Dehydroepiandrosterone: An Ancestral Ligand of Neurotrophin Receptors

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

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

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

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received July 14, 2014. Accepted October 14, 2014.
First Published Online October 20, 2014

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

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

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

Materials and Methods

Plasmids, antibodies, and proteins

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

Tissue culture and cell transfection

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

[^{3}H]DHEA binding assays

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

Immunoprecipitation and immunoblotting

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

Fluorescence-activated cell sorting (FACS) analysis

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

Phylogenetic equivalent

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

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

Statistical analysis

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

Results

DHEA interacted with both vertebrate and invertebrate Trk receptors

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

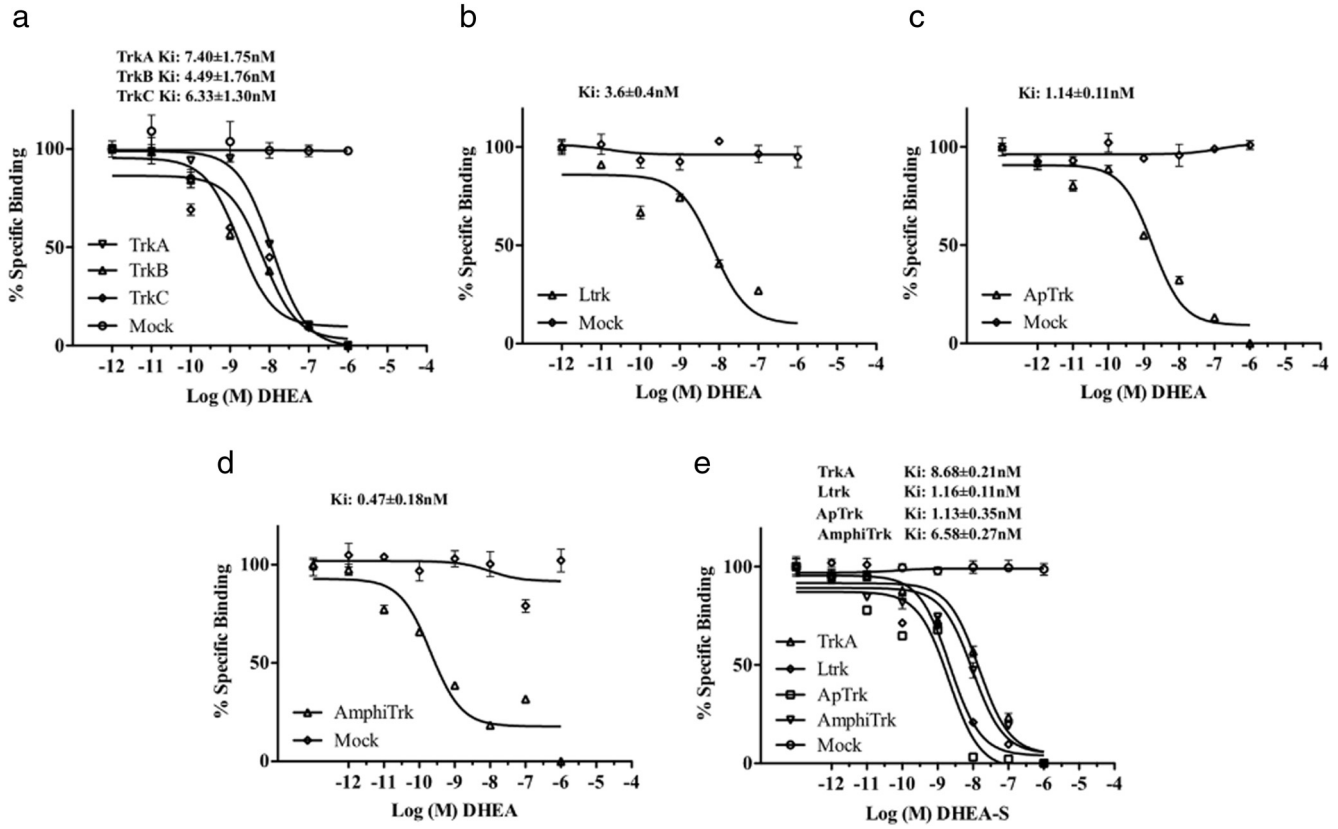

Figure 1. [^{3}H]DHEA binds to cell membranes from HEK293 cells transfected with the cDNAs of mammalian and invertebrate Trk receptors. Competition binding assays of tritiated [^{3}H]DHEA in the presence of increasing concentrations of nonlabeled DHEA (A–D) or DHEAS (E) were performed using membranes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

