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Programming Neuroendocrine Stress Axis Activity by Exposure to Glucocorticoids during Postembryonic Development of the Frog, *Xenopus laevis*

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Exposure to elevated glucocorticoids during early mammalian development can have profound, long-term consequences for health and disease. However, it is not known whether such actions occur in nonmammalian species, and if they do, whether the molecular physiological mechanisms are evolutionarily conserved. We investigated the effects of dietary restriction, which elevates endogenous corticosterone (CORT), or exposure to exogenous CORT added to the aquarium water of *Xenopus laevis* tadpoles on later-life measures of growth, feeding behavior, and neuroendocrine stress axis activity. Dietary restriction of prometamorphic tadpoles reduced body size at metamorphosis, but juvenile frogs increased food intake, showed catch-up growth through 21 d after metamorphosis, and had elevated whole-body CORT content compared with controls. Dietary restriction causes increased CORT in tadpoles, so to mimic this increase, we treated tadpoles with 100 nM CORT or vehicle for 5 or 10 d and then reared juvenile frogs to 2 months after metamorphosis. Treatment with CORT decreased body weight at metamorphosis, but juvenile frogs showed catch-up growth and had elevated basal plasma (CORT). Immunohistochemical analysis showed that CORT exposure as a tadpole led to decreased glucocorticoid receptor immunoreactivity in brain regions involved with stress axis regulation and in the anterior pituitary gland of juvenile frogs. The elevated CORT in juvenile frogs, which could result from decreased negative feedback owing to down-regulation of glucocorticoid receptor, may drive the hyperphagic response. Taken together, our findings suggest that long-term, stable phenotypic changes in response to elevated glucocorticoids early in life are an ancient and conserved feature of the vertebrate lineage. (*Endocrinology* 149: 5470–5481, 2008)

THERE IS GROWING evidence from both epidemiological studies and work with experimental animal models that exposure to environmental stressors during postembryonic development leads to intrauterine growth retardation and low birth weight (1, 2), with subsequent profound and persistent effects on later life phenotypic expression (3–7). For example, low birth weight and premature birth are correlated with increased risk of cardiovascular and metabolic diseases expressed later in life (1–4, 7). These studies suggest that precocious or prolonged activation of the neuroendocrine stress axis during fetal or early postnatal life in mammals may be the causal link between exposure to environmental stressors and the expression of adult disease (1, 3).

The hypothalamo-pituitary-adrenal (HPA) axis [hypothalamo-pituitary-interrenal (HPI) axis in amphibians] mediates the release of glucocorticoids (GCs) in response to diurnal cues and stressors (8). Activity of the HPA/HPI axis is regulated by negative feedback by GCs, which inhibit the biosynthesis and release of hypothalamic corticotropin-releasing factor (CRF) and pituitary ACTH, thus terminating the stress response (8–10). GC actions are mediated by glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), both of which reside within the cell and function as ligand-activated transcription factors. There is also evidence for membrane-associated receptors that mediate rapid GC-negative feedback, among other actions (11). Negative feedback in the HPA/HPI axis occurs through GCs acting directly on anterior pituitary corticotropes and hypophysiotropic CRF neurons [located in the paraventricular nucleus (PVN) in mammals or the anterior preoptic area (POA) in frogs] or indirectly via descending pathways from the hippocampus (homologous to the amphibian medium pallium) and the amygdala and bed nucleus of the stria terminalis (BNST) (5, 10, 12–14). The GR is expressed in each of these brain structures in mammals and in the frog (15, 16), suggesting that GC-negative feedback on the HPA axis is an evolutionarily conserved physiological mechanism involved in recovery from stress axis activation.

In mammals, the functioning of the HPA axis can be permanently altered by early-life events that activate the HPA axis or by exposure to exogenous GCs (5, 6, 17–19). Studies in rodents have shown that maternal malnutrition, exposure to stressors or exogenous GCs during prenatal or neonatal life can permanently alter the basal or stimulated activity of the HPA axis of offspring (5, 14, 20, 21). Prenatal or neonatal exposure to stressors typically causes hyperactivity of the

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Abbreviations: BNST, Bed nucleus of the stria terminalis; CORT, corticosterone; CRF, corticotropin-releasing factor; GC, glucocorticoid; HPA, hypothalamo-pituitary-adrenal; HPI, hypothalamo-pituitary-interrenal; IHC, immunohistochemistry; ir, immunoreactivity; LSD, least significant differences; MeA, medial amygdala; mp, medial pallium; NF, Nieuwkoop and Faber; POA, preoptic area; PVN, paraventricular nucleus.

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HPA axis, including elevated basal expression of hypothalamic CRF, elevated basal plasma GC concentration (21, 22), magnified or prolonged responses of CRF and cortisol to acute stressors (23), and increased food intake associated with higher probabilities of obesity and metabolic dysfunction (24, 25).

Rodent studies suggest that prenatal or neonatal exposure to stressors leads to altered HPA activity through modified GC-negative feedback at the level of the hippocampus, hypothalamus, and anterior pituitary gland (12, 15, 17, 26). Exposure to exogenous GCs during prenatal life results in increased numbers of CRF neurons in the rodent PVN and reduced GR expression in the hippocampus (5, 27). Natural elevations in plasma GC concentration caused by exposure to prenatal or neonatal stressors produces similar effects on CRF and GR expression as that observed following exposure to exogenous GCs (17, 19, 28, 29).

Despite the potential for profound fitness consequences of early-life exposure to stress hormones on later life phenotypic expression, virtually nothing is known about such actions and their consequences in nonmammalian species. In amphibians, the effects of environmental stressors on tadpole growth and development parallel those of intrauterine stressors on fetal growth and development in mammals. Like the mammalian fetus, development of the amphibian tadpole is strongly influenced by environmental conditions (for reviews see Refs. 30–32). For example, food availability, habitat desiccation, conspecific density, and presence of predators all have significant effects on juvenile morphology, growth, and survival, and all are known to elevate GCs (33–42). These findings suggest that the consequences of early-life exposure to GCs, and the mechanisms of GC action on development may be evolutionarily conserved.

In the present study, we investigated whether dietary restriction, or corticosterone (CORT) added to the aquarium water of prometamorphic tadpoles leads to long-term effects on juvenile growth, feeding, and HPI axis activity in the South African clawed frog, *Xenopus laevis*. We subjected prometamorphic tadpoles to dietary restriction and then measured growth, food intake, and whole-body CORT content 21 d after metamorphosis (juvenile frog stage). Dietary restriction and other environmental stressors cause elevations in CORT in tadpoles (31, 35, 39–41, 43). To test whether CORT mediates effects of dietary restriction on growth and endocrine function, we treated early prometamorphic tadpoles for 5 or 10 d with a dose of CORT that caused elevated whole-body CORT within the physiological range. We reared animals through 2 months after metamorphosis, monitored growth rate during this time, and then measured basal and stressor-induced plasma (CORT). We also analyzed changes in GR immunoreactivity (ir) by immunohistochemistry (IHC) in discrete regions of the frog brain that are known or hypothesized to be involved in the regulation of the HPI axis and in the anterior pituitary gland.

