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Supplementary Information for

Isolation and characterization of adrenocortical progenitors involved in the adaptation to stress

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This PDF file includes:

Supplementary Materials and Methods
Figs S1 to S5
Tables S1 to S2
Captions for movies S1 to S2
References for SI reference citations

Other supplementary materials for this manuscript include the following:

Movies S1 to S2

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Supplementary Information Materials and Methods

Animals

Heterozygous Nestin–GFP transgenic mice (1) on the C57BL/6N genetic background were generated as described previously (2). For lineage tracing studies, Nes–CreERT mice (3) (The Jackson Laboratory, stock 012906) were bred with R26R-eYFP mice (4) also on the C57BL/6N background.

In general, induction of Cre in adult Nes-CreERT/R26R-eYFP mice was carried out by intraperitoneal injections of 100 µl tamoxifen (20 mg/ml in sunflower oil/10% ethanol (v/v)) for 5 consecutive days. All mouse colonies were maintained under 12:12 h light/dark cycle and fed ad libitum. Mice were sacrificed by cervical dislocation or, in the case of stress experiments, by CO_2/O_2 anesthesia.

Lineage tracing

In two months old Nes-CreERT/R26R-eYFP mice of both sexes Cre recombination was induced as described above. The mice were sacrificed at different time points after the last injection of tamoxifen (2 days, 1 month, 10 weeks, 20 weeks and 1 year). Adrenals were excised and processed for immunofluorescent staining as described below.

CLARITY protocol

Adrenals free of fat were washed 3x in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 4 h. After washing 3x in PBS, adrenals were incubated in 4% acrylamide monomer solution containing 0.25% of the thermal initiator VA-044 for 1-2 days at 4°C. Hybridization was then performed at 37°C for 4 h without movement. The tissue was recovered and washed 3x for 5min in PBS followed by embedding in 4% LMP agar and sectioning in 2-5 mm blocks. Passive clarification was then performed by incubation in 4% SDS, 250 mM boric acid, pH 8.5 for 4 weeks at 37°C with buffer change once per week. After proper clearing, when tissue was transparent, immunostaining was performed by washing 5x for 30 min in PBS containing 1% Triton X-100 (PBST), followed by blocking ON at 4°C in 4% BSA in PBST (PBSTB). After washing 3x in PBSTB, incubation with primary antibody for at least 48 h was performed. Tissue sections were then washed 3x for 3-6 h with PBST. After 3x washing with PBSTB tissue was incubated with secondary antibody in PBSTB for at least 48 h followed by nuclear staining using Hoechst reagent and washing 3x for 3-6 h in PBS. For refractive index matching, samples were incubated in 70% (w/v) sorbitol in PBS 3x for 4 h followed by mounting with the same solution for microscopy.

Immobilization stress

Adult male Nestin–GFP mice, aged 2.5–4 months, were divided into control and experimental (stress) groups (n=6 per group). Mice from the stress group were placed in individual cages, whereas control mice were not disturbed. Two days later, restraining for 2 h was initiated during 6 consecutive days at 9am using mouse restrainers (Braintree Scientific). Mice were sacrificed on the last day and adrenals and blood were collected. Nes-CreERT/R26R-eYFP mice, injected with tamoxifen as described above, were subjected to the same restraining protocol and also sacrificed and studied after 6 days of immobilization stress (n=5 per group). The adrenals from both experiments were processed for immunostaining.

Immunofluorescence

Adrenal glands were fixed (4% PFA, 4 h), cryoprotected (30% sucrose in PBS, 4°C overnight), embedded in Tissue-Tek Medium (OCT; Sakura Finetek), and stored at -80°C. Cryosections were sliced to 7-11 µm thickness (Leica CM 1900; Leica Biosystems) and mounted (Superfrost Plus slides; Thermo Scientific). Cultured cells were fixed in 4% PFA in PBS for 15 minutes. Slides or

cells were then immunostained using specific antibodies (Table S1). Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) and slides were mounted with fluorescent mounting medium (Aqua-Poly/Mount; Polysciences).

Confocal laser scanning microscopy and fluorescence microscopy

Confocal imaging was performed with a Zeiss LSM 780 inverted confocal laser scanning microscope and ZEN 2010 software (Zeiss). Fluorescence microscopy was done with a Zeiss Axiovert 200M fluorescence microscope and AxioVision software (Zeiss). Image processing and analysis were carried out using ImageJ software.

