Supporting Information

Close et al. 10.1073/pnas.0914026107

SI Materials and Methods

Materials. Radiolabeled steroids were obtained from American Radiolabeled Chemicals. Synthetic steroids were obtained from Steraloids and Sigma. All other reagents were obtained from Sigma unless otherwise noted.

Animal Maintenance and Blood Collection. Sea lampreys (*Petromyzon marinus*) were collected in landlocked streams by US Fish and Wildlife Service employees and transported to Michigan State University or to the US Geological Survey Hammond Bay Biological Station. Lampreys were held at 10–13 °C. Blood was obtained by cardiac puncture using Vacutainers containing EDTA (Becton Dickinson), placed on ice for 15 min, and then centrifuged at 1,000 × *g* at 4 °C for 15 min. The plasma was removed and stored at −80 °C. Tissues collected from fish were frozen in liquid nitrogen and held at −80 °C until processed for cytosolic fractions. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 05/04-077-00).

Plasma Extraction. Initial screening (20 mL) and LH-20 isolation (800 mL) of plasma from male and female lampreys was diluted 1:1 with 0.9% saline, passed through a 0.45-μM filter (Millipore), and loaded onto an activated Sep-Paks (Waters). The Sep-Paks were washed with deionized water and eluted with methanol. The methanol elute was evaporated under reduced pressure using a CentriVap Concentrator (Labconco).

HPLC. Samples were dissolved in 1 mL acetonitrile/water/TFA (28/72/0.01, vol/vol/vol) and loaded onto a C18 reverse-phase HPLC column (Nova-Pak, 3.9 mm × 300 mm, Waters) fitted with a guard module. Two solvents were used to deliver a gradient to the column. Solvent A was 0.01% TFA in deionized water, and solvent B was 70% acetonitrile and 0.01% TFA in deionized water. The pattern of development was as follows: 0 → 10 min, 28% B; 10 → 60 min, 28 → 100% B; 60 → 80 min, 100% B. The eluate was monitored for UV absorption with a photodiode array detector (Waters). Fractions were collected in 1.5-mL tubes at 1-min intervals between 11 and 70 min.

RIA and Binding-Protein Assay Procedures. RIA and binding-protein assay procedures were conducted in glass culture tubes (10 mm × 75 mm, Fisher Scientific). Briefly, the assay buffer consisted of 50 mM sodium phosphate, pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, and 0.77 mM sodium azide. Nine standards were made in duplicate over the range of 1.95–500 pg/100 μL per tube. The tubes containing samples also had a volume of 100 μL RIA buffer. Binding reagent was made by adding radiolabel (American Radiolabeled Chemicals) and antiserum (Chemicon; 1:100) or rabbit sera (Sigma-Aldrich; 1:40) for binding-protein assay to 20 mL of assay buffer in amounts such that when 100 μl was dispensed to all tubes, each tube contained 5,000 dpm and, in the absence of any standard, 50% of the radiolabel was bound to the antiserum or cortisol binding protein. Blank tubes, to which no antibody was added, and tubes necessary to determine the total and maximum dpm counts were also included in the assay. All tubes were incubated overnight at 4 °C, after which 500 μL ice-cold charcoal solution at 0 °C [50 mM sodium phosphate, pH 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal (DCC)] was added to each tube. The tubes were kept on ice for 20 min and then centrifuged in an Allegra 6R (Beckman Coulter) at 1, 000 × *g* for 12 min. The supernatants were poured into 8-mL scintillation vials and mixed with 6 mL scintillation fluid; dpm were counted with an LS-6500 (Beckman Coulter) scintillation counter.

LH-20 Column Chromatography. Each sample was dissolved in 2 mL 98% dichloromethane and 2% methanol and loaded onto a glass column (450 mm × 15 mm) packed with 20.0 g Sephadex LH-20 (Amersham). Two solvents were used to deliver sample through the column. A solution containing 98% dichloromethane and 2% methanol was pumped through the column at 4 mL/min. Fractions were collected in 16 × 100-mm glass culture tubes at 1-min intervals between 1 and 60 min. Elute was dried down and resuspended with 0.5 mL methanol per tube.

