BBA - Molecular Cell Research 1864 (2017) 1382–1392

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

# Dehydroepiandrosterone sulfate augments blood-brain barrier and tight junction protein expression in brain endothelial cells

Dimitrios Papadopoulos, Georgios Scheiner-Bobis*

*Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Germany*

ARTICLE INFO

*Keywords:*
Blood-brain-barrier
Claudin
DHEAS
Gnα11
Tight junction
ZO-1

ABSTRACT

Tight junctions (TJ) between brain endothelial cells are essential for formation and maintenance of the blood-brain barrier (BBB). Although loss of BBB integrity is associated with several neuropathological disorders, treatments that augment or stabilise the BBB are scarce. Here we show that physiological concentrations of dehydroepiandrosterone sulfate (DHEAS) stimulate the expression of the TJ proteins zonula occludens-1 (ZO-1) and claudin-3 in the brain-derived endothelial cell line bEnd.3 and promote TJ formation between neighbouring cells, demonstrated by augmented transendothelial resistance across cell monolayers. Silencing androgen receptor expression by siRNA does not prevent DHEAS-induced stimulation of ZO-1 expression, indicating that conversion of DHEAS into testosterone is not required for its actions. Suppression of Gnα11 expression by siRNA prevents DHEAS actions, pointing towards a G-protein-coupled receptor as being a mediator of the DHEAS effects. These results are consistent with the idea that DHEAS, acting as a hormone in its own right, supports the integrity of the BBB. The current findings might help in developing new strategies for the prevention or treatment of neurological disorders associated with BBB defects.

## 1. Introduction

Dehydroepiandrosterone sulfate (DHEAS) is the most abundant circulating steroid. DHEAS is produced mainly in adrenal cortex but also in brain [1] and in gonads [2–4]. In primates its production in the zona reticularis of the adrenals begins at early developmental stages and declines immediately after birth [5]. Its serum concentrations peak again during adrenarche [6]. DHEAS is derived from dehydroepiandrosterone (DHEA), which is almost entirely converted to DHEAS by the enzyme sulfotransferase [7]. The sulfated steroid is then secreted into the serum [8]. Young adults maintain a DHEAS plasma concentration ranging between 1.3 and 6.8 μM [9]. The plasma concentration of DHEA (7–31 nM) is 200-fold lower than the concentration of DHEAS [9]. With aging, DHEAS in blood and cerebrospinal fluid continuously decline to reach minimum concentrations at around age 70 [10,11]. The fact that age-related illnesses steeply increase as DHEAS concentrations decline prompted the conclusion and hope that restoring DHEAS concentration in elderly to levels seen in young adults would benefit their everyday feeling of well being and eventually, by preventing the ravages of aging, prolong life [7,12].

Neither the (patho)physiological significance of DHEAS (or DHEA) nor its molecular mechanisms have been studied well enough or are sufficiently understood. This may be associated with the long-held notion that sulfated steroids like DHEAS are physiologically inactive waste products of steroid hormone metabolism. The identification of cytosolic steroid sulfatases [13], however, has prompted the new idea that the sulfated steroids constitute a reservoir that upon desulfation can serve as precursors for the biosynthesis of other biologically active steroid hormones. In analogy, DHEAS has been considered as a pro-androgen that has to be converted to DHEA and then to testosterone or other steroid hormones in order to exert its biological activity [14].

Newer findings, including those from our laboratory, call into question the heretofore generally accepted idea of DHEAS being simply a pro-androgen [15,16]. They rather suggest that DHEAS, by interacting with a G-protein-coupled receptor (GPCR), might act as a steroid hormone in its own right. Thus, in spermatogenic cells DHEAS induces signaling pathways that correspond to the non-classical actions of steroid hormones [15]. In Sertoli cells this signaling pathway stimulates claudin expression and formation of tight junctions (TJ), thus indicating that DHEAS might be of physiological significance for the formation and maintenance of the blood-testis barrier [16].

TJ at the blood-brain barrier (BBB) protect the brain from harmful factors or cells of the immune system by limiting the paracellular flow of ions and molecules. Aging-associated dysfunction or disruptions of

* Corresponding author at: Institut für Veterinär-Physiologie und -Biochemie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Str. 100, D-35392 Giessen, Germany.
*E-mail address:* Georgios.Scheiner-Bobis@vetmed.uni-giessen.de (G. Scheiner-Bobis).

http://dx.doi.org/10.1016/j.bbamcr.2017.05.006
Received 11 January 2017; Received in revised form 4 May 2017; Accepted 6 May 2017
Available online 08 May 2017

the BBB have been connected with the onset of dementia, cerebral microvascular, or Alzheimer's disease [17–20]. In view of the exceptional physiological importance of this tissue barrier, little if anything is known about measures that might stimulate its formation and maintenance. Thus far, glucocorticosteroid treatment is the only applicable and most widely used therapeutic approach to improve BBB integrity [21,22].

Motivated by the effects of DHEAS that we identified on the expression of TJ-forming proteins of the blood-testis barrier [16] and the coinciding decline in circulating DHEAS concentrations and the increase in cognitive disorders with age, we investigate here the effects of DHEAS on the expression of proteins involved in the formation and maintenance of the BBB in a mouse brain endothelial cell line that is a model of the BBB.

## 2. Material and methods

### 2.1. Cell culture

The mouse brain endothelial cell line bEnd.3 [23] was cultured in gelatin-coated 10-cm culture dishes (Greiner, Frickenhausen, Germany). The culture medium was phenol red-free DMEM high glucose with 2 mM L-glutamine and 25 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% *v/v* standardized fetal bovine serum (Biochrom GmbH, Berlin, Germany), 1 mM Na-pyruvate (Thermo Fisher Scientific), 1% non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin combination (5000 units penicillin and 5 mg streptomycin/ml) (Sigma-Aldrich). The cells were kept in a humidified incubator at 37 °C under 5% $CO_2$. The culture medium was renewed every three days. The passages used for experiments were always between 20 and 27, as monolayers of this cell line are known to show increased permeability with increasing passage number [24].

### 2.2. Preparation of cell lysates

bEnd.3 cells were seeded at a density of $2.5 \times 10^5$ cells in gelatin-coated 5-cm culture dishes (Greiner) and left to grow, as described above, for 4 days. The medium was afterwards replaced with a 2% FBS medium, containing either 1 μM DHEAS or 10 nM testosterone (T) each dissolved in absolute ethanol and cells were incubated for 48 h. Control cells received vehicle alone. The final concentration of ethanol in the samples was kept at 0.1% (by vol.). The medium was then removed by aspiration and cells were washed once with ice-cold phosphate-buffered saline (PBS; without $Ca^{2+}$ or $Mg^{2+}$; GE Healthcare, Munich, Germany) and lysed in 400 μl of a commercially available cell lysis buffer (New England Biolabs GmbH, Frankfurt, Germany) according to the protocol of the provider. The lysis buffer contained the phosphatase inhibitors vanadate and leupeptin. Immediately before use, 1 μM PMSF (Carl Roth GmbH, Karlsruhe, Germany) was added to the lysis buffer. All lysis steps were carried out on ice. After 10 min of incubation, cells were harvested with a scraper, transferred into plastic tubes, and sonicated 5 times for 5 s each. The reaction tubes were then centrifuged at 13,000 × *g* for 15 min at 4 °C. The protein content of the supernatants was determined at 540 nm using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA) and a Labsystems (Helsinki, Finland) plate reader. The lysis buffer was included in the bovine serum albumin protein standard. Aliquots of the supernatant were taken and stored at − 20 °C for further analysis.

### 2.3. Protein electrophoresis and western blotting

In most cases a total of 10 μg of the cell lysate protein was separated by SDS-PAGE on 6.5–12% acrylamide gels. In order to detect claudin-3 also in control cells, SDS-PAGE was run with 15 μg of lysate protein. In the case of claudin-5, 6 μg of lysate protein were used in SDS-PAGE, because of its high abundance in control and DHEAS-treated cells. Biotinylated molecular weight markers (Cell Signaling Technology, Frankfurt a.M., Germany; cat. # 7727) were used to determine the relative molecular mass of the separated proteins and a pre-stained protein ladder (Thermo Fisher Scientific; cat. # 26619) was additionally used during the electrophoresis for zonula occludens-1 (ZO-1), to pinpoint the position of the protein on the gel. After the electrophoresis, proteins were blotted onto hydrophobic PVDF transfer membranes (Merck Chemicals GmbH, Schwalbach, Germany) for 30 min at 0.5 V/$cm^2$. The membranes were then blocked for 1 h by incubation in TBS with 0.1% *v/v* Tween 20 and 5% *w/v* nonfat milk at room temperature. The membranes were subsequently incubated overnight at 4 °C with the appropriate primary antibodies (Anti-ZO-1, Santa Cruz Biotechnology, Heidelberg, Germany, cat. # sc-10804, 1:1000; anti-claudin 3, Novus Biologicals, Cambridge, United Kingdom, cat. # NBP1-67517, 1:1000; anti-claudin 5, Santa Cruz Biotechnology, cat. #sc-28670, 1:2000; anti-androgen receptor (AR), Santa Cruz Biotechnology, cat. # sc-13062, 1:1000; anti-pan-Actin, Cell Signaling Technology, cat. # 4968, 1:2000) diluted in blocking solution. The membranes were afterward incubated with the appropriate HRP-conjugated secondary antibody for 60 min at room temperature. An anti-biotin, HRP-conjugated antibody (Cell Signaling Technologies) at a dilution of 1:20,000 was also included in the mixture containing the secondary antibody in order to detect the biotinylated molecular weight marker. An enhanced chemiluminescence solution made by mixing the buffer with p-coumaric acid, luminol, and $H_2O_2$ [25] was then used to visualize the protein bands. The chemiluminescence obtained was visualized by exposure to film. Films were analyzed by the GelAnalyzer 1D gel electrophoresis image analysis software. The PVDF membranes were occasionally reused for the detection of additional proteins. Primary and secondary antibodies were thus stripped by incubating the membrane 3 times for 15 min in 50 ml of 100 mM glycine, pH 2.0. To ensure the successful removal of the antibodies, the membranes were incubated in the enhanced chemiluminescence solution and controlled for any residual fluorescence signals by exposure to film for 5 min. The membranes were then washed once in 25 ml TBS-Tween 20 for 5 min. After blocking in 5% nonfat milk, western blotting for the detection of further proteins of interest was carried out as described above.