Isolation and culture of adrenal cells

Adrenals of 10-15 mice (age 2-5 months, both sexes) per experiment were excised and placed in petri dishes with ice-cold PBS. Fat tissue surrounding the adrenals was carefully removed and afterwards the adrenal cortex was thoroughly isolated from the medulla. All cortical and medullary tissues, respectively, were pooled, pelleted (350 x *g*, 5 min), and digested for 20 min at 37°C while shaking (1.8 mg/ml collagenase, 10 mg/ml BSA, 0.18 mg/ml DNAse in PBS; all from Sigma-Aldrich). The digestion was stopped by washing twice in PBS and adrenocortical cells were resuspended in 1 ml Dulbecco's modified Eagle medium (DMEM/F12)–high glucose (Gibco, Thermo Fisher Scientific) containing 10% steroid-free fetal bovine serum (charcoal/dextran treated; Hyclone Laboratories), 1% antibiotic-antimycotic solution (Gibco, Thermo Fisher Scientific), 1% L-Glutamine (PAA Laboratories) and 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich). Adrenomedullary cells were cultured in Neurobasal Medium (Gibco) containing 2% B27, 1% antibiotic-antimycotic solution, 1% L-Glutamine (PAA Laboratories) and 20 ng/ml basic bFGF (Sigma-Aldrich). Isolated cells were cultured in Ultra-Low Attachment Surface plates (Corning) at 37°C in a humidified atmosphere (95% O_2, 5% CO_2). To induce Cre recombination *in vitro* in cells isolated from Nes-CreERT/R26R-eYFP mice, 1 µM 4OH-tamoxifen (Sigma-Aldrich) was added to the culture the first 3 days after isolation.

Cell differentiation

To assess *in vitro* differentiation of isolated adrenal cells, spheres (after 6 days of proliferation), were plated into 24-well plates (Corning) or 8-well chamber plates (ibidi) coated with 1 mg/ml poly-D-lysine (Merck Millipore) and 3 µl/ml bFibronectin (R&D Systems) and cultured in the absence of bFGF. The medium was replaced by fresh medium every 2-3 days.

Fluorescent activated cell sorting

For *in vitro* proliferation and differentiation of Nestin-positive cells alone, GFP-positive and GFP-negative cells from the adrenal medulla and cortex of Nestin-GFP mice were flow sorted on a FACSAriaII flow cytometer (BD Biosciences) using FACSDiva Software using Propidium Iodide (BioLegend) as a live/dead cell marker. Cells were sorted directly in growth medium containing bFGF. After forming spheres, cells were transferred to 8-well chamber plates and differentiated as described above.

Flow cytometry

Flow cytometric analysis was performed on adrenal cells isolated as described above. After washing cells in ice-cold PBS containing 2% FCS, the appropriate antibodies were added, and the staining was performed in 50 µl cell suspension for 30 min at 4°C for fluorescently labelled antibodies. For unlabeled antibodies, cells were washed and stained with fluorescently labelled secondary antibodies for 20 min at 4°C. The antibodies used are listed in Table S1. After washing with PBS/2% FCS, cells were acquired on an LSRII flow cytometer (BD Biosciences). Cell viability staining was performed using PI (BioLegend). Data were analyzed using FlowJo software (BD Biosciences).

Real-Time PCR
Total RNA was isolated using the Direct-Zol RNA Miniprep Kit (Zymo Research) and reverse transcription was performed using the Moloney MLV Reverse Transcription system (Promega). qRT-PCR was performed using a Roche Light Cycler 1.5 System and the SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen) for all genes except *Cyp11b1* and *Cyp11b2*, which were measured using the Light Cycler Taq Man Master system (Roche Life Science). Primers used are shown in Table S2. qRT-PCR analyses were performed in triplicates, and C_t values were normalized against the internal control gene *beta-Actin*. Fold-differences in expression levels were calculated according to the comparative C_t method (5).

Steroid profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and ELISA
The medium of *in vitro* cultured cells was changed and after 24 h a conditioned medium sample was collected. Aldosterone and corticosterone in these samples were measured by ELISA using the instructions of the manufacturer (IBL International). Cell culture supernatants for measurement of steroid hormones (aldosterone, corticosterone, 11-desoxycorticosterone, cortisol, 11-desoxycortisol, cortisone, progesterone, 17-OH-progesterone and pregnenolone) by LC-MS/MS were also collected and stored frozen until the end of the experiment. All supernatants were finally batch analyzed by LC-MS/MS as described previously (6, 7). Quantification of steroid levels was done by comparisons of ratios of analyte peak area obtained from samples to the respective peak area of stable isotope labelled internal standard calibrators.

ACTH and Ang II stimulation
To cells differentiated for 7 days, 3 ng/ml $ACTH_{1-24}$ (Synacthen, Sigma-tau Arzneimittel GmbH) or 100 nM Ang II (Sigma-Aldrich) was added for 24 h before the medium was collected. Aldosterone and corticosterone in these samples were measured by ELISA as described above. Triptorelin was dissolved in DMSO and added to the culture medium to provide a concentration of 10 µM. Synthetic bombesin (Bachem) was dissolved in 0.9% NaCl before it was added to the culture medium at 0.5 µg/ml.

