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Evolutionarily Conserved Glucocorticoid Regulation of Corticotropin-Releasing Factor Expression

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Glucocorticoids (GCs) exert feedback regulation on corticotropin-releasing factor (CRF) neurons in mammals. The nature of GC actions is cell-type specific, being either inhibitory (*e.g.* paraventricular nucleus) or stimulatory (*e.g.* amygdala and bed nucleus of the stria terminalis). Nothing is known about differential regulation of CRF gene expression by GCs in nonmammalian vertebrates. We studied the actions of GCs on CRF expression in discrete brain regions of the frog *Xenopus laevis*. Treatment with corticosterone (CORT) decreased, whereas the corticosteroid synthesis inhibitor metyrapone increased CRF expression in the anterior preoptic area (homolog of the mammalian paraventricular nucleus), as measured by CRF primary transcript, mRNA, and CRF immunoreactivity (ir) (by immunocytochemistry). By contrast to the preoptic area, CORT increased CRF-ir in the medial amygdala and bed nucleus of the stria terminalis, whereas metyrapone decreased CRF-ir in the medial amygdala. CRF-ir and glucocorticoid receptor-ir were colocalized in cells in the frog brain. In transient transfection assays in PC-12 cells, GCs decreased forskolin-induced activation of the frog CRF promoters. Treatment with CORT also reduced CRF promoter activity in transfected tadpole brain *in vivo*. Frog glucocorticoid receptor bound with high-affinity *in vitro* to regions in the proximal promoters of frog CRF genes that are homologous with the human CRF gene. Our findings suggest that the neural cell-type specificity and molecular mechanisms of GC-dependent regulation of CRF are phylogenetically ancient, and that the limbic pathways mediating behavioral and physiological responses to stressors were likely present in the earliest land-dwelling vertebrates. (*Endocrinology* 149: 2352–2360, 2008)

HYPOPHYSIOTROPIC corticotropin-releasing factor (CRF) neurons located in the paraventricular nucleus (PVN) of the hypothalamus of mammals are subject to negative feedback regulation by glucocorticoids (GCs). GCs modulate the activity of the hypothalamo-pituitary-adrenal axis, thereby limiting the magnