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**Direct effect of dehydroepiandrosterone sulphate (DHEAS) on PC-12 cell
differentiation processes**

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Abstract

Dehydroepiandrosterone sulphate (DHEAS) is classically seen as an inactive reservoir for the production of dehydroepiandrosterone (DHEA). Steroid sulfatase (STS) is the enzyme that catalyzes the hydrolysis of DHEAS to DHEA, which can then be further metabolized to other steroid hormones. Recent studies, however, indicate that DHEAS can mediate biological effects without being converted to DHEA.

This study aims to evaluate whether DHEAS itself influences the differentiation of PC-12 cells or if its desulfation to DHEA is required. DHEA and DHEAS both influence the differentiation of chromaffin PC-12 cells. Blocking steroid sulfatase activity and thereby the conversion of DHEAS to DHEA by the enzyme blocker estrone sulfamate (EMATE) showed that the effect of DHEAS is independent of its conversion to DHEA. DHEAS, similar to DHEA, reduced nerve growth factor (NGF)-induced neurite outgrowth of PC-12 cells and the expression of synaptosomal-associated membrane protein of 25 kDa (SNAP-25), increased the expression of chromogranin A (CGA) and significantly increased dopamine release of PC-12 cells. In addition, DHEAS, DHEA and membrane impermeable DHEA-BSA all significantly reduced NGF-induced MAPK ERK1/2 signaling after 5 minutes.

In summary, this study provides evidence that DHEAS, independent of its conversion to DHEA, directs PC-12 cells' differentiation to a neuroendocrine direction. Furthermore, employing membrane-impermeable DHEA-BSA indicates the involvement of plasma-membrane bound receptors.

Key words: Dehydroepiandrosterone-sulfate (DHEAS), steroid sulfatase, estrone sulfamate (EMATE)

1. Introduction

Dehydroepiandrosterone (DHEA) and its sulfate ester DHEA-sulfate (DHEAS) are the major androgens produced by the human adrenal cortex. More than 99% (3.5 to 20 mg/daily) of DHEA is sulfated to DHEAS prior to secretion (Burger, 2002). The normal range of DHEA plasma levels in humans is 7–31 nm. DHEAS ranges from 1.3 to 6.8 μ m (Chen et al., 2005) representing the primary circulating form of DHEA with concentrations more than 100-fold higher than any other steroid hormone. Steroid sulfatase (STS) is the enzyme that catalyzes the hydrolysis of steroid sulfates to their unconjugated, biologically active forms (Stanway et al., 2007). In the periphery, STS converts DHEAS to DHEA, which can then be further metabolized to other steroid hormones; conversely, DHEA can be inactivated via sulfonation to DHEAS by cytosolic hydroxysteroid sulfotransferase SA1 (SULT2A1) (Hammer et al., 2005). Due to its rapid metabolic clearance rate, DHEA has a rather short half-life of 1–3 hours, while the half-life of DHEAS is 10–20 hours (Rosenfeld et al., 1975).

DHEA and DHEAS are widely distributed throughout the body and DHEAS has long been seen merely as a circulating storage pool for DHEA regeneration (Chen et al., 2005). Numerous recent studies, however, have demonstrated that DHEAS indeed can have a direct impact on various cell systems. DHEAS can be synthesized *de novo* in the CNS and in brain glial cells from cholesterol or from steroid precursors through mechanisms partly independent of peripheral steroidogenic endocrine glands, gonads or adrenals and was found to directly increase the length of neurites (Compagnone and Mellon, 1998). Among other major biological actions of DHEAS are those associated with neuroprotection, neuronal survival, apoptosis, catecholamine synthesis and secretion, as well as antioxidant, anti-inflammatory and anti-glucocorticoid effects (for review (Maninger et al., 2009). Furthermore, DHEAS was found to inhibit vascular smooth muscle cell (VSMC) proliferation (Li et al., 2009) and to directly increase superoxide formation in neutrophils (Radford et al., 2010). Our own recent data show that DHEA and DHEAS differentially modulate growth factor-induced proliferation of bovine adrenomedullary chromaffin cells in an age-dependent manner. While DHEA reduced the proliferation of adrenal cells from young and adult animals, DHEAS exclusively increased the proliferation in adult-cell cultures. These effects were not mediated by androgen or estrogen receptors (Sicard et al., 2007). Our studies on PC-12 cells further demonstrated that DHEA (Ziegler et al., 2008) and DHEAS (Krug et al., 2009) promote a neuroendocrine phenotype of these cells.