Electron microscopy
Adrenocortical cells were cultured on ACLAR foil in 24 well plates and after differentiation they were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 2 h. Afterwards the cells were washed in 1% osmium tetroxide in PB for 1 h. Dehydration in 50%, 70%, 90%, 96%, 3x 100% EtOH, each for 10 min was performed. A mixture of 100% EtOH and Epon mixed 1:1 with propyleneoxide was then added for 2 h. Pure Epon was added ON followed by polymerization for 48 h at 60°C. For the immunostaining cells were fixed in 4% PFA in PB followed by dehydration as described above and then embedded in LR White. Samples were then incubated with anti-GFP and subsequently with gold-labelled secondary antibody. Ultrathin sections (70 nm) were stained with 2% uranyl acetate for 10 min and 0.4% lead citrate for 5 min and examined at 80 kV in a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Statistical analysis.
Statistical analysis was performed using the GraphPad Prism program version 5.01 (GraphPad Software Inc.), and statistical significance was determined using one-way ANOVA followed by a Dunnett's multiple comparison test or two-way ANOVA followed by a Bonferroni multiple comparison test correction where appropriate. A Student's t test was performed when only two means were compared. The significance was defined as: not significant (ns) $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

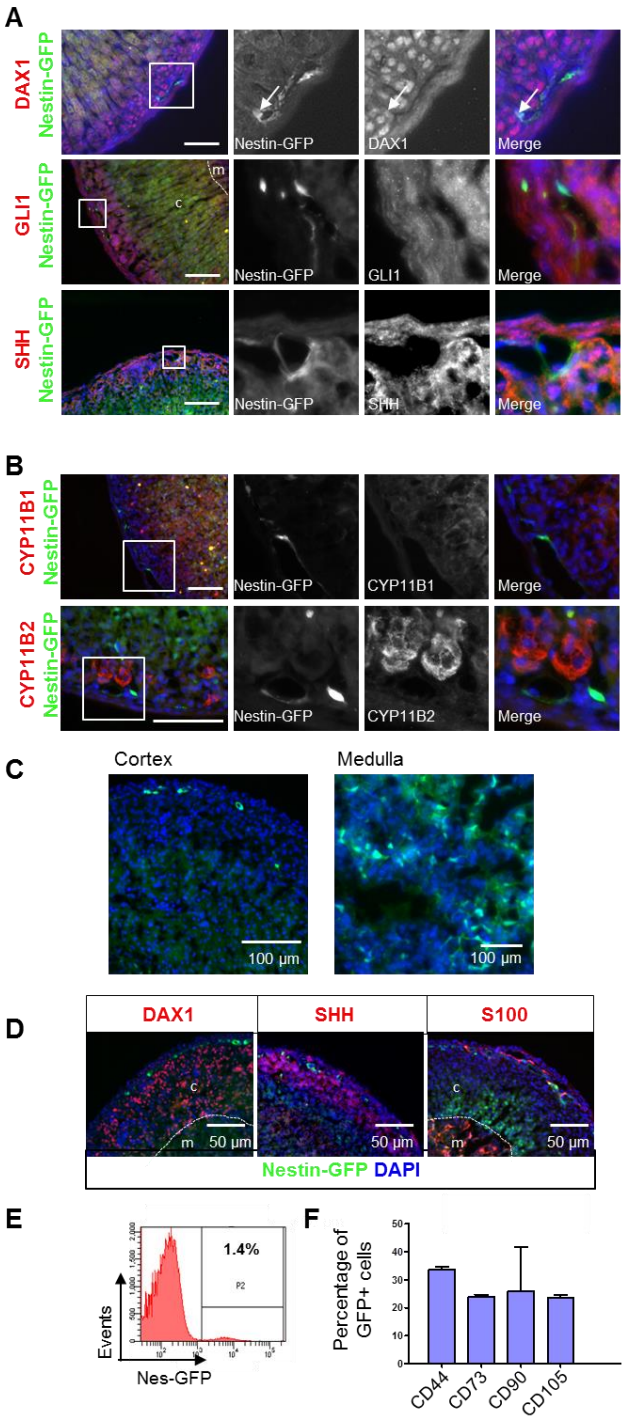

Fig. S1. Nestin-positive cells in the adrenal. (A) Immunofluorescent staining of Nestin-GFP adrenals with a panel of known progenitor/stem cell markers and **(B)** steroidogenic enzymes. Double positive cells are marked with arrows. Scale bars, 100 µm. **(C)** Nestin-positive cells in the adrenal cortex and medulla of P14 mice **(D)** Double-staining with DAX1, SHH or S100 of adrenals from P14 mice. **(E)** Flow cytometry of adrenocortical Nestin-GFP from adult mice cells showing Nestin-positive cells in the adrenal cortex. **(F)** Flow cytometric analysis of mesenchymal stem cell markers on cells isolated from whole adrenals. Representative images are shown. Related to Fig. 1.