### 2.4. Silencing AR expression via siRNA

bEnd.3 cells were seeded at a density of $1.5 \times 10^5$ cells in gelatine-coated 6-well plates (Greiner) and left to grow for 48 h before siRNA treatment. The expression of AR was silenced by using commercially available siRNA and by following the protocol of the provider (Stealth™ RNAi; Invitrogen, Karlsruhe, Germany). The oligonucleotides used were 5′ACUCGAUCGCAUCAUUGCAUGCAAA3′ and 5′UUUGCAUGCAAUGA UGCGAUCGAGU3′ at a final concentration of 150 nM. Control cells were treated with OptiMem plus Lipofectamine RNAiMAX plus the siRNA Negative Control as supplied by the provider. Transfection efficiency was estimated by the Block-iT™ Transfection Kit.

After 72 h of incubation with the siRNA, preparation of samples for western blots or immunofluorescence experiments were carried out as described in previous or subsequent paragraphs.

### 2.5. Silencing Gnα11 expression via siRNA

Silencing expression of Gnα11 was carried out by using commercially available siRNA (Silencer® Select siRNA; Invitrogen) as described earlier [16]. Here briefly: silencing was achieved by using the oligonucleotides 5′CAAGAUCCUCUACAAGUAUtt3′ and 5′AUACUUGUAGAG-GAUCUUGag3′ at a final concentration of 150 nM. They specifically suppress expression of Gnα11 without affecting the expression of the closely related Gnαq [16]. Control cells were treated with OptiMem, Lipofectamine RNAiMAX and the siRNA Negative Control as supplied by the provider. All other steps were the same as for the silencing of AR expression described above.

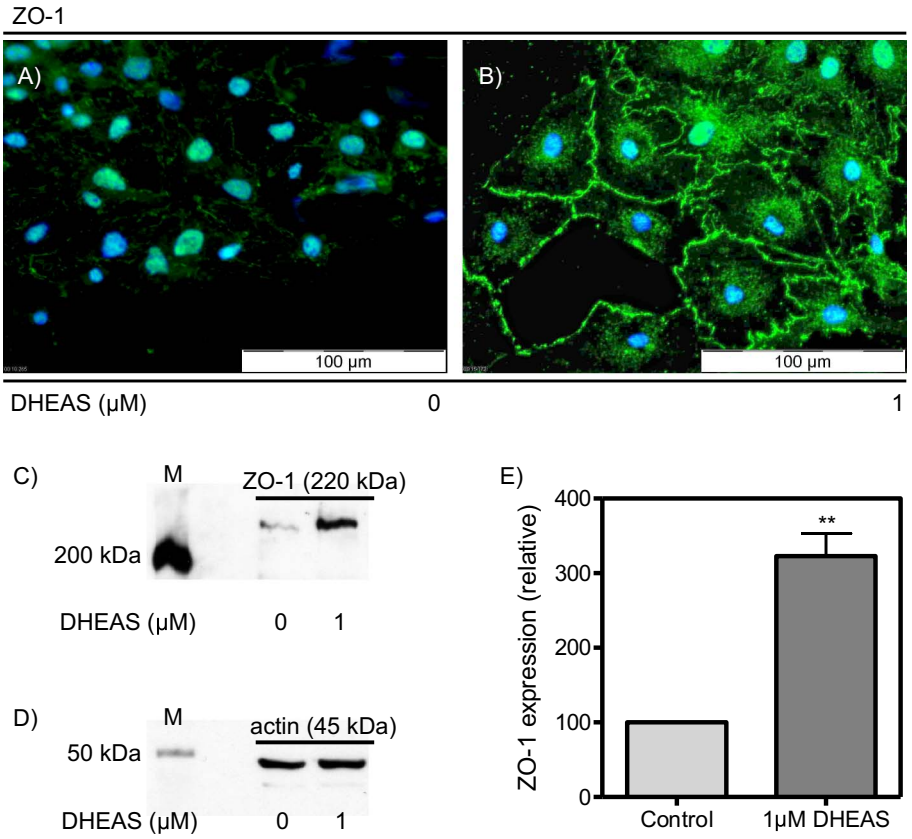


**Fig. 1.** DHEAS stimulates expression of ZO-1 as detected by immunofluorescence and in western blots. Cells were incubated for 48 h with 1 µM DHEAS. Controls received vehicle alone. (A) In the absence of DHEAS green fluorescence indicating the presence of ZO-1 was rather low. Nuclei of cells were stained with DAPI (blue). (B) Incubation of the bEnd.3 cells with 1 µM DHEAS stimulated the expression of ZO-1, found to be localized at the boundaries between neighbouring cells, around nuclei and within nuclei. (C) The western blots show ZO-1 expression in lysates of cells that had been exposed for 48 h to vehicle alone or to 1 µM DHEAS. DHEAS stimulated ZO-1 expression. (D) The expression of total actin was not affected by the incubation with DHEAS. (E) For statistical analysis, data were corrected for the amount of total actin as shown in D ($n = 4$; means ± SEM; $^{**}p < 0.0001$).

### 2.6. RT-PCR

RT-PCR was carried out as described earlier [16]. Gnα11-specific mRNA/cDNA was amplified in 35 PCR cycles at an annealing temperature of 58 °C. Forward and reverse primers were the oligonucleotides 5′GAACCGGGAAGAGGTAGGG3′ and 5′GACCAAGTGTGAGTGCAGGA3′, respectively. These amplify a 917-bp fragment of mouse Gnα11-specific mRNA localized between bases 70 and 986. GAPDH-specific mRNA/cDNA was amplified similarly with the exemption that the annealing temperature was set at 57 °C. Forward primer was the oligonucleotide 5′GGAGATTGTTGCCATCAACG3′ and reverse primer the oligonucleotide 5′CACAATGCCAAAGTTGTCA3′. These amplify a fragment of 430 bp between bases 128 and 557 of mouse GAPDH-specific mRNA.

### 2.7. Immunofluorescence

A total of $8 \times 10^4$ cells were added to gelatin-coated 12-well plates (Greiner) for the detection of ZO-1 or commercially available pre-coated 4-well chamber slides (Sigma-Aldrich; cat. # S6690) for the detection of claudin-3, claudin-5, AR or Gnα11 and were subsequently left to grow for 4 days. The cells were afterwards incubated for 48 h in 2% FBS medium containing either 1 µM DHEAS or 10 nM T, each dissolved in ethanol. Control cells received vehicle only. The final concentration of ethanol in the samples was kept at 0.1% (by vol.).

Every media change, which will be described below, was followed by the cells being washed three times with PBS. Unless otherwise specified, the following procedures were carried out at room temperature. All incubation steps with UV-sensitive reagents were performed in the dark. Following the 48 h DHEAS or T incubation, the cells were fixed in 3.7% formaldehyde for 15 min for the detection of ZO-1 or in 100% ice-cold methanol for 10 min for the detection of claudin-3, claudin-5, AR or Gnα11. The cells were then incubated in a blocking solution (3% BSA, 0.3% Triton-X 100 in PBS) for 1 h. Afterwards, the blocking solution was replaced with PBS containing 1% BSA and 0.1% Triton-X 100 along with the primary antibody against either ZO-1 (1:250), claudin-3 (1:200), claudin-5 (1:300), AR (1:200) or Gnα11 (Santa Cruz Biotechnology, cat. # sc-390382, 1:300). Incubation continued in a humidified chamber for 24 h at 4 °C.

Staining was achieved by incubating for 60 min at room temperature with an Alexa Fluor 488-labelled goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) diluted at 1:300 in 1% BSA, 0.1% Triton-X 100 in PBS. The solution was then removed and the cell nuclei were stained by incubating the cells for 10 min with PBS containing DAPI (4′,6-diamidino-2-phenylindole) in a concentration of 0.5 µg/ml. The DAPI solution was subsequently aspirated and the cells were overlaid with PBS and stored at 4 °C for further investigation. All fluorescence images were obtained with an inverse Olympus microscope (model IX81; Olympus, Hamburg, Germany) using a 40 × objective with numerical apertures of 0.3 and 0.6, respectively. The excitation filters were 360–370 nm (DAPI images) and 470–495 nm (Alexa Fluor 488 images). The corresponding emission filters were 420–460 nm and 510–550 nm, respectively.

Green fluorescence corresponding to Gnα11 expression was measured by using the software program ImageJ (freely available at http://rsbweb.nih.gov/ij/). All cells in the optical field were considered. Data points from three similar experiments were transferred to and analyzed by the software program GraphPad Prism4 (GraphPad Software, Inc., La Jolla, CA, USA).

