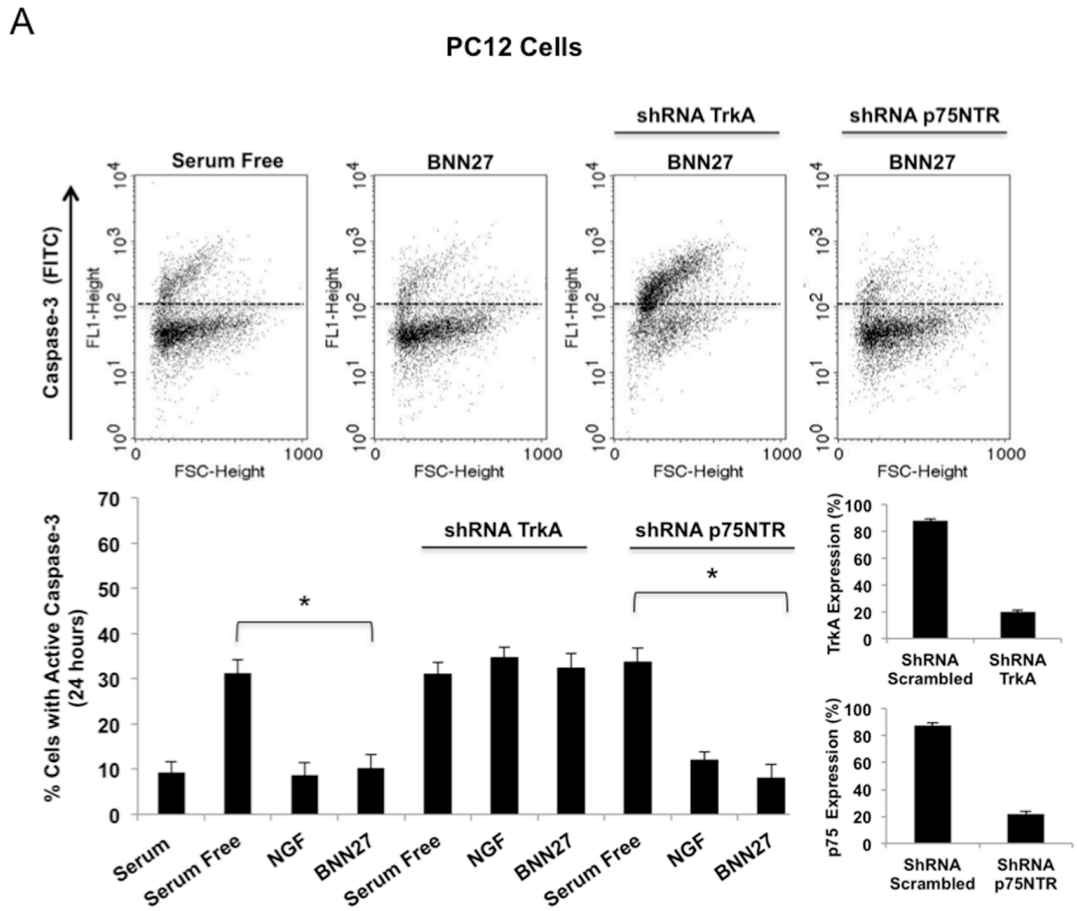


Figure 3.12. A. BNN27 decreases serum-deprivation induced apoptosis in PC12 cells. Diminution of TrkA expression by shRNA reversed the anti-apoptotic effect of NGF and BNN27. In contrast, the decrease of $p75^{NTR}$ expression by shRNA did not significantly affect the anti-apoptotic effect of both agents. PC12 cells were transfected with shRNAs of TrkA or $p75^{NTR}$. Cells were treated for 24 h in serum-supplemented or serum-starved conditions in the absence or the presence of NGF (100 ng/ml) or BNN27 (100 nM). FACS analysis shows the percentage of cells with active Caspase-3, in measures of 10.000 cells. Mean±SEM of triplicate measurements. Right panel shows the efficacy of transfection.

FACS analysis for caspase-3 positive cells showed that BNN27 at 100 nM diminished the apoptotic cell death of serum deprived PC12 cells from 34%±1.15 to 15.33%±0.88 (NGF decreased cell death to 10.66%±1,45) (Figure 3.12.A). Knock-down of TrkA expression using shRNAs resulted in the almost complete reversal of the anti-apoptotic effects of NGF and BNN27 increasing apoptosis to 36±1.2 and 33±0.88 respectively (Figure 3.12.A). Transfection of PC12 cells with shRNAs against $p75^{NTR}$ receptor did not have any significant effect on the anti-apoptotic actions of both NGF and BNN27, suggesting that their anti-apoptotic effects are primarily afforded by TrkA receptors. Furthermore, the anti-apoptotic effects of both NGF and BNN27 were not affected by the transfection of scrambled shRNAs.

We have also tested the anti-apoptotic effects of BNN27 in a classical NGF-dependent cellular model, sympathetic neurons from Superior Cervical Ganglia (SCG). Sympathetic neurons depend on NGF for their survival during the late embryonic and the early postnatal life (*Crowley et al., 1994; Smeyne et al., 1994*). Isolated sympathetic neurons from P1 rats were maintained for 7d in medium containing 100 ng/ml NGF and the antimitotic agent FdU, in order to obtain an almost pure (>90%) neuronal culture. SCGs cultures were then washed and incubated for 48 h in culture medium containing NGF (100 ng/ml) or in NGF-

free medium with the supplementation of a polyclonal rabbit anti-NGF neutralizing antibody, in the absence or the presence of 100 nM BNN27. NGF deprivation resulted in a dramatic increase of the number of apoptotic neurons (quantified as TUNEL positive cells with condensed nuclei) after 48 h, which was significantly decreased by the addition 100 nM BNN27 (63%±3.6 vs 43%±3.2) (Figure 3.12.B). Moreover the anti-apoptotic effect of BNN27 was abolished with the addition of a selective TrkA inhibitor (64%±3.17), and, it was further increased by the addition of an inhibitor of pro-apoptotic $p75^{NTR}$ receptor (data not shown). The number of surviving neurons was also higher, compared to anti-NGF condition, normalized to the number of neurons in the presence of NGF (27%±3 vs 52%±1) (Figure 3.12.B).

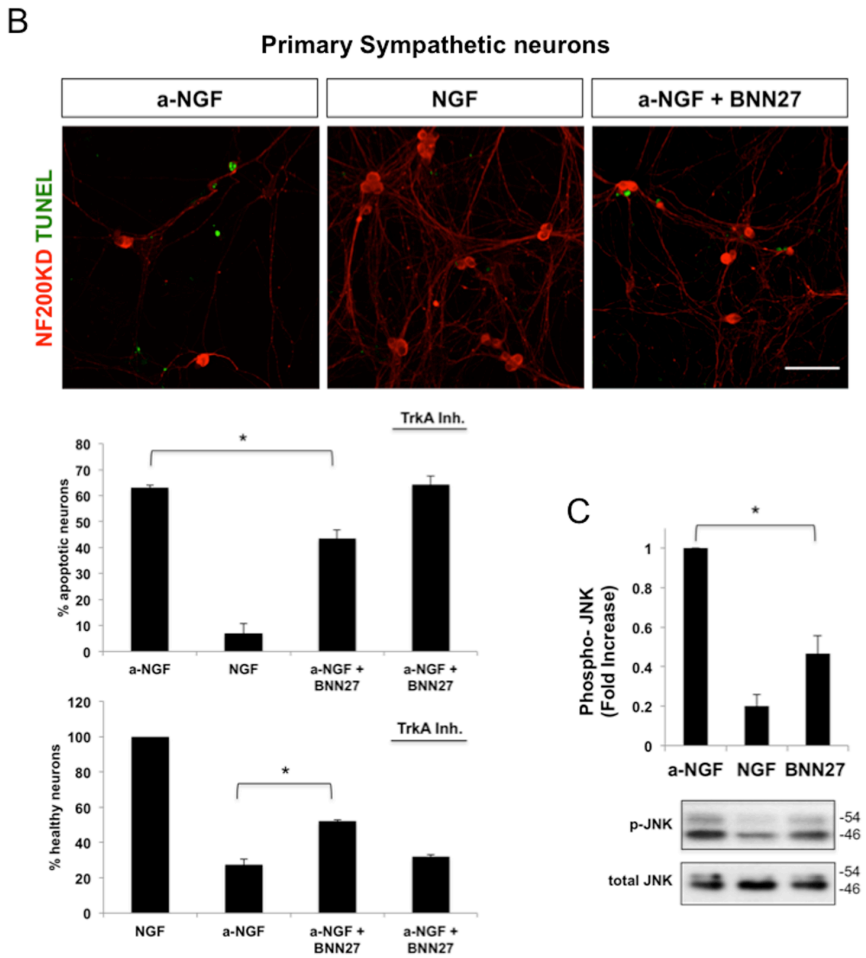


Figure 3.12. B. Sympathetic neurons, from superior cervical ganglia (SCG) isolated from P1 rats were cultured for 7 days in the presence of NGF (100 ng/ml), then they washed and cultured for 48h in either in the same medium or in NGF-free medium in the presence of a NGF polyclonal neutralizing antibody with or without 100 nM BNN27 and in the absence or the presence of TrkA inhibitor. Neurons were stained with an antibody against NF200kD (Red) and with TUNEL (Green). Over 300 neurons were counted in six to seven randomly selected optical fields and the percentage of $TUNEL^+$, apoptotic neurons for each condition is presented as mean from three (3) separate experiments (*$*P<0.01$* relative to anti-NGF condition). **C.** In different cultures treated in the same way the activity of JNK was assessed by western blot analyses, 18 h after NGF deprivation. Scale bar represents 100 μM.

BNN27 also decreased the levels of p-JNK (Jun-amino-terminal kinase) after 18 h of NGF deprivation (Figure 3.12.C). JNK, a kinase of the mitogen activated protein kinases, is activated upon NGF deprivation, phosphorylating c-jun, and triggering apoptotic signaling pathways by the up-regulation of pro-apoptotic genes (*Estus et al., 1994; Palmada et al., 2002*).

3.9. BNN27 delays apoptosis of $TrkA^+$ sensory neurons in *ngf* null mice

To investigate whether BNN27 could mimic NGF in its anti-apoptotic properties *in vivo*, heterozygous mice for the deletion of *ngf* gene were crossed and pregnant mothers were carefully intraperitoneal (IP) injected with 3 mg BNN27 per day from day E6.5 to E13.5 or from E13.5 to E17.5. At this embryonic age, TrkA is abundantly expressed in a subpopulation of the DRGs, which become dependent to NGF for their survival. As it was previously described, NGF null mice showed dramatic loss of sensory neurons in DRG, through apoptosis (*White et al., 1996*). By embryonic day E17.5, apoptotic signals could not be detected and TrkA expression was absent in DRG sensory neurons.

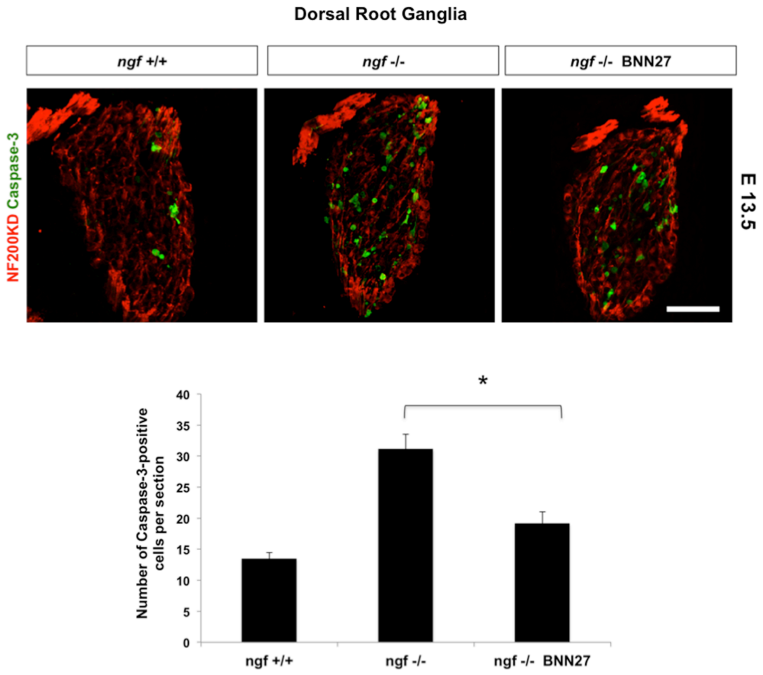


Figure 3.13. Mice heterozygous for NGF deletion were crossed and pregnant mothers were treated from E6.5 until the respective embryonic day with either saline or 3 mg BNN27 per day. At the end of the treatment embryos were collected and immunostained against activated caspase-3 and NF200kD to localize the area of DRG and the apoptotic neurons per section were quantified. Massive apoptotic death of neurons was observed within the area of DRGs in *ngf-/-* mice starting at age E12.5, as expected, which it was significantly decreased in animals treatment with BNN27. Scale bar represents 75 μM. All data in graphs are represented as mean ±SEM. Statistical analysis was done using Student's t test. N=7,*$P < 0.05$ relative to *null mice*.

