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Susanne Lemcke, Christoph Hönnscheidt, Gustav Waschatko, Anita Bopp, Dieter Lütjohann, et al..  
DHEA-Bodipy—a functional fluorescent DHEA analog for live cell imaging. *Molecular and Cellular  
Endocrinology*, 2009, 314 (1), pp.31. 10.1016/j.mce.2009.09.006 . hal-00529009

**HAL Id: hal-00529009**

**<https://hal.science/hal-00529009>**

Submitted on 24 Oct 2010

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## Accepted Manuscript

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PII: S0303-7207(09)00489-4  
DOI: doi:10.1016/j.mce.2009.09.006  
Reference: MCE 7318

To appear in: *Molecular and Cellular Endocrinology*

Received date: 12-2-2009  
Revised date: 31-7-2009  
Accepted date: 9-9-2009

Please cite this article as: Lemcke, S., Hönnscheidt, C., Waschatko, G., Bopp, A., Lütjohann, D., Bertram, N., Gehrig-Burger, K., DHEA-Bodipy—a functional fluorescent DHEA analog for live cell imaging, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2009.09.006

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**DHEA-Bodipy – a functional fluorescent DHEA analog for live cell imaging**

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**Key words:** DHEA-Bodipy; genomic; non-genomic; fluorescence microscopy; live cell imaging; trafficking.

## ABSTRACT

The androgen dehydroepiandrosterone (DHEA) has been reported to protect neuronal cells against dysfunction and apoptosis. Several signaling pathways involved in these effects have been described but little is known about the intracellular trafficking of DHEA. We describe design, synthesis and characterization of DHEA-Bodipy, a novel fluorescent DHEA analog. DHEA-Bodipy proved to be a functional DHEA derivative: DHEA-Bodipy (i) induced estrogen receptor  $\alpha$ -mediated gene activation, (ii) protected PC12 rat pheochromocytoma cells against serum deprivation-induced apoptosis, and (iii) induced stress fibers and focal adhesion contacts in SH-SY5Y human neuroblastoma cells. DHEA-Bodipy bound rapidly and specifically to plasma membranes of living PC12 cells. We analyzed metabolism and trafficking of DHEA-Bodipy in human neuroblastoma cells. DHEA-Bodipy is the first functional fluorescent DHEA derivative suitable for live cell imaging of intracellular DHEA transport and localization.

## 1. Introduction

Dehydroepiandrosterone (DHEA) is an androgen produced by the adrenals and the central nervous system (CNS). Declining DHEA and DHEA sulfate (DHEAS) levels during aging are associated with pathophysiological effects such as neuronal degeneration (Leblhuber et al., 1993). Recent studies show that DHEA protects rat chromaffin cells and PC12 rat pheochromocytoma cells against serum-deprivation induced apoptosis (Charalampopoulos et al., 2004). In PC12 cells, DHEA binds to and activates a  $G\alpha$  coupled receptor that in turn leads to an induction of anti-apoptotic Bcl proteins (Charalampopoulos et al., 2006) and to an activation of Akt kinase (Charalampopoulos et al., 2008). Besides, several other receptors have been proposed as possible DHEA receptors, for example the pregnane X receptor (PXR), estrogen receptor  $\alpha$  ( $ER\alpha$ ) and  $\beta$  and the sigma-1 receptor subtype (Goodwin et al., 2002; Maurice et al., 2006; Webb et al., 2006).

To our knowledge, little is known about the intracellular trafficking and localization of DHEA. Therefore, we developed a DHEA derivative suitable for fluorescence microscopy of living cells. DHEA-Bodipy was compared with DHEA regarding its ability (i) to induce  $ER\alpha$ -mediated gene activation, (ii) to protect PC12 rat pheochromocytoma cells against serum deprivation-induced apoptosis, and (iii) to induce stress fibers and focal adhesion contacts in SH-SY5Y human neuroblastoma cells. Metabolism of DHEA and DHEA-Bodipy in neuroblastoma cells were analyzed. We explored trafficking and subcellular localization of DHEA-Bodipy in living neuroblastoma cells. Structure-function analysis demonstrates the specific binding of DHEA-Bodipy to plasma membranes of PC12 cells. In all aspects tested, DHEA-Bodipy proved to be a functional DHEA derivative suitable for live-cell imaging of intracellular DHEA transport and localization.

## 2. Materials and methods

### 2.1. Materials

Human SH-SY5Y neuroblastoma cells were from the Interlab Cell Line Collection (Genova, Italy). Rat PC12 pheochromocytoma cells were obtained from ATCC (Rockville, U.S.A.). SK-ER $\alpha$  cells were a generous gift of Christian Behl (University of Mainz, Germany). Trilostane (2(5 $\alpha$ )-androstene-3-cyano-4 $\alpha$ ,5 $\alpha$ -epoxy-3-ol-17-one), 7-keto-DHEA-7-CMO (5-androstene-3 $\beta$ -ol-7,17-dione-7-O-carboxymethylloxime) and DHEA sulphate were from Steraloids (Rhode Island, U.S.A.). Steroids were prepared as 1000x stock solutions in 100% ethanol or DMSO (for DHEAS) or 10,000x stock solution in DMSO (for Trilostane). Thin layer chromatography plates and solvents were from Merck (Darmstadt, Germany). Bodipy<sup>®</sup> FL EDA (4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine, hydrochloride), MitoTracker<sup>®</sup> Red CMXRos, ER-Tracker<sup>™</sup> Blue-White DPX, CellMask<sup>™</sup> Deep Red plasma membrane stain and Lipofectamine<sup>™</sup> 2000 transfection reagent were from Invitrogen (Karlsruhe, Germany). Cell culture media were from PAA (Germany). APOPercentage Apoptosis Assay was from Tebu-Bio (Offenbach, Germany). ER firefly luciferase reporter vector pERE-luc was from BioCat (Heidelberg, Germany), Renilla luciferase vector phRL was from Promega (Mannheim, Germany). Fluorescein isothiocyanate (FITC) labeled phalloidin, monoclonal anti-vinculin antibody (clone hVIN-1, mouse ascites fluid), Cy3 labeled anti-mouse IgG, DAPI (2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride) and all other chemicals and steroids were from Sigma (Munich, Germany).

### 2.2. Synthesis of DHEA-Bodipy (3 $\beta$ -Hydroxy-(7-ylidene-aminoxy-acetamido-ethyl)-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene)-3-propionamide)-17-oxo-5-androsten)

N,N'-Dicyclohexylcarbodiimide-Pentafluorophenol ("F-complex") was synthesized as described (Kovacs et al., 1967). All subsequent reactions were performed in the dark. Bodipy<sup>®</sup> FL EDA (1.5 mg, 4.047  $\mu$ mol) and 7-keto-DHEA-7-CMO (7 mg, 18.65  $\mu$ mol) were dissolved in dimethylformamide (3 ml). 1 ml HCO<sub>3</sub><sup>-</sup>/CO<sub>3</sub><sup>2-</sup> buffer (pH 8.3) was added. The solution was cooled down to 0°C and 61.39 mg (80.94  $\mu$ mol) F-complex were added for activation of the carboxyl group of DHEA-CMO (Kisfaludy et al., 1973). The solution was stirred at room temperature for 72 hours.

The products of the synthesis were fractionated and purified by semipreparative thin layer chromatography with benzol/ethylacetate/acetone (1/8/1, v/v) as solvent system. Detection was carried out by iodine vapor or ultraviolet light. The R<sub>F</sub>-values were as follows: 0.34 (DHEA-Bodipy), 0.48 (DHEA-CMO) and 0.53 (Bodipy<sup>®</sup> FL EDA). The yield for DHEA-Bodipy was 2 mg (2.89  $\mu$ mol, 71.2%). The identity of the prepared sample was confirmed by ESI mass spectrometry.

### 2.3. Cell cultures

Human SH-SY5Y cells were grown in phenol red-free DMEM/nutrient mixture F-12 Ham supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and penicillin/streptomycin. PC12 rat pheochromocytoma cells were cultured on poly-L-lysine coated cell culture dishes in phenol red-free RPMI medium 1640 containing 10% horse serum, 5% FCS, 2 mM glutamine and penicillin/streptomycin. SK-ER $\alpha$  cells were grown in phenol red-free DMEM supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate and penicillin/streptomycin. All cell lines were cultured at 37°C, 5% CO<sub>2</sub> and 100% humidity.

