UNIVERSITY OF CRETE, SCHOOL OF MEDICINE
DEPARTMENT OF PHARMACOLOGY

NEUROSTEROIDS WITH NEUROPROTECTIVE AND NEUROREGENERATIVE PROPERTIES: ASSESSMENT OF THEIR *IN VIVO* EFFECTS IN ANIMAL MODELS.

Ph.D thesis

IAKOVOS LAZARIDIS

INTERDEPARTMENTAL GRADUATE PROGRAM
"BRAIN AND MIND SCIENCE"

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ΕΡΓΑΣΤΗΡΙΟ ΦΑΡΜΑΚΟΛΟΓΙΑΣ

ΝΕΥΡΟΣΤΕΡΟΕΙΔΗ ΜΕ ΝΕΥΡΟΠΡΟΣΤΑΤΕΥΤΙΚΕΣ ΙΔΙΟΤΗΤΕΣ: ΑΞΙΟΛΟΓΗΣΗ ΤΩΝ ΒΙΟΛΟΓΙΚΩΝΚΑΙ ΦΑΡΜΑΚΟΛΟΓΙΚΩΝ ΙΔΙΟΤΗΤΩΝ ΤΟΥΣ ΣΕ IN VIVO ΜΟΝΤΕΛΑ ΝΕΥΡΟΕΚΦΥΛΙΣΤΙΚΩΝ ΑΣΘΕΝΕΙΩΝ

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ΕΓΚΕΦΑΛΟΣ ΚΑΙ ΝΟΥΣ

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ABSTRACT

Neurosteroid DHEA is biosynthesized in neurons and glia, regulating neuronal survival and neurogenesis during development and aging. We provide evidence that DHEA acts as a neurotrophic factor, protecting neuronal cells against apoptosis via activation of TrkA and $p75^{NTR}$, membrane receptors of neurotrophin NGF. Specifically, we have shown that siRNA against prosurvival TrkA receptors blocked the anti-apoptotic effect of DHEA. Radiolabeled [^{3}H]DHEA bound with high affinity to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and $p75^{NTR}$ receptors. DHEA-polyethylene-glycol beads effectively pulled down recombinant TrkA and $p75^{NTR}$ proteins, and precipitated both proteins from extracts prepared from cells expressing both receptors. DHEA effectively activated NGF receptor-mediated signaling; Shc, Akt, and ERK1/2 kinases down-stream to TrkA receptors and TRAF6, RIP2 and RhoGDI effectors of $p75^{NTR}$ receptor. Finally, DHEA rescued sensory neurons of dorsal root ganglia from apoptosis in NGF null embryos and compensated NGF in rescuing sympathetic neurons of embryonic superior cervical ganglia. Our findings suggest that DHEA and NGF cross-talk via their activation of NGF receptors to afford brain shaping and maintenance. Phylogenetic findings on the evolution of neurotrophins, their receptors and CYP17, the enzyme responsible for DHEA biosynthesis, combined with our data support the hypothesis that DHEA served as a phylogenetically ancient neurotrophic factor.

ΠΕΡΙΛΗΨΗ

Το νευροστεροειδές Διϋδροεπιανδροστερόνη (DHEA) συντίθεται στον εγκέφαλο απο νευρώνες και μικρογλιακά κύτταρα και ρυθμίζει την νευρική επιβίωση και νευρογένεση κατά τη διάρκεια της ανάπτυξης και της γήρανσης. Στη παρούσα μελέτη δείχνουμε ότι η DHEA ενεργεί ως νευροτροφικός παράγοντας, προστατεύοντας νευρικά κυττάρα από αποπτωτικό θάνατο μέσω της ενεργοποίησης των υποδοχέων TrkA και $p75^{NTR}$, που είναι οι υποδοχέις του NGF. Συγκεκριμένα, έχουμε δείξει ότι siRNAs εναντίον του TrkA αναστέλλουν την αντι-αποπτωτική δράση της DHEA. Ραδιοσημασμένη [3H]DHEA προσδένεται με υψηλή συνάφεια σε μεμβράνες απομονωμένες από HEK293 κύτταρα διαμολυσμένα με cDNA's των υποδοχέων TrkA και $p75^{NTR}$. DHEA-polyethylene-glycol beads κατακρημνίζουν αποτελεσματικά απομονωμένους υποδοχείς TrkA και $p75^{NTR}$. Επίσης, η DHEA όπως και ο NGF φωσφορυλιώνει τον TrkA και ενεργοποιεί το ενδοκυττάριο σηματοδοτικό μονοπάτι του (Shc, Akt και Erk1/2) και οδηγεί στην αλληλεπίδραση του $p75^{NTR}$ με τους τελεστές του (TRAF6, RIP2 και RhoGDI). Τέλος, η DHEA φάνηκε να αναστρέφει τον αποπτωτικό θάνατο λόγω έλλειψης NGF τοσο σε πρωτογενείς καλλιέργειες συμπαθητικών νευρώνων, όσο και των αισθητικών νεύρων των γαγγλίων της ραχιαίας ρίζας σε έμβρυα *ngf-/-* διαγονιδιακών ποντικών , *in vivo*. Τα ευρήματά μας δείχνουν ότι η DHEA και ο NGF αλληλεπιδρούν μέσω των υποδοχέων του NGF συμβάλλοντας στην διαμόρφωση και διατήρηση του ΝΣ. Φυλογενετική ανάλυση της εξέλιξης των νευροτροφινών των υποδοχέων τους και του υπεύθυνου ενζύμου για τη σύνθεση της DHEA (CYP17) σε συνδυασμό με τα αποτελέσματα της παρούσας μελέτης στηρίζουν την υπόθεση ότι η DHEA πιθανόν να κατείχε το ρόλο ενός φυλογενετικά αρχέγονου νευροτροφικού παράγοντα.

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This project was both enjoyable and informative. It has enabled development of skills, which will most certainly prove invaluable in my future work. I would like to thank everyone in my surrounding who have made this project possible.

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1. Introduction

1.1 Dehydroepiandrosterone (DHEA)

DHEA is synthesized from pregnenolone, that is produced with the cleavage of cholesterol side chain by cytochrome P450scc. Pregnenolone is metabolized to DHEA by the 17α-hydroxylase / c17 and 20-lyse activity of cytochrome P450c17 enzyme. Further on DHEA can be bidirectionally converted in to each sulfated derivative DHEAS by hydroxysteroid sulfotransferase and back to DHEA with a sulphatase (Walter L. Miller 2002; H. Mellon 2001).

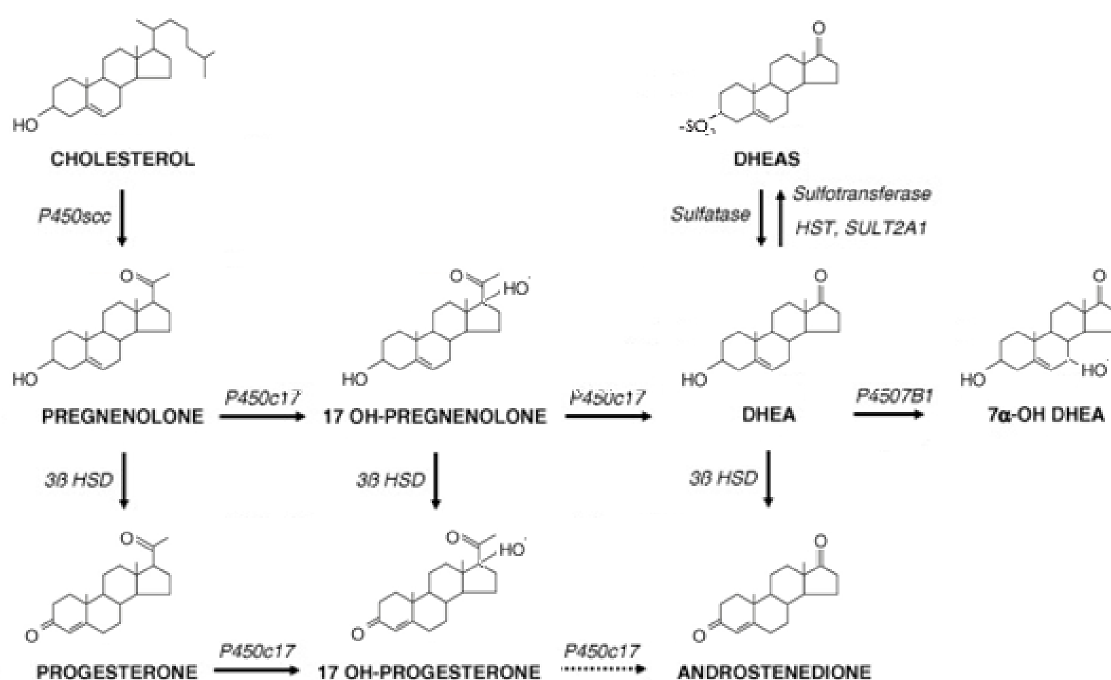


Figure 1.1 Biosynthesis and metabolism of DHEA(S). *The pathway and the enzymes involved in the biosynthesis and metabolism of DHEA from cholesterol are shown. P450scc, cholesterol side chain cleavage; 3βHSD, 3β-hydroxysteroid dehydrogenase; P450c17, 17α-hydroxylase/c17,20-lyase; P4507B1, 25-hydroxycholesterol 7-alpha-hydroxylase. The reaction indicated by the doted arrow does not occur in humans (the figure was modified from Maninger N. et al 2009).*

Dehydroepiandrosterone (DHEA) and its sulfated derivative (DHEAS) are the major neurosteroids in the brain (Baulieu EE 1997). From the time that the local synthesis of DHEA in the brain was reported (Corpechot C 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 NA 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. (Chie Kohchi et al 1998). Furthermore, P450c17 has been identified in both neurons and astrocytes in hypothalamic and cortical cultures (Zwain IH and Yen SS 1999[1]; Zwain IH and Yen SS 1999[2]) and in neurons, astrocytes and oligodendrocytes. in the spinal cord of adult rats (Cherkaouia 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 H et al 2006; Hojo Y et al 2004). The presence of P450c17 in neurons and glial cells has been also described in the brain and pituitary of frog (Jean Luc Do 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 C et al 2000).

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, 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 (for review see Charalampopoulos I 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 (N.A. Compagnone and S.H. Mellon 2000).

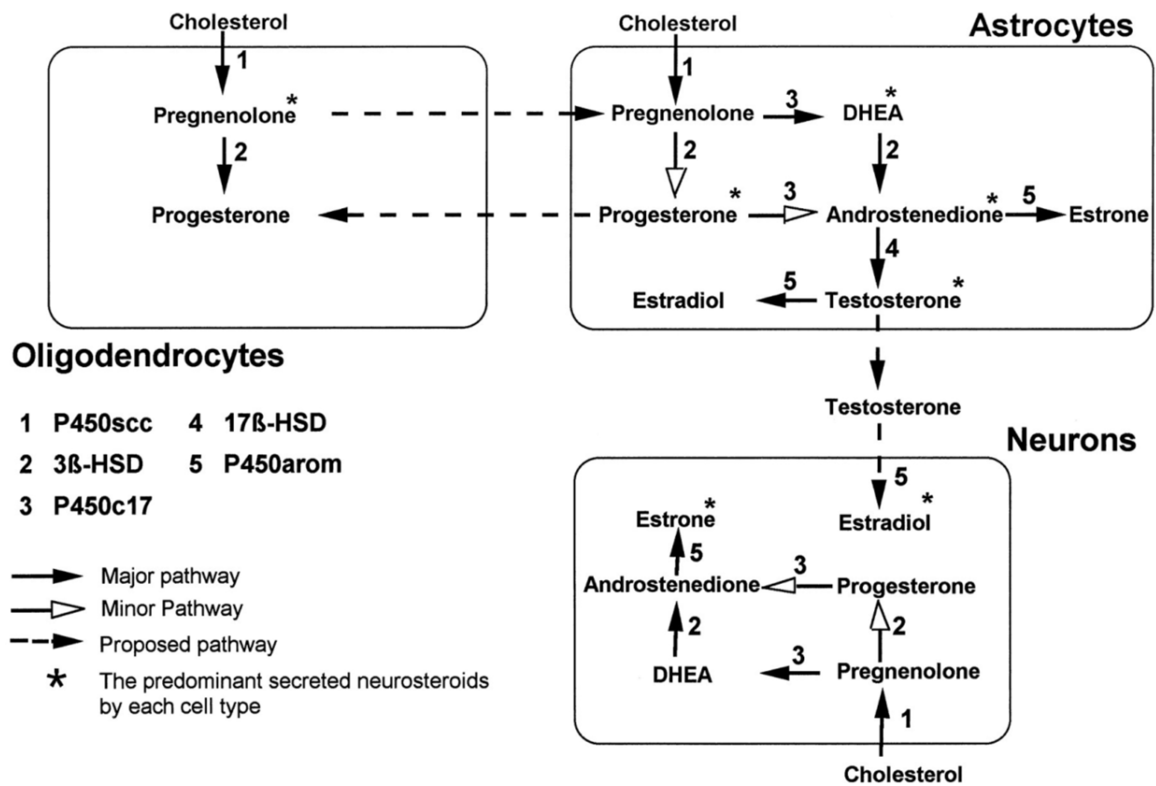


Figure 1.2 Neurosteroid biosynthesis in the brain. *Diagram shows pathways and enzymes of neurosteroidogenesis that are present in neurons astrocytes and oligodendrocytes (Ismail H. et al 1999)*

It is now well established that some of these effects of DHEA are mediated through modulation of various neurotransmitter receptors (N.A. Compagnone and S.H. Mellon 2000). Indeed, DHEA and DHEAS are important regulators of neural function and fate, through their modulatory effects on $GABA_A$ (Majewska MD et al 1990), NMDA (Johansson T et al 2005), Sigma-1 (Maurice T et al 1996), MAP2 (Laurine E et al 2003) and other neurotransmitter receptors (for review see Iván Pérez-Neri et al 2008 and N.A. Compagnone and S.H. Mellon 2000).

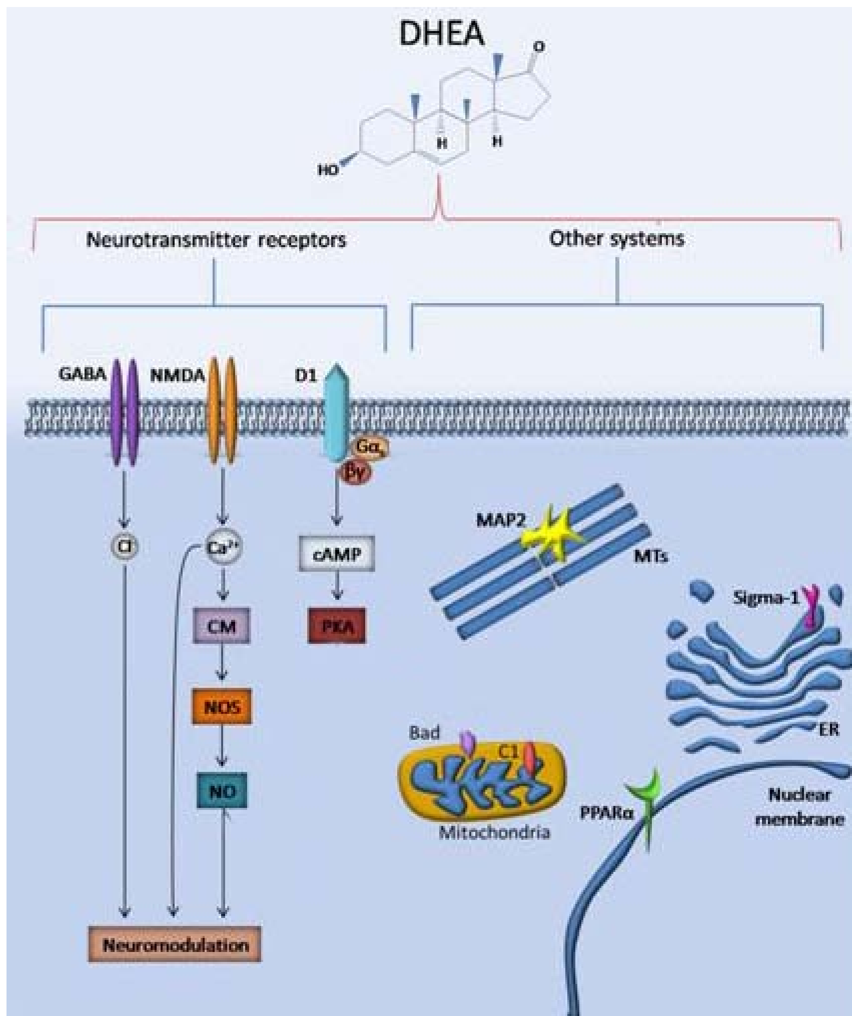


Figure 1.3 Signaling pathways involved in the neuroprotective effects of DHEA. *DHEA is a multifaceted neurohormone, affecting neural tissues with multiple mechanisms of action. DHEA and DHEAS are important regulators of neural function through their modulatory effects on* $GABA_A$*, NMDA, or Sigma-1 receptors. Additionally DHEA may affect neuronal cell fate interacting with cytoskeleton protein MAP, or with Dopamine-1receptor (D1).*

1.2 Nerve growth factor (NGF)

Nerve growth factor (NGF) is a major prosurvival molecule exerting its effects mainly in the nervous system, although it has pleiotropic effects in several other systems. NGF is a member of the neurotrophin family, which further includes Brain Derived Neurotrophin Factor (BDNF), neurotrophin 3 and 4/5 (NT3 and NT4/5) (Dechant G. and Neumann H. 2002). Neurotrophins mediate their actions by interacting with two receptor types: the first one is the tyrosine kinase (Trk) family of receptors including TrkA, TrkB

and TrkC which show high specificity for NGF, BDNF and NT4/5, and NT3, respectively and the second one is $p75^{NTR}$ that binds all the neurotrophins with lower affinity.

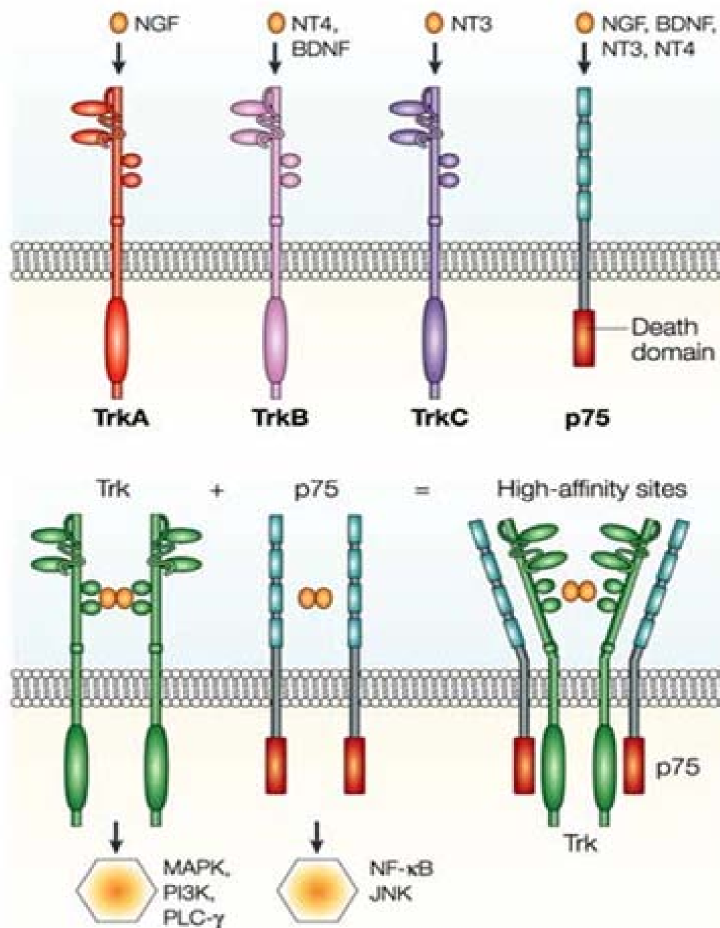


Figure 1.4 Schematic representation of neurotrophin receptors. *Upper diagram shows the specificity of each neurotrophin receptor for neurotrophins. TrkA binds NGF, TrkB binds both NT4 and BDNF, TrkC binds NT3 and* $p75^{NTR}$ *binds all the neurotrophins (NGF, NT4 BDNF and NT3). Lower diagram shows the interaction of Trk receptors with* $p75^{NTR}$ *and neurotrophins (Chao MV 2003)*

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, and lower levels were found in the cerebellum and spinal cord. In the PNS, high levels of NGF mRNA were found 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 M. et al 1986).

NGF and its receptors are synthesized and produced by 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. 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 reactivated under pathological conditions as a protective mechanism (Tsong-Hai Lee et al 1998)

1.3 Neurotrophins and neurosteroids in neurodegeneration

1.3.1 Aging: Alzheimer disease and dementia

The incidence of age related dementias, including Alzheimer disease (AD), is increasing dramatically in industrialized countries, with AD been the most common among them effecting half of the demented patients. Age related dementias are characterized by progressive loss of memory and global cognitive decline. The histological hallmarks of AD are protein aggregates that form senile plaques from the disposition of misfolded amyloid-beta (Aβ) and hyperphosphorylated Tau in neurofibrillary tangles, apoptotic loss of hippocampal and cerebral cortex neurons and neuroinflammation. The mechanisms underlying neural lose include oxidative stress, energy depletion, excitotoxicity and damage due to neuroinflammation. A number of *in vivo* and *in vitro* models resembling the behavioral and/or cellular pathology of AD have been developed by the use of either of these insults.

1.3.1.1 DHEA

During aging there is a progressive decline in the levels of circulating DHEA(S) to an extend where at age of 70 the circulating levels have reached 20% of their peak values. Furthermore reduced levels of DHEA(S) have been found in brain regions of patients with AD compared with age matched none demented controls (Weill-Engerer S et al 2002, Shumacher M et al 2003). This association between dementia and DHEA(S) concentration shows that DHEA may act as a neuroprotective factor. Recent experimental findings support this hypothesis.

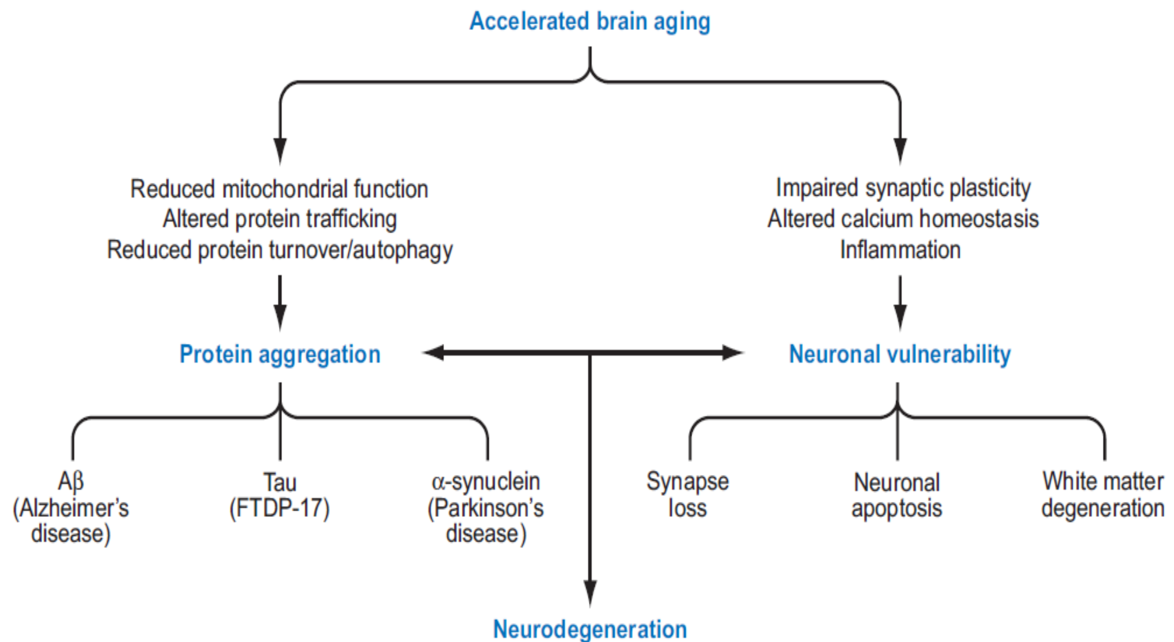


Figure 1.5 Cellular and molecular mechanisms of brain aging. *Dysregulation of molecular and cellular processes due to environmental and/or genetic factor results in protein misfolding and aggregation, reduced neuronal survival and neurodegeneration. (Bruce A. Yankner et al 2008)*

Indeed, DHEAS showed to enhance working memory in a win-shift task in aged mice using water escape motivation (Markowski M et al 2001). Augmenting effects of DHEA have been reported also in spontaneous movement, spatial learning and memory ability in a model of chronic mild stress in mice (Zhang X et al 2007). Furthermore DHEA(S) showed to enhance memory retention as it was demonstrated in foot-shock active avoidance test in mice. In this study DHEA(S) administration reversed the amnestic effects of both the inhibitor of protein synthesis anisomycin and the muscarinic cholinergic antagonist scopolamine (Flood JF et al 1988). These attenuating effects of DHEA on learning impairments produced by scopolamine were blocked by the sigma-1 receptor antagonist NE-100 (Alexandre Urani et al 1998). DHEA treatment resulted in reduction of the neurodegeneration in CA1 area of the hippocampus and in the amelioration of learning deficits due to hypoxic insult with repeated exposure to carbon monoxide here the effects were not affected by the sigma-1 antagonist NE-100 (Tangui Maurice et al). Peripheral benzodiazepine receptors are known to play a role in protecting cells from reactive oxygen species and their density is reduced during aging. DHEA

showed to reverse the reduction of peripheral benzodiazepine receptor density of mitochondria from cerebral cortex and the impairment in learning/memory ability in a model of aging with D-galactose induced neurodegeneration. (Chen C et al 2008).

There is also evidence indicating a protective role of DHEA(S) against the pathogenic effects of Abeta overproduction. DHEA dose-dependently attenuates the loss of newborn hippocampal neurons induced by Abeta (25-35)-infusion in mice and this effect is blocked by the sigma-1 receptor antagonist NE100 and is mimicked by the sigma-1 receptor agonist PRE084 (Li L. et al 2010). These protective actions of DHEA are sensitive to the PI3K inhibitor LY294002. Abeta (25-35) decreases the levels of Akt phosphorylation, in a sigma-1 receptor-dependent manner. DHEA was shown to reverse the Abeta (25-35)-induced decrease of the dendritic density and length of doublecortin positive cells in the dentate gyrus. It appears that DHEA prevents the Abeta (25-35)-impaired survival and dendritic growth of newborn hippocampal neurons through a sigma-1 receptor-mediated modulation of PI3K kinase signaling. DHEA may also prevent Abeta neurotoxicity, mediated by elevated levels of Ca(2+). Abeta forms Ca(2+)-permeable pores on membranes of rat hippocampal neurons, causing a marked increase in intracellular calcium level, and neuronal death. Pre-administration of DHEA or DHEAS significantly inhibit the increase of intracellular calcium levels, induced by Abeta. These findings suggest that supplementation of reduced levels in the elderly may be effective in preventing Abeta neurotoxicity (Kato-Negishi M. and Kawahara, M. 2008). Oligodendrocytes and astrocytes may produce DHEA via a P450c17 alternative, Fe^{2+} dependent pathway. Exposure of oligodendrocytes to beta-amyloid increases DHEA formation (Brown R.C. et al 2000), suggesting that DHEA synthesis can be regulated by neurotoxic beta-amyloid, possibly as a reactive rescue response to the beta-amyloid challenge. Furthermore, DHEA treatment decreased the levels of lipid proxidation products in cerebral cortex, hippocampus, cerebellum and brain stem, in both 12 and 22 months old rats, with the decrease been higher in the older group (Sinha N et al 2005).

Neuroprotective effects of DHEA(S) have been also demonstrated by *in vitro* studies. DHEAS supplementation of rat cultured cerebellar granule cells reversed the levels of apoptosis that was induced due to oxygen-glucose deprivation. The neuroprotective effects of DHEAS were blocked with the addition of pentobarbital, a $GABA_A$ agonist

(Kaasik A et al 2001). Supplementation of cultured hippocampal neurons with DHEA(S) protected them also from NMDA, AMPA and Kainic acid neurotoxicity. Furthermore pretreatment with DHEA was more effective than posttreatment and DHEA was protective at 10 times lower concentration than DHEAS. In the same study they also demonstrated that *in vivo* treatment with DHEA protected hippocampal CA1/2 neurons from NMDA infusion (Kimonides V.G. *et al* 1998). Furthermore, DHEA showed to attenuate the translocation of stress-activated protein kinase 3 to the nucleus in primary culture of hippocampal neurons (Kimonides V.G. et al 1999). In addition, DHEA treatment significantly increased the dose of NMDA necessary to induce seizures in mice (Budziszewska B et al 1998). Pretreatment of the mouse hippocampal cell line HT-22 with DHEA for 24h completely reversed the effects of glutamate treatment on the translocation of glucocorticoid receptor (GR) in the nucleolus (Cardounel A et al 1999) and protected from oxidative stress induced by H_20_2 and sodium nitroprusside (Bastianetto S et al 1998). DHEA treatment reversed the NMDA induced suppression of the PI3K/Akt pathway in a P19-N neuronal culture (Xilouri M and Papazafiri P 2008). Moreover in a human neuroblastoma cell line (SH-SY5Y), DHEA and DHEAS (at lower concentration than DHEA) inhibited the staurosporine-induced toxicity by reducing the activity of Caspase-3 and apoptotic cell loss and increasing mitochondrial membrane potential. The increased viability produced by DHEA and DHEAS on staurosporine treated cells was abolished by inhibitors of PI3K (wortmannin) and ERK/MAPK (PD98059) (Leskiewicz M. et al 2008). Finally, DHEAS potentiated the NGF induced neurite outgrowth and these effects were blocked by the selective sigma-1 antagonist NE-100 (Nishimura T et al 2008)

In addition to the beneficial effects of DHEA(S) in memory due to its neuroprotective efficacy, there is also evidence for neuromodulatory actions playing a role in the physiological process of memory. Chronic treatment with DHEAS resulted in lowering the threshold pulse number required for induction of activity- dependent long-term potentiation (LTP) in the hippocampal Schaffer collateral CA1 synapse of rats. These effects were abolished with co-administration of the sigma-1 antagonist NE100, although, acute administration did not effect the induction of DHEAS facilitated LTP, showing that sigma-1 plays a role in the chronic effects but not in the induction of LTP facilitation by DHEAS (Chen L. et al 2006). Furthermore, sub-threshold high frequency stimulation in

the presence of DHEAS triggered the phosphorylation of Src and ERK2 and administration of the Src family kinase inhibitor PP2 resulted in oblation of the DHEAS facilitating effects, showing that the Src and ERK2 activation is required for the induction of LTP facilitating effects of DHEAS (Chen L. et al 2006; Kaminska M. et al 2000). In addition, injection of DHEA in gonadectomized male and female rats resulted in a large increase (106%) of the hippocampal CA1 spine synapse. (Hajszan T *et al* 2004; MacLusky N.J. et al 2004). DHEAS increased the number of NMDA receptors in the CA1, CA3 and dentate gyrus of hippocampus and in the layer IV of the cortex (Wen S. et al 2001). In a study where the effects of DHEAS on synaptic transmission was studied in a hippocampal slice preparation, it was shown that DHEAS enhanced the synaptic evoked population spike in the somatic region of CA1 neurons without effecting the dentritic synaptic potential. This augmentation of the somatic population spike was shown to be mediated by antagonizing effects of DHEAS on $GABA_A$ receptor-mediated inhibition (Meyer J.H. et al 1999; Meyer JH and Gruol DL. 1994). Moreover it has been shown that DHEA(S) inhibit GABA-stimulated chloride uptake (Imamura M and Prasad C 1998). DHEA potentiated the excitatory response of NMDA and this effect was blocked by the sigma-1 antagonist NE-100 (Debonnel G. et al 1996; Bergeron R. et al 1996). DHEAS administration reduced the paired pulse inhibition (GABA-mediated recurrent inhibition) in the dentate gyrus and CA1 area of the hippocampus. These effects were mimicked by the muscarinic M2 agonist *cis*-dioxolane and were blocked by the muscarinic M2 receptor antagonist gallamine (Steffensen S.C. et al 2006). Additionally, it has been shown that DHEA can induce increased neurogenesis in dentate gyrus of the hippocampus. Treatment with the precursor, pregnenolone, or the metabolite androstenediol, of DHEA had no effect in neurogenesis and DHEA protected the existing neurons antagonizing the effects of corticosterone (Karishma K.K. and Herbert J. 2002).

Finally, DHEA may also play a role in the development and connectivity of hippocampus. Neonatal administration of DHEA resulted in increased immunoreactivity of the neuronal dentritic marker microtubule-associated protein 2 (MAP2) in the hippocampus and nucleus accumbens (Iwata M. et al 2005) and synapsin I and NPY in the hippocampus of post-puberty rats. Increased numbers and processes of astrocytes were found in the hippocampus in both pre- and post-puberty rats as it was demonstrated by the immunoreactivity of glial fibrilary acidic protein (GFAP) (Shirayama Y. et al

2005). The same group has also shown the involvement of DHEA in the formation of synaptic inputs into the striatum as it was demonstrated by the increase in the amount of synapsin I and in the development of DA and NPY systems in the nigrostriatal system. (Muneoka K. et al 2009).

1.3.1.2 NGF

A large body of work has shown the implication and importance of NGF in Alzheimer's disease. The levels of NGF have been shown to be reduced in both animal models and in human patients of Alzheimer's disease and furthermore, reduction of the levels of NGF experimentally, in animal models, induces deficits that resemble both the cellular and the clinical hallmarks of the disease (Calissano P. et al 2010). NGF deprivation causes amyloid-beta (Aβ) production and apoptotic cell death in both differentiated PC12 cells (Matrone C. et al 2008) and hippocampal neurons in primary cultures (Matrone C. et al 2008). Furthermore, in these studies addition of an antibody against Aβ resulted in the ablation 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 reduction of NGF, the levels of the precursor of NGF (proNGF) are increased in AD patients. The importance of this dysregulation of the levels of NGF and proNGF in the pathology of AD, have been demonstrated by the development of the AD11 transgenic mice that produces neutralizing antibodies against NGF resulting in an unbalance of the NGF/proNGF ration that produces a very similar pathology with AD (Ruberti F. et al 2000, Capsoni S. et al 2010) .

Moreover, a large body of evidence demonstrates the importance of NGF as a therapeutic tool for AD. Implantation of NGF-loaded microspheres in the basal forebrain of rats with fimbria-fornix lesion significantly improved the spatial learning and memory by promoting survival of axotomized cholinergic neurons in the medial septum and ventral diagonal branch (Gu H. et al 2009). Furthermore, 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 Alzheimer disease (Ernfors P. et al 1989). NGF overexpressing

fibroblasts implantation also rescued basal forebrain and medial septum cholinergic neurons in a model of unilateral fimbria-fornix-lesioned rats (Strömberg I. et al 1990) Genetically modified NGF overexpressing hamster kidney cells transplanted into the lateral ventricle of monkeys after unilateral transection of the fornix rescued cholinergic neurons within the septum and ventral limb of the diagonal band (Emerich D.F. et al 1994, Kordower J.H. et al 1994). Injection of NGF overexpressing monocytes in organotypic brain slices protected cholinergic neurons of the basal forebrain from NMDA-induced neurotoxicity (Zassler B. and Humpel C. 2006).