The present study aims to clarify whether the effects of DHEAS on PC-12 cell differentiation are indeed mediated directly by DHEAS or are exerted by DHEA after

conversion by STS. By blocking this enzyme we were able to pin down direct effects of DHEAS on chromaffin PC12 cells.

2. Materials and methods

2.1 Cell culture

Rat PC12 cells were sustained in Kaighn's modified Ham's F-12 medium (ATCC, Manassas, USA) with 2 mmol/l l-glutamine and 1500 mg/l sodium bicarbonate supplemented with 15% horse serum, 2.5% fetal calf serum (FCS) and 20 U/ml penicillin-streptomycin (Gibco, Gaithersburg MD, USA) in a humidified 5% CO₂/95% O₂ atmosphere at 37 °C. The culture medium was changed every other day. Cells were grown in this culture medium for 72 h, and left in serum-free medium overnight prior to the experiments. DHEA and DHEA-BSA were dissolved in ethanol, DHEAS and NGF were dissolved in DMSO and 10 mmol/l sodium acetate (pH 5), respectively. The final volume of ethanol or DMSO in each well, including controls, was 0.01% for all assays performed. The solvents had no effect in control experiments.

2.2 RNA isolation and RT-PCR

RNA was isolated using the Qiagen RNeasy Plus kit, according to the manufacturer's instructions (Qiagen, Germany). This kit includes DNA binding columns to exclude genomic DNA. Furthermore, for avoidance of genomic DNA amplification we used the exon-spanning primers for STS detection.

For reverse transcriptase (RT)-PCR, 500 ng RNA from each sample was reverse transcribed into cDNA by Moloney murine leukemia (MML) virus reverse transcriptase using random primers (Promega) in a final volume of 20 µl. One µl of cDNA was amplified in a 10 µl solution containing 1.5 mM MgCl₂, 1xPCR buffer (Invitrogen, CA, USA), 0.25 mM of each deoxynucleotide (Promega), 1 unit of Taq DNA polymerase (Invitrogen, CA, USA) and 5 pmol of each of the different primers. Samples were denatured for 5 min at 94°C, then subjected to 15 s 94°C, 15 s 65°C, 15 s 72°C and min 72°C for 40 cycles altogether. After agarose gel electrophoresis and ethidium bromide staining, bands were visualized under UV light.

Exon-spanning primer were found through data base search and checked for right length and position using BLAST (basic local alignment search tool). Primer sequences (5'-3'): ACTCCAACTCAGCTACAACCTGGTG and CACAGAGAAAGTTGAGCTGGGAGAG.

2.3 Neurite outgrowth

Single cell suspensions were plated in 200 mm² wells at a density of 2×10^4 cells per well in serum-free culture medium, then treated with NGF (20 ng/ml) and DHEAS or DHEA (10^{-6}

mol/l), alone and in combination as well as w/wo EMATE (10^{-6} mol/l). After 24 h incubation, the percentage of cells showing neurite outgrowth was determined by light microscopy. Cells with one or more neurites whose lengths were at least twice the diameter of the cell body were scored as positive. Neurite outgrowth was determined from at least three different regions of interest (ROI) in three independent experiments (Kim et al., 2004; Xiao et al., 2002; Ziegler et al., 2008).

2.4 High-performance liquid chromatography (HPLC)

Dopamine concentrations in culture medium were determined as previously described (Ziegler et al., 2008). Briefly, catecholamines were extracted by solid-phase extraction after 24 h stimulation of cells with NGF (20 ng/ml) and DHEA as well as DHEAS (10^{-6} mol/l), alone and in combination as well as w/wo EMATE (10^{-6} mol/l). The cell medium samples were processed according to the sample preparation protocol for urine (Chromsystems, Munich, Germany). 6 ml neutralization buffer and 100 μ l internal standard (3,4-dihydroxybenzylamine) were added to 3 ml cell medium. This mixture was run on a sample clean-up column by vacuum. The eluate was discarded. Subsequently, the solid-phase extraction column was washed twice with pure water (10 ml) and eluted with 6 ml elution buffer by vacuum. Finally, the eluate was collected and acidified with 180 μ l 5N HCl. An aliquot of the purified eluate was applied to a HPLC reverse-phase column, and dopamine concentrations were measured using an electrochemical detector (Bio-Rad, Munich, Germany). Dopamine measurements were quantified in correlation to the added internal standard.