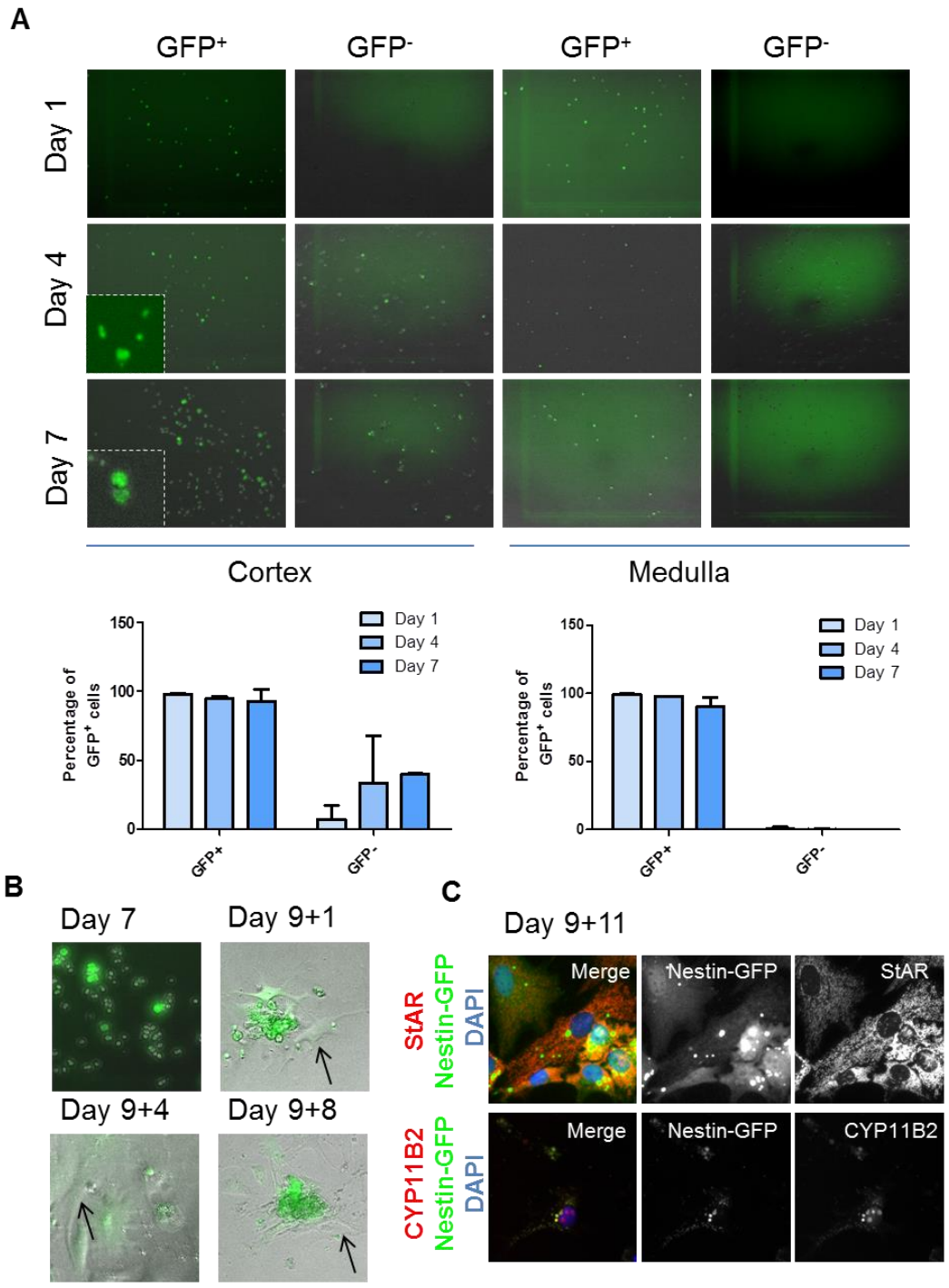

Fig. S2. FACS sorting of Nestin-GFP$^{+/-}$ cells in the adrenal cortex and medulla. (A) FACS sorted cells were cultured under conditions promoting proliferation. The percentage of green cells was counted at day 1, 4 and 7 of culture (n=3). **(B)** After 9 days of proliferation, the cells were transferred to conditions promoting differentiation. Cells with diminished GFP-signal are marked with arrows. **(C)** After 11 days of differentiation, cells were fixed and stained for StAR and CYP11B2. Representative images are shown. Related to Figs 2 and 3.