In addition, NGF implementation by intraseptal infusion of NGF enhanced cholinergic neuronal markers and the induction of long term potentiation (LTP) in the hippocampus, where's decrease of endogenous NGF levels by intraseptal infusion of anti-NGF neutralizing antibodies, resulted in reduced LTP and spatial memory (Conner J.M. et al 2009). NGF administration in a model of basal forebrain cholinergic neurons lesion with specific neurotoxin for cholinergic neurons (AF64A) reversed the memory deficits as measured by both passive avoidance test and water maze and redused the apoptotic loss of basal forebrain cholinergic neurons (Fernández C.I. et al 1996). Intranasal administration of NGF rescued cholinergic neurons of basal forebrain and improved performance in memory and object recognition tests in the AD11 anti-NGF mouse model of Alzheimer disease (De Rosa R. et al 2005, Capsoni S. et al 2002) Furthermore, NGF treatment increased the peripheral benzodiazepine receptor levels in PC12 cells (Miller L.G. et al 1988). Finally NGF induces neurite outgrowth of hippocampal neurons in culture by increasing ceramide formation through p75^{NTR} activation (Brann A.B. et al 1999).

1.3.2 Parkinson disease

Parkinson's disease (PD) is a very common neurodegenerative disorder 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. This distraction leads to the clinical hallmarks of PD such as tremor, bradykinesia and posture instability (for review see Lewis S.J. and Barker R.A. 2009).

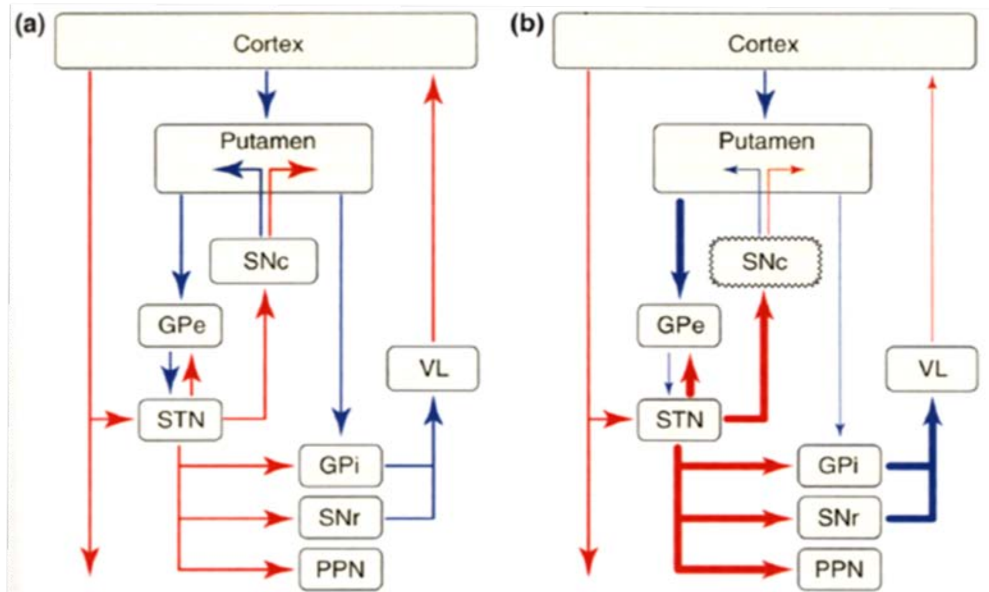


Figure 1.6 Schematic representation of the dysregulation of the basal ganglia in Parkinson disease. *(a) Physiologic processes, (b) dysregulation in Parkinson. Red arrows indicate excitatory and blue inhibitory pathways. Because of the differential effects of dopamine in the putamen through D2 (inhibition) and D1 (excitation) receptors, the reduction of its levels due to apoptotic loss of dopaminergic neurons in the SNc results in the augmentation (disinhibition) of the indirect pathway (cortex→ putamen→ GPe→ SNT→ GPi and SNr) and in the inhibition of the direct pathway (cortex→ putamen→ GPi and SNr). This dysregulation leads in overexcitation of SNT, GPi and SNr neurons resulting in over-inhibition of thalamic-cortical and brainstem motor centers producing the clinical hallmarks of PD. Abbreviations: pedunculopontine nuclei (PPN), substantia nigra pars compacta (SNc), ventralis lateralis (VL), globus pallidus pars intema (GPi), substantia nigra pars reticularis (SNr), globus pallidus pars externa (GPe), subthalamic nucleus (STN). (José A. Obesoa, et al 2000)*

1.3.2.1 DHEA

A chemical compound, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) resembles the pathology of PD when administered to experimental animals. Systemic administration of MPTP leads to severe apoptotic damage in the nigrostriatal

dopaminergic system with similar behavioral consequences as in PD (Tatton et al 1997, Kuhn et al 2003, Schmidt and Ferger 2001). DHEA treatment of MPTP mice resulted in reversing the levels of dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) to comparable levels with the intact animals. Furthermore, DHEA prevented the decline of the mRNA levels of dopamine transporter and tyrosine hydroxylase that caused from MPTP toxicity (D'Astous M. et al 2003). In MPTP monkeys DHEA improved the mean clinical score and potentiated locomotor activity in both moderately and severely impaired subjects (B´elanger N. et al 2006) Administration of DHEA together with MPP^+ (the effectors metabolite of MPTP) in the striatum of rats resulted in reduced loss of DA concentration and tyrosine hydroxylase and acetylcholinesterase positive fibers density in the striatum (Tomas-Camardiel M. et al 2002). Moreover, DHEA has been shown to induced the formation of synaptic inputs into the striatum, increasing the expression of synapsin-I and dopamine production in the nigrostriatal system (Muneoka K. et al 2009).

These findings were corroborated by *in vitro* data, showing that DHEA and DHEAS increased acutely (peak effect between 10–30 min) and dose dependently (EC_{50} in the nanomolar range) the release of catecholamines from dopaminergic PC12 cells (Charalampopoulos I. et al 2005). It appears that the acute effect of these steroids involves actin depolymerization and submembrane actin filament disassembly, a fast-response cellular system regulating trafficking of catecholamine vesicles. DHEAS but not DHEA also affected catecholamine synthesis, increasing both the mRNA and protein levels of tyrosine hydroxylase. Furthermore, DHEAS was also neuroprotective with reduced apoptosis in cultured cerebellar granule cells against MPP^+. (Kaasik A. et al 2001).

DHEAS may also affect brain areas associated with non-motor behavioral manifestations of PD, like the prelimbic cortex. Recent experimental findings have shown that DHEAS enhances the excitatory postsynaptic currents in both the prelimbic cortex and the hippocampus. In this study it was shown that glutamate release due to DHEAS, although, in the hippocampus it was completely blocked with sigma-1 antagonists (BMY14802), in the prelimbic cortex there was partial blockage. In the prelimbic cortex the effects were completely blocked with D1 receptor antagonist (SCH23390), adenylyl

cyclase inhibitor (MDL-12330A) and protein kinase A (PKA) inhibitor (H89). Furthermore, the activating effect of DHEA on PKA in the prelimbic cortex was completely blocked by the D1 antagonist showing that the glutamate releasing effects of DHEAS are mediated through D1 receptor in this area (Dong L.Y. et al 2006).

1.3.2.2 NGF

Both NGF and TrkA are expressed by a large number of dopaminergic neurons in the substantia nigra in humans (Nishio T. et al 1998). Reduced levels of NGF were found in the serum of both hemiparkinsonian rats in MPTP-treated monkeys and in human parkinsonian patients (Lorigados L. et al 1996). Moreover, increased levels of pro-NGF are produced after 6-OHDA which induces apoptotic loss of dopaminergic neurons through its interaction with $p75^{NTR}$/sortilin co-receptor system (Wang T. et al 2010). Reactive astrocytes in the striatum of MPTP treated mice produce NGF, NT3 and GDNF (Chen L.W. et al 2006).

A number of studies have demonstrated the therapeutic potential of NGF in PD. 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 E. et al 1992). NGF administration in parallel with ventral mesencephalic cells (VMC) transplantation in the striatum had a beneficial effect for both the transplanted VMC cells and substantia nigra dopaminergic neurons in 6-OHDA mice. In particular, NGF enhanced survival and neurite outgrowth of grafted TH positive VMC cells, increased the number of dopaminergic neurons in the substantia nigra and restored the levels of D-amphetamine induced rotation, nigrostriatal content of dopamine and 3,4-dihidroxy-phenyl acetic acid (DOPAC) and spontaneous locomotor activity (Chaturvedi R.K. et al 2006). Systemic administration of NGF protected striatal dopaminergic neurons from MPP^{+} neurotoxicity in neonatal rats (Kirschner P.B. et al 1996). Furthermore, NGF had a protective effect on cultured mesencephalic neurons against rotenone- or colchicines-induced neurotoxicity by preventing microtubule depolymerization and death of TH positive neurons through MEK (microtubule-associated protein kinase) (Jiang Q. et al 2006).

1.3.3 Multiple sclerosis

Multiple sclerosis (MS) is an important autoimmune disease of the central nervous system. The component responsible for the initiation and progression of the disease is adaptive immunity and the breakdown of tolerance, with autoreactive T cells and autoantibodies against protein and lipid components of myelin sheath. This attack results in degeneration of oligodendrocytes that produce the myelin and the underline axons leading to degeneration of neural cell bodies in the gray matter, that as the disease progresses results in the significant cerebral atrophy that characterize MS.

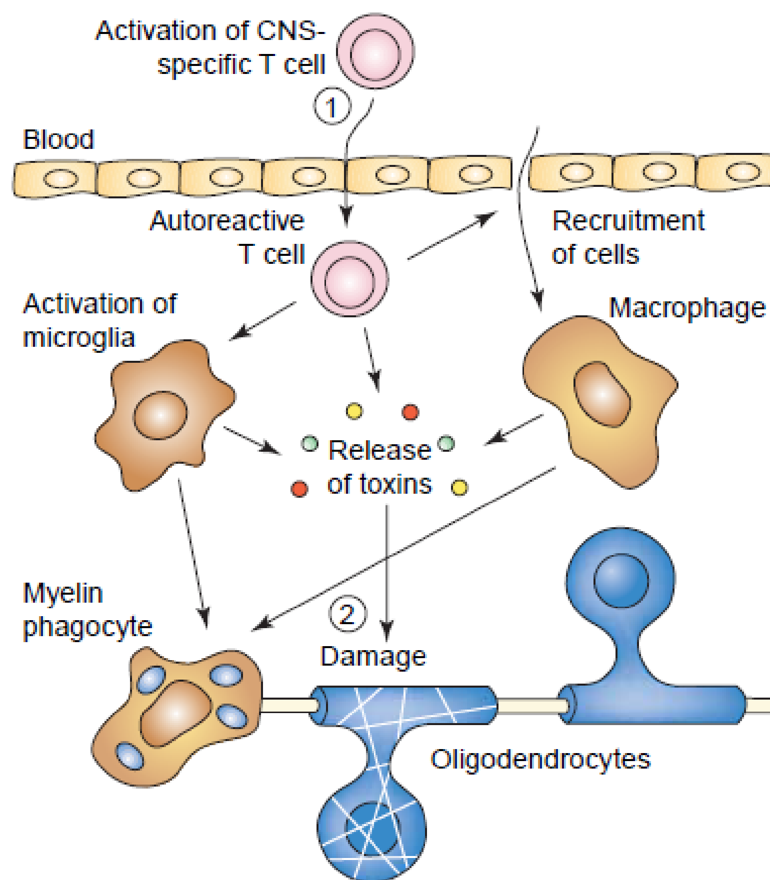


Figure 1.7 Schematic representation of the mechanism of lesion formation in MS. *Autoreactive T cells infiltrate in the CNS attaching myelin and initiating inflammation. This process includes the recruitment of peripheral macrophages and the activation of resident microglia, the release of toxins that all together lids in myelin distraction and neuronal dysfunction. (Carlos Matute and Fernando Pe´rez-Cerda 2005)*

1.3.3.1 DHEA

In human clinical studies low serum levels of DHEA(S) have been associated with severity of fatigue, one of the most limiting symptoms in (MS) (Téllez N. et al 2005). Furthermore a large number of studies have demonstrated immunoregulatory actions of DHEA(S) (for review see Regelson W. et al 1994). Administration of DHEA in a model of experimental autoimmune neuritis (EAN) resulted in a significant delay in the onset of disease with decreased infiltration of inflammatory cells in the PNS. Furthermore there was significant decrease in the numbers of IFN-gamma, TNF-alpha expressing cells in the PNS, and reduction of the secreted levels of IFN-gamma and TNF-alpha and proliferation of autoreactive T cells in the spleen (Tan X.D. et al 2009). DHEA treatment significantly reduced the incidence and severity of Experimental Autoimmune Encephalomyelitis (EAE) and reduced the Th1-mediated response in mice. Furthermore in the same study it was shown that supplementation of myelin basic protein (MBP)-primed splenocytes with DHEA significantly decreased the activation and proliferation of T lymphocytes as well as NF-κB translocation, secretion of nitric oxide (NO) and proinflammatory cytokines such as TNF-alpha, IFN-gamma and interleukin 12 (IL12) (Du C. et al 2001). The synthetic fluorinated DHEA analog fluasterone significantly delayed the onset, reduced the peak clinical score and cumulative disease index of EAE mice, and prevented or significantly attenuated relapses (Offner H. et al 2002). Moreover, T cells from treated EAE mice had significantly reduced proteolipid protein139–151-specific T cell proliferation responses and reduced numbers of TNF-alpha- and IFN-gamma-producing cells in the CNS. This DHEA analog, which has been reported to have weak androgenic or estrogenic side effects, appears to have a potent inhibitory activity in EAE.

In vitro studies in cell cultures also showed that DHEA supplementation resulted in a decrease of the protein and mRNA levels of nitric oxide synthase (iNOS) from LPS stimulated BV2, a microglial cell line (Wang M.J. et al 2001), and reduction of the TNF production in LPS stimulated astrocytes, microglia and peripheral monocytes (THP-1) (Di Santo E. et al 1996). Furthermore, DHEA(S) reduced the proliferation of astrocytes after stimulation with MBP as it was demonstrated by 3H-thymidine incorporation (Muntwyler R. and Bologa L. 1989).

1.3.3.2 NGF

The immunoregulatory role of NGF and its possible implication and importance in autoimmune disease have been demonstrated by a large number of studies (Aloe L. et al 1994). In fact, NGF and its receptors are expressed in many lymphoid organs by both structural and immune cells (Labouyrie E. et al 1997). Cultured monocytes/macrophages purified from healthy human peripheral blood mononuclear cells (PBMC) express NGF and its receptors TrkA and $p75^{NTR}$. The levels of NGF and TrkA, but not of $p75^{NTR}$, were enhanced after lipopolysaccharide (LPS) stimulation. Furthermore, blocking NGF with neutralizing antibodies resulted in an up-regulation of the levels of $p75^{NTR}$ and eventually in the apoptotic death of cultured monocytes/macrophages (Caroleo M.C. et al 2001). Microglia also secretes NGF after LPS stimulation (Mallat M. et al 1989).

The implication of NGF in autoimmune demyelination has also been demonstrated (Linker R. et al 2009). Astrocytes secrete NGF in an antigen recognition dependent manner after their cell-cell encounter with MBP-specific Th1 and Th2 cells. These effects were shown to be mediated by the synapse between MHC-II of the astrocytes and the T Cell Receptor (TCR) of T cells rather than from secreted cytokines. Neutralizing antibodies for IFN-γ, IL4 and IL10 were unable to block these effects; in contrast obstruction of cell-cell contact with antibodies against MHC-II was able to significantly diminish them (Barthel C. et al 2009). TrkA and $p75^{NTR}$ was found on perivascular cells and a subset of inflammatory cells within the lesions in multiple sclerosis. Furthermore, high levels of $p75^{NTR}$ were found in reactive astrocytes in the borders of the lesion and on microglia within the lessoned area. In this study $p75^{NTR}$ was not expressed in most of mature or oligodendrocyte precursors (Valdo P. et al 2002). NGF mRNA and protein levels are up-regulated in astrocytes and oligodendrocytes of the white mater in the brain of EAE rats (Micera A. et al 1998). $p75^{NTR}$ protein is also up-regulated in the endothelial and perivascular cells (Nataf S. et al 1998) and the levels of both TrkA and $p75^{NTR}$ were upregulated in radial glia of the white and gray matter in EAE rats (Oderfeld-Nowak B. et al 2001).

Additionally, an increasing body of evidence highlights the possible therapeutic

potential of NGF in autoimmune disease. Intracerebroventricular administration of NGF in EAE rats results in an up-regulation in the expression levels of MHC-I, IFN-γ and IRF-1 (interferon regulatory factor 1) in neurons, and adversely in a down-regulation of MHC-II in glial cells (Stampachiacchiere B. and Aloe L. 2005). These effects result in delaying the onset and the progression of EAE in C-jacchus marmosets by reducing IFNγ secretion from T cells and inducing the levels of IL10 from glia cells (Villoslada P. et al 2000). More importantly, it was demonstrated that NGF-overexpressing MBP-T cells (myelin basic protein specific T cells) not only didn't induce EAE when transferred into mice, but were able to suppress EAE development when transferred together with non NGF transdused MBP-T cells (Flügel A. et al 2001, Kramer R. et al 1995). In contrast NGF-overexpressing OVA-T cells (ovalbumin specific T cells) were unable to suppress EAE induction. The inhibition of EAE was associated with a general suppression of the inflammatory response in the CNS with reduced numbers of infiltrating monocytes and macrophages (Flügel A. et al 2001). In addition, NGF neutralizing antibody overexpression peripherally in rats exacerbated the severity of EAE (Micera A. et al 2000).

There are evidences that NGF may farther protect by inducing oligodendrocyte regeneration and remyelination (Althaus H.H. et al 2008). Intracerebroventricular injection of NGF further increased the number of proliferating cells (BrdU incorporation) in the subventricular zone (SVZ) and olfactory bulb (OB) of EAE rats. The expression of TrkA was increased in the SVZ of EAE rats compared to the control animals, these levels were unaffected by the administration of NGF instead they were increased by administration of neutralizing antibodies against NGF. Furthermore, co-localization of both TrkA and NGF was shown in radial glial cells associated with migrating oligodendrocytes (Triaca V. et al 2005).

1.3.4 Ischemia and brain trauma

Damage of neural tissue from ischemia or trauma results in a secondary propagation of the lesion with degeneration of the surrounding area and distal neuronal axons. This secondary phase is driven by mechanisms such as blood brain barrier disruption, edema

with infiltrating immune cells and neuroinflammation, degeneration of myelin, excitotoxicity and oxidative stress (Greve M.W and Zink B.J. 2009). The therapeutic strategy for ischemic and traumatic brain injury involves amelioration of these secondary events, reducing sequential injure, together with enhancement of regenerating mechanisms in order to promote tissue rebuilt.

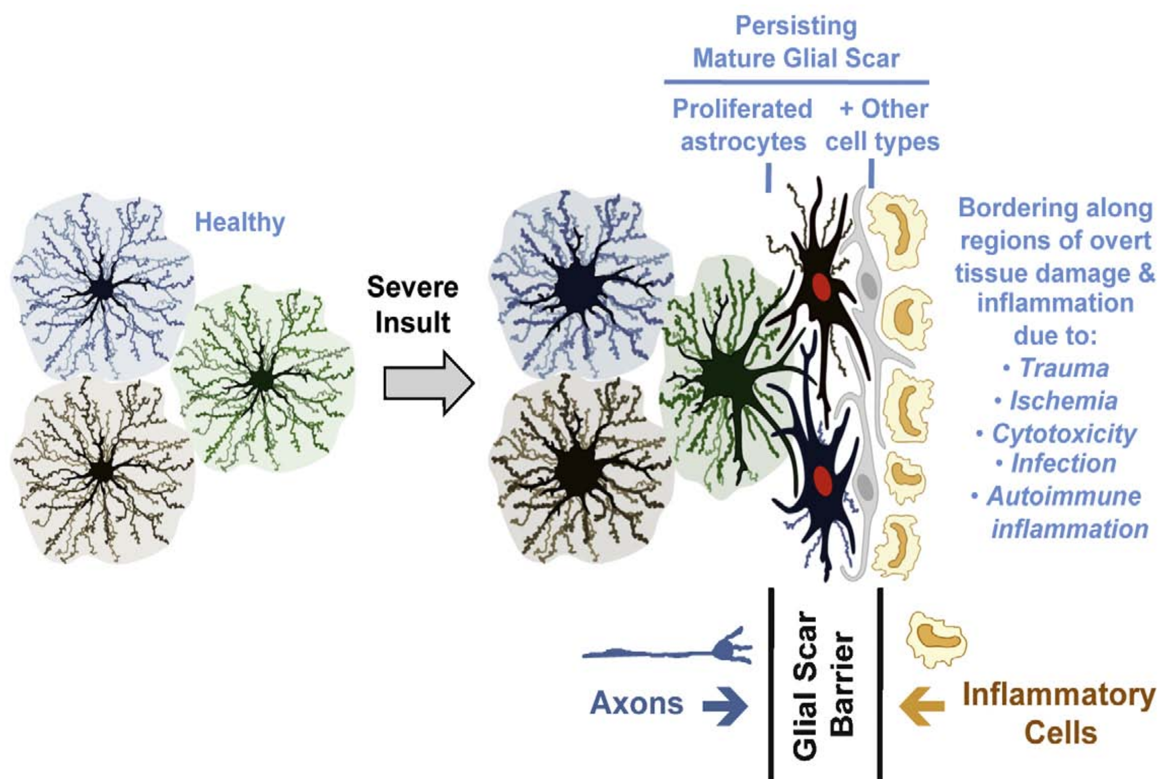


Figure 1.8 Schematic representation of gliotic scar formation after brain injury. *Astrogliosis is formed by activated astrocytes that migrate in the borders of the insulted area forming a physical barrier between the damaged, inflamed area and healthy tissue. This astrogliotic barriers persists for long periods protecting healthy unaffected tissue from the ongoing inflammation that occurs within the damaged areas (Michael V. Sofroniew 2009).*

1.3.4.1 DHEA

There is now strong experimental evidence that DHEA exerts neuroprotective effects in a number of animal models of brain ischemia. In a model of transient global cerebral ischemia in rats a single administration of DHEA during 3 to 48 h after ischemia

induction resulted in decreasing levels of neuronal death in the hippocampal CA1 and improvement of spatial learning performance. In contrast, administration 1 h before or after the induction of ischemia resulted in exacerbation of both the neuronal death and the spatial learning performance. This exacerbation was postulated that was due to activation of NMDA and sigma-1 receptors by DHEA because administration of an NMDA blocker or an antagonist of sigma-1 receptor prevented these effects (Zhen L. et al 2009). On the other hand, DHEAS treatment decreased the probability of permanent paraplegia in a model of reversible spinal cord ischemia in rabbits. These neuroprotective effects were abolished with the administration of $GABA_A$ agonist bicuculline (Lapchak P.A. et al 2000)

In the model of transient forebrain ischemia administration, DHEAS reversed the impairment of long term potentiating (LTP) in CA1 produced by ischemia. Sigma-1 antagonist NE100 completely abolished the effects of DHEAS (Zhen L. et al 2009). Furthermore, DHEAS prevented the biochemical changes in the ischemic injury of rat retina (Bucolo C. and Drago F. 2004) and decreased the threshold shift of the compound action potential that was produced after transient cochlear ischemia. (Tabuchi K. et al 2003). Finally, in a model of ischemia with bilateral carotid artery occlusion in both healthy and diabetic rats, DHEA restored the reactive oxygen species, the lactate dehydrogenase release and the activity of Na/K-ATPase (Aragno M. et al 2000)**.** The above *in vivo* studies were further supported by *in vitro* data showing that DHEAS supplementation of rat cerebellar granule cell cultures decreases the levels of apoptosis, induced by oxygen-glucose deprivation (Kaasik A. et al 2001), and this effect is blocked by pentobarbital, a $GABA_A$ receptor agonist. DHEAS inhibits persistent sodium currents, as measured with whole-cell patch clamp in rat medial prefrontal cortex slices, showing that may protect neurons under ischemia conditions. These effects were abolished with the addition of inhibitors for Gi protein and protein kinase C and with a sigma-1 blocker. Furthermore these effects were mimicked by sigma-1 agonists (Cheng Z.X. et al 2008).

DHEA and DHEAS were proven effective in protecting against brain trauma. In the model of focal cortical cold lesion, DHEAS significantly decreased the area of the lesion when administered either before or after the induction of the injury (Juhasz-Vedres G. et al 2006). DHEA improved performance in the passive avoidance test and in the forced

swimming test in mild traumatic brain injury, reversing the cognitive and behavioral damages induced by brain trauma (Milman A. et al 2008). DHEAS facilitated the recovery of the cortices in the surrounding area of focal cold brain injury and enhanced the amplitude of the cortical evoked responses (Lür G. et al 2006). Furthermore, DHEA facilitated the recovery of motor control and coordination after spinal cord injury in mice, increasing white matter and reducing gliosis in the surrounding area of the lesion (Fiore, C., Inman et al 2004). DHEA was equally effective even 7 days after traumatic brain injury, improving the performance in both sensory-motor and cognitive tasks (Hoffman, S.W. et al 2003). DHEA minimized the damage produced after transection of the sciatic nerve, by increasing the number and the diameter of myelinated axons and reducing atrophy of the gastrocnemius muscle (Ayhan S. et al 2003). DHEA subepineurial treatment of rats after transaction of the sciatic nerve resulted in increased number of myelinated axons, larger average fiber diameter and reduced atrophy of the gastrocnemius muscle (Ayhan S. et al 2003). Additionally, DHEA treatment in a model of penetrating brain trauma resulted in a significant decrease of gliotic tissue formation. In this study DHEA showed to decrease the accumulation of astrocytes near the wounded area and in decreased proliferation of reactive astrocytes (García-Estrada J. et al 1999). DHEA inhibited the activation of astrocytes due to increased levels of potassium in hippocampal slice cultures (Del Cerro S. et al 1996). The fluorinated DHEA analog fluasterone facilitated functional recovery of traumatic brain injury in rats, improving beam walk performance, neurological reflexes and declarative memory (Malik A. et al 2003). Both DHEA and its fluorinated analog inhibited IL-1beta-induced cyclooxygenase-2 (COX2) mRNA and prostaglandin (PGE2) production in cultured mesangial cells. Finally, both DHEA and its analog relaxed middle cerebral artery in ex vivo preparation (Malik A.S. et al 2003)

1.3.4.2 NGF

NGF mRNA is upregulated in microglia, astrocytes neurons and Schwann cells in the injured spinal cord (Brown A. et al 2004). In hippocampus the concentration of NGF was significantly reduced in CA3 of the dentate gyrus (DG) and the septum, after 2 and 7 days respectively, after forebrain ischemia. The levels of NGF in the DG areas were recovered 30 days after ischemia (Shozuhara H. et al 1992). In the CA1 area there was a massive

increase (50%) in the expression levels of NGF 2 weeks after ischemia (Hashimoto Y. et al 1992). Furthermore, there was an increase in the infiltrating astrocytes in the hippocampus suggesting their participation in the increase of NGF levels (Shozuhara H. et al 1992). This idea is farther supported by *in vitro* data showing that treatment of cultured cortical astrocytes with hydrogen peroxide (H_2O_2) increased the production of NGF and basic fibroblast growth factor (bFGF) (Pechan P.A. et al 1992). In contrast with the levels of NGF, there was a massive increase in the expression levels of $p75^{NTR}$ in the CA1, CA3 and DG as early as 1h after transient forebrain ischemia. These high levels of $p75^{NTR}$ were present in the remaining neurons of CA1 and in reactive astrocytes 7 days after transient forebrain ischemia but not in the other hippocampal areas (Lee T.H. et al 1995). The levels of the high affinity NGF receptor TrkA were reduced in CA1 degenerating pyramidal neurons after global cerebral ischemia. In contrast the levels of both of the NGF receptors' $p75^{NTR}$ and TrkA were strongly upregulated on reactive astrocytes in the same area (Oderfeld-Nowak B. et al 2003). The levels of NGF were also increased in the cortex after unilateral cortical infraction. Furthermore, there was an 8 fold increase in the levels of NGF after intraserebroventricular administration of acidic fibroblast growth factor (aFGF) (Figueiredo B.C. et al 1995). Expression of NGF was shown to be upregulated in the DG 4h after cerebral ischemia and as early as 1min after hippocampal coma induced buy administration of 2 units of insulin per kg in Wistar rats (Lindvall O. et al 1992).

The protective role of NGF in neuronal injury is well documented for many years, promoting neuronal survival, neurite regrowth and neural regeneration (Lykissas M.G. et al 2007). Intraventricular administration of NGF rescued pyramidal neurons of the CA1 area of the hippocampus from delayed neuronal death (DND) due to transient global cerebral ischemia in Mongolian gerbil (Shigeno T. et al 1991, Tanaka K. et al 1994). Intracerebroventricular administration of NGF prevented the neuronal atrophy in the cholinergic pathway of nucleus basalis magnocellularis (NBM) after infarction of the frontoparietal cortical area in the rat (Cuello A.C. et al 1992) and ameliorated the damage in both the striatum and the cortex in a neonatal model of hypoxia-ischemia in 7 days old rats (Holtzman D.M. et al 1996).

Implantation of genetically modified fibroblasts that produce high levels of NGF in the CA1 and CA2 areas of the hippocampus 7 days prior transient cerebral ischemia resulted in increased viability of the pyramidal neurons in this areas, compared with the contralateral non implanted hemisphere (in rats) (Pechan P.A. et al 1995). In a similar study, implantation of immortalized genetically modified neural stem cells, which produced high levels of NGF, resulted in a large reduction of the apoptotic loss of both projection neurons and the total number of neurons in the striatum (Andsberg G. et al 1998). Transplanted NGF overexpressing monocytes either *in vivo* in the lateral ventricle or *in vitro* into organotypic brain slices of the basal nucleus of Meynert (nBM) protected cholinergic neurons from NMDA-induced neurotoxicity (Zassler B. and Humpel C. 2006) NGF-expressing human NT2N neurons ameliorated the cognitive deficits (improving learning ability in Morris water maze) that are present after traumatic brain injury (TBI) when transplanted 24h after the insult in to the medial septum of mice (Longhi L. et al 2004).

1.4 Aims and objectives

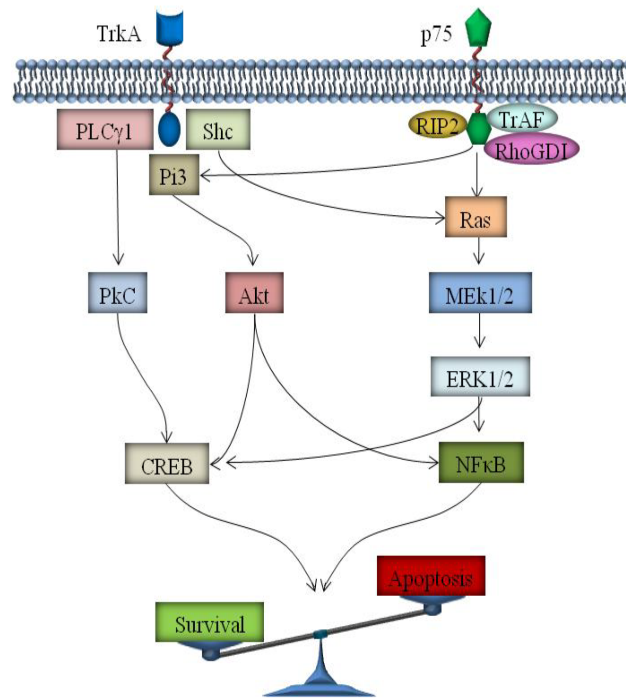
The decline of circulating concentrations of DHEA and DHEAS during human aging has been associated with age-related degenerative processes (Belanger A. et al 1994, Migeon C.J. et al 1957, Vermeulen A. et al 1982), leading to the hypothesis that DHEA supplementation may improve the course of age-related health conditions, including neuronal loss (Charalampopoulos I. et al 2008). Some of the potential anti-aging properties of DHEA are attributed to its cytoprotective actions. Indeed, *in vitro* studies have shown that DHEA exerts potent anti-apoptotic and prosurvival effects in various cells, including keratinocytes, lymphocytes, thymocytes and endothelial cells, (Liu D. et al 2002, Liu D. et al 2006, Liu D. et al 2007, Yan C. et al 1999, Takahashi H. et al 2004, Liang J. et al 2009). In most of these cell systems, DHEA transmits its prosurvival signals via specific membrane binding sites of unknown nature, preventing cell apoptosis, through induction of anti-apoptotic proteins. DHEA was shown to activate similar signaling pathways and protect neuronal cells against various apoptotic challenges (Gil-ad I. et al 2001, Charalampopoulos I. et al 2004, Leskiewicz M. et al 2008). DHEA protects neural crest-derived cells against serum or NGF deprivation-induced apoptosis at nanomolar concentrations (Charalampopoulos I. et al 2004). The anti-apoptotic effects of

DHEA are mediated via binding to specific plasma membrane receptors (K_d:0.9 nM) (Charalampopoulos I. et al 2006), rapid activation of prosurvival kinases Shc/MEK1/2/ERK1/2, and PI3K/Akt, the induction of transcription factors CREB and NFkappaB and the subsequent production of anti-apoptotic Bcl-2 proteins (Charalampopoulos I. et al 2004, 2008).

In fact, the prosurvival signaling pathways induced by DHEA in neuronal cells are initiated at the plasma membrane level, and have major common characteristics with those activated by nerve growth factor (NGF), the key neurotrophic factor of neural crest-derived cells. Neurotrophin NGF binds with high affinity (K_d:0.1 nM) to the transmembrane tyrosine kinase TrkA receptor and with lower affinity (K_d:1.0 nM) to the $p75^{NTR}$ receptor, a membrane protein belonging to TNF receptor super family (Reichardt L.F. 2006). In the presence of TrkA receptors, $p75^{NTR}$ participates in the formation of high affinity binding sites, leading to cell survival signals. In the absence of TrkA, $p75^{NTR}$ generates cell death signals. Docking of TrkA by NGF initiates receptor dimerization, and phosphorylation of tyrosine residues on the receptor, interacting with Shc and other adaptor proteins, resulting in activation of PI3K/Akt and MEK/ERK signaling kinase pathways. These signals lead to the activation of prosurvival transcription factors CREB and NFkappaB, the subsequent production of anti-apoptotic Bcl-2 proteins and prevention of apoptotic neuronal death.

Based on the similarities in the intracellular signaling pathways of DHEA and NGF, in the present study we set out to examine whether the anti-apoptotic effects of DHEA are mediated through the receptors of NGF, TrkA and $p75^{NTR}$. To address this issue we designed the following experiments: we used RNA interference (RNAi) to define the involvement of TrkA and $p75^{NTR}$ receptors in the anti-apoptotic action of DHEA; we assessed membrane binding of DHEA in HEK293 cells transfected with the TrkA and $p75^{NTR}$ plasmid cDNAs, using binding assays, confocal laser microscopy and flow cytometry; to investigate the potential direct physical interaction of DHEA with NGF receptors, we tested the ability of immobilized DHEA to pull-down recombinant or naturally expressed TrkA and $p75^{NTR}$ receptors; finally, we examined the ability of DHEA 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 provide evidence that DHEA directly binds to NGF receptors to protect neuronal cells against apoptosis, acting as a neurotrophic factor.