#### 2.4. Dual Luciferase Assay

SK-N-MC cells stably expressing the ER $\alpha$  (SK-ER $\alpha$  cells) were grown in 24-well cell culture plates in culture medium without antibiotics for 24 hours. At 90% confluency, cells were transiently transfected with endotoxin-free phRL Renilla luciferase reporter vector (phRL) and ER firefly luciferase reporter vector (pERE-luc) or empty control vector. In brief, per well 0.1  $\mu$ l phRL (100 ng) and 0.4  $\mu$ l pERE-luc (400 ng) or 0.4  $\mu$ l control vector (400 ng) were added to 100  $\mu$ l serum-free culture medium. 1  $\mu$ l Lipofectamine™ 2000 in 100  $\mu$ l serum-free culture medium was added. Diluted vectors and diluted Lipofectamine™ were combined and incubated for 20 min. Cells were incubated with the DNA/transfection complex at 37°C in a CO<sub>2</sub> incubator. 5 hours and 29 hours after transfection, cells were treated with 30 nM 17 $\beta$ -estradiol, 30  $\mu$ M DHEA, 30  $\mu$ M DHEA-Bodipy, 30  $\mu$ M 5-androstene-3 $\beta$ ,17 $\beta$ -diol, or 0.1% ethanol (vehicle control). 15 min before the addition of the steroids, cells were treated or not with 20  $\mu$ M trilostane in culture medium with 10% charcoal-stripped FCS. After addition of steroids, the trilostane concentration was kept at 10  $\mu$ M. 48 hours after transfection, cells were washed with PBS and incubated with lysis buffer (Promega) according to the manufacturer's instructions. Renilla and firefly luciferase activity were measured in Lumitrac™ 96 well plates (Greiner, Germany) in a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenburg, Germany).

#### 2.5. DHEA and DHEA-Bodipy metabolism in SK-ER $\alpha$ cells

SK-ER $\alpha$  cells were seeded on 10 cm culture dishes and grown to 90% confluency in complete medium. 24 hours before the experiment, medium was changed against culture medium containing 10% charcoal-stripped FCS. The experiment was initiated by adding 30  $\mu$ M DHEA, 30  $\mu$ M DHEA-Bodipy, 1  $\mu$ Ci of [<sup>3</sup>H]DHEA plus 30  $\mu$ M DHEA or 0.1% ethanol (vehicle control) in 5 ml medium per dish. In some experiments, cells were treated with 20  $\mu$ M trilostane, an inhibitor of 3 $\beta$ -hydroxysteroid dehydrogenase, 15 min before the addition of the steroids. After the indicated times lipids were extracted from medium and cells according to the method of Bligh and Dyer (Bligh and Dyer, 1959). Reference steroids and extracted steroids were applied on silica gel thin layer chromatography (TLC) plates and developed with toluene/acetone/chloroform (8/2/5, vol/vol) (Godin et al., 1999). [<sup>3</sup>H]Steroids were visualized with a BAS-1800 (Bioimaging Analyzer System, Fujifilm). In addition, steroids were visualized by spraying the plates with H<sub>2</sub>SO<sub>4</sub>/methanol (1/1) and heating up to 120°C until colored spots appeared.

In addition, lipid extracts from cells and media were analyzed by gas chromatography-mass spectrometry (GC-MS) as described (Steckelbroeck et al., 2002). GC-MS analysis of the DHEA metabolites was performed after derivatisation of the hydroxy groups of the extracted steroids to trimethylsilyl (TMSi) ethers by adding 1.5 ml TMSi-reagent (pyridine-hexamethyldisilazan-trimethylchlorosilane 9/3/1, v/v/vol) and incubation at 65°C for 1 h. The solvents were evaporated under a stream of nitrogen. The residues were dissolved in 50  $\mu$ l n-decane and transferred into microvials for GC-MS analysis. Extracted compounds were separated on a cross-linked methyl silicone DB-XLB 122–1232 capillary column (30 m x 0.25 mm i.d. x 0.25  $\mu$ m film thickness; J & W, Folsom, CA, USA) in a Hewlett Packard gas chromatograph 6890 after splitless injection by an HP 7683 injector at 280°C. Hydrogen was used as carrier gas with an inlet pressure of 13.4 psi, resulting in a total gas-flow of 1.0 ml/min. The oven temperature was kept for 3 min at 150°C, then raised at a rate of 30°C/min to a final temperature of 290°C and kept at this temperature for 10.33 min. The injector of the GC and detector temperature of an HP5972 mass selective detector were set to 280°C.

Multiplier voltage was set to 2700 eV. The emission current was 220  $\mu$ A. Electron impact ionization was employed at 70 eV ionization energy. Total ion chromatogram was established by scanning between  $m/z$  50 and 500. The identities of the reaction products were qualified by comparison of their retention times and spectra with those of 5-androstene-3 $\beta$ ,17 $\beta$ -diol, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 17 $\beta$ -estradiol and DHEA.

Because of its high molecular mass (691.6) DHEA-Bodipy had to be hydrolyzed before GC-MS analysis. Lipid extracts of DHEA-Bodipy treated cells and media were incubated in 1 ml hydrolysis solution (1 M NaOH dissolved in 90% ethanol) at 37°C for 1 h. Distilled water (0.5 ml), and cyclohexane (3 ml) were added, vortexed and centrifuged at 3,500 rpm for 10 min. The organic phase was transferred into a new vial, and the extraction was repeated with 3 ml of cyclohexane. Extracts were pooled and evaporated under a stream of nitrogen.

## 2.6. Apoptosis assay

PC12 cells were seeded on poly-L-lysine coated 24-well cell culture plates (60,000 cells per well) and grown to 95% confluency in complete medium. 24 hours before the assay, cells were cultured in medium with or without serum, 30 nM DHEA, 30 nM DHEA-Bodipy or vehicle (0.1% ethanol). The APOPercentage Apoptosis Assay was used to quantify apoptosis, according to the manufacturer's instructions.

## 2.7. Fluorescence imaging of fixed cells

SH-SY5Y cells were seeded on poly-L-lysine-coated 10 mm-coverslips in 24-well cell culture plates. 24 hours before the experiment, at a cell density of about 80%, cells were cultivated in phenol red-free DMEM containing 10% charcoal-stripped FCS, glutamine and penicillin/streptomycin. Cells were washed twice with phenol red-free DMEM without supplements and treated for 30 min with 30 nM DHEA, 30 nM DHEA-Bodipy or 0.1% ethanol (vehicle control) at 37°C. Cells were fixed in 3.7% paraformaldehyde/PBS for 15 min and permeabilized in 0.05% Triton X-100 for 10 min. Vinculin was visualized with a mouse anti-vinculin antibody (1:400 in PBS/5% FCS, 30 min) followed by a Cy3-conjugated anti-mouse antibody (1:500 in PBS/5% FCS, 30 min). F-actin was stained with FITC-phalloidin (1:500 in PBS/5% FCS, 30 min). Nuclei were counterstained with DAPI. Cells were visualized under a fluorescence microscope (Axiovert S100, Zeiss Germany) equipped with a MicroMax CCD camera (Princeton Instruments, USA) and MetaView™ Imaging software (Universal Imaging Corporation, USA).