Administration of BNN27 to pregnant mothers reduced the number of Caspase-3 positive cells within the area of the DRG compared to *ngf-/-* at E13.5 (31±2 vs 19±2.3) (Figure 3.13). However, BNN27 was not able to fully rescue these neurons at E17.5 (data not shown). We did not observed TrkA+ positive neurons in the DRGs of *ngf-/-* embryos, treated with BNN27.

3.10. BNN27 exerts synergistic effects with NGF in the axonal outgrowth of PC12 cells

In order to investigate if BNN27 can mimic NGF in its other functions besides its pro-survival actions, we used the classical NGF dependent cell model of PC12 cells which respond to NGF by extending neurites, acquiring a neuronal phenotype (*Drubin et al., 1985*).

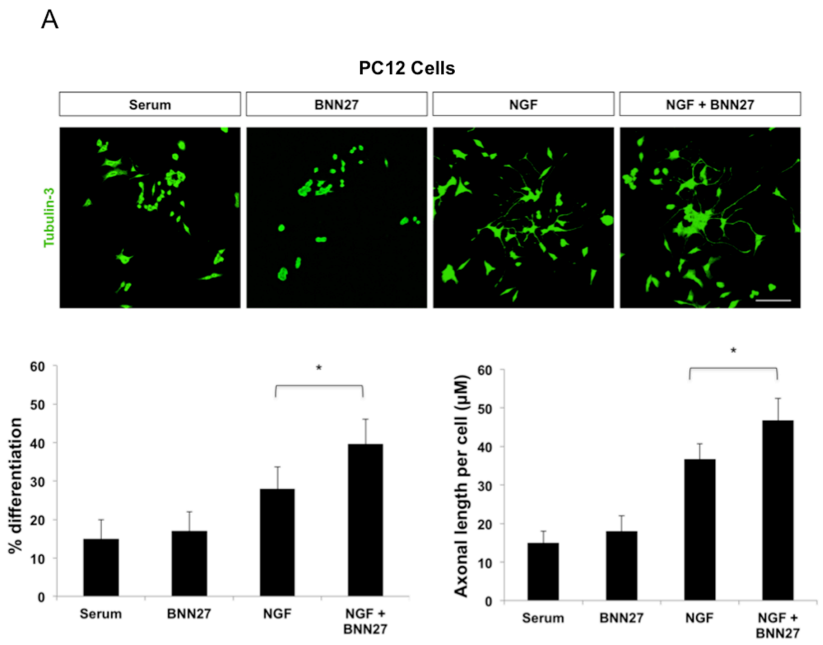


Figure 3.14. A. PC12 cells were incubated for 24h in medium with either NGF alone or with 100 nM BNN27 and stained with Tubulin-3 antibody. The addition of BNN27 increased the percentage of differentiated cells and the axonal length of these cells. Means are the percentage of cells having neurites, longer than the soma size. The length of each neurite from 4 optical fields in each condition was counted in three (3) independent experiments. Scale represents 75µM. N=3,*P <0.05 relative to NGF condition.

BNN27 at 100 nM was unable alone to induce the differentiation of PC12 cells. However, BNN27 increased the percentage of differentiated PC12 cells cultured for 48 h in the presence of low concentrations of NGF (20 ng/ml), compared to NGF alone (28%±5.6 vs 39.6%±5.6). Equally, BNN27 enhanced the

effects of NGF on the maximum length of neurites (39.6±4 μM vs 46.8±5.7 μM) (Figure 3.14.A).

Similar synergistic effects between BNN27 and NGF were also observed in primary cultures of DRG sensory neurons, another NGF-dependent neuronal cell model. Indeed, BNN27 increased the axonal length of DRG cells (99±20 μM vs 145±8.9 μM) in the presence of 20 ng/ml NGF within the first 24 h of culture (Figure 3.14.B).

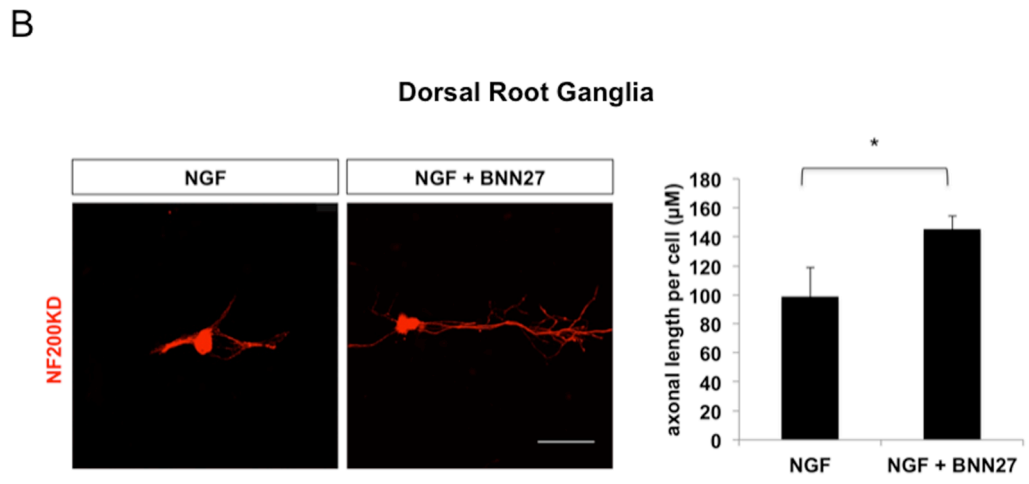


Figure 3.14. B. Sensory neurons of dissociated DRGs were cultured for 24h in medium containing 20 ng/ml NGF with or without the addition of 100 nM BNN27. Neurons were stained with NF200kD antibody and the mean of maximum axonal length was assessed from approximately 50 neurons per condition in three (3) independent experiments. N=3, *$P<0.05$.

3.11. Kinetics of TrkA receptor internalization in BNN27-treated cells

Given the differential effect of BNN27 in cell survival and differentiation, compared to NGF, we examined whether some of these differences may stem from a differential turn-over of the TrkA receptor in cell membranes. It is well established that lipid raft micro-domains are cholesterol-rich islands in the membrane which facilitate signaling by bridging molecules and providing a

fostering milieu for certain protein-protein interactions (*Limpert et al., 2007*). MβCD (methyl-β-cyclodextrin) treatment is a common method of examining lipid raft function, efficiently removing cholesterol from the plasma membrane (*Christian et al., 1997*). To define if the activity of TrkA is affected via modulation of cholesterol intramembrane levels, we monitored the effect of cholesterol depletion in the levels of the phosphorylated TrkA following BNN27 stimulation. CHO cells transfected with a TrkA plasmid were treated with 10 mM MβCD for 20 min prior to BNN27 stimulation (100 nM) for the indicated times. Cell viability was unaffected by MβCD throughout this time course (Figure 3.15.A). Interestingly, the levels of TrkA phosphorylation by BNN27 were significantly reduced after MβCD treatment, compared to untreated cells.

Furthermore, we sought to determine if BNN27 stimulation affects the levels of raft-associated TrkA receptors. We separated lipid raft micro-domains from non-raft membranes by sucrose gradient and analyzed the distribution of TrkA in control, NGF-treated and BNN27-treated conditions, in HEK293 cells transfected with the TrkA cDNA plasmid. BNN27 caused TrkA to effectively segregate into lipid raft fractions, specifically marked by the presence of flotillin-1. In contrast, NGF resulted in TrkA receptors shifting from lipid rafts into non-raft domains (Figure 3.15.B). These findings suggest that binding of BNN27 to TrkA initiates a distinct signaling cascade compared to NGF, starting from the localization of the receptor in different membrane compartments.

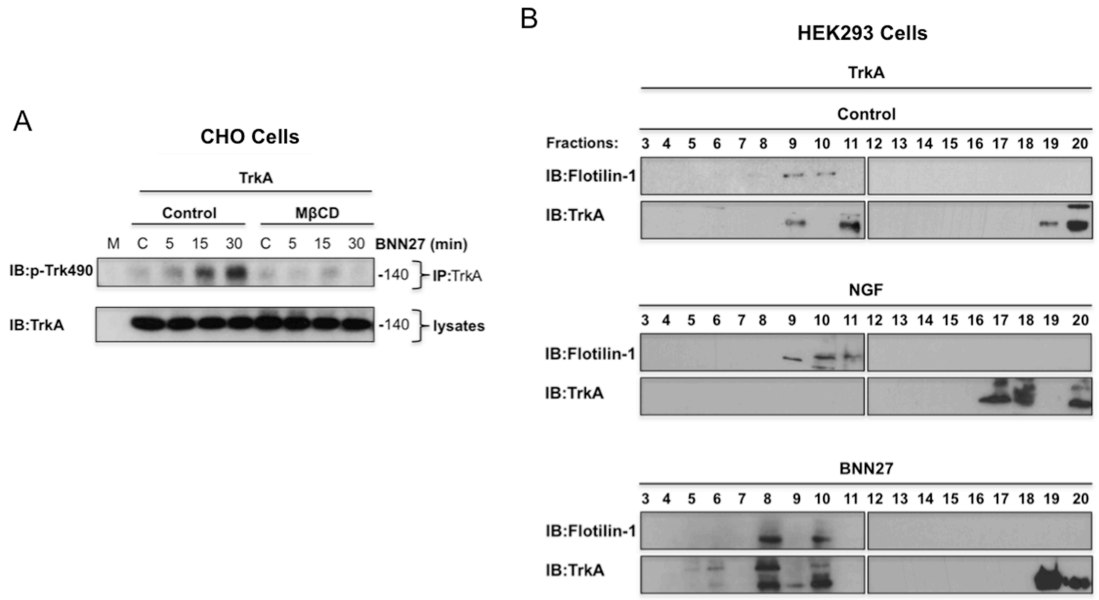


Figure 3.15. A. MβCD treatment decreases TrkA phosphorylation. CHO cells transfected with the TrkA cDNA plasmid were treated with 10 mM MβCD, depleting cholesterol from cell membranes, for 20 min prior to BNN27 stimulation (100 nM) for the indicated times. Western blots were probed for TrkA pY490. The blots were stripped and subsequently reprobed for TrkA to assess for total TrkA protein loading. **B.** TrkA segregates to lipid raft microdomains upon BNN27 treatment. Detergent resistant membranes isolated from control, BNN27 or NGF-treated HEK293 cells were fractionated by sucrose gradient to separate cholesterol-rich lipid raft fractions, marked by the presence of flotillin-1, from non-raft domains. Note that BNN27 treatment causes TrkA to preferentially segregate into lipid rafts, compared to control. Conversely, NGF treatment promotes the localization of TrkA into non-raft fractions.

It is well documented that endocytosis of the NGF-pTrkA complex into endosomes and signaling within the endosome is necessary for NGF-induced neurite outgrowth (*Grimes et al., 1996; Zhang et al., 2000; Liu et al., 2007*). To assess whether BNN27 modulates the internalization rate of TrkA, we performed internalization assays in PC12 using biotynilation. Surface receptors were biotinylated. Subsequently, NGF and BNN27 were added for 5, 15, and 30 min to drive endocytosis of biotinylated receptors. Then, biotin was stripped of the surface leaving only the internal biotinylated receptors. A representative experiment in Figure 3.16.A showed that TrkA was rapidly internalized in PC12

cells after 15 and 30 min of NGF and BNN27 stimulation. However, there was no significant internalization of TrkA after 5 min of BNN27 stimulation. To confirm the internalization efficiency of TrkA receptors in response to BNN27 stimulation, we performed FACS analysis on PC12 cells in order to measure the TrkA surface levels. PC12 cells were incubated for different time periods (15, 30, 60, and 180 min) with both NGF and BNN27. Consistent with the results presented in Figure 3.16.A, TrkA surface levels were diminished after 15 and 30 min in response to both NGF and BNN27 stimulation, highlighting the internalization of TrkA receptors within the cell. Particularly, after NGF stimulation for 15 min, TrkA surface levels values were 45% (±7.7%), whereas after BNN27 stimulation surface levels were 63% (±2.8%) in comparison to unstimulated cells. At 30 min after NGF stimulation TrkA surface levels values were 44% (±9%), whereas after BNN27 stimulation surface levels were 55% (±5%) of non-stimulated cells, Figure 3.16.B. Interestingly, the regulation of the TrkA surface levels was clearly different after 60 and 180 min between NGF and BNN27. Whereas the TrkA surface levels decreased rapidly and were almost depleted within 60 and 180 min in response to NGF because of TrkA lysosomal degradation, TrkA surface levels were comparatively high in response to BNN27. At 60 min after NGF stimulation TrkA surface levels values were 30% (±3.6%), whereas after BNN27 stimulation surface levels were 74% (±9.4%) of unstimulated cells. Finally, at 180 min after NGF TrkA surface levels values were 29% (±7.7%), whereas after BNN27 stimulation surface levels were 89% (±6.8%) of non-stimulated cells (Figure 3.16.B). In addition, we revealed a similar endocytic sorting of $p75^{NTR}$ receptor in response to NGF or BNN27 in PC12 cells that incubated for different time periods (15, 30, 60, and 180 min) (Figure 3.16.C).