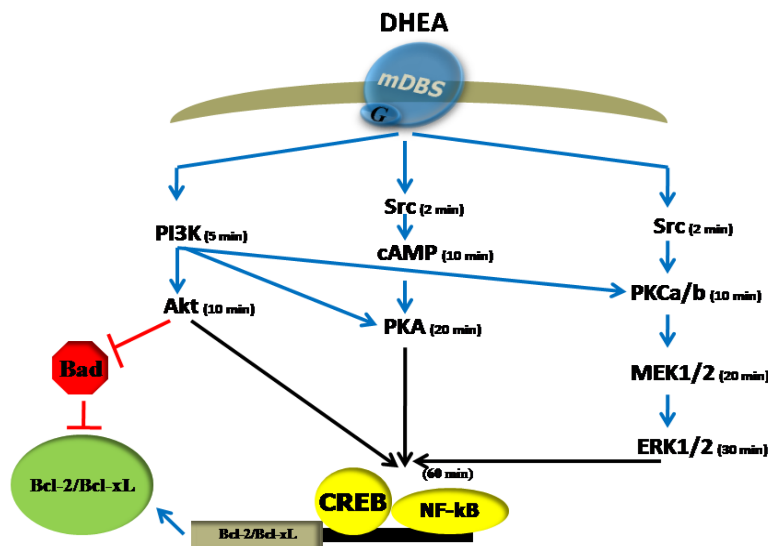


Figure 1.9 Comparison of intracellular signaling induced by DHEA and NGF. *Diagrams show the intracellular signaling pathways of NGF (upper diagram) and DHEA (lower diagram). (Diagram for DHEA was adopted from* Charalampopoulos I. *et al* 2008)

2. Methods

2.1 Cell cultures.

PC12 cells were obtained from Dr. M. Greenberg (Children's Hospital, Boston, MA). NNR5 cells were obtained from Carlos F. Ibáñez. Both cell types were grown in RPMI 1640 containing 2mM 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. HEK293 cells were obtained from LGC Promochem (LGC Standards GmbH, Germany). 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°C.

2.2 siRNAs, plasmids and antibodies.

HEK293 and PC12 cells were transfected with Lipofectamine 2000 (InVitrogen) according to manufacturer's instructions. Transfected cells were typically used on the 2nd day after transfection. PC12 cells were transfected with specific si/shRNAs for blocking the expression of TrkA and/or $p75^{NTR}$ receptors. More specifically, three siRNAs and two shRNAs for TrkA and $p75^{NTR}$ respectively were obtained. The sequences for TrkA siRNAs (Ambion) were: GCCUAACCAUCGUGAAGAG (siRNA ID 191894), GCAUCCAUCAUAAUAGCAA (siRNA ID 191895) and CCUGACGGAGCUCUAUGUG (siRNA ID 191893). Sequences for $p75^{NTR}$ (Qiagen) were: GACCUAUCUGAGCUGAAA (CatNo SI00251090) and GCGUGACUUUCAGGGAAA (CatNo SI00251083).

Rat TrkA was expressed from the pHA vector backbone and rat $p75^{NTR}$ was expressed from the pCDNA3 vector backbone (InVitrogen) using a full length coding sequence flanked by an N-terminal hemagglutinin (HA) epitope tag. Plasmids to express RIP2 [19] and RhoGDI [36] were myc-tagged, while TRAF6 [19] was FLAG-tagged, as previously described.

The origin of antibodies was as follows: Bcl-2 (Cat.No. C-2, sc-7382, Santa Cruz Biotechnology Inc.), phospho TrkA (Cat.No. 9141, Cell Signaling), TrkA (CatNo. 2505, Cell Signaling, was used for Western Blotting and Cat.No. 06-574, Upstate, was used for immunostainings), $p75^{NTR}$ (Cat.No. MAB365R, Millipore), c-myc (CatNo 9E10, sc-40, Santa Cruz Biotechnology Inc.), phospho ERK1/2 (Cat.No. 9106, Cell Signaling), Erk1/2 (Cat.No. 9102, Cell Signaling), phospho-Shc (Tyr239/240) Antibody (Cat.No. 2434, Cell Signaling), Shc (Cat.No. 2432, Cell Signaling), phospho-Akt (Ser473) (Cat.No. 9271, Cell Signaling), Akt (Cat.No 9272, Cell Signaling), anti-FLAG (M2) mouse monoclonal (Cat.No. F1804, Sigma), pTyr (Cat.No. sc-508, Santa Cruz Biotechnology Inc.), active Caspase-3 (Cat. No. ab13847, Abcam), Tyrosine Hydroxylase (Cat. No. ab6211, Abcam), 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), anti-rabbit Alexa Fluor 546 (Cat.No. A10040), and GAPDH (Cat.No. 2118, Cell Signaling) Olig-2 (Chemicon AB9610) MAP-2 (abcam ab32454) Ki67 (abcam ab15580) NF 200kD (abcam ab4680) .

2.3 Quantitative Measurement of Apoptosis using Flow Cytometry Analysis (FACS)

PC12 or $PC12^{nnr5}$ cells were cultured in 6well plates, 24h later they were transfected with siRNAs for TrkA and/or $p75^{NTR}$ or cDNAs of TrkA ($PC12^{nnr5}$) or for GAPDH (CatNo 4605, Ambion) as a control. After 24h the medium was aspirated and replaced either with complete media (serum supplemented) or serum free media in the presence or absence of DHEA. Apoptosis was quantified 24h later with annexin V-FITC and PI (BD Pharminogen Cat.No. 556547) by following manufacturer instructions. Briefly, cells were washed twice with cold PBS and then harvested in Binding Buffer (BB, provided in the kit) in a concentration of 10^6 cells/ml. Annexin V-FITC (5μl) and PI (5μl) was added in 100μl/10^5 cells and incubated for 15min at room temperature in the dark. After the incubation 400μl of BB was added and the cells were analyzed at a Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes, NJ) and analyzed with the CELLQuest (Beckton Dickinson).

2.4 NGF secretion levels of PC12 cells

NGF protein secretion from PC12 cells was tested with ELISA by the use of a sandwich enzymeimmunoassay (EIA), which measures NGF *(ChemiKin* NGF kit, millipore CYT304) following manufacturer's instructions.

2.5 [^{3}H]DHEA binding assays

HEK293 cells transfected with an empty vector (HEK293^{Vector}) or overexpressing p75^{NTR} (HEK293^{p75NTR}) or TrkA (HEK293^{TrkA}), and PC12 cells wild type or shRNA knocked down for p75^{NTR} and TrkA, were cultured, collected by scrapping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7.4. After centrifugation at 1200 rpm, cells were homogenized 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 aprotinin). Crude membrane fractions were isolated by differential centrifugation at 2500×g (10 min at 4°C, to remove unbroken cells and nuclei) and 102,000×g (1 h, at 4°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 3min on ice, washed once, resuspended in PBS (pH 7.4) with protease inhibitors, at a concentration of 2 mg/ml and used immediately for binding experiments.

Fifty μl of membrane suspension (2 mg/ml) were incubated with 10 μl of 1-30 nM [^{3}H]-DHEA (Perkin Elmer, Boston MA) in the presence or absence of 500-fold molar excess of DHEA, in PBS, pH 7.4 with protease inhibitors, in a final volume of 100 μl. After an overnight incubation at 4°C on a rotating plate, membranes were collected on GF/B filters, prewetted in 0.5% PEI solution at 4°C. Filters were washed three 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. For saturation curves specific binding (Bound, B) was calculated as the difference of Total Binding - Non Specific Binding. KDs were calculated from B/F over B Scatchard plots. For displacement experiments, a constant concentration of [^{3}H]DHEA (1 nM) was

incubated with increasing concentrations of competitors (10^{-11}-10^{-6}M), under the same conditions as for saturation binding.

2.6 Fluorescence microscopy

HEK293 cells were allowed to grow on gelatin coated glass coverslips for 24hr in culture medium, and 24h later they were transfected with the cDNAs of TrkA, and $p75^{NTR}$ receptors or the vector alone. Stainings were performed 48h after transfection. Culture medium was aspirated and transfectants were washed twice with PBS buffer. Primary antibodies against TrkA (rabbit, Upstate, No. 06-574) (1/100) or $p75^{NTR}$ (mouse monoclonal ab, Chemicon, MAB365) (1/500) were added for 30min at 37°C. Transfectants were also incubated with the DHEA-BSA-FITC or the BSA-FITC conjugates (10^{-7}M) for 15min at 37°C in the dark, then they were washed with serum free culture medium and incubated for another 15min in serum free culture medium containing 4% BSA. Secondary antibodies, anti-rabbit-R-phycoerythrin conjugated (Sigma, No. P9537) and anti-mouse-fluorescein conjugated (Chemicon No. AP124F) were added at 1/100 dilution and transfectants were incubated for 30min at 37°C, then they were washed three times with PBS, and counterstained with Hoechst nuclear stain (Molecular Probes) for 5min. Coverslips were mounted to slides with 90% glycerin and were analyzed with a confocal laser scanning microscope (Leica TCS-NT, Leica Microsystems GmbH, Heidelberg, Germany).

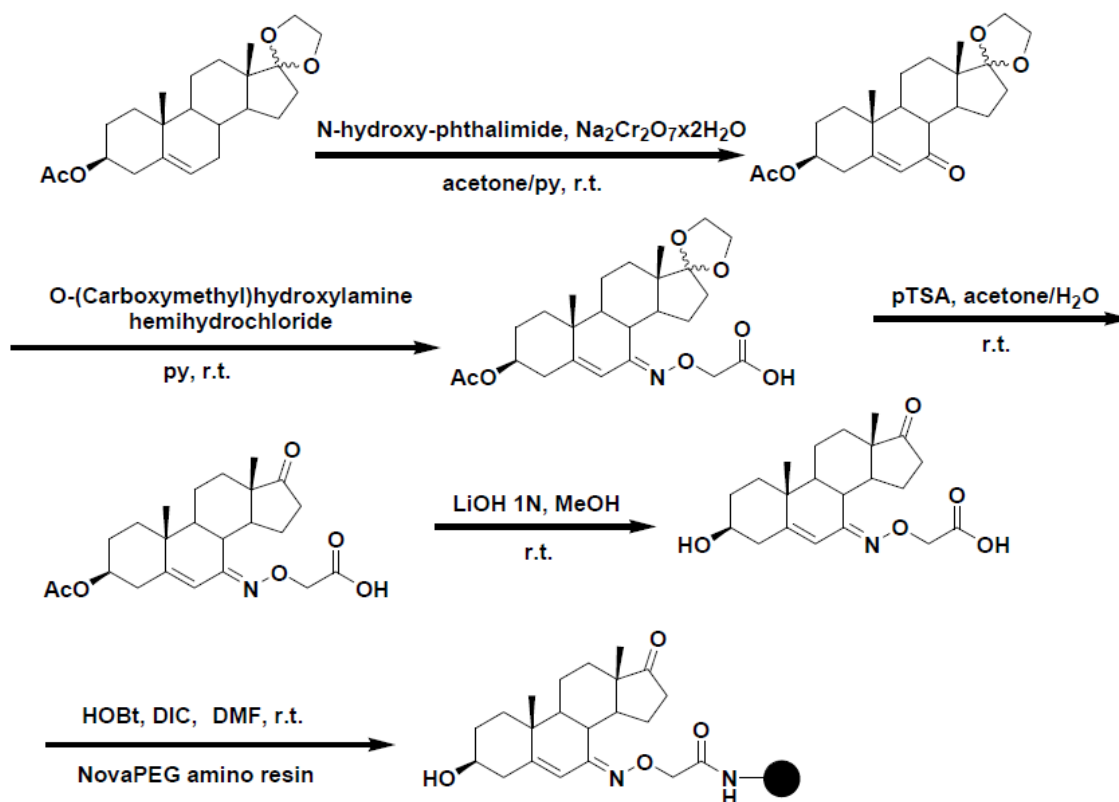
2.7 Flow cytometry

HEK293 cells were cultured in 12-well plates, 24h later they were transfected with the cDNAs of TrkA and/or $p75^{NTR}$ receptors, or the vector alone. Staining was performed 48h after transfection. Transfectants ($5x10^{5}$ cells) were pelleted and incubated with 20μl of the primary antibodies against TrkA (rabbit, Upstate, No. 06-574) (1/100) or $p75^{NTR}$ receptors (mouse, Chemicon MAB365, (1/500), or with 20μl of DHEA-BSA-FITC (10^{-7}M) for 30min at 4°C. Then, they were washed three times with PBS and 20μl of the secondary antibodies, anti-rabbit-Alexa-488 conjugated or anti-mouse-Alexa-488, were added at 1/1000 dilution and cells were incubated for 30min at 4°C. Transfectants were

washed twice with PBS and resuspended in 500µl of PBS, and were analyzed by a Beckton-Dickinson FACSArray apparatus and the CELLQuest software (Beckton-Dickinson, Franklin Lakes, NJ).

2.8 DHEA-polyethylglycol resin synthesis

NovaPEG amino resin (loading value 0.78 mmol/g) was purchased from Novabiochem. NMR spectra were recorded on a Varian 300 spectrometer operating at 300 MHz for 1H and 75.43 MHz for 13C or on a Varian 600 operating at 600 MHz for 1H. 1H NMR spectra are reported in units of □relative to the internal standard of signals of the remaining protons of deuterated chloroform, at 7.24 ppm. 13C NMR shifts are expressed in units of δ relative to CDCl3 at 77.0 ppm. 13C NMR spectra were proton noise decoupled. IR spectra was recorded at Bruker Tensor 27. Absorption maxima are reported in wavenumbers (cm-1).



3β-Acetoxy-17,17-ethylenedioxyandrost-5-ene (0.74 g, 1.98 mmol) and N-hydroxy phthalimide (0.71 g, 2.2 mmol) were dissolved in acetone (39 mL) containing 1 mL of pyridine. The mixture was stirred vigorously at room temperature and sodium dichromate dihydrate (0.89 g, 3 mmol) was added. Additional portions of solid sodium dichromate dihydrate (0.89 g, 3 mmol) were added after 10 and 20 hours stirring at room temperature. After reaction completion (48hrs), the mixture was diluted with dichloromethane, filtered through a bed of celite and the filtrate was washed with water, saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate, the solvent evaporated in vacuo and the residue purified by flash column chromatography using hexane/acetone/ 25% NH4OH (85:15:0.1 mL) as eluent to afford 3β-acetoxy-17,17-ethylenedioxyandrost-5-ene-7-one (0.6 g, yield: 78%). 1H NMR (CDCl3, 300MHz) δ: 0.87 (s, 3H), 1.21 (s, 3H), 1.26-2.00 (m, 14H), 2.05 (s, 3H), 2.20-2.51 (m, 3H), 3.84-3.92 (m, 4H), 4.68-4.76 (m, 1H), 5.70 (d, J = 1.58 Hz, 1H).

To a solution of 3β-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one (0.1 g, 0.26 mmol) in pyridine (1.9 mL) was added O-(carboxymethyl)hydroxylamine hemihydrochloride (0.11 g, 0.52 mmol) and the reaction mixture was stirred overnight under argon. After completion of the reaction, the solvent was evaporated and the residue was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate and the solvent was evaporated in vacuo to afford 3β-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one7-(O-carboxymethy1) oxime as a white foam (0.12 g, yield: 100 %). 1H NMR (CDCl3, 300MHz) δ: 0.88 (s, 3H), 1.13 (s, 3H), 1.16-1.95 (m, 12H), 2.04 (s, 3H), 2.25-2.59 (m, 5H), 3.84-3.95 (m, 4H), 4.59 (d, J = 2.29 Hz, 2H), 4.62-4.73 (m, 1H), 6.51 (d, J = 1.47 Hz, 1H).

To a solution of 3β-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one-7-(O-carboxymethy1) oxime (0.12 g, 0.26 mmol) in a mixture of acetone/water (5:1, 6.3 mL) was added p-toluenesulfonic acid monohydrate (0.019 g, 0.10 mmol) and the reaction mixture was stirred until the starting material was consumed (48 hrs). The solvent was evaporated in vacuo and the residue was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate and the solvent was evaporated in vacuo to afford 3β-acetoxy-androst-5-en-7,17-dione 7-(O-carboxymethy1) oxime as a white foam (0.11 g, yield: 100 %). 1H NMR (CDCl3, 600MHz) δ: 0.90 (s,

3H), 1.15 (s, 3H), 1.20-1.95 (m, 12H), 2.05 (s, 3H), 2.09-2.68 (m, 5H), 4.63 (d, J = 4.18 Hz, 2H), 4.65-4.71 (m, 1H), 6.56 (d, J = 1.39 Hz, 1H).

To a solution of 3β-acetoxy-androst-5-en-7,17-dione 7-(O-carboxymethy1) oxime (0.11 g, 0.26 mmol) in methanol (3.9 mL) was added LiOH (1.5mL, 1.5 mmol, 1N solution) and the reaction mixture was stirred until the starting material was consumed (4 hrs). The solvent was evaporated in vacuo and the residue was diluted with water. The solution was acidified with 10% hydrochloric acid and DHEA-7-CMO precipitated as a white solid, which was isolated by filtration (0.097 g, yield: 100 %). 1H NMR (CDCl3/CD3OD, 600MHz) δ: 0.90 (s, 3H), 1.14 (s, 3H), 1.20-2.75 (m, 17H), 3.49-3.54 (m, 1H), 4.54 (s, 2H), 6.54 (s, 1H).

3β-Hydroxy-17-oxoandrost-5-en-7-O-(carboxymethyl)oxime (DHEA-7-CMO) (192 mg, 0.511 mmol) in DMF (5 mL) was treated with HOBt (69 mg, 0.511 mmol) and DIC (0.08 mL, 0.511 mmol) and the resulting mixture was stirred at room temperature for 30 min. This solution was added to NovaPEG amino resin (130 mg, 0.102 mmol, 0.78 mmol/gr) (pre-swollen with DMF for 1 h) and the slurry was shaken at room temperature overnight. The mixture was filtered, the resin was sequentially washed with dichloromethane (3x), methanol (3x), and diethyl ether (3x) and was dried in vacuo overnight. Yield 175 mg (100%), loading value 0.61 mmol/gr. 13C NMR (gel phase, CDCl3) δ: 220.66, 170.15, 157.10, 154.15, 113.11, 72.57, 66.59, 49.92, 47.86, 42.15, 38.46, 37.08, 36.53, 35.49, 31.20, 30.71, 24.96, 20.15, 18.05, 13.95; IR: νmax/cm-1 2865 (s), 1735 (m), 1669 (w), 1653 (w), 1637 (w), 1456 (m), 1348 (w), 1289 (w), 1247 (w), 1093 (s), 946 (w).

2.9 Co-Immunoprecipitation

Co-Immunoprecipitation (coIP) was used to identify the physical interaction of two or more proteins, using their reaction specifically with an antibody from mixture of proteins. In brief, HEK293 cells (co)transfected with the appropriate plasmids (TrkA, $p75^{NTR}$, RIP2, TRAF-6 and RhoGDI) by using Lipofectamine 2000 (Invitrogen). Cells were harvested 48h after transfection, suspended in lysis buffer (50 mM Tris-HCl, 0.15 M

NaCl, 1% Triton-X100, pH 7.4) supplemented with protease inhibitors. Lysates were precleared for 1h with Protein A-Sepharose beads (Amersham) and immunoprecipitated with the appropriate antibody (pTyr or c-myc) overnight at 4°C. Protein A Sepharose beads were incubated with the lysates for 4h at 4°C with gentle shaking. In the case of DHEA-resin beads, HEK293 or PC12 cells lysates or purified receptors (both from R&D Systems, Recombinant Mouse NGF R/TNFRSF16/Fc Chimera, Catalog Number: 1157-NR and Recombinant Rat Trk A/Fc Chimera, Catalog Number: 1056-TK),were incubated onernight at 4°C with the resin beads alone or conjugated with DHEA. Beads were collected by centrifugation, washed four times with lysis buffer, and resuspended in SDS loading buffer. Proteins were separated by SDS/PAGE, followed by immunoblotting with specific antibodies.

2.10 Western Blot Analysis

PC12 or HEK293 cells lysates were electrophoresed through a 12% SDS-polyacrylamide gel, then proteins were transferred to nitrocellulose membranes, which were processed according to standard Western blotting procedures, as previously described (Charalampopoulos I. et al, 2004). To detect protein levels, membranes were incubated with the appropriate antibodies: Bcl-2 (dilution 1:500), phospho TrkA (dilution 1:500), total TrkA (dilution1:500), $p75^{NTR}$ (dilution 1:500), phospho ERK1/2 (dilution 1:500) and total ERK1/2 (dilution 1:500). Proteins were visualized using the ECL Western blotting kit (ECL Amersham Biosciences, UK) and blots were exposed to Kodak X-Omat AR films. A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis, Inc., Ontario, Canada).

To normalize for protein content the blots were stripped and stained with GAPDH antibody (dilution 1:1000); the concentration of each target protein was normalized versus GAPDH. Where phosphorylation of TrkA or ERK1/2 was measured, membranes were first probed for the phosphorylated form of the protein, then stripped and probed for the total protein. The quantity of the phosphorylated proteins was expressed as the ratio of the phosphorylated divided by the total protein in each case.

2.11 Superior cervical ganglia (SCG) and Dorsal root ganglia (DRG) neuronal cultures.

SCGs and DRGs were removed from newborn (P0-P1) rat pups and dissociated in 0.25% trypsin (Gibco, 15090) for 30 minutes at 37C^o. After dissociation SCG and DRG neurons were resuspended in culture medium (Gibco, Neurobasal Cat.No. 21103) containing 1% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 3μg/ml araC antimitotic, and 100ng/ml NGF (Millipore, 01-125). Cells were plated on collagen coated 24 well plates and cultured for 5 days prior to use. For NGF withdrawal experiments, cells were washed twice with Neurobasal containing 1% FBS, and fresh culture medium lacking NGF and containing anti-NGF antibody at 1:50 dilution (Millipore, AB1526). DHEA, TrkA-inhibitor (Calbiochem, 648450) and anti-p75^{NTR} (mouse, MAB365R Millipore) were used at 100nM, 100nM and 1:50 respectively. For SCG and DRG explants culture the ganglions were plated directly after dissection either in the presence of NGF for 7 days prior experimentation (figure 3.14) or directly in the tested conditions (figure 3.16).

2.12 *In vivo* experiments: *ngf+/-* mice

ngf+/- mice (Crowley et al, 1994) were obtained from the Jackson Laboratory and maintained on C57BL/6 background. All procedures described below were approved by the Animal Care Committee of the University of Crete School of Medicine, Heraklio, Crete, Greece and from the Veterinary Department of the Heraklion Prefecture, Heraklio, Crete, Greece. Animals were housed in cages maintained under a constant 12 h light–dark cycle at 21–23°C, with free access to food and tap water. Genotyping was performed on tail DNA as previously described (Jose Maria Frade and Yves-Alain Barde, 1999) 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 seconds)/59°C (30 seconds)/72°C (1 minute).

Mice heterozygous for the NGF null mutation were interbred to obtain mice homozygous for the NGF gene disruption and the first day of gestation determined by the

discovery of a copulation plug. The mothers were treated daily with a subcutaneous injection of DHEA (120 mg/kg) or vehicle (4.5% ethanol in 0.9% saline) starting from the third day after gestation. A group of animals were collected at E14 and a second at E18. At the day of collection the mothers were deeply anesthetized with sodium pentobarbital (Dolethal 0,7 ml/kg i.p) and were perfused transcardially with saline solution for about 15min and then undergone perfusion in 4% PFA, 15% Picric Acid, 0.05% GA in PB 0.1M, for another 15min. After the perfusion the embryos were collected and maintained in the same fixative over night at 4°C. Embryos were then washed in 0.1M PB and cryoprotected by using 10% sucrose followed by 20% sucrose over night at 4°C. Finally embryos were frozen in OCT in iso-pentane over liquid nitrogen for five minutes and the frozen tissues were stored for later use at -80°C. The samples were sectioned (20μm) and mounted onto Superfrost plus slides (Menzel-Glaser J1800AMNZ). Slides left to air-dry overnight at room temperature (RT), and were then either used immediately or were fixed in cold acetone for 1 minute and stored at -80°C for later use.

2.12.1 Immunostaining

Stored or fresh slides were fixed for 15 minutes in cold acetone at 4oC and left to dry for 10 minutes at room temperature. They were then washed in PB 0.1 M then in TBS and incubated for 45 min with 10% horse serum in TBS-T 0.1%. The normal serum was drained off and the primary antibodies (anti-TrkA diluted 1:400 and active Caspase-3 diluted 1:50), diluted in TBS-T 0.1% with 1% horse serum, were added. Sections were incubated for 4h at RT and overnight at 4oC; they were then washed in TBS-T 0.1% and the anti-rabbit secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546, 1:1000 in TBS-T 0.1%) were added for 6h at RT. Sections washed in TBS-T, TBS and in PB 0.1 M and were coverslipped with Vectashield (Vector, H-1400) and visualized in a confocal microscope. TUNEL (Roche, Cat.No 12156792910) staining of apoptotic was performed according to the manufacturer’s instructions.

2.12.2 Staining with Fluoro-Jade C

Slides immersed in 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed in 70% ethanol, in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Following a water rinse, the slides were then transferred for 10 min to a 0.0001% solution of Fluoro-Jade C (Millipore, Cat.No. AG325) in 0.1% acetic acid. The slides were then washed three times in distilled water. Slides were then air dried at 50°C for 5 min. The air dried slides were then cleared in xylene and then coverslipped with Clarion mounting medium (Sigma, C0487)

2.13 Statistical Analysis

Results are presented either as the ratio of OD in Western blot experiments normalized to GAPDH or as percentage of parallel control cells that were treated with the vehicle only. For the statistical evaluation of our data we have used 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 RNA interference against TrkA receptors reverses the anti-apoptotic effect of DHEA.

To test the involvement of NGF receptors in the anti-apoptotic effect of DHEA in serum deprived PC12 cells we have used the RNAi technology. A combination of three different sequences of siRNAs for TrkA and two different shRNAs for $p75^{NTR}$ transcripts (Brown, R.-C *et al* 200) were selected. The effectiveness of si/shRNAs was shown by the remarkable decrease of TrkA and $p75^{NTR}$ protein levels in PC12 cells, observed by immunobloting analysis, using GAPDH as reference standard (Figure 3.2). Scrambled siRNAs were ineffective in decreasing TrkA and $p75^{NTR}$ protein levels and did not significantly alter the effect of DHEA (data not shown). FACS analysis of apoptotic cells (stained with Annexin V-FITC) has shown that DHEA and membrane impermeable DHEA-BSA conjugate at 100 nM diminished the number of apoptotic cells in serum deprived PC12 cell cultures from 53.5±17.6% increase of apoptosis in serum free condition (control) to 6±1.4% and 13±5.2%, respectively (n:8, $P<0.01$ versus control) (Figure 3.1). Decreased TrkA expression in serum-deprived PC12 cells with siRNAs resulted in the almost complete reversal of the anti-apoptotic effect of DHEA and DHEA-BSA conjugate (Figure 3.1). Co-transfection of serum deprived PC12 cells with the si/shRNAs for TrkA and $p75^{NTR}$ receptors did not modify the effect of the TrkA deletion alone. Furthermore, transfection of serum deprived PC12 cells with the shRNAs against $p75^{NTR}$ receptor alone did not significantly alter the anti-apoptotic effect of DHEA, suggesting that the anti-apoptotic effect of DHEA is primarily afforded by TrkA receptors.

In accordance with the above observations, transfection of serum-deprived PC12 cells with the siRNAs against the TrkA transcript fully annulled the ability of DHEA to maintain elevated the levels of anti-apoptotic Bcl-2 protein (Figure 3.2). Again, transfection with the shRNA against $p75^{NTR}$ receptor alone did not significantly affect Bcl-2 induction by DHEA, further supporting the hypothesis that TrkA is the main mediator of the anti-apoptotic effect of DHEA in this system.

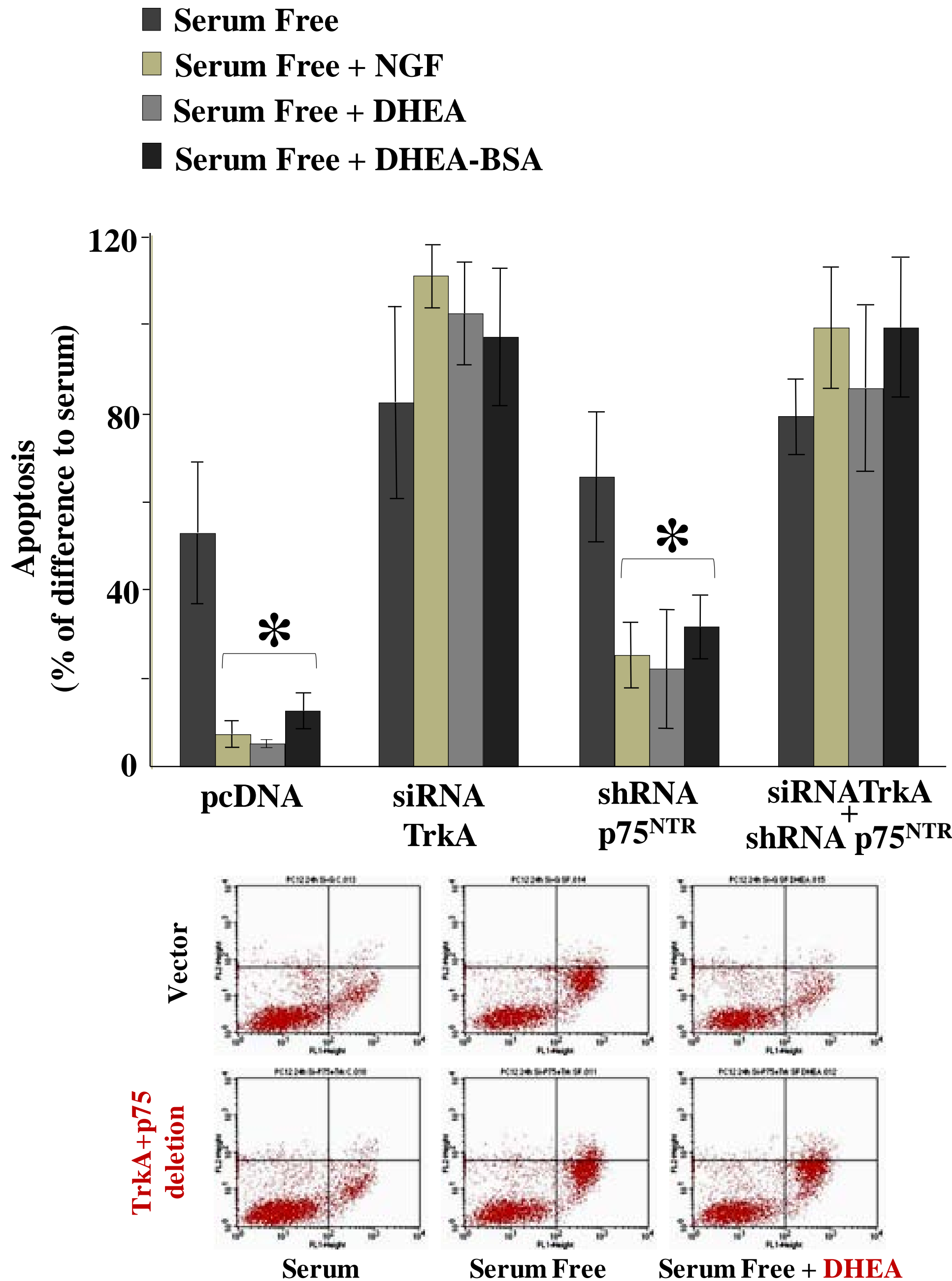


Figure 3.1 RNA interference against NGF receptors affects the anti-apoptotic effect of DHEA. *PC12 cells were transfected with si/shRNAs of TrkA and/or p75^{NTR} . Twenty four hours later, the medium was replaced either with complete medium (serum supplemented) or serum free medium in the absence or the presence of DHEA, DHEA-BSA (100 nM) or NGF (100 ng/ml). Apoptosis was quantified 24h later by FACS using Annexin V-FITC and PI.* ***a)*** *Upper panel: levels of apoptosis expressed as % of difference from serum supplemented cells (see supplementary files)[* P<0.01 versus control (serum free conditions), n:8]. Lower panel: representative FACS analysis of Annexin V-FITC and PI staining. [Transfection eficasy for each condition was asesed by western blot analisis (see lower panel in 3.2)]*

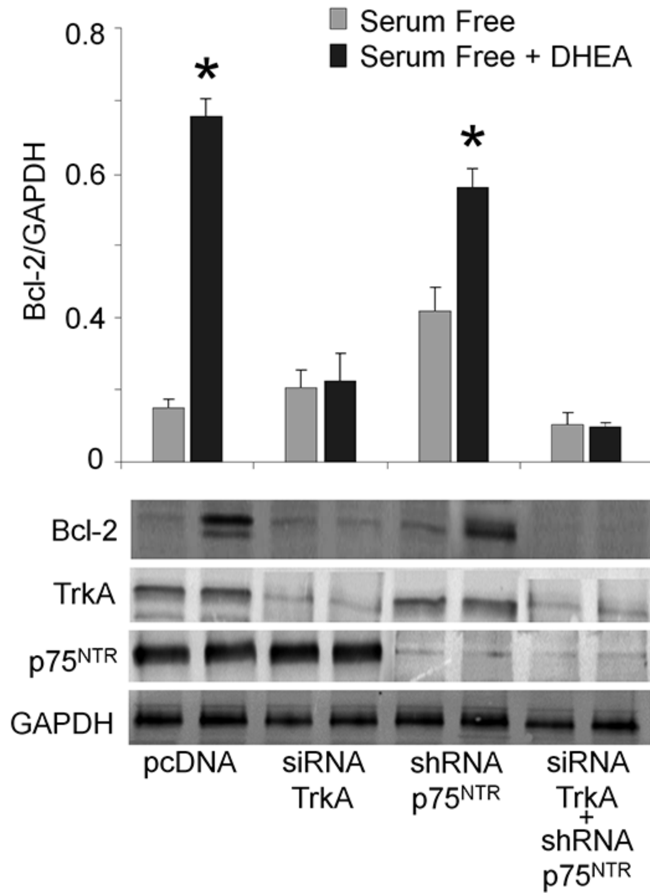


Figure 3.2 Levels of Bcl-2 protein in serum deprived PC12 cells with or without DHEA treatment. *Cellular extracts containing total proteins were collected and levels of Bcl-2 protein were measured by western blot, and normalized per GAPDH protein content. Upper panel: mean±SE of Bcl-2 levels, normalized against GAPDH (*P<0.01 versus control, n:4), lower panel: representative western blots of Bcl-2, TrkA, $p75^{NTR}$ and GAPDH proteins.*

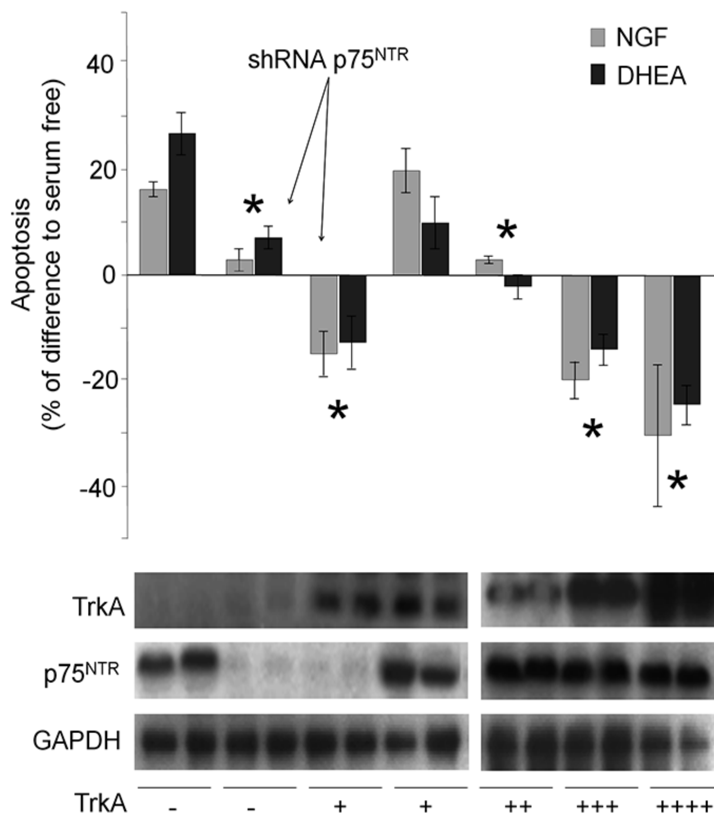


Figure 3.3 The ratio of TrkA and $p75^{NTR}$ receptors determines the effect of DHEA. *Upper panel: levels of apoptosis in $PC12^{nnr5}$ cells, transfected with si/shRNAs for $p75^{NTR}$ and/or expressing vectors for TrkA, expressed as % of difference from serum deprivation condition. (*P<0.01 versus control-naive cells, n=4). Lower panel: western blots of TrkA, $p75^{NTR}$ and GAPDH proteins for each condition*

It appears that the ratio of TrkA and $p75^{NTR}$ receptors determines the effect of DHEA on cell apoptosis and survival. Indeed, both NGF and DHEA induced apoptosis of nnr5 cells, a clone of PC12 cell line known to express only pro-death $p75^{NTR}$ receptors (Figure 3.3), confirming the pro-apoptotic function of this receptor. Blockade of $p75^{NTR}$ expression by shRNA almost completely reversed the pro-apoptotic effect of both agents. The anti-apoptotic effect of NGF and DHEA was remarkably restored after transfection of nnr5 cells with the TrkA cDNA, the efficacy of reversal being proportionally dependent on the amount of transfected TrkA cDNA (Figure 3.3).