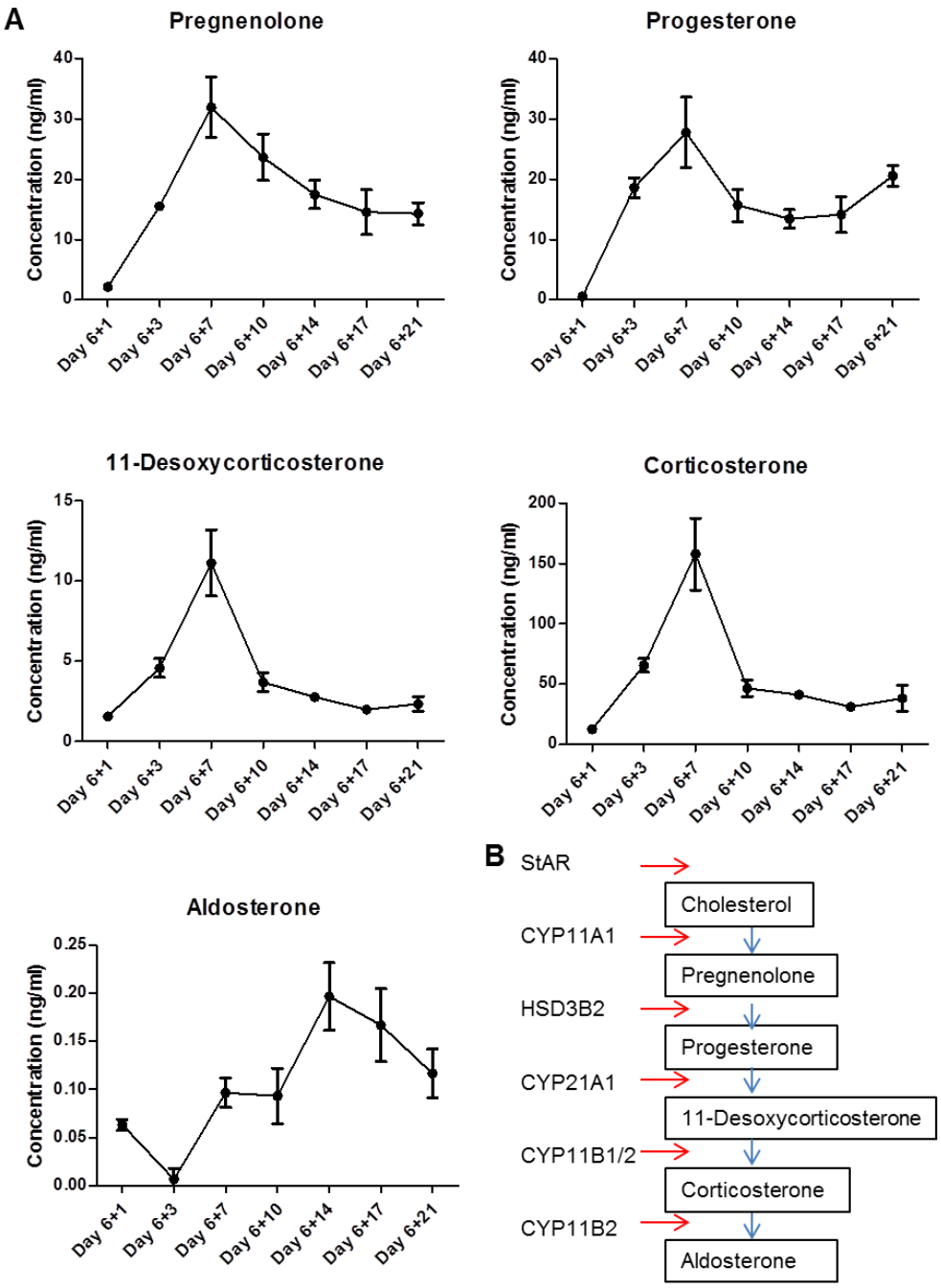

Fig. S3. Steroid profiling. (A) Adrenocortical cells isolated from C57BL/6N mice were allowed to proliferate for 6 days before differentiation was induced. During differentiation conditioned medium samples were collected 24 h after a medium change at the indicated time points and the levels of pregnenolone, progesterone, 11-desoxycorticosterone, corticosterone and aldosterone in these medium samples were measured using LC-MS/MS (n=3). **(B)** Diagram showing steroidogenesis in mice. Related to Fig. 3.