## 2.8. Live-cell imaging on PC12 and SK-ER $\alpha$ cells

For live-cell imaging, cells were seeded on 18 mm coverslips coated with poly-L-lysine (SK-ER $\alpha$  cells) or poly-L-lysine plus collagen (PC12 cells) in 3.5 cm cell culture dishes. 24 hours before the experiment, cells were cultivated in phenol red-free medium containing charcoal-stripped FCS, glutamine and penicillin/streptomycin. For live cell imaging, the coverslip was mounted on a thermostated chamber (37°C) on an Axiovert S100 microscope (Zeiss, Germany). For colocalization experiments, PC12 cells were treated with the plasma membrane marker CellMask™ Deep Red (5  $\mu$ g/ml). After 5 min, cells were washed twice with medium and 1  $\mu$ M DHEA-Bodipy was added. For displacement experiments, PC12 cells were incubated with 1  $\mu$ M DHEA-Bodipy  $\pm$  30  $\mu$ M DHEA, DHEAS, 4-androstene-3,17-dione, 17 $\beta$ -estradiol, testosterone or pregnenolone. For analysis of the intracellular transport and localization of DHEA-Bodipy, SK-ER $\alpha$  cells were treated with 1  $\mu$ M or 30  $\mu$ M DHEA-



Bodipy for various times. Colocalization experiments were performed with MitoTracker<sup>®</sup> Red CMXRos (25 nM, 15 min) or ER-Tracker<sup>™</sup> Blue-White DPX (100 nM, 30 min), respectively. Pictures were taken at an excitation wavelength of  $\lambda = 490$  nm for DHEA-Bodipy,  $\lambda = 374$  nm for ER-Tracker<sup>™</sup>,  $\lambda = 560$  nm for MitoTracker<sup>®</sup> or  $\lambda = 620$  nm for CellMask<sup>™</sup>, respectively. Fluorescence emission was measured using the following filters: Q505LP (DHEA-Bodipy), 400DCLP (ER-Tracker<sup>™</sup>), Q570LP (MitoTracker<sup>®</sup>) and 630DCXR (CellMask<sup>™</sup>). Cells were checked for autofluorescence at all wavelengths used. In some experiments, cells were fixed with 3.7% paraformaldehyde/PBS for 15 min after the staining of the mitochondria to prevent the rapid movement of these organelles.

### *2.9. Statistical analysis*

Values are expressed as the mean  $\pm$  SD. Statistical differences between mean values were determined by Student's t-test. All of the experiments were reproduced at least three times.

### 3. Results

#### 3.1 Design and synthesis of DHEA-Bodipy

The aim of this study was to create a functional fluorescent DHEA analog suitable for live-cell imaging. We chose 7-keto-DHEA-7-CMO for derivatization with Bodipy in order to retain an unaltered DHEA core structure with a hydroxyl group at C3, a  $\Delta 5$  double bond and a keto group at C17 (Fig. 1).

#### 3.2. DHEA-Bodipy induces the expression of luciferase reporter gene under the control of an estrogen response element

Up to date, no specific nuclear receptor for DHEA is known. However, it has been reported that micromolar concentrations (50  $\mu\text{M}$ ) of DHEA activate the estrogen receptor  $\alpha$  and  $\beta$  in human hepatoma HepG2 cells (Webb et al., 2006). To test whether DHEA-Bodipy is able to control transcriptional activation via the classical nuclear estrogen receptor, we transfected SK-ER $\alpha$  cells with a vector for the firefly luciferase gene under the control of an estrogen response element or an empty control vector, respectively. As internal standard for transfection efficiency and cell number, cells were transfected in parallel with a vector for constitutive expression of Renilla luciferase. None of the steroid hormones used in this study interfered with the luminescence or activity of the firefly or Renilla luciferase (data not shown).

Repeated treatments of SK-ER $\alpha$  cells with 30 nM 17 $\beta$ -estradiol (5 and 29 hours after transfection) induced a 19fold ( $\pm 3.4$ ) expression of the firefly luciferase compared to ethanol (0.1%) treated cells. 30  $\mu\text{M}$  DHEA enhanced the expression of luciferase 40.5fold ( $\pm 7.4$ ). Similar results were obtained for DHEA-Bodipy which stimulated the expression of luciferase 37fold ( $\pm 1.6$ ) (Fig. 2). Cells transfected with empty control vector showed no enhanced chemiluminescence upon treatment with steroid hormones or ethanol, respectively (data not shown). These results demonstrate that both DHEA-Bodipy and DHEA activate the ER $\alpha$  and induce the expression of firefly luciferase under control of an estrogen response element, i.e. both DHEA and DHEA-Bodipy are able to induce genomic effects.

It was also possible that DHEA and DHEA-Bodipy activate the ER $\alpha$  after their conversion to 17 $\beta$ -estradiol and 17 $\beta$ -estradiol-Bodipy, respectively. In an additional experiment, we treated SK-ER $\alpha$  cells with trilostane, an inhibitor of 3 $\beta$ -hydroxysteroid dehydrogenase, prior to the addition of the steroids. Trilostane inhibits both, the conversion of DHEA to 5-androstene-3,17-dione and the conversion of 5-androstene-3 $\beta$ ,17 $\beta$ -diol to testosterone. Consequently, trilostane inhibits the metabolization of DHEA to 17 $\beta$ -estradiol (see also Fig. 3, B1). We found that trilostane did not inhibit the activation of ER $\alpha$  by DHEA or DHEA-Bodipy (Fig. 2). This indicates that DHEA and DHEA-Bodipy are not converted to 17 $\beta$ -estradiol or 17 $\beta$ -estradiol-Bodipy prior to the activation of the ER $\alpha$ .

5-Androstene-3 $\beta$ ,17 $\beta$ -diol, a direct metabolite of DHEA, has also been reported to stimulate the endogenous ER $\alpha$  in breast cancer cells (Maggiolini et al., 1999). The results of Maggiolini *et al.* were corroborated by our findings that 5-androstene-3 $\beta$ ,17 $\beta$ -diol activates the ER $\alpha$  29.8fold ( $\pm 2.7$ ) in SK-ER $\alpha$  cells (Fig. 2). In summary, our results suggest that DHEA might activate the ER $\alpha$  either directly or after its conversion to 5-androstene-3 $\beta$ ,17 $\beta$ -diol.

### 3.3 DHEA and DHEA-Bodipy metabolism in SK-ER $\alpha$ cells

To analyze the metabolism of DHEA in SK-ER $\alpha$  cells, we treated cells on 10 cm culture dishes with 1  $\mu$ Ci of [ $^3$ H]DHEA plus 30  $\mu$ M DHEA for 1, 24, 48 and 72 hours and analyzed the lipid composition of cells and culture media by TLC (toluene / acetone / chloroform (8/2/5)). As steroid standards we applied DHEA ( $R_f$  = 0.37) and its possible metabolites 5-androstene-3 $\beta$ ,17 $\beta$ -diol ( $R_f$  = 0.21), 4-androstene-3,17-dione ( $R_f$  = 0.47), testosterone ( $R_f$  = 0.26), 17 $\beta$ -estradiol ( $R_f$  = 0.28), estrone ( $R_f$  = 0.51), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol ( $R_f$  = 0.21), dihydrotestosterone ( $R_f$  = 0.38) and DHEA sulfate ( $R_f$  = 0.00). Spraying the plate with H<sub>2</sub>SO<sub>4</sub>/methanol and heating yielded colored spots, e.g. yellow spots for estrogens, green for androstenediol and mauve for DHEA and androstenediol (see also Fig. 3, B2, where some of the steroid standards are shown). After 24 hours [ $^3$ H]DHEA is partially converted to a tritiated lipid with  $R_f$  (0.21) identical to 5-androstene-3 $\beta$ ,17 $\beta$ -diol and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol and color identical to 5-androstene-3 $\beta$ ,17 $\beta$ -diol (Fig. 3, A1 and A2). This tritiated steroid was found both in cells and culture medium. The ratio of total (cells plus medium) 5-androstene-3 $\beta$ ,17 $\beta$ -diol to total DHEA increased from 0.203 (after 1 h of incubation) to 0.633 (after 72 h) (Fig. 3, A3). No additional tritiated spots could be observed for the duration of the experiment, e. g. we could not detect [ $^3$ H]17 $\beta$ -estradiol in cells or culture media (Fig. 3, A1).

In a similar experiment, we analyzed the effect of the 3 $\beta$ -hydroxysteroid dehydrogenase inhibitor trilostane on the DHEA metabolism of SK-ER $\alpha$  cells. 15 min before the addition of 30  $\mu$ M DHEA, cells were treated or not with 20  $\mu$ M trilostane or 0.01% DMSO (vehicle). The lipid composition of culture media was analyzed 24 and 48 hours after the addition of DHEA, whereas cells were analyzed after 48 hours. In parallel, we incubated cells with 0.1% ethanol (vehicle) alone to analyze the endogenous steroid hormone composition of SK-ER $\alpha$  cells. Lipid composition was analyzed by TLC which provided the following results (Fig. 3, B2): (i) SK-ER $\alpha$  cells have no endogenous steroid hormones. In control (i.e. ethanol-treated) cells, cholesterol was the only steroid that could be detected. (ii) DHEA is partially converted to 5-androstene-3 $\beta$ ,17 $\beta$ -diol which is secreted into the medium. 17 $\beta$ -estradiol could not be detected. (iii) Trilostane did not influence the DHEA metabolism in SK-ER $\alpha$  cells. This indicates further that 5-androstene-3 $\beta$ ,17 $\beta$ -diol and not 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol is the only DHEA metabolite in SK-ER $\alpha$  cells.