DHEA was also controlling the response of NGF receptor positive cells, by regulating TrkA and $p75^{NTR}$ receptor levels, mimicking NGF. Serum deprived PC12 cells were exposed to 100 nM of DHEA or 100 ng/ml of NGF for 12, 14 and 48 hours, TrkA and $p75^{NTR}$ protein levels were measured in cell lysates with immunoblotting, using specific antibodies against TrkA and $p75^{NTR}$ proteins and were normalized against GAPDH. Both NGF and DHEA significantly increased pro-survival TrkA receptor levels in the time frame studied, i.e. from 12 to 48 hours (n:5, P<0.01) (Figure 3.4). Furthermore, DHEA and NGF significantly decreased $p75^{NTR}$ receptor levels between 24 to 48 hours of exposure (n:5, P<0,01).

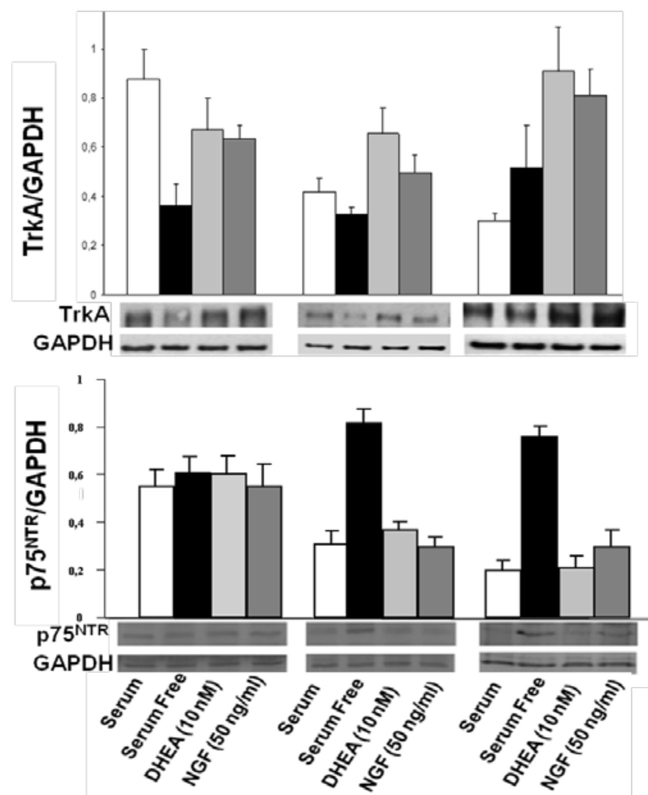


Figure 3.4 DHEA regulates the expression levels of TrkA and $p75^{NTR}$. *Serum deprived PC12 cells were exposed to 100 nM of DHEA or 100 ng/ml of NGF for 12, 14 and 48 hours, TrkA and $p75^{NTR}$ protein levels were measured in cell lysates with immunoblotting, using specific antibodies against TrkA and $p75^{NTR}$ proteins and were normalized against GAPDH (* P<0.01 versus control-Serum Free, n:5)*

3.2 [^{3}H]DHEA binds with high affinity to HEK293^{TrkA} and HEK293^{p75NTR} cell membranes.

We have previously shown the presence of specific DHEA binding sites to membranes isolated from PC12, primary human sympathoadrenal, and primary rat hippocampal cells, with Kd 0.9, 0.1 and 0.06 nM, respectively [9]. The presence of DHEA-specific membrane binding sites on PC12 cells has been confirmed by flow cytometry and confocal laser microscopy of cells stained with the membrane impermeable DHEA-BSA-FITC conjugate. In contrast to estrogens, glucocorticoids and androgens displaced [3H]DHEA from its membrane binding sites, acting as pure antagonists by blocking the anti-apoptotic effect of DHEA in serum deprived PC12 cells (Kibaly, K. *et al* 2005). In the present study, we repeated this series of experiments using membranes isolated from HEK293 cells transfected with the plasmid cDNAs of TrkA or p75^{NTR}.

HEK293 cells not expressing TrkA or p75^{NTR} were transfected with an empty vector (control) or a specific TrkA or p75^{NTR} vector; transfection efficiency was controlled by western blot (Figure 3.5 a and b, inserts), confocal laser microscopy and flow cytometry (Figure 3.6 detailed description in section 3.3). Saturation binding experiments have shown that [^{3}H]-DHEA bound with high affinity to membranes isolated from HEK293 cells, transfected with the cDNAs of TrkA or p75^{NTR} receptors (Figures 3.5). Membranes isolated from HEK293 cells transfected with the empty vector shown no specific binding. The KD values calculated after Scatchard analysis of saturation curves were 7.8±3.1 nM and 5.9±1.7 nM for TrkA or p75^{NTR} respectively (n:3).

Transfection of PC12 cells, endogenously expressing NGF receptors, with shRNAs against both TrkA and p75^{NTR} receptors resulted in a complete loss of [^{3}H]-DHEA specific membrane binding (Figure 3.7a). To rule out the possibility the loss of specific binding might be due to the transfection process, we tested binding of [^{3}H]-DHEA to membranes isolated from PC12 cells transfected with siRNA against GAPDH. Saturation binding and Scatchard analysis have shown that [^{3}H]-DHEA bound to membranes from PC12-siRNAGAPDH cells with a KD = 1.068 ± 0.43 nM (Figure 3.7a).

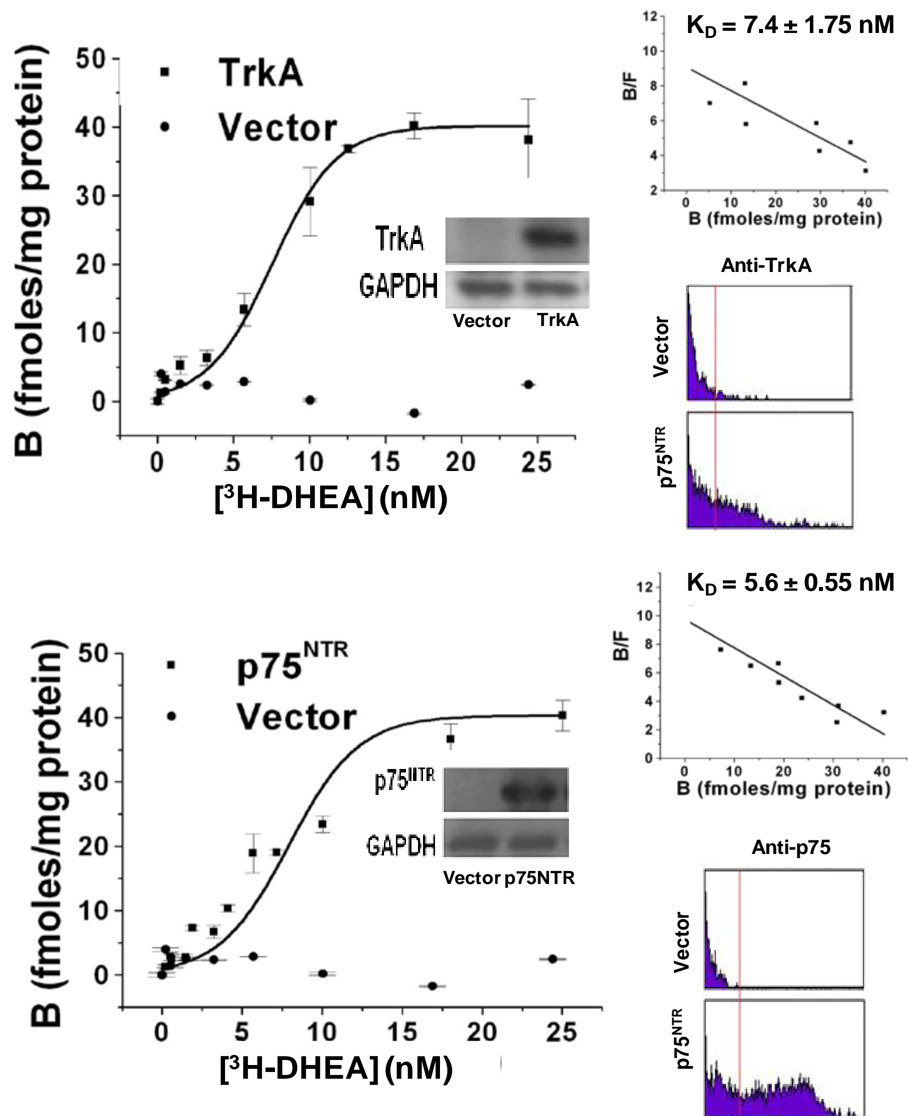


Figure 3.5 DHEA binds with high affinity to HEK293^{TrkA} and HEK293^{p75NTR} cell membranes. *[3H]-DHEA saturation binding assays and Scatchard blots in HEK293 cells, transfected with the plasmid cDNAs of TrkA and p75^{NTR} receptors. Western blot and FACS inserts show the efficacy of transfection (KD represents the mean±SE of 3 experiments)*

The selectivity of DHEA binding to HEK293^{TrkA} and HEK293^{p75NTR} cell membranes was examined by performing heterologous [^{3}H]-DHEA displacement experiments using a number of non-labeled steroids or NGF. Binding of [^{3}H]-DHEA to membranes isolated from both HEK293^{TrkA} and HEK293^{p75NTR} cells was effectively displaced by NGF (IC_{50}: 0.8±0.2 and 1.19±0.45 nM, respectively) (Figure 3.8). NGF was also effectively displacing [^{3}H]-DHEA binding on membranes isolated from PC12 cells (IC_{50}: 0.92±0.32

nM, data not shown). Estradiol failed to displace [^{3}H]-DHEA from its binding on membranes from HEK293^{TrkA} and HEK293^{p75NTR} cells at concentrations ranging from 0.1 to 1000 nM. In contrast, displacement of [^{3}H]-DHEA binding to membranes from both HEK293^{TrkA} and HEK293^{p75NTR} cells was shown by testosterone (Testo) (IC_{50}: 5.3±2.1 and 7.4±3.2 nM, respectively). Glucocorticoid dexamethasone (DEX) effectively competed [^{3}H]-DHEA binding to membranes from HEK293^{TrkA} (IC_{50}: 9.5±4.6 nM) but was ineffective in displacing DHEA binding to membranes from HEK293^{p75NTR} cells. Homologous [^{125}I]-NGF displacement experiments with unlabeled NGF confirmed the presence of specific NGF binding on membranes from both HEK293^{TrkA} and HEK293^{p75NTR} cells with IC_{50} =0.03±0.009 and 1.7±0.38 nM respectively. It is of note that in contrast to unlabeled NGF, DHEA was unable to displace binding of [^{125}I]-NGF to membranes isolated from HEK293^{TrkA} and HEK293^{p75NTR} transfectants (data not shown).

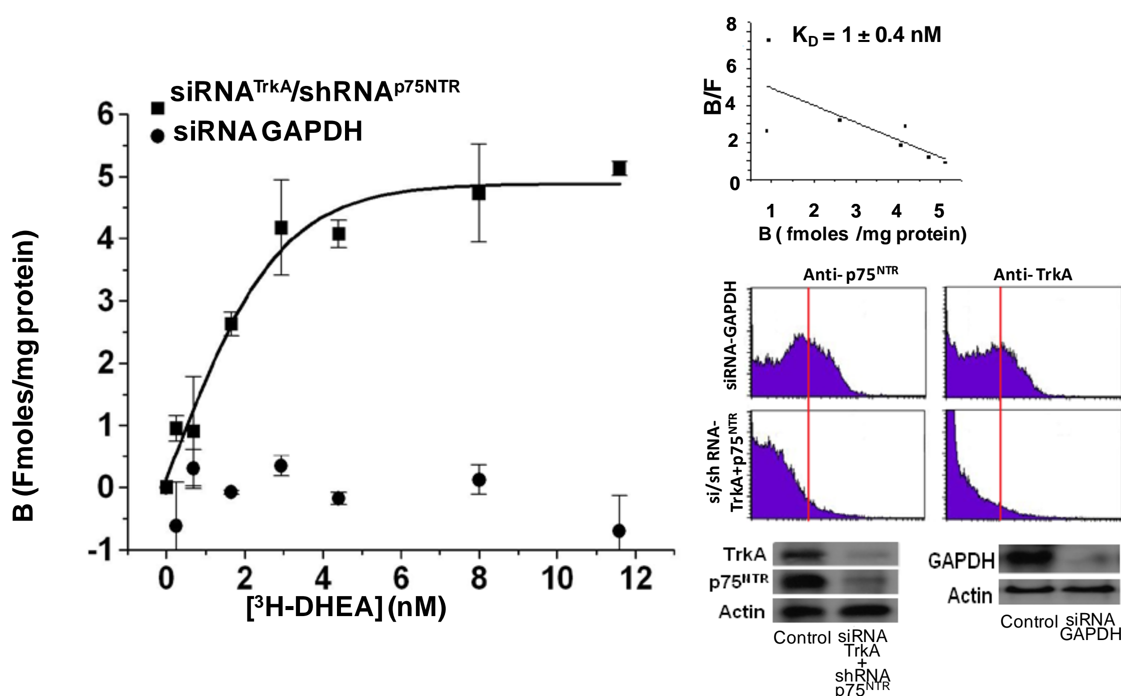


Figure 3.7 Withdrawal of TrkA and p75^{NTR} from PC12 cells results in complete lose of DHEA specific binding. ***a***) *[^{3}H]-DHEA saturation binding assays in PC12 cells, transfected with the si/shRNAs against TrkA and p75^{NTR} receptors or with the siRNA against GAPDH. The efficacy of transfection was shown by western blot (inserts in a) and by FACS analysis (**b**), (KD represents the mean±SE of 3 experiments).*

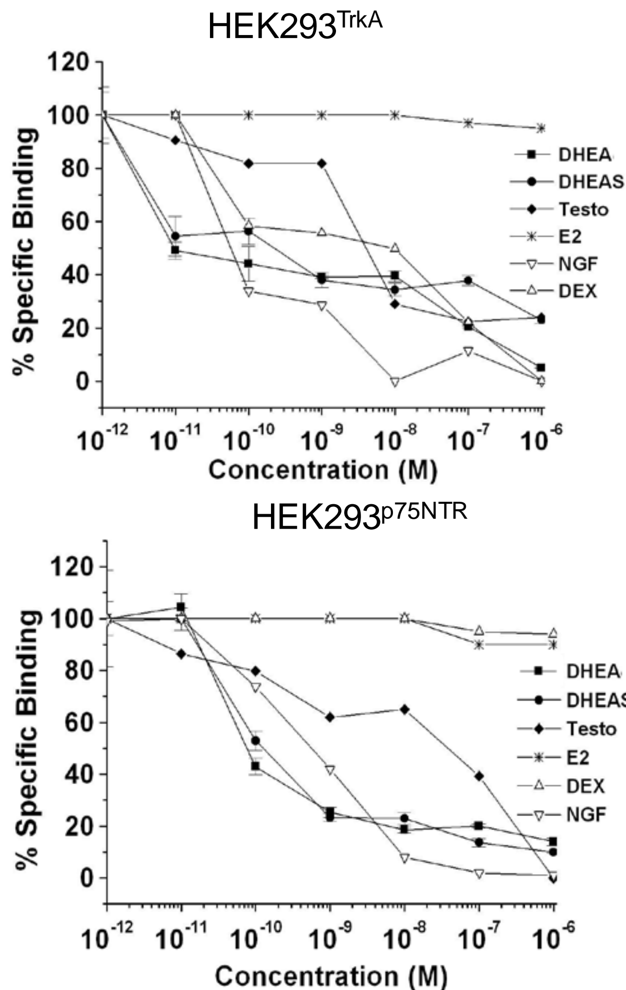


Figure 3.8 Representative curves of [³H]-DHEA displacement experiments. *Competition binding experiments were performed using isolated membranes (at a final concentration 2 mg protein/ml) from HEK293 cells transfected with the plasmid cDNAs of TrkA and $p75^{NTR}$ receptors, incubated for 30 min with 5 nM [³H]DHEA in the absence (for total binding) or the presence of various unlabeled steroids (DHEA, testosterone (Testo), estradiol (E2) dexamethasone (Dex) or NGF at concentrations varying from 0.01 to 1000 nM.*

3.3 DHEA-BSA-FITC conjugate stains $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cell membranes.

Incubation of PC12 cells with the membrane impermeable, fluorescent DHEA-BSA-fluorescein conjugate results in a specific spot-like membrane fluorescent staining (Kibaly, K. *et al* 2005). In the present study, we have tested the ability of DHEA-BSA-FITC conjugate to stain $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ transfectants. Fluorescence microscopy analysis revealed that DHEA-BSA-FITC clearly stained the membranes of $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells (Figure 3.6 upper panels). No such staining was found in non-transfected HEK293 cells (data not shown) or in HEK293 cells transfected with the vectors empty of TrkA and $p75^{NTR}$ cDNAs (Figure 3.6 upper panels). Furthermore, BSA-FITC conjugate was ineffective in staining both transfectants (data not shown). We have further confirmed the presence of membrane DHEA-BSA-FITC staining of $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells with flow cytometry (FACS) analysis

(Figure 3.6 lower panels). Specific staining was noted in both transfectants. No such staining was seen in non-transfected HEK293 cells (data not shown) or in HEK293 cells transfected with the empty vectors (Figure 3.6 lower panels). In both fluorescence microscopy and FACS experiments membrane staining of TrkA or $p75^{NTR}$ proteins in $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells was also shown using specific antibodies for each protein (Figure 3.6 lower panels).

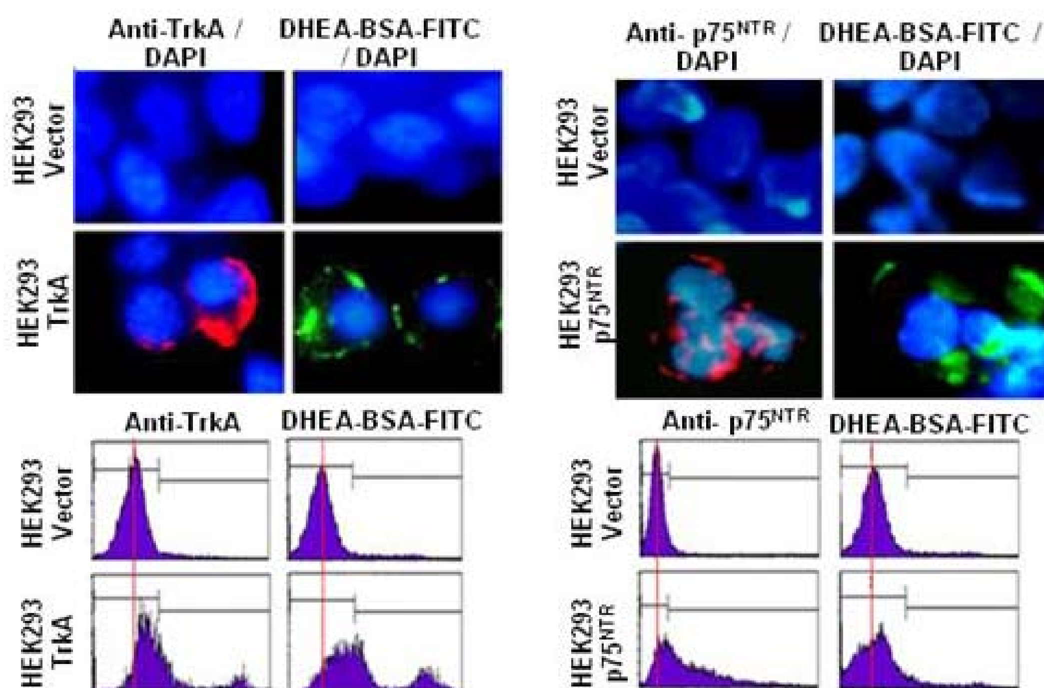


Figure 3.6 Fluorescence localization of DHEA membrane binding on HEK293 cells transfected with the plasmid cDNAs of TrkA and $p75^{NTR}$ receptors. *Transfectants were incubated with either the membrane impermeable DHEA-BSA-FITC conjugate (100 nM), BSA-FITC (100 nM) or with specific antibodies against TrkA and $p75^{NTR}$ proteins. Transfectants were analyzed under the confocal laser scanning microscope or by FACS analysis. Blue staining depicts Hoechst nuclear staining.*

3.4 Immobilised DHEA pulls down TrkA and $p75^{NTR}$ receptors.

Our binding assays with radiolabeled DHEA suggest that DHEA physically interacts with NGF receptors. To test this hypothesis we covalently linked DHEA-7-O-

(carboxymethyl) oxime DHEA-7-CMO) to polyethylene glycol amino resin (NovaPEG amino resin) and we tested the ability of immobilized DHEA to pull down TrkA and $p75^{NTR}$ proteins. Precipitation experiments and western blot analysis of precipitates with specific antibodies against TrkA and $p75^{NTR}$ proteins (Figure 3.9) showed that immobilized DHEA effectively precipitated recombinant TrkA and $p75^{NTR}$ proteins. Similar results were obtained when cell extracts isolated from HEK293 cells transfected with TrkA and $p75^{NTR}$ cDNAs, PC12 cells and whole rat brain were treated with immobilised DHEA (Figure 3.9, panels marked with A). No precipitation of TrkA and $p75^{NTR}$ proteins was shown with polymer-supported DHEA-7-CMO incubated with cell extracts from untransfected HEK293 cells or HEK293 cells transfected with the empty vectors. A control experiment was performed with NovaPeg amino resin (no DHEA-7-CMO present) which was found ineffective in precipitating TrkA and $p75^{NTR}$ proteins (Figure 3.9). The presence of TrkA and $p75^{NTR}$ receptors in $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ transfectants and in PC12 and fresh rat brain was confirmed with western blot analysis using specific antibodies against TrkA and $p75^{NTR}$ proteins and GAPDH as reference standard (Figure 3.9, panels marked with B).

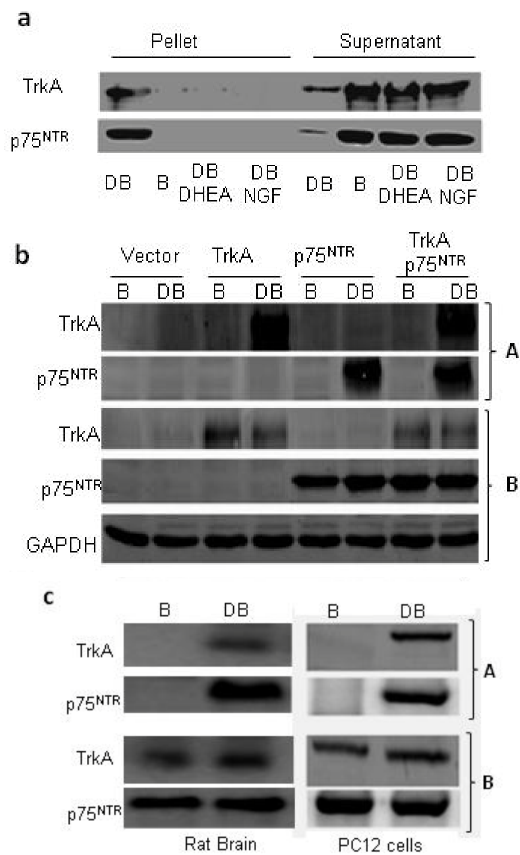


Figure 3.9 Immobilised DHEA pulls down TrkA and $p75^{NTR}$ receptors. *Covalently linked DHEA-7-O-(carboxymethyl) oxime DHEA-7-CMO) to polyethylene glycol amino resin (NovaPEG amino resin) was incubated with recombinant TrkA and $p75^{NTR}$ proteins (a) or with cell extracts isolated from $HEK293^{TrkA}$ $HEK293^{p75NTR}$ transfectants (b), PC12 cells and whole rat brain (c). Precipitation experiments (panels marked with A) and western blot analysis (panels marked with B) of precipitates with specific antibodies against TrkA and $p75^{NTR}$ proteins were performed as described in Materials and Methods. DB: DHEA-7-O-(carboxymethyl) oxime DHEA-7-CMO) polyethylene glycol amino resin, B: polyethylene glycol amino resin, P: pellet, S: supernatant.*

3.5 DHEA induces TrkA- and p75^{NTR}-mediated signaling.

Previous findings have shown that NGF controls the responsiveness of sensitive cells through induction of TrkA phosphorylation and regulation of the levels of each own receptors (Vermeulen, A *et al* 1982). We compared the ability of NGF and DHEA to induce phosphorylation of TrkA in HEK293 cells transfected with the cDNAs of TrkA receptors. HEK293^{TrkA} transfectants were exposed for 10 and 20 min to 100 nM of DHEA or 100 ng/ml of NGF, and cell lysates were immunoprecipitated with anti-tyrosine antibodies and analyzed by western blotting, using specific antibodies against TrkA receptors. Both NGF and DHEA strongly increased phosphorylation of TrkA as early as 10 min, effect which was also maintained at 20 min (Figure 3.11). We also tested the effects of DHEA and NGF in PC12 cells, endogenously expressing TrkA receptors. Naive or siRNA-TrkA transfected PC12 cells were incubated for 10 min with DHEA or NGF, and cell lysates were analyzed with western blotting, using specific antibodies against Tyr490-phosphorylated TrkA and total TrkA. Both NGF and DHEA strongly induced the phosphorylation of TrkA in naive PC12 cells, effects which were diminished in siRNA-TrkA transfected PC12 cells (Figure 3.11). The stimulatory effect of DHEA on TrkA phosphorylation might be due to an increase of NGF production. To test this hypothesis, we measured with ELISA the levels of NGF in culture media of PC12 cells exposed for 15min to 3h to 100 nM of DHEA.

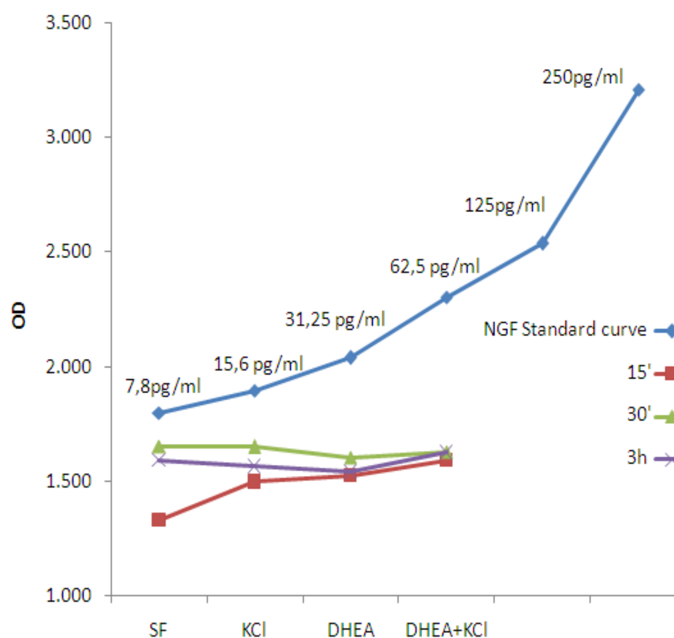


Figure 3.10 Undetectable levels of NGF secretion from PC12 cells. *PC12 cells were cultured in serum free (SF) conditions in the presence of DHEA and/or KCl for 15' and 30' and 3h, the levels of NGF were measured in supernatant by ELISA. NGF was undetectable in all conditions.*

NGF levels in culture media of control and DHEA-treated PC12 cells were undetectable, indicating that DHEA-induced TrkA phosphorylation was independent of NGF production (Figure 3.10). Furthermore, we compared the ability of NGF and DHEA to induce phosphorylation of TrkA-sensitive Shc, ERK1/2 and Akt kinases. Serum deprived naive or siRNA-TrkA transfected PC12 cells were incubated for 10 min with 100 nM DHEA or 100 ng/ml NGF and cell lysates were analyzed with western blotting, using specific antibodies against the phosphorylated and total forms of kinases mentioned above. Both DHEA and NGF strongly increased phosphorylation of Shc, ERK1/2 and Akt kinases in naive PC12 cells, effects which were almost absent in siRNATrkA transfected PC12 cells, suggesting that both DHEA and NGF induce Shc, ERK1/2 and Akt phosphorylation via TrkA receptors (Figure 3.11).

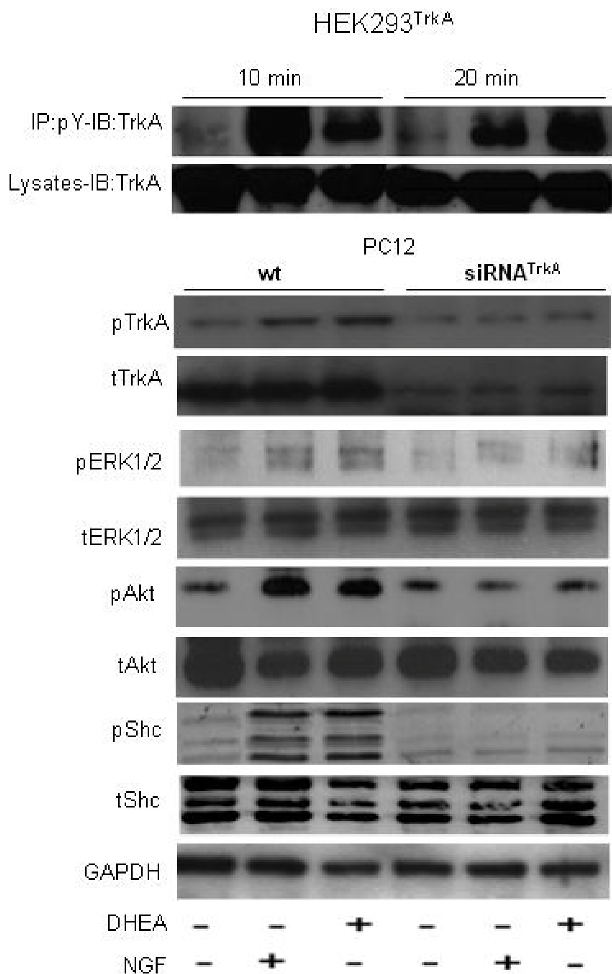


Figure 3.11 DHEA induces TrkA-mediated signaling. *$HEK293^{TrkA}$ transfectants were exposed for 10 and 20 min to 100 nM of DHEA or 100 ng/ml of NGF, and cell lysates were immunoprecipitated with anti-tyrosine antibodies and analyzed by Western Blotting, using specific antibodies against TrkA receptors (upper panel). Serum deprived naive or siRNATrkA transfected PC12 cells were incubated for 10 min with 100 nM of DHEA or 100 ng/ml of NGF and cell lysates were analyzed with western blotting, using specific antibodies against the phosphorylated and total forms of TrkA receptor and of Shc, ERK1/2 and Akt kinases (lower panel).*

The effectiveness of DHEA to promote the interaction of $p75^{NTR}$ receptors with its effector proteins TRAF6, RIP2 and RhoGDI was also assessed. It is well established that

NGF induces the association of p75^{NTR} receptors with TNF receptor-associated factor 6 (TRAF6), thus, facilitating nuclear translocation of transcription factor NFκB (Charalampopoulos, I. *et al* 2008). Furthermore, p75^{NTR} receptors associate with receptor-interacting protein 2 (RIP2) in a NGF-dependent manner (Compagnone, N.A., and Mellon, S.-H. 2000). RIP2 binds to the death domain of p75^{NTR} via its caspase recruitment domain (CARD), conferring nuclear translocation of NFκB. Finally, naive p75^{NTR} interacts with RhoGDP dissociation inhibitor (RhoGDI), activating small GTPase RhoA (Majewska, M.D. *et al* 1990). In that case, NGF binding abolishes the interaction of p75^{NTR} receptors with RhoGDI, thus, inactivating RhoA. We co-transfected HEK293 cells with the plasmid cDNAs of p75^{NTR} and of each one of the effectors TRAF6, RIP2 or RhoGDI, tagged with the flag (TRAF6) or myc (RIP2, RhoGDI) epitopes. Transfectants were exposed to 100 nM DHEA or 100 ng/ml NGF, and lysates were immunoprecipitated with antibodies against flag or myc, followed by immunoblotting with p75^{NTR} specific antibodies. Both DHEA and NGF efficiently induced the association of p75^{NTR} with effectors TRAF6 and RIP2, while facilitated the dissociation of RhoGDI from p75^{NTR} receptors (Figure 3.12).

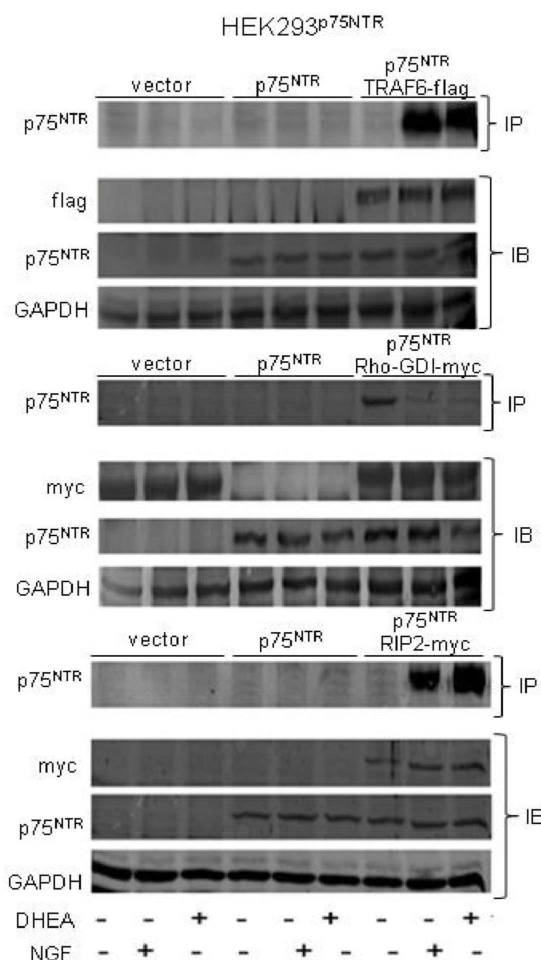


Figure 3.12 DHEA induces p75^{NTR}-mediated signaling. *HEK293 cells were co-transfected with the plasmid cDNAs of p75^{NTR} and of each one of the effectors TRAF6, RIP2 or RhoGDI, tagged with the flag (TRAF6) or myc (RIP2, RhoGDI) epitopes. Transfectants were exposed for 30 min to 100 nM of DHEA or 100 ng/ml of NGF, and lysates were immunoprecipitated with antibodies against flag or myc, followed by immunoblotting with p75^{NTR} specific antibodies. (c), Serum deprived PC12 cells were exposed to 100 nM of DHEA or 100 ng/ml of NGF for 12, 14 and 48 hours, TrkA and p75^{NTR} protein levels were measured in cell lysates with immunoblotting, using specific antibodies against TrkA and p75^{NTR} proteins and were normalized against GAPDH (* P<0.01 versus control-Serum Free, n:5).*

3.6 DHEA rescues TrkA positive primary sympathetic neurons.

We have also tested the anti-apoptotic effects of DHEA in neural crest deriving superior cervical ganglia (SCG), a classical NGF/TrkA sensitive mammalian neural tissue, containing primarily one class of neurons, principal sympathetic neurons. Indeed, NGF and TrkA receptors are absolutely required for SCG sympathetic neuron survival during late embryogenesis and early postnatal development (Cascio, C. *et al* 2000,; Belanger, A. *et al* 1994). TrkC receptors are barely detectable after E15.5, and no significant TrkB receptors are present in the SCG at any developmental stage (Migeon, C.J. *et al* 1957).