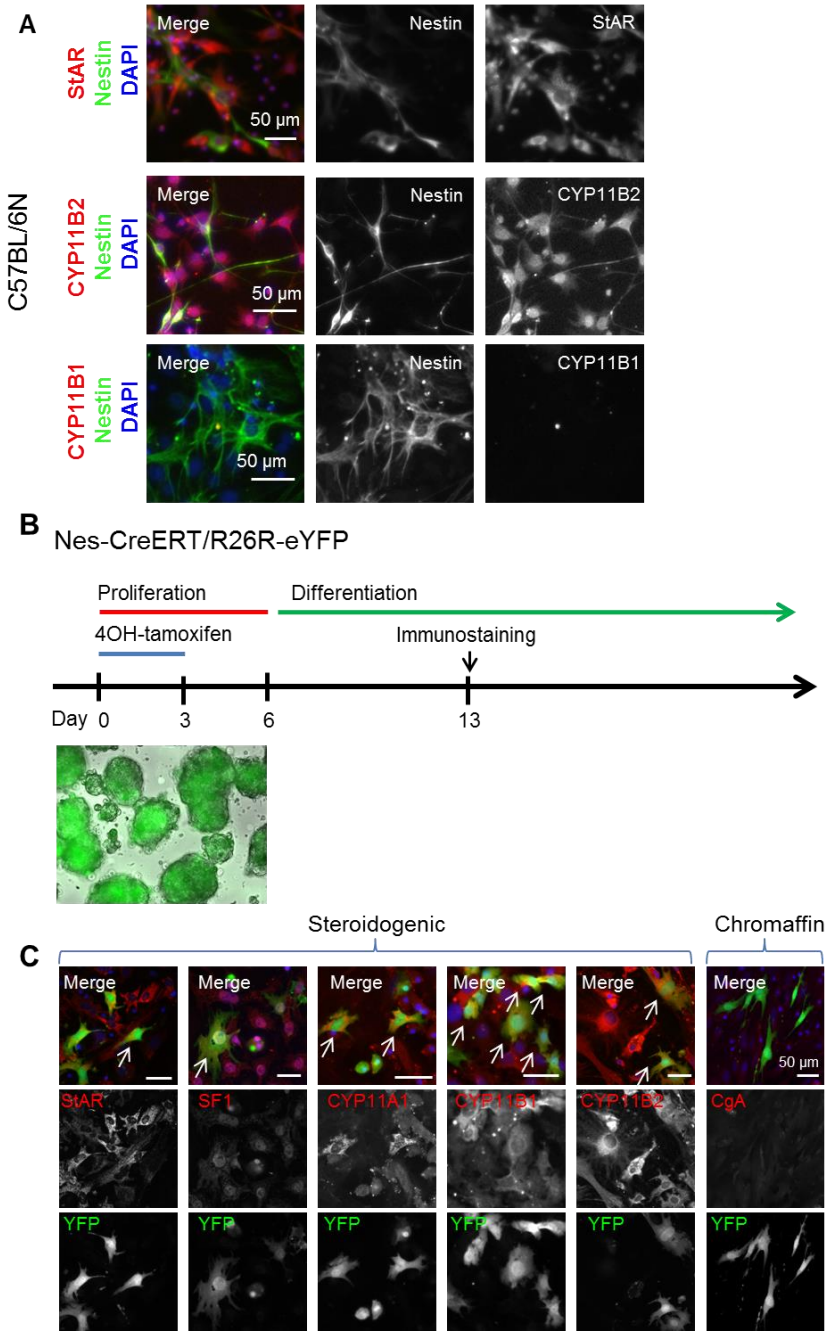

Fig. S4. ***In vitro*** **proliferation and differentiation of adrenocortical cells. (A)** Cells isolated from the adrenal cortex of C57BL/6N mice after proliferation for 6 days and differentiation for 4 weeks, fixed and stained for Nestin, StAR, CYP11B1 and CYP11B2. **(B)** Cells isolated from the adrenal cortex of Nes-CreERT/R26R-eYFP mice treated with 4OH-tamoxifen *in vitro* after 6 days of proliferation **(C)** *In vitro* tracing of Nestin-positive cells isolated from Nes-CreERT/R26R-eYFP mice, where recombination was induced *in vitro*. After differentiation for 7 days cells were immunostained for steroidogenic markers and a marker for chromaffin cells. Double positive cells are indicated with arrows. Scale bars, 50 µm. Representative images are shown. Related to Figs 2 and 3.