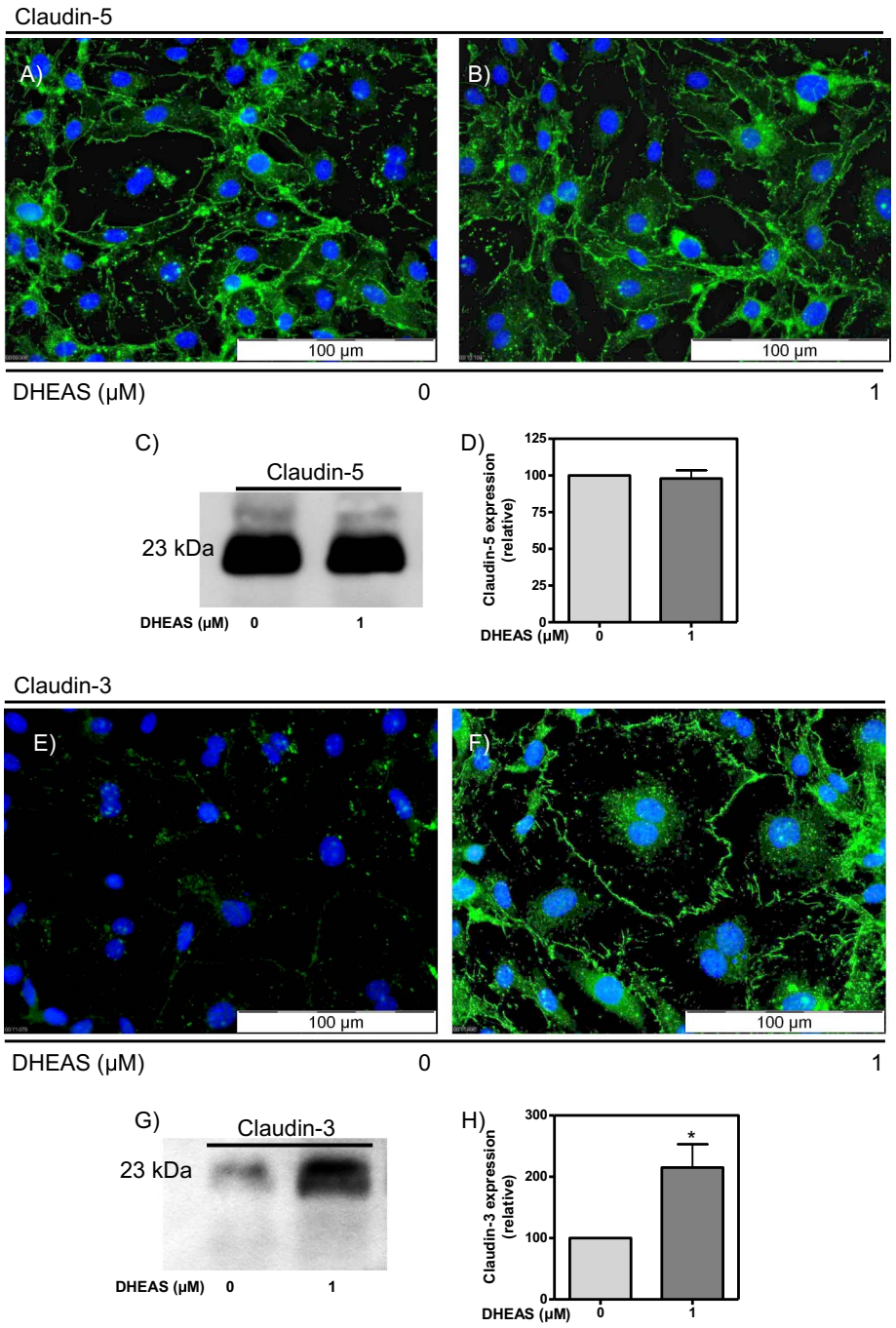


**Fig. 2.** Effect of DHEAS on the expression of claudin-5 and claudin-3. Cells were incubated for 48 h with or without DHEAS. Nuclei of cells were stained with DAPI (blue). Green fluorescence corresponds to either claudin-5 or claudin-3. (A) Detection of claudin-5 by immunofluorescence in the absence of DHEAS. (B) Incubation with 1 µM DHEAS does not affect the expression level of claudin-5. (C) Detection of claudin-5 by western blotting in the absence or presence of DHEAS. (D) Analysis of western blots like the one shown in (C) did not reveal any significant differences in the expression levels of claudin-5 in the presence or absence of DHEAS ($n = 3$; means ± SEM, $p = 0.7348$). (E) Detection of claudin-3 by immunofluorescence in the absence of DHEAS. (F) Incubation with 1 µM DHEAS stimulated the expression of claudin-3, visible at the boundaries between adjacent cells and around the nuclei (ER). (G) Confirmation of the results shown in (A) and (B) by western blot analysis. (H) Analysis of western blots like the one shown in (G) reveal a statistically significant stimulation of claudin-3 expression by DHEAS ($n = 4$; means ± SEM; $^*p = 0.023$).

### 2.8. Measurement of transendothelial resistance (TER)

A total of $1 \times 10^5$ cells/insert were seeded on collagen-coated ThinCert inserts with a 0.4-µm pore diameter and 0.33 $cm^2$ culture surface for use with 24-well plates (both from Greiner) and cultured as described above. The cells were considered confluent enough, when the initial electrical resistance measurement exceeded 70 Ω. At that point, incubation in fresh media was carried out for the indicated periods of time in the presence or absence of 1 µM DHEAS. Measurement of TER was carried out with a Millicell ERS-2 epithelial Volt-Ohm meter (Merck Millipore, Darmstadt, Germany). TER in Ω/$cm^2$ was calculated according to the protocol of the Volt-Ohm meter manufacturer and by subtracting the resistance of cell-free filters. The electrode of the Volt-Ohm meter was mounted on an adjustable stand to ensure its precise depth positioning in each of the wells.

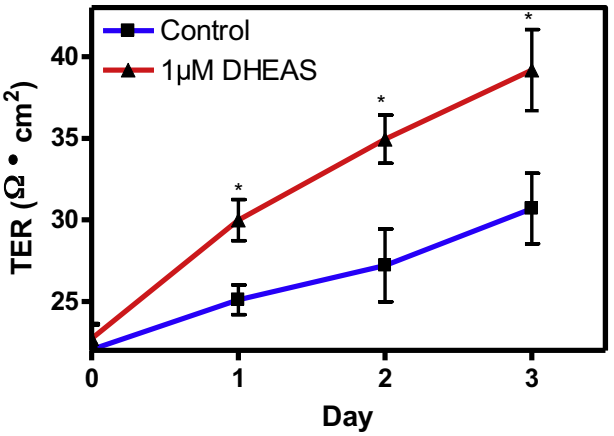


**Fig. 3.** DHEAS increases transendothelial resistance across cell monolayers. After accomplishing the first TER (in Ω/cm$^2$) measurements (Day 0), vehicle alone or 1 μM DHEAS was added to the cells and incubation was continued for 3 more days. As early as day 1 after addition of 1 μM DHEAS, TER across cell monolayers was significantly higher than that measured across untreated monolayers. For each data point: $n = 5$; means ± SEM; day 0: $p = 0.7100$; *day 1: $p = 0{,}0144$; *day 2: $p = 0.0200$; *day 3: $p = 0.0223$.

### 2.9. Statistical analysis

Loading differences in the various western blots were corrected by taking into consideration the optical density of total actin bands. Data were analyzed either by Student's unpaired, two-tailed *t*-test (Figs. 1, 2, 3, 6 and 7) or by applying one-way ANOVA with repeated measures and Dunnett's comparison of all data to the control (Figs. 4, 5 and 8). Differences were considered to be significant when $p < 0.05$. Immunofluorescence and western blot experiments were repeated at least three times.

## 3. Results

### 3.1. Stimulation of ZO-1 expression by DHEAS

The initial focus of the current investigation was aimed at possible effects of DHEAS on the expression of zonula occludens-1 (ZO-1) protein, a scaffold protein that is a critical component of TJ of the BBB. Whereas ZO-1 expression after 48 h of incubation of bEnd.3 cells with vehicle alone remained low (Fig. 1A), incubation over the same period of time with 1 μM DHEAS led to a clear stimulation of ZO-1 expression (Fig. 1B). Green immunofluorescence corresponding to ZO-1 protein was clearly visible at membrane contacts between cells but also in the periphery of nuclei (likely in the endoplasmic reticulum, ER) and within nuclei (Fig. 1B). Western blotting confirmed that treatment of cells with 1 μM DHEAS stimulated the expression of ZO-1 protein (Fig. 1C) without affecting the expression of actin (Fig. 1D). Within 48 h of incubation ZO-1 levels in the presence of DHEAS were > 3 times higher than the levels of the protein detected in the absence of the sulfated steroid (Fig. 1E).

### 3.2. DHEAS effects on the expression of claudin-5 and claudin-3

Claudins are important components of all blood-tissue barriers. The focus of the current investigation concentrated, however, on effects of DHEAS on claudins 3 and 5 whose altered expression has been connected to various neurological disorders [26]. Incubation of bEnd.3 cells with DHEAS did not influence the expression of claudin-5 as assessed by immunofluorescence and western blotting (Fig. 2A–D). DHEAS had marked effects, however, on the expression of claudin-3. Green fluorescence corresponding to claudin-3 was very low after 48 h in the absence of DHEAS (Fig. 2E), although cells had reached confluence at this time point. Incubation of the bEnd.3 cells for the same time span with 1 μM DHEAS had a strong stimulatory effect on the expression of claudin-3 (Fig. 2F), which was confirmed by western blot experiments (Fig. 2G and H). The localization of claudin-3 at the boundaries between cells and also around the nuclei (Fig. 2F; probably ER) is in good agreement with results published previously [27].