In addition, the lipid composition of untreated, DHEA-treated and DHEA-Bodipy-treated cells was analyzed by GC-MS (not shown). GC-MS confirmed that (i) SK-ER $\alpha$  cells have no endogenous steroid hormones and (ii) DHEA is partially converted to 5-androstene-3 $\beta$ ,17 $\beta$ -diol which is secreted into the medium, but not to 17 $\beta$ -estradiol. To a minor extent, DHEA is metabolized to a steroid hormone that could not be identified (TMS derivative:  $m/z$  = 409) and to a hydrogenated derivative of this steroid. DHEA-Bodipy is partly metabolized to two steroid-Bodipy conjugates that could not be identified. The steroid moiety of both conjugates is identical to the unknown steroid hormones of DHEA-treated cells. DHEA-Bodipy is not converted to 5-androstene-3 $\beta$ ,17 $\beta$ -diol-Bodipy or 17 $\beta$ -estradiol-Bodipy.

### 3.4. DHEA-Bodipy protects rat pheochromocytoma PC12 cells against serum-deprivation induced apoptosis

Rat pheochromocytoma PC12 cells are an established model for cell apoptosis and survival. It has been shown that DHEA protects PC12 cells against serum-deprivation induced apoptosis (Charalamopoulos et al., 2004). Based on these findings, we tested whether DHEA-Bodipy would exert a similar antiapoptotic effect on PC12 cells. As positive control (100%

apoptosis), PC12 cells were grown in serum-supplemented medium for 24 hours and treated with 1 mM H<sub>2</sub>O<sub>2</sub> for 2 hours. PC12 cells grown in serum-supplemented medium for 24 hours showed an apoptosis rate of 1.5 % ( $\pm$  0.29 %). In contrast, 24 hours after serum deprivation, the apoptosis rate increased to 65.8 % ( $\pm$  1.29 %). 30 nM DHEA as well as 30 nM DHEA-Bodipy significantly decreased the apoptosis rate of PC12 cells grown for 24 hours in serum-free medium to 52.4 % ( $\pm$  1.42 %) and 54.6 % ( $\pm$  2.74 %), respectively (Fig. 4), indicating that the antiapoptotic effect of DHEA-Bodipy is similar to that of DHEA.

### *3.5. DHEA-Bodipy induces stress fibers and focal adhesion contacts in SH-SY5Y neuroblastoma cells*

Compagnone and Mellon found that DHEA induces morphological changes in primary cultures of embryonic neocortical neurons by increasing the length of neurites containing the axonal marker Tau (Compagnone and Mellon, 1998). Axonal guidance is mediated by the highly motile and actin-rich growth cone at the terminus of the axon (Rodriguez et al., 2003). Based on these findings, we tested the influence of DHEA and DHEA-Bodipy on the actin cytoskeleton of SH-SY5Y neuroblastoma cells.

Almost all of the SH-SY5Y cells exhibited thin cortical and central stress fibers and small focal adhesion contacts (Fig. 5, control). Some of these cells had short processes and/or a few filopodia. 30 min incubation with 30 nM DHEA increased stress fiber assembly and the formation of focal adhesion contacts in SH-SY5Y cells. Both effects were mimicked by DHEA-Bodipy (Fig. 5).

### *3.6. DHEA-Bodipy binds rapidly and specifically to the plasma membrane of PC12 cells*

In PC12 cells, DHEA has been reported to bind to specific plasma membrane binding sites (Charalampopoulos et al., 2006). We incubated living PC12 cells with 1  $\mu$ M DHEA-Bodipy. DHEA-Bodipy bound rapidly (within 2 min) to the plasma membrane as could be shown by its colocalization with CellMask™ Deep Red plasma membrane stain (Fig. 6). In addition, a weaker and uniform staining of the cytoplasm could be observed. In contrast, the nuclei of PC12 cells were never stained even after a long-term incubation for up to 72 hours (data not shown). The specificity of these DHEA plasma membrane binding sites was studied by performing displacement experiments of DHEA-Bodipy with a 30fold excess (30  $\mu$ M) of different steroids (Fig. 7). DHEA, DHEAS and 4-androstene-3,17-dione completely displaced DHEA-Bodipy from the plasma membrane. In contrast, 17 $\beta$ -estradiol, testosterone and pregnenolone did not compete with DHEA-Bodipy for plasma membrane binding sites. The weak and uniform staining of the cytoplasm was never displaced completely by an excess of steroids.

### *3.7. DHEA-Bodipy is suitable for live cell imaging of intracellular DHEA transport*

Finally, we tested the suitability of DHEA-Bodipy for fluorescence microscopy of living SK-ER $\alpha$  cells. We incubated SK-ER $\alpha$  cells with 30  $\mu$ M DHEA-Bodipy, i.e. the same concentration we used for the luciferase assay, or with 1  $\mu$ M DHEA-Bodipy (not shown). At both concentrations, we found that DHEA-Bodipy accumulates rapidly (within a few minutes) in the endoplasmic reticulum as could be shown by its colocalization with ER-Tracker™ Blue-White DPX (Fig. 8). Colocalization with the endoplasmic reticulum was complete after 60 min. In contrast to our results with PC12 cells, we observed a weak staining of the nucleus which remained during the complete time course of the experiment (72 hours). After 22

hours, DHEA-Bodipy started to colocalize with MitoTracker<sup>®</sup> Red CMXRos, a fluorescent marker for mitochondria. After 72 hours, DHEA-Bodipy was situated in both mitochondria and the endoplasmic reticulum. The staining of the endoplasmic reticulum with DHEA-Bodipy was reversible and disappeared 10 min after washing the cells twice with medium whereas the staining of the mitochondria remained unchanged (not shown). Colocalization of DHEA-Bodipy and mitochondria is shown in cells fixed with paraformaldehyd after staining of the mitochondria (Fig. 8, row 5).

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#### 4. Discussion

During the past ten years important progress has been made concerning the mechanism(s) of action of DHEA. Nevertheless, little is known about the intracellular trafficking and subcellular localization of this important steroid hormone. For the analysis of the subcellular localization of DHEA, anti-DHEA antibodies are commercially available. The advantage of anti-steroid antibodies over fluorescent steroid hormone analogs is that antibodies recognize unaltered steroid hormone molecules whereas fluorescent steroid hormones are chemically modified. With this in mind, we chose 7-keto-DHEA-7-CMO for derivatization with the fluorescent molecule Bodipy in order to retain an unaltered DHEA core structure. Bodipy is frequently used for labeling of lipids (Pagano and Chen, 1998). It is small, nonpolar and membrane permeable and therefore suitable for live cell imaging of steroid hormones. The long spacer between DHEA and Bodipy provides a flexible link between the steroid hormone and the fluorescent dye. Thereby, potentially interfering effects of Bodipy on the functions of DHEA should be minimized. Moreover, the advantages of fluorescent steroid hormones outweigh the use of anti-steroid antibodies: (i) steroid hormones often have very similar structures. For example, DHEA differs from 5-androstene-3 $\beta$ ,17 $\beta$ -diol only at position C17. Therefore, cross-reactivity might easily occur. (ii) In steroidogenic cell lines such as SH-SY5Y neuroblastoma cells (Guarneri et al., 2000), antibodies permit no discrimination between steroid hormones made by the cell itself and steroid hormones added to the cells. (iii) For analysis of the subcellular distribution of steroid hormones with antibodies, cells need to be fixed and permeabilized which might lead to artifacts.