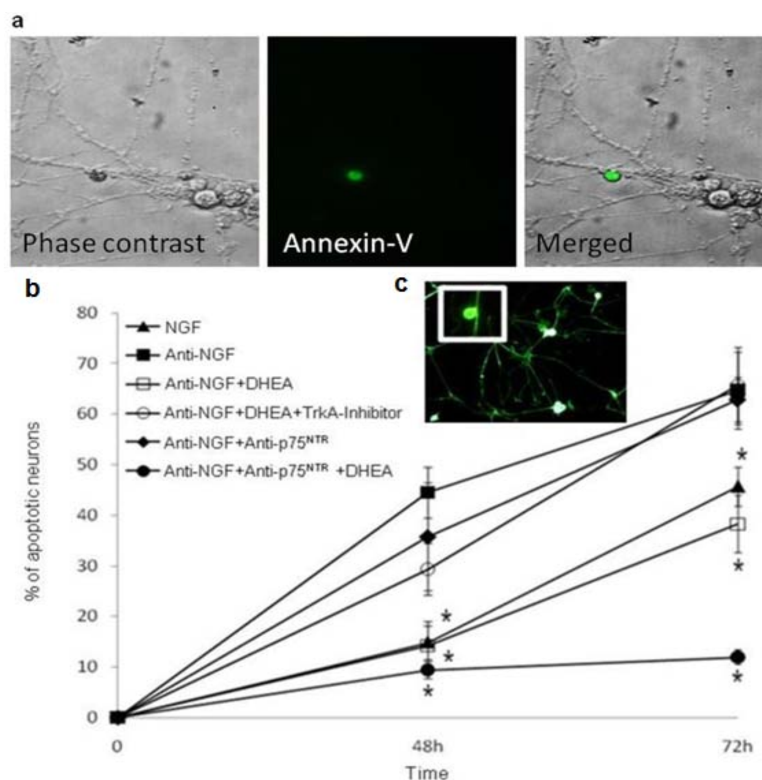


Figure 3.13 DHEA rescues TrkA positive primary sympathetic neurons from NGF deprivation-induced apoptosis, in a TrkA dependent manner. *(a) Light and Annexin V-FITC stained fluorescence microscopy photographs of dispersed primary sympathetic neurons in culture, isolated from rat SCG at P1. (b) Sympathetic neurons were incubated in the presence of 100 ng/ml NGF, or in the same medium but lacking NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum and or 100 nM DHEA in the absence or the presence of TrkA-inhibitor or a mouse anti-p75*^{NTR}*-neutralizing antibody. The results shown are the mean±SE from 3 separate experiments where over 300 neurons were counted in 6 to 7 randomly selected optical fields (*P<0.01 versus anti-NGF condition). Photograph in c depicts tyrosine hydroxylase (TH) staining of sympathetic neurons*

Single cell cultures of rat SCG at P1 were incubated in the presence of 100ng/ml NGF, or in the same medium as above but lacking NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum in the absence or the presence of 100nM DHEA. Deprivation of NGF strongly increased the number of apoptotic sympathetic neurons stained with Annexin V, while DHEA effectively compensated NGF by decreasing the levels of apoptotic neurons, effect which was blocked by a specific TrkA inhibitor thus, suggesting the involvement of TrkA receptors as the main mediator of the anti-apoptotic action of DHEA (Figure 3.13). Moreover, inhibition of $p75^{NTR}$ by a specific antibody (MAB365R, Millipore) against its extracellular domain, strongly induced a DHEA- or NGF-mediated anti-apoptotic effect, clearly indicating that $p75^{NTR}$ receptor serves a pro-apoptotic role in SCGs also, effect which is apparent only in the absence of TrkA receptor, as it was also shown in nnr5 cells (Figure 3.3).

3.7 DHEA treatment prevents the retraction of axons in NGF depraved DRG explants.

Showing the ability of DHEA to reverse the apoptotic loss of TrkA positive neurons, we went further to test its effects in axonal growth and maintenance. Organotypic cultures of rat DRGs at P1 were incubated in the presence of 100ng/ml NGF for 7 days. After indicating that all the explants had establish growth of axons in the periphery around them, the medium was aspirated and the explants were either remained in the same medium or lacking NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum in the absence or the presence of 100nM DHEA. Deprivation of NGF induced axonal retraction in a degree that at 48h after treatment there was almost complete loss of axons. DHEA was able to compensate NGF deprivation preventing axonal retraction. There was no sign of axonal retraction after 48h in DHEA treated explants (figure 3.14). Although, these results were obtained after a single experiment and for this reason further confirmation is needed.

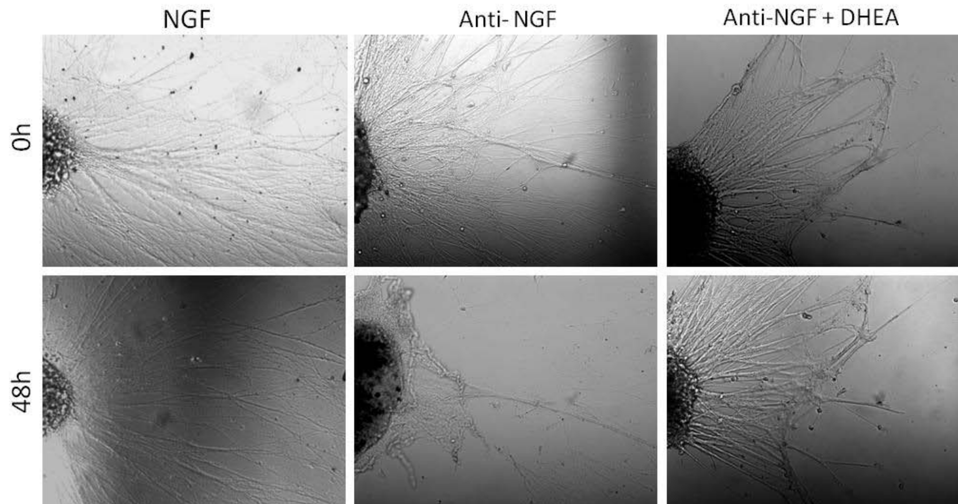


Figure 3.14 DHEA prevents axonal retraction due to NGF deprivation. *Dorsal root ganglion dissected from P1 rat pups were maintained in culture for 5 days in the presence of 100ng/ml NGF. Then NGF removed from the last two conditions and cells were incubated with Anti-NGF neutralizing antibody 10nM DHEA was added at the last condition. DHEA completely reversed the axonal retraction induced by NGF deprivation for 48h.*

3.8 DHEA treatment induces migration of non neuronal cells in the surrounding area of SCG explants.

Organotypic cultures of rat SCGs at P1 were incubated immediately after plating in the presence of 100ng/ml NGF or in the same medium lacking NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum in the absence or the presence of 100nM DHEA. As expected, NGF induced neurite outgrowth that resulted in a dense axonal nest surrounding the explants. There was no axonal growth in the explants growning in the absence of NGF, and DHEA was unable to induce neurite outgrowth. Although there was an increase in the number of non neuronal cells in the surrounding area of the explants in the presence of DHEA as compared with the NGF deprivation condition (figure 3.16). Further experimental procedures are in progress in order to specify the cell phenotype of these cells.

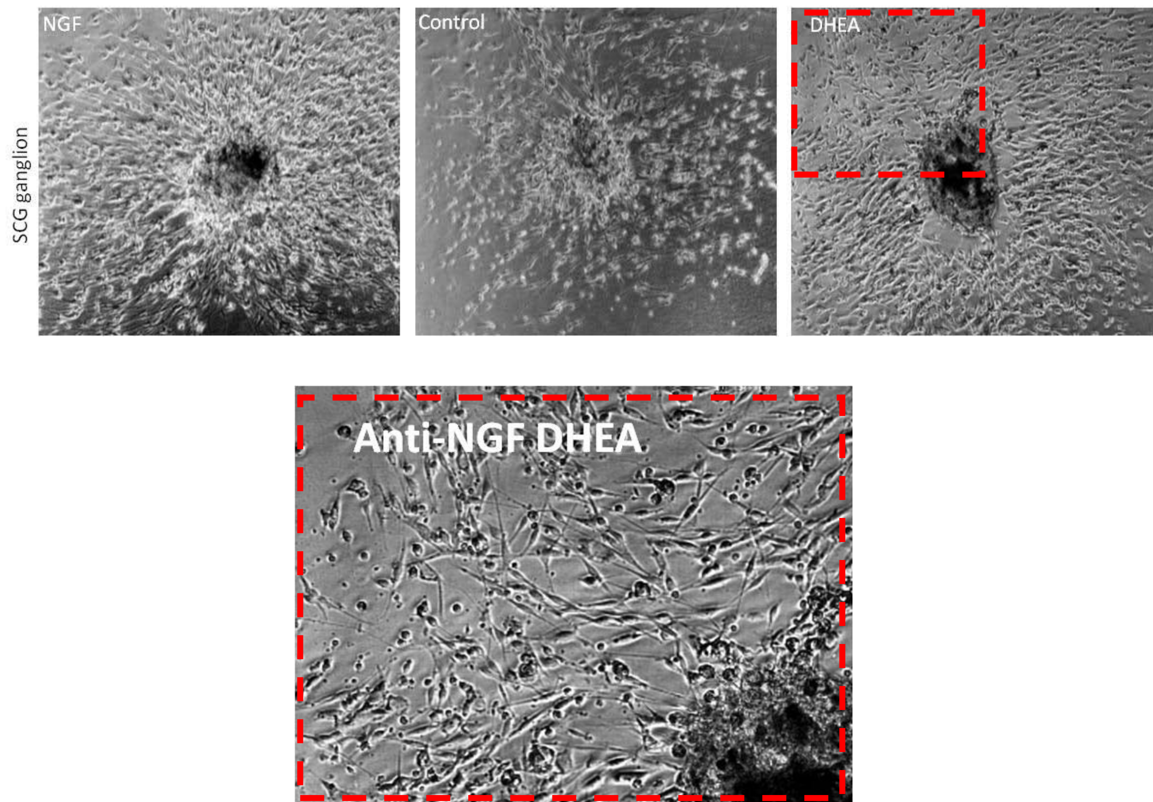


Figure 3.16 DHEA increased the number of non-neuronal cells in the surrounding of SCG explants. *Explants of superior cervical ganglia dissected from P1 rat pups and directly cultured in the presence of 100ng/ml NGF, anti-NGF neutralizing antibody and 10nM DHEA. NGF and DHEA induced the proliferation and migration of resident non-neuronal cells around the SCG explants.*

3.9 DHEA reverses the apoptotic loss of TrkA positive sensory neurons in dorsal root ganglia of NGF null mouse embryos.

NGF null mice have less sensory neurons in dorsal root ganglia (DRG) due to their apoptotic loss (Cascio, C. *et al* 2000). Heterozygous mice for the NGF deletion were interbred to obtain mice homozygous for the NGF gene disruption. The mothers were treated daily with an intraperitoneal injection of DHEA (2 mg) or vehicle (4.5% ethanol in 0.9% saline). Embryos were collected at E14 day of pregnancy and sections were stained for Caspase 3 and Fluoro jade C, markers of apoptotic and degenerative neurons, respectively. *ngf-/-* embryos at E14 showed a dramatic increase in the number of Fluoro Jade C and Caspase-3 positive neurons in the DRG compared to the *ngf+/-* embryos (Figures 3.17a and b).

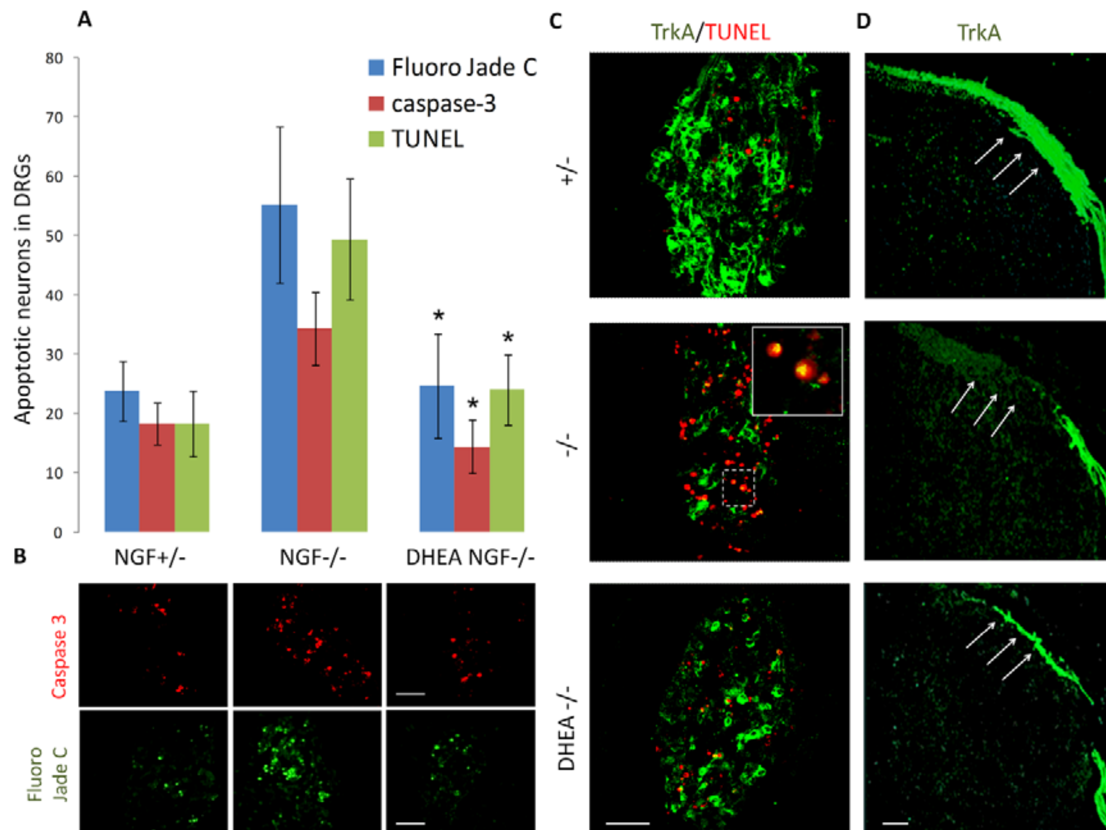


Figure 3.17 DHEA decreases the apoptotic loss of TrkA positive sensory neurons in dorsal root ganglia of NGF null mouse embryos. *Heterozygous mice for the NGF deletion were interbred to obtain mice homozygous for the NGF gene disruption. The mothers were treated daily with an intraperitoneal injection of DHEA (2 mg) or vehicle (4.5% ethanol in 0.9% saline). Embryos were collected at E14 day of pregnancy and sections were stained for various apoptotic and neuronal markers: (a) Caspase 3, Fluoro Jade C and TUNEL positive neurons were counted. The results shown are the mean±SE from 3 embryos in each group. In each embryo apoptotic neurons were counted in at least 8 sections from different DRGs (* P<0.01 versus NGF null mice). (b) staining for Caspase 3 and Fluoro jade C, markers of apoptotic and degenerative neurons, respectively, (c) double staining for TrkA positive and TUNEL apoptotic neurons, (d) TrkA positive collaterals of DRG sensory neurons and (c) neurofilament 200kD positive axons. Scale bars at b, c and d: 200μm.*

DHEA treatment significantly reduced Fluoro Jade C and Caspase 3 positive neurons in the DRG to levels of *ngf+/-* embryos. Furthermore, TrkA and TUNEL double staining of DRGs has shown that in *ngf+/-* embryos, numbers of TUNEL-positive apoptotic neurons were minimal, while TrkA positive staining was present in a large number of neuronal cell bodies of the DRG and their collaterals were extended within the marginal

zone to the most dorsomedial region of the spinal cord. On the contrary, in DRG of *ngf-/-* embryos levels of TUNEL-positive apoptotic neurons were dramatically increased while TrkA neuronal staining was considerably decreased and DRG collaterals of the dorsal funiculus were restricted in the dorsal root entry zone (Figure 3.17c). DHEA treatment resulted in a significant increase of TrkA positive staining and the extension of TrkA staining within the marginal zone to the most dorsomedial region of the spinal cord similarly to the *ngf+/-* embryos but with reduced width (Figure 3.17d), while staining of TUNEL-positive apoptotic neurons was decreased to levels shown in *ngf+/-* embryos.

3.10 DHEA restores the network of NF 200kd positive fibers and reverses the apoptotic los of motor neurons in NGF$^{-/-}$ E14 embryos.

In *ngf+/-* mice, Neurofilament 200Kd was present in the white matter, commissural axons, dorsal and ventral root filaments, DRGs and the spinal nerve. Also, some collaterals from the dorsal funiculus were entering the dorsal spinal cord from the dorsomedial region of the dorsal funiculus. Furthermore, positive axons were present in the gray matter with a higher density in the anterior gray horn in the region of motor neurons (Figure 3.18 1st, 2nd and 4th row). TH was observed in the dorsal root filaments and in a large number of cell bodies in the DRGs (figure 3.18 4th row). *ngf-/-* mice showed a decrease in the density of neurofilament 200Kd network in the gray matter and in DRGs, compared with the *ngf+/-* mice. A decrease was also observed in the number and florescence intensity of TH positive cells in the DRGs. Furthermore, in *ngf+/-* mice a number of TUNEL positive cells were present in the SC in the area of motor neurons that were absent in *ngf+/-* mice. n*gf-/-* mice from mothers that were treated with DHEA showed an increase in the density of neurofilament 200Kd network in both DRGs, the anterior gray horn and the collaterals from the dorsal funiculus that enter the dorsal spinal cord from the dorsomedial region of the dorsal funiculus to a degree that was indistinguishable from the *ngf+/-* mice. Furthermore, as in *ngf+/-*, there were no TUNEL positive cells in the SCs of DHEA treated *ngf-/-* embryos. There was no increase in the number of TH positive cell bodies of the DRGs, although there was an amplification in the intensity of florescence of positive cells and their axons in the dorsal funiculus. TAG-1 positive axons were reduced in NGF$^{-/-}$ mice compared to those of the *ngf+/-* mice. DHEA had no effect in the density of TAG-1 positive axons.

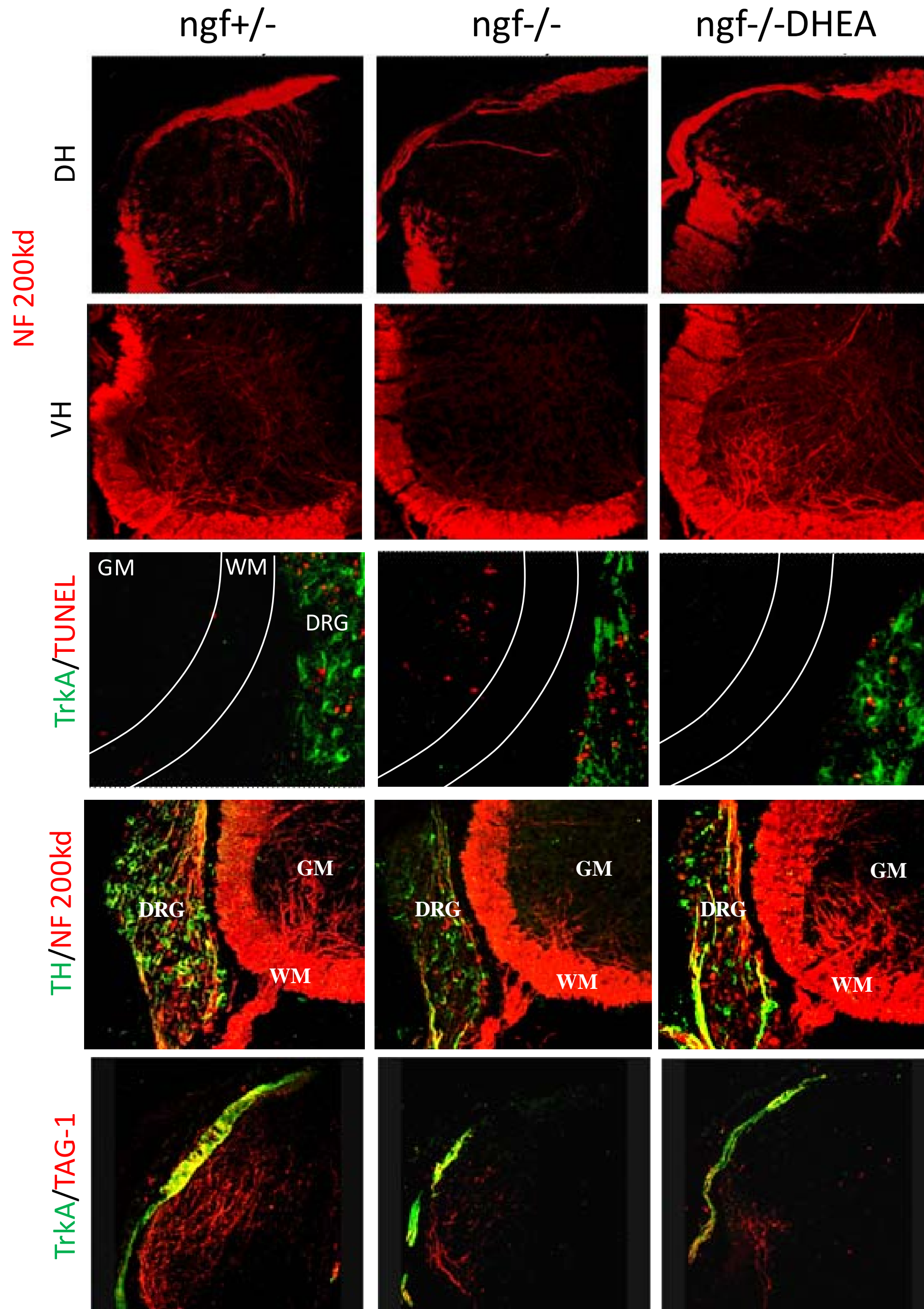


Figure 3.18 DHEA increased the density of neurofilament 200kd network in the SC and reversed the apoptotic loss of motor neurons. *Heterozygous mice for the NGF deletion were interbred to obtain mice homozygous for the NGF gene disruption. The mothers were treated daily with an intraperitoneal injection of DHEA (2 mg) or vehicle (4.5% ethanol in 0.9% saline). Embryos were collected at E14 day of pregnancy and sections were stained for neurofilament 200kD (ventrolateral view 1st row, dorsolateral view 2nd row), TrkA and TUNEL (3rd row), NF 200kd and TH (4th row) and TrkA and TAG-1 (last row).*

4. Discussion

DHEA exerts multiple actions in the central and peripheral nervous system; however no specific receptor has been reported to date for this neurosteroid. Most of its actions in the nervous tissue were shown to be mediated via modulation, at micromolar concentrations, of membrane neurotransmitter receptors, such as NMDA, $GABA_A$ and Sigma1 receptors (figure 1.3). DHEA may also influence brain function by direct binding at micromolar concentrations to dendritic brain microtubule-associated protein MAP2 (Laurine E. et al 2003)

Our findings suggest that the neuroprotective, antiapoptotic effects of DHEA are mediated through its interaction with the NGF receptors, TrkA and $p75^{NTR}$. Indeed, blocking of TrkA expression by RNAi almost completely reversed the ability of DHEA to protect PC12 cells from serum deprivation-induced apoptosis (figure 3.1) and to maintain elevated levels of the anti-apoptotic Bcl-2 protein (figure 3.2). In contrast removal of $p75^{NTR}$ had no effect indicating that TrkA is the mediator of the antiapoptotic effects of DHEA (figure 3.1). Furthermore, DHEA and NGF had the tendency to increase the levels of apoptosis in the absence of TrkA. However, these effects were not statistically important, and a possible explanation was that the deletion of TrkA was not complete. For this reason we repeated the same experiments in $PC12^{nnr5}$ cells, representing a clone of PC12 cells which have lost their ability to express TrkA thus expressing only $p75^{NTR}$. Both DHEA and NGF induced apoptosis of $PC12^{nnr5}$ cells. The pro-death effects of both agents were completely blocked by $p75^{NTR}$ shRNA and were remarkably restored after simultaneous transfection with the TrkA cDNA (figure 3.3). In contrast, addition of TrkA in the presence of $p75^{NTR}$ had no effect. It appears that the decision between survival and death among NGF-responsive cells is determined by the ratio of TrkA and $p75^{NTR}$ receptors. In fact increasing the amount of TrkA cDNA in $PC12^{nnr5}$ cells restored the antiapoptotic efficacy of both DHEA and NGF in a concentration dependent manner (figure 3.3).

To further test the hypothesis that TrkA/$p75^{NTR}$ ratio controls the effects of both DHEA and NGF we examined the effects of DHEA on the expression levels of these receptors in PC12 cells. DHEA like NGF had differential effects, increasing the levels of TrkA and reducing those of $p75^{NTR}$ (figure 3.4). However, it has been shown that the

expression of these receptors during development is independent of the presence of NGF (Davies A. et al 1995). Thus, these effects may not reflect direct effects on the expression levels; instead it is possible that this change in the levels of TrkA and $p75^{NTR}$ reflect the effects of NGF and DHEA to induce cell survival. Indeed, neurotrophic agents may induce prosurvival while inhibiting pro-death pathways. It is of note that during brain development the ratio of TrkA to $p75^{NTR}$ varies tempospatially (Barrett G.L. and Bartlett P.F. 1994), suggesting that the ability of DHEA to act as a positive or negative regulator of neuronal cell survival may depend upon the levels of the two receptors during different stages of neuronal development.

We provide evidence that DHEA binds with high affinity to NGF receptors. The efficacy of DHEA to bind with high affinity to both membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and $p75^{NTR}$ receptors (K_D: 7.8±3.1 nM and 5.9±1.7 nM for TrkA or $p75^{NTR}$, respectively) showed by saturation experiments and Scatchard analysis of [^{3}H]-DHEA binding to membranes (figure 3.5). Non-radioactive NGF was able to displace [^{3}H]-DHEA from both membrane preparations, with IC_{50}: 0.8±0.2 and 1.19±0.45 nM, respectively. Moreover, pull down experiments using DHEA covalently immobilized on NovaPEG amino resin suggest that DHEA binds directly to TrkA and $p75^{NTR}$ proteins (figure 3.9). Polymer-supported DHEA-7-CMO effectively precipitated both receptors from extracts prepared from cells expressing them ($HEK293^{TrkA}$, $HEK293^{p75NTR}$ and PC12 cells and freshly isolated rat brain) and pulled down recombinant TrkA and $p75^{NTR}$ proteins. However, DHEA was unable to displace binding of [^{125}I]-NGF from $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ transfectants. A possible explanation is that dissociation of NGF from its receptors lasts longer due to the multiple sites of interaction within the binding site of this large peptidic molecule compared to smaller in volume steroid. Another explanation might be that NGF and DHEA du not share the same binding site on NGF receptors. It is possible that DHEA due to its lipophilic nature can penetrate and bind in the intramembrane region of NGF receptors. Mutagenesis assays combined with NMR spectroscopy are planned to map the domains of both receptors related to DHEA binding.

Displacement assays showed that the binding of DHEA on both TrkA and $p75^{NTR}$ receptors was effectively competed by testosterone (IC_{50}: 3.3 and 7.4 nM, respectively)

while synthetic glucocorticoid dexamethasone displaced DHEA binding only from TrkA receptor (IC_{50}: 10.5 nM) (figure 3.8). In a previous study we had shown that both steroids effectively displaced DHEA from its specific membrane binding sites of PC12 cells, acting as DHEA antagonists by blocking both the anti-apoptotic effect and the induction of anti-apoptotic Bcl-2 proteins (Charalampopoulos I. et al 2006). Testosterone and glucocorticoids may antagonize endogenous DHEA and NGF for their binding to NGF receptors thus acting as neurotoxic factors (Yang S.H. et al 2002; Nuñez J.L. et al 2008). Glucocorticoids show a bimodal effect on hippocampal neurons causing acutely an increase in performance of spatial memory tasks, while chronic exposure has been associated with decreased cognitive performance, and neuronal atrophy (Lupien S.J. et al 2009). During prolonged periods of stress overexposure to glucocorticoids is detrimental to central nervous system neurons, especially in aged animals, affecting mainly the hippocampus. It is possible that part of these neurotoxic effects is due to the antagonistic effect of glucocorticoids on the neuroprotective effect of endogenous DHEA and NGF. Furthermore, glucocorticoid neurotoxicity becomes more pronounced in aged subjects since cortisol levels in the cerebrospinal fluid increase in the course of normal aging, as well as in relatively early stages of Alzheimer's disease (Lupien S.J. et al 2009). During aging and in Alzheimer's disease both DHEA and NGF brain levels are reduced, this decline might exacerbate this phenomenon, rendering neurons more vulnerable to glucocorticoid toxicity.

The pro-survival signaling pathways that are initiated by DHEA at the membrane level have been demonstrated by previous work of our lab (Charalampopoulos I. et al 2008). These pathways include MEK1/2/ERK1/2, and PI3K/Akt pro-survival kinases. Here we provide evidence that DHEA activates these kinases via NGF receptors (figure 3.11). Furthermore, down regulation of TrkA receptor by siRNAs, resulted in an almost complete ablation of the ability of DHEA to increase the phosphorylation of kinases Shc, Akt and ERK1/2. In addition to TrkA receptor, DHEA induced the activation of $p75^{NTR}$ receptor (figure 3.12). Signal transduction by $p75^{NTR}$ proceeds via ligand-dependent recruitment and release of cytoplasmic effectors to and from the receptor. DHEA-mediated activation of $p75^{NTR}$ led to the dissociation of bound RhoGDI, a protein belonging to small GTPases and interacting with RhoA and facilitated the recruitment of

two major cytoplasmic interactors of $p75^{NTR}$, TRAF6 and RIP2 proteins (Yamashita T. et al 2003).

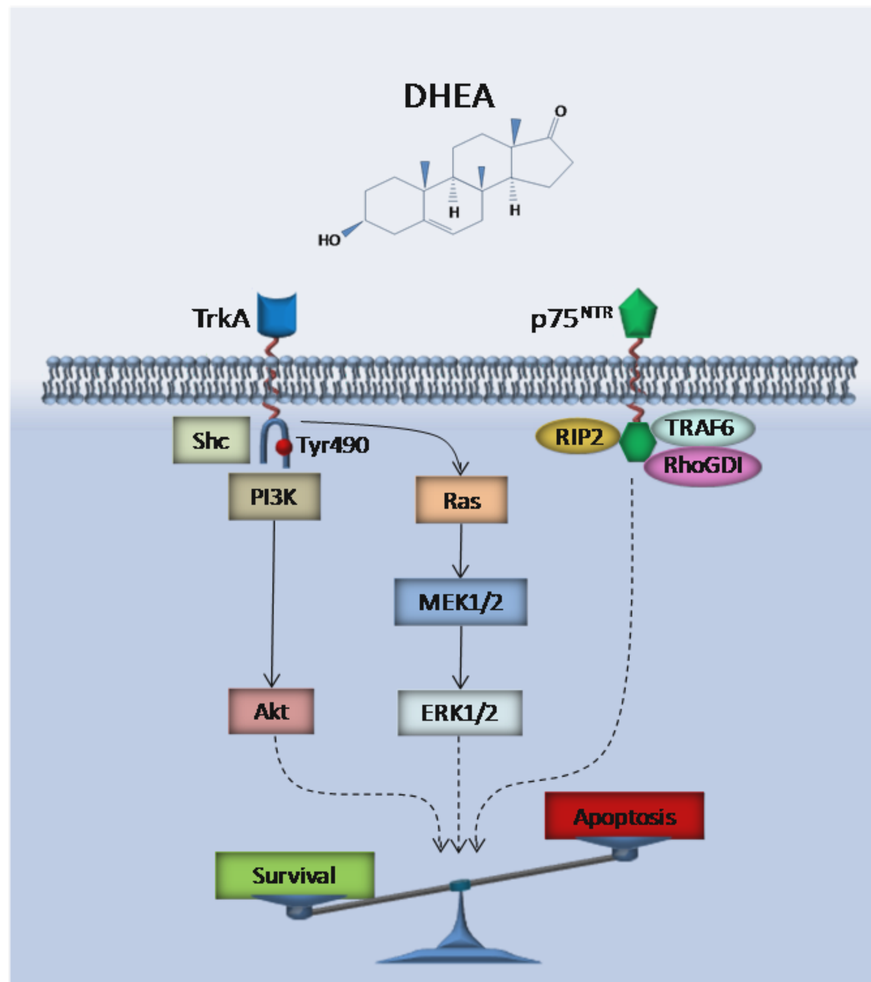


Figure 4.1 Proposed model of NGF receptor-mediated signaling pathways involved in the effects of DHEA on neuronal cell fate. *DHEA binds with high affinity to TrkA and* $p75^{NTR}$ *receptors, initiating the following sequence of events: 1) DHEA induces TrkA-mediated Tyr490-phosphorylation of Shc, ERK1/2 and Akt kinases, controlling the expression and function of apoptotic Bcl-2 proteins, 2) DHEA promotes the interaction of* $p75^{NTR}$ *receptors with effector proteins TRAF6, RIP2 and RhoGDI affecting neuronal cell apoptosis.*

Finally we tested the ability of DHEA to compensate NGF deprivation in both primary cultures of sympathetic and sensory neurons and *in vivo* in NGF null mouse. Indeed, in cultures of dispersed primary sympathetic neurons from SCGs, DHEA effectively compensated NGF deprivation by decreasing the levels of apoptotic neurons.

These effects were abolished by a specific inhibitor of TrkA receptor and were augmented by a blocker antibody for $p75^{NTR}$ (figure 3.13), further supporting the involvement of NGF receptors in the anti-apoptotic action of DHEA. Additionally DHEA effectively rescued from apoptosis TrkA-positive dorsal root ganglia sensory neurons of NGF null mouse embryos (figure 3.17). In contrast DHEA was not able to completely restore the innervation of the spinal cord by these neurons. These results show that even though DHEA has the ability to mimic the antiapoptotic effects of NGF, it does not share the same properties on neurite outgrowth. Recent findings showed that axonal growth from sensory neurons was promoted by keratinocytes when the two cell types are co-cultured (Ulmann L. et al 2009). The neuritogenic effect of keratinocytes was suppressed when the activity of NGF receptors was blocked with TrkA inhibitor K252a or by inhibitors of steroidogenesis, and it was mimicked by DHEA. Our findings also support that DHEA was able to prevent axonal retraction due to NGF deprivation in cultured DRG explants (figure 3.14). A possible explanation for this discrepancy may be that the concentration of DHEA that reaches DRGs *in vivo* is lower than that supplied in cell culture, due to its metabolism. It is possible that in the study of Ulmann L. et al the concentration used (1μM) was high enough to activate other receptor systems involved in the process.