### 3.3. DHEAS effects on transendothelial resistance

In the absence of DHEAS, TER across the cell monolayer increased continuously over the course of 4 days (Fig. 3). Addition of 1 μM DHEAS had a significant stimulatory effect on TER values at all time points investigated, consistent with the sulfated steroid triggering additional sealing of the TJ.

### 3.4. Silencing AR expression by siRNA

In order to evaluate whether the conversion of DHEAS into testosterone and mediation of the AR are responsible for the effects described above, DHEAS and testosterone effects were investigated in the presence of the AR or after abrogation of its expression by siRNA. Cells that had been treated with negative control siRNA (nc-siRNA) were incubated for 48 h with vehicle alone, with 1 μM DHEAS, or with 10 nM testosterone. AR expression was significantly stimulated by testosterone but was not influenced by DHEAS (Fig. 4, A and B), indicating that conversion of the latter into testosterone under these conditions is unlikely. Treatment of cells with AR-specific siRNA led to the complete abrogation of AR expression (Fig. 4A). Neither nc-siRNA

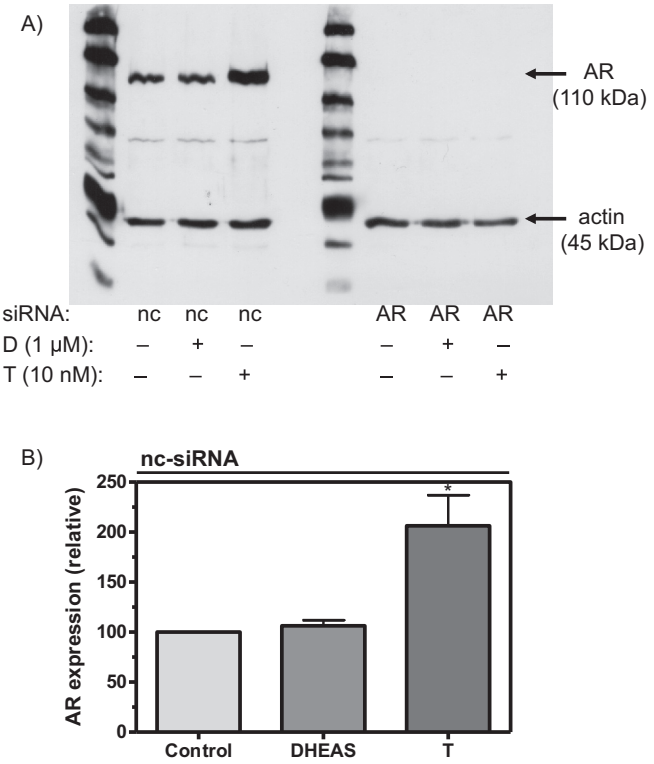


**Fig. 4.** Silencing AR expression by siRNA. Cells that had been first treated with negative control siRNA (nc-siRNA) or with siRNA against AR (AR-siRNA) were exposed to either vehicle alone, 1 μM DHEAS, or 10 nM testosterone (T). Simultaneous detection of AR and actin was accomplished by incubating the PVDF membranes with AR and actin antibodies at the same time. (A) In nc-siRNA-treated cells expression of AR was not affected by DHEAS but was considerably stimulated by T. In AR-siRNA-treated cells, AR-specific bands were not detectable in any of the samples. Expression of actin was not affected by nc-siRNA, AR-siRNA, DHEAS, or T. (B) Statistical analysis of the effects of DHEAS and T on the expression of AR. 10 nM T stimulated AR expression > 2-fold over its expression observed in control or DHEAS-treated cells ($n = 3$; means ± SEM; *$p = 0.0254$).

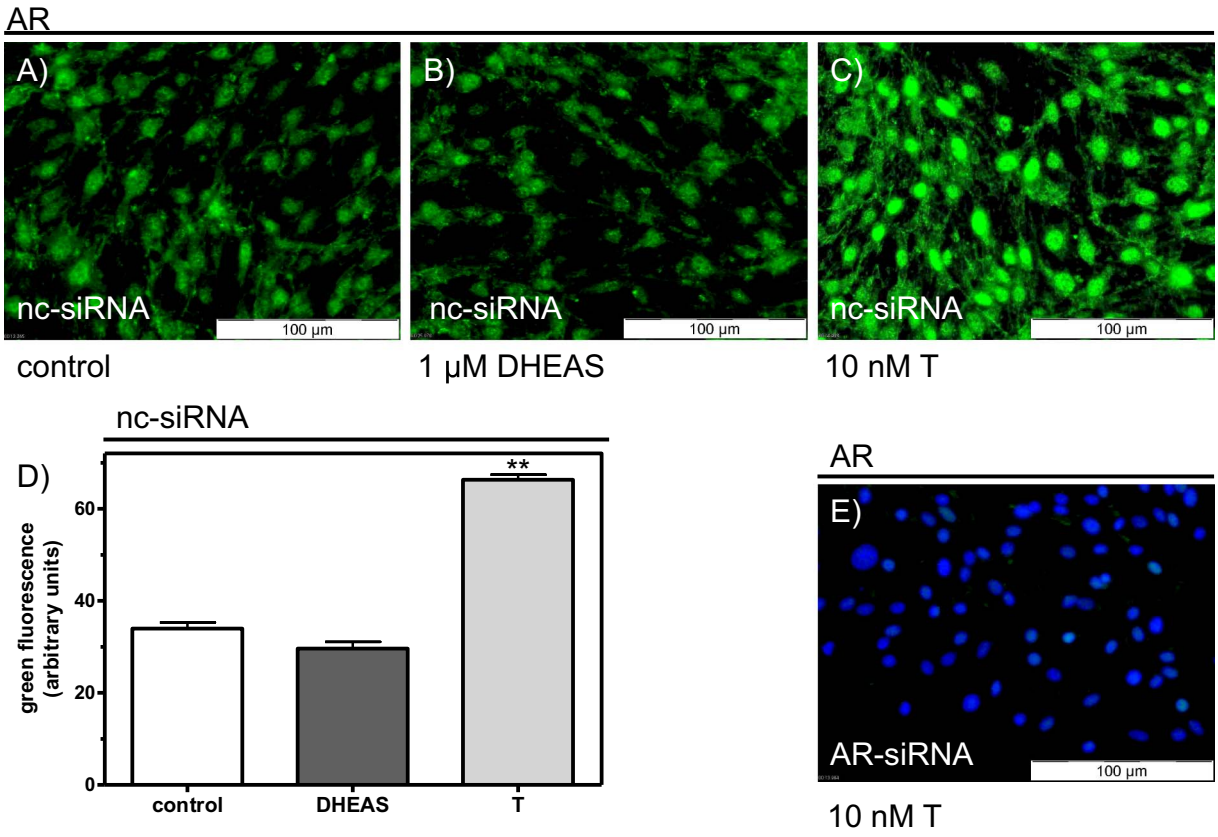


**Fig. 5.** Stimulation of AR expression and AR translocation into the nuclei by T but not by DHEAS. Cells that had been treated with negative control siRNA (nc-siRNA) were incubated for two days with either 1 µM DHEAS or 10 nM T. Control cells received only vehicle. Green fluorescence corresponds to AR. (A) Detection of AR in control, (B) in cells treated with DHEAS, and (C) in cells treated with T. (D) Statistical analysis reveals a significant stimulation of AR expression in the entire cells and increased nuclear localization of AR in the presence of T. DHEAS influenced neither the expression nor the nuclear localization of AR ($n = 20$; means ± SEM; $^{**}p < 0.0001$). (E) When cells were treated with AR-specific siRNA (AR-siRNA), green fluorescence indicating the presence of AR was not detectable. The figure shows results in the presence of T; similar results were also obtained when cells were treated with either vehicle or DHEAS (not shown). The figure shows blue DAPI-stained nuclei to demonstrate the presence of cells.

nor AR-siRNA had any effect on the expression of actin, which was detected concurrently with the AR by the simultaneous use of specific antibodies against each of the two proteins.

The apparent testosterone-specific effect on the expression of the AR was confirmed in immunofluorescence experiments. Here, too, testosterone stimulated the expression of the AR and triggered its translocation into the nuclei of bEnd.3 cells in the presence of nc-siRNA (Fig. 5, C and D). The level of expression of AR and its localization in response to DHEAS (Fig. 5, B and D) were the same as that observed in control incubations (Fig. 5, A and D). Green fluorescence indicating the presence of AR was not visible in cells that had been treated with AR-siRNA to silence AR expression, even in the presence of testosterone (Fig. 5E). Similar results were obtained with AR-siRNA-treated cells that were exposed to either vehicle or DHEAS.

### 3.5. DHEAS effects on ZO-1 expression in the absence of AR

Although the results described thus far clearly show different effects of testosterone or DHEAS on the expression and translocation of the classical AR into the nuclei, they cannot exclude a possible involvement of this receptor in the DHEAS-induced effects on BBB proteins. Therefore, we investigated whether abrogation of AR expression by siRNA might influence the DHEAS-induced effects on the expression or localization of ZO-1. Incubation with 1 µM DHEAS of bEnd.3 cells that had been treated with AR-siRNA to silence AR expression clearly stimulated the expression of ZO-1 over control (Fig. 6, A and B), which was similar to the response displayed in Fig. 1. Western blotting (Fig. 6C) confirmed the AR-independent, significant stimulation of ZO-1 expression by DHEAS (Fig. 6D).