Materials and Methods

Animal husbandry

Premetamorphic *X. laevis* tadpoles were purchased from Xenopus I (Dexter, MI) or generated by in-house breeding by injecting sexually mature male and female *X. laevis* with human chorionic gonadotropin (Sigma-Aldrich Chemical, St. Louis, MO) dissolved in 0.9% NaCl via the dorsal lymph sac. Tadpoles were maintained in dechlorinated tap water with aeration on a 12-h light,12-h dark regimen at 20 ± 2 C and fed Frog Brittle powder (Nasco, Fort Atkinson, WI). Postmetamorphic frogs were reared as described below. All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan.

Dietary restriction

We housed tadpoles in 30 × 20 × 20 cm aquaria containing 10 liters water (eight tadpoles/tank) and fed them once a day from Nieuwkoop and Faber (NF) (44) developmental stages 43–44 (just after tadpoles start to feed independently) until NF 56–57. At this stage tadpoles were subjected to one of three feeding regimens: fed once a day (control: one time per day), fed twice a day (increased food: two times per day), or fed once every other day (reduced food: one time per 2 d). Body weight at metamorphosis (NF stage 66) was measured, and frogs were raised individually in 2-liter tanks through 21 d after metamorphosis. Daily food intake (grams food per gram body weight per day) was measured by weighing about 20 mg of calf liver pieces and placing them into the tank; after 1 h the remaining liver pieces were removed and weighed. After 21 d, frogs were anesthetized by submersion in 0.01% benzocaine solution, weighed, and snap frozen for whole-body CORT analysis (animals were too small to collect plasma for RIA). The size specific, or instantaneous growth rate [$\Delta \ln(\text{body weight})/\Delta t$], was analyzed as described by Kaufmann (45) and Sinervo and Adolph (46). Sample sizes ranged from six to eight for growth parameters and CORT RIA, and five to eight for food intake measures.

Corticosterone treatment of tadpoles

Prometamorphic tadpoles at NF stages 52–54 were housed in 30 × 20 × 20 cm aquaria containing 10 liters water (30 tadpoles/tank) and fed daily. We noninvasively manipulated plasma CORT concentrations in tadpoles by adding the hormone to the rearing water to a final concentration of 100 nM. We dissolved CORT in 100% EtOH, and controls received EtOH vehicle; the final concentration of EtOH in control and CORT treatments was 0.00025%. We treated tadpoles with CORT for 5 or 10 d and changed the aquarium water and added fresh CORT daily over the treatment period. After the CORT treatment periods ended, tadpoles were transferred to fresh aquaria and raised in dechlorinated tap water until metamorphosis. The experiment was repeated for the 5 d CORT treatment.

Over the course of the experiment, tadpole development and growth were measured weekly. Twenty tadpoles from each group were individually weighed, and whole-body length, hind-limb length, and snout-vent length were measured with a caliper. At metamorphosis (NF stage 66), frogs were weighed and transferred to 30 × 10 × 20 cm aquaria containing 3 liters of dechlorinated tap water and fed juvenile frog brittle daily. Juvenile frogs were housed four to five per aquarium, with five aquaria per treatment group. The aquaria were shielded throughout to minimize disturbance. Approximately 2 months after metamorphosis, a subset of juvenile frogs from each treatment was subjected to shaking/confinement stressor (39, 47) for 2 h before the animals were killed and tissue collection. We chose to rear frogs to 2 months after metamorphosis so that they would be large enough to collect plasma for circulating CORT measurements (see below).

Shaking/confinement stressor

We determined the effects of CORT treatment as a tadpole on subsequent basal and stressor-induced activity of the HPI axis in frogs at 2 months after metamorphosis. Half of the frogs from each treatment group (vehicle or CORT, 5 or 10 d exposure) were subjected to shaking/confinement stressor as described previously (39, 47); the other half were left undisturbed until the animals were killed. Briefly, two to three frogs were placed into 32-oz white polypropylene containers containing 100 ml water. The containers were placed on an orbital shaker and shaken continuously at 120 rpm for 2 h. The shaking intensity was sufficient to require constant spatial adjustment by the frogs but not enough to cause physical damage (39, 47). All frogs were rapidly killed by decapitation, and blood was collected into heparinized capillary tubes and plasma

separated for CORT RIA (RIA; see below). The brains were removed and fixed in 4% paraformaldehyde for immunohistochemistry (described below).

Measurement of whole-body and plasma CORT

Juvenile frogs (21 d old) or tadpoles were extracted for whole-body CORT analysis following the methods of Denver (35) with modifications described by Glennemeier and Denver (39). Briefly, animals were homogenized in ethyl acetate, and a trace amount of [H^3]CORT was added to each homogenate (and to plasma; see below) for later estimation of recoveries. This tracer did not interfere with the potency estimates in the RIA because it represented only 2–3% of the total counts added to the assay tubes. Individual frogs were analyzed, but for tadpoles, three animals were pooled per replicate (n = 4/treatment). The CORT was separated from the lipid fraction by thin-layer chromatography, and the region of the thin-layer chromatography lane containing the CORT (as determined by calibration with both radiolabeled and radioinert CORT) was scraped and the silica extracted with diethyl ether. The extract was dried under nitrogen and then reconstituted in PBS-gelatin [0.2 M PBS with 1% gelatin (pH 7.4)] for determination of recovery and for RIA. Plasma collected from juvenile frogs was extracted once with 5 ml diethyl ether and the extract dried under nitrogen and reconstituted with 500 μl PBS-gelatin buffer. We measured whole-body CORT content or plasma (CORT) by RIA as described (48). We purchased the CORT antiserum from Esoterix (Calabasas Hills, CA). Intra- and interassay coefficients of variation were less than 10 and 12%, respectively.

IHC

We used IHC to analyze changes in GR-ir in discrete regions of the frog brain as described previously (16) with minor modifications. We fixed brains in 4% paraformaldehyde overnight at 4 C and then submerged them in 30% sucrose before snap freezing and preparing 10-μm transverse cryosections. We immersed the slides in heated 0.01 M citric acid (pH 6.0) for 5 min for antigen retrieval and then blocked with Tris Superblock blocking buffer (Pierce Chemical Co., Rockford, IL) to which normal goat serum (Sigma) was added to a final concentration of 5%. For IHC, we used an affinity-purified polyclonal antiserum that we generated in a rabbit to a synthetic peptide corresponding to a unique region of the *X. laevis* GR (0.5 μg/ml IgG) (16). To detect immune complexes, we used the Vectastain elite ABC (rabbit) and Vector VIP kits (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions. We captured micrographic images using an IX81 inverted microscope (Olympus, Tokyo, Japan) and a Retiga 1300R Fast digital video camera (QImaging, Tucson, AZ). We adjusted brightness, contrast, and evenness of illumination uniformly for images shown in the figures using Photoshop CS2 (Adobe, San Jose, CA). The images used for morphometric analysis (see below) were not adjusted.