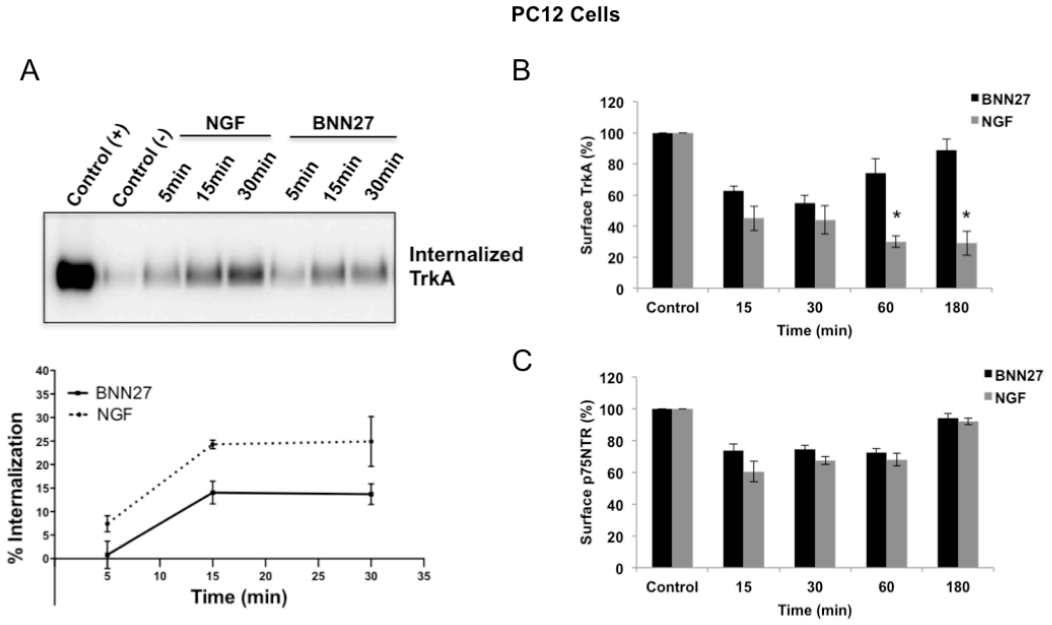


Figure 3.16. A. PC12 cells were biotinylated and then treated with NGF (100ng/mL) or BNN27 (100nM) for 5, 15 and 30min. Biotin from non-internalized proteins was removed using reducing conditions. Cell lysates were prepared; surface proteins were subjected to precipitation with neutroavidin and western blot analysis was performed with antibodies against TrkA receptor. Positive control (+): Total labeled-TrkA at surface (no treatment and no biotin stripping), negative control (-) :Total TrkA at surface with biotin stripped and non stimulated. Quantification of internalized TrkA receptor. Data are presented as mean±SEM (N=3). **B, C.** PC12 cells were treated with NGF (100 ng/ml) or BNN27 (100 nM) for 15min, 30min, 60min, or 180min. After incubation, surface TrkA or $p75^{NTR}$ receptors were stained with anti-TrkA or anti-$p75^{NTR}$ extracellular domain antibodies and then exposed to FITC-labeled secondary antibodies. Surface expression of TrkA or $p75^{NTR}$ receptors was detected using a FACS flow cytometer (Beckton & Dickinson) and fluorescence data were analyzed with Cellquest Software.

A striking observation was that TrkA phosphorylation/activation was enhanced by BNN27 in PC12 cells, remaining active for a longer time period (60 min), in comparison with NGF (Figure 3.17). Whereas NGF mediates sustained activation of the Erk kinases, BNN27 mediates only transient activation. This transient signaling by BNN27 is possibly due to the differential kinetics of TrkA endocytosis. To evaluate the kinetics of the effect of BNN27 in TrkA localization,

we performed co-immunoprecipitation assays, employing organelle marker proteins. PC12 cells were treated with NGF or BNN27 for 0, 15, 30 and 60min. TrkA was immunoprecipitated and its interactions with endosomes were examined by blotting with well-characterized organelle-specific markers: Rab5, Rab7 and Rab11 (Figure 3.17).

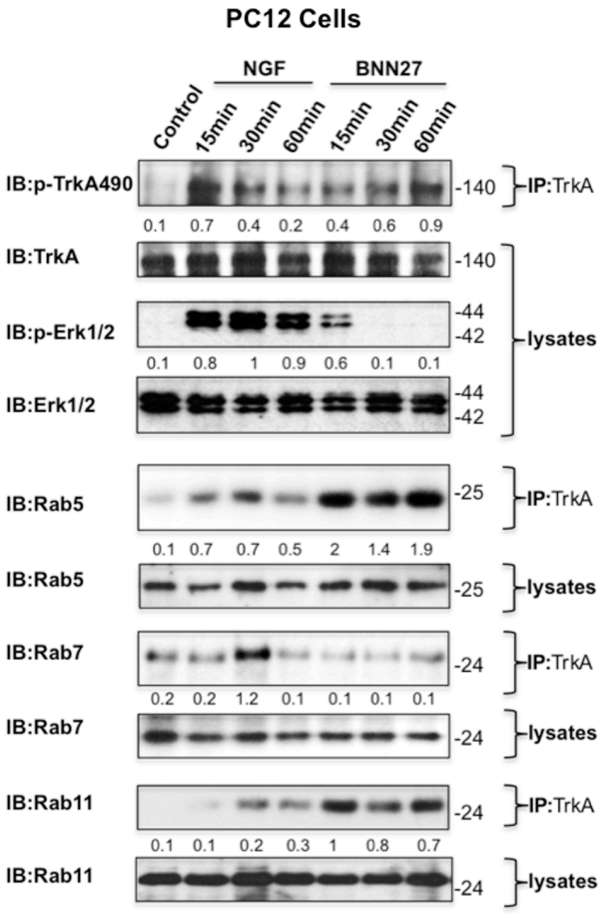
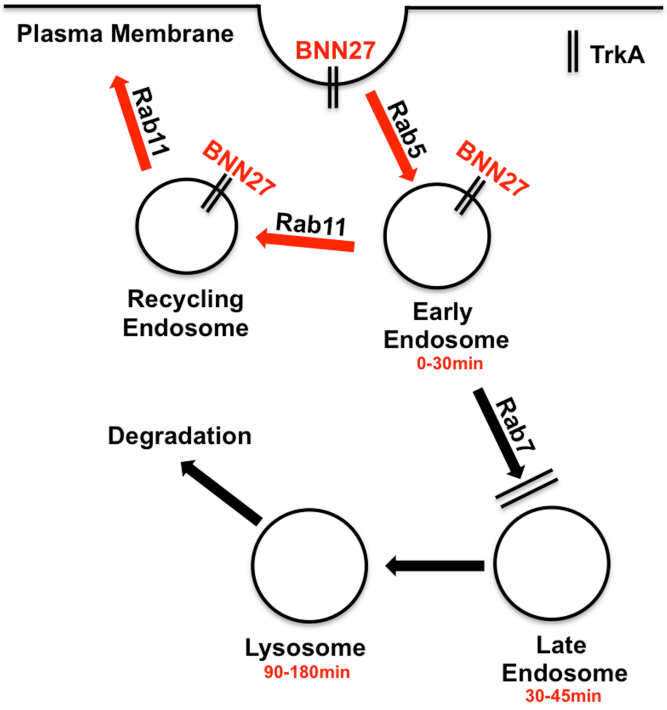


Figure 3.17. BNN27 signaling enhances Rab5 activity, pTrkA recycling (Rab 11) and surface levels of TrkA, whereas blocks the transition to late endosomes (Rab7). PC12 cells were treated with NGF (100 ng/ml) or BNN27 (100 nM) for 15, 30 or 60min. Cells were lysed, immunoprecipitated with TrkA specific antibodies, then immunoblotted with antibodies against Rab5, Rab7, Rab11, and p-TrkA490. Mean±SEM of triplicate measurements.

Based on these findings, we hypothesize that BNN27 fails to differentiate PC12 cells because it fails, upon binding to TrkA receptors to induce its internalization into the appropriate signaling endosomes. TrkA was shown to interact with Rab5 at 15–60 min, post-treatment with BNN27. These findings suggest that BNN27-induced Rab5 activity might explain the ineffectiveness of



BNN27 to induce differentiation of PC12 cells (*Liu et al., 2007*). Longer lasting association of Rab5 with TrkA might be refractory to further Rab7-mediated lysosomal processing of the receptor. To test this hypothesis, we examined whether Rab7, a late endosomal marker, interacts with TrkA at 30 min, the time-point at which delivery to late endosomes occurs (*Geetha et al., 2003*). NGF induced within 30 min the interaction of TrkA with Rab7, unlike BNN27, which to reproduce this effect. An additional step of TrkA turn-over is its escape from lysosomal processing through trafficking of TrkA from the early endosomes back to the plasma membrane, via recycling endosomes, and its interaction with Rab11 endosomal protein (*Chen et al., 2005*). BNN27 modulated the fast recycling of TrkA receptors to the plasma membrane within 60 min, inducing Rab11 protein, within 30 min, in contrast to late recycling by NGF of TrkA receptors within 180 min, (Figure 3.16.B).

3.12. Synthesis of new microneurotrophins

Based on our previous data, indicating that the presence of a 17-spiro-oxirane group in DHEA derivatization leads to compounds which interact with TrkA and exhibit neuroprotective activity, we enriched our chemical libraries of

synthetic neurosteroids by altering the ring size and polarity at position C17 (cycloalkyl, heterocyclic or heteroaromatic rings) and their substituents (polar, hydrophobic, hydrogen bond donors or acceptors, or with ion chelating ability). A new 17-spiro DHEA derivative is BNN237 (Dr T. *Calogeropoulou, personal communication*), exhibits strong neuroprotective actions (Figure 3.18.A) and selective agonist activity for TrkA and $p75^{NTR}$ receptors (Figure 3.18.B).

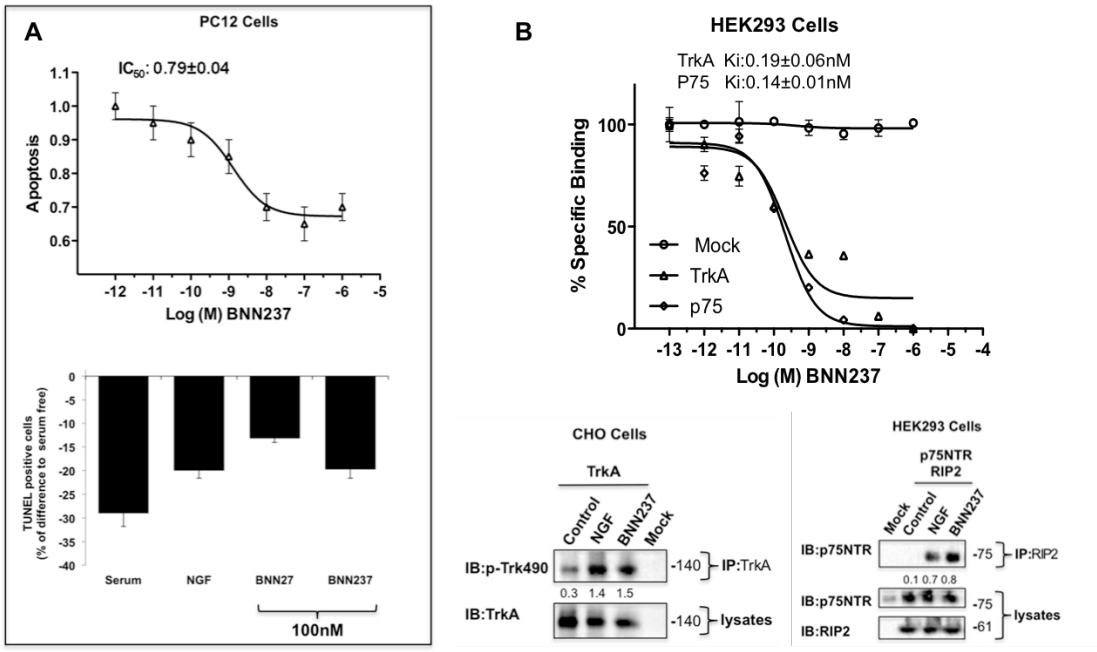


Figure 3.18. A. BNN237 protected PC12 cells against serum deprivation-induced apoptosis in a dose-dependent manner. Cells were cultured for 24 h either in complete media (serum supplemented) or serum-free media in the presence of BNN237 at concentrations ranging from 10^{-12} to 10^{-6} M. Apoptosis was quantified by the TUNEL assay, depicted as ratio to control (serum-free media in the absence of steroids). Values represent mean±SEM of four (4) independent experiments. **B.** Competitive binding assays of [^{3}H]-DHEA in the presence of increasing concentrations of BNN237 using membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or $p75^{NTR}$ receptors. BNN237 induces TrkA tyrosine phosphorylation (pY490). CHO cells were transfected with the plasmid cDNA of TrkA. Transfectants were then exposed for 15 min to NGF (100 ng/ml) or to BNN237 (100 nM), and lysates were immunoprecipitated with TrkA-specific antibodies, and then immunoblotted with p-Trk490. Total lysates were analysed for TrkA expression by immunobloting. BNN237 efficiently induced the association of $p75^{NTR}$ with its effector RIP2. HEK293 cells were co-tranfected with the plasmid cDNAs of $p75^{NTR}$ and RIP2.

