11-Deoxycortisol is a corticosteroid hormone in the lamprey

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Corticosteroid hormones are critical for controlling metabolism, hydromineral balance, and the stress response in vertebrates. Although corticosteroid hormones have been well characterized in most vertebrate groups, the identity of the earliest vertebrate corticosteroid hormone has remained elusive. Here we provide evidence that 11-deoxycortisol is the corticosteroid hormone in the lamprey, a member of the agnathans that evolved more than 500 million years ago. We sequenced lamprey gill cytosol. The receptor was highly specific for 11-deoxycortisol and exhibited corticosteroid binding characteristics, including DNA binding. Furthermore, we observed that 11-deoxycortisol was regulated by the hypothalamus-pituitary axis and responded to acute stress. 11-Deoxycortisol implants reduced sex steroid concentrations and up-regulated gill Na+, K*-ATPase, an enzyme critical for ion balance. We show here that 11-deoxycortisol functioned as both a glucocorticoid and a mineralocorticoid in the lamprey. Our findings indicate that a complex and highly specific corticosteroid signaling pathway evolved at least 500 million years ago with the arrival of the earliest vertebrate.

Results

Identification of Circulating Corticosteroids. To determine whether corticosteroids are present in lamprey, we assumed that the structure of a lamprey corticosteroid might be related to modern vertebrate GCs. We isolated putative corticosteroids (Fig. L1A) by screening lamprey plasma with RIAs for cortisol and corticosterone and with a binding protein assay for cortisol after HPLC fractionation. These assays showed no cortisol and corticosterone in the plasma (Fig. L1 B and C), but revealed cross-reactivity in fractions where 11-deoxycortisol and 11-deoxycorticosterone standards eluted on HPLC (Fig. 1 B and C). To isolate sufficient quantities of steroids for mass spectrometry (MS) analysis, we fractionated extracted plasma by group partition chromatography (LH-20) and screened it with 11-deoxycortisol and 11-deoxycorticosterone RIAs (Fig. S1) and isolated by HPLC fractionation followed by MS analysis.

To confirm the identity of the compounds in the immunoreactive fractions, we subjected the analyte ions to tandem MS (MS/MS) product ion analyses. The product ion spectra of authentic 11-deoxycortisol [M+H]+ ion at m/z 347 and 11-deoxycorticosterone [M+H]+ ion at m/z 331, obtained by direct infusion of standards (Fig. 1D, Inset, and E, Inset), matched the fragmented daughter ions of the plasma samples (Fig. 1D and E). Taken together, the binding–assay–guided isolation and chromatographic and MS analyses demonstrated that 11-deoxycortisol and 11-deoxycorticosterone were...
present in circulation and thus potential corticosteroids in the sea lamprey. These results were intriguing because 11-deoxycortisol and 11-deoxycorticosterone are precursor steroids to cortisol and corticosterone in the biosynthetic pathway.

Identification and Characterization of CR. To characterize the cognate receptor for 11-deoxycortisol, we performed radioligand binding experiments using tritiated 11-deoxycortisol and the cytosol fraction of gill tissue. We confirmed the presence of a highly specific 11-deoxycortisol receptor with glucocorticoid receptor (GR) binding characteristics. Linear transformation of saturation data revealed a single population of binding sites for 11-deoxycortisol in the cytosolic fraction of gill homogenate (Fig. 2A, Inset). The receptor had a high affinity (Kd = 2.66 ± 0.47 nM, mean ± SEM) and low capacity (Bmax = 58.10 ± 3.33 fmol/mg protein) for 11-deoxycortisol (Fig. 2A), consistent with GR affinity and concentration of binding sites in previous studies with teleosts (9, 10).

Kinetic studies of the 11-deoxycortisol binding moiety showed that its association rate (T1/2) was 2.11 ± 0.32 min and that its specific binding remained constant during the experiment (Fig. 2B). The specific binding was reversible with a dissociation rate (T1/2) of 26.44 ± 8.41 min during the 2-h experiment (Fig. 2B, Inset). Both association and dissociation rates of 11-deoxycortisol were generally faster than GR characteristics in salmonids (9, 11, 12).