Morphometric analysis of GR-ir

We quantified GR-ir in discrete regions of the frog brain using MetaMorph software (version 6. 2r4; Universal Imaging Corp., Downingtown, PA) following the methods described by Yao *et al.* (47). All samples were processed simultaneously under identical conditions. We analyzed three to five transverse sections from each animal that contained the anterior POA, medial pallium (mp), medial amygdala (MeA), BNST, and anterior pituitary following the anatomical definitions of Tuinof *et al.* (49) and Marín *et al.* (50). These brain regions were selected because of their robust GR-ir, suggesting that they are important targets for GC actions, and their known roles in the regulation of the HPA axis (10, 16). All sections were carefully matched for anatomical level, and digital images were captured at ×100 magnification for morphometric analysis. Image analysis was conducted in a blinded manner. We isolated brain regions on the captured images using a hand-made frame that covered the area of interest. Each selected brain region analyzed on adjacent sections and from different brains was roughly equivalent in total area, but because each sample differed slightly in shape, we hand drew boxes around each region of interest. Using the autothreshold for dark objects tool in the MetaMorph software, we adjusted the threshold to eliminate background staining. This was repeated on five to eight sections, and a mean threshold was established that was then set for analysis of all sections. The signal density within a selected area was then counted automatically. The signal density was divided by the total area of the selected brain region to obtain a mean signal density, which allowed for correction for size differences between brains and between adjacent sections. The average of the mean densities in a given brain region on replicate brain sections was calculated, summed for all animals in the treatment, and divided by the number of animals in the treatment to obtain the average signal density for each brain region (16, 47).

Statistical analysis

All continuous variables (days to metamorphosis, body weight and size, plasma CORT, and GR-ir mean signal density) were examined for homogeneity of variance using Bartlett's test. Data with heterogeneous variances were first $\log_{10}$ transformed before parametric analyses (Student's *t* test or one-way ANOVA followed Fisher's least significant differences (LSD) multiple comparisons; $P < 0.05$). The interaction of shaking/confinement stress and CORT treatment was analyzed by two-way ANOVA using stressor and treatment as independent factors. All statistical analyses were conducted using the SYSTAT version 10 software (SPSS Inc., Chicago, IL). Data are reported as mean ± SEM.

Results

Food restriction during the prometamorphic tadpole stage influences postmetamorphic growth and HPI activity

Tadpoles fed every other day (food restricted) had reduced body weight at metamorphosis compared with tadpoles fed once a day (controls) or tadpoles fed twice a day (extra food) from NF stage 56–57 to the completion of metamorphosis (NF stage 66; $F_{(2, 19)} = 14.523$, $P < 0.0001$, ANOVA; Fig. 1A). After metamorphosis, juvenile frogs that were food restricted as tadpoles exhibited catch-up growth and were not different from controls at 21 d after metamorphosis, but they remained smaller than frogs that had received extra food as tadpoles ($F_{(2,19)} = 8.292$, $P = 0.0026$; Fig. 1B). The food-restricted animals exhibited significantly greater food intake ($F_{(2,18)} = 7.382$, $P = 0.0046$; Fig. 1C), size-specific growth rates ($F_{(2,15)} = 11.299$, $P = 0.001$; Fig. 1D), and whole-body CORT content ($F_{(2,15)} = 5.538$, $P = 0.0158$; Fig. 2) compared with the other two treatments.

Corticosterone treatment of early prometamorphic tadpoles elevates whole-body CORT content within the physiological range

Treatment of tadpoles with 100 nM CORT for 5 d increased whole-body CORT content by approximately 3-fold ($P = 0.0008$ *vs.* control; 897.66 ± 213 pg/mg body weight for control and 2914.09 ± 239 pg/mg body weight for 5 d CORT, n = 4/treatment; Student's *t* test). These results are consistent with our prior studies that showed that 100 or 125 nM CORT caused a 2- to 3-fold increase in whole-body CORT content (41, 51), which is within the physiological range of CORT levels achieved in tadpoles after exposure to shaking/confinement stressor (39). We observed similar elevations in whole-body CORT content when tadpoles were exposed for 5 d or more to simulated pond drying (31, 35), intraspecific competition (increased tadpole density or decreased food availability) (36, 40, 52), or predators (Middelemis-Maher, J., F. Hu, and R. J. Denver, unpublished results). We treated tadpoles in early prometamorphosis (*X. laevis*: NF stage 52–54) because at this stage of development, tadpoles are capable of mounting a robust and sustained HPI response to a stressor (39). Also, the tadpole brain undergoes dramatic changes in