We employed genomic and non-genomic assays to test the functionality of DHEA-Bodipy. Up to date, no specific receptor is known that mediates genomic effects of DHEA. However, Webb et al. found that in HepG2 cells, micromolar concentrations of DHEA activate ER $\beta$  nearly to the same and ER $\alpha$  to a lesser extent as compared to nanomolar concentrations of 17 $\beta$ -estradiol (Webb et al., 2006). In SK-ER $\alpha$  cells we even found a twofold stronger activation of ER $\alpha$  by 30  $\mu$ M DHEA as compared to 30 nM 17 $\beta$ -estradiol. This action of DHEA was mimicked faithfully by DHEA-Bodipy demonstrating that DHEA-Bodipy is able to induce genomic effects similar to DHEA. These results are supported by the observed staining of the nuclei of living SK-ER $\alpha$  cells after incubation with DHEA-Bodipy. Neuronal cells express steroidogenic enzymes such as cytochrome P450 side-chain cleavage enzyme (P450<sub>sc</sub>), 17 $\alpha$ -hydroxylase/C17-20-lyase (P450<sub>c17</sub>), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD), cytochrome P450 aromatase (P450<sub>arom</sub>) and 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD) types 1 and 3 and produce pregnenolone, DHEA, androstenedione, androstenediol, testosterone and 17 $\beta$ -estradiol (Mukai et al., 2006; Zwain and Yen, 1999).

When we analyzed the lipid composition of SK-ER $\alpha$  cells by TLC and GC-MS we did not detect endogenous steroid hormones. We conclude that SK-ER $\alpha$  cells are not capable of *de novo* biosynthesis of steroid hormones. This makes SK-ER $\alpha$  cells particularly suitable for the investigation of effects of steroid hormones, because these effects are not influenced by endogenous steroid hormones.

Nevertheless, it was possible that DHEA and DHEA-Bodipy activate the ER $\alpha$  after their conversion to 17 $\beta$ -estradiol and 17 $\beta$ -estradiol-Bodipy in SK-ER $\alpha$  cells, respectively. We analyzed the DHEA metabolism in SK-ER $\alpha$  cells by TLC and GC-MS and found that DHEA is converted to 5-androstene-3 $\beta$ ,17 $\beta$ -diol, a direct metabolite of DHEA, and two other steroid hormones that could not be identified. DHEA was not metabolized to 17 $\beta$ -estradiol. In contrast, DHEA-Bodipy is partly metabolized to two steroid-Bodipy conjugates. The steroid moiety of both conjugates is identical to the unknown steroid hormones of DHEA-treated

cells. DHEA-Bodipy is not converted to 5-androstene-3 $\beta$ ,17 $\beta$ -diol-Bodipy or 17 $\beta$ -estradiol-Bodipy. This suggests that at least in SK-ER $\alpha$  cells, the metabolisms of DHEA and DHEA-Bodipy is similar but not identical. In contrast to DHEA, DHEA-Bodipy seems not to be accepted by the enzyme 17 $\beta$ -hydroxysteroid dehydrogenase which reduces DHEA to 5-androstene-3 $\beta$ ,17 $\beta$ -diol. In line with these results, inhibition of 3 $\beta$ -hydroxysteroid dehydrogenase which converts 5-androstene-3 $\beta$ ,17 $\beta$ -diol to testosterone, a direct precursor of 17 $\beta$ -estradiol, had no effect on the activation of ER $\alpha$  in DHEA or DHEA-Bodipy-treated cells.

Interestingly, 5-androstene-3 $\beta$ ,17 $\beta$ -diol has a high affinity to the estrogen receptor, translocates the receptor to the nucleus and induces the expression of an estrogen-dependent secreted glycoprotein and a transfected ER reporter gene (Adams et al., 1981; Maggiolini et al., 1999). Therefore, DHEA might activate the ER $\alpha$  after its conversion to 5-androstene-3 $\beta$ ,17 $\beta$ -diol. However, because DHEA-Bodipy is not converted to androstenediol-Bodipy and activates the ER $\alpha$  to the same extent as DHEA, it seems likely that the ER $\alpha$  might be activated also by DHEA itself.

Some of the steroidogenic enzymes reside in mitochondria, most of them in the endoplasmic reticulum (Miller, 2008). Therefore, the staining of the endoplasmic reticulum and mitochondria following incubation of SK-ER $\alpha$  cells with DHEA-Bodipy could reflect the binding of DHEA-Bodipy and its unknown fluorescent metabolites to steroidogenic enzymes. In general, the predominant accumulation of DHEA-Bodipy in the endoplasmic reticulum and mitochondria indicates a specific interaction of DHEA with microsomal and mitochondrial lipids and/or proteins. Besides steroidogenic enzymes, one candidate protein could be the sigma-1 receptor which is highly concentrated on the endoplasmic reticulum (McCann and Su, 1990) and interacts with several neuroactive steroids including DHEA (Maurice et al., 2006). It plays a role in cellular Ca<sup>2+</sup> mobilization and the formation of lipid droplets and lipid microdomains (Maurice et al., 2006). Another candidate protein is ER $\beta$  because it has been found in mitochondria of neurons (Yang et al., 2004) and binds DHEA (Webb et al., 2006). ER $\beta$  protects cells from UV irradiation stress (Pedram et al., 2006) or oxidative stress (Razmara et al., 2007) in part due to activation of manganese superoxide dismutase which catalyzes superoxide radical breakdown. Moreover, steroid hormone receptors in mitochondria play a role in mitochondrial transcription and OXPHOS biosynthesis (Psarra and Sekeris, 2008). A third candidate protein is GPR30, a G protein-coupled receptor for 17 $\beta$ -estradiol. In cells transfected with GPR30-GFP the fluorescent 17 $\beta$ -estradiol derivative E2-Alexa 546 colocalizes with the endoplasmic reticulum and Golgi (Revankar et al., 2005). Activation of GPR30 results in intracellular calcium signalling and synthesis of phosphatidylinositol-3,4,5-trisphosphate in the nucleus.

We can only speculate why DHEA-Bodipy disappears rapidly from endoplasmic reticulum but less from mitochondria after washing. DHEA binding sites at the endoplasmic reticulum might have a lower affinity for DHEA as compared with mitochondrial binding sites. Alternatively, if DHEA-Bodipy binds to the inner mitochondrial membrane it might not be washed away easily. In contrast, the endoplasmic reticulum has a very large surface (up to 50% of total cellular membranes) facilitating a rapid exchange with the cytosol.

Using primary cultures of mouse embryonic neocortical neurons, Compagnon and Mellon showed that DHEA selectively increases the length of neurites containing the axonal marker Tau (Compagnone and Mellon, 1998). In PC12 cells, Charalampopoulos et al. showed that DHEA stimulates actin depolymerization and actin filament disassembly (Charalampopoulos et al., 2005). Therefore, we tested the influence of DHEA and DHEA-Bodipy on the morphology of the actin cytoskeleton of SH-SY5Y neuroblastoma cells. We focused our

study on stress fibers, i.e. contractile bundles of actin and the motor protein myosin, and focal adhesion contacts, i.e. protein complexes through which the cytoskeleton connects to the extracellular matrix. We found that treatment of the cells with DHEA or DHEA-Bodipy, respectively, rapidly (within 30 min) increased the amount and thickness of both stress fibers and focal adhesion contacts in SH-SY5Y cells. Unpublished results from our lab indicate that the small GTPase Rho might be involved in these morphological changes. In contrast, the small GTPases Cdc42 and Rac1 act antagonistically to RhoA and promote neurite outgrowth (Koh, 2006). The reason for the apparently contradictory results of our lab compared to those of Compagnone and Mellon and Charalampopoulos et al. might originate from the use of different cell types and/or (concerning the work of Compagnone and Mellon) different incubation times (20 hours vs. 30 min).

The neuroactive steroid DHEA is produced in the brain and the adrenals. Serum concentrations of DHEA peak between the ages of 20 and 30 years. With advancing age, the production rates and levels of these steroids in serum and adrenals decrease gradually (Nawata et al., 2004). This is associated with neuronal dysfunction and degeneration, most probably because DHEA protects CNS neurons against noxious agents. Recently, Charalampopoulos et al. showed that DHEA protects rat pheochromocytoma PC12 cells against serum deprivation-induced apoptosis. They demonstrated that DHEA induced both genomic and non-genomic effects in PC12 cells: DHEA binds to and activates a  $G\alpha$  coupled receptor that in turn activates prosurvival tyrosine kinase Src, protein kinase C (PKC) and MEK1/2/ERK1/2. These kinases activate the prosurvival transcription factors CREBP and NF-kappaB which stimulate the expression of antiapoptotic Bcl-proteins Bcl-2 and Bcl-xL. In addition, PKC phosphorylates Bcl-2 which is necessary for its antiapoptotic function (Charalampopoulos et al., 2006; Charalampopoulos et al., 2004). Moreover, DHEA induces phosphorylation of Akt kinase which inactivates pro-apoptotic Bad (Charalampopoulos et al., 2008).