### 3.6. DHEAS effects on ZO-1 and claudin-3 expression in the absence of Gnα11

Many steroid hormones elicit their actions not only through cytosolic but also through membrane-bound GPCRs [28–31]. Earlier findings demonstrated the involvement of Gnα11 in DHEAS-induced signaling in spermatogenic and Sertoli cells [15,16]. We therefore addressed a possible participation of Gnα11 in the effects of DHEAS on bEnd.3 cells. This was done by incubating cells with DHEAS after silencing Gnα11 expression. RT-PCR experiments revealed that after 72 h of incubation of cells with siRNA oligonucleotides against Gnα11 (Gnα11–siRNA), the expression of Gnα11-specific mRNA/cDNA was greatly reduced when compared with the presence of the same mRNA/cDNA fragment in cells that had been treated with nc-siRNA (Fig. 7A). GAPDH-specific mRNA/cDNA was not affected by either of the two siRNAs (Fig. 7A), indicating that the suppression of Gnα11-specific mRNA by Gnα11-siRNA is specific and not the result of an overall inhibition of mRNA expression. Immunofluorescence demonstrated that green fluorescence indicating the presence of Gnα11 protein, clearly visible in cells treated with nc-siRNA (Fig. 7B), was markedly reduced following treatment of cells with Gnα11-siRNA, verifying the successful suppression of the expression of Gnα11 protein (Fig. 7, C and D).

To test the effects of abrogation of Gnα11 protein expression on DHEAS-elicited responses, we utilized cells treated with either Gnα11-siRNA or nc-siRNA. As in the results shown in Figs. 1B and 6B, exposure of nc-siRNA-treated cells to 1 µM DHEAS led to the stimulation of ZO-1 protein expression that can be visualized as green fluorescence at the borders between neighbouring cells and around or within the nuclei (Fig. 8, A and B). When Gnα11 expression was suppressed by incuba-

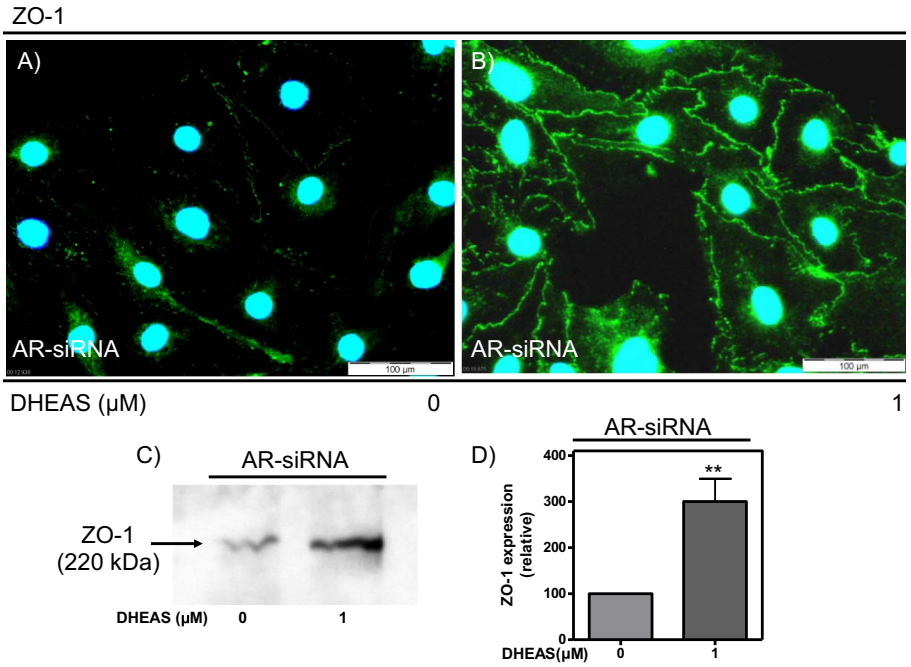


**Fig. 6.** Abrogation of AR expression by siRNA does not influence DHEAS-induced stimulation of ZO-1 expression. (A) Cells treated with AR-siRNA express only modest quantities of ZO-1 in the absence of DHEAS. (B) Incubation of AR-siRNA-treated cells with DHEAS led to increased expression of ZO-1 protein. The localization of ZO-1 protein (green fluorescence) is similar to its localization depicted in Fig. 1. (C) The western blot confirms the results of the immunofluorescence experiments and demonstrates (D) a statistically significant stimulation of ZO-1 expression by DHEAS, even in the absence of AR ($n = 4$; means ± SEM; $^{**}p = 0.00045$).

tion with Gnα11-specific siRNA, however, the stimulatory effect of DHEAS on ZO-1 expression was no longer apparent (Fig. 8, C and D). The confirmation of these results by western blot experiments (Fig. 8, E–G) is consistent with Gnα11 mediating the DHEAS-induced expression of ZO-1 protein.

Similar results were obtained with respect to the involvement of Gnα11 in the DHEAS-triggered stimulation on claudin-3 expression. Thus, treatment of bEnd.3 cells with nc-siRNA did not affect the stimulatory effect of DHEAS on the expression of claudin-3 (Fig. 9, A and B). In cells that were exposed to Gnα11-specific siRNA, however,

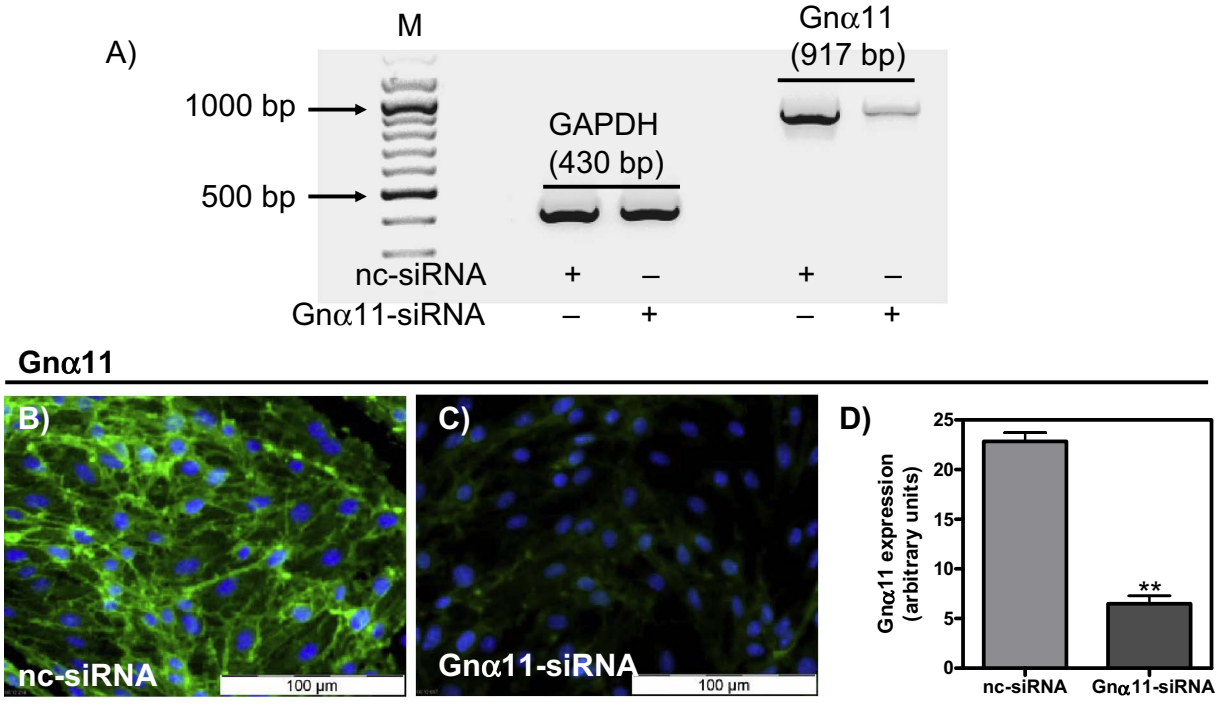


**Fig. 7.** Silencing expression of Gnα11 by siRNA. (A) Treatment of cells with negative-control siRNA (nc-siRNA) did not affect the expression of Gnα11-specific mRNA/cDNA. Application of specific siRNA against Gnα11 (Gnα11-siRNA) considerably reduced the RT-PCR signals for Gnα11 but did not affect the expression of the related Gαq-specific mRNA/cDNA. (B) The immunofluorescence experiments confirmed that nc-siRNA does not affect expression of Gnα11, whereas (C) treatment with Gnα11-siRNA leads to the complete abrogation of Gnα11 expression. (D) Statistical analysis of green fluorescence ($n = 31$; means ± SEM; $^{*}p < 0.0001$).