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

DHEA was able to activate all types of invertebrate Trk homologs

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

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

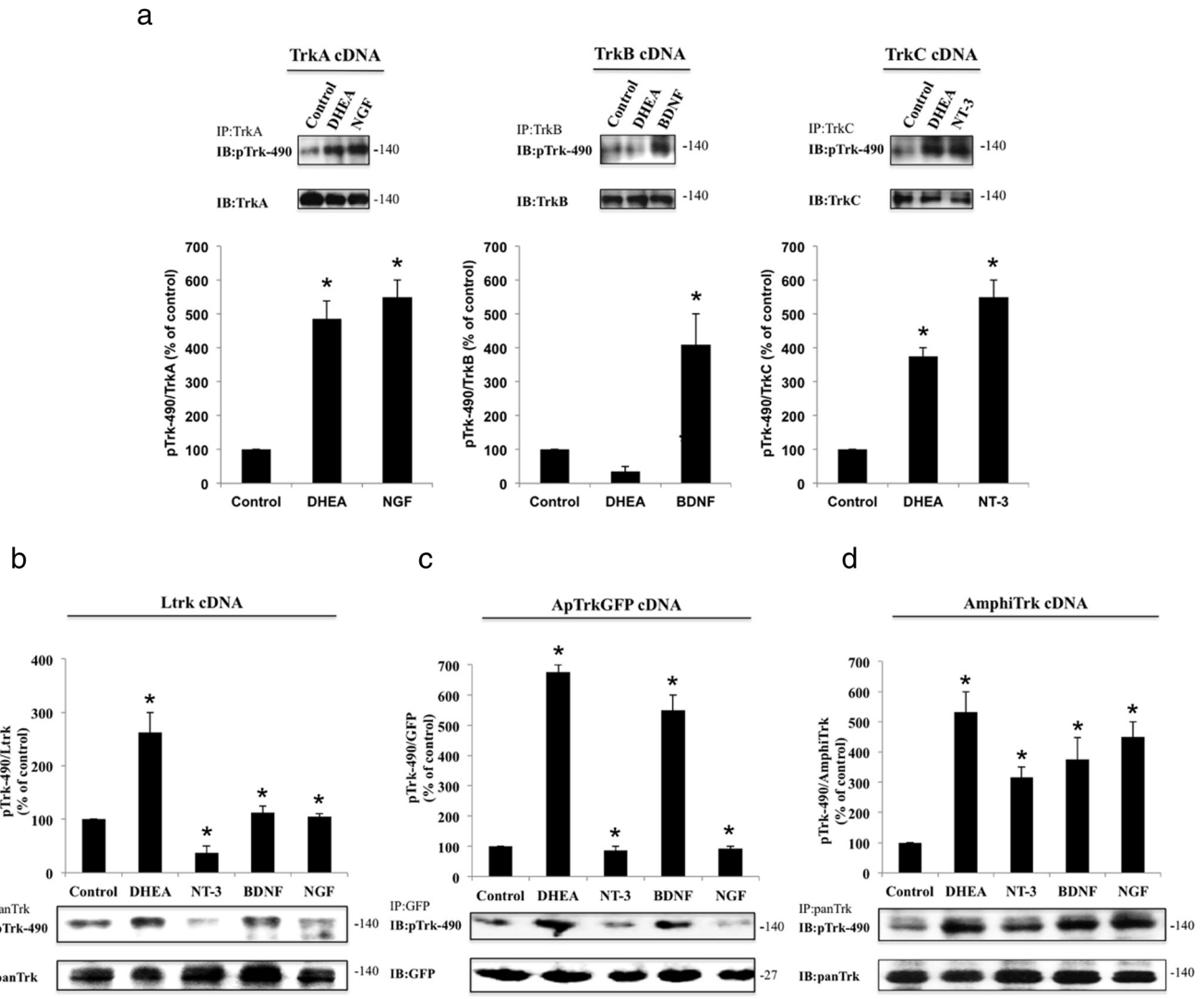

Figure 2. DHEA induces tyrosine phosphorylation of Trk receptors. A, CHO cells were transfected with the plasmid cDNAs of mammalian TrkA, TrkB, or TrkC receptors. Transfectants were then exposed for 15 minutes to 100 ng/mL of mammalian