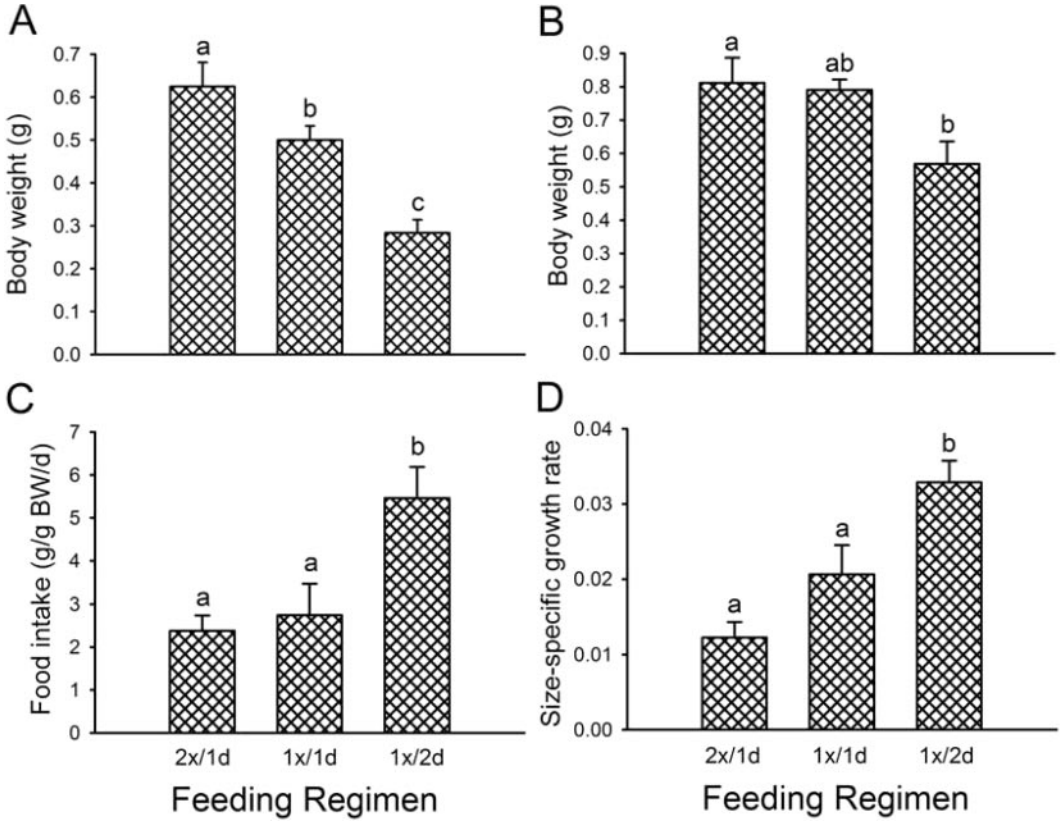

FIG. 1. Dietary restriction of prometamorphic tadpoles decreases body weight (BW) at metamorphosis, but postmetamorphic frogs eat more and reach a similar body size to controls (*i.e.* they show catch-up growth). Tadpoles were fed once a day from NF stage 43–44 until NF stage 56–57 (prometamorphosis). At this stage tadpoles were subjected to one of three feeding regimens until metamorphosis: fed once a day (control: 1×/1d), fed twice a day (increased food: 2×/1d), or fed once every other day (reduced food: 1×/2d). After metamorphosis juvenile frogs were raised through 21 d before they were killed (see *Materials and Methods*). Body weights at metamorphosis (NF stage 66) (A) or 21 days after metamorphosis (B) were determined. Daily food intake was measured (C), and size-specific growth rates ($\Delta\ln(BW)/\Delta t$) were calculated (D). *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 6–8/treatment).

gene expression and morphology during this period of development, which may be influenced by GCs (52–55).

Corticosterone treatment decreases size at metamorphosis, but juvenile frogs show catch-up growth

Tadpoles treated with CORT for 5 or 10 d took significantly longer to reach metamorphic climax ($F_{(2,73)} = 6.649$, $P = 0.002$, ANOVA; Fig. 3A) and exhibited lower body weights at metamorphosis ($F_{(2,73)} = 7.49$, $P = 0.001$); Fig. 3B) compared with controls. However, 2 months after metamorphosis, there were no significant differences in body weight among the treatments (Fig. 3C), *i.e.* the smaller animals showed catch-up growth. Juveniles that had been treated with CORT as tadpoles exhibited a greater magnitude increase in mean body weight than that of controls (13.2- and 12.4-fold increases in 5 and 10 d CORT group, respectively, *vs.* 11.0-fold increase in controls). In this experiment we did not raise frogs individually, and thus, we were unable to calculate size-specific growth rates.

Corticosterone treatment during the tadpole stage leads to increased basal plasma (CORT) in juvenile frogs

The basal (unstressed) plasma (CORT) of 2-month postmetamorphic juvenile frogs was significantly greater in animals that had been treated with CORT for 10 d ($F_{(2,21)} = 5.43$, $P = 0.013$; ANOVA; n = 5–10/treatment) compared with the control group (Fig. 4A). There was a trend toward increased basal plasma (CORT) in the 5-d-treatment group, but this was not statistically significant, perhaps owing to the small sample size in this group (n = 5). There was no significant difference in the mean stressor-induced plasma (CORT) among the treatments (Fig. 4B). However, whereas the stressor caused significant increases in plasma (CORT) in the control ($P = 0.005$; Student's *t* test) and 5-d CORT treatments ($P = 0.012$), it did not in the 10-d CORT treatment owing to the already high basal plasma (CORT) in this group (Fig. 4C). The magnitude change in plasma (CORT) after exposure to the stressor was lower in the CORT-treated groups compared with controls (5.42-, 3.35-, and 1.53-fold for control, 5-d CORT, and 10-d CORT, respectively; Fig. 4C).

Corticosterone treatment during the tadpole stage led to decreased GR-ir in discrete regions of the frog brain and pituitary gland

Treatment of tadpoles with CORT for 5 or 10 d caused significant decreases in mean GR-ir signal densities in the POA of juvenile frogs ($F_{(3,16)} = 5.05$, $P = 0.002$ for 5d;

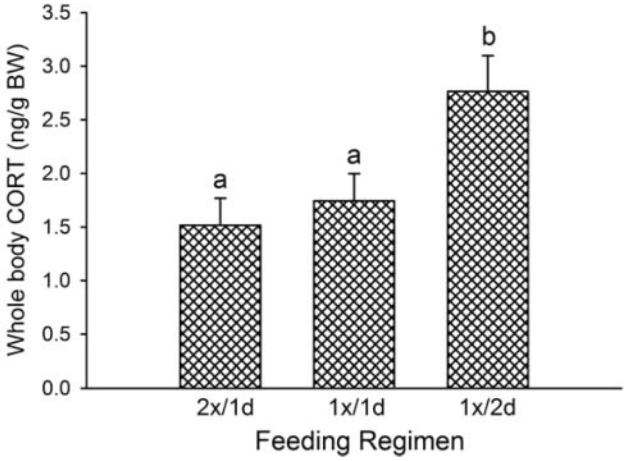

FIG. 2. Dietary restriction of prometamorphic tadpoles leads to elevated whole body CORT content in 21-d postmetamorphic frogs. Tadpoles were fed, and frogs were reared as described in the legend of Fig. 1 and *Materials and Methods*. At 21 d after metamorphosis, frogs were euthanized and whole-body CORT determined. *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 5–8/treatment).

$F_{(3,16)} = 11.16$, $P = 0.001$ for 10 d; ANOVA; Fig. 5). In the MeA there was a trend toward lower GR-ir mean signal density in both CORT-treated groups (Fig. 6, *top panel*). In the BNST (Fig. 6, *middle panel*) and mp (Fig. 6, *bottom panel*), GR-ir showed a trend toward lower mean signal density in the 5-d CORT treatment and was significantly decreased in the 10-d CORT group (BNST: $F_{(3,14)} = 6.55$, $P = 0.012$; mp: $F_{(3,16)} = 4.77$, $P = 0.026$). Mean GR-ir signal density was significantly decreased in the anterior pituitary gland of frogs treated as tadpoles with CORT for 5 or 10 d ($F_{(3,16)} = 6.66$, $P = 0.003$ for 5-d; $F_{(3,15)} = 5.61$, $P = 0.008$ for 10-d; Fig. 7).

Exposure to shaking/confinement stressor for 2 h caused significant decreases in mean GR-ir signal densities relative to unhandled controls in all brain regions examined and in the pituitary gland of animals from the control group ($P < 0.05$; Figs. 5–7). However, exposure to the stressor did not decrease GR-ir in the brain or pituitary gland of animals from the 5- or 10-d CORT treatments owing to the already low GR-ir in the basal (unhandled) state (Figs. 5–7).

Discussion

Here we report that exposure to GCs during postembryonic development in a nonmammalian species causes changes in feeding behavior and the functioning of the neuroendocrine stress axis that persist into the juvenile adult stage. We found that exposure to CORT during the early prometamorphic tadpole stage led to elevated basal plasma (CORT) and decreased mean GR-ir signal density in several brain regions involved with HPI axis function and in the rostral pars distalis, the location of corticotropes in the frog (56, 57). Such changes in HPI axis activity may be associated with increased food intake and thus increased growth rate, as suggested by the catch-up growth that we observed in frogs that were food restricted or treated with CORT as tadpoles.