Tranfectants were exposed for 20 min to NGF(100 ng/ml) or BNN237 (100 nM), and lysates were immunoprecipitated with RIP2-specific antibodies and then immunoblotted with antibodies against $p75^{NTR}$. Total lysates were analysed for $p75^{NTR}$ or RIP2 expression by immunobloting. Mean±SEM of triplicate measurements.

The therapeutic potential of TrkB activation using BDNF has been demonstrated in several preclinical models of CNS diseases (*Frisen et al., 1993; Leeds et al., 2005*), validating TrkB as a promising drug target. Therefore, we aimed to develop TrkB-specific receptor agonists that show highly selective binding to TrkB, inducing robust activation of TrkB signaling. Recent findings demonstrate that our spiro-neurosteroid derivative BNN20 induces TrkB tyrosine phosphorylation confirming its specificity compared to other members of our chemical library, as TrkB receptor ligands (Figure 3.19).

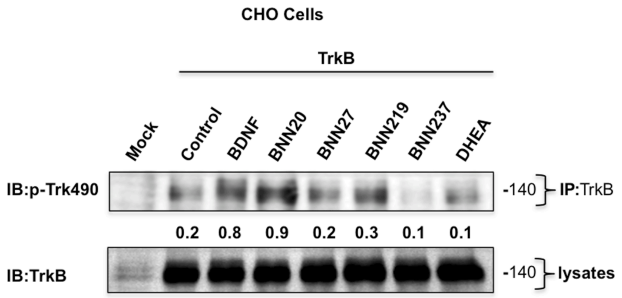


Figure 3.19. BNN20 induces TrkB tyrosine phosphorylation (pY490). CHO cells were transfected with the plasmid cDNA of TrkB. Transfectants were then exposed for 15 min to BDNF (100 ng/ml) BNN20 (100 nM), BNN27 (100 nM), BNN219 (100 nM), BNN237 (100 nM) or DHEA (100 nM), and lysates were immunoprecipitated with TrkB-specific antibodies, and then immunoblotted with pY490. Total lysates were analysed for TrkB expression by immunobloting. Mean±SEM of triplicate measurements.

4. DISCUSSION

Neurotrophins have been well characterized in terms of their ability to positively and negatively regulate cell survival, growth, selection and differentiation (*Barbacid et al., 1994*). There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Despite the demonstrated beneficial effects, neurotrophins have been shown to be ineffective pharmacological agents, because of their polypeptide nature and their subsequent poor penetration across the blood-brain-barrier (BBB). Recently, we provided evidence that a highly lipophilic small molecule, dehydroepiandrosterone (DHEA), prevents neuronal apoptosis (*Charalampopoulos et al., 2004*), through activation and binding to NGF receptors, TrkA and $p75^{NTR}$ membrane proteins (*Lazaridis et al., 2011*). These findings suggest that DHEA could serve as a new pharmacological agent in the treatment of neurodegenerative diseases. In the present study, we show that DHEA may interact with all forms of vertebrate Trk receptors (Figure 4.1), although with affinities approximately two orders of magnitudes lower compared to that of polypeptide neurotrophins (0.01–0.1 nM). DHEA effectively displaced binding of [^{3}H]-DHEA to membranes isolated from HEK293 cells (not expressing endogenous Trk receptors) transfected with the cDNAs of TrkA, TrkB or TrkC receptors (Ki: 7.40±1.7 nM, 4.49±1.7 nM and 6.33±1.3 nM, N=3, respectively).

DHEA was proven equally effective in displacing binding of [^{3}H]-DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of various invertebrate Trk receptor homologs, such as Ltrk, ApTrk or AmphiTrk receptors (Ki: 3.6 ±0.4 nM, 1.14±0.11 nM, 0.47±0.18 nM, N=3, respectively) from Lemnea,

Aplysia and Amphioxus (Figure 3.1). Furthermore, DHEA showed to activate the post-Trk receptor pro-survival pathway mediated by the kinases ERK1/2 and Akt, key mediators of neuronal survival (*Huang et al., 2003; Sofroniew et al., 2001*). However, DHEA was ineffective in mimicking the effects of polypeptide neurotrophins on Trk receptor-mediated neuronal functions including differentiation and neurite elongation (results summarized in Figure 4.1). Our observations further support the pleiotropic, "sticky" properties of DHEA in the nervous system across a variety of species. The early evolutionary presence of CYP17, the enzyme responsible for its biosynthesis, points towards an important role of DHEA, early in evolution.

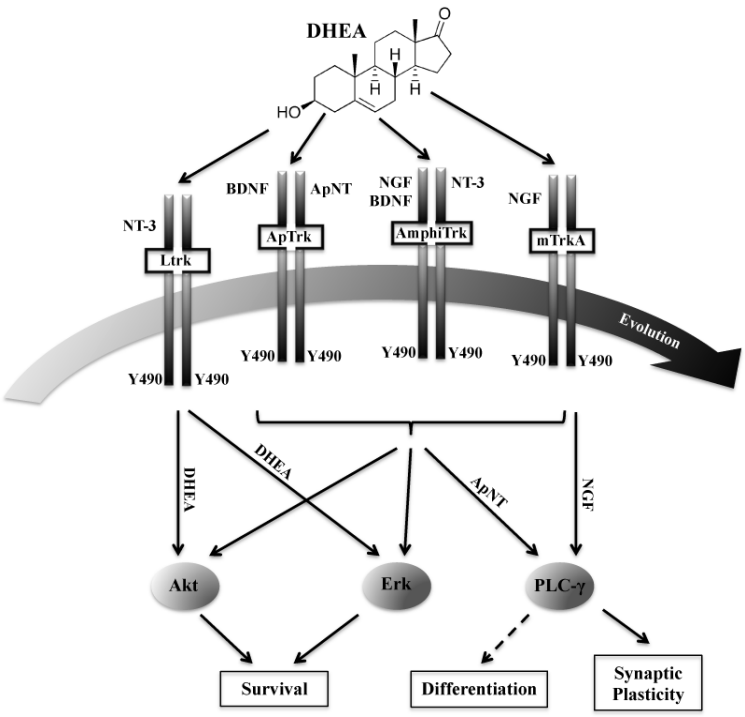


Figure 4.1. Schematic illustration of DHEA signaling features in the Trk-related molluscan and Amphioxus receptors: DHEA activates invertebrate LTrk and ApTrk, Ampioxus AmphiTrk, and mammalian TrkA/C, sequentially inducing the phosphorylation of prosurvival kinases Akt and ERK1/2, preventing cellular apoptosis. However, DHEA is ineffective (in contrast to NGF for mammalian TrkA or to Aplysia neurotrophin for its receptor) in inducing phospholipase C (PLC)-γ activation, a signaling pathway strongly related to higher functions of the nervous system (memory, plasticity, etc).

Based on these findings we hypothesize that as neuronal complexity emerged during evolution, ancient neurotrophic factors (like DHEA or other ancient molecules) were gradually replaced by more specific and fast-acting flash-like peptide factors, which could afford a more effective tempospatial regulation of neural development in a complex and compartmentalized neural system. It is conceivable that, in the ancient diffused and less complicated nervous system, small and highly lipophilic, longer-lasting molecules could more effectively support primordial neuronal development, differentiation, and survival, a property that is partially sustained in more evolved organisms.

A hypothetical model is now generally accepted suggesting that after two rounds of genome duplication during the evolution of vertebrates, the ancestral *Trk* gene gave rise to four different copies, TrkA, TrkB, TrkC, and TrkD, the latter being lost thereafter (*Benito-Gutierrez et al., 2006*). A tree generated using the full-length sequences of human and mouse Trks including AmphiTrK, Ltrk, and ApTrk (Figure 4.2) supports this hypothesis that the first duplication generated a *TrkA/D* gene (ancestor of TrkA and TrkD) and a *TrkB/C* gene (ancestor of TrkC and TrkB) (*Benito-Gutierrez et al., 2006; Hallböök et al., 2006*). Although TrkB and TrkC group together phylogenetically, TrkA and TrkC group together from a functional point of view regarding the ability of DHEA to activate them. The BDNF/TrkB system is considered to be the most evolved neurotrophin signal, suggested by its localization within the CNS, in contrast to NGF/TrkA and NT-3/ TrkC, which are mainly located in peripheral neurons. It is of note that TrkA and TrkC were shown to induce cell death in the absence of their ligands, in contrast to TrkB, suggesting higher specificity of the latter for its potential ligands (*Nikoletopoulou et al., 2010*). Because the two rounds of genome duplication

occurred in vertebrates after the cephalochordate split from the vertebrate stem lineage (*Holland et al., 2008; Furlong et al., 2002*), the *AmphiTrk* gene appears to be more conserved, resembling the ancestral *Trk* gene compared with the three mammalian Trks (*Putnam et al., 2008*).

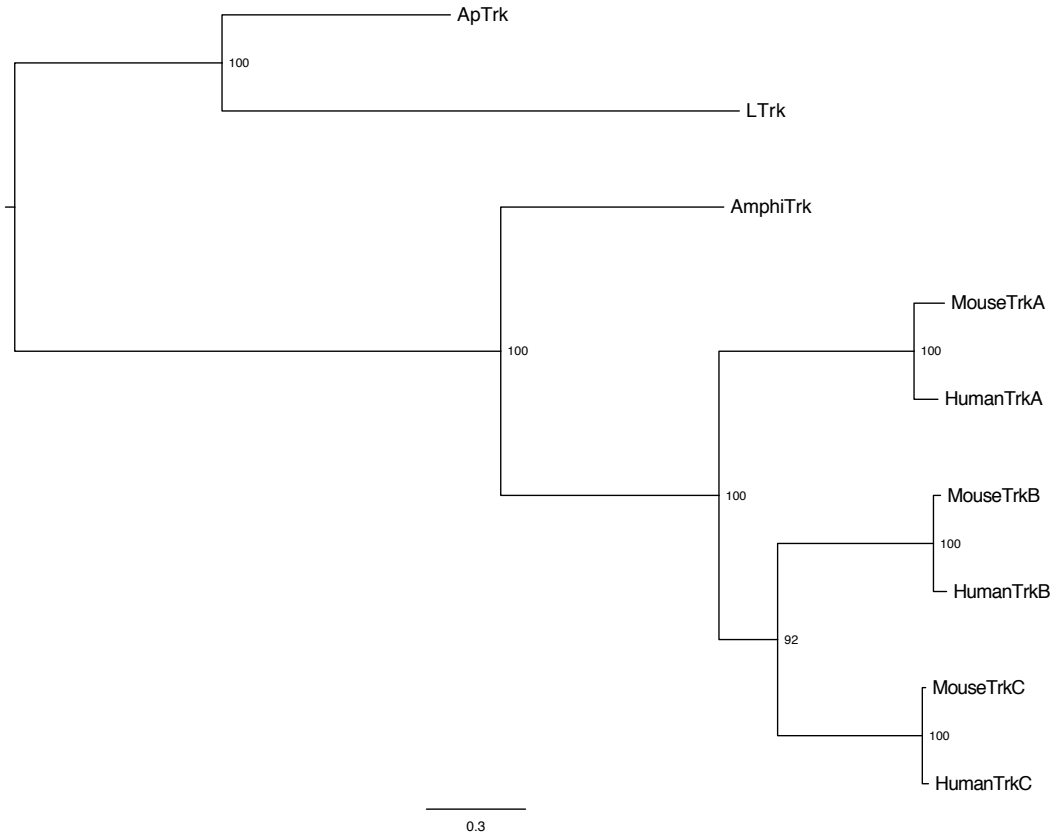


Figure 4.2. Maximum likelihood tree of the *Trk* genes among human, mouse AmphiTrk, Ltrk, and ApTrk. Numbers of nodes indicate bootstrap values after 1000 replications.

This hypothesis is further supported by the observation that duplicated genes tend to evolve faster compared to singletons (*Papanikolaou et al., 2009*). This can explain why DHEA exhibits higher affinity for the cephalochordate Trk than for its mammalian counterparts. We believe that DHEA played a role as a crucial molecule in the survival of neuronal cells before the emergence of more complex nervous systems. Our data support the hypothesis that during evolution DHEA

may have served as a primordial neurotrophic factor, promoting neuronal survival in the less ancient complex nervous systems. Indeed, our data show that DHEA interacts with all neurotrophin receptors in a more or less promiscuous manner, offering new insights into the largely unknown roles of DHEA within the nervous system as well as in other tissues including the immune, endocrine, and cardiovascular systems, which also express neurotrophin receptors (*Scuri et al., 2010; Caporali et al., 2009; Anagnostopoulou et al., 2013*). The vast number of experimental and clinical findings involving this abundant but also highly controversial and multifaceted steroid in the reproductive, immune, endocrine, vascular, and cancer cell physiology and dysfunction should be reassessed in view of our findings.