We also found that DHEA decreases significantly serum deprivation induced apoptosis. Moreover, DHEA-Bodipy decreased the apoptosis rate to the same extent as DHEA suggesting further that DHEA-Bodipy induces the same genomic and non-genomic effects as DHEA. It has been shown that the antiapoptotic effect of DHEA is structure specific, e.g. hydroxylation at C7 results in loss of antiapoptotic activity of DHEA (Charalampopoulos et al., 2004). In contrast, we found that DHEA-Bodipy is as effective as DHEA in preventing serum deprivation-induced apoptosis albeit DHEA-Bodipy is a DHEA derivative modified at position C7. Probably, the hydrophobic fluorescent group in combination with a long spacer is better tolerated by the putative G protein-coupled receptor for DHEA than a hydrophilic hydroxyl group. This further emphasizes that the modification of DHEA in DHEA-Bodipy does not interfere with the functions of DHEA.

DHEA-Bodipy binds rapidly to plasma membranes of living PC12 cells. Similar results were obtained by Charalampopoulos et al. with DHEA-BSA-FITC, a membrane impermeable fluorescein isothiocyanat labeled bovine serum albumin conjugate of DHEA (Charalampopoulos et al., 2006). In contrast to DHEA-BSA-FITC, DHEA-Bodipy is nonpolar similar to DHEA. A small portion of DHEA-Bodipy rapidly entered the cells and led to a weak and apparent uniform staining of the cytoplasm which could not be displaced by an excess of DHEA or other steroids. In contrast, DHEA-Bodipy did not translocate to the nuclei of PC12 cells even after three days of incubation. This corroborates recent findings that genomic effects of DHEA in PC12 cells are membrane-mediated and involve PI3K/Akt pathway, PKA and MAPK pathways but not a nuclear receptor (Charalampopoulos et al., 2008).



The specificity of the membrane staining of DHEA-Bodipy was examined by competition experiments with a 30fold excess of different steroid hormones. We found that the 17-ketosteroids DHEA and 4-androstene-3,17-dione were able to compete with DHEA-Bodipy for putative DHEA membrane binding sites on PC12 cells regardless of the chemical group on position C3. Even DHEAS bearing a negatively charged sulfate group at position C3 instead of a 3 $\beta$ -hydroxy-group was able to displace DHEA-Bodipy. In contrast, 17 $\beta$ -estradiol, pregnenolone (5-pregnen-3 $\beta$ -ol-20-one) and testosterone (4-androstene-17 $\beta$ -ol-3-one) were not able to compete with DHEA-Bodipy for plasma membrane binding sites on PC12 cells. We conclude that the C17-keto group of DHEA interacts specifically with a putative plasma membrane receptor for DHEA whereas the C3- $\beta$ -hydroxy function is not necessary for plasma membrane binding of DHEA. Our results are in line with the results of displacement experiments of [<sup>3</sup>H]DHEA on isolated PC12 membranes (Charalampopoulos et al., 2006). DHEA and DHEAS competed for [<sup>3</sup>H]DHEA binding on plasma membranes whereas the progestagen allopregnanolon failed to displace [<sup>3</sup>H]DHEA. Testosterone was a weaker competitor for [<sup>3</sup>H]DHEA than DHEA and DHEAS. Even at 200fold excess over [<sup>3</sup>H]DHEA it displaced only 60% of the specific binding of [<sup>3</sup>H]DHEA.

We have shown in this work that DHEA-Bodipy is a functional DHEA analog with regard to both genomic and non-genomic effects and that DHEA-Bodipy is suitable for live cell imaging of intracellular transport of DHEA. The investigation of the exact itinerary and trafficking mechanism of DHEA-Bodipy in different cell types will be the focus of our future work.

**Acknowledgements**

We thank Silvia Wienken, Julia Reimann, Peter Bachon, Daniel Jänsch and Anja Kerksiek for technical assistance. We thank Gerald Gimpl for helpful comments and discussions and Kristina Endres for help with the luciferase assay. We express our grateful thanks to Christian Behl (University of Mainz, Germany) for generously providing the SK-ER $\alpha$  cells. This work was supported by the Bundesministerium für Bildung und Forschung (BMBF), Germany.

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## Figure legends

**Fig. 1.** Chemical structures of DHEA (A) and DHEA-Bodipy (B).

**Fig. 2.** DHEA and DHEA-Bodipy activate the estrogen receptor  $\alpha$ .

SK-N-MC cells stably expressing the estrogen receptor  $\alpha$  (SK-ER $\alpha$  cells) were transiently transfected with firefly luciferase reporter vector under control of an estrogen response element and Renilla luciferase reporter vector. 5 hours and 29 hours after transfection, cells were treated with 0.1% ethanol (vehicle control, 100%), 30 nM 17 $\beta$ -estradiol, 30  $\mu$ M DHEA, 30  $\mu$ M DHEA-Bodipy, or 30  $\mu$ M 5-androstene-3 $\beta$ ,17 $\beta$ -diol, respectively. 15 min before the addition of the steroids cells were treated or not with 20  $\mu$ M of trilostane. After the addition of steroids, the trilostane concentration was kept at 10  $\mu$ M. 48 hours after transfection, activation of ER $\alpha$  was measured indirectly via the activity of firefly luciferase (normalized to Renilla luciferase). The data are the mean  $\pm$  SD (bars) of three experiments in duplicate. \*\*\*  $P < 0.001$  (17 $\beta$ -estradiol *versus* ethanol, DHEA, DHEA-Bodipy, and 5-androstene-3 $\beta$ ,17 $\beta$ -diol, respectively), n.s. not significant.

**Fig. 3:** Metabolism of DHEA and DHEA-Bodipy in SK-ER $\alpha$  cells.

(A) SK-ER $\alpha$  cells were treated with 1  $\mu$ Ci of [ $^3$ H]DHEA plus 30  $\mu$ M DHEA. Lipids were extracted from cells or media and separated by TLC with toluene/acetone/chloroform (8/2/5). Lipids were visualized by H<sub>2</sub>SO<sub>4</sub>/methanol (1/1) and heating (A2). A1 shows the same TLC as A2. In A1, [ $^3$ H]steroids were visualized with a BAS-1800 (Bioimaging Analyzer System, Fujifilm). The following samples were applied: 1, 6 = DHEA, 2-5 = cells after 1, 24, 48 or 72 h incubation with steroids, 7-10 = media after 1, 24, 48 or 72 h incubation with steroids. (A3) The amount of [ $^3$ H]DHEA and [ $^3$ H]5-androstene-3 $\beta$ ,17 $\beta$ -diol was analyzed densitometrically. The ratio of total (cells + medium) androstenediol to total DHEA was plotted against the incubation time. (B1) Possible metabolites of DHEA. HSD = hydroxysteroid dehydrogenase, P450aro = aromatase. There are several isoforms of HSDs (not shown). (B2) Effect of the 3 $\beta$ -hydroxysteroid dehydrogenase inhibitor trilostane on the DHEA metabolism of SK-ER $\alpha$  cells. 15 min before the addition of 30  $\mu$ M DHEA, cells were treated or not with 20  $\mu$ M trilostane or 0.01% DMSO (vehicle). The lipid composition of culture media were analyzed 24 and 48 hours after the addition of DHEA (M24, M48), whereas cells were analyzed after 48 hours (C48). In parallel, cells were incubated with 0.1% ethanol (vehicle) alone. Lipid extraction and TLC were performed as in (A). Standards: 1 = 5-androstene-3 $\beta$ ,17 $\beta$ -diol, 2 = DHEA, 3 = 17 $\beta$ -estradiol, 4 = cholesterol, 5 = 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol.

**Fig. 4.** DHEA and DHEA-Bodipy protect PC12 cells against serum-deprivation induced apoptosis.

PC12 cells were grown in serum-free medium supplemented with 30 nM DHEA-Bodipy, 30 nM DHEA, 0.1% ethanol (vehicle control) or without drugs. As additional controls, cells were grown in serum-supplemented medium for 24 hours and were treated (100% apoptosis) or not with 1 mM H<sub>2</sub>O<sub>2</sub> for 2 hours. The data are the mean  $\pm$  SD (bars) of four experiments in duplicate. \*\*\*  $P < 0.001$  (DHEA *versus* ethanol), \*\*  $P < 0.01$  (DHEA-Bodipy *versus* ethanol), n.s. not significant.