As reported previously (39, 40, 58–60), we found that

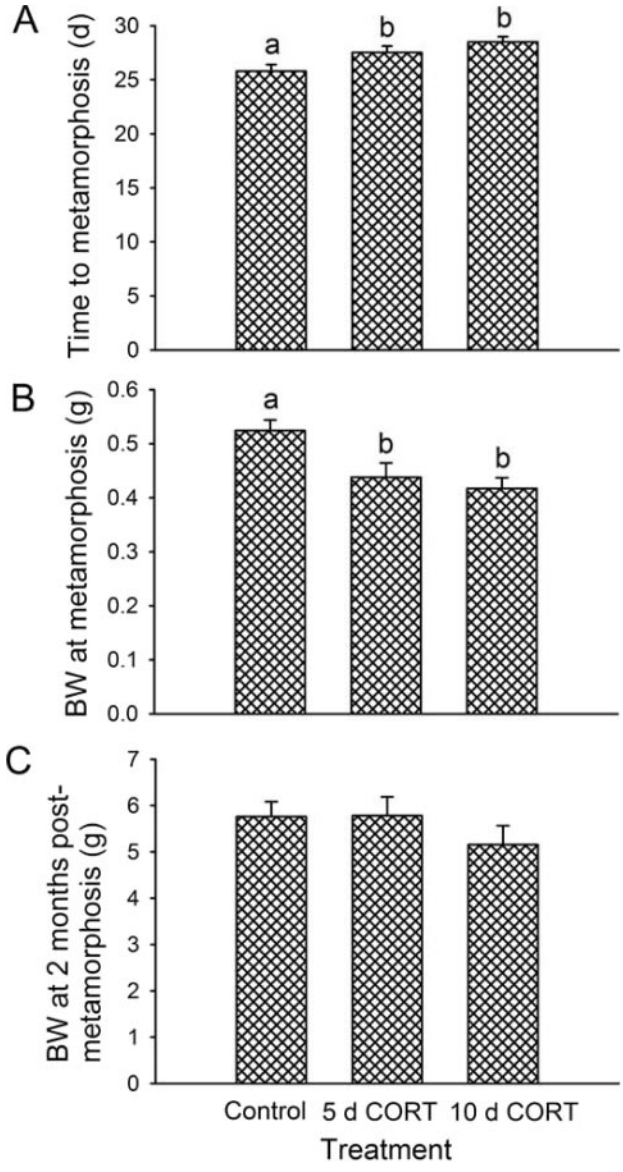

FIG. 3. Treatment of prometamorphic tadpoles with CORT slows the timing of metamorphosis (A) and decreases body weight at metamorphosis (B), but juvenile frogs achieve similar body weight 2 months after metamorphosis (C) (*i.e.* they show catch-up growth). Prometamorphic tadpoles were treated with vehicle or CORT (100 nM) for 5 or 10 d by adding the hormone or EtOH vehicle to the aquarium water (see *Materials and Methods*). *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 19–30/treatment). The experiment was repeated for the 5-d CORT treatment with similar results.

dietary restriction or CORT treatment slowed tadpole development, reduced growth, and decreased body weight at metamorphosis. It is important to note that these effects of CORT depend on the stage of development, in which CORT inhibits growth and development during pre- and early prometamorphic stages (39, 40, 58–60) but accelerates metamorphosis during mid- to late prometamorphosis (30, 37, 61). Exposure of tadpoles to a diversity of environmental stressors (*e.g.* pond drying, food restriction, high conspecific density, and predators) leads to elevations in whole-body CORT

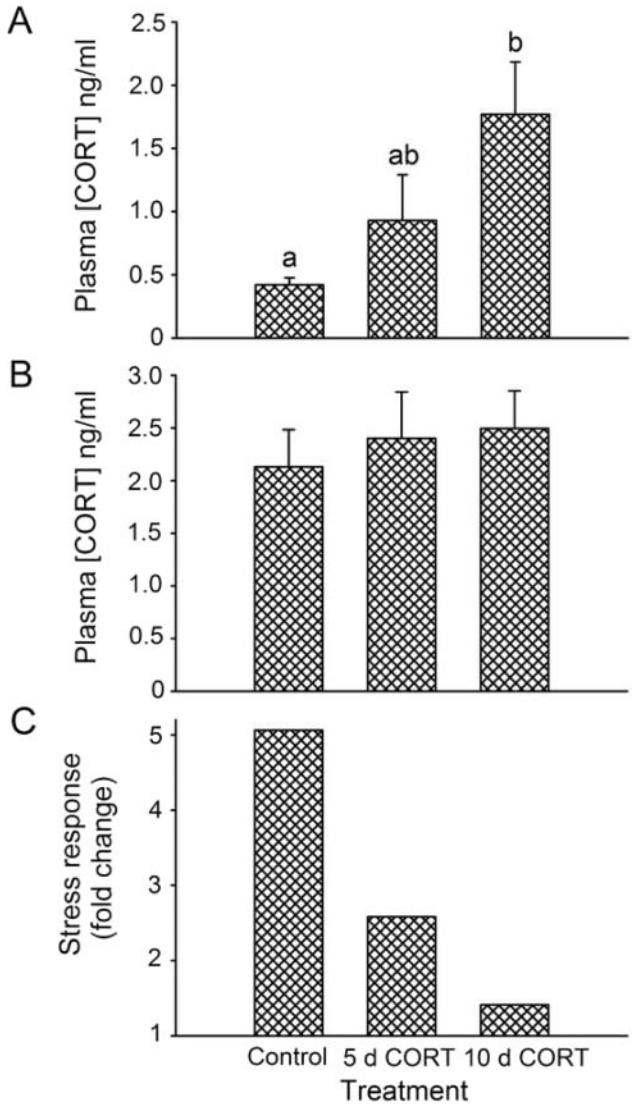

FIG. 4. Treatment of prometamorphic tadpoles with CORT leads to elevated basal HPI axis activity in 2-month-old juvenile frogs. Shown are basal (A) and stressor-induced plasma (CORT) (B and C) in 2-month-old juvenile frogs. Prometamorphic tadpoles were treated as described in the legend of Fig. 3 and *Materials and Methods*. Two-month-old juvenile frogs in each treatment were then divided into two groups and one was exposed to shaking/confinement stressor for 2 h, whereas the other group was left undisturbed (unhandled). *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 9–14/treatment).

[simulated pond drying (35); food restriction and conspecific density (40, 43); predators (Middlemis-Maher, J., unpublished data)] and decreased body weight at metamorphosis (30, 31, 43, 62).

It is well established that tadpole growth and development rates are highly plastic and that changes in these rates are considered to be an adaptive strategy for responding to natural environmental conditions (30, 63, 64). Tadpoles reared in hostile environments metamorphose at a smaller body size, and the resultant juvenile frogs are more likely to exhibit slower growth rates, inferior locomotor abilities, greater susceptibility to starvation, and higher mortality (33, 34, 37, 38, 42). Thus, one predicts that the reduced body size at metamorphosis caused by elevated CORT during the tadpole stage would lead to physiological, immunological, and behavioral outcomes in juveniles that could reduce fitness. Similar consequences of adverse environmental conditions during embryonic and fetal development in mammals have been reported. For example, maternal malnutrition and elevated GCs caused by exposure to stressors during gestation leads to intrauterine growth retardation and low body weight at birth (1–4). This can result in permanent changes in the brain, particularly the neuroendocrine system, which contributes to disease-related changes in metabolism and behavior, leading to increased risk for obesity, cardiovascular disease, and diabetes (1, 3).

We found that although dietary restriction or CORT treatment reduced body weight at metamorphosis, the body weights of juvenile frogs did not differ from controls 2 months after metamorphosis. These results suggest that, when fed *ad libitum*, small metamorphic frogs increase their growth rate by increasing food intake and achieve a similar body size to that of animals that metamorphosed at a larger size, *i.e.* they exhibit catch-up growth. Juvenile frogs that had been dietary restricted or treated with CORT as tadpoles had elevated basal CORT levels, which could have driven the increased food intake and growth seen in these animals. Corticosteroids are known to stimulate feeding in vertebrate species (65, 66), including amphibians (43, 67). In rodents, catch-up growth and hyperphagia are observed in maternally undernourished offspring, which also have increased basal PVN CRF content and increased plasma CORT concentration (25).

Thus, our findings support the hypothesis that exposure to elevated CORT during tadpole life can affect the development of central nervous system feeding controls, perhaps leading to permanent alterations in appetite and feeding behavior in later life stages. Compensatory growth could quickly reverse any competitive disadvantage that smaller individuals have at metamorphosis (or birth). On the other hand, although an organism might appear to recover through catch-up growth, early life nutritional deficits can result in profound and permanent changes in adult physiology and behavior (68).

In addition to changes in basal stress axis activity, the rodent studies also found that prenatally stressed offspring show pronounced changes in behavior (69, 70) and stress reactivity after acute stimuli because they display significantly higher circulating plasma (ACTH and CORT) and prolonged secretion of CORT (28, 69–74). In the frog, we observed decreased reactivity of the stress axis of juvenile frogs exposed to CORT as tadpoles, as measured by the magnitude increase in plasma (CORT) after exposure to a shaking/confinement stressor for 2 h. Owing to the small size of the juvenile frogs, we could not obtain serial blood samples and thus chose only the 2-h time point for analysis. Thus, we do not know the kinetics of CORT secretion after exposure to the stressor and whether there are differences among treatments in the magnitude of the response and the time required to return to basal. It is noteworthy that, although prenatally stressed rats did not differ from controls in their

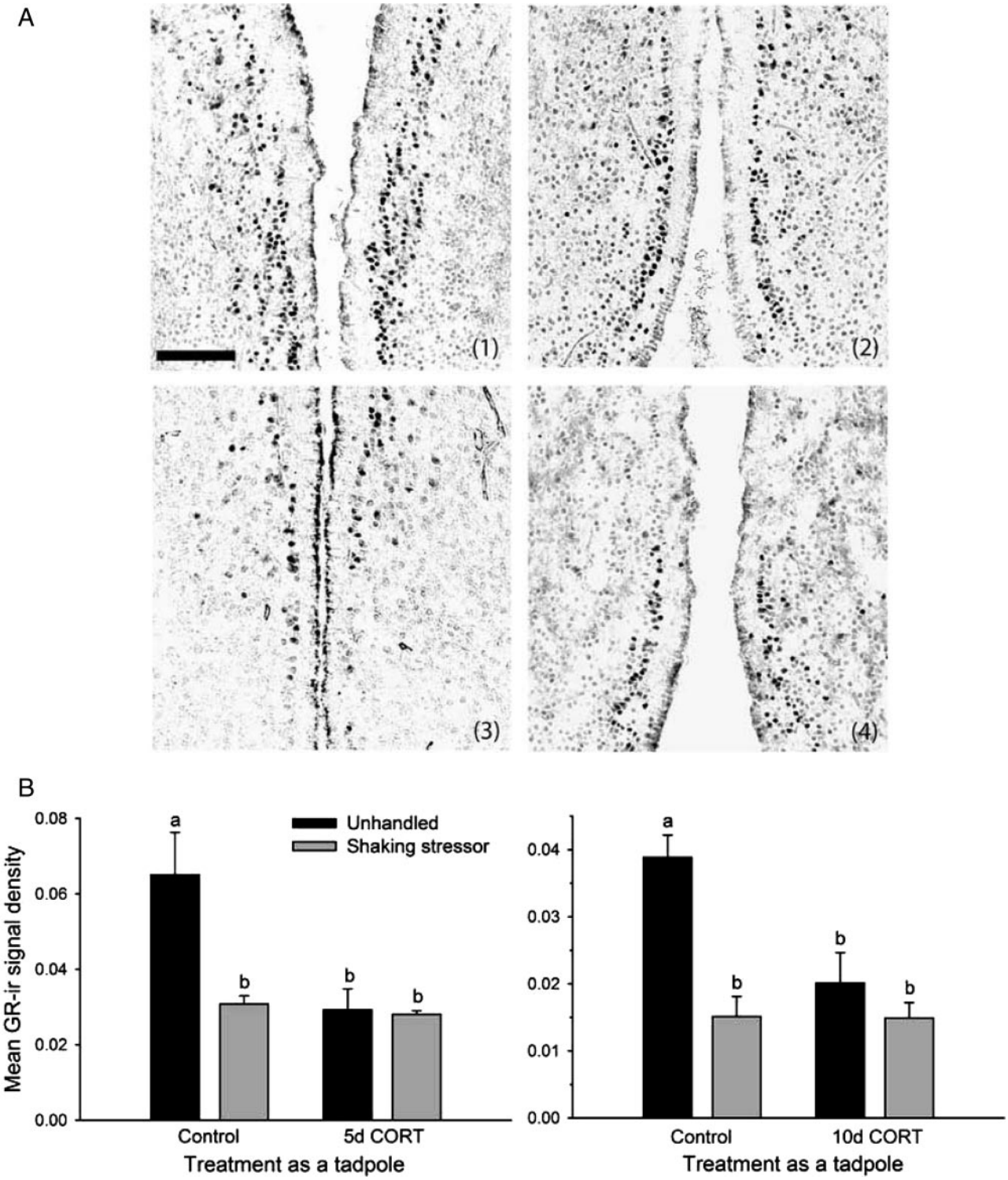

FIG. 5. Treatment of prometamorphic tadpoles with CORT leads to decreased GR-ir in the anterior POA of 2-month-old juvenile frogs. Prometamorphic tadpoles were treated as described in the legend of Fig. 3 and *Materials and Methods*. Two-month-old juvenile frogs in each treatment were then divided into two groups and one was exposed to shaking/confinement stressor for 2 h, whereas the other group was left undisturbed (unhandled). A, Photomicrographs of representative transverse sections through the POA of juvenile frogs: 1, control, unhandled; 2, control, shaking stressor; 3, 5 d CORT treatment, unhandled; 4, 5 d CORT treatment, shaking stressor. B, Quantitative morphometric analysis showing the mean GR-ir signal density in the POA of juvenile frogs. *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 5/treatment; *scale bar*, 100 μm).