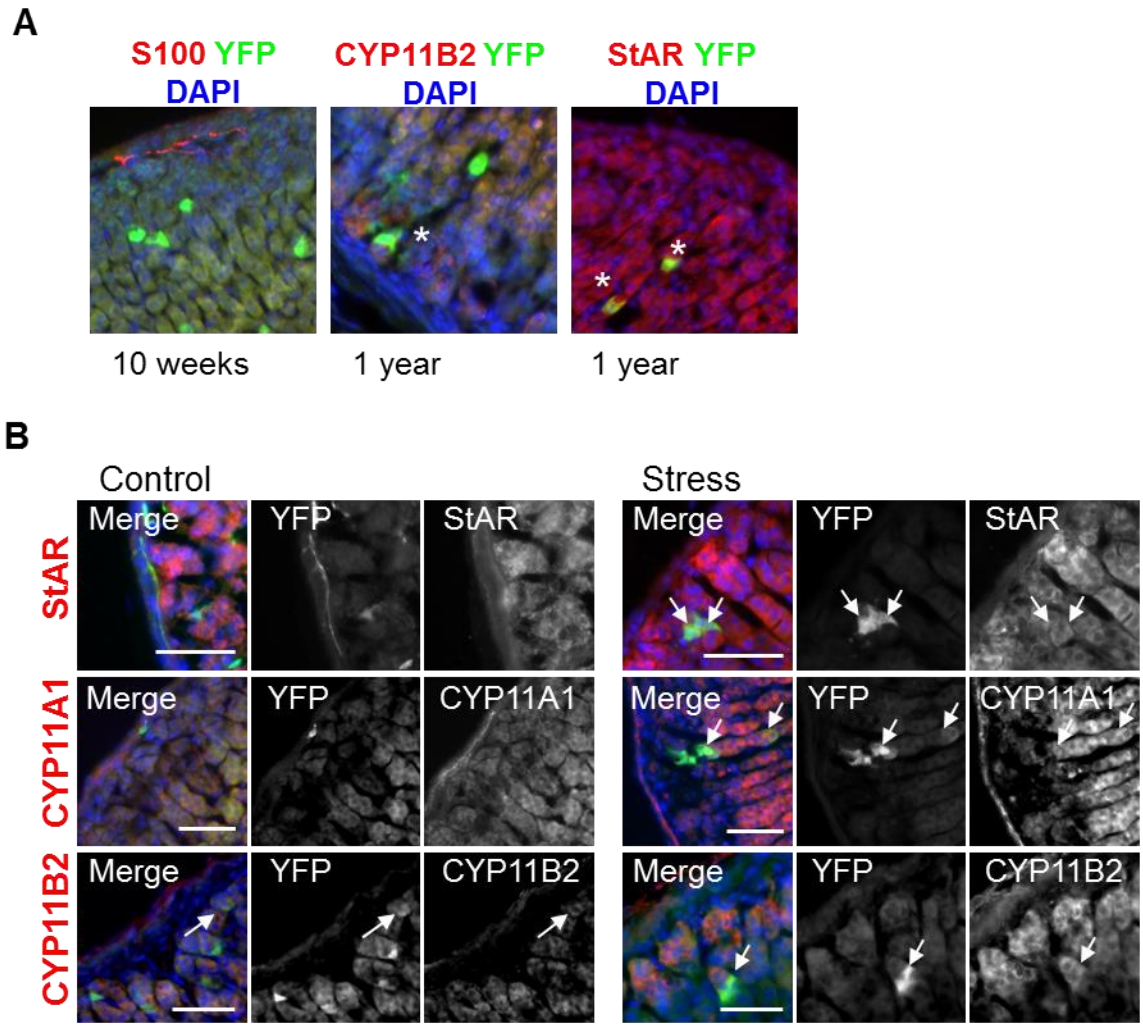

Fig. S5. Nes-CreERT/Rosa26-eYFP mice. (A) Lineage tracing. Mice were injected with tamoxifen for 5 consecutive days to induce recombination in Nestin-expressing cells. At different time points, mice were sacrificed. Adrenals were immunostained with antibodies against S100, CYP11B2 and StAR. Double positive cells are indicated with asterisks. Representative images are shown. Related to Fig. 4. **(B) Stress.** Immunostaining of adrenals from control mice and mice injected with tamoxifen and subjected to immobilization stress (n = 5). Double positive cells are marked with arrows. Representative images are shown. Related to Figs 4 and 5.

Movie S1

3D imaging of the whole adrenal gland showing interconnections between S100-positive (red) Nestin-positive (green) cells under the adrenal capsule. Related to Fig. 1.

Movie S2

3D imaging of the whole adrenal gland showing ß-III tubulin (red) interconnections between Nestin-positive (green) cells in the cortex and the medulla. Related to Fig. 1.