We also show that 100nM DHEA was unable to induce neuritogenesis in SCG explants. However in these experiments DHEA increased the outward migration of non-neuronal cells from the explants to the surrounding area. It is of note that DHEA was shown to increase the proliferation and migration of oligodendrocyte precursors in E14 embryos (data not shown). Interestingly, the role of NGF on non-neuronal supporting cells both during development and after injury is well documented (Zimmermann A. and Sutter A. 1983; Marchetti D. et al 1987; Taniuchi M. et al 1988; Houle J.D. 1992; Anton E.S. et al 1994; Cornelia A. Bentley C.A. and Kuo-Fen Lee 2000; Anton B. et al 2008; Althaus H.H. et al 2008: Junhua Xiao et al 2009). NGF and its receptors are expressed in astrocytes, myelinating cells and fibroblasts that all participate in the neuritogenesis. During development, a large number of Schwann cells express NGF and its receptors. Although receptor levels are reduced later on, NGF and its receptors are upregulated after injury (Zimmermann A. and Sutter A. 1983). These observations suggest that DHEA may contribute in the development and maintenance of CNS by affecting non-neuronal

supporting cells. We are now performing experiments using compartmental cultures of sympathetic and sensory neurons either alone or cocultured with Schwann or oligodendrocytes, in order to differentiate the effects of DHEA in the cell bodies, axons and myelinating cells. In addition, we plan to use labeled and fluorescent DHEA to further test its ability to internalize TrkA receptors, a mechanism involved in both the survival and neuritogenic effects of NGF.

We have also observed a reduction in the network density of NF 200kD positive axons in the spinal cord. This reduction was present both in the dorsal part with reduced Ia muscle projections and in the ventral gray horn in the area of motor neurons that was further accompanied with apoptotic loss of motor neurons. DHEA treatment resulted in a complete reversal of this phenotype. Although, neither Ia muscle afferents nor MN are TrkA positive, there is evidence suggesting that they are indirectly dependent on NGF during development and nerve injury. Indeed, anti-NGF administration in rat embryos reduced the action potentials of hind limb nerves (Kuno M. et al 1985). Additionally, treatment with NGF increased the number of muscle spindles and monosynaptic excitatory postsynaptic potentials (EPSPs) of spinal MN through Ia muscle afferents, after nerve injury in neonatal rats (Miyata Y. et al 1986, Sekiya S. et al 1986). Myoblasts are known to express TrkA and $p75^{NTR}$ both during development and after injury. NGF promotes their differentiation and development (Rende M. et al 2000), while chronic deprivation of NGF results in muscular dystrophy (Capsoni S. et al 2000). Complementary, other findings are showing that NGF does not prevent the apoptotic loss of Ia neurons in DRGs (Yip H.K. et all 1984). Summarizing all the aforementioned information regarding NGF participation in spinal cord networks, it's proposed that NGF may hold a role in muscle and/or spinal cord innervation. However, it is known that in mice the innervation of MN by Ia fibers is proceeding and required for the phenotypic establishment and the former peripheral innervation of Ia afferents (Kucera J. et al 1995), and later on it is followed by the innervation and functionality of muscles. These, together with the fact that muscle innervation and muscle spindle formation are not yet established at E14 embryos, favor a different explanation which is that NGF may be required for the establishment of the proper innervation of MN by Ia afferents in the spinal cord.

Evidence supporting the above hypothesis came from studies on $NT3^{-/-}$, $TrkC^{-/-}$ and

NT3 overexpression (Ernsberger U. 2009). Interestingly the phenotype of NT3 overexertion is very similar to that of $NT3^{-/-}$ and $TrkC^{-/-}$ with notable limb ataxia, associated to dysfunction of the limb proprioceptive system (Ringstedt T. et al 1997; Zhang L. et al 1994). Furthermore NT3 overexpression results in abnormalities in the innervation of spinal cord by Ia afferents and in incomplete maturation of proprioceptive phenotype without influencing their survival. In particular, the afferents fail to reach the ventral gray horn and form synaptic contacts with MN; instead, they are restricted in the dorsal part of the spinal cord indicating differential requirement of NT3 for survival and target innervation. These morphological changes have a great resemblance with our observations in NGF null mice, suggesting a possible crosstalk between various neurotrophins. In these studies it is postulated that a proper gradient of NT3 towards MN is required for the proper sprouting of Ia afferents. This hypothesis is further supported by the observation of ectopic fibers towards NT3 rich sites (Ringstedt T. et al 1997). However, neither NT3/BAX null mice (Patel T.D. et al 2003; Genc B. et al 2004) nor neutralization of NT3 (Oakley et al 1995) prevent Ia afferents from reaching the ventral gray horn, indicating that even though NT3 is required for proper innervation of MN, other mechanisms may also contribute to their proper projection in the ventral gray horn.

It is possible that NGF has indirect effects through the regulation of the expression of repellent factors and/or the sensitivity of growing axons to them. Indeed the innervation site of different sensory neurons is governed by a combination of positive and negative signals by neurotrophins and repellent factors respectively (Tear G. 1999; Koeberle P.D. and Bähr M. 2004; Masuda T. and Shiga T 2005). One of these factors is semaphorin 3A that binds on neuropilin-1 on NGF dependent neurons and acts as a repellent factor. Early in embryogenesis, semaphorin 3A is expressed throughout the spinal cord preventing premature sensory innervation. At later stages this expression is restricted to the ventral part of the spinal cord allowing TrkA positive nociceptors to innervate the dorsal gray horn. Although TrkC positive proprioceptive fibers are also prevented from entering the gray matter at earlier stages, they became insensitive to semaphorin 3A by downregulating the expression of neuropilin-1 at later stages thus projecting to the ventral horn (Pond A. et al 2002). The exact area of innervation and synapses formation for the different types of sensory neurons is established by the combined signaling of neurotrophins and repellant factors. For instance, NGF administration blocks the repellant

effects of semaphorin 3A without preventing the inhibition of neurite outgrowth of TrkA positive sensory neurons (Dontchev V.D. et al 2002; Ben-Zvi A. et al 2008). Thus, as the axons enter the gray matter semaphorin 3A prevents them from entering the ventral horn by inducing retraction, while the NGF secreted by the innervation targets in combination with semaphorin 3A “slows down” the extending axons over the targets and induces axonal sprouting and synapse formation.

Another possible explanation for the observed restriction of Ia fibers in the dorsal gray horn in NGF null mice is that except from the chemoattractant effects of NT3, the opposing effects of other neurotrophins on growing axons may also play a role. In particular, in addition to the role of NT3 gradient, the reason that normally TrkC Ia afferents do not stop in the dorsal gray matter, which normally is the end point of TrkA positive afferents, may be the opposing effects of NGF through $p75^{NTR}$. This hypothesis is further supported by evidence showing that Ia afferents fail to project in the ventral horn in $NT3^{+/-}$ mice only when $p75^{NTR}$ was also deleted (Fan G. et al 1999). However deletion of $p75^{NTR}$ alone did not affect the projection of Ia afferents, at least in E14 embryos, suggesting that in the absence of $p75^{NTR}$, NT3 becomes necessary for proper innervation of Ia afferents. In summary: 1) neurotrophins/repellent factors ratio acts as a “brake” that stops axons and induces synapses formation, 2) NT3 is not required for the projection of Ia afferents in the ventral horn, 3) NT3 becomes necessary in the absence of $p75^{NTR}$ and 4) NT3 overexpression induces a similar phenotype as NGF null mice at E14 embryos. These observations taken together postulate that NGF may contribute in neurite outgrowth of Ia afferents through $p75^{NTR}$. It is possible that the action of the non-preferred neurotrophins through $p75^{NTR}$ participate in the avoidance of incorrect innervation by accelerating neurite outgrowth (figure 4.2). This hypothesis is further supported by evidence showing that NT3 controls the innervation of the layers of cerebral cortex not only by inducing sprouting of the preferred axons but also by exerting opposing effects on different neuronal subtypes (Castellani V. and Bolz J. 1999). Our future studies of neurite behavior in compartmental cultures, using combinations of neurotrophin gradients in the presence or absence of specific inhibitors for their receptors and/or repellent factors, will provide evidence on possible interactions between different neurotrophins and repellent factors and their functional role in the innervation process.

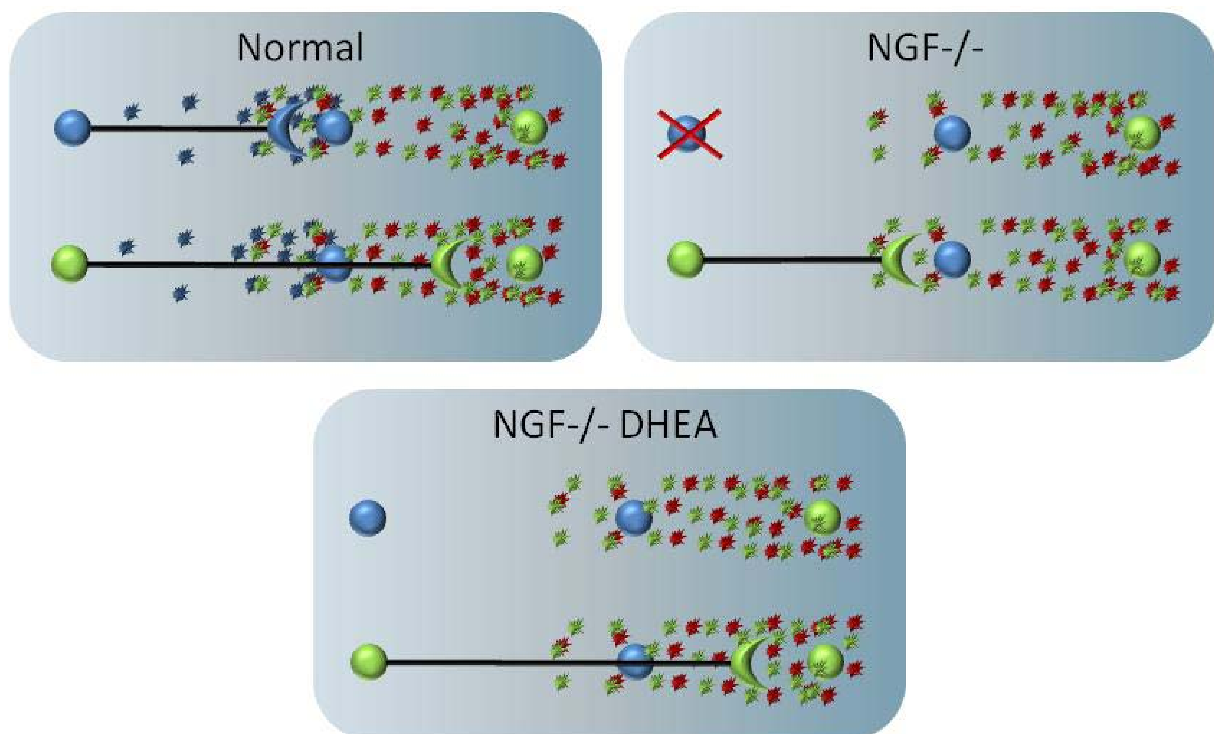


Figure 4.2 Crosstalk between neurotrophins during central innervation of sensory neurons. *In normal mice the ratio NGF/semaphorin 3A controls the innervation of NGF dependent neurons in the dorsal gray horn. TrkC positive neurons that are insensitive to semaphorin 3A project towards the ventral gray horn where NT3 induces innervation of MN. NGF promotes neurite outgrowth of NT3 dependent neurons through* $p75^{NTR}$*. In* $ngf^{/-}$ *mice TrkA positive fibers fail to enter the spinal cord due to their apoptotic loss. NT3 dependent axons lag their projection to the ventral gray horn due to the absence of the promoting signal of NGF. DHEA treatment reverses the apoptotic loss of TrkA positive neurons without completely reversing their innervation and mimics the neurite outgrowth signal of NGF on TrkC positive neurons through* $p75^{NTR}$*. Bleu: TrkA positive cells and NGF, Grin: TrkC positive cells and NT3, Red: semaphorin 3A.*

The interaction of DHEA with the NGF system was first suggested fifteen years ago by Compagnone et al, showing co-localized staining of CYP17, the rate limiting enzyme of DHEA biosynthesis, and NGF receptors in mouse embryonic DRGs (Compagnone et al 1995). About one fifth of CYP17-immunopositive DRG neurons in the mouse were found to be also TrkA-immunopositive. Among the TrkA-expressing cells, about one third also expresses CYP17, while $p75^{NTR}$-expressing neurons represent only 13% of the cells in the DRG. Thus, about one fifth of CYP17-immunopositive neurons may be able

to respond to both DHEA and NGF stimulation. Furthermore, the expression of CYP17 starts as early as E10.5 in neural crest derived cells, and coincides with the expression of TrkA expression (Marmigere F. and Ernfors P. 2007), suggesting a possible role of DHEA in the induction of TrkA expression during embryogenesis. This hypothesis is supported by our *in vitro* findings, showing a stimulatory effect of DHEA on TrkA levels in PC12 cells.

Recent studies have shown the expression of CYP17 in invertebrate cephalochordata Amphioxus (Mizuta T. and Kubokawa K. 2007). Amphioxus is also expressing the TrkA receptor homologous AmphiTrk (Benito-Gutierrez E et al 2005). Although this receptor has been shown to bind and be activated by all neurotrophins, till now no ligand has been found in Amphioxus. Phylogenetic analysis of neurotrophins revealed that they emerged with the appearance of vertebrates (530-550 million years ago), when complexity of neural tissue increased (Hallbook F. 1999). Interestingly CYP17 is also considered to be an innovation of vertebrates (Mizuta T. and Kubokawa K. 2007). Invertebrate cephalochordata like Amphioxus are positioned on the phylogenetic boundary with vertebrates (600 million years ago). It is thus tempting to hypothesize that DHEA contributed as one of the "prehistoric" neurotrophic factors in an ancestral, simpler structurally invertebrate nervous system (Miller G. 2009), then, when a strict tempospatial regulation of evolving nervous system of vertebrates was needed, peptidic neurotrophins emerged to afford rigorous and cell specific neurodevelopmental processes. Furthermore the flexibility was further increased by $p75^{NTR}$ which evolved together with neurotrophins. It is thus, possible that DHEA was the main ligand for the proto Trk receptor, AmphiTrk. Vertebrate Trk receptors evolved by domain and exon shuffling, thus it is possible that DHEA might have preserved its ability to bind to all Trk's. We are now testing the binding affinity of DHEA to TrkB, TrkC and AmphiTrk receptors.

In conclusion, our findings suggest that DHEA and NGF cross-talk via their binding to NGF receptors. This cross-talk may play important roles both during development affording brain shaping, and during adulthood effectively protecting neurons from various challenges. It is of note that a number of neurodegenerative conditions are associated with lower production or action of both DHEA and NGF (Weill-Engerer S. et al 2002; Capsoni S. et al 2000). Animal studies suggest that NGF may reverse or slow down the

progression of Alzheimer's related cholinergic basal forebrain atrophy (Capsoni S. et al 2000). Furthermore, the neurotrophic effects of NGF in experimental animal models of neurodegenerative conditions, like MPTP (Parkinson's disease), experimental allergic encephalomyelitis (multiple sclerosis) or ischemic retina degeneration mice (Villoslada P. et al 2000; Shimoke K. and Chiba H. 2001; Sivilia S. et al 2009) support its potential as a promising neuroprotective agent. However, the use of NGF in the treatment of these conditions is limited because of its poor brain blood barrier permeability. It is of interest that DHEA also exerts neuroprotective properties in some of these animal models (Belanger N. et al 2006; Du C. et al 2001).

The circulating concentrations of DHEA decrease markedly during aging, and have been associated to age-related cognitive decline. This has led to the hypothesis that DHEA supplementation during aging may improve mental health by preventing neuronal loss (Maninger N. et al 2009). In rodents, DHEA possesses important neuroprotective and neurogenic properties *in vitro* and *in vivo*, but it is unclear whether these effects are mediated indirectly through its conversion to estrogens or to androgens. Indeed, naturally occurring DHEA is metabolized in humans into estrogens or androgens which are known to exert important generalized endocrine side effects, including hormone-dependent neoplasias, thus, making the long term clinical use of DHEA dubious. These findings suggest that synthetic DHEA analogs, deprived of endocrine effects, may represent a new class of brain blood barrier permeable NGF receptor agonists with neuroprotective properties. We have recently reported the synthesis of 17-spiro-analogs of DHEA, with strong anti-apoptotic and neuroprotective properties, deprived of endocrine effects (Calogeropoulou T. et al 2009), which are now being tested for their ability to bind and activate NGF receptors. These compounds are also tested as potential neuroprotective and neurogenic agents, using various animal models of neurodegenerative diseases, such as the experimental allergic encephalomyelitis mice (Multiple Sclerosis), the MPTP mice (Parkinson disease) and the retina degeneration in diabetic mice.

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QUALIFICATIONS

2007- 2011 **University of Crete (Greece)**
PhD Department of Pharmacology

- Title:
Neurosteroids with neuroprotective and neuroregenerative properties: assessment of their in vivo efficacy in animal models. Supervisor: Achille Gravanis

2004- 2006 **University of Crete (Greece)**
MSc. "Brain and Mind"
Neuroscience interdepartmental program

- Key modules included:
Structure of the nervous system, Systems neuroscience, Computational neuroscience, Psychology-Neurophilosophy, Synaptic interactions in the cortex, Oculomotor control, Cerebral cortex and cognition , Analysis and modelling of signals, Anthropology of the symbolic systems

2001- 2002 **University of Manchester (UK)**
MSc. "Immunology and immunogenetics"

- Dissertation title:
Effects of local denervation on the migration of antigen specific T and B cells in the spleen. Supervisor: Eric B. Bell

1999-2001 **University of Hertfordshire (UK)**
BSc. "Molecular Biology"

- Dissertation title:
Activation of HLA class I surface expression on Acute Monocytic Leukaemia cells (THP1) by interferon in the presence of dexamethasone. Supervisor: Niall McMullan

WORKING EXPERIENCE

2004 – until now **University of Crete**
Department of Pharmacology

- Duties involved the study of the role of neurosteroids in the neurodegeneration, working both on *in vitro* (primary cultures of DRGs, SCGs and hippocampal neurons and cell lines) and *in vivo* models (EAE, MPTP, NGF-/- and cryolesion animal models). The study involved FACS analysis, immunohistochemistry and immunofluorescence. Supervisor: Achille Gravanis

2002 - 2003 **Hellenic Pasteur Institute**
Department of neurobiology and immunology

- Duties involved the study of myasthenia gravis: ELISA, FACS analysis, RIA, immunohistochemistry and immunofluorescence. Supervisor: Socrates J. Tzartos

2001-2002 **University of Manchester**
Department of immunology

- *Effects of local denervation on the migration of antigen specific T and B cells in the spleen.* The approach was to denervate the spleen using chemical methods and to examine the effects on the migration, localisation and interaction of T and B-lymphocytes after antigen stimulation. The study involved FACS analysis and immunohistochemistry**.** Supervisor: Eric B. Bell

TECHNIQUES

- In vivo models (mice and rats: EAE, MPTP, NGF-/- and cryolesion)
- Perfusion, tissue preparation and sectioning (cryostat and *vibrotom)*
- Immunofluorescence and immunohistochemical staining (Confocal laser scanning microscopy)
- FACS analysis
- Cell cultures (cell lines and primary cultures)
- Transfection, Western blotting, PCR, RIA, ELISA

PUBLICATIONS

Ioannou M, Alissafi T, **Lazaridis I**, Deraos G, Matsoukas I, Gravanis A, Mastorodemos V, Plaitakis A, Boumpas D, Verginis P, Crucial role of granulocytic myeloid-derived suppressor cells in the regulation of the central nervous system autoimmune disease. *Immunity.* Submitted

Rassouli O, Liapakis G, **Lazaridis I**, Sakellaris G, Gkountelias K, Gravanis K, Margioris AN, Karalis KP, and Venihaki M. A novel role of peripheral corticotropin-releasing hormone (crh) on dermal fibroblasts. *PLoS ONE.* Under revision.

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Chapter 4.2 Neuroprotective and neurogenic properties of dehydroepiandrosterone and its synthetic analogs (Charalampopoulos I, **Lazaridis I** and Gravanis A)

ORAL COMMUNICATIONS

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Neurosteroid Dehydroepiandrosterone Interacts with Nerve Growth Factor (NGF) Receptors, Preventing Neuronal Apoptosis

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Abstract

The neurosteroid dehydroepiandrosterone (DHEA), produced by neurons and glia, affects multiple processes in the brain, including neuronal survival and neurogenesis during development and in aging. We provide evidence that DHEA interacts with pro-survival TrkA and pro-death $p75^{NTR}$ membrane receptors of neurotrophin nerve growth factor (NGF), acting as a neurotrophic factor: (1) the anti-apoptotic effects of DHEA were reversed by siRNA against TrkA or by a specific TrkA inhibitor; (2) [^{3}H]-DHEA binding assays showed that it bound to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and $p75^{NTR}$ receptors (K_D: 7.4±1.75 nM and 5.6±0.55 nM, respectively); (3) immobilized DHEA pulled down recombinant and naturally expressed TrkA and $p75^{NTR}$ receptors; (4) DHEA induced TrkA phosphorylation and NGF receptor-mediated signaling; Shc, Akt, and ERK1/2 kinases down-stream to TrkA receptors and TRAF6, RIP2, and RhoGDI interactors of $p75^{NTR}$ receptors; and (5) DHEA rescued from apoptosis TrkA receptor positive sensory neurons of dorsal root ganglia in NGF null embryos and compensated NGF in rescuing from apoptosis NGF receptor positive sympathetic neurons of embryonic superior cervical ganglia. Phylogenetic findings on the evolution of neurotrophins, their receptors, and CYP17, the enzyme responsible for DHEA biosynthesis, combined with our data support the hypothesis that DHEA served as a phylogenetically ancient neurotrophic factor.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CYP17, cytochrome P450 17-hydroxylase/17,20-lyase; DHEA, dehydroepiandrosterone; DHEA-BSA, DHEA–Bovine Serum Albumin; DHEA-BSA-FITC, DHEA-BSA–Fluorescein Isothiocyanate; DHEA-PEG, DHEA–polyethylene glycol amino resin; DHEAS, dehydroepiandrosterone sulfate; DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK293, human embryonic kidney cell line 293; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF, nerve growth factor; $p75^{NTR}$, p75 neurotrophin receptor; PTX, pertussis toxin; RhoGDI, rho GDP dissociation inhibitor; RIP2, receptor-interacting protein 2; SCG, superior cervical ganglia; TRAF6, TNF receptor-associated factor 6; Trk, tropomyosin related kinase

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Introduction

Dehydroepiandrosterone (DHEA) is a steroid, produced in adrenals, in neurons and in glia [1]. The physiological role of brain DHEA appears to be local, i.e. paracrine, while that produced from adrenals, which represents the almost exclusive source of circulating DHEA, is systemic. The precipitous decline of both brain and circulating DHEA with advancing age has been associated with aging-related neurodegenerative diseases [1,2]. It is experimentally supported that DHEA protects neurons against noxious conditions [3–6]. DHEA exerts its multiple pro-survival effects either directly modulating at micromolar concentrations γ-aminobutiric acid type A ($GABA_A$), N-methyl-D-aspartate (NMDA), or sigma1 receptors, or following its conversion to estrogens and androgens. We have recently shown that nanomolar concentrations of DHEA protect sympathoadrenal PC12 cells from apoptosis [7]. PC12 cells do not express functional $GABA_A$ or NMDA receptors and cannot metabolize DHEA to estrogens and androgens [8]. The anti-apoptotic effect of DHEA in PC12 cells is mediated by highly affinity (K_D at nanomolar levels) specific membrane binding sites [9]. Activation of DHEA membrane binding sites results in an acute, transient, and sequential phosphorylation of the pro-survival MEK/ERK kinases, which, in turn, activate transcription factors CREB and NFκB, which afford the transcriptional control of anti-apoptotic Bcl-2 proteins. In parallel, activation of DHEA membrane binding sites induces the phosphorylation of PI3K/Akt kinases, leading to phosphorylation/deactivation of the pro-apoptotic Bad protein and protection of PC12 cells from apoptosis [10].

In fact, the anti-apoptotic pathways in sympathoadrenal cells initiated by DHEA at the membrane level strikingly resemble those sensitive to neurotrophin nerve growth factor (NGF). NGF promotes survival and rescues from apoptosis neural crest–derived sympathetic neurons (including their related sympathoadrenal cells) and sensory neurons involved in noniception. NGF binds with high affinity (K_D: 0.01 nM) to transmembrane tyrosine kinase

Author Summary

Dehydroepiandrosterone (DHEA) and its sulphate ester are the most abundant steroid hormones in humans, and DHEA was described as the first neurosteroid produced in the brain. DHEA is known to participate in multiple events in the brain, including neuronal survival and neurogenesis. However, to date no specific cellular receptor has been described for this important neurosteroid. In this study, we provide evidence that DHEA exerts its neurotrophic effects by directly interacting with the TrkA and $p75^{NTR}$ membrane receptors of nerve growth factor (NGF), and efficiently activates their downstream signaling pathways. This activation prevents the apoptotic loss of NGF receptor positive sensory and sympathetic neurons. The interaction of DHEA with NGF receptors may also offer a mechanistic explanation for the multiple actions of DHEA in other peripheral biological systems expressing NGF receptors, such as the immune, reproductive, and cardiovascular systems.

TrkA receptor and with lower affinity (K_D: 1.0 nM) to $p75^{NTR}$ receptor, a membrane protein belonging to the TNF receptor superfamily [11]. In the presence of TrkA receptors, $p75^{NTR}$ participates in the formation of high affinity binding sites and enhances NGF responsiveness, leading to cell survival signals. In the absence of TrkA, $p75^{NTR}$ generates cell death signals. Indeed, docking of TrkA by NGF initiates receptor dimerization and phosphorylation of cytoplasmic tyrosine residues 490 and 785 on the receptor. Phosphotyrosine-490 interacts with Shc and other adaptor proteins resulting in activation of PI3K/Akt and MEK/ERK signaling kinase pathways [11]. These signals lead to the activation of prosurvival transcription factors CREB and NFκB, the subsequent production of anti-apoptotic Bcl-2 proteins, and prevention of apoptotic cell death of sympathetic neurons and sympathoadrenal cells, including PC12 cells [12].

Intrigued by the similarities in the prosurvival membrane signaling of DHEA and NGF, we set out to examine in the present study whether the anti-apoptotic effects of DHEA are mediated by NGF receptors. To address this issue we employed a multifaceted approach, designing an array of specific experiments: we used RNA interference (RNAi) to define the involvement of TrkA and $p75^{NTR}$ receptors in the anti-apoptotic action of DHEA; we assessed membrane binding of DHEA in HEK293 cells transfected with the TrkA and $p75^{NTR}$ plasmid cDNAs, using binding assays, confocal laser microscopy, and flow cytometry; to investigate the potential direct physical interaction of DHEA with NGF receptors, we tested the ability of immobilized DHEA to pull-down recombinant or naturally expressed TrkA and $p75^{NTR}$ receptors; finally, we examined the ability of DHEA 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 [13]. We provide evidence that DHEA directly binds to NGF receptors to protect neuronal cells against apoptosis, acting as a neurotrophic factor.

Results

RNA Interference against TrkA Receptors Reverses the Anti-Apoptotic Effect of DHEA

To test the involvement of NGF receptors in the anti-apoptotic effect of DHEA in serum deprived PC12 cells we have used a combination of three different sequences of siRNAs for TrkA and two different shRNAs for $p75^{NTR}$ transcripts [14]. The effectiveness of si/shRNAs was shown by the remarkable decrease of TrkA and $p75^{NTR}$ protein levels in PC12 cells, observed by immunoblotting analysis, using GAPDH as reference standard (Figure 1B). Scrambled siRNAs were ineffective in decreasing TrkA and $p75^{NTR}$ protein levels and did not significantly alter the effect of DHEA (unpublished data). FACS analysis of apoptotic cells (stained with Annexin V) has shown that DHEA and membrane impermeable DHEA-BSA conjugate at 100 nM diminished the number of apoptotic cells in serum deprived PC12 cell cultures from 53.5%±17.6% increase of apoptosis in serum free condition (control) to 6%±1.4% and 13%±5.2%, respectively ($n=8$, $p<0.01$ versus control) (Figure 1A). Decreased TrkA expression in serum deprived PC12 cells with siRNAs resulted in the almost complete reversal of the anti-apoptotic effects of NGF and DHEA or DHEA-BSA membrane-impermeable conjugate (Figure 1A). Co-transfection of serum deprived PC12 cells with the si/shRNAs for TrkA and $p75^{NTR}$ receptors did not modify the effect of the TrkA deletion alone. Furthermore, transfection of serum deprived PC12 cells with shRNAs against $p75^{NTR}$ receptor alone did not significantly alter the anti-apoptotic effects of NGF and DHEA, suggesting that their anti-apoptotic effects are primarily afforded by TrkA receptors.

Transfection of serum deprived PC12 cells with the siRNAs against the TrkA transcript fully annulled the ability of DHEA to maintain elevated levels of anti-apoptotic Bcl-2 protein (Figure 1B). Again, transfection with the shRNA against $p75^{NTR}$ receptor alone did not significantly affect Bcl-2 induction by DHEA, further supporting the hypothesis that TrkA is the main mediator of the anti-apoptotic effect of DHEA in this system.

It appears that the ratio of TrkA and $p75^{NTR}$ receptors determines the effect of DHEA or NGF on cell apoptosis and survival. Indeed, both NGF and DHEA induced apoptosis of nnr5 cells, a clone of PC12 cell line, known to express only pro-death $p75^{NTR}$ receptors (Figure 1C), confirming the pro-apoptotic function of this receptor. Blockade of $p75^{NTR}$ expression by shRNA almost completely reversed the pro-apoptotic effect of both agents. The anti-apoptotic effect of NGF and DHEA was remarkably restored after transfection of nnr5 cells with the TrkA cDNA, the efficacy of reversal being proportionally dependent on the amount of transfected TrkA cDNA (Figure 1C).

DHEA was also controlling the response of NGF receptor-positive cells, by regulating TrkA and $p75^{NTR}$ receptor levels, mimicking NGF. Serum deprived PC12 cells were exposed to 100 nM of DHEA or 100 ng/ml of NGF for 12, 24, and 48 h; TrkA and $p75^{NTR}$ protein levels were measured in cell lysates with immunoblotting, using specific antibodies against TrkA and $p75^{NTR}$ proteins, and were normalized against GAPDH. Both NGF and DHEA significantly increased pro-survival TrkA receptor levels in the time frame studied, i.e. from 12 to 48 h ($n=5$, $p<0.01$) (Figure S1). Furthermore, DHEA and NGF significantly decreased $p75^{NTR}$ receptor levels between 24 and 48 h of exposure ($n=5$, $p<0.01$).

We have also tested the anti-apoptotic effects of DHEA in neural crest deriving superior cervical ganglia (SCG), a classical NGF/TrkA sensitive mammalian neuronal tissue, containing primarily one class of neurons, principal sympathetic neurons. Indeed, NGF and TrkA receptors are absolutely required for SCG sympathetic neuron survival during late embryogenesis and early postnatal development [13,15]. TrkC receptors are barely detectable after E15.5, and no significant TrkB receptors are present in the SCG at any developmental stage [16]. Dispersed rat sympathetic SCG neurons at P1 were isolated and cultured for at least 7 d in the presence of 100 ng/ml NGF before the experiments are performed, in order to obtain an enriched,

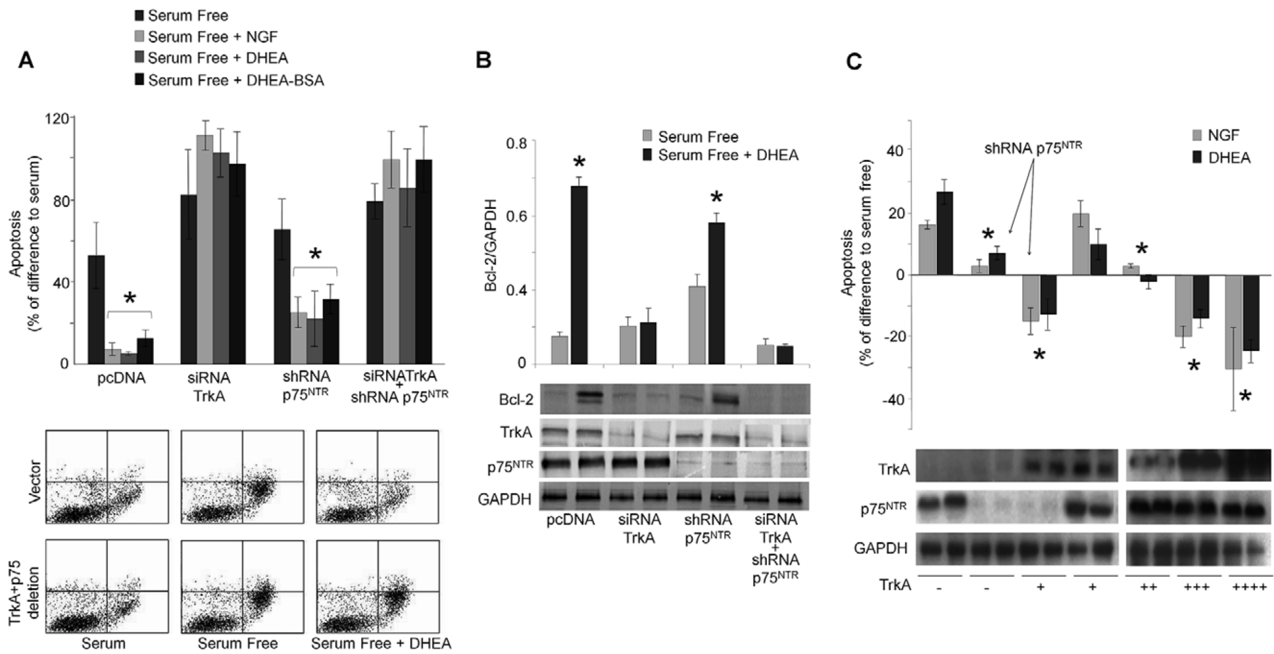

Figure 1. RNA interference against NGF receptors reverses the anti-apoptotic effect of DHEA. PC12 or PC12^{nnr5} cells were transfected with si/shRNAs of TrkA and/or p75^{NTR} (A and B) and/or expressing vectors of TrkA (c). Twenty-four hours later the medium was replaced either with complete medium (serum supplemented) or serum free medium, in the absence or the presence of DHEA, DHEA-BSA (100 nM), or NGF (100 ng/ml). Apoptosis was quantified 24 h later by FACS using Annexin V-FITC and PI. (A) *Upper panel*: levels of apoptosis expressed as % of difference from serum supplemented cells [* $p<0.01$ versus control (serum conditions), $n=8$]. *Lower panel*: representative FACS analysis of Annexin V-FITC and PI staining. (B) Levels of Bcl-2 protein in serum deprived PC12 cells with or without DHEA treatment. Cellular extracts containing total proteins were collected and levels of Bcl-2 protein were measured by Western blot, and normalized per GAPDH protein content. *Upper panel*: mean ± SE of Bcl-2 levels, normalized against GAPDH (* $p<0.01$ versus control, $n=4$), *lower panel*: representative Western blots of Bcl-2, TrkA, p75^{NTR}, and GAPDH proteins. (C) *Upper panel*: levels of apoptosis in PC12^{nnr5} cells expressed as % of difference from serum deprivation condition. (* $p<0.01$ versus control-naive cells, $n=4$). *Lower panel*: Western blots of TrkA, p75^{NTR}, and GAPDH proteins for each condition.
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quasi-homogenous (95%) neuronal cell culture. Enriched SCGs were then incubated in the presence of 100 ng/ml NGF or in the same medium as above but lacking NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum in the absence or the presence of 100 nM DHEA. Withdrawal of NGF strongly increased the number of apoptotic sympathetic neurons stained with Annexin V, while DHEA effectively compensated for NGF by decreasing the levels of apoptotic neurons. This effect was blocked by a specific TrkA inhibitor, thus suggesting the involvement of TrkA receptors as the main mediator of the anti-apoptotic action of DHEA (Figure 2).