2.5 Western blot analysis

Western blot analysis was performed as previously described (Krug et al., 2003). PC-12 cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold Cell Lytic Mammalian Cell Lysis/Extraction reagent (Sigma) containing 1% protease inhibitor cocktail (Sigma). Cell lysates were matched for protein content. Protein concentrations were determined by BCA assay (PIERCE, Rockford, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. After blocking, membranes were immunostained using the following antibodies: rabbit anti-phospho-ERK1/2 (1:1000, Cell Signaling, Danvers, USA), rabbit anti-SNAP25 (1:5000, Sigma-Aldrich, Steinheim, Germany), goat anti-chromogranin A (CgA) (1:500, Santa Cruz, Heidelberg, Germany) and rabbit anti- β -actin (1:500, Cell Signaling, Danvers, USA). The bands were visualized using peroxide solution and luminol solution (PIERCE, Rockford, USA). Chemoluminescence signals were read with the GeneGnome Chemoluminescence detector (Syngene, Frederick, USA). As a loading control we used the house-keeping gene β -actin

2.6 Quantification of ERK1/2 phosphorylation by ELISA

We performed in situ cell-based ELISA as described previously, in a slightly modified way (Krug et al., 2009). Cells were seeded in 96-well plates at a concentration of 20,000 cells per 0.32 cm^2 and after reaching approximately 80% confluence, were serum-starved for 24 h. Subsequently, cells were equilibrated in 1xHEPES-Ringer solution (130.0 mmol/l NaCl, 5.4 mmol/l KCl, 1.0 mmol/l CaCl_2 , 1.0 mmol/l MgCl_2 , 1.0 mmol/l NaH_2PO_4 , 10 mmol/l HEPES, and 5 mmol/l glucose; pH 7.4) at 37 °C for 30 min, and then stimulated in the same buffer w/o the hormones and growth factors of interest. After fixation of cells with 8% formaldehyde in PBS, endogenous peroxidases were quenched with freshly prepared 1% H_2O_2 and 0.1% azide. Cells were washed again three times in the same buffer, blocked by 5% FCS in PBS/Triton for 1 h and, finally, incubated overnight with the primary antibody (pERK, Cell Signaling, 1:1000) in PBS/Triton containing 5% bovine serum albumin at 4 °C. The next day, cells were washed three times with PBS/Triton and incubated with the secondary antibody (anti-rabbit HRP-linked IgG, Cell Signaling, 1:3000) in PBS/Triton containing 5% bovine serum albumin for 1 h at room temperature. After one washing step, chemoluminescence signals were read using a Mithras multi-well reader (Berthold Technologies, Bad Wildberg, Germany). After two washing steps, air-dried wells were stained with 100 μl of crystal violet solution (0.25% in PBS) for 30 min at room temperature. Following three PBS washing steps, 100 μl of 1% SDS solution was added and the plate was incubated on a shaker for 1 h at room temperature. Finally, absorbance was measured at 595 nm. Chemoluminescence signals were normalized to the protein content in each well as determined by crystal violet staining. This step allows normalizing ERK1/2 phosphorylation to total protein content per well.

2.7 Statistical analysis

In all experiments, statistical differences between experimental groups relative to appropriate controls were determined by analysis of variance (ANOVA). Data are presented as means $\pm$ S.E.M. Significance of differences was tested by analysis of variance with Bonferroni's as a secondary test. Differences were considered significant at values of $p < 0.05$. Cells from at least two different passages were used for each experimental series; n represents the number of cells or tissue culture dishes investigated.

3. Results

The prerequisite for the conversion of DHEAS to DHEA is the expression of the enzyme steroid sulfatase which catalyzes the conversion of DHEAS to DHEA. RT-PCR analysis revealed the expression of STS in PC-12 cells (Fig. 1).

To identify possible direct effects of DHEAS, intracellular STS activity was blocked by addition of the enzyme blocker estrone sulfamate (EMATE). The effects of both DHEA and DHEAS were evaluated in the presence and absence of EMATE. We focused on various neuronal and neuroendocrine differentiation parameters in accordance with our previous studies on chromaffin PC-12 cells (Krug et al., 2009; Ziegler et al., 2008; Ziegler et al., 2006).