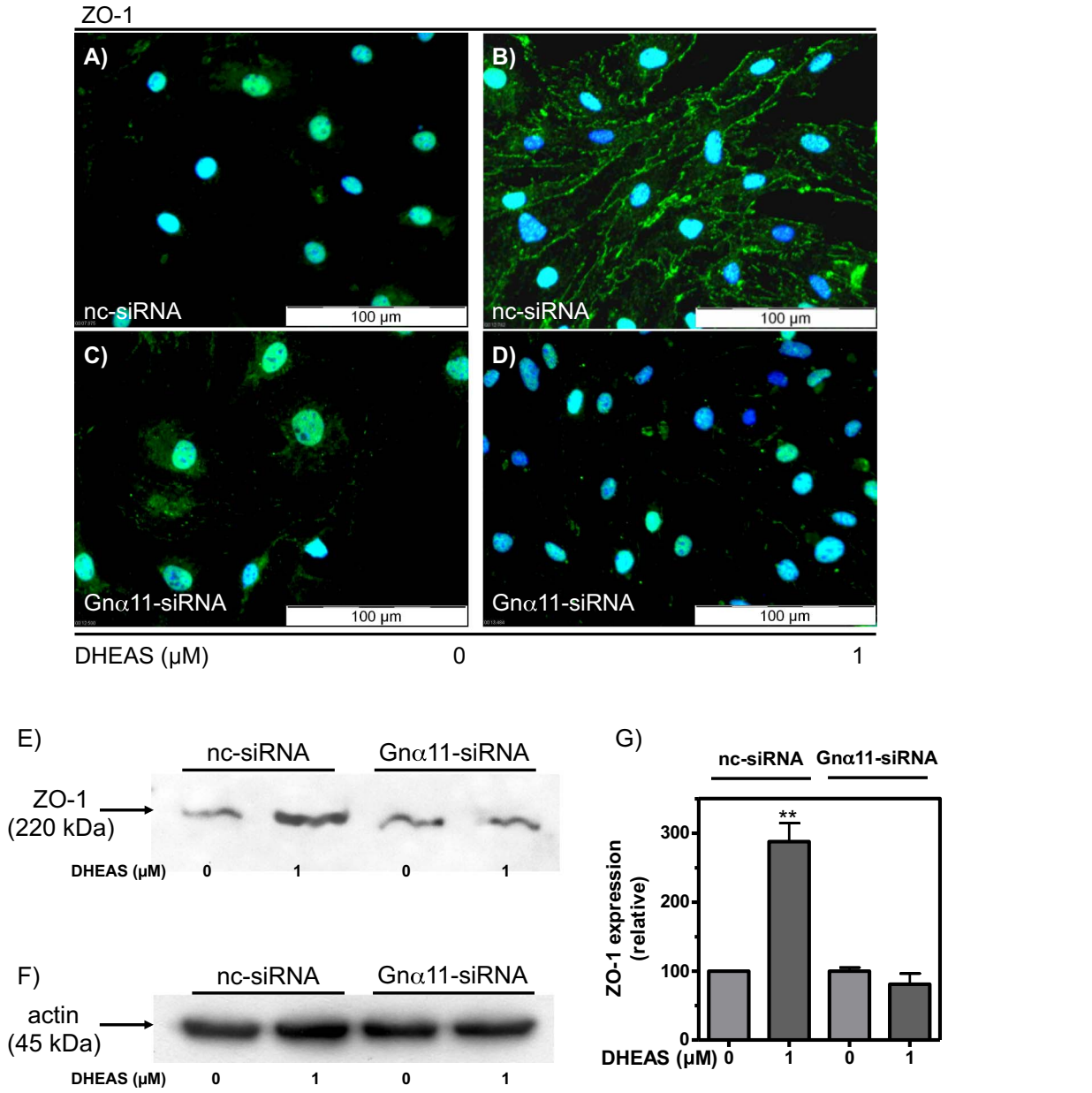


**Fig. 8.** Effects of DHEAS on ZO-1 expression in the absence of Gnα11. Green fluorescence reflects expression of ZO-1. Nuclei are stained blue. (A) Detection of ZO-1 in bEnd.3 cells that were treated with nc-siRNA in the absence of DHEAS. (B) DHEAS (1 µM) stimulated ZO-1 expression in cells that had been treated with nc-siRNA. (C) Green fluorescence indicating the expression of ZO-1 in bEnd.3 cells treated with Gnα11-siRNA. (D) Treatment of cells with Gnα11-siRNA completely inhibited the DHEAS-induced stimulation of ZO-1 expression. (E) Western blot analysis of ZO-1 expression. In cells treated with Gnα11-siRNA, DHEAS failed to stimulate ZO-1 expression. (F) Expression of total actin was not influenced by nc-siRNA, Gnα11-siRNA, or DHEAS. (G) The data were corrected for the amount of total actin, which was used as a gel loading control. ($n = 5$; means ± SEM; $^{**}p < 0.0001$).

the stimulatory effect of DHEAS on claudin-3 expression was completely suppressed (Fig. 9, C and D): fluorescence corresponding to claudin-3 was at the same level as the fluorescence measured in the absence of the steroid (Fig. 9, C and D). The western blot experiments shown in Fig. 9, E–G confirm the results of the immunofluorescence experiments and support the idea of Gnα11 being the mediator of the stimulatory effect of DHEAS on claudin-3 expression.

## 4. Discussion

Our results derived from brain endothelial cells constitute the first demonstration of the stimulatory effects of DHEAS on the expression of the TJ and BBB proteins ZO-1 and claudin-3. As a corollary, DHEAS also increases TER across endothelial cell monolayers. We further determined that DHEAS does not need to be converted into a different steroid hormone to exert its actions; it appears to act as a steroid hormone in its own right.

The BBB consists primarily of the TJ of endothelial cells of the capillaries. Occludin and various claudins, proteins spanning the intercellular cleft, and junctional adhesion molecules are the building blocks of TJ at the BBB. On the cytosolic side occludin and claudins are linked to the cytoskeleton through the mediation of zonula occludens (ZO) proteins [32–34]. ZO proteins are members of the family of membrane-associated guanylate kinase (MAGUK)-like proteins. As scaffold proteins they recruit various other proteins to the cytosolic surface of epithelial or endothelial cells and contribute to the formation of TJ [35]. From the various ZO proteins, ZO-1 regulates formation of the junctional complex by interacting via its PDZ domain with the C-terminal sequences of claudins and via its GK domain with occludin [36–39]. ZO proteins not only contribute to the formation of TJ; they also associate with proteins

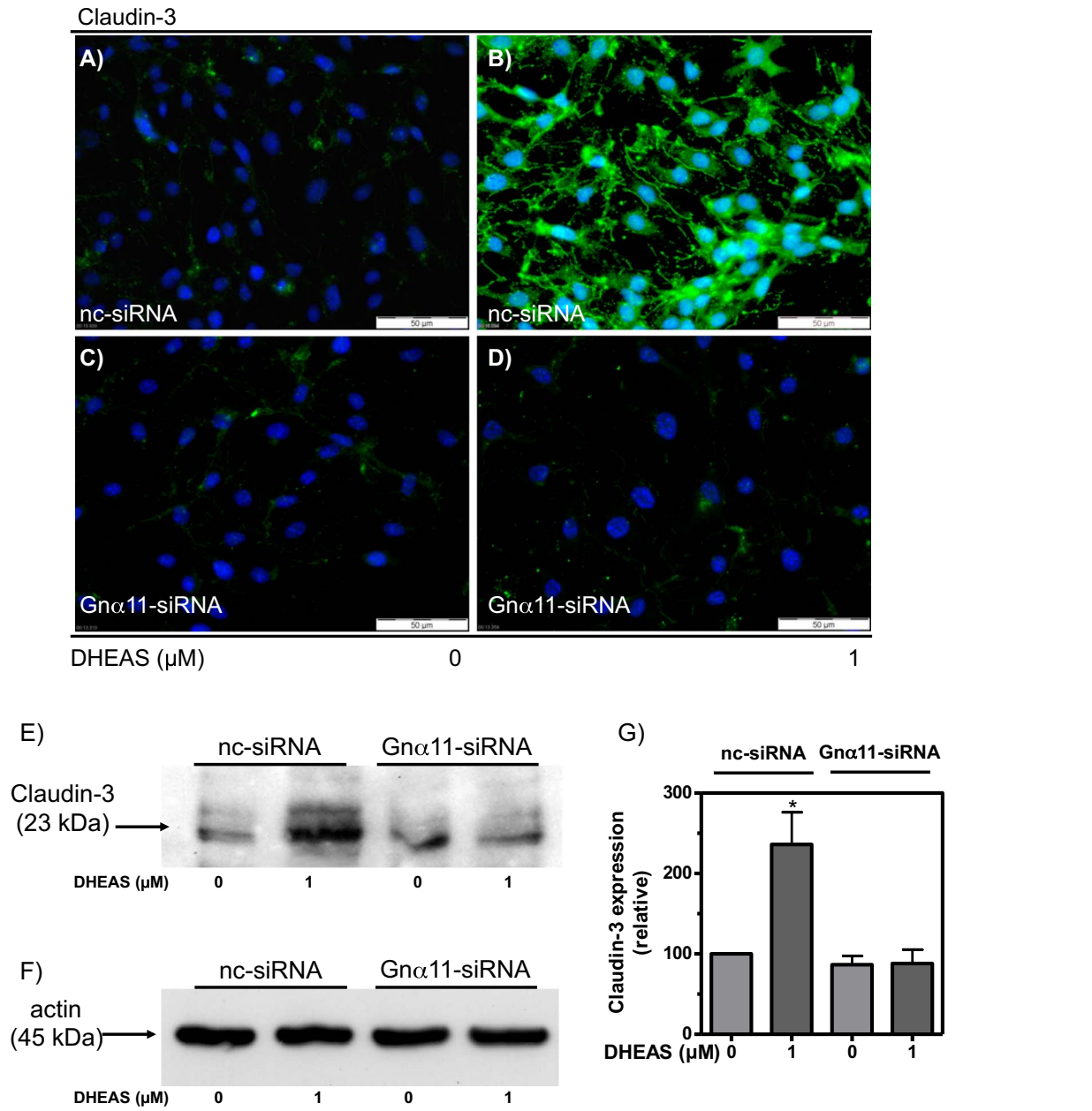


**Fig. 9.** Effects of DHEAS on claudin-3 expression in the absence of Gnα11. Green fluorescence reflects expression of claudin-3. Nuclei are stained blue. (A) Detection of claudin-3 in bEnd.3 cells that were treated with nc-siRNA in the absence of DHEAS. (B) In cells that had been treated with nc-siRNA, DHEAS (1 μM) stimulated claudin-3 expression. (C) Green fluorescence indicating the expression of claudin-3 in bEnd.3 cells treated with Gnα11-siRNA. (D) In cells that had been treated with Gnα11-siRNA, DHEAS (1 μM) failed to stimulate claudin-3 expression. (E) Western blot analysis of claudin-3 expression. In cells treated with nc-siRNA, DHEAS stimulated claudin-3 expression. This DHEAS effect was abrogated when cells were treated with Gnα11-siRNA. (F) Expression of total actin was not affected by nc-siRNA, Gnα11-siRNA, or DHEAS. (G) The data were corrected for the amount of total actin, which was used as a gel loading control. ($n = 3$; means ± SEM; $^*p = 0.0193$).