MS Analysis of Plasma. LH-20 fractions corresponding to immunoreactivity of 11-deoxycortisol and 11-deoxycorticosterone were collected and dried down under reduced pressure. Samples were then fractionated by HPLC and reassayed to identify fractions. Fractions were dried down under reduced pressure and then subjected to atmospheric pressure chemical ionization MS analysis. Mass spectra were obtained by using a LCQ-Deca ion trap (Thermo Scientific). The vaporizer temperature was 300 °C and the capillary temperature was 250 °C. Samples were compared against authentic 11-deoxycortisol and 11-deoxycorticosterone standards (Sigma-Aldrich). MS analysis was performed at the Mass Spectrometry Facility, Research Technology Support Facility at Michigan State University.

Preparation of Cytosolic Fractions of Tissues. Preparation of cytosolic fractions for binding studies was performed as described elsewhere (1). Frozen tissue was ground in liquid nitrogen with a mortar and pestle. Frozen tissue was mixed 1:5 (weight:volume) in Hepes buffer (25 mM Hepes, 10 mM NaCl, 1 mM monothioglycerol, pH 7.4) and kept on ice while being homogenized. The homogenate was centrifuged at 1,000 × *g* for 15 min at 4 °C. The supernatant containing the cytosolic fraction was collected and the pellet discarded. The supernatant was centrifuged at 40,000 × *g* for 1 h at 4 °C. The supernatant was removed and glycerol (10% vol/vol) was added. Cytosolic fractions were immediately used in subsequent assays.

Saturation Curve and Scatchard Analysis. Radiolabeled 11-deoxycortisol (0.2–20 nM) in ethanol was added to each assay tube with or without 1 μg cold 11-deoxycortisol (to determine nonspecific binding). The ethanol was evaporated under nitrogen at 40 °C, after which 200 μL gill cytosol was added to the assay tubes and incubated at 0 °C for 2 h. After incubation, 500 μL ice-cold DCC solution was added to assay tubes and incubated on ice for 5 min. The samples were then centrifuged at 1,000 × *g* for 5 min at 4 °C and the supernatants were poured into scintillation vials. A 6-mL quantity of scintillation mixture was added to each vial, and dpm were counted by a scintillation counter. The concentration of binding sites (B_{max}) and the dissociation constant (K_D) were determined by hyperbolic regression using Sigmaplot v9.0 (SYSTAT). Protein concentrations were determined by *DC* Protein Assay Kit for microplates (Bio-Rad) with BSA as a standard.

Association and Dissociation Kinetics. The rate of association was determined by incubating gill cytosol (200 μL) with 3.3 nM of [^{3}H] 11-deoxycortisol with or without 1 μg of 11-deoxycortisol at 0 °C for 0–4 h. To determine the dissociation rate, we incubated gill cytosol (200 μL) with 3.3 nM of [^{3}H] 11-deoxycortisol in the presence or absence of 1 μg of 11-deoxycortisol at 0 °C for 2 h and then initiated the dissociation by adding 1 μg of 11-deoxycortisol to

all assay tubes for another 0–2 h at 0 °C. Bound and free steroid were separated by addition of DCC. The tubes were kept on ice for 5 min and then centrifuged at 1,000 × *g* for 5 min. The supernatants were poured into 8-mL scintillation vials and mixed with 6 mL scintillation fluid; dpm were counted with a scintillation counter.

Steroid and Tissue Binding Specificity. To determine steroid specificity of the binding moiety, we used cold steroids for their ability to compete with [^{3}H] 11-deoxycortisol binding. Gill cytosol preparations (200 μL) were incubated at 0 °C for 2 h with 3.3 nM [^{3}H] 11-deoxycortisol in the presence of different amounts of cold steroid (0.1–1,000 nM). Specificity was examined for 11-deoxycortisol, 11-deoxycorticosterone, cortisol, corticosterone, aldosterone, dexamethasone, androstenedione, 17β-estradiol, 17α-hydroxyprogesterone, and progesterone.

Relative binding was measured in the gill, intestine, testis, liver, kidney, heart, and muscle by binding assays. Radiolabeled 11-deoxycortisol (5 nM) in ethanol was added to each tube in the presence or absence of 1 μg cold 11-deoxycortisol and dried down. Each tube received an aliquot of gill cytosol (200 μL) and was incubated for 2 h at 0 °C. The reaction was stopped by the addition of DCC. The tubes were kept on ice for 5 min and then centrifuged at 1,000 ×*g* for 5 min. The supernatants were poured into 8-mL scintillation vials and mixed with 6 mL scintillation fluid; dpm were counted with a scintillation counter.