neurotrophins NGF (left panel), BDNF (middle panel), NT-3 (right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

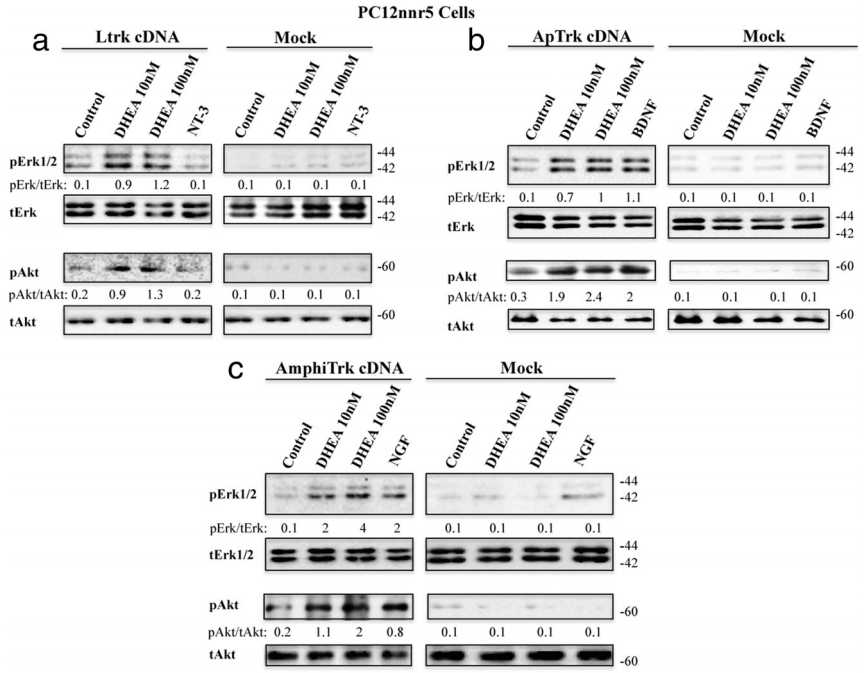

Figure 3. Invertebrate Trk receptors mediate activation of prosurvival kinases ERK and Akt by DHEA (A–C). PC12nnr5 cell were transfected with the plasmid cDNAs of Ltrk, ApTrk, and AmphiTrk receptors and control plasmid (mock), treated for 15 minutes with DHEA at various concentrations or 100 ng/mL NT-3,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