of cadherin-based adherens junctions and with gap junction-forming connexins [17,40–43]. Nevertheless, new findings provide evidence that the function of ZO proteins goes beyond the regulation of barrier formation between endothelial or epithelial cells. ZO proteins, including ZO-1, are also found in the nuclei where they regulate gene transcription and influence cell proliferation by interacting with transcription factors [36,44–47]. Our finding that stimulation of ZO-1 expression by DHEAS leads to an increase in the presence of the protein at the boundaries of neighbouring cells as well as in the nuclei and in perinuclear areas is consistent with DHEAS promoting synthesis of new ZO-1 within the ER. We do not know at the current stage whether nuclear ZO-1 associates with transcription factors in the endothelial cells, but this would be an interesting topic for future investigations. The peripherally localized ZO-1 likely contributes to the dynamics of the endothelial TJ by interacting with occludin and various claudins. DHEAS, however, did not have any effects on the expression of occludin, neither at the mRNA/cDNA nor at the protein level (not shown).

Claudins are the main constituents of all blood-tissue barriers. Claudins 1, 2, 3, 5, 11, 12, and 18 have been identified in the mammalian brain microvascular endothelial cell system [48]. Selective loss of claudin-3 expression has been associated with experimental autoimmune encephalomyelitis and glioblastoma [49], and in human endothelial cell models claudin-3 constitutes a potential marker for BBB characteristics in vitro [27]. Claudin-5 knockout mice are characterized by size-selective BBB defects [50], and in an experimental mouse model of autoimmune encephalomyelitis (an animal model of CNS inflammatory disease), down-regulation of claudin-5 expression correlates with the collapse of BBB function [51].

Unstimulated bEnd.3 cells express detectable quantities of claudin-5, as we observed by immunofluorescence and western blotting. This and the localization of the claudin-5 protein we observed at the contact sites between neighbouring cells are in good agreement with previously published data [24,52]. Its expression is not increased by DHEAS, however, not even after two days of incubation, possibly because claudin-5 presence in these cells is constitutively high [52]. In contrast, treatment of bEnd.3 cells with DHEAS under the same conditions does significantly increase the expression of claudin-3 over a basal level, suggesting that DHEAS may regulate the development of TJ and not the already established junctions. These findings may have physiological relevance. Although several claudins show a highly tissue-specific expression pattern, claudin-3 is expressed in TJ of intestinal, lung, liver, kidney, or skin epithelia as well as in blood vessel endothelium. This omnipresence in various blood-tissue barriers supports the idea that claudin-3 is basic and critical component of TJ. Consistent with this, in the BBB, the central component regulating TJ integrity is not claudin-5 but rather claudin-3 [49], and in the BBB it acts as a sealing component of TJ that prevents the passage of mono- and divalent cations, anions, and uncharged solutes [53]. Taking these findings into account, we propose that the significant increase in TER across DHEAS-treated bEnd.3 cell monolayers that we observed is due to the DHEAS-induced stimulation of claudin-3 expression. The participation of other TJ proteins, however, cannot be excluded at the current stage.

DHEAS is considered by many as a proandrogen that needs to be converted into testosterone to exert its effects. The conversion of DHEAS to testosterone is not required for its actions described here. Incubation of bEnd.3 cells for 2 days with testosterone stimulates AR expression and its translocation into the nuclei. Replacement of testosterone by a 100-fold higher concentration of DHEAS does not generate similar effects, thus indicating that even after 2 days there is no significant conversion of DHEAS to testosterone and that DHEAS is probably acting on its own. This conclusion is further supported by the fact that silencing AR expression does not prevent the stimulatory effect of DHEAS on ZO-1 expression.

The results obtained by immunofluorescence as well by western blotting clearly show that an involvement of the classical AR in the stimulatory effects of DHEAS on ZO-1 expression is very unlikely. The effects of DHEAS appear to be mediated rather by a different signaling route involving the G-protein Gnα11. Gnα11 is a member of the Gq/11 family of heterotrimeric G proteins [54]. In the mast cell line RBH-2H3 the Gq/11 protein was shown to interact with DHEAS [55]; however, a participation of Gnαq in the DHEAS-induced signaling in Sertoli cells could be excluded [16]. Gnα11 is ubiquitously expressed across tissues and is also present in bEnd.3 cells, as we demonstrate here. Silencing Gnα11 expression in bEnd.3 cells with Gnα11-specific siRNA prevents the DHEAS-induced stimulation of ZO-1 or claudin-3 expression, indicating that the DHEAS receptor involved in the effects described here is likely to be a GPCR interacting with Gnα11. Although this conclusion is supported by earlier findings demonstrating the involvement of Gnα11 in DHEAS-induced signaling in spermatogenic and Sertoli cells [15,16], the actual DHEAS-specific GPCR has not yet been identified. Taken together with earlier findings [15,16,55], however, the results of the current investigation suggest that DHEAS, acting as a steroid hormone in its own right, may be directly involved in the formation and maintenance of the BBB.

Whether the decline of DHEAS availability at high age can be connected to various well-documented dysfunctions of the BBB in the aging brain [17], and whether DHEAS administration might be beneficial for the treatment of age-related cognitive disabilities, has not yet been comprehensively investigated. Considering the undisputed fact that BBB integrity is a prerequisite for normal brain function and in view of the paucity of remedies that stimulate BBB formation, maintenance or repair, further research in this direction is required and might help to develop new strategies for the prevention or treatment of age-related neurological disorders linked to BBB defects.

## Transparency document

The http://dx.doi.org/10.1016/j.bbamcr.2017.05.006 associated with this article can be found, in the online version.

## Acknowledgements

Supported in part through DFG, SCHE 307/7-1.