[^{3}H]-DHEA Binds to HEK293^{TrkA} and HEK293^{p75NTR} Cell Membranes

We have previously shown the presence of specific DHEA binding sites to membranes isolated from PC12, primary human sympathoadrenal, and primary rat hippocampal cells, with K_D at the nanomolar level [9]. The presence of DHEA-specific membrane binding sites on PC12 cells has been confirmed by flow cytometry and confocal laser microscopy of cells stained with the membrane impermeable DHEA-BSA-FITC conjugate. In contrast to estrogens, glucocorticoids and androgens displaced [^{3}H]DHEA from its membrane binding sites, acting as pure antagonists by blocking the anti-apoptotic effect of DHEA in serum deprived PC12 cells [9]. In the present study, we repeated this series of experiments using membranes isolated from HEK293 cells transfected with the plasmid cDNAs of TrkA or p75^{NTR} receptors.

HEK293 cells (not expressing TrkA or p75^{NTR}) were transfected with an empty vector (control) or a specific TrkA or p75NTR vector; transfection efficiency was assessed by Western blot (Figure 3A and C,F inserts), confocal laser microscopy, and flow cytometry (Figure 3B,D). Saturation binding experiments have shown that [^{3}H]-DHEA bound to membranes isolated from HEK293 cells, transfected with the cDNAs of TrkA or p75^{NTR} receptors. Membranes isolated from HEK293 cells transfected with the empty vector showed no specific binding. The K_D values calculated after Scatchard analysis of saturation curves were, for incubation of membranes at 25°C for 30 min, 7.4±1.75 nM and 5.6±0.55 nM for TrkA or p75^{NTR}, respectively ($n=3$) (Figure 3A,C), and for overnight incubation of membranes at 4°C, 7.8±3.1 nM and 5.9±1.7 nM for TrkA or p75^{NTR}, respectively ($n=3$) (Figure S2). DHEA was previously shown to bind with low affinity (K_D: 2 μM) to androgen receptors (AR) [17]. We have thus tested the hypothesis that specific binding of DHEA to membranes of HEK293 cells transfected with the TrkA and p75^{NTR} cDNAs might be due to the presence of AR receptors, induced by the transfection with NGF receptors. However, RT-PCR analysis showed no detectable levels of androgen receptors mRNA in RNA preparations isolated from naïve and TrkA or p75^{NTR} transfected HEK23 cells (Figure S3).

Transfection of PC12 cells, endogenously expressing NGF receptors, with shRNAs against both TrkA and p75^{NTR} receptors resulted in a complete loss of [^{3}H]-DHEA specific membrane binding (Figure 3E,F). To rule out the possibility that the loss of specific binding might be due to the transfection process, we tested

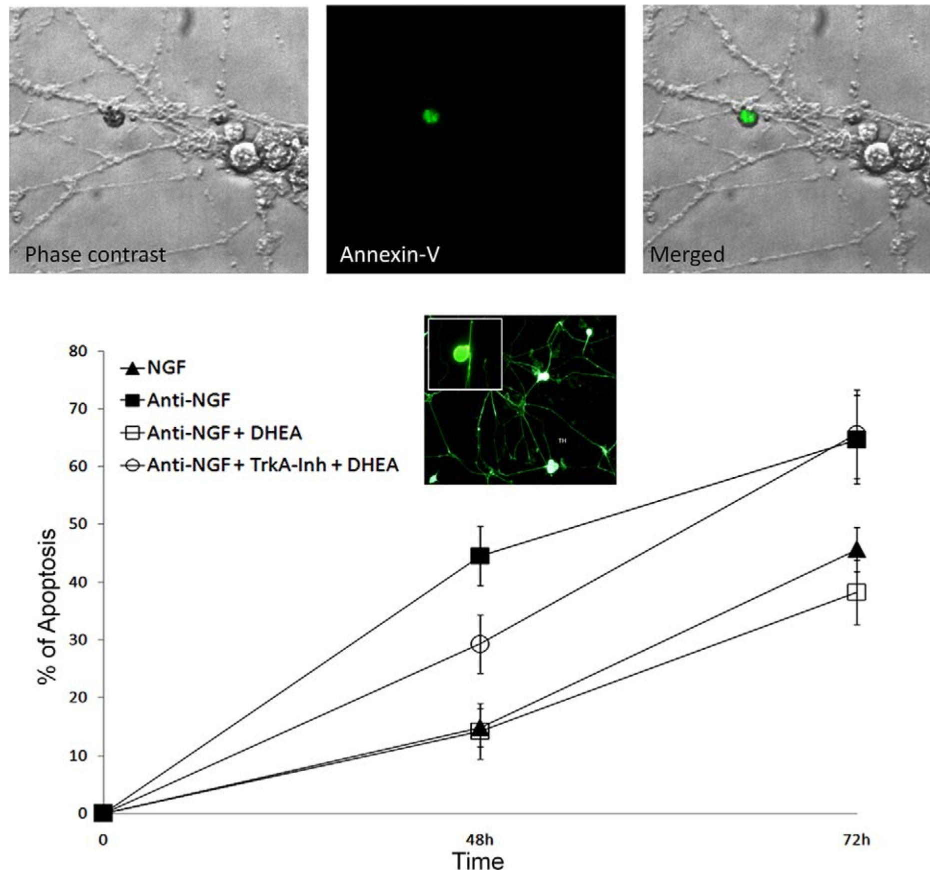

Figure 2. DHEA rescues TrkA positive primary sympathetic neurons from NGF deprivation-induced apoptosis, in a NGF receptor dependent manner. Light and Annexin V-FITC-green stained fluorescence microscopy photographs of dispersed primary sympathetic neurons in culture, isolated from rat superior cervical ganglia (SCG) at P1. SCG dispersed neurons were isolated and cultured for at least 7 d in the presence of the antimitotic drug cytosine-beta-D-arabinofuranoside (AraC) and of NGF before the experiments are performed, in order to obtain an enriched, quasi-homogenous (95%) neuronal cell culture. Sympathetic neurons were cultured in the presence of 100 ng/ml NGF or in the same medium without NGF and containing a polyclonal rabbit anti-NGF-neutralizing antiserum and/or 100 nM DHEA, in the absence or the presence of TrkA-inhibitor. The results shown are the means from three separate experiments where over 300 neurons were counted in six to seven randomly selected optical fields (*$p<0.01$ versus anti-NGF condition). Inserted photograph depicts tyrosine hydroxylase (TH) staining of sympathetic neurons.
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binding of [^{3}H]-DHEA to membranes isolated from PC12 cells transfected with siRNA against GAPDH. Saturation binding and Scatchard analysis have shown that [^{3}H]-DHEA bound to membranes from PC12-siRNA GAPDH cells with a $K_D = 1.068 \pm 0.43$ nM (Figure 3E).

The selectivity of DHEA binding to HEK293^{TrkA} and HEK293^{p75NTR} cell membranes was examined by performing heterologous [^{3}H]-DHEA displacement experiments using a number of non-labeled steroids or NGF. Binding of [^{3}H]-DHEA to membranes isolated from both HEK293^{TrkA} and HEK293^{p75NTR} cells was effectively displaced by NGF (IC_{50}: 0.8 ± 0.2 and 1.19 ± 0.45 nM, respectively) (Figure S4). NGF was also effective in displacing [^{3}H]-DHEA binding on membranes isolated from PC12 cells (IC_{50}: 0.92 ± 0.32 nM, unpublished data). Estradiol failed to displace [^{3}H]-DHEA from its binding to membranes from HEK293^{TrkA} and HEK293^{p75NTR} cells at concentrations ranging from 0.1 to 1000 nM. In contrast, displacement of [^{3}H]-DHEA binding to membranes from both HEK293^{TrkA} and HEK293^{p75NTR} cells was shown by sulfated ester of DHEA, DHEAS (IC_{50}: 6.1 ± 1.1 and 8.1 ± 1.2 nM, respectively, $n = 3$), and testosterone (Testo) (IC_{50}: 5.3 ± 2.1 and 7.4 ± 3.2 nM, respectively). Glucocorticoid dexamethasone (DEX) effectively competed [^{3}H]-DHEA binding to membranes from HEK293^{TrkA} (IC_{50}: 9.5 ± 4.6 nM) but was ineffective in displacing DHEA binding to membranes from HEK293^{p75NTR} cells. Homologous [^{125}I]-NGF displacement experiments with unlabeled NGF confirmed the presence of specific NGF binding on membranes from both HEK293^{TrkA} and HEK293^{p75NTR} cells with IC_{50} 0.3 ± 0.09 and 1.7 ± 0.38 nM, respectively. It is of note that in contrast to unlabeled NGF, DHEA was unable to displace binding of [^{125}I]-NGF to membranes isolated from HEK293^{TrkA} and HEK293^{p75NTR} transfectants (unpublished data).

DHEA-BSA-FITC Conjugate Stains HEK293^{TrkA} and HEK293^{p75NTR} Cell Membranes

Incubation of PC12 cells with the membrane impermeable, fluorescent DHEA-BSA-fluorescein conjugate results in a specific spot-like membrane fluorescent staining [9]. In the present study, we have tested the ability of DHEA-BSA-FITC conjugate to stain HEK293^{TrkA} and HEK293^{p75NTR} transfectants. Fluorescence microscopy analysis revealed that DHEA-BSA-FITC clearly stained the membranes of HEK293^{TrkA} and HEK293^{p75NTR} cells (Figure 3B,D). No such staining was found in non-transfected HEK293 cells (unpublished data) or in HEK293 cells transfected

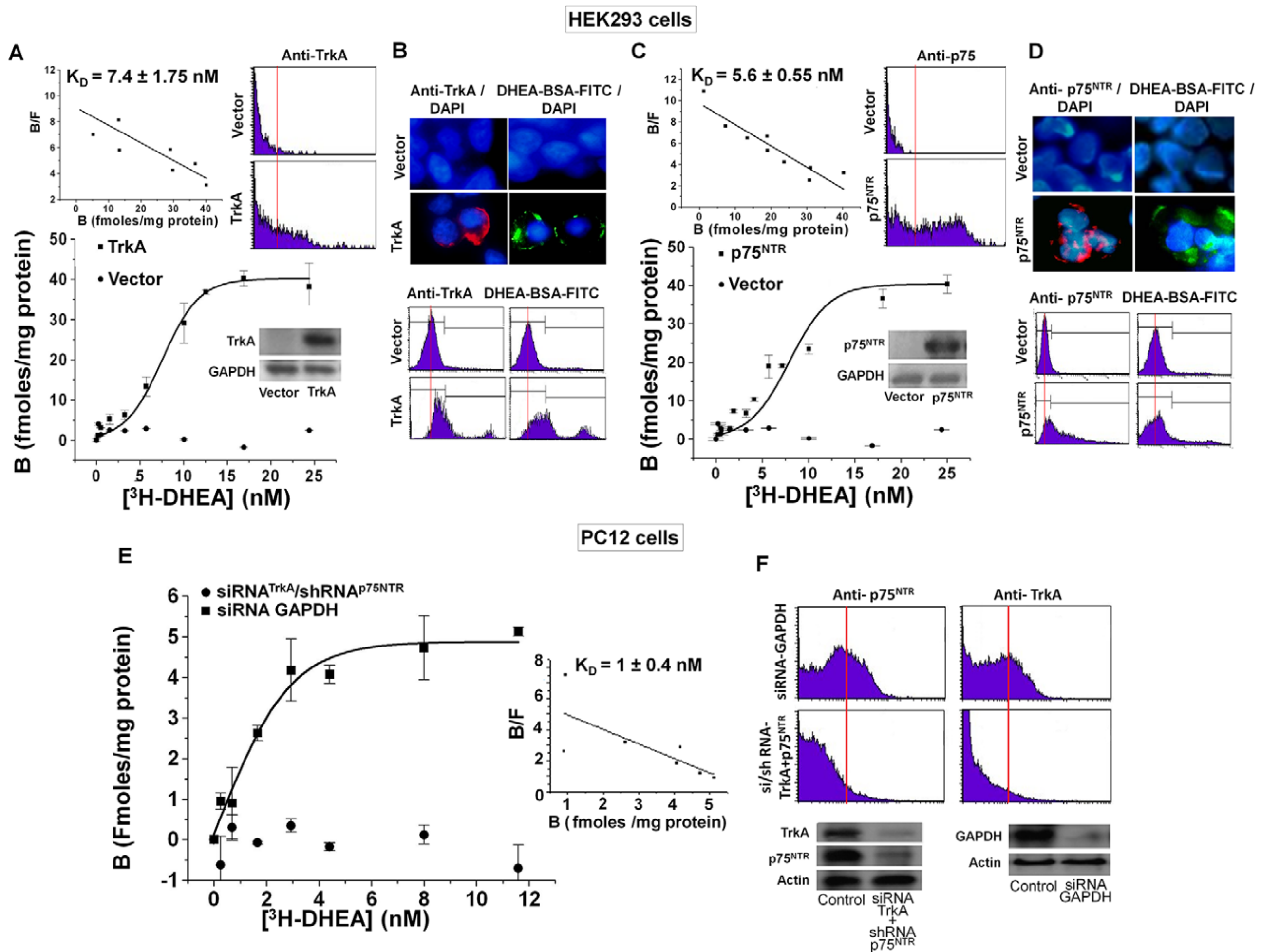

Figure 3. DHEA binds with high affinity to HEK293^{TrkA} and HEK293^{p75NTR} cell membranes. (A, C) [^{3}H]-DHEA saturation binding assays and Scatchard blots in HEK293 cells, transfected with the plasmid cDNAs of TrkA and p75^{NTR} receptors. Fifty µl of cell membrane suspension in triplicate were incubated for 30 min at 25°C with 1–30 nM [^{3}H]-DHEA in the presence or absence of 500-fold molar excess of DHEA. Western blot inserts show the efficacy of transfection (K_D represents the mean ± SE of 3 experiments) (E) [^{3}H]-DHEA saturation binding assays in PC12 cells, transfected with the si/shRNAs against TrkA and p75^{NTR} receptors or with the siRNA against GAPDH. The efficacy of transfection is shown in Western blot inserts and in FACS analysis (F), (K_D represents the mean ± SE of three experiments). (B, D) Fluorescence localization of DHEA membrane binding on HEK293 cells transfected with the plasmid cDNAs of TrkA and p75^{NTR} receptors. Transfectants were incubated with either the membrane impermeable DHEA-BSA-FITC conjugate (100 nM), BSA-FITC (100 nM), or with specific antibodies against TrkA and p75^{NTR} proteins. Transfectants were analyzed under the confocal laser scanning microscope (B) or by FACS analysis (C). Blue staining depicts Hoechst nuclear staining.
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with the vectors empty of TrkA and p75^{NTR} cDNAs (Figure 3B,D). Furthermore, BSA-FITC conjugate was ineffective in staining both transfectants (unpublished data). We have further confirmed the presence of membrane DHEA-BSA-FITC staining of HEK293^{TrkA} and HEK293^{p75NTR} cells with flow cytometry (FACS) analysis (Figure 3B,D). Specific staining was noted in both transfectants. No such staining was seen in non-transfected HEK293 cells (unpublished data) or in HEK293 cells transfected with the empty vectors (Figure 3B,D). In both fluorescence microscopy and FACS experiments membrane staining of TrkA or p75^{NTR} proteins in HEK293^{TrkA} and HEK293^{p75NTR} cells was also shown using specific antibodies for each protein (Figure 3B,D).

Immobilised DHEA Pulls Down TrkA and p75^{NTR} Receptors

Our binding assays with radiolabeled DHEA suggest that DHEA physically interacts with NGF receptors. To test this hypothesis we covalently linked DHEA-7-*O*-(carboxymethyl) oxime (DHEA-7-CMO) to polyethylene glycol amino resin (NovaPEG amino resin) and tested the ability of immobilized DHEA to pull down TrkA and p75^{NTR} proteins. Precipitation experiments and Western blot analysis of precipitates with specific antibodies against TrkA and p75^{NTR} proteins (Figure 4A) showed that immobilized DHEA effectively precipitated recombinant TrkA and p75^{NTR} proteins, while pre-incubation of the recombinant proteins with DHEA or NGF in excess abolished the ability of DHEA-PEG to pull down both receptors. Similar results were obtained when cell extracts isolated from HEK293 cells transfected with TrkA and p75^{NTR} cDNAs, PC12 cells, and whole rat brain were treated with immobilized DHEA (Figure 4B, panels marked with A). No precipitation of TrkA and p75^{NTR} proteins was shown with polymer-supported DHEA-7-CMO incubated with cell extracts from untransfected HEK293 cells or HEK293 cells transfected with the empty vectors. A control experiment was

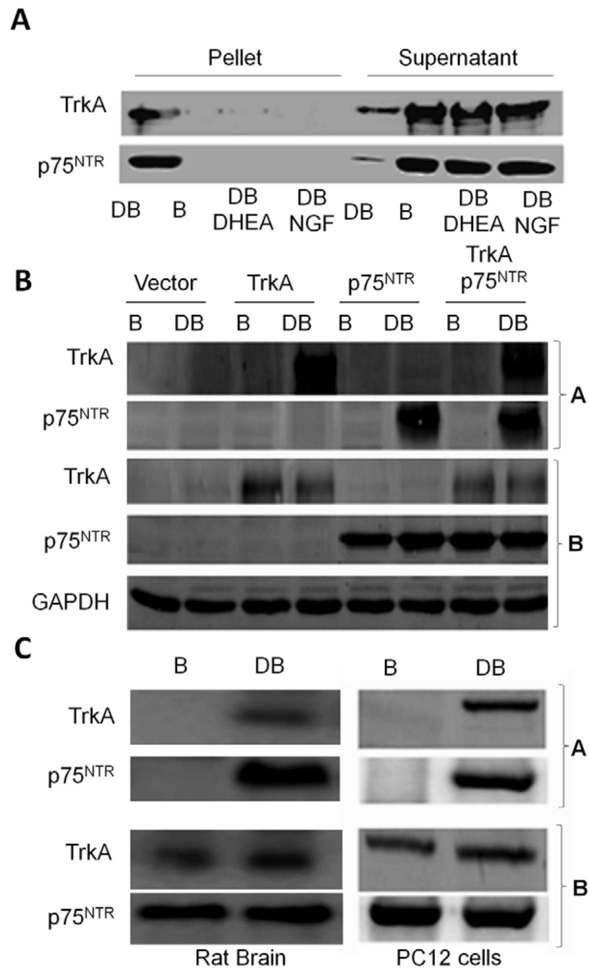

Figure 4. Immobilised DHEA pulls down TrkA and p75^{NTR} receptors. Covalently linked DHEA-7-*O*-(carboxymethyl) oxime (DHEA-7-CMO) to polyethylene glycol amino resin (NovaPEG amino resin) was incubated with recombinant TrkA and p75^{NTR} proteins (A) or with cell extracts isolated from HEK293^{TrkA} HEK293^{p75NTR} transfectants (B), PC12 cells, and whole rat brain (C). Precipitation experiments (panels marked with A show Western blot analysis of precipitates while panels marked with B show Western blot analysis of total lysates) with specific antibodies against TrkA and p75^{NTR} proteins were performed as described in Materials and Methods. DB, DHEA-7-*O*-(carboxymethyl) oxime (DHEA-7-CMO) polyethylene glycol amino resin; B: polyethylene glycol amino resin, DB-DHEA, DB-NGF pre-incubation of the recombinant proteins with DHEA or NGF, respectively; P, pellet; S, supernatant.
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performed with NovaPeg amino resin (no DHEA-7-CMO present), which was found ineffective in precipitating TrkA and p75^{NTR} proteins (Figure 4). The presence of TrkA and p75^{NTR} receptors in HEK293^{TrkA} and HEK293^{p75NTR} transfectants and in PC12 and fresh rat brain was confirmed with Western blot analysis using specific antibodies against TrkA and p75^{NTR} proteins and GAPDH as reference standard (Figure 4, panels marked with B).

DHEA Induces TrkA- and p75^{NTR}-Mediated Signaling

Previous findings have shown that NGF controls the responsiveness of sensitive cells through induction of TrkA phosphorylation and regulation of the levels of each one's receptors [18]. We compared the ability of NGF and DHEA to induce phosphorylation of TrkA in HEK293 cells transfected with the cDNAs of TrkA receptors. HEK293^{TrkA} transfectants were exposed for 10 and 20 min to 100 nM of DHEA or 100 ng/ml of NGF, and cell lysates were immunoprecipitated with anti-tyrosine antibodies and analyzed by Western blotting, using specific antibodies against TrkA receptors. Both NGF and DHEA strongly increased phosphorylation of TrkA as early as 10 min, an effect which was also maintained at 20 min (Figure 5A). We also tested the effects of DHEA and NGF in PC12 cells, endogenously expressing TrkA receptors. Naive or siRNA^{TrkA} transfected PC12 cells were incubated for 10 min with DHEA or NGF, and cell lysates were analyzed with Western blotting, using specific antibodies against Tyr490-phosphorylated TrkA and total TrkA. Both NGF and DHEA strongly induced the phosphorylation of TrkA in naive PC12 cells, effects which were diminished in siRNA^{TrkA} transfected PC12 cells (Figure 5A). The stimulatory effect of DHEA on TrkA phosphorylation might be due to an increase of NGF production. To test this hypothesis, we measured with ELISA the levels of NGF in culture media of HEK293 and PC12 cells exposed for 5 to 30 min to 100 nM of DHEA. NGF levels in culture media of control and DHEA-treated HEK293 and PC12 cells were undetectable, indicating that DHEA-induced TrkA phosphorylation was independent of NGF production. DHEAS mimicked the effect of DHEA and rapidly induced (within 10 min) the phosphorylation of TrkA receptors in HEK293 transfected with the TrkA cDNA expression vector (Figure S5). On the other hand, testosterone, while capable of displacing DHEA binding to TrkA receptors, was unable to increase phosphorylation of TrkA in the same system (Figure S5).

We compared the ability of NGF and DHEA to induce phosphorylation of TrkA-sensitive Shc, ERK1/2, and Akt kinases. Serum deprived naive or siRNA^{TrkA} transfected PC12 cells were incubated for 10 min with 100 nM DHEA or 100 ng/ml NGF and cell lysates were analyzed with Western blotting, using specific antibodies against the phosphorylated and total forms of kinases mentioned above. Both DHEA and NGF strongly increased phosphorylation of Shc, ERK1/2, and Akt kinases in naive PC12 cells, effects which were almost absent in siRNA^{TrkA} transfected PC12 cells, suggesting that both DHEA and NGF induce Shc, ERK1/2, and Akt phosphorylation via TrkA receptors (Figure 5A).

The effectiveness of DHEA to promote the interaction of p75^{NTR} receptors with its effector proteins TRAF6, RIP2, and RhoGDI was also assessed. It is well established that NGF induces the association of p75^{NTR} receptors with TNF receptor-associated factor 6 (TRAF6), thus facilitating nuclear translocation of transcription factor NFκB [19]. Furthermore, p75^{NTR} receptors associate with receptor-interacting protein 2 (RIP2) in a NGF-dependent manner [20]. RIP2 binds to the death domain of p75^{NTR} via its caspase recruitment domain (CARD), conferring nuclear translocation of NFκB. Finally, naive p75^{NTR} interacts with RhoGDP dissociation inhibitor (RhoGDI), activating small GTPase RhoA [21]. In that case, NGF binding abolishes the interaction of p75^{NTR} receptors with RhoGDI, thus inactivating RhoA. We co-transfected HEK293 cells with the plasmid cDNAs of p75^{NTR} and of each one of the effectors TRAF6, RIP2, or RhoGDI, tagged with the flag (TRAF6) or myc (RIP2, RhoGDI) epitopes. Transfectants were exposed to 100 nM DHEA or 100 ng/ml NGF, and lysates were immunoprecipitated with antibodies against flag or myc, followed by immunoblotting with p75^{NTR} specific antibodies. Both DHEA and NGF efficiently induced the association of p75^{NTR} with effectors TRAF6 and RIP2, while facilitating the dissociation of RhoGDI from p75^{NTR} receptors (Figure 5B).

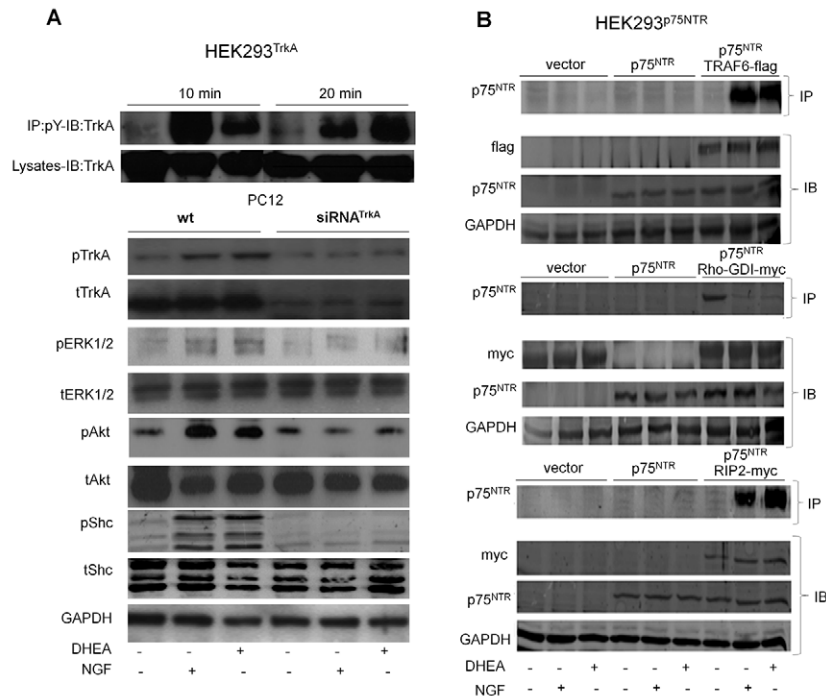

Figure 5. DHEA induces TrkA- and p75^{NTR}-mediated signaling. (A) *upper panel*: HEK293^{TrkA} transfectants were exposed for 10 and 20 min to 100 nM of DHEA or 100 ng/ml of NGF, and cell lysates were immunoprecipitated with anti-tyrosine antibodies and analyzed by Western blotting, using specific antibodies against TrkA receptors. *lower panel*: Serum deprived naive or siRNA^{TrkA} transfected PC12 cells were incubated for 10 min with 100 nM of DHEA or 100 ng/ml of NGF and cell lysates were analyzed with Western blotting, using specific antibodies against the phosphorylated and total forms of TrkA receptor and of Shc, ERK1/2, and Akt kinases. (B) HEK293 cells were co-transfected with the plasmid cDNAs of p75^{NTR} and of each one of the effectors TRAF6, RIP2, or RhoGDI, tagged with the flag (TRAF6) or myc (RIP2, RhoGDI) epitopes. Transfectants were exposed for 30 min to 100 nM of DHEA or 100 ng/ml of NGF, and lysates were immunoprecipitated with antibodies against flag or myc, followed by immunoblotting with p75^{NTR} specific antibodies.
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DHEA Reverses the Apoptotic Loss of TrkA Positive Sensory Neurons in Dorsal Root Ganglia of NGF Null Mouse Embryos

NGF null mice have fewer sensory neurons in dorsal root ganglia (DRG) due to their apoptotic loss [13]. Heterozygous mice for the NGF deletion were interbred to obtain mice homozygous for the NGF gene disruption. The mothers were treated daily with an intraperitoneal injection of DHEA (2 mg) or vehicle (4.5% ethanol in 0.9% saline). Embryos were collected at E14 day of pregnancy and sections were stained for Caspase 3 and Fluoro jade C, markers of apoptotic and degenerative neurons, respectively. *ngf*−/− embryos at E14 showed a dramatic increase in the number of Fluoro Jade C and Caspase 3 positive neurons in the DRG compared to the *ngf*+/− embryos (Figure 6A,B). DHEA treatment significantly reduced Fluoro Jade C and Caspase 3 positive neurons in the DRG to levels of *ngf*+/− embryos. Furthermore, TrkA and TUNEL double staining of DRGs has shown that in *ngf*+/− embryos, numbers of TUNEL-positive apoptotic neurons were minimal, while TrkA positive staining was present in a large number of neuronal cell bodies of the DRG and their collaterals were extended within the marginal zone to the most dorsomedial region of the spinal cord. On the contrary, in DRG of *ngf*−/− embryos levels of TUNEL-positive apoptotic neurons were dramatically increased, while TrkA neuronal staining was considerably decreased and DRG collaterals of the dorsal funiculus were restricted in the dorsal root entry zone (Figure 6C). DHEA treatment resulted in a significant increase of TrkA positive staining and the extension of TrkA staining within the marginal zone to the most dorsomedial region of the spinal cord similarly to the *ngf*+/− embryos (Figure 6D), while staining of TUNEL-positive apoptotic neurons was decreased to levels shown in *ngf*+/− embryos.

Discussion

DHEA exerts multiple actions in the central and peripheral nervous system; however, no specific receptor has been reported to date for this neurosteroid. Most of its actions in the nervous tissue were shown to be mediated via modulation, at micromolar concentrations, of membrane neurotransmitter receptors, such as NMDA, $GABA_A$, and sigma1 receptors. DHEA may also influence brain function by direct binding, also at micromolar concentrations, to dendritic brain microtubule-associated protein MAP2C [22]. In the present study we provide evidence that DHEA binds to NGF receptors. This is the first report showing a direct binding of a steroid to neurotrophin receptors. Saturation experiments and Scatchard analysis of [^{3}H]-DHEA binding to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and p75^{NTR} receptors showed that DHEA binds to both membranes (7.4±1.75 nM and 5.6±0.55 nM for TrkA or p75^{NTR}, respectively). Non-radioactive NGF effectively displaced [^{3}H]-DHEA binding to both membrane preparations, with IC_{50}: 0.8±0.2 and 1.19±0.45 nM, respectively. Furthermore, pull-down experiments using DHEA covalently immobilized on NovaPEG amino resin suggest that DHEA binds directly to TrkA and p75^{NTR} proteins. Indeed, polymer-supported DHEA-7-CMO effectively pulled down recombinant TrkA and p75^{NTR} proteins

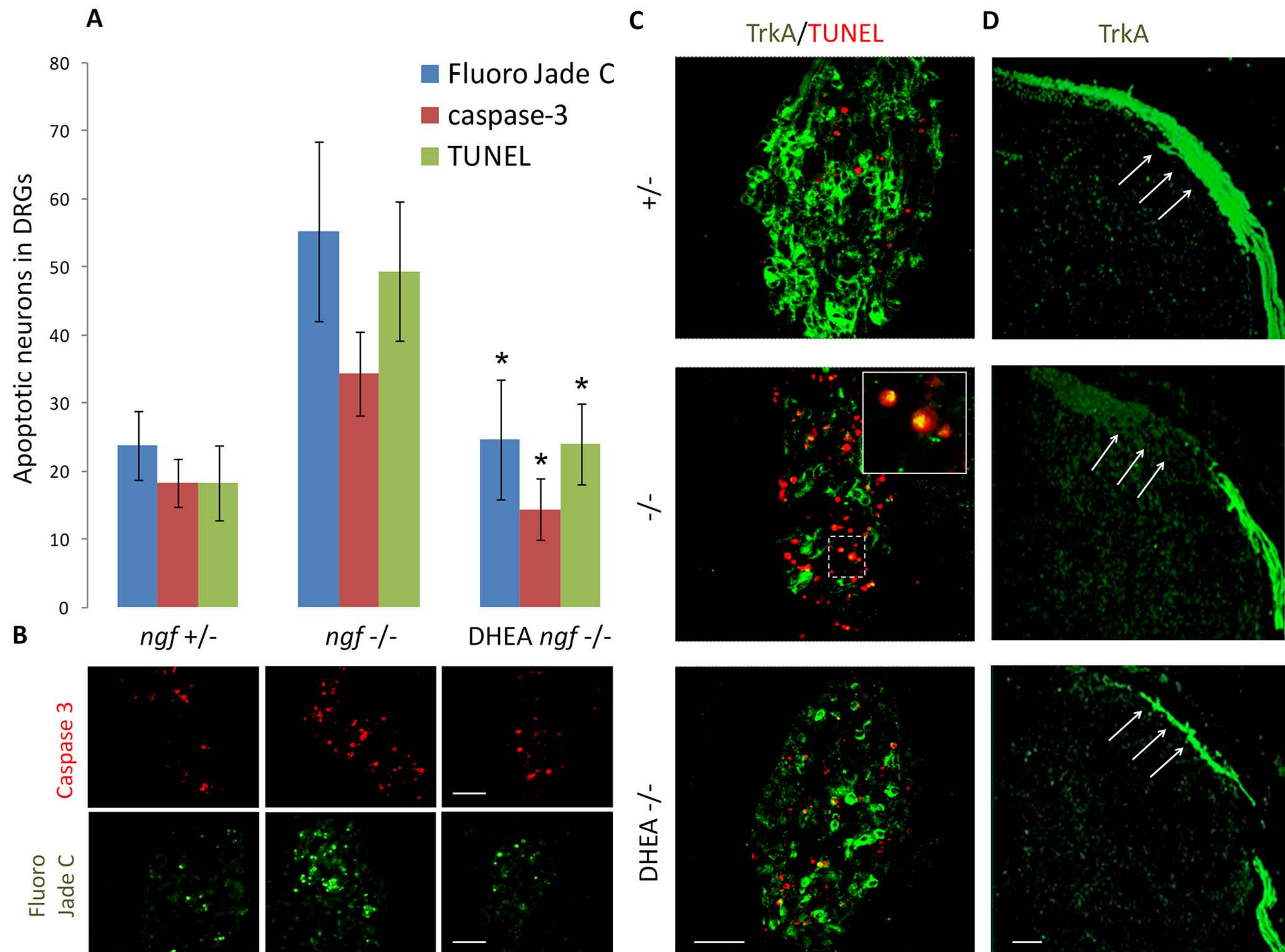

Figure 6. DHEA decreases the apoptotic loss of TrkA positive sensory neurons in dorsal root ganglia of NGF null mouse embryos. Heterozygous mice for the NGF deletion were interbred to obtain mice homozygous for the NGF gene disruption. The mothers were treated daily with an intraperitoneal injection of DHEA (2 mg) or vehicle (4.5% ethanol in 0.9% saline). Embryos were collected at E14 day of pregnancy as described in Material and Methods, and sections were stained for various apoptotic and neuronal markers: (A) Caspase 3, Fluoro Jade C,and TUNEL positive neurons were counted. The results shown are the mean ± SE from three embryos in each group. In each embryo apoptotic neurons were counted in at least eight sections from different DRGs (* $p<0.01$ versus NGF null mice). (B) Staining for Caspase 3 and Fluoro jade C, markers of apoptotic and degenerative neurons, respectively; (C) double staining for TrkA positive and TUNEL apoptotic neurons (note the co-staining of apoptotic neurons for both TrkA and TUNNEL in NGF null embryos); and (D) TrkA positive collaterals of DRG sensory neurons. Scale bars at B, C, and D: 200 µm.

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and precipitated both proteins from extracts prepared from cells expressing both receptors (HEK293^{TrkA}, HEK293^{p75NTR}, and PC12 cells and freshly isolated rat brain). Interestingly, DHEA was unable to effectively displace binding of [^{125}I]-NGF on membranes isolated from HEK293^{TrkA} and HEK293^{p75NTR} transfectants. It is possible that dissociation of binding of peptidic NGF from its receptors lasts longer due to the multiple sites of interaction within the binding cleft of this large peptidic molecule compared to smaller in volume steroid. Another explanation might be that NGF and DHEA bind to different domains of NGF receptors, the NGF domain being non-recognizable by DHEA. It is of note that antidepressant amitryptiline cannot chase NGF from TrkA receptors because it binds to a different domain on TrkA protein compared to NGF. Indeed, other small molecules, like antidepressant amitriptyline and gamboge's natural extract gambogic amide, bind in the extracellular and the cytoplasmic juxtamembrane domains of TrkA receptor, although with much lower affinity compared to DHEA (K_d 3 µM and 75 nM, respectively) [23,24]. The domains of TrkA and p75^{NTR} proteins involved in DHEA binding were not defined in the present study. Mutagenesis assays combined with NMR spectroscopy are planned to map the domains of both receptors related to DHEA binding.