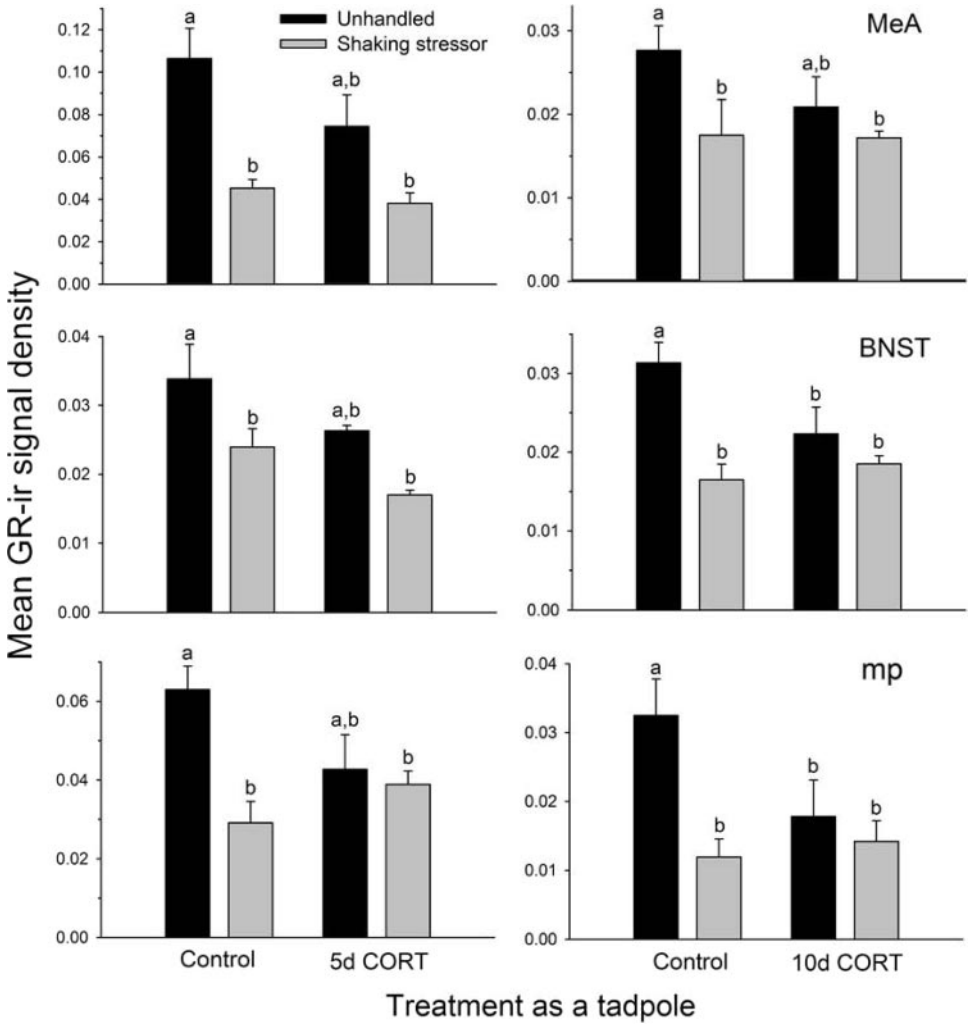

FIG. 6. Treatment of prometamorphic tadpoles with CORT leads to decreased GR-ir in limbic structures (MeA, BNST, and mp) in 2-month-old juvenile frogs. Prometamorphic tadpoles were treated as described in the legend of Fig. 3 and *Materials and Methods*. Two-month-old juvenile frogs in each treatment were then divided into two groups and one was exposed to shaking/confinement stressor for 2 h, whereas the other group was left undisturbed (unhandled). Shown are the quantitative morphometric analyses of mean GR-ir signal density in each brain region. *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 5–6/treatment).

plasma (CORT) after 30 min exposure to restraint stress, their plasma (CORT) remained high 2 h after the stress, whereas the controls had returned to basal (28, 74). The duration of the stressor used in our studies (2 h) and our inability to obtain serial blood samples at multiple time points could have caused us to miss critical differences in the reactivity of the stress axis in frogs, and this requires further study.

The activity of the HPA/HPI axis is regulated by GC-negative feedback at the central nervous system and pituitary gland, which is mediated in large part by the GR. We found that GR-ir was decreased in the brain and pituitary gland of juvenile frogs that had been exposed to CORT as tadpoles. The largest decrease in GR-ir was observed in the POA (homolog of the mammalian PVN), the location of hypophysiotropic CRF neurons in the frog brain (47, 75), and the rostral pars distalis, the location of corticotropes (56, 57). We also found decreases in GR-ir in limbic structures that include the MeA, BNST, and mp (homologous to the mammalian hippocampus). In mammals, these limbic structures are implicated in negative feedback regulation of PVN CRF neurons, and similar relationships may exist in the frog (8, 10, 16). Our findings are in general agreement with rodent studies that showed that prenatal stress decreased GR expression in the hypothalamus as measured by Western blotting (76) and in the hippocampus as measured by radioreceptor binding assay (20, 77), Western blotting (76), and *in situ* hybridization histochemistry (78, 79). In addition, prenatal GC treatment decreased inhibitory actions of GCs on ACTH secretion and reduced ACTH content and the numbers of corticotrope cells (80). The decreased GR-ir that we observed in the frog pituitary after CORT treatment as a tadpole could result from decreased GR expression, a decrease in the number of corticotrope cells, or both.

An important mechanism for regulating the responsiveness of the HPA axis during chronic stress is the autoregulation of GR expression by circulating GCs. Exposure to repeated or chronic stressors can decrease GR expression in the rat hippocampus and PVN (81–85). This down-regulation of GR expression by GCs can reduce inhibition of HPA activity by elevated circulating GCs, which is hypothesized to maintain the responsiveness of the HPA axis to further stimulation (86, 87). We recently found that this regulatory mechanism is conserved in *Xenopus* in which exposure to elevated GCs decreases GR-ir in the brain and pituitary gland (16). Our current findings also show that exposure to a physical stressor for 2 h rapidly down-regulates GR. The GR