## References

[1] A.G. Mensah-Nyagan, J.L. Do-Rego, D. Beaujean, V. Luu-The, G. Pelletier, H. Vaudry, Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system, Pharmacol. Rev. 51 (1999) 63–81.
[2] E. Nieschlag, D.L. Loriaux, H.J. Ruder, I.R. Zucker, M.A. Kirschner, M.B. Lipsett, The secretion of dehydroepiandrosterone and dehydroepiandrosterone sulphate in man, J. Endocrinol. 57 (1973) 123–134.
[3] A. Vermeulen, Sex hormone status of the postmenopausal woman, Maturitas 2 (1980) 81–89.
[4] S.J. Webb, T.E. Geoghegan, R.A. Prough, K.K. Michael Miller, The biological actions of dehydroepiandrosterone involves multiple receptors, Drug Metab. Rev. 38 (2006) 89–116.
[5] S. Mesiano, R.B. Jaffe, Developmental and functional biology of the primate fetal adrenal cortex, Endocr. Rev. 18 (1997) 378–403.
[6] J.C. Havelock, R.J. Auchus, W.E. Rainey, The rise in adrenal androgen biosynthesis: adrenarche, Semin. Reprod. Med. 22 (2004) 337–347.
[7] J. Young, B. Couzinet, K. Nahoul, S. Brailly, P. Chanson, E.E. Baulieu, G. Schaison, Panhypopituitarism as a model to study the metabolism of dehydroepiandrosterone (DHEA) in humans, J. Clin. Endocrinol. Metab. 82 (1997) 2578–2585.
[8] H.G. Burger, Androgen production in women, Fertil. Steril. 77 (Suppl. 4) (2002) S3–S5.
[9] F. Chen, K. Knecht, E. Birzin, J. Fisher, H. Wilkinson, M. Mojena, C.T. Moreno, A. Schmidt, S. Harada, L.P. Freedman, A.A. Reszka, Direct agonist/antagonist functions of dehydroepiandrosterone, Endocrinology 146 (2005) 4568–4576.
[10] M. Muller, I. den Tonkelaar, J.H. Thijssen, D.E. Grobbee, Y.T. van der Schouw, Endogenous sex hormones in men aged 40–80 years, Eur. J. Endocrinol. 149 (2003) 583–589.
[11] C. Tannenbaum, E. Barrett-Connor, G.A. Laughlin, R.W. Platt, A longitudinal study of dehydroepiandrosterone sulphate (DHEAS) change in older men and women: the Rancho Bernardo Study, Eur. J. Endocrinol. 151 (2004) 717–725.
[12] W. Leowattana, DHEA(S): the fountain of youth, J. Med. Assoc. Thail. 84 (Suppl. 2) (2001) S605–S612.
[13] M.J. Reed, A. Purohit, L.W. Woo, S.P. Newman, B.V. Potter, Steroid sulfatase: molecular biology, regulation, and inhibition, Endocr. Rev. 26 (2005) 171–202.
[14] P. Ebeling, V.A. Koivisto, Physiological importance of dehydroepiandrosterone, Lancet 343 (1994) 1479–1481.
[15] M. Shihan, U. Kirch, G. Scheiner-Bobis, Dehydroepiandrosterone sulfate mediates activation of transcription factors CREB and ATF-1 via a Galpha11-coupled receptor in the spermatogenic cell line GC-2, Biochim. Biophys. Acta 1833 (2013) 3064–3075.
[16] D. Papadopoulos, R. Dietze, M. Shihan, U. Kirch, G. Scheiner-Bobis, Dehydroepiandrosterone sulfate stimulates expression of blood-testis-barrier proteins claudin-3 and -5 and tight junction formation via a Gnalpha11-coupled receptor in Sertoli cells, PLoS One 11 (2016) e0150143.
[17] B.O. Popescu, E.C. Toescu, L.M. Popescu, O. Bajenaru, D.F. Muresanu, M. Schultzberg, N. Bogdanovic, Blood-brain barrier alterations in ageing and dementia, J. Neurol. Sci. 283 (2009) 99–106.
[18] A.J. Farrall, J.M. Wardlaw, Blood-brain barrier: ageing and microvascular disease—systematic review and meta-analysis, Neurobiol. Aging 30 (2009) 337–352.
[19] J.M. Starr, A.J. Farrall, P. Armitage, B. McGurn, J. Wardlaw, Blood-brain barrier permeability in Alzheimer's disease: a case-control MRI study, Psychiatry Res. 171 (2009) 232–241.
[20] N. Maninger, O.M. Wolkowitz, V.I. Reus, E.S. Epel, S.H. Mellon, Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), Front. Neuroendocrinol. 30 (2009) 65–91.
[21] D.H. Miller, A.J. Thompson, S.P. Morrissey, D.G. MacManus, S.G. Moore, B.E. Kendall, I.F. Moseley, W.I. McDonald, High dose steroids in acute relapses of multiple sclerosis: MRI evidence for a possible mechanism of therapeutic effect, J. Neurol. Neurosurg. Psychiatry 55 (1992) 450–453.
[22] B. Obermeier, R. Daneman, R.M. Ransohoff, Development, maintenance and disruption of the blood-brain barrier, Nat. Med. 19 (2013) 1584–1596.
[23] R. Montesano, M.S. Pepper, U. Mohle-Steinlein, W. Risau, E.F. Wagner, L. Orci, Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene, Cell 62 (1990) 435–445.
[24] R.C. Brown, A.P. Morris, R.G. O'Neil, Tight junction protein expression and barrier properties of immortalized mouse brain microvessel endothelial cells, Brain Res. 1130 (2007) 17–30.
[25] D.D. Mruk, C.Y. Cheng, Enhanced chemiluminescence (ECL) for routine immunoblotting: an inexpensive alternative to commercially available kits, Spermatogenesis 1 (2011) 121–122.

[26] R.F. Haseloff, S. Dithmer, L. Winkler, H. Wolburg, I.E. Blasig, Transmembrane proteins of the tight junctions at the blood-brain barrier: structural and functional aspects, Semin. Cell Dev. Biol. 38 (2015) 16–25.
[27] A. Schrade, H. Sade, P.O. Couraud, I.A. Romero, B.B. Weksler, J. Niewoehner, Expression and localization of claudins-3 and -12 in transformed human brain endothelium, Fluids Barriers CNS 9 (2012) 6.
[28] E. Grazzini, G. Guillon, B. Mouillac, H.H. Zingg, Inhibition of oxytocin receptor function by direct binding of progesterone, Nature 392 (1998) 509–512.
[29] S.J. Evans, T.F. Murray, F.L. Moore, Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain, J. Steroid Biochem. Mol. Biol. 72 (2000) 209–221.
[30] P.P. Rojas-Garcia, M.P. Recabarren, L. Sarabia, J. Schon, C. Gabler, R. Einspanier, M. Maliqueo, T. Sir-Petermann, R. Rey, S.E. Recabarren, Prenatal testosterone excess alters Sertoli and germ cell number and testicular FSH receptor expression in rams, Am. J. Physiol. Endocrinol. Metab. 299 (2010) E998–E1005.
[31] D.G. Romero, M.Y. Zhou, L.L. Yanes, M.W. Plonczynski, T.R. Washington, C.E. Gomez-Sanchez, E.P. Gomez-Sanchez, Regulators of G-protein signaling 4 in adrenal gland: localization, regulation, and role in aldosterone secretion, J. Endocrinol. 194 (2007) 429–440.
[32] H. Wolburg, A. Lippoldt, Tight junctions of the blood-brain barrier: development, composition and regulation, Vasc. Pharmacol. 38 (2002) 323–337.
[33] H. Wolburg, S. Noell, A. Mack, K. Wolburg-Buchholz, P. Fallier-Becker, Brain endothelial cells and the glio-vascular complex, Cell Tissue Res. 335 (2009) 75–96.
[34] N.J. Abbott, A.A. Patabendige, D.E. Dolman, S.R. Yusof, D.J. Begley, Structure and function of the blood-brain barrier, Neurobiol. Dis. 37 (2010) 13–25.
[35] J.M. Anderson, B.R. Stevenson, L.A. Jesaitis, D.A. Goodenough, M.S. Mooseker, Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells, J. Cell Biol. 106 (1988) 1141–1149.
[36] H. Bauer, J. Zweimueller-Mayer, P. Steinbacher, A. Lametschwandtner, H.C. Bauer, The dual role of zonula occludens (ZO) proteins, J. Biomed. Biotechnol. 2010 (2010) 402593.
[37] K. Ebnet, C.U. Schulz, M.K. Meyer Zu Brickwedde, G.G. Pendl, D. Vestweber, Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1, J. Biol. Chem. 275 (2000) 27979–27988.
[38] Y. Hamazaki, M. Itoh, H. Sasaki, M. Furuse, S. Tsukita, Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule, J. Biol. Chem. 277 (2002) 455–461.
[39] S.L. Muller, M. Portwich, A. Schmidt, D.I. Utepbergenov, O. Huber, I.E. Blasig, G. Krause, The tight junction protein occludin and the adherens junction protein alpha-catenin share a common interaction mechanism with ZO-1, J. Biol. Chem. 280 (2005) 3747–3756.
[40] B.N. Giepmans, W.H. Moolenaar, The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein, Curr. Biol. 8 (1998) 931–934.
[41] P.J. Kausalya, M. Reichert, W. Hunziker, Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells, FEBS Lett. 505 (2001) 92–96.
[42] A.W. Hunter, R.J. Barker, C. Zhu, R.G. Gourdie, Zonula occludens-1 alters connexin43 gap junction size and organization by influencing channel accretion, Mol. Biol. Cell 16 (2005) 5686–5698.
[43] A.K. Rajasekaran, M. Hojo, T. Huima, E. Rodriguez-Boulan, Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions, J. Cell Biol. 132 (1996) 451–463.
[44] M.S. Balda, K. Matter, The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression, EMBO J. 19 (2000) 2024–2033.
[45] M.S. Balda, M.D. Garrett, K. Matter, The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density, J. Cell Biol. 160 (2003) 423–432.
[46] E. Takai, X. Tan, Y. Tamori, M. Hirota, H. Egami, M. Ogawa, Correlation of translocation of tight junction protein zonula occludens-1 and activation of epidermal growth factor receptor in the regulation of invasion of pancreatic cancer cells, Int. J. Oncol. 27 (2005) 645–651.
[47] E. Norsted, B. Gomuc, B. Meister, Protein components of the blood-brain barrier (BBB) in the mediobasal hypothalamus, J. Chem. Neuroanat. 36 (2008) 107–121.
[48] F.L. Cardoso, D. Brites, M.A. Brito, Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches, Brain Res. Rev. 64 (2010) 328–363.
[49] H. Wolburg, K. Wolburg-Buchholz, J. Kraus, G. Rascher-Eggstein, S. Liebner, S. Hamm, F. Duffner, E.H. Grote, W. Risau, B. Engelhardt, Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme, Acta Neuropathol. 105 (2003) 586–592.
[50] T. Nitta, M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, S. Tsukita, Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice, J. Cell Biol. 161 (2003) 653–660.
[51] A.T. Argaw, B.T. Gurfein, Y. Zhang, A. Zameer, G.R. John, VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1977–1982.
[52] T. Watanabe, S. Dohgu, F. Takata, T. Nishioku, A. Nakashima, K. Futagami, A. Yamauchi, Y. Kataoka, Paracellular barrier and tight junction protein expression in the immortalized brain endothelial cell lines bEND.3, bEND.5 and mouse brain endothelial cell 4, Biol. Pharm. Bull. 36 (2013) 492–495.
[53] S. Milatz, S.M. Krug, R. Rosenthal, D. Gunzel, D. Muller, J.D. Schulzke, S. Amasheh, M. Fromm, Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes, Biochim. Biophys. Acta 1798 (2010) 2048–2057.
[54] K.B. Hubbard, J.R. Hepler, Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins, Cell. Signal. 18 (2006) 135–150.
[55] K. Mizota, A. Yoshida, H. Uchida, R. Fujita, H. Ueda, Novel type of Gq/11 protein-coupled neurosteroid receptor sensitive to endocrine disrupting chemicals in mast cell line (RBL-2H3), Br. J. Pharmacol. 145 (2005) 545–550.