**Fig. 5.** DHEA and DHEA-Bodipy increase stress fibers and focal adhesion contacts in SH-SY5Y neuroblastoma cells.

Cells were treated with 0.1% ethanol (control), 30 nM DHEA, or 30 nM DHEA-Bodipy for 30 min at 37°C. F-actin was stained with FITC-phalloidin (green). Focal adhesion contacts were visualized with an antibody against vinculin, followed by a Cy3 labeled secondary antibody (orange). Nuclei were counterstained with DAPI (blue). The scale bar represents 10  $\mu\text{m}$ .

**Fig. 6.** DHEA-Bodipy binds rapidly to the plasma membrane of PC12 cells.

Living PC12 cells were stained with the plasma membrane marker CellMask™ Deep Red (5  $\mu\text{g}/\text{ml}$ ). After 5 min cells were washed twice with medium and DHEA-Bodipy (1  $\mu\text{M}$ ) was added. Pictures were taken 2 min after addition of DHEA-Bodipy. The scale bar represents 10  $\mu\text{m}$ .

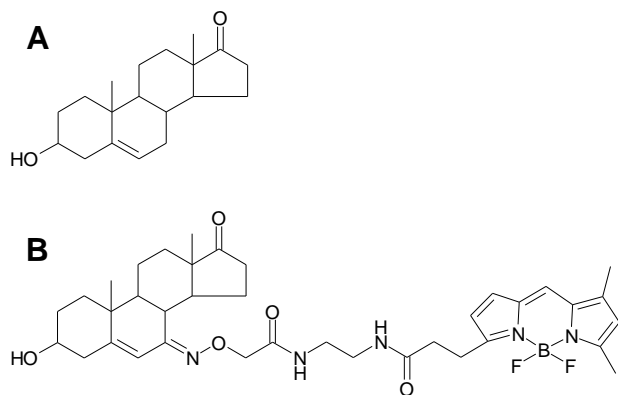
**Fig. 7.** DHEA-Bodipy binds specifically to the plasma membrane of PC12 cells.

PC12 cells were incubated simultaneously with 1  $\mu\text{M}$  DHEA-Bodipy  $\pm$  30  $\mu\text{M}$  steroid as indicated. Pictures of DHEA-Bodipy were taken 3 min after addition of the steroids. The scale bar represents 10  $\mu\text{m}$ .

**Fig. 8.** Itinerary of DHEA-Bodipy in SK-ER $\alpha$  cells.

Living SK-ER $\alpha$  cells were incubated with DHEA-Bodipy (30  $\mu\text{M}$ , i.e. the same concentration that we used in the luciferase assay) for the times indicated. Endoplasmic reticulum was visualized with ER-Tracker™ Blue-White DPX, mitochondria were visualized with MitoTracker® Red CMXRos. To prevent the rapid movement of mitochondria, cells were fixed after staining of the mitochondria with paraformaldehyde (row 5). The scale bar represents 10  $\mu\text{m}$ .

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Figure 1\_new





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Figure 2\_new

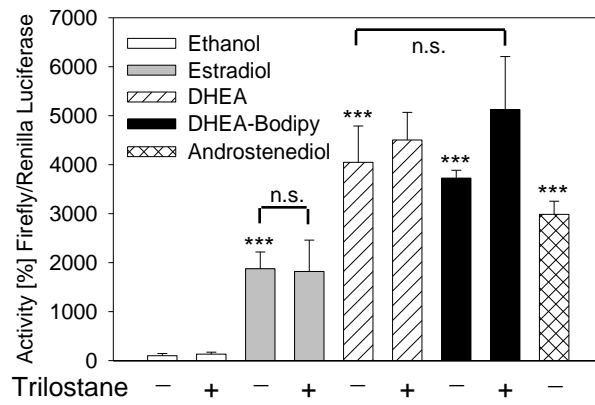
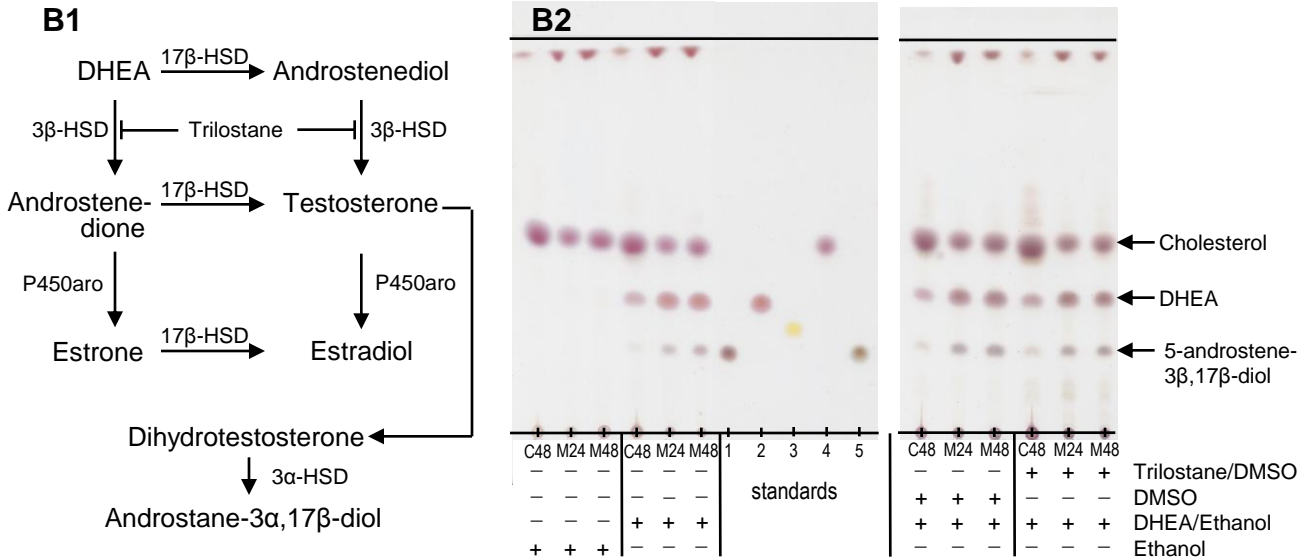
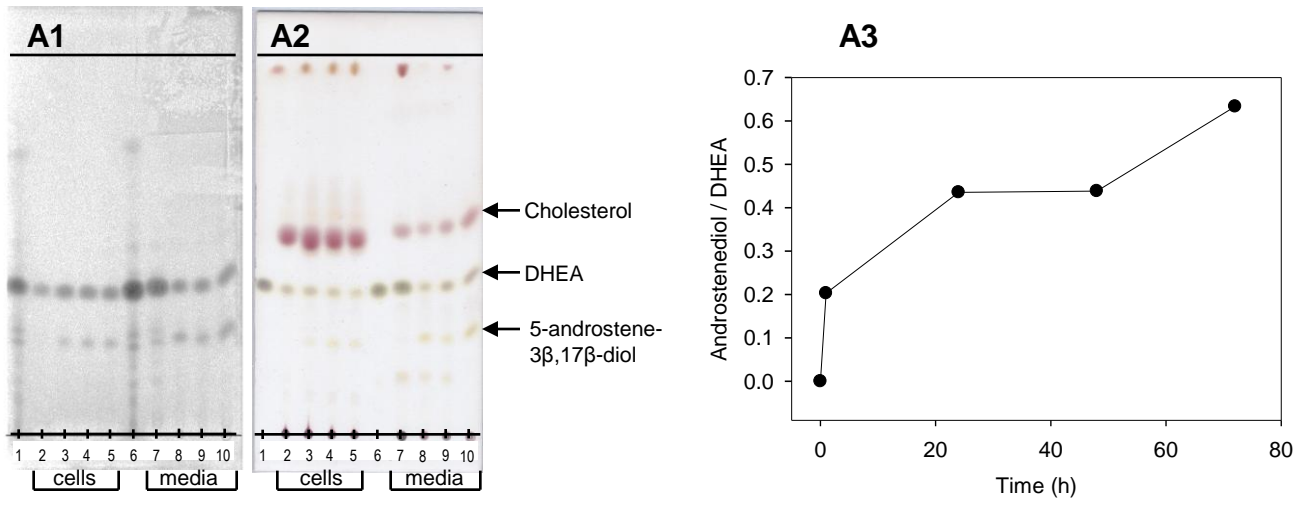
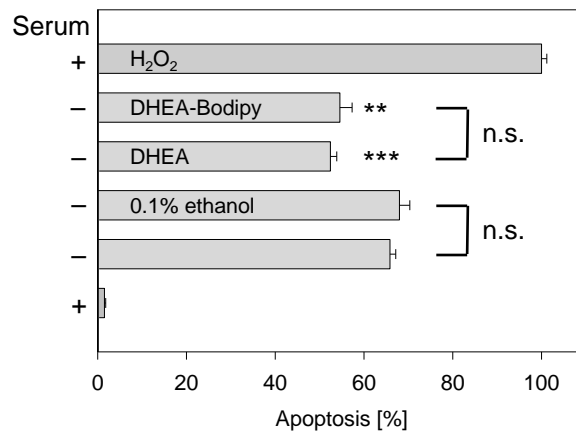