DHEA is metabolized *in vivo* to estrogens, androgens, and their related metabolites, affecting the endocrine system, and altering the hormonal microenvironment in the brain. Therefore, the long-term use of DHEA as a potential treatment or chemio-prophylaxis in neurodegeneration is problematic, particularly in patients with genetic predisposition to hormone-dependent tumors (breast, endometrium, ovaries, prostate). To overcome these effects of DHEA, we have recently developed synthetic C17-derivatives of DHEA (BNNs) (*Calogeropoulou et al., 2009*), with anti-apoptotic properties, deprived of androgenic/estrogenic actions. The present study provides evidence that BNNs act as synthetic microneurotrophins, interacting with both NGF receptors, TrkA and $p75^{NTR}$ (Figure 4.3.). Specifically, competition assays using [^{3}H]-DHEA showed that 17 spiro-DHEA derivative BNN27 effectively displaced binding of [^{3}H]-DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and $p75^{NTR}$ receptors (IC_{50}: 1.86 and 3.9 nM respectively). In addition,

pull-down assays and NMR studies confirmed the direct physical interaction of BNN27 with recombinant TrkA and $p75^{NTR}$ receptors: polyethylene-BNN27 beads effectively pull-down recombinant TrkA and $p75^{NTR}$ proteins, while NMR studies revealed a specific interaction of BNN27 to TrkA receptor, (ineffective with recombinant TrkB and TrkC), as well as with $p75^{NTR}$ receptors. The NMR signal of the interaction of NGF with the purified TrkA receptor was significantly enhanced in the presence of BNN27, suggesting a synergistic conformational effect of these two molecules. Mutagenesis assays showed that binding of BNN27 to TrkA receptors does not require its extracellular, the binding domain affording NGF binding. In contrast, BNN27 needed the extracellular domain of p75NTR receptors, shown by completion assays in mutated $p75NTR^{\Delta ECD}$ receptor, lacking its extracellular part.

BNN27 induced dose-dependently TrkA tyrosine phosphorylation in the functionally relevant tyrosine residues (Tyr490, Tyr674/675 and Tyr785), affecting the downstream signaling pathways Akt and MAPKs in primary sympathetic neurons: BNN27 at 100nM induced TrkA phosphorylation even 6h latter, compared to short acting NGF within 15min. Furthermore, by measuring TrkA phosphorylation at 490-tyrosine residue, we showed that BNN27 - in contrary to NGF- was able to induce phosphorylation of TrkA receptor in cells transfected with the $TrkA^{C302S}$ mutant, in the absence of the extracellular domain.

Our internalization experiments suggest that BNN27 shows a different pattern of TrkA turn-over compared to NGF: it induced internalization and fast return of the receptor into the membrane through activation of Rab5 protein and docking of the receptor in the early endosomes. These results are compatible not only with the longer lasting of TrkA phosphorylation/activation, but also with the

transient activation of Erk kinases. Interestingly enough, BNN27 appears to secure high levels of surface TrkA, due to its faster recycling into the membranes. This phenomenon may partially explain to synergistic effect of this cound with NGF, and its potential modulatory effect of limiting, low concentrations of NGF. Cholesterol depletion from membranes and separation of lipid raft microdomains, showed that BNN27 –in contrast to NGF- caused TrkA to segregate into lipid raft fractions, suggesting that phospholipid membrane micro-environment may affect cell responsiveness to BNN27. It is worth noticing here that various techniques (Biacore electron plasmon resonance, Isometric Calorimetry) failed to detect binding of BNN27 to recombinant TrkA receptors. It appears that, in contrast to NGF, phospholipid membrane microstructures are important factors to afford effective and high affinity binding of these small molecules to NGF receptors.

BNN27 efficiently induced the association of $p75^{NTR}$ with its effector proteins RIP2 and TRAF6 in Schwann cells, known to endogenously express $p75^{NTR}$ receptors. In addition, binding of BNN27 enabled the release of RhoGDI from $p75^{NTR}$ in MEF cells, transfected with the plasmid cDNA of $p75^{NTR}$, and subsequently decreasing the activity of RhoA, due to its association-blocking by its inhibitor, RhoGDI. Binding assays with two $p75^{NTR}$ mutants, $p75NTR^{\Delta ECD}$ lacking the whole extracellular domain, and $p75NTR^{C257A}$ ineffective for RIP2 recruitment, due to ineffective dimerization (*Vilar et al., 2009*), showed that the extracellular domain of $p75^{NTR}$ and its dimerized conformation are necessary for BNN27 binding. It is of special note, that endogenous neurosteroids like DHEA, E2, ADIOL, and synthetic BNN compounds, have differential effects in neurotrophin receptors activation. As previously mentioned, DHEA binds to all three Trk (TrkA, TrkB, TrkC) and $p75^{NTR}$ receptors, induces tyrosine

phosphorylation of TrkA and TrkC receptors but does not appear to affect the phosphorylation of TrkB receptors. E2 has no affinity for neurotrophin receptors, and ADIOL, a metabolite of DHEA, does not bind neither activates TrkA, while it propagates cell death (*Charalampopoulos et al., 2004*) through the activation of $p75^{NTR}$ receptor. Synthetic BNN compounds like BNN27 and BNN237 bind and activate TrkA and $p75^{NTR}$ but not TrkB or TrkC receptors, while BNN20 appears to activate all forms of Trk receptors. Thus, it becomes evident that chemical structural of steroids determines their binding and activation ability, as it is known for classical steroids receptors (ERalpha, ERbeta, AR) too. The dependence of differential binding capacity of steroids for neurotrophin receptors upon their chemical structure supports the selectivity and biological importance of this binding. Our findings are also in agreement with the recently described properties of Trk and $p75^{NTR}$ receptors to differentially signal upon binding to different polypeptide ligands (proneurotrophins, Aβ-amyloid, PrP, MAG, for $p75^{NTR}$) (*Nykjaer et al., 2004; Della-Bianca et al., 2001; Yaar et al., 2002; Wang et al., 2002; Wong et al., 2002*). The conformational analysis of the interactions of small molecules and polypeptide agents with neurotrophin receptors offers a useful theoretical basis for *in silico* studies and the development of a pharmacophore model, for rational design of more active and selective neurotrophin receptor agonists and antagonists.

BNN27 was effectively mimicking NGF and DHEA in inhibiting apoptosis of various neuronal cell types. The anti-apoptotic actions of BNN27 were mediated by TRKa receptors, since inhibition of its expression by specific shRNA completely reversed its neuroprotective actions. It is of note that $p75^{NTR}$-shRNA did not modify the anti-apoptotic effect of both BNN27 and NGF. On the contrary

it enhanced their neuroprotective activity, most probably by decreasing the intrinsic pro-apoptotic actions of this death receptor. The anti-apoptotic actions of BNN27 *in vitro* were confirmed *in vivo*. Indeed, BNN27 appears to rescue from apoptosis TrkA positive sensory neurons of Dorsal Root Ganglia (DRG) in *ngf-/-* mouse embryos at the age of E13.5 when naturally occurs programmed cell death.

We have also tested the ability of BNN27 to mimic NGF in other of its functions beyond the pro-survival ones. For this purpose, we used PC12 cells, which respond to NGF by extending neurites and acquiring a neuronal phenotype (*Drubin et al., 1985*). BNN27 given alone was ineffective in inducing differentiation and neurite outgrowth of PC12 cells. However, when it was given with low concentrations of NGF (20 ng/ml) it further increased the percentage of cells that differentiate, enhancing also the axonal length in both PC12 and dorsal root ganglion cells (DRGs). These findings are in agreement with our NMR studies, showing the BNN27 potentiates binding of NGF to TrkA receptors. Furthermore, they support our hypothesis from the TrkA turn-over, internalizations studies, showing that BNN27 facilitates the fast returning of the receptor into the membrane, enhancing cellular sensitivity to NGF. Indeed, It is well documented that endocytosis of the NGF-pTrkA complex into endosomes and signaling within the endosome is necessary for NGF-induced neurite outgrowth (*Zhang et al., 2000*). In addition, NGF signaling suppresses Rab5 to help establish the signaling endosomes and sustain the differentiation signals (*Liu et al., 2007*). Contrary to NGF, BNN27 enhances Rab5 activity, pTrkA recycling (Rab11) and surface expression of TrkA, blocking in parallel the transition of the receptor towards the late endosomes (Figure 4.2).

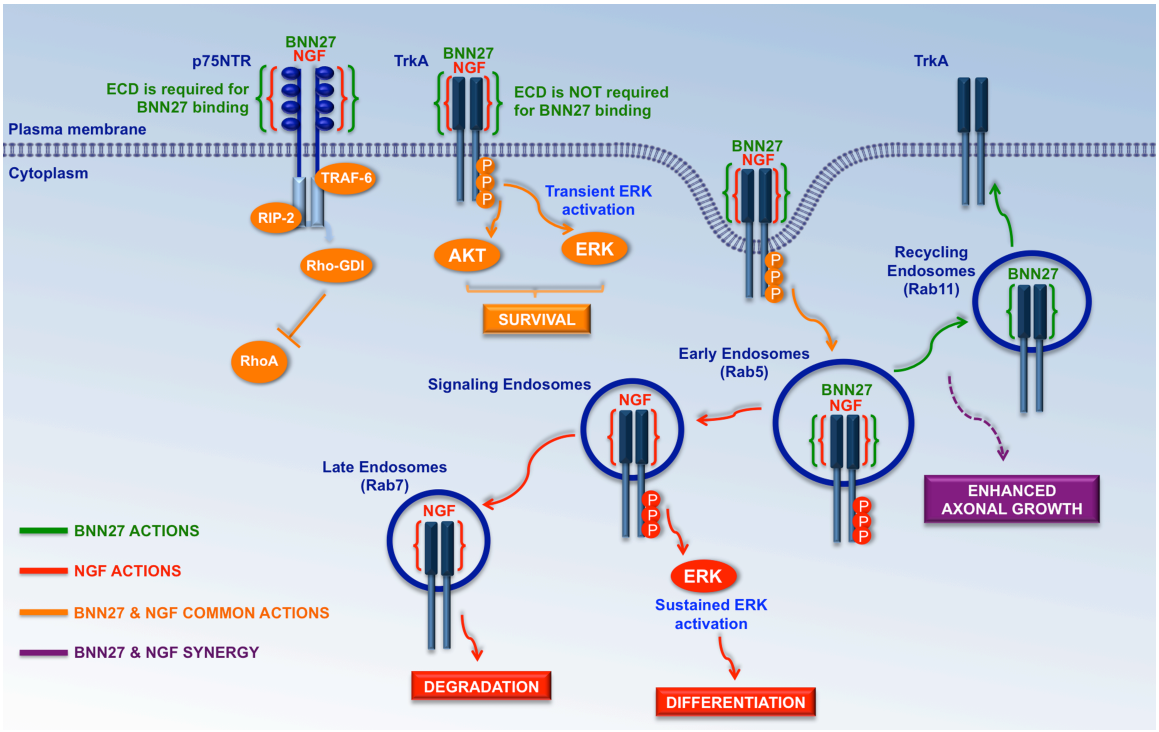


Figure 4.3. Schematic representation of the molecular proposed mechanisms of action of BNN27.

A large number of experimental and clinical studies involve NGF in the pathophysiology of various neurodegenerative diseases and its potential use in their therapeutics, targeting neural tissue protection and repairing (*Aloe et al., 2012*). Recent studies have highlighted abnormalities in intracellular trafficking in many neurodegenerative disorders, including Alzheimer's disease and amyotrophic lateral sclerosis (*Gunawardena et al., 2001; Williamson et al., 1999*). In one animal model of Alzheimer's disease, where defective retrograde NGF transport was observed together with degeneration of basal forebrain cholinergic neurons, application of exogenous NGF reversed these defects (*Cooper et al., 2001*). However, NGF does not pass the blood brain barrier because of its polypeptidic nature. Intra-cerebro-ventricular (ICV) infusion of NGF was tested in

AD patients in late 90s, early 2000, with very limited beneficial effects (*Olson et al., 1992; Eriksdotter et al., 1998*). Important and severely limiting side effects were observed, such as weight loss during the NGF infusion period and, most importantly, development of back pain symptoms after the beginning of ICV infusion, most probably reflecting the NGF-mediated hyper-activation of nociceptive transmission system in the spinal cord. Thus, utilization of NGF, especially when systemically administered, remains hampered because of its adverse events on pain system and the difficulty to achieve pharmacological concentrations in therapeutic relevant targets without affecting non-target areas. Recently, intranasal delivery of NGF was tested in animal models of AD (*Capsoni et al., 2012; Capsoni et al., 2011*). Our 17-spiro synthetic derivatives of DHEA, like BNN27, mimic some of the neuroprotective effects of neurotrophins (named because of this property as Microneurotrophins, MNT), are small, lipophilic molecules, which effectively pass the blood-brain-barrier (BBB) reaching the suffering neurons. Indeed, we have recently detected BNN27 by Mass-Spectrometry in brain and retina extracts, 10-30 minutes after its intraperitoneal administration in mice (data not shown). Synthetic compounds like BNN27 may serve as lead molecules to develop BBB-permeable small agonists and antagonists for neurotrophin receptors with selective neuroprotective and neurogenic actions and potential applications in therapeutics of neurodegenerative diseases and brain trauma.