Incubation of PC-12 cells with NGF lead to neurite outgrowth of the cells as early as 24h after stimulation (Ziegler et al., 2008). Both androgens (10^{-6} mol/l) were found to reduce this NGF (20 ng/ml)-induced neurite outgrowth by 72% for DHEA and 58% for DHEAS after 24h. Addition of EMATE and thus inhibiting the conversion of DHEAS to DHEA had no significant effect on DHEAS-induced reduction of neurite outgrowth. DHEA, DHEAS and EMATE alone did not affect neurite outgrowth (Fig. 2). Furthermore, lower concentrations of DHEA (10^{-7} - 10^{-10} mol/l) or DHEA-S (10^{-7} - 10^{-9} mol/l) were also checked and not found to induce neurite outgrowth in our experimental setting. This is in accordance with our previous publications (Krug et al., 2009; Ziegler et al., 2008).

Associated with NGF-induced neurite outgrowth of PC-12 cells is the expression of neuronal marker proteins, such as synaptosome-associated protein of 25kDa (SNAP-25) (Krug et al., 2009). DHEAS and DHEA (10^{-6} mol/l) both reduced NGF (20 ng/ml)-induced expression of SNAP-25 after 24h by 14% for DHEA and 18% for DHEAS respectively, as demonstrated by Western blotting. The conversion of DHEAS to DHEA was blocked by EMATE. DHEA, DHEAS and EMATE alone (10^{-6} mol/l) did not influence SNAP-25 expression (Fig. 3). DHEA and DHEAS at concentrations of 10^{-6} mol/l significantly reduced NGF-induced expression of SNAP-25. Lower concentrations of the androgens were also tested and not as effective (Krug et al., 2009; Ziegler et al., 2008).

Catecholamine secretion and the expression of neuroendocrine marker proteins, such as chromogranin A (CGA) are features of the neuroendocrine differentiation processes of PC-12 cells. Concordantly with the reduction of neuronal cell differentiation parameters, both androgens (10^{-6} mol/l) directly increased the expression of the neuroendocrine marker protein CGA after 24h by 115% for DHEA and 120% for DHEAS, as demonstrated by Western blotting. Co-incubation with NGF (20 ng/ml) reduced the androgens' effects; NGF alone did not influence CGA expression. Blocking the conversion of DHEAS to DHEA by EMATE had no effect on the DHEAS-induced increase of CGA concentrations within the cells (Fig. 4). The most effective concentration was 10^{-6} mol/l for DHEA, DHEAS also at 10^{-7} mol/l was significantly influencing CGA expression (Krug et al., 2009; Ziegler et al., 2008).

Furthermore, DHEA and DHEAS (10^{-6} mol/l) similarly and significantly increased dopamine release from NGF (20 ng/ml)-induced PC-12 cells by 370% for DHEA and 320% for DHEAS, after 24h; no significant effect was observed when these hormones were given alone. The enzyme blocker (10^{-6} mol/l) alone had no effect on dopamine secretion (Fig. 5). The most effective concentration was 10^{-6} mol/l for DHEA; DHEAS also at 10^{-7} mol/l was significantly influencing NGF (20 ng/ml)-induced dopamine release (Krug et al., 2009; Ziegler et al., 2008).

Our results provide evidence for direct effects of DHEAS on PC-12 cell differentiation processes independent of its conversion to DHEA as demonstrated by blocking the conversion with EMATE. In accordance to our previous studies, both androgens seem to shift PC-12 cells towards a more neuroendocrine phenotype, as revealed by reduced neurite outgrowth and reduced neuronal marker protein expression. Furthermore, DHEAS and DHEA significantly influenced CGA expression and catecholamine secretion.

Recently, Charalampopoulos and co-workers provided a mechanism for DHEA action, concluding that this androgen might mediate its action via a fast non-genomic effect including plasma-membrane binding as a first step of action (Charalampopoulos et al., 2006). In accordance, our data reveal that DHEA as well as the membrane impermeable DHEA-BSA exerts an identical reduction of NGF-induced ERK1/2 phosphorylation. The involvement of a membrane component is also indicated by the rapid onset of DHEA and DHEAS effects within 5 min (Fig. 6).