Supplementary Table S1. List of antibodies

Antibody	SOURCE	Identifier
Chicken polyclonal anti-GFP	Abcam	Cat# ab13970; RRID:AB_300798
Rabbit polyclonal anti-DAX-1, clone H-300	Santa Cruz Biotechnology	Cat# sc-13064; RRID:AB_2154485
Rabbit polyclonal anti-Gli1, clone H-300	Santa Cruz Biotechnology	Cat# sc-20687; RRID:AB_2111764
Goat polyclonal anti-SHH	R&D Systems	Cat# AF464; RRID: AB_355373
Rabbit polyclonal anti-S100	Abcam	Cat# ab868; RRID:AB_306716
Rabbit polyclonal anti-SF1	Sigma-Aldrich	Cat# HPA018883;, RRID:AB_1856763
Rabbit polyclonal anti-StAR, clone FL-285	Santa Cruz Biotechnology	Cat# sc-25806; RRID:AB_2115937
Rabbit polyclonal anti-ßIII-tubulin	BioLegend	Cat# 802001; RRID:AB_2564645
Goat polyclonal anti-CYP11A1	Santa Cruz Biotechnology	Cat# sc-18043; RRID: AB_2245659
Sheep polyclonal anti-CYP11B1	Gift from Celso Gomez-Sanchez	N/A
Rabbit polyclonal anti-CYP11B2	Gift from Celso Gomez-Sanchez	N/A
Rat monoclonal anti-CD44-Alexa Fluor 647	BioLegend	Cat# 103017; RRID:AB_493680
Rat monoclonal anti-CD73-Alexa Fluor A647	BioLegend	Cat# 127207; RRID:AB_2154093
Rat monoclonal anti-CD90.2-Alexa Fluor 647	BioLegend	Cat# 105317; RRID:AB_492889
Rat monoclonal anti-CD105-APC	BioLegend	Cat# 120413; RRID:AB_2277915
Goat polyclonal anti-Chromogranin A	Santa Cruz Biotechnology	Cat# sc-1488; RRID:AB_2276319
Cy3-goat-anti-rabbit	Jackson ImmunoResearch	Cat# 111-165-144; RRID:AB_2338006
Cy3-donkey-anti-goat	Jackson ImmunoResearch	Cat# 705-165-147; RRID:AB_2307351
Alexa Fluor 488-donkey-anti-chicken	Jackson ImmunoResearch	Cat# 703-546-155; RRID:AB_2340376
Alexa Fluor 647-goat-anti-rabbit	Thermo Fisher Scientific	Cat# A21246; RRID:AB_2535814
Rhodamine-donkey anti-sheep	Jackson ImmunoResearch	Cat# 713-025-147; RRID:AB_2340707

Supplementary Table S2. List of primers and amplification conditions for RT-PCR

Gene	Primer sequence	Product size (base pairs)
Actb	F: GAGCACAGCTTCTTTGCAGCTCCTT	280
	R: TGCCATGTTCAATGGGGTACTTCAG	
Nes	F: CTCTGCTGGAGGCTGAGAAC	176
	R: ATTAGGCAAGGGGGAAGAGA	
Gli1	F: TGGATCGGATGGGAGGTCTT	298
	R: ACCTCTGGCTCCTCCTGTAG	
Shh	F: TGAACGGACCTTCAAGAGCC	190
	R:GCAGGAGCATAGCAGGAGAG	
Nr0b1 (*Dax1*)	F: ATGGAGATCCCGGAGACCAA	236
	R: AAGAGCACGGTCCCTTTCAG	
Mc2r	F: CAAAGCCAAGGAGAGGAGCATTATT	149
	R: GGTGTTTGCCGTTGACTTACAGAAA	
Cyp11a1	F: TGGGTGGCCTATCACCAGTATTATC	103
	R: CCATCACCTCTTGGTTTAGGACGAT	
Cyp21a1	F: AACAGAACCATTGAGGAGGCCTTGA	305
	R: TCTCCAAAAGTGAGGCAGGAGATGA	
Actb TaqMan	F: TATTGGCAACGAGCGGTT	75
	R: ATGCCACAGGATTCCATACCC	
Actb TaqMan probe	CCTGAGGCTCTTTTTCCAGCCTTCCTTCT	
Cyp11b1 TaqMan	F: AGAGCTGGCAGAGGGTCGT	79
	R: TGGCATCCATTGACAGAGTTCT	
Cyp11b1 TaqMan probe	CACAGTCCTGGAGTGTCACAGCAGAGCT	
Cyp11b2 TaqMan	F: CAGACTCGGCAGCTCTCAGA	77
	R: ATGGCGTCGAGAGGCAAA	
Cyp11b2 TaqMan probe	CTACAGTGGCATTGTGGCGGAACTAATATCTCA	

F: forward primer, R: reverse primer.

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