Figure 3

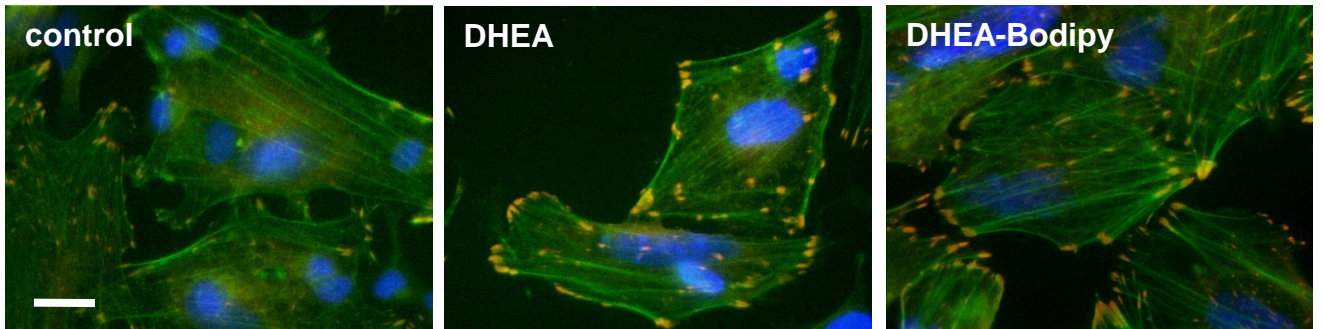
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**Figure 3\_new**



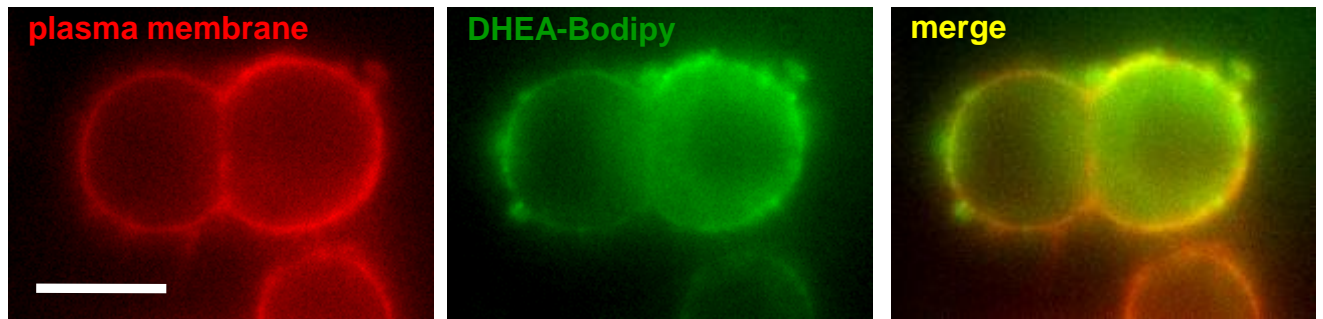
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Figure 4\_new



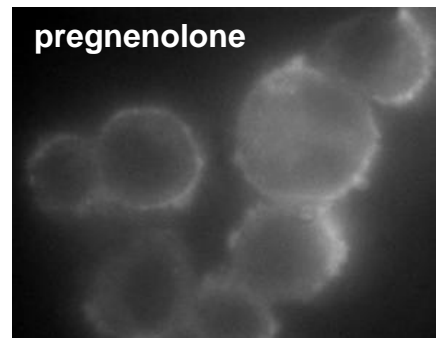
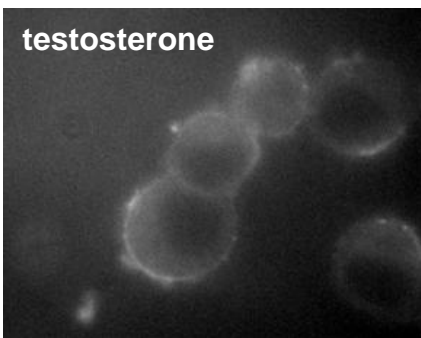
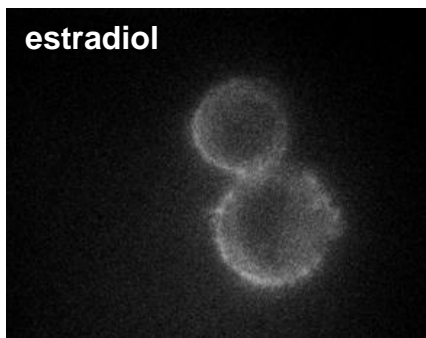
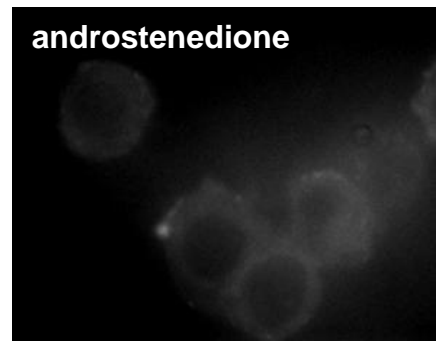
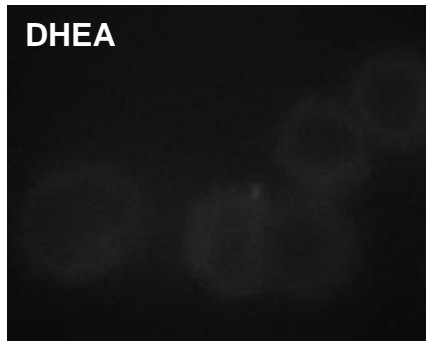
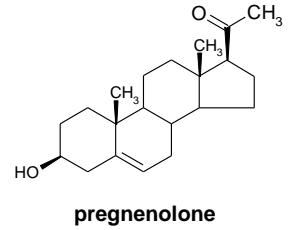
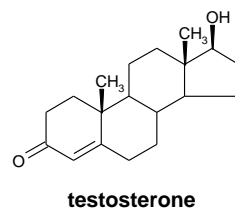
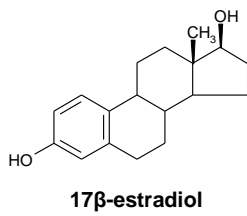
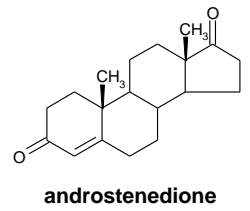
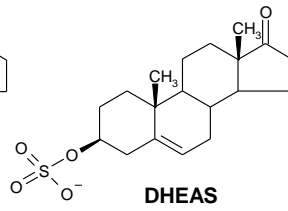
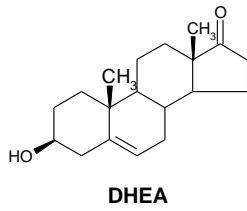
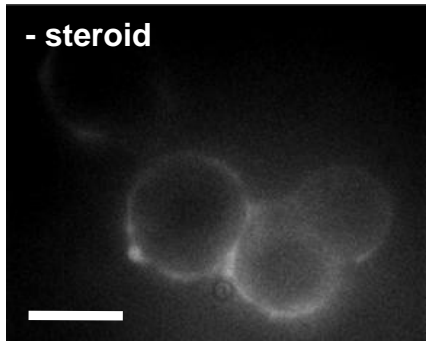
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**Figure 5\_new**



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Figure 6\_new



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Figure 7\_new



**Figure 8**

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**Figure 8\_new**