Our findings suggest that binding of DHEA to NGF receptors is functional, mediating its anti-apoptotic effects. Indeed, blocking of TrkA expression by RNAi almost completely reversed the ability of DHEA to protect PC12 cells from serum deprivation-induced apoptosis and to maintain elevated levels of the anti-apoptotic Bcl-2 protein. Additionally, in dispersed primary sympathetic neurons in culture, DHEA effectively compensated NGF deprivation by decreasing the levels of apoptotic neurons, an effect which was reversed by a specific TrkA inhibitor, further supporting the involvement of TrkA receptors in the anti-apoptotic action of DHEA. Finally, DHEA effectively rescued from apoptosis TrkA-

positive dorsal root ganglia sensory neurons of NGF null mouse embryos.

It appears that the decision between survival and death among DHEA-responsive cells is determined by the ratio of TrkA and $p75^{NTR}$ receptors. In fact, DHEA and NGF induced apoptosis of nnr5 cells, a clone of PC12 cells expressing only pro-death $p75^{NTR}$ receptors. The pro-death effects of both agents were completely blocked by $p75^{NTR}$ shRNA and were remarkably restored after transfection of nnr5 cells with the TrkA cDNA. It is of note that during brain development the ratio of TrkA to $p75^{NTR}$ varies tempospatially [25]. Thus, the ability of DHEA to act in a positive or negative manner on neuronal cell survival may depend upon the levels of the two receptors during different stages of neuronal development.

Binding of DHEA on both TrkA and $p75^{NTR}$ receptors was effectively competed by sulfated DHEA, DHEAS (IC_{50}: 6.1 ± 1.1 and 8.1 ± 1.2 nM, respectively), suggesting that DHEAS may also bind to NGF receptors. Testosterone displaced DHEA binding to TrkA and $p75^{NTR}$ (IC_{50}: 5.3 ± 2.1 and 7.4 ± 3.2 nM, respectively), while synthetic glucocorticoid dexamethasone displaced DHEA binding only to pro-survival TrkA receptors (IC_{50}: 9.5 ± 4.6 nM). In a previous study we had shown that both steroids effectively displaced DHEA from its specific membrane binding sites of sympathoadrenal cells, acting as DHEA antagonists by blocking its anti-apoptotic effect and the induction of anti-apoptotic Bcl-2 proteins [9]. Our findings suggest that testosterone and glucocorticoids may act as neurotoxic factors by antagonizing endogenous DHEA and NGF for their binding to NGF receptors, explaining previously published data. Indeed, testosterone was shown to increase NMDA and $GABA_A$-mediated neurotoxicity [26,27]. Our findings suggest that testosterone may act as a neurotoxic factor by also antagonizing the neuroprotective effects of endogenous DHEA. Furthermore, glucocorticoids show a bimodal effect on hippocampal neurons causing acutely an increase in performance of spatial memory tasks, while chronic exposure has been associated with decreased cognitive performance and neuronal atrophy [28]. Acute administration of glucocorticoids results in a glucocorticoid receptor-mediated phosphorylation and activation of hippocampal TrkB receptors, exerting trophic effects on dentate gyrus hippocampal neurons [29], via an increase in the sensitivity of hippocampal cells to neurotrophin BDNF, the endogenous TrkB ligand known to promote memory and learning [30]. However, overexposure to glucocorticoids during prolonged periods of stress is detrimental to central nervous system neurons, especially in aged animals, affecting mainly the hippocampus. It is possible that part of neurotoxic effects of glucocorticoids may be due to their antagonistic effect on the neuroprotective effect of endogenous DHEA and NGF, via TrkA receptor antagonism. The decline of brain DHEA and NGF levels during aging and in Alzheimer's disease [28] might exacerbate this phenomenon, rendering neurons more vulnerable to glucocorticoid toxicity. Indeed, glucocorticoid neurotoxicity becomes more pronounced in aged subjects since cortisol levels in the cerebrospinal fluid increase in the course of normal aging, as well as in relatively early stages of Alzheimer's disease [28].

A number of neurodegenerative conditions are associated with lower production or action of both DHEA and NGF [31,32]. Animal studies suggest that NGF may reverse, or slow down the progression of Alzheimer's related cholinergic basal forebrain atrophy [32]. Furthermore, the neurotrophic effects of NGF in experimental animal models of neurodegenerative conditions, like MPTP (Parkinson's disease), experimental allergic encephalomyelitis (multiple sclerosis), or ischemic retina degeneration mice [33–35] support its potential as a promising neuroprotective agent. However, the use of NGF in the treatment of these conditions is limited, because of its poor brain blood barrier permeability. It is of interest that DHEA also exerts neuroprotective properties in some of these animal models [7,36]. These findings suggest that synthetic DHEA analogs, deprived of endocrine effects, may represent a new class of brain blood barrier permeable NGF receptor agonists with neuroprotective properties. We have recently reported the synthesis of 17-spiro-analogs of DHEA, with strong anti-apoptotic and neuroprotective properties, deprived of endocrine effects [37], which are now being tested for their ability to bind and activate NGF receptors.

We have previously defined the pro-survival signaling pathways that are initiated by DHEA at the membrane level [3]. These pathways include MEK1/2/ERK1/2 and PI3K/Akt pro-survival kinases. We now provide experimental evidence that DHEA activates these kinases via TrkA receptors. Down-regulation of TrkA receptors using siRNAs resulted in an almost complete reversal of the ability of DHEA to increase the phosphorylation of kinases Shc, Akt, and ERK1/2. In addition to TrkA receptors, binding of DHEA to the low affinity NGF receptor was also functional, affording the activation of $p75^{NTR}$ receptors. Unlike TrkA receptors, $p75^{NTR}$ lacks any enzymatic activity. Signal transduction by $p75^{NTR}$ proceeds via ligand-dependent recruitment and release of cytoplasmic effectors to and from the receptor. Indeed, DHEA like NGF facilitated the recruitment of two major cytoplasmic interactors of $p75^{NTR}$, TRAF6 and RIP2 proteins. Additionally, DHEA-mediated activation of $p75^{NTR}$ led to the dissociation of bound RhoGDI, a protein belonging to small GTPases and interacting with RhoA [21]. A schematic representation of our findings is shown in Figure 7.

Previous findings suggest that DHEA protects PC12 cells against apoptosis via pertussis toxin (PTX) sensitive, G protein-associated specific plasma membrane-binding sites [9]. Indeed, PTX was shown to partially reverse the anti-apoptotic effects of DHEA and its membrane impermeable DHEA-BSA conjugate, as well as their effects on prosurvival kinases PI3K/Akt, the activation of transcription factor NFkappaB, and the phosphorylation and inactivation of apoptotic protein Bad [10]. Interestingly, the prosurvival effects of NGF in sympathetic neurons and PC12 cells are also partially reversed by PTX [38]. Furthermore, the NGF-dependent activation of Akt is partially attenuated by PTX, indicating the participation of G(i/o) proteins. In the same study, NGF-induced phosphorylation of Bad and transcriptional activity of NFkappaB were also shown to be sensitive to PTX [38]. It appears that other NGF-driven pathways are sensitive to PTX too. For instance, in PC12 cells and primary cortical neurons the NGF-induced phosphorylation of tuberin (a critical translation regulator holding a central role in NGF-promoted neuronal survival) is partially blocked by PTX, suggesting the participation of G(i/o) proteins [39]. Finally, NGF-dependent activation of the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) pathway in PC12 cells was effectively blocked by PTX [40]. However in HEK293 cells transfected with TrkA receptors, PTX was unable to affect the induction of TrkA phosphorylation by NGF or DHEA (Figure S5). These findings considered together suggest that TrkA receptors may use down-stream G protein-coupled receptor pathways, after binding and activation by NGF or DHEA, to control neuronal cell survival.

It is worth noticing that the interaction of DHEA with the NGF system was first suggested 15 years ago by Compagnone et al., showing co-localized staining of CYP17, the rate limiting enzyme of DHEA biosynthesis, and NGF receptors in mouse embryonic DRGs [41]. About one-fifth of CYP17-immunopositive DRG neurons in the mouse were found to be also TrkA-immunoposi-

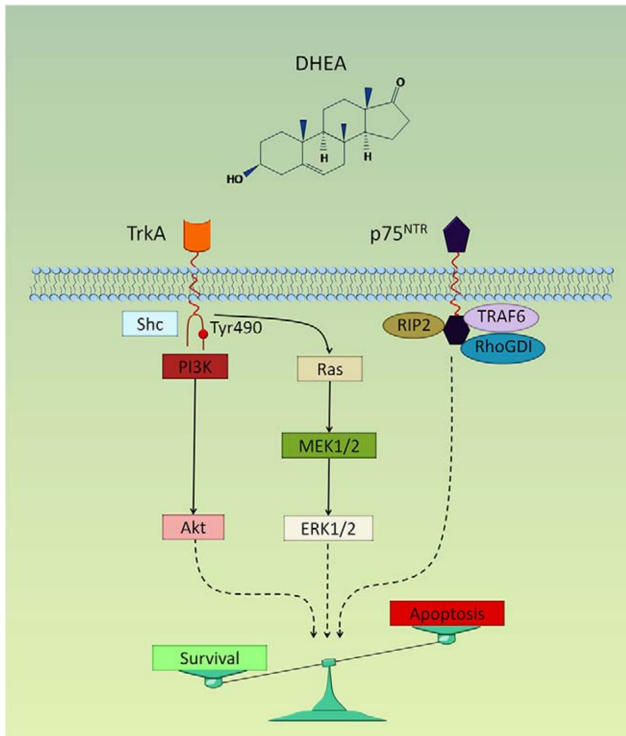

Figure 7. Hypothetical model of NGF receptor-mediated signaling pathways involved in the effects of DHEA on neuronal cell fate. DHEA binds with high affinity to TrkA and $p75^{NTR}$ receptors, initiating the following sequence of events: 1) DHEA induces TrkA-mediated Tyr490-phosphorylation of Shc, ERK1/2, and Akt kinases, controlling the expression and function of apoptotic Bcl-2 proteins; and 2) DHEA promotes the interaction of $p75^{NTR}$ receptors with effector proteins TRAF6, RIP2, and RhoGDI affecting neuronal cell apoptosis. Our findings suggest that the decision between survival and death among DHEA-responsive cells is determined by the balance of its interactions with TrkA and $p75^{NTR}$ receptors.
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tive. Among the TrkA-expressing cells, about one-third also express CYP17, while $p75^{NTR}$-expressing neurons represent only 13% of the cells in the DRG. Thus, about one-fifth of CYP17-immunopositive neurons may be able to respond to both DHEA and NGF stimulation, an observation compatible with our data, presented in Figure 6C. A recent report further supports the interaction of DHEA with NGF receptors. Indeed, DHEA was shown to act as a keratinocyte-deriving neurotrophic signal, mimicking NGF in promoting axonal outgrowth of NGF non-producing but TrkA positive sensory neurons, an effect blocked by TrkA inhibitor K252a [42].

CYP17 is expressed in invertebrate cephalochordata Amphioxus [43]. Amphioxus is also expressing TrkA receptor homologous AmphiTrk, which effectively transduces signals mediated by NGF [44]. Phylogenetic analysis of neurotrophins revealed that they emerged with the appearance of vertebrates (530–550 million years ago), when complexity of neural tissue increased [45]. Invertebrate cephalochordata like Amphioxus are positioned on the phylogenetic boundary with vertebrates (600 million years ago). It is thus tempting to hypothesize that DHEA contributed as one of the ‘‘prehistoric’’ neurotrophic factors in an ancestral, simpler structurally invertebrate nervous system [46]; then, when a strict tempospatial regulation of evolving nervous system of vertebrates was needed, peptidic neurotrophins emerged to afford rigorous and cell specific neurodevelopmental processes.

In conclusion, our findings suggest that DHEA and NGF cross-talk via their binding to NGF receptors to afford brain shaping and maintenance during development. During aging, the decline of both factors may leave the brain unprotected against neurotoxic challenges. This may also be the case in neurodegenerative conditions associated with lower production or action of both factors. DHEA analogs may represent lead molecules for designing non-endocrine, neuroprotective, and neurogenic micromolecular NGF receptor agonists.

Materials and Methods

si/shRNAs, Plasmids, and Antibodies

PC12 cells were transfected with specific si/shRNAs for blocking the expression of TrkA and/or $p75^{NTR}$ receptors. More specifically, three siRNAs and two shRNAs for TrkA and $p75^{NTR}$, respectively, were obtained. The sequences for TrkA siRNAs (Ambion) were: GCCUAACCAUCGUGAAGAG (siRNA ID 191894), GCAUCCAUCAUAAUAGCAA (siRNA ID 191895), and CCUGACGGAGCUCUAUGUG (siRNA ID 191893). Sequences for $p75^{NTR}$ (Qiagen) were: GACCUAUCUGAGCU-GAAA (Cat. No. SI00251090) and GCGUGACUUUCAGG-GAAA (CatNo SI00251083).

Rat TrkA was expressed from the pHA vector backbone and rat $p75^{NTR}$ was expressed from the pCDNA3 vector backbone (InVitrogen) using a full length coding sequence flanked by an N-terminal hemagglutinin (HA) epitope tag. Plasmids to express RIP2 [19] and RhoGDI [36] were myc-tagged, while TRAF6 [19] was FLAG-tagged, as previously described.

The origin of antibodies was as follows: Bcl-2 (Cat. No. C-2, sc-7382, Santa Cruz Biotechnology Inc.), phospho TrkA (Cat. No. 9141, Cell Signaling), TrkA (Cat. No. 2505, Cell Signaling, was used for Western Blotting and Cat. No. 06-574, Upstate, was used for immunostainings), $p75^{NTR}$ (Cat. No. MAB365R, Millipore), c-myc (Cat. No. 9E10, sc-40, Santa Cruz Biotechnology Inc.), phospho ERK1/2 (Cat. No. 9106, Cell Signaling), Erk1/2 (Cat. No. 9102, Cell Signaling), phospho-Shc (Tyr239/240) Antibody (Cat. No. 2434, Cell Signaling), Shc (Cat. No. 2432, Cell Signaling), phospho-Akt (Ser473) (Cat. No. 9271, Cell Signaling), Akt (Cat. No. 9272, Cell Signaling), anti-FLAG (M2) mouse monoclonal (Cat. No. F1804, Sigma), pTyr (Cat. No. sc-508, Santa Cruz Biotechnology Inc.), active Caspase-3 (Cat. No. ab13847, Abcam), Tyrosine Hydroxylase (Cat. No. ab6211, Abcam), 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), anti-rabbit Alexa Fluor 546 (Cat. No. A10040), and GAPDH (Cat. No. 2118, Cell Signaling).

Cell Cultures and Transfection

PC12 cells were obtained from LGC Promochem (LGC Standards GmbH, Germany) and nnr5 cells from Dr. C.F. Ibáñez (Karolinska Institute). Both cell types were grown in RPMI 1640 containing 2 mM L-glutamine, 15 mM 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. HEK-293 cells were obtained from LGC Promochem. 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 3°C. HEK-293 and PC12 cells were transfected with Lipofectamine 2000 (InVitrogen) according to manufacturer's instructions. Transfected cells were typically used on the 2nd day after transfection.

Measurement of Apoptosis

PC12 cells were cultured in 12-well plates, and 24 h later they were transfected with the si/shRNAs for TrkA and/or p75^{NTR}. Twenty-four hours later the medium was aspirated and replaced either with complete medium (serum supplemented) or serum free medium in the absence or the presence of DHEA or DHEA-BSA conjugate at 100 nM. Apoptosis was quantified 24 h later with annexin V-FITC and PI (BD Pharmingen) according to our protocol [8].

[^{3}H]-DHEA Binding Assays

Membrane preparation. HEK293 cells transfected with an empty vector (HEK293-Vector) or overexpressing p75^{NTR} (HEK293-p75) or TrkA (HEK293-TrkA), and PC12 cells wild type or shRNA knocked down for p75^{NTR} and TrkA, were cultured, collected by scraping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7.4. After centrifugation at 1,200 rpm, cells were homogenized 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 aprotinin). Crude membrane fractions were isolated by differential centrifugation at 2,500×g (10 min at 4°C, to remove unbroken cells and nuclei) and 102,000×g (1 h, at 4°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 elute membrane adsorbed proteins, washed once, resuspended in PBS (pH 7.4) with protease inhibitors, at a concentration of 2 mg/ml, and used immediately for binding experiments.

Binding conditions. Fifty µl of membrane suspension (2 mg/ml) in triplicate were incubated with 10 µl of 1–30 nM [^{3}H]-DHEA (Perkin Elmer, Boston, MA) in the presence or absence of 500-fold molar excess of DHEA, in PBS, pH 7.4 with protease inhibitors, in a final volume of 100 µl. Membranes were incubated for 30 min at 25°C or overnight at 4°C, on a rotating plate; then they were collected on GF/B filters, prewetted in 0.5% PEI solution at 4°C. Filters were washed three 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. For saturation curves, specific binding (Bound, B) was calculated as the difference of Total Binding − Non Specific Binding. K_{Ds} were calculated from B/F over B Scatchard plots. Non-specific binding was ranging from 30% to 50% of total binding shown in the micromolar concentrations of ligand. Relatively higher levels of non-specific binding have been typically reported in binding assays for specific membrane binding of various steroid hormones, due to the highly lipophilic nature of phospholipid containing cell membranes combined with the strong lipophilicity of steroids. For displacement experiments, a constant concentration of [^{3}H]-DHEA (1 nM) was incubated with increasing concentrations of competitors (10^{-12}–10^{-6} M), under the same conditions as for saturation binding.

Fluorescence Microscopy

HEK293 cells were allowed to grow on gelatin-coated glass coverslips for 24 h in culture medium, and 24 h later they were transfected with the cDNAs for TrkA, and p75^{NTR} receptors or the vector alone. Staining was performed 48 h after transfection. Culture medium was aspirated and transfectants were washed twice with PBS buffer. Primary antibodies against TrkA (rabbit, Upstate, No. 06-574, diluted 1:100) or p75^{NTR} (mouse monoclonal ab, MAB365R, Millipore, dilution 1:500) were added for 30 min at 37°C. Secondary antibodies, anti-rabbit-R-phycoerythrin conjugated (Sigma, No. P9537), and anti-mouse-fluorescein conjugated (No. AP124F, Millipore) were added at 1:100 dilution and transfectants were incubated for 30 min at 37°C; then they were washed three times with PBS and counterstained with Hoechst nuclear stain (Molecular Probes) for 5 min. Transfectants were also incubated with the DHEA-BSA-FITC or the BSA-FITC conjugates (10^{-6}M) for 15 min at room temperature in the dark; then they were washed with serum free culture medium and incubated for another 15 min in serum free culture medium containing 4% BSA. Coverslips were mounted to slides with 90% glycerin and were observed with a confocal laser scanning microscope (Leica TCS-NT, Leica Microsystems GmbH, Heidelberg, Germany), mounted with a digital camera.

Flow Cytometry

HEK293 cells were cultured in 12-well plates, and 24 h later they were transfected with the cDNAs for TrkA and/or p75NTR receptors, or the vector alone. Staining was performed 48 h later. Transfectants (5×10^5 cells) were pelleted and incubated with 20 µl of the primary antibodies against TrkA or p75^{NTR} receptors for 30 min over ice. Afterwards, transfectants were washed three times with PBS and 20 µl of the secondary antibodies, and anti-rabbit-R-phycoerythrin conjugated and anti-mouse-fluorescein conjugated were added, as described above. For DHEA-BSA-FITC binding on cells, 20 µl (100 nM) were added on the pelleted cells for 10 min at RT, and then they were washed with serum free culture medium and incubated for another 15 min in serum free culture medium containing 4% BSA. Transfectants were washed twice with PBS, resuspended in 500 µl of PBS, and were analyzed in a Beckton-Dickinson FACSArray apparatus and the CELL-Quest software (Beckton-Dickinson, Franklin Lakes, NJ).

Synthesis of Immobilised DHEA-7-CMO

NovaPEG amino resin (loading value 0.78 mmol/g) was purchased from Novabiochem. NMR spectra were recorded on a Varian 300 spectrometer operating at 300 MHz for ^{1}H and 75.43 MHz for ^{13}C or on a Varian 600 operating at 600 MHz for ^{1}H. ^{1}H NMR spectra are reported in units of δ relative to the internal standard of signals of the remaining protons of deuterated chloroform, at 7.24 ppm. ^{13}C NMR shifts are expressed in units of δ relative to $CDCl_3$ at 77.0 ppm. ^{13}C NMR spectra were proton noise decoupled. IR spectra was recorded at Bruker Tensor 27. Absorption maxima are reported in wavenumbers (cm^{-1}).

3*β*-Acetoxy-17,17-ethylenedioxyandrost-5-ene (0.74 g, 1.98 mmol) and N-hydroxy phthalimide (0.71 g, 2.2 mmol) were dissolved in acetone (39 mL) containing 1 mL of pyridine. The mixture was stirred vigorously at room temperature and sodium dichromate dihydrate (0.89 g, 3 mmol) was added. Additional portions of solid sodium dichromate dihydrate (0.89 g, 3 mmol) were added after 10 and 20 h stirring at room temperature. After reaction completion (48 h), the mixture was diluted with dichloromethane, filtered through a bed of celite, and the filtrate was washed with water, saturated sodium bicarbonate solution, and brine. The organic layer was dried over anhydrous sodium sulfate, the solvent evaporated *in vacuo*, and the residue purified by flash column chromatography using hexane/acetone/25% NH_4OH (85:15:0.1 mL) as eluent to afford 3*β*-acetoxy-17,17-ethylenedioxyandrost-5-ene-7-one (0.6 g, yield: 78%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.87 (s, 3H), 1.21 (s, 3H), 1.26–2.00 (m, 14H), 2.05 (s, 3H), 2.20–2.51 (m, 3H), 3.84–3.92 (m, 4H), 4.68–4.76 (m, 1H), 5.70 (d, J = 1.58 Hz, 1H).

To a solution of 3*β*-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one (0.1 g, 0.26 mmol) in pyridine (1.9 mL) was added O-(carboxymethyl)hydroxylamine hemihydrochloride (0.11 g, 0.52 mmol) and the reaction mixture was stirred overnight under

argon. After completion of the reaction, the solvent was evaporated and the residue was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and the solvent was evaporated *in vacuo* to afford 3β-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one7-(O-carboxymethy1) oxime as a white foam (0.12 g, yield: 100%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.88 (s, 3H), 1.13 (s, 3H), 1.16–1.95 (m, 12H), 2.04 (s, 3H), 2.25–2.59 (m, 5H), 3.84–3.95 (m, 4H), 4.59 (d, J = 2.29 Hz, 2H), 4.62–4.73 (m, 1H), 6.51 (d, J = 1.47 Hz, 1H).

To a solution of 3β-acetoxy-17,17-ethylenedioxyandrost-5-en-7-one-7-(O-carboxymethy1) oxime (0.12 g, 0.26 mmol) in a mixture of acetone/water (5:1, 6.3 mL) was added *p*-toluenesulfonic acid monohydrate (0.019 g, 0.10 mmol), and the reaction mixture was stirred until the starting material was consumed (48 h). The solvent was evaporated *in vacuo* and the residue was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and the solvent was evaporated *in vacuo* to afford 3β-acetoxy-androst-5-en-7,17-dione 7-(O-carboxymethy1) oxime as a white foam (0.11 g, yield: 100%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 0.90 (s, 3H), 1.15 (s, 3H), 1.20–1.95 (m, 12H), 2.05 (s, 3H), 2.09–2.68 (m, 5H), 4.63 (d, J = 4.18 Hz, 2H), 4.65–4.71 (m, 1H), 6.56 (d, J = 1.39 Hz, 1H).

To a solution of 3β-acetoxy-androst-5-en-7,17-dione 7-(O-carboxymethy1) oxime (0.11 g, 0.26 mmol) in methanol (3.9 mL) was added LiOH (1.5 mL, 1.5 mmol, 1N solution), and the reaction mixture was stirred until the starting material was consumed (4 h). The solvent was evaporated *in vacuo* and the residue was diluted with water. The solution was acidified with 10% hydrochloric acid and DHEA-7-CMO precipitated as a white solid, which was isolated by filtration (0.097 g, yield: 100%). ^{1}H NMR ($CDCl_3$/CD_3OD, 600 MHz) δ: 0.90 (s, 3H), 1.14 (s, 3H), 1.20–2.75 (m, 17H), 3.49–3.54 (m, 1H), 4.54 (s, 2H), 6.54 (s, 1H).

3β-Hydroxy-17-oxoandrost-5-en-7-*O*-(carboxymethyl)oxime (DHEA-7-CMO) (192 mg, 0.511 mmol) in DMF (5 mL) was treated with HOBt (69 mg, 0.511 mmol) and DIC (0.08 mL, 0.511 mmol), and the resulting mixture was stirred at room temperature for 30 min. This solution was added to NovaPEG amino resin (130 mg, 0.102 mmol, 0.78 mmol/gr) (pre-swollen with DMF for 1 h) and the slurry was shaken at room temperature overnight. The mixture was filtered, the resin was sequentially washed with dichloromethane (3×), methanol (3×), and diethyl ether (3×), and was dried *in vacuo* overnight. Yield 175 mg (100%), loading value 0.61 mmol/gr. ^{13}C NMR (gel phase, $CDCl_3$) δ: 220.66, 170.15, 157.10, 154.15, 113.11, 72.57, 66.59, 49.92, 47.86, 42.15, 38.46, 37.08, 36.53, 35.49, 31.20, 30.71, 24.96, 20.15, 18.05, 13.95; IR: v_{max}/cm^{-1} 2865 (s), 1735 (m), 1669 (w), 1653 (w), 1637 (w), 1456 (m), 1348 (w), 1289 (w), 1247 (w), 1093 (s), 946 (w).

Co-immunoprecipitation and Pull-Down Assays

HEK293 cells were transfected with the appropriate plasmids (TrkA, $p75^{NTR}$, RIP2, TRAF-6, and RhoGDI) by using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection and suspended in lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4) supplemented with protease inhibitors. Lysates were precleared for 1 h with Protein A-Sepharose beads (Amersham) and immunoprecipitated with the appropriate antibody (pTyr, Flag, or c-myc) overnight at 4°C. Protein A Sepharose beads were incubated with the lysates for 4 h at 4°C with gentle shaking. In the case of immobilized DHEA-7-CMO, HEK293 or PC12 cells lysates or purified receptors (both from R&D Systems, Recombinant Mouse NGF R/TNFRSF16/Fc Chimera, Cat. No.: 1157-NR and Recombinant Rat Trk A/Fc Chimera, Cat. No.: 1056-TK) were incubated overnight at 4°C with the NovaPEG amino resin alone or conjugated with DHEA. Beads were collected by centrifugation, washed four times with lysis buffer, and resuspended in SDS loading buffer. Proteins were separated by SDS/PAGE, followed by immunoblotting with specific antibodies.

Western Blot Analysis

PC12 or HEK293 cells lysates were electrophoresed through a 12% SDS-polyacrylamide gel, and then proteins were transferred to nitrocellulose membranes, which were processed according to standard Western blotting procedures, as previously described [8]. To detect protein levels, membranes were incubated with the appropriate antibodies: Bcl-2 (dilution 1:500), phospho TrkA (dilution 1:500), total TrkA (dilution1:500), $p75^{NTR}$ (dilution 1:500), phospho Shc (dilution 1:1000), total Shc (dilution 1:1000), phospho Akt (dilution 1:500), total Akt (dilution 1:500), phospho ERK1/2 (dilution 1:500), and total ERK1/2 (dilution 1:500). Proteins were visualized using the ECL Western blotting kit (ECL Amersham Biosciences, UK), and blots were exposed to Kodak X-Omat AR films. A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis, Inc., Ontario, Canada).

To normalize for protein content the blots were stripped and stained with GAPDH antibody (dilution 1:1000); the concentration of each target protein was normalized versus GAPDH. Where phosphorylation of TrkA or kinases was measured, membranes were first probed for the phosphorylated form of the protein, then stripped, and probed for the total protein.

Superior Cervical Ganglia Neuronal Cultures

Superior cervical ganglia (SCG) were removed from newborn (P0–P1) rat pups and dissociated in 0.25% trypsin (Gibco, 15090) for 30 min at 37°C. After dissociation SCG neurons were re-suspended in culture medium (Gibco, Neurobasal Cat. No. 21103) containing 1% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 3 μg/ml araC antimitotic, and 100 ng/ml NGF (Millipore, 01-125). Cells were plated on collagen coated 24-well plates and cultured for 5 d prior to use. For NGF withdrawal experiments, cells were washed twice with Neurobasal containing 1% FBS and fresh culture medium lacking NGF and containing anti-NGF antibody at 1:50 dilution (Millipore, AB1526). DHEA, TrkA-inhibitor (Calbiochem, 648450) and anti-$p75^{NTR}$ (mouse, MAB365R Millipore) were used at 100 nM, 100 nM, and 1:50, respectively.

In Vivo Experiments with the NGF Null Mice

$ngf^{+/-}$ mice [13] were obtained from the Jackson Laboratory and maintained on C57BL/6 background. All procedures described below were approved by the Animal Care Committee of the University of Crete, School of Medicine. Animals were housed in cages maintained under a constant 12 h light–dark cycle at 21–23°C, with free access to food and tap water. 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 null mutation were interbred to obtain mice homozygous for the NGF gene disruption and the first day of gestation determined by the discovery of a copulation plug. The mothers were treated daily with a subcutaneous injection of DHEA (2 mg/day) or vehicle (4.5% ethanol in 0.9% saline) starting from the third day after gestation. Animals were collected at E14. At the day of collection the mothers were deeply anesthetized

with sodium pentobarbital (Dolethal 0.7 ml/kg i.p.) followed by transcardial perfusion with saline solution containing heparin for about 7 min, and with 4% PFA, 15% Picric Acid, 0.05% GA in phosphate buffer 0.1 M, for another 7 min. After the perfusion the embryos were collected and maintained in the same fixative overnight at 4°C. Embryos were then washed in 0.1 M phosphate buffer and cryoprotected by using 10% sucrose followed by 20% sucrose overnight at 4°C. Finally, embryos were frozen in OCT in iso-pentane over liquid nitrogen for 5 min and the frozen tissues were stored for later use at −80°C. The samples were sectioned (20 µm) and mounted onto Superfrost plus slides (Menzel-Glaser J1800AMNZ). Slides were left to air-dry overnight at room temperature (RT) and were then either used immediately or were fixed in cold acetone for 1 min and stored at −80°C for later use.

Stored or fresh slides were fixed for 15 min in cold acetone at 4°C and left to dry for 10 min at room temperature. They were then washed in PB 0.1 M, then in TBS, and incubated for 45 min with 10% horse serum in TBS-T 0.1%. The normal serum was drained off and the primary antibodies (anti-TrkA diluted 1:400 and active Caspase-3 diluted 1:50), diluted in TBS-T 0.1% with 1% horse serum, were added. Sections were incubated for 4 h at RT and overnight at 4°C; they were then washed in TBS-T 0.1% and the anti-rabbit secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546, 1:1000 in TBS-T 0.1%) were added for 6 h at RT. Sections were washed in TBS-T, TBS, and in PB 0.1 M and were coverslipped with Vectashield (Vector, H-1400) and visualized in a confocal microscope. TUNEL (Roche, Cat. No. 12156792910) and Fluoro-Jade C (Millipore, Cat. No. AG325) staining of apoptotic and degenerating neurons, respectively, was performed according to the manufacturer's instructions.

Statistical Analysis

For the statistical evaluation of our data we have used 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.

Supporting Information

Figure S1 DHEA regulates the levels of TrkA and $p75^{NTR}$ receptors, mimicking NGF. Serum deprived PC12 cells were exposed to 100 nM of DHEA or 100 ng/ml of NGF for 12, 14, and 48 h. TrkA and $p75^{NTR}$ protein levels were measured in cell lysates with immunoblotting, using specific antibodies, and were normalized against GAPDH (* $p<0.01$ versus control-Serum Free, $n = 5$).
(TIF)

Figure S2 [^{3}H]-DHEA saturation binding assays and Scatchard blots in HEK293 cells, transfected with the plasmid cDNAs of TrkA and $p75^{NTR}$ receptors. Fifty µl of cell membrane suspension in triplicate were incubated overnight at 4°C with 1–30 nM [^{3}H]-DHEA in the presence or absence of 500-fold molar excess of DHEA.
(TIF)

Figure S3 RT-PCR of androgen receptors (AR) mRNA in HEK23 cells, transfected with the plasmid cDNAs of TrkA and $p75^{NTR}$ receptors. Total RNA was extracted from LNCaP and naïve HEK293 cells or HEK293 cells transfected with the plasmid cDNAS of TrkA, $p75^{NTR}$, or both plasmids, by using the Trizol Reagent (InVitrogen). One microgram of total RNA was reverse transcribed by using the Thermo-Script RT-PCR System (InVitrogen). The cDNA was amplified by PCR. PCR was performed in a Perkin–Elmer DNA Thermal Cycler with the following conditions: 60 s at 94°C, 60 s at 60°C, and 3 min at 72°C (for Androgen Receptor, AR) or 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C (for GAPDH), for 30 cycles to detect the product at the exponential phase of the amplification. Ten microliters of the amplified products (1.4 Kb for AR and 483 bp for GAPDH) were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Primers for AR were 5′-AGCTACTCCG-GACCTTACG-3′ (sense) and 5′-AGGTGCCATGGGAGGGT-TAG-3′ (antisense) and primers for GAPDH were 5′-GCCA-CATCGCTCAGACACCA-3′ (sense) and 5′-GATGACCCTTT-TGGCTCCCC-3′ (antisense).
(TIF)

Figure S4 Representative curves of [^{3}H]-DHEA displacement experiments. The selectivity of DHEA binding to $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cell membranes was examined by performing heterologous [^{3}H]-DHEA displacement experiments using a number of non-labeled steroids or NGF. Membranes (at a final concentration of 2 mg protein/ml) isolated from HEK293 cells transfected with the plasmid cDNAs of TrkA and $p75^{NTR}$ receptors were incubated with 1 nM [^{3}H]-DHEA in the absence or the presence of various unlabeled steroids or NGF at concentrations varying from 0.01 to 1,000 nM. Binding of [^{3}H]-DHEA to membranes isolated from both $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells was effectively displaced by NGF (IC_{50}: 0.8±0.2 and 1.19±0.45 nM, respectively, $n = 4$). Estradiol failed to displace [^{3}H]-DHEA from its binding on membranes from $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells at concentrations ranging from 0.1 to 1,000 nM. In contrast, displacement of [^{3}H]-DHEA binding to membranes from both $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ cells was shown by sulfated ester of DHEA, DHEAS (IC_{50}: 6.1±1.1 and 8.1±1.2 nM, respectively, $n = 3$), and testosterone (Testo) (IC_{50}: 5.3±2.1 and 7.4±3.2 nM, respectively, $n = 4$). Glucocorticoid dexamethasone (DEX) effectively competed [^{3}H]-DHEA binding to membranes from $HEK293^{TrkA}$ (IC_{50}: 9.5±4.6 nM, $n = 4$) but was ineffective in displacing DHEA binding to membranes from $HEK293^{p75NTR}$ cells.
(TIF)

Figure S5 Effects of PTX, DHEAS, and testosterone on TrkA phosphorylation in $HEK293^{TrkA}$ cells. $HEK293^{TrkA}$ transfectants cultured in serum free conditions were exposed for 10 min to 100 nM of DHEA or 100 ng/ml of NGF, in the absence or the presence of 100 ng/ml PTX, or to 100 nM of DHEAS or testosterone. Cell lysates were then immunoprecipitated overnight at 4°C with anti-tyrosine antibody and analyzed by Western blotting, using specific antibodies against TrkA receptors.
(TIF)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: IC EC AG. Performed the experiments: IL IC VIA IP PE NA. Analyzed the data: IL IC VIA EC AG. Contributed reagents/materials/analysis tools: NA TC. Wrote the paper: AG.

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