We demonstrated that the lamprey CR was highly specific for 11-deoxycortisol, as determined by a competitive binding assay. 11-Deoxycortisol had the highest affinity to the cytosolic binding moiety among the nine steroids tested (Fig. 2C). Relative binding affinity of 11-deoxycorticosterone, compared with 11-deoxycortisol, was ≈5% for the CR. All other steroids, at concentrations up to 1 μM, failed to displace 50% of 3.3 nM of [3H]11-deoxycortisol. Specific binding of 11-deoxycortisol to the binding moiety was found in all tissues tested, with highest specific binding levels in gill, intestine, and testis (Fig. S2). In addition, there was no
11-Deoxycortisol Decreases Sex Steroids and Increases Na⁺, K⁺-ATPase Activity. Acute and chronic stress is known to suppress reproductive functions through GCs; therefore, we investigated whether sex steroids in circulation would decrease in response to chronic elevation of 11-deoxycortisol. 11-Deoxycortisol implants increased plasma concentrations of 11-deoxycortisol and 11-deoxycorticosterone (Fig. 4A) and resulted in lowered circulatory concentrations of dehydroepiandrosterone sulfate, dehydroepiandrosterone, testosterone, and estradiol in both male and female lampreys (Fig. 4B and C). Our previous measurements of 11-deoxycortisol concentrations in the metamorphosing sea lamprey showed that the concentrations achieved by the implants are physiologically relevant. Stress or cortisol implants have been shown to decrease testosterone and estradiol in fish, amphibians, and reptiles (17–23). To investigate whether 11-deoxycortisol regulates MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24). 11-Deoxycortisol implants nearly doubled gill Na⁺, K⁺-ATPase activity in both male and female lampreys (Fig. 4D). This effect is similar to that of cortisol, a GC in teleosts, in up-regulating gill MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24). 11-Deoxycortisol implants nearly doubled gill Na⁺, K⁺-ATPase activity in both male and female lampreys (Fig. 4D). This effect is similar to that of cortisol, a GC in teleosts, in up-regulating gill MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24). 11-Deoxycortisol implants nearly doubled gill Na⁺, K⁺-ATPase activity in both male and female lampreys (Fig. 4D). This effect is similar to that of cortisol, a GC in teleosts, in up-regulating gill MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24). 11-Deoxycortisol implants nearly doubled gill Na⁺, K⁺-ATPase activity in both male and female lampreys (Fig. 4D). This effect is similar to that of cortisol, a GC in teleosts, in up-regulating gill MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24). 11-Deoxycortisol implants nearly doubled gill Na⁺, K⁺-ATPase activity in both male and female lampreys (Fig. 4D). This effect is similar to that of cortisol, a GC in teleosts, in up-regulating gill MC homeostasis, we measured Na⁺, K⁺-ATPase activity in the gills after exogenous treatment with 11-deoxycortisol. In lampreys, this ion-translocating enzyme is involved both in salt uptake in freshwater and in salt secretion in seawater (24).
**Discussion**

The present study demonstrates that 11-deoxycortisol functions as both a GC and an MC through its highly specific CR in lamprey. 11-Deoxycortisol was regulated by the HPI axis and responded to acute stress. Moreover, implanted 11-deoxycortisol suppressed the sex steroid concentrations and stimulated ion transport capacity in the gills. Collectively, these data demonstrate that 11-deoxycortisol is a biologically active corticosteroid hormone in lamprey. This identification of the corticosteroid in the living representative of the most basal vertebrate provides crucial insight into the evolution of corticosteroid signaling.

In the present study, we used a combination of RIAs, HPLC, and MS analysis to isolate and identify corticosteroid-like molecules in the sea lamprey. On the basis of the assumption that the corticosteroids in the lamprey share structural features with the steroids found in other vertebrates such as fish and tetrapods, we used antibodies raised against cortisol and corticosterone and a cortisol binding protein in RIAs and a binding assay to screen HPLC fractions. We were able to isolate two steroid compounds that are direct precursors to cortisol and corticosterone. This approach enabled us to separate steroid compounds of interest on the basis of their chromatographic and MS characteristics, ruling out the possibility of misinterpretation caused by cross-reactivity of antibodies (27). Lamprey possesses 15 hydroxy steroids, including 15α-OH progesterone (15α-OH P), which shares some chemical features with the known vertebrate corticosteroids. Although 15α-OH P exists in the circulation of the sea lamprey (28–30), previous studies in our laboratory found no evidence for 15α-OH P binding to cytosolic, nuclear, and membrane preparations of various sea lamprey tissues. Further research would be required to test whether there are any other corticosteroids that are not structurally related to the common vertebrate corticosteroids.