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

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

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

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

The invertebrate Trk receptors mediated the antiapoptotic effects of DHEA

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

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

Discussion

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

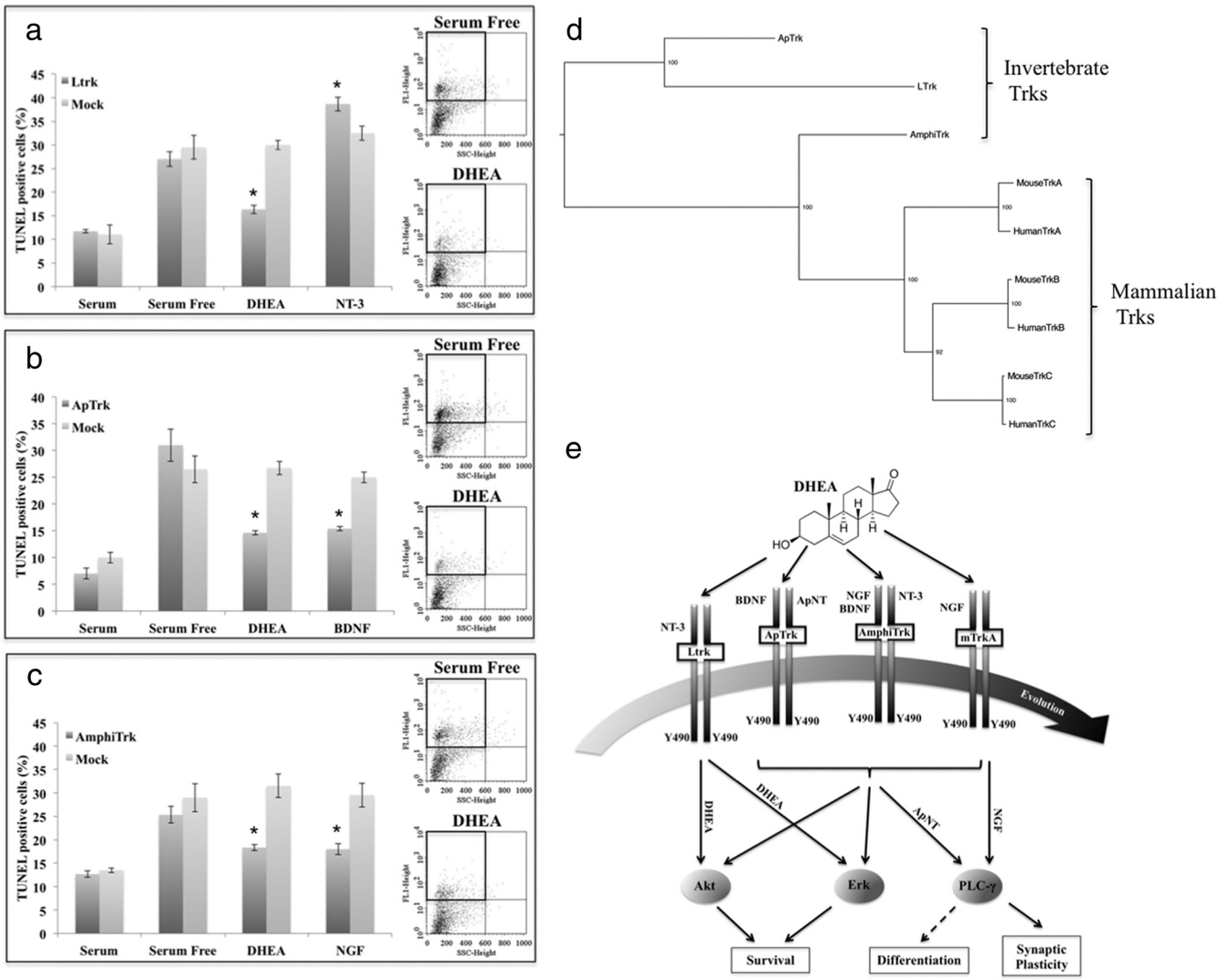

Figure 4. Antiapoptotic effects of DHEA in serum-deprived PC12nnr5 cells transfected with the invertebrate Trk receptors (A–C). PC12nnr5 cells were transfected with the plasmid cDNAs of Ltrk, invertebrate ApTrk, AmphiTrk receptors, or empty vectors (mock) and cultured for 24 hours in serum-starved conditions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

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

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

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

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

Acknowledgments

We thank Drs Carlos F. Ibáñez, Yves-Alain Barde, Mike Fainzilber, and Eric Kandel for providing the Trk plasmids; Marcial Vilar for providing the PC12nnr5 cell line; and Stefan R. Kassabov for his valuable suggestions. We also thank our graduate student, Marileta Grozi, for her technical assistance; and the Graduate Program in Cellular and Genetic Etiology of Human Diseases of the Medical School, University of Crete.

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Disclosure Summary: The authors have nothing to disclose.

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