Future work should concentrate on the *in vivo* testing of BNN analogs in experimental animal models of neurodegenerative diseases (proof of concept studies *in vivo*). Alzheimer's related mouse models such as, transgenic mouse models (5xFAD, AD11, etc) which experience extensive cortical cell loss,

cholinergic deficit in the basal forebrain and behavioral deficits or familial AD (FAD)-based transgenic mouse models, where altered amyloid precursor protein (APP) processing leads to Aβ accumulation correlating with hippocampal-dependent memory deficits, MPTP mouse and Weaver mouse models of Parkinson's disease, Experimental Autoimmune Encephalomyelitis (EAE) and neurotroxin Cuprizone mouse models of Multiple Sclerosis and demyelinating diseases or ischemic retina degeneration mice, are very interesting animals models now under testing.

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07/2010-03/2015 **University Of Crete**
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Specialization: **The neurotrophic effects of endogenous and synthetic Neurosteroids: structure-function analysis of their interactions with neurotrophins receptors and their biological role in neuroprotection.**

09/2008-07/2010 **University Of Crete**
MSc in Medicine School
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09/2002-06/2007 **University Of Crete**
Department of Biology
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09/1998- 07/2001 **7th Lyceum of Heraklion, Greece**
Greek Apolytirion Grade 17.6 out of 20, Rating: Very Good

PUBLICATIONS

Dehydroepiandrosterone: an ancestral ligand of neurotrophin receptors.
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PLoS Biol. 2011 Apr;9(4):e1001051.

LABORATORY AND WORK EXPERIENCE

04/2009-current **University of Crete**
Medicine School
Department of Pharmacology
Laboratory of Professor **A. Gravanis.**
My responsibilities included:

- Primary Neuronal Cultures,
- Isolation and characterization of Neural Stem Cells,
- Flow Cytometry (FACs),
- Immunocytochemistry (ICC),
- Competitive Binding Assays,

07/2004- 06/2007 **Institute of Molecular Biology & Biotechnology, Crete**
Laboratory of Professor **J. Papamatheakis**.
My responsibilities included:

- projects concerning enhanceosome of MHC class II genes,
- various laboratory projects using molecular techniques such as isolation of plasma DNA in bacteria, cloning and DNA ligation, DNA transformation in bacteria, DNA transfection in eukaryotic cells, Polymerase Chain Reaction (PCR) and Western Blot.

AWARDS

2011-2012 **"Manassaki" Scholarship**
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2010-2011 **"Manassaki" Scholarship**
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Dehydroepiandrosterone: An Ancestral Ligand of Neurotrophin Receptors

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Dehydroepiandosterone (DHEA), the most abundant steroid in humans, affects multiple cellular functions of the endocrine, immune, and nervous systems. However, up to quite recently, no receptor has been described specifically for it, whereas most of its physiological actions have been attributed to its conversion to either androgens or estrogens. DHEA interacts and modulate a variety of membrane and intracellular neurotransmitter and steroid receptors. We have recently reported that DHEA protects neuronal cells against apoptosis, interacting with TrkA, the high-affinity prosurvival receptor of the neurotrophin, nerve growth factor. Intrigued by its pleiotropic effects in the nervous system of a variety of species, we have investigated the ability of DHEA to interact with the other two mammalian neurotrophin receptors, ie, the TrkB and TrkC, as well as their invertebrate counterparts (orthologs) in mollusks Lymnaea and Aplysia and in cephalochordate fish Amphioxus. Amazingly, DHEA binds to all Trk receptors, although with lower affinity by 2 orders of magnitude compared with that of the polypeptidic neurotrophins. DHEA effectively induced the first step of the TrkA and TrkC receptors activation (phosphorylation at tyrosine residues), including the vertebrate neurotrophin nonresponding invertebrate Lymnaea and Aplysia receptors. Based on our data, we hypothesize that early in evolution, DHEA may have acted as a nonspecific neurotrophic factor promoting neuronal survival. The interaction of DHEA with all types of neurotrophin receptors offers new insights into the largely unidentified mechanisms of its actions on multiple tissues and organs known to express neurotrophin receptors. **(*Endocrinology* 156: 16–23, 2015)**

Polypeptide neurotrophins (NTs) and their high-affinity tyrosine kinase membrane receptors (tropomyosin related kinase [Trks]) are key effectors of neuronal differentiation, growth and survival (1, 2). Most vertebrates express three types of transmembrane Trk receptors, namely the TrkA, TrkB, and TrkC, all sharing a common structural organization of their extra- and intracellular domains (3), although they do exhibit differences in their affinity toward the polypeptide neurotrophic ligands. More specifically, TrkA is primarily activated by nerve growth factor (NGF), TrkB mainly by brain-derived neurotrophic factor (BDNF) and the NT-4/5, whereas the TrkC receptor is preferentially activated by NT-3 (4). Finally, the immature proneurotrophins are selective ligands for the pan-neurotrophin p75 receptor (5). It should be noted here that NT-3 can also bind to TrkA and TrkB receptors (6, 7).

Although Trk receptors are evolutionarily conserved, they are missing in some species including *Caenorhabditis elegans* and *Drosophila melanogaster*, which do not have the genes encoding the Trk receptors and their ligands (8, 9). Based on these observations, it has been speculated that an alternative form of neurotrophic signaling should be present in invertebrates, possibly including several Trk-related receptors like the mollusk *Lymnaea stagnalis* (Ltrk) (5), the marine mollusk *Aplysia californica* (ApTrk)

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Abbreviations: AmphiTrk, amphioxus Trk; ApTrk, *Aplysia* Trk; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; FACS, fluorescence-activated cell sorting; Ki, inhibitory constant; Ltrk, *Lymnaea* Trk; NGF, nerve growth factor; NT, neurotrophin; Trk, tropomyosin related kinase.

(10), and the cephalochordate amphioxus *Branhiostoma floridae* (AmphiTrk) (11). However, to date, researchers have been unable to identify neurotrophin homologs outside the vertebrate lineage (1, 12, 13), suggesting the presence of other types of molecules acting as activators of neurotrophin receptors. The only known example of such an invertebrate ligand, binding to fully conserved Trk receptor (ApTrk), is the ApNT, which is expressed exclusively in the central nervous system (CNS) of the marine mollusk *A californica*, playing a central role in learning-related synaptic plasticity (10). ApTrk is the only invertebrate Trk receptor with the structural and signaling features of its vertebrate Trk counterparts (14). In addition to Trk invertebrate ligands, the molluscan neurotrophic factor cysteine-rich neurotrophic factor has been found to interact with the pan-neurotrophin receptor $p75^{NTR}$ (15).

We have recently shown that dehydroepiandrosterone (DHEA), the most abundant steroid in humans, interacts and activates the mammalian TrkA receptor (16), exerting a strong neuroprotective effect (17). DHEA is also produced in neuronal and glial cells of the nervous system (18) in addition to its production by the adrenals. It is well documented that DHEA affects multiple cellular functions of the endocrine, immune, and nervous systems. However, no specific receptor has been described for DHEA and most of its effects have been attributed to its conversion into either androgens or estrogens. DHEA is also known to interact and modulate a variety of membrane and intracellular neurotransmitter and steroid receptors (19). Intrigued by its pleiotropic effects on the nervous system across a variety of species and the early evolutionary presence of CYP17, the enzyme responsible for its biosynthesis (20, 21), we have examined, in the present study, the ability of DHEA to interact with the two other mammalian neurotrophin receptors (TrkB and TrkC) as well as with their invertebrate counterparts (orthologs), ie, those present in mollusks Ltrk, ApTrk, and AmphiTrk.

Materials and Methods

Plasmids, antibodies, and proteins

The TrkA expression plasmid has been previously described (16). Plasmids were kindly provided by the following researchers: AmphiTrk plasmid by Dr C. F Ibáñez (Karolinska Institutet, Stockholm, Sweden), TrkB and TrkC plasmids by Dr Yves-Alain Barde (University of Cardiff, Cardiff, United Kingdom), ApTrk and ApTrk-GFP plasmids by Dr Eric R. Kandel (Columbia University, New York, New York), and the expression plasmid for Ltrk by Dr Mike Fainzilber (Weizmann Institute, Rehovot, Israel). All information about the antibodies used is described in Supplemental Table 1.

Tissue culture and cell transfection

Human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) cells were obtained from LGC Promochem (LGC Standards GmbH) and were grown in DMEM medium containing 10% fetal bovine serum (charcoal stripped for removing endogenous steroids), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 5% CO_2 and 37°C. PC12nnr5 cells were kindly provided by Dr Marçal Vilar (Instituto de Salud Carlos III-ISCII, Madrid, Spain). PC12nnr5 cells were grown in RPMI 1640 containing 10% horse serum, 5% fetal calf serum, 2 mM L-glutamine, 15 mM HEPES, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 5% CO_2 and 37°C. Cells were transfected with TurboFect (Fermentas) or Lipofectamine LTX (Invitrogen) according to the manufacturers' instructions. Transfected cells were typically used on the second day after transfection.

[^{3}H]DHEA binding assays

HEK293 cells transfected with the cDNA expression plasmids encoding for TrkA, TrkB, TrkC, Ltrk, ApTrk, or AmphiTrk were cultured, collected by scraping on ice, and washed twice with cold PBS (pH 7.4). Membranes were isolated and bindings experiments using tritiated DHEA were performed as previously described (16). The efficacy of transfection and expression of TrkA, TrkB, TrkC, Ltrk, ApTrk, or AmphiTrk plasmids in HEK293 cells were analyzed in a Beckton-Dickinson FACSArray apparatus and the CELL-Quest software, using the relevant antibodies.

Immunoprecipitation and immunoblotting

Forty-eight hours after transfection with the cDNA plasmids of the various Trk receptors, CHO cells were starved from serum for 4–6 hours and stimulated with 100 nM DHEA or 100 ng/mL of the appropriate neurotrophin for 15 minutes as indicated. Cells were then lysed and cell extracts were immunoprecipitated as previously described (16) with the appropriate antibody. Twenty-four hours after transfection, PC12nnr5 cells were starved from serum for 12 hours and stimulated with different concentrations of DHEA or 100 ng/mL of neurotrophins for 15 minutes. Cells were then lysed and immunoblotted appropriately (16). Image analysis and quantification of band intensities were measured with ImageQuant (GE Healthcare).

Fluorescence-activated cell sorting (FACS) analysis

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (Roche) staining of apoptotic PC12nnr5cells was performed according to the manufacturer's instructions and analyzed in a Beckton-Dickinson FACSArray apparatus and the CELL-Quest software.

Phylogenetic equivalent

Amino acid sequences of the orthologous and paralogous Trk genes from *Homo sapiens*, *Mus musculus*, *A californica*, *B floridae*, and *Lymnaea stagnalis* were obtained from GenBank (accession numbers are shown in Supplemental Table 2) and were aligned with the program ClustalX2 creating amino acid sequence data of 955 positions. The evolutionary equivalent was assessed using the MEGA version 6.05 software (22). According to the Akaike's information criterion Jones-Taylor-Thornton aminoacid substitution model with γ(G) distributed rate of vari-

ation among sites model was selected. The maximum likelihood tree was constructed with MEGA version 6.05 by performing 1000 bootstrap replications and setting the γ-parameter of the Jones-Taylor-Thornton aminoacid substitution model with γ (G) distributed rate of variation among sites model to 2.

Statistical analysis

Statistical analysis of our data was performed using an ANOVA and post hoc comparison of means, followed by the Fisher's least significance difference test. For data expressed as percentage changes, we have used the nonparametric Kruskal-Wallis test for several independent samples.