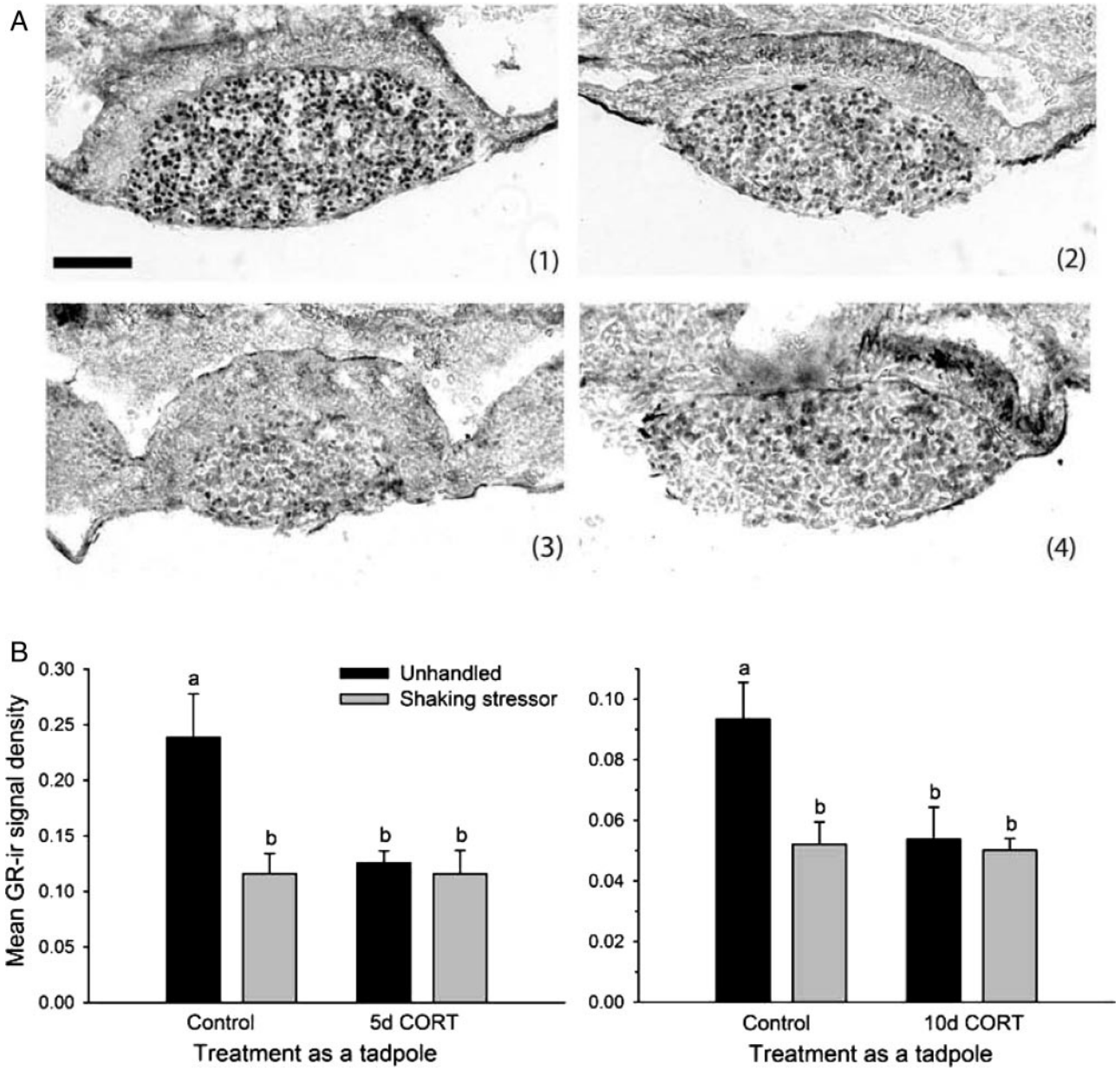

FIG. 7. Treatment of prometamorphic tadpoles with CORT leads to decreased GR-ir in the anterior pituitary gland of 2-month-old juvenile frogs. A, Photomicrographs of representative transverse sections through the rostral pars distalis of juvenile frogs: 1, control, unhandled; 2, control, shaking stressor; 3, 5 d CORT treatment, unhandled; 4, 5 d CORT treatment, shaking stressor. B, Quantitative morphometric analysis showing the mean GR-ir signal density in the rostral pars distalis of juvenile frogs. *Bars*, Means ± SEM. *Letters*, Significant differences among treatments based on one-way ANOVA followed by Fisher's LSD multiple comparison tests ($P < 0.05$; n = 5/treatment; *scale bar*, 100 μm).

rapidly translocates to the nucleus upon hormone binding, and the protein is then degraded through a proteasome-mediated mechanism (88, 89). Our findings suggest that the decrease in GR-ir in the brain and pituitary gland caused by early-life exposure to CORT may result in decreased negative feedback by GCs and thus account for the elevation in basal plasma (CORT) that we observed in juvenile frogs.

The negative feedback regulation of HPA axis activity involves both direct and indirect modes of action. Glucocorticoids act directly on CRF neurons in the hypothalamus and on pituitary corticotropes to limit ACTH secretion (65, 90). An indirect mode of action is mediated by a complex limbic circuit that involves the hippocampus, amygdala, and BNST (8). The mammalian hippocampus sends inhibitory projections to the PVN, which are activated by GC binding primarily to the GR (29). Given the high expression of GR-ir in the amphibian mp, we hypothesize that a similar limbic circuit that regulates HPI activity exists in the frog (16). By contrast to the hippocampus, activation of the amygdala leads to the stimulation of the HPA axis in response to stressors (15, 91). As in mammals, the amphibian BNST acts as a relay for signals from the amygdala to hypophysiotropic neurons in the POA (PVN in mammals) (92). As for the mp, the functional role of the amphibian amygdala and BNST in

HPI axis regulation is not known. However, our findings that CORT increased CRF-ir (10) but decreased GR-ir (16) in the MeA and BNST show that these structures are influenced by circulating GCs and thus are likely to be involved in regulation of the frog HPI axis.

The molecular mechanisms by which early-life exposure to GCs affects HPA axis activity and GR expression are poorly understood. Recent studies showed that early-life experience may cause epigenetic modifications of the GR gene through DNA methylation, resulting in altered GR gene expression (93–95). The rodent GR gene has a CpG island in the first exon, which becomes hypermethylated after exposure to stressors/GCs early in life (94). It is hypothesized that this hypermethylation leads to suppression of GR gene expression (93, 94). Although we have not yet examined this possibility in the frog, it is noteworthy that the frog GR gene also has a CpG island in the first exon (Kyono Y., and R. J. Denver, unpublished data).

In conclusion, our results show that both reduced food availability, which increases endogenous CORT levels, and exposure to exogenous CORT during the prometamorphic tadpole stage decreases growth, and although this results in smaller body size at metamorphosis, juvenile frogs eat more and thus exhibit catch-up growth. Early-life exposure to CORT leads to long-term changes in the activity of the HPI axis in juvenile frogs, as evidenced by elevated basal plasma (CORT) and decreased GR-ir in the brain and pituitary gland. The elevated CORT in juvenile frogs may have driven the hyperphagic response and could result from decreased negative feedback on the HPI axis owing to decreased GR expression in key brain areas involved in HPI axis function and in the anterior pituitary gland. Future studies in frogs and other vertebrate model species can help to identify the molecular developmental mechanisms by which GCs program the HPI axis (and other neural/endocrine systems) and elucidate whether such responses have positive or negative fitness consequences, which can have important consequences for the health of human and wildlife populations.

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