4. Discussion

This study provides evidence of a direct effect of DHEAS on chromaffin cell differentiation processes independent of a conversion of DHEAS to DHEA. Together with our recent work where we showed that these androgens regulate proliferation of primary chromaffin cells (Sicard et al., 2007), differentiation of chromaffin progenitors (Chung et al., unpublished data) and of chromaffin PC12 cells (Ziegler et al., 2008), these data suggest significant direct effects of both androgens on adrenal biology in different stages of adrenal development.

Classically, DHEAS, which circulates in micromolar amounts in the human body, is assumed to have no independent biological function other than an inactive storage pool for DHEA (Arlt and Stewart, 2005). However, different lines of evidence recently suggested direct DHEAS effects independent of its conversion to DHEA. Thus, the two androgens *in vitro* differentially modulated growth factor-induced proliferation of bovine adrenomedullary chromaffin cells (Sicard et al., 2006) in an age-dependent manner (Sicard et al., 2007). While

DHEA reduced the proliferation of adrenal cells from young and adult cattle, DHEAS exclusively increased the proliferation in adult cell cultures. These effects were not mediated by androgen or estrogen receptors (Sicard et al., 2007). Furthermore, while DHEAS and DHEA stimulated catecholamine secretion from PC12 cells (Charalampopoulos et al., 2005; Krug et al., 2009; Ziegler et al., 2008) only DHEAS but not DHEA stimulated catecholamine synthesis (Charalampopoulos et al., 2005). DHEA and DHEAS also had different effects on the growth of embryonic rodent cortical neuronal neurite growth (Compagnone and Mellon, 1998). In addition, it was recently shown that DHEAS, but not DHEA, increases superoxide generation in primed human neutrophils, thereby impacting on a key bactericidal mechanism (Radford et al., 2010). These studies indicate that DHEAS not solely represents a circulating storage pool for DHEA regeneration but indeed mediates direct effects.

By blocking the DHEAS converting enzyme steroid sulfatase, we provide evidence for direct effects of DHEAS on chromaffin cell differentiation processes, independent of its conversion to DHEA. Thus, DHEAS directed PC12 cells to a more endocrine differentiation by inhibiting NGF-induced PC-12 cells neurite outgrowth, influencing neuronal and neuroendocrine marker protein expression as well as the MAPK ERK1/2 signaling pathway. These data adds to recent studies demonstrating important direct effects of DHEAS on various biological parameters (for review (Maninger et al., 2009).

PC-12 cells are a widely used model to study adrenal catecholamine metabolism, chromaffin cell proliferation, and differentiation processes as well as the underlying signaling events (Vaudry et al., 2002). PC-12 cells also harbor features of early chromaffin progenitor cells which can differentiate into either chromaffin cells or sympathetic neurons under the influence of different growth factors and adrenocortical steroid hormones (Beaujean et al., 2003; Eaton and Duplan, 2004). They are thus a useful model for studying adrenal chromaffin cell development.

In the adrenal gland, adrenocortical and adrenomedullary systems are intimately linked both anatomically and functionally (Ehrhart-Bornstein et al., 1998), suggesting a close bi-directional crosstalk. Human adrenal chromaffin cells are in direct contact with the inner androgen producing zone, the *zona reticularis*, and are thus exposed to high concentrations of adrenal androgens, predominantly DHEA and DHEAS. This is especially relevant during fetal development where DHEA and DHEAS are the major products of the adrenal premordium when the neural crest derived sympathoadrenal progenitor cells invade these adrenal anlagen (Ehrhart-Bornstein et al., 1997; Mesiano and Jaffe, 1997). There they

acquire the features of neuroendocrine catecholamine-producing cells, suggesting a direct influence of DHEA and DHEAS in the development and maintenance of the adrenal medulla.

Our previous work on PC-12 cells (Ziegler et al., 2008; Ziegler et al., 2006) demonstrated that DHEA and DHEAS promote a neuroendocrine differentiation while reducing neuronal cell parameters, possibly via ERK1/2 MAPK signaling; ERK1/2 MAPK pathway is involved in PC-12 cell proliferation and differentiation processes. NGF rapidly induces this pathway, an effect significantly reduced by DHEA (Ziegler et al., 2008) and DHEAS (Krug et al., 2009). Since DHEA and DHEAS are not cytotoxic for PC-12 cells (data not shown), this indicates a shift of PC-12 cells towards differentiation (Ziegler et al., 2008). Blocking the conversion of DHEAS to DHEA now proves that these effects were indeed mediated by DHEAS directly.