DNA-Cellulose Chromatography. DNA-cellulose chromatography procedures were performed as described (2). Hepes buffer with 0.2 mg/mL BSA consisted of three concentrations of NaCl: 0.05 M (column buffer), 0.4 M (elution buffer), and 2.0 M (wash buffer). Gill cytosol (1.0 mL) was incubated for 2 h at 0 °C with 20 nM [^{3}H] 11-deoxycortisol with or without 1 μg cold 11-deoxycortisol. Samples were placed on a laboratory table for 30 min at 25 °C and then cooled with ice for 5 min. The samples were then diluted in 3 mL of column buffer (total volume 4 mL) and added to a 20-mL column (Bio-Rad) containing 5 mL DNA-cellulose (Amersham) in column buffer. The sample was allowed to flow into the DNA-cellulose, and then the flow was stopped to allow absorption for 20 min. The column was then washed with 20 mL of column buffer to remove free radiolabeled steroid. To elute the bound receptor complex from the DNA, we used 7 mL 0.4 M NaCl elution buffer followed by 7 mL wash buffer (2.0 M NaCl). Fractions (1 mL) were collected and dpm were counted with a scintillation counter.

Corticotropin-Releasing Hormone Injections. Mammalian corticotropin-releasing hormone (CRH) (Sigma-Aldrich) was dissolved in 0.9% saline and injected i.p. with a dose of 100 μg/kg. Saline solution was used as a control. Blood samples were collected 1 h after injections by cardiac puncture using Vacutainers (Becton Dickinson). Blood samples were centrifuged at 1,000 × *g* for 15 min; plasma was collected and stored at −80 °C until analyzed by RIA for 11-deoxycortisol.

Pituitary Extract Injections. To obtain pituitary extract, we collected pituitary glands from 400 adult sea lampreys in June at Hammond Bay Biological Station. The frozen pituitary glands were homogenized in 20 mL 20-mM Tris buffer, pH 7, containing protease inhibitor mixtures (Roche). This mixture was centrifuged at 1,000 × *g* for 20 min, allowing recovery of the supernatant. The protein concentration was determined by using a BCA protein analysis kit (Pierce). The protein concentration for the extract was 6.7 mg/mL. A 1-mL quantity of the extract was equivalent to 20 lamprey pituitary glands. Lampreys were given a single i.p. equivalent to 1, 5, or 10 pituitaries or a 0.9% saline as a control (four treatments total, 10 lampreys/treatment). Blood was sampled at 0, 6, 12, 24, and 48 h after the injection. Blood samples were centrifuged at 1,000 × *g* for 15 min; plasma was collected and stored at −80 °C until analyzed by RIA for 11-deoxycortisol.

Handling and Salinity Stressors. Adult lampreys were acclimated in flow-through tanks (254 L) at least 2 wk before stress tests were conducted. Tanks were isolated to keep people from disturbing the lampreys during acclimation. In the first stress experiment (1–48 h recovery), 140 lampreys were distributed in tanks at a density of 7 lampreys/tank, with replicate tanks for each treatment at each time. No lampreys were sampled more than once. In the second stress experiment (1- to 24-h recovery), 80 parasitic lampreys were distributed in tanks at a density of five lampreys/tank, with replicate tanks for each treatment at each time. No lampreys were sampled more than once. Lampreys were netted out of tanks, placed in a dry bucket for 5 min, and then transferred to 3% saltwater for 10 min. To obtain plasma, we netted lampreys out of tanks and immersed them in an anesthetic dose of 400 mg/L tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. We collected blood samples at 1, 4, 8, 24, and 48 h after stressors to measure steroid levels. For parasitic lampreys, blood was sampled at 1 and 24 h after stressors. Lampreys were then euthanized with a lethal dose of MS-222. Blood was centrifuged at 1,000 × *g* at 4 °C for 15 min and plasma removed. Plasma was stored at −80 °C until analysis.