Results

DHEA interacted with both vertebrate and invertebrate Trk receptors

In this set of experiments, we have tested the ability of DHEA to bind to various representative members of the Trk receptor evolutionary forms and more specifically to the vertebrate TrkA, TrkB, and TrkC receptors and to the invertebrate Trk orthologs, Ltrk and ApTrk (mollusks *Lymnaea* and *Aplysia*, respectively), and AmphiTrk (cephalochordate fish *Amphioxus*). DHEA effectively displaced binding of [^{3}H]DHEA to membranes isolated from HEK293 cells (not expressing endogenous Trk receptors) transfected with the cDNAs of TrkA, TrkB, or TrkC receptors [inhibitory constant (Ki): 7.40 ± 1.7 nM, 4.49 ± 1.7 nM, and 6.33 ± 1.3 nM, n = 3, respectively], suggesting that DHEA appears to interact with all forms of vertebrate Trk receptors (Figure 1A), although with affinities approximately 2 orders of magnitudes lower compared with that of polypeptide neurotrophins (0.01–0.1 nM). Moreover, DHEA was proved equally effective in displacing binding of [^{3}H]DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of Ltrk, ApTrk, or AmphiTrk receptors (Ki: 3.6 ± 0.4 nM, 1.14 ± 0.11 nM, and 0.47 ± 0.18 nM, n = 3, respectively) (Figure 1, B–D). Membranes isolated from HEK293 cells transfected with the empty vector (mock) showed no specific binding of tritiated DHEA. We have previously shown that DHEA sulfate (DHEAS), the sulfated ester of DHEA, binds to TrkA with an affinity comparable with that of DHEA (16). We report now that DHEAS also binds to all four invertebrate receptor types at nanomolar concentrations (Figure 1E). However, the affinity of DHEAS for AmphiTrk was significantly lower compared with that of

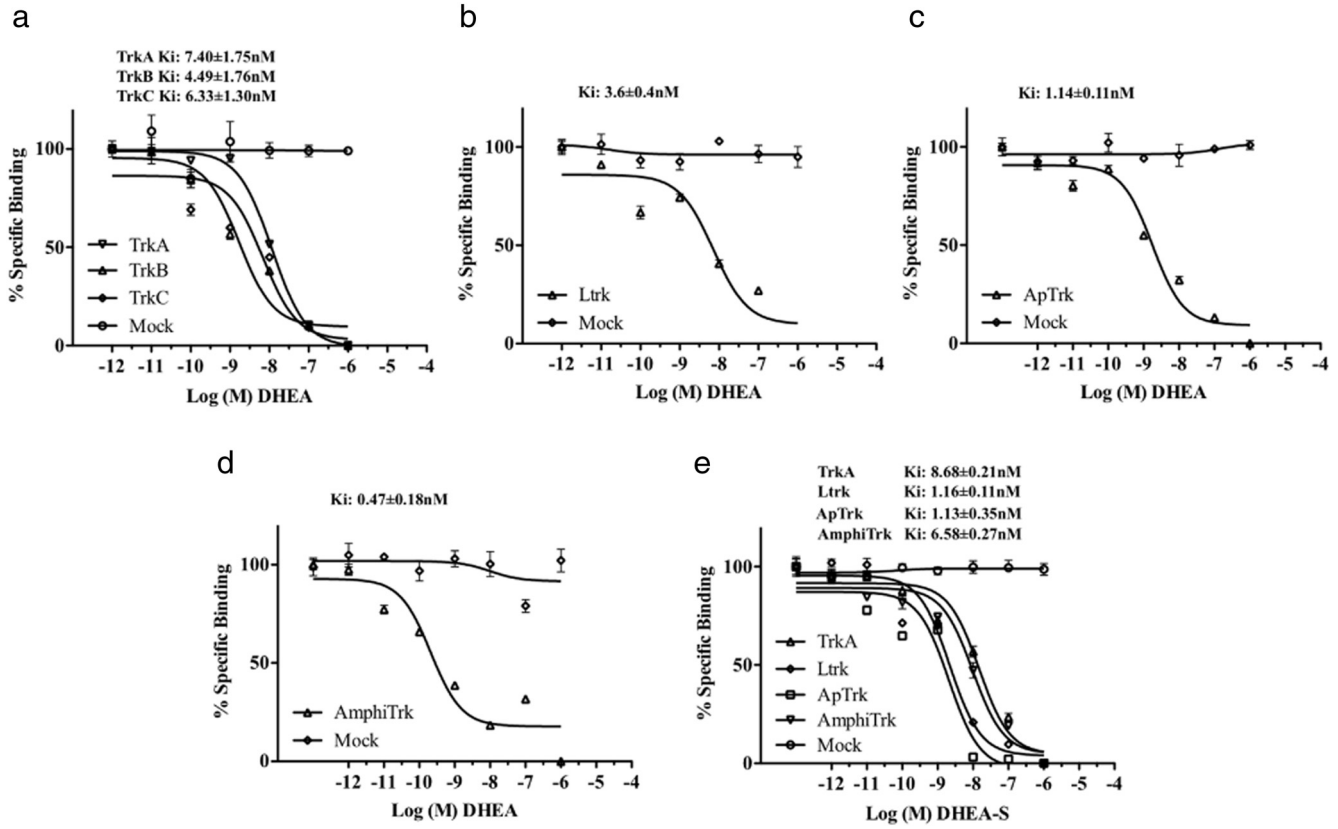

Figure 1. [^{3}H]DHEA binds to cell membranes from HEK293 cells transfected with the cDNAs of mammalian and invertebrate Trk receptors. Competition binding assays of tritiated [^{3}H]DHEA in the presence of increasing concentrations of nonlabeled DHEA (A–D) or DHEAS (E) were performed using membranes isolated from HEK293 cells transfected with the plasmid cDNAs of mammalian TrkA (A and E), TrkB and TrkC receptors (A), or invertebrate Ltrk, ApTrk, and AmphiTrk receptors (B–E). (Ki represents the mean ± SEM of three independent experiments).

DHEA, suggesting that even small structural differences significantly affect binding of these steroids to neurotrophin receptors.

DHEA was able to activate all types of invertebrate Trk homologs

To examine whether binding of DHEA to Trk receptors is functional, we assessed its effectiveness in inducing tyrosine phosphorylation of Trk receptors, the sine qua non proof in assessing the activation of Trk receptors. We have found that DHEA induced tyrosine phosphorylation of TrkA and TrkC receptors in CHO cells transfected with the appropriate cDNAs but did not affect the phosphorylation of TrkB receptors (Figure 2A). To our knowledge, DHEA is the only molecule that successfully binds to all three vertebrate Trk receptors, activating two of them.

The invertebrate Ltrk receptor has a conserved transmembrane and intracellular tyrosine kinase domain but lacks the immunoglobulin-like domains at the extracellular part of the protein (7). Ltrk has been shown to bind mammalian NT-3 (but not NGF or BDNF), however with no apparent signaling response to this neurotrophin (7). It is interesting that DHEA was also able to induce tyrosine phosphorylation of the Ltrk receptors in CHO cells transfected with the Ltrk cDNA, an effect not seen in any polypeptide neurotrophin (Figure 2B).

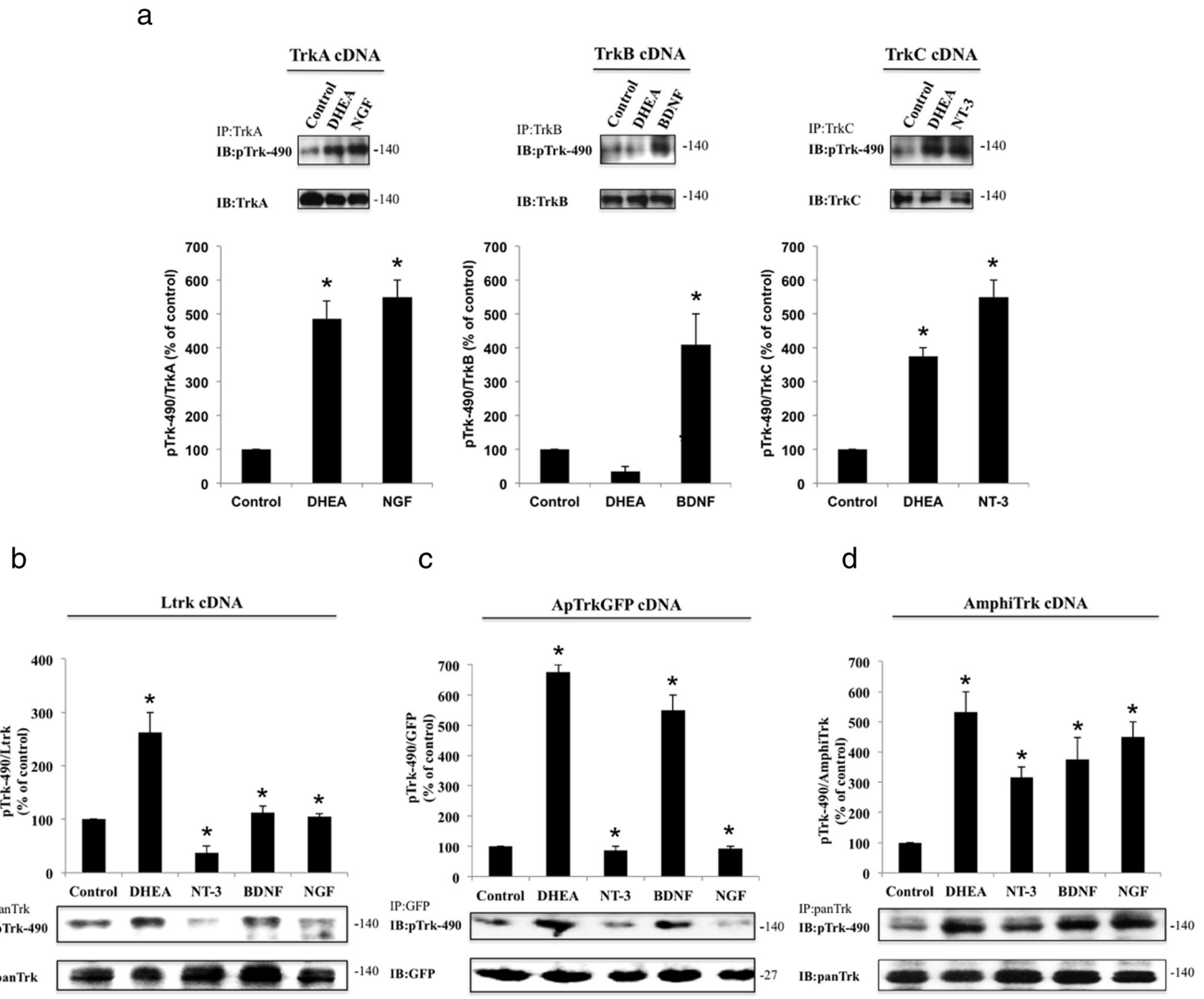

Figure 2. DHEA induces tyrosine phosphorylation of Trk receptors. A, CHO cells were transfected with the plasmid cDNAs of mammalian TrkA, TrkB, or TrkC receptors. Transfectants were then exposed for 15 minutes to 100 ng/mL of mammalian neurotrophins NGF (left panel), BDNF (middle panel), NT-3 (right panel), or to 100 nM of DHEA. Cell lysates were immunoprecipitated with specific antibodies against TrkA, TrkB, or TrkC and then immunoblotted with pTyr-490 antibodies. Total lysates were analyzed for TrkA, TrkB, or TrkC expression by immunoblotting. B–D, CHO cells were transfected with the plasmid cDNAs of invertebrate Ltrk, ApTrk, or AmphiTrk receptors and then exposed for 15 minutes to DHEA or to mammalian neurotrophins, as above. Cell lysates were immunoprecipitated with pan-Trk or GFP antibodies and then immunoblotted with phosphorylated Trk-490 antibodies. Total lysates were analyzed for Trk expression by immunoblotting (mean ± SEM of three independent experiments). *, $P < .05$ relative to control, serum free condition). GFP, green fluorescent protein; IB, immunoblot; IP, immunoprecipitation.

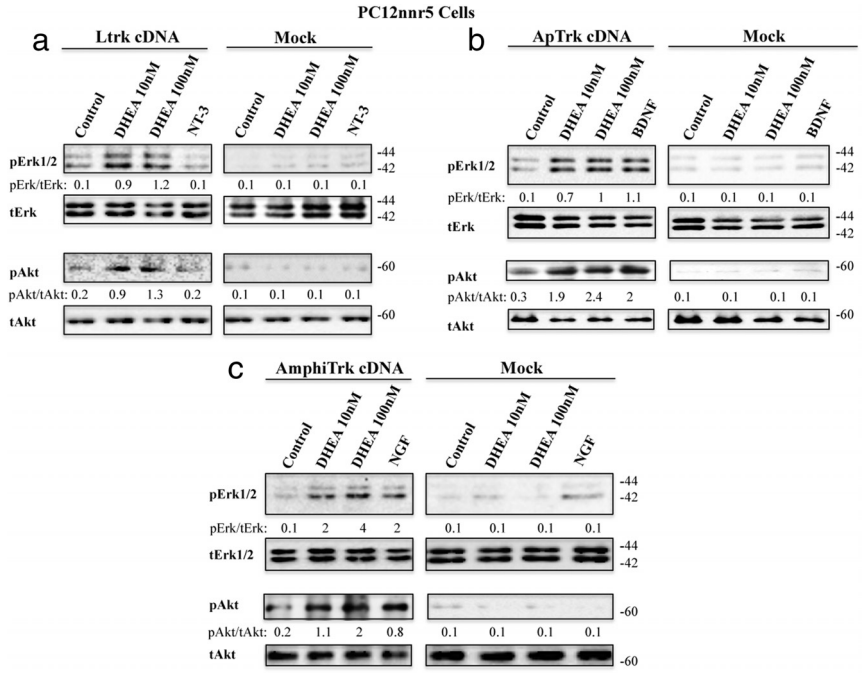

Figure 3. Invertebrate Trk receptors mediate activation of prosurvival kinases ERK and Akt by DHEA (A–C). PC12nnr5 cell were transfected with the plasmid cDNAs of Ltrk, ApTrk, and AmphiTrk receptors and control plasmid (mock), treated for 15 minutes with DHEA at various concentrations or 100 ng/mL NT-3, 100 ng/mL BDNF, and 100 ng/mL NGF, respectively, and then lysed and analyzed by Western blotting with the indicated antibodies.