A specific receptor for DHEA and DHEAS has not been identified to date. In our previous studies we were able to exclude the action via classical steroid receptors since neither the estrogen receptor antagonist ICI 182,780 nor the androgen receptor antagonist flutamide inhibited DHEA/DHEAS effects (Sicard et al., 2007; Ziegler et al., 2008). Recent studies have indicated that plasma membrane-associated receptors can mediate fast steroid effects, therefore being involved in important physiological and pathological processes (Charalampopoulos et al., 2006; Liu and Dillon, 2002; Liu et al., 2003). In accordance with these studies, the inhibition of NGF-induced differentiation process also by a membrane impermeable DHEA-BSA complex provides further evidence for the involvement of plasma-membrane bound receptors in DHEA and possibly also DHEAS effects. Furthermore, the rapid onset of DHEA and DHEAS effects within 5 min supports the hypothesis that a membrane component is implicated in the action of these steroids. In a recent study, a photoreactive, biotinylated DHEA analogue was successfully synthesized; this compound might help to further identify plasma membrane DHEA binding sites and eventually also to purify a possible receptor (Liu et al., 2010). In contrast to the lipophilic DHEA, DHEAS, due to its hydrophilic nature does not enter the cell (Hammer et al., 2005) indicating a membrane bound receptor for this steroid. The suggested regulation of PC-12 differentiation is summarized in Figure 7.

Our combined recent data reveals decisive roles of both DHEA and DHEAS in the differentiation and function of adrenal medullary chromaffin cells. Both androgens seem to influence the fate of the neighboring adrenomedullary chromaffin cells during different developmental steps.

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Legends to the figures

Figure 1. RT-PCR demonstrates expression of steroid sulfatase mRNA in PC-12 cells. The PCR product has the expected length of 229 bp.

Figure 2. Neurite outgrowth of PC12 cells after 24h. (A) 20 ng/ml NGF induced neurite outgrowth, an effect significantly reduced by 10^{-6} mol/l DHEAS and DHEA after 24h. DHEAS or DHEA alone did not influence neurite outgrowth. Addition of the blocker estrone sulfamate (EMATE) had no significant effect. (B-D) Representative bright field microscopy images

demonstrate the reduction of NGF (20 ng/ml)-induced neurite outgrowth by DHEA and DHEAS (10^{-6} mol/l), respectively in PC12 cells.

Figure 3. Expression of the neuronal marker protein SNAP-25. 20 ng/ml NGF significantly induced expression of SNAP-25, an effect significantly reduced by 10^{-6} mol/l DHEAS and DHEA, respectively after 24h. DHEAS or DHEA alone did not influence SNAP-25 expression. The combinations with EMATE had the same effects as combinations without the blocker.

Figure 4. Expression of the neuroendocrine marker protein CGA. 10^{-6} mol/l DHEAS and DHEA significantly induced the expression of CGA after 24h. NGF (20 ng/ml) alone had no effect on CGA expression but reduced DHEAS and DHEA induced CGA-expression. Combinations including EMATE had the same effects as combinations without the blocker.

Figure 5. DHEAS and DHEA significantly increased dopamine release of NGF-induced PC12 cells. NGF (20 ng/ml) alone lead only to a slight dopamine secretion compared to control cells. The effect on dopamine secretion was similar for DHEAS and DHEA. Combinations including EMATE had the same effects as combinations without the blocker.

Figure 6. DHEA and membrane-impermeable DHEA-BSA (10^{-6} mol/l) similarly affect NGF (20 ng/ml)-induced ERK1/2 phosphorylation after 5 min.

Figure 7. DHEA and DHEAS influence PC-12 cell differentiation towards an endocrine phenotype with increased dopamine secretion and chromogranin A expression. DHEA and DHEAS might mediate its effect via a fast non-genomic mechanism, docking initially at the plasma membrane.

Figure 1: Ziegler et al.

Figure 2: Ziegler et al.

Figure 3: Ziegler et al.

Figure 4: Ziegler et al.

Figure 5: Ziegler et al.

Figure 6: Ziegler et al.

Figure 7: Ziegler et al.