Steroid Implants. 11-Deoxycortisol (Sigma-Aldrich) time-release pellets were made by Innovative Research of America. The 21-d slow-release steroid implants (5 mg/pellet) were injected between the muscle and the skin near the front dorsal fin of the sea lamprey. A total of 48 lampreys were distributed in flow-through tanks (254 L) at a density of six lampreys/tank, with replicate tanks for each treatment. On day 21, blood samples and gill tissues were collected. Plasma was analyzed by RIAs for 11-deoxycortisol, 11-deoxycorticosterone, dehydroepiandrosterone-sulfate, dehydroepiandrosterone, testosterone, and estradiol.

Gill Na^+, K^+-ATPase Activity. A gill pouch was removed and placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen immediately at −80 °C. Na^+, K^+-ATPase activity was determined with a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min as previously described (3). Gill tissue was homogenized in 500 μL SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5,000 × *g* for 30 s. Samples (10-μL) were run in two sets of duplicates, one set containing the assay mixture and the other containing the assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity is expressed as μmoles ADP/mg protein per hour. Protein concentrations were determined with a BCA protein assay kit (Pierce). Both assays were run on a THERMOmax microplate reader using SoftMax software (Molecular Devices).

Statistical Analysis. Data expressed are mean ± SEM. For the CRH experiment, a two-tailed Student *t* test was used in assessing the differences. Analysis of the pituitary extract experiment was done with repeated measures two-way ANOVA in which pituitary equivalent dosage and time were factors. Analysis of the acute stress experiments was done using ANOVA, in which time was an independent variable. Time intervals were compared using Bonferroni multiple comparison tests. Analysis of the implant experiments was done using the Student *t* test. Males and females were analyzed separately.

1. Patiño R, Thomas P (1990) Characterization of membrane receptor activity for 17 alpha, 20 beta, 21-trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). *Gen Comp Endocrinol* 78:204–217.

2. Knoebl I, Fitzpatrick MS, Schreck CB (1996) Characterization of a glucocorticoid receptor in the brains of chinook salmon, *Oncorhynchus tshawytscha*. *Gen Comp Endocrinol* 101:195–204.

3. McCormick SD (1993) Methods for nonlethal gill biopsy and measurement of Na, K-ATPase activity. *Can J Fish Aquat Sci* 50:656–658.

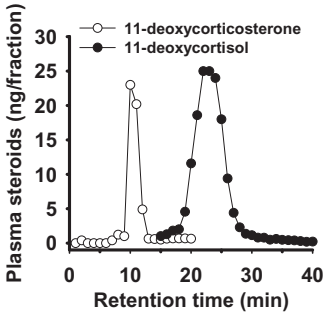

Fig. S1. Isolation of putative corticosteroids in lamprey plasma. An 800-mL quantity of plasma was subjected to solid phase extraction and then fractionated by LH-20 chromatography. Concentrations of putative corticosteroids are based on RIAs of 20 μL of each fraction and back calculated to the fraction volume.

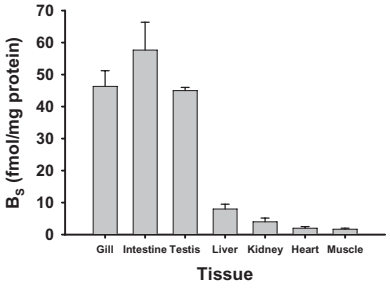

Fig. S2. Corticosteroid receptor distribution among various tissues. Specific binding of [^{3}H] 11-deoxycortisol (5 nM) to corticosteroid receptors. B_S, specific binding. Vertical bars represent mean ± SE (n = 3).

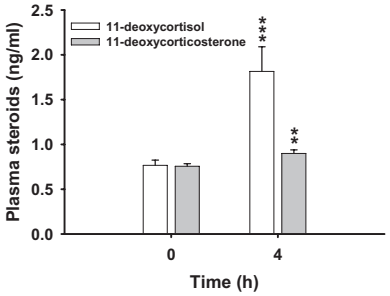

Fig. S3. Effects of acute stress on plasma concentrations of 11-deoxycortisol and 11-deoxycorticosterone. Results are mean ± SE. Asterisks indicate significant (*P < 0.05; **P < 0.01; ***P < 0.001) difference with Student's t test.