In the steroid biosynthetic pathway (Fig. L4), 11-deoxycorticosterone and 11-deoxycortisol are direct precursors to corticosterone and cortisol, respectively, the major GCs in more derived vertebrates. Previous studies have provided mixed results regarding the presence of corticosteroids in lamprey, a question that has remained unresolved for many years (31–35). The most recent of these studies reported the presumptive identification of cortisol, corticosterone, and 11-deoxycortisol, but not 11-deoxycorticosterone in sea lamprey sera (35), in contrast to our findings that identified 11-deoxycortisol and 11-deoxycorticosterone with no evidence for cortisol or corticosterone in the plasma. In retrospect, the difference between the previous identification studies and our study can likely be attributed to technological advances that provide more sensitive and precise HPLC and MS analyses. The absence of cortisol and corticosterone in sea lampreys suggests that the enzyme CYP11B1 may not have been present early in vertebrate evolution. Our search of the lamprey genome database for CYP11B1 orthologs was unsuccessful. However, we confirmed the presence of a CYP 21 ortholog—necessary to produce 11-deoxycorticosterone and 11-deoxycortisol—with 55% sequence identity to the zebra fish CYP21 (XP_001919231).

Our characterization of the lamprey CR revealed high specificity and affinity for only one corticosteroid, 11-deoxycortisol. The low affinity of aldosterone, 11-deoxycorticosterone, corticosterone, and cortisol shown in our study contradicts a previous study that showed promiscuous activation by these corticosteroids in a reporter gene assay (6). In the previous study, a luciferase reporter gene with GAL4-DBD and the LBD of lamprey CR was used to measure both transcriptional and ligand-binding activities of the receptor (6). Such promiscuous activation may be an artifact because of the absence of the whole receptor, lack of lamprey-specific chaperone proteins, or may be a result of assay conditions such as incubation temperature and/or the use of heterologous cell lines. The affinity and specificity of a steroid receptor for a given ligand is critically determined by the hsp-90 complex, which chaperones the unliganded receptor while maintaining an open binding conformation (36, 37). In addition, recent studies have shown that regions outside the LBD of an MR affects ligand binding selectivity (38). Although it is possible that more than one CR exists in lampreys and could explain differences among studies, this seems unlikely. PC-R-based cloning found a single CR from liver (6) and gill tissue (39), and there is only one detectable gene with high homology to CR from the lamprey genome database (39), which was also found to be expressed in the lamprey gill tissue (39). Furthermore, identification and characterization of a single population of binding sites in our study indicates that we have characterized the only CR present. The data that we derived with our classical approach indicate that the native CR in lamprey gill cytosol is highly specific for 11-deoxycortisol, and thus a corticosteroid hormone signaling pathway with a highly specific binding capability likely evolved in ancestral vertebrates.
In response to a stressful stimulus, animals elicit a stress response that includes the release of GCs and other hormones from the adrenal glands—in fish from interrenal cells—to maintain homeostatic conditions. In the sea lamprey, we demonstrated that 11-deoxycortisol is part of the acute stress response, as evidenced by a 2-fold increase of 11-deoxycorticosterone concentrations in response to stressors. In jawed vertebrates, the GC response is largely regulated by the HPI/hypothalamic–pituitary–adrenal (HPA) axis, represented by the secretion of the hypothalamic hormone CRH and the pituitary hormone ACTH. A previous study has shown CRH to be highly conserved among jawed vertebrates (40). We conducted a lamprey genome database search and found a single CRH sequence with 88% identity to human CRH. Thus, studies using human CRH and lamprey pituitary extract demonstrated that 11-deoxycortisol is regulated by the HPI axis, consistent with HPI/HPA axis regulation of the GCs cortisol and corticosterone in jawed vertebrates.

GCs are known to suppress the sex steroid biosynthetic pathway, thus affecting reproductive functions in vertebrates. The effect of GCs on circulating sex steroids can be mediated at several levels of the hypothalamus–pituitary–gonadal axis, including up-regulation of gonadotropin inhibitory hormone (41) and down-regulation of steroidogenic enzymes (42). In the rat, acute stress disrupts the steroid biosynthetic pathway in testes by inhibiting 17α-hydroxylase and 17, 20 lyase (42). Consistent with these results, our findings demonstrated that 11-deoxycortisol implants reduced plasma concentrations of sex steroids. The biosynthetic pathway for production of sex steroids requires CYP17 (17α-hydroxylase/17, 20 lyase) activity for conversion to C19 steroids. In addition, we found that 11-deoxycorticoesterone levels also increased in response to 11-deoxycortisol implants, likely because of inhibition of 17α-hydroxylase activity that effectively blocked conversion of 11-deoxycorticoesterone to 11-deoxycortisol. More work is necessary, however, to determine the precise mechanisms by which 11-deoxycortisol regulates reproductive functions in the lamprey.