In *Aplysia*, ApTrk is highly conserved compared with vertebrate Trk receptors possessing an identical domain architecture that includes two IgG domains in its ligand-binding extracellular region and the two main signaling sites. BDNF and Aplysia's neurotrophin ApNT were able to bind and activate ApTrk, whereas NGF and NT-3/4 failed to activate ApTrk (14). We have found that DHEA also induced tyrosine phosphorylation of the ApTrk receptor in CHO cells transfected with the ApTrk cDNA (Figure 2C).

Amphioxus AmphiTrk receptors share the basic protein domain structure of the mammalian Trk receptors at both the intracellular and extracellular level, interacting with mammalian neurotrophins NGF, BDNF, NT-3, and NT-4 (11). We have found that DHEA also induced tyrosine phosphorylation of AmphiTrk receptors in CHO cells transfected with the AmphiTrk cDNA (Figure 2D).

DHEA induced the activation of ERK1/2 and Akt kinases via all types of invertebrate Trk receptors

To evaluate whether activation of invertebrate Trk receptors by DHEA do activate downstream signaling pathways involving the prosurvival ERK1/2 and Akt kinases, we used PC12nnr5 cells, a clone of PC12 cells not expressing endogenous Trk receptors but expressing the pan-neurotrophin receptor $p75^{NTR}$ (23). DHEA effectively induced the phosphorylation of both ERK1/2 and Akt kinases in PC12nnr5 cells transfected with the cDNAs of Ltrk, ApTrk, or AmphiTrk receptors (Figure 3, A–C). It should be stressed, however, that in the same transfectants, DHEA failed to induce phosphorylation of phospholipase C-γ (data not shown), a protein that is implicated in neuronal differentiation and synaptic plasticity (11, 24).

The invertebrate Trk receptors mediated the antiapoptotic effects of DHEA

Neurotrophins support two major physiological functions of neuronal cells, ie, their survival and neurite outgrowth (2, 25, 26). We have recently shown that vertebrate TrkA receptors mediate the strong antiapoptotic effects of DHEA in serum-deprived PC12 and neuronal cells in culture, whereas it is ineffective in inducing neurite outgrowth in PC12 in culture (16). In this set of experiments, the ability of DHEA was tested in controlling these two effects in PC12nnr5 cells, transfected with the cDNAs of invertebrate Trk receptors. We have found that DHEA effectively reversed the serum deprivation-induced apoptosis in Ltrk- or ApTrk-transfected PC12nnr5 cells (from 0.31 ± 0.03 in serum free conditions to 0.163 ± 0.008 and 0.146 ± 0.003, respectively, $n = 3$, $P < .05$) or AmphiTrk-transfected PC12nnr5 cells (from 0.253 ± 0.008 in serum free conditions to 0.183 ± 0.016, $n = 3$, $P < .05$) (Figure 4, A–C). Mammalian neurotrophins NGF or BDNF exerted similar antiapoptotic actions in ApTrk-or AmphiTrk-transfected PC12nnr5 cells. However, mammalian NT-3 increased further the apoptosis induced by serum deprivation in Ltrk-transfected PC12nnr5 cells, possibly due to the inability of NT-3 to activate Ltrk on the one hand, and on the other, it can activate the proapoptotic $p75^{NTR}$ receptor (which is also expressed) and thus increase apoptosis.

Finally, we have found that DHEA was unable to induce neurite outgrowth in Ltrk-, ApTtrk-, or AmpiTrk-transfected PC12nnr5 cells, confirming our previously published observations in endogenously TrkA expressing PC12 cells (16).

Discussion

DHEA, a pleiotropic steroid hormone, is mainly produced by the mammalian adrenal cortex and by a number of

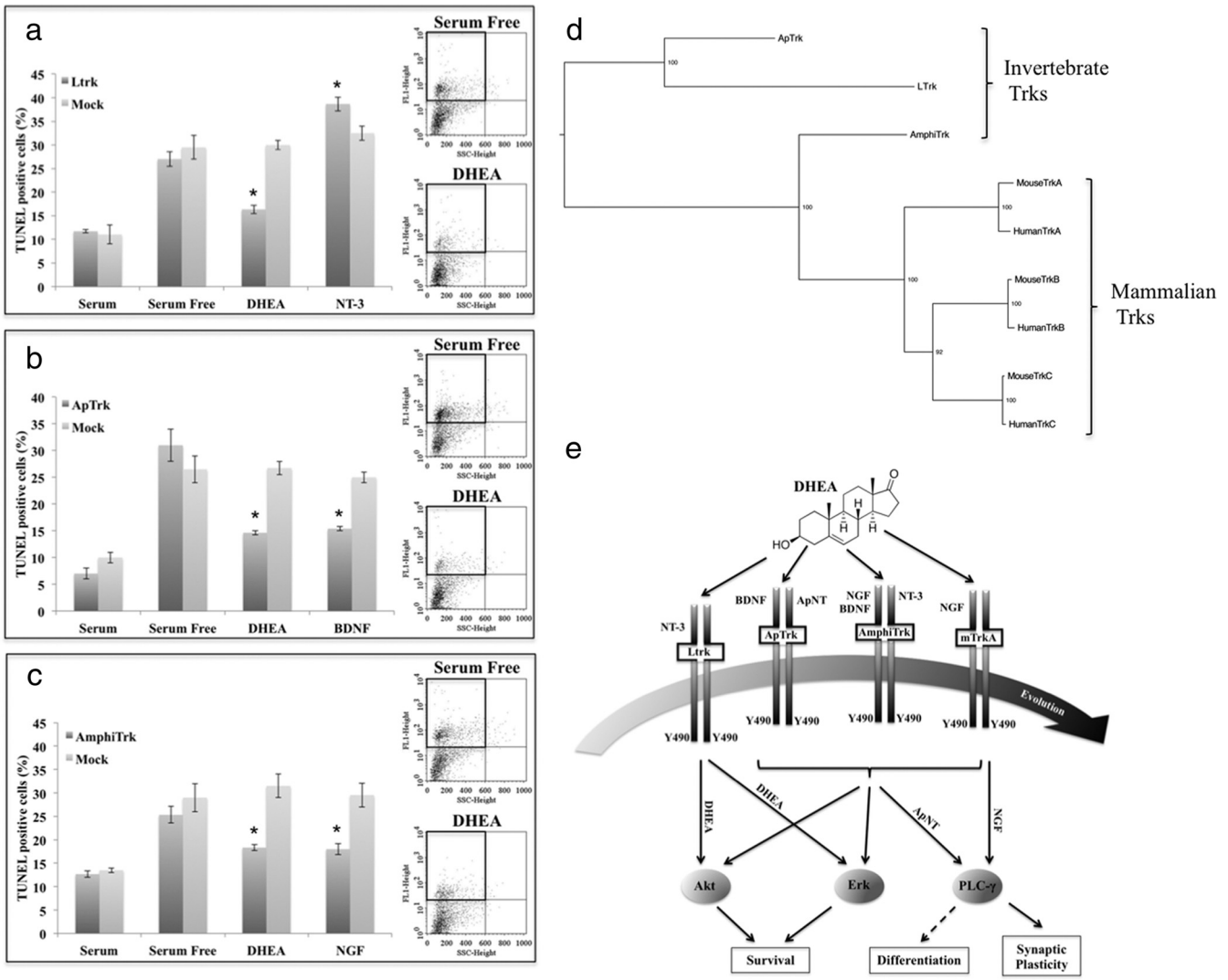

Figure 4. Antiapoptotic effects of DHEA in serum-deprived PC12nnr5 cells transfected with the invertebrate Trk receptors (A–C). PC12nnr5 cells were transfected with the plasmid cDNAs of Ltrk, invertebrate ApTrk, AmphiTrk receptors, or empty vectors (mock) and cultured for 24 hours in serum-starved conditions in the absence or the presence of 100 nM DHEA or 100 ng/mL of mammalian neurotrophins. A FACS analysis shows the percentage of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling-positive cells compared with the serum-free condition, measuring 10 000 cells. (mean ± SEM of three independent experiments. *, $P < .05$, FACS diagrams on the right panel show only the results from transfected cells). D, Maximum likelihood tree of the *Trk* genes among human, mouse AmphiTrk, Ltrk, and ApTrk. Numbers of nodes indicate bootstrap values after 1000 replications. E, Schematic illustration of DHEA signaling features in the Trk-related molluscan and Amphioxus receptors: DHEA activates invertebrate LTrk and ApTrk, Ampioxus AmphiTrk, and mammalian TrkA/C, sequentially inducing the phosphorylation of prosurvival kinases Akt and ERK1/2, preventing cellular apoptosis. However, DHEA is ineffective (in contrast to NGF for mammalian TrkA or to Aplysia neurotrophin for its receptor) in inducing phospholipase C (PLC)-γ activation, a signaling pathway strongly related to higher functions of the nervous system (memory, plasticity, etc).

neurons and glia in the CNS (18, 19). It is interesting that an evolutionary early gene codes CYP17, the most important enzyme of its production (20, 21), whereas *YP* genes are conserved from yeast and fungi up to humans, arising from a single ancestor molecule (25) and implicated in multiple physiological and pathological conditions (reviewed in reference 26). Our results, summarized in Figure 4E, suggest that DHEA effectively binds and activates a vast array of invertebrate neurotrophin receptors including the Ltrk receptor, which has been known not to be activated by any mammalian neurotrophin, including NT-3, although it binds to this receptor. Furthermore, our data suggest that DHEA activates the post-Trk receptor prosurvival pathway mediated by the kinases ERK1/2 and Akt, key mediators of neuronal survival (2, 27). However, DHEA was ineffective in mimicking the effects of polypeptide neurotrophins on Trk receptor-mediated neuronal functions including differentiation and neurite elongation. Based on these data, we hypothesize that because neuronal complexity emerged during evolution, ancient neurotrophic factors like the DHEA were gradually replaced by more specific and fast-acting flash-like peptide

factors, which could afford a more effective tempospatial regulation of development in a complex and compartmentalized neural system. It is conceivable that, in the ancient diffused and less complicated nervous system, small and highly lipophilic, longer-lasting molecules could more effectively support primordial neuronal development, differentiation, and survival.

A hypothetical model is now generally accepted proposing that after two rounds of genome duplication during the evolution of vertebrates, the ancestral *Trk* gene gave rise to four different copies, TrkA, TrkB, TrkC, and TrkD, the latter being lost thereafter (3). A tree generated using the full-length sequences of human and mouse Trks including AmphiTrK, Ltrk, and ApTrk (Figure 4D) supports this hypothesis and in particular that the first duplication generated a *TrkA/D* gene (ancestor of TrkA and TrkD) and a *TrkB/C* gene (ancestor of TrkC and TrkB) (3, 28). Although TrkB and TrkC group together phylogenetically, TrkA and TrkC group together from a functional point of view regarding the ability of DHEA to activate them. The BDNF/TrkB system is considered to be the most evolved neurotrophin signal, suggested by its localization within the CNS, in contrast to NGF/TrkA and NT-3/TrkC, which are mainly located in peripheral neurons. It is of note that TrkA and TrkC were shown to induce cell death in the absence of their ligands, in contrast to TrkB, suggesting higher specificity of the latter for its potential ligands (29). Because the two rounds of genome duplication occurred in vertebrates after the cephalochordate split from the vertebrate stem lineage (30, 31), the *AmphiTrk* gene appears to be more conserved resembling the ancestral *Trk* gene compared with the three mammalian Trks (32). This can be further supported by the well-known fact that duplicated genes tend to evolve faster compared to singletons (33). This can explain that DHEA exhibits higher affinity to the cephalochordate Trk than to its mammalian counterparts (Figure 1, A–D), and thus, we believe that DHEA played a role as a crucial molecule in the survival of neuronal cells before the emergence of more complex nervous systems.

In conclusion, our data support the hypothesis that during evolution DHEA may have served as a primordial neurotrophic factor, promoting neuronal survival in the less ancient complex nervous systems. Indeed, our data show that DHEA interacts with all neurotrophin receptors in a more or less promiscuous manner, offering new insights into the largely unknown roles of DHEA within the nervous system as well as in other tissues including the immune, endocrine, and cardiovascular systems, which also express neurotrophin receptors (34–36). The vast number of experimental and clinical findings involving this abundant but also highly controversial and multifaceted steroid in the reproductive, immune, endocrine, vascular, and cancer cell physiology and dysfunction should be reassessed in view of our findings.

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