In a phylogenetic analysis of CRs, Stolte et al. (7) grouped lamprey CR with other vertebrate MRs, suggesting that the MR gene is ancestral to the GR gene. Using a heterologous expression system, Bridgham et al. (6) showed that the lamprey CR was activated by several chemically related corticosteroids, which is similar to MR binding characteristics in recent vertebrates. It was further suggested that the consanguineous hormone retained by the MR after the genome duplication between jawless and jawed vertebrates, whereas the specificity of the duplicated GR was a derived phenotype (6). In contrast, the highly specific binding of the lamprey CR for 11-deoxycortisol demonstrated in our study indicates that the promiscuity of later vertebrate MRs is a derived trait. Furthermore, we postulate that the highly specific binding characteristic of the ancestral CR was retained by the GR in later vertebrates. Therefore, it is likely that the evolution of promiscuous binding of the MR played an important role in the eventual divergence of ancestral corticosteroid functions in later vertebrates.

Taken together, our experiments support a model in which 11-deoxycortisol and its CR functioned as both an MC and a GC in the lamprey. Following the genome duplication event between jawless and jawed vertebrates (6), the ancestral CR functions were subfunctionalized between the MR and GR in later vertebrates (Fig. 4E). Thus, the duplicated MR retained part of the ancestral function of regulating ion homeostasis, whereas the duplicated GR retained the GC function regulated by the HPI/HPA axis. The regulation of GC and MC functions by a single corticosteroid hormone thus appears to be the early vertebrate condition, which is consistent with a single corticosteroid hormone having dual function in teleosts (43). Although the timing of the partitioning is unknown, the divergence of these functions by MR and GR may have occurred in the terrestrial vertebrates with the arrival of CYP11B2, necessary for the biosynthesis of aldosterone as an MC (44). Our findings strongly indicate that subfunctionalization occurred during the evolution of the corticosteroid signaling system, thus leading to increased complexity.

It seems likely that complexity in recent corticosteroid signaling systems occurred in a Darwinian stepwise process through co-evolution of CYP enzymes and receptor proteins. Point mutations in the ligand binding pocket, coupled with the arrival of new corticosteroids produced by steroidogenic enzymes, likely occurred after the genome duplication between jawless and jawed vertebrates. This idea is supported by the evolution of MR and GR from ancestral CR (6), along with corticosterone and possibly cortisol production by CYP11B1 in elasmobranchs (45). A recent study has shown that steroidogenic CYPs were crucial in the evolution of lipophilic molecules through the mechanism of hydroxylation (46). In the basal vertebrates, the addition of an 11-position hydroxyl (CYP11B1) to 11-deoxycortisol may have arisen as a necessity to clear excess hormone, which then led to the production of cortisol and corticosterone as deactivated metabolites. These compounds would then be available to interact with the duplicated CRs, eventually leading to cortisol/corticosterone–GR coupling, along with aldosterone–MR coupling, after CYP11B2 and 11β-HSD2 arose in later vertebrates (44) (Fig. 4E).

Our studies, which combined chemical identification of a corticosteroid, biochemical characterization of its cognate receptor, and establishment of biological actions, demonstrate that 11-deoxycortisol is a functional corticosteroid hormone in the closest living relative of the earliest vertebrate, the sea lamprey. In addition, the dual roles of the lamprey corticosterone as an MC and a GC indicates that the corticosteroid signaling system evolved through the mechanism of subfunctionalization. We hypothesize that 11-deoxycortisol and its highly specific receptor represents the primitive condition of vertebrates, and our findings contribute to a more complete understanding of the evolution of corticosteroid signaling.

Materials and Methods

Experimental Subjects. Adult lampreys were acclimated in flow-through tanks (254 L) at 10–13 °C for at least 2 wk before stress tests. For the CRH experiment, saline-injected (n = 8) controls and CRH-injected (n = 8) lampreys were used. For the pituitary extract study, saline-injected (n = 9) or extract-injected (n = 9–10) lampreys were used. For the acute stress study (1 and 24 h), parasitic lampreys were stressed (n = 40) and bled after being anesthetized. Controls (n = 40) were left in tanks undisturbed until bleeding. For the second acute stress study (1–48 h), lampreys were stressed (n = 70) and controls (n = 70) were left undisturbed until bleeding. All animal care and procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Acute Stress Treatment. Lampreys were stressed by netting them out of tanks, placing them in a dry bucket for 5 min, and then transferring them to 3% saline-injected (n = 40) controls and CRH-injected (n = 8) lampreys before returning them to a freshwater tank.

Plasma Steroid Sampling. In all in vivo experiments, blood was collected by cardiac puncture. Blood samples were centrifuged at 1,000 × g for 15 min and plasma was stored at −80 °C until assayed. Corticosteroids and sex steroids were measured by using RIAs.

Chromatography and MS. Plasma was extracted (solid phase extraction) and dissolved in 1 mL acetonitrile/water/trifluoroacetic acid (28/72/0.01, vol/vol/vol) and loaded onto a C18 HPLC column (Nova-Pak, 3.9 mm × 300 mm; Waters). Two solvents were used to deliver a gradient to the column. Fractions were collected at 1-min intervals between 11 and 70 min. LH-20 chromatography was performed on a glass column containing Sephadex LH-20 (Amersham) resin by using a mixture of dichloromethane and methanol (98:2, vol/vol) as the eluting solution. Fractions from each chromatography step were screened by RIAs. Fractions were dried down under reduced pressure and then subjected to atmospheric pressure chemical ionization MS analysis. Mass spectra were obtained with an LCQ-Deca ion trap (Thermo Scientific). Samples were compared against authentic 11-deoxycortisol and 11-deoxycorticosterone standards (Sigma-Aldrich).

Characterization of CR. Tissues collected from fish were frozen in liquid nitrogen and held at −80 °C until processed for cytosolic fractions. To characterize the CR, we performed receptor binding assays with [3H] 11-
deoxycortic. The concentration of binding sites ($B_{max}$) and the dissociation constant ($K_d$) were determined by hyperbolic regression using Sigmaplot 10.0 (SYSTAT). For DNA-cellulose chromatography, gill cytosol (1.0 mL) was incubated for 2 h at 0 °C with 20 nM [3H] 11-deoxycortisol with or without 1 μg cold 11-deoxycortisol. The sample was allowed to flow into the DNA cellulose (Amersham), and then the flow was stopped to allow absorption for 20 min. 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).

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. Gill tissue was homogenized in 5 mL resulting SI SEI buffer (2.0% deoxycholic acid) and centrifuged at 5,000 × g for 30 s. The resulting ouabain-sensitive ATPase activity was expressed as micromoles ADP/mg protein per hour. Protein concentrations were determined by using BCA (bicinchoninic acid) Protein Assay (Pierce). Additional details are provided in SI Materials and Methods.

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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 for 15 min. The plasma was removed and stored at −80 °C. Tissues collected from fish were frozen in liquid nitrogen and held at −13 °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 absorbance 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 reanalyzed 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 LCO-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 (Bmax) and the dissociation constant (Kd) 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 [3H] 11-deoxycortisol with or without 1 μg of 11-deoxycortisol at 0 °C for 4 h. To determine the dissociation rate, we incubated gill cytosol (200 μL) with 3.3 nM of [3H] 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 [3H] 11-deoxycorticisol binding. Gill cytosol preparations (200 μL) were incubated at 0 °C for 2 h with 3.3 nM [3H] 11-deoxycorticisol in the presence of different amounts of cold steroid (0.1–1,000 nM). Specificity was examined for 11-deoxycorticisol, 11-deoxycorticoctosterone, 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-deoxycorticisol (5 nM) in ethanol was added to each tube in the presence or absence of 1 μg cold 11-deoxycorticisol 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 [3H] 11-deoxycorticisol with or without 1 μg cold 11-deoxycorticisol. 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-deoxycorticisol.

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-deoxycorticisol.

Handling and Salinity Stressors. Adult lampreys were acclimatized 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-Deoxycorticisol (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-deoxycorticisol, 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.


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 [3H] 11-deoxycortisol (5 nM) to corticosteroid receptors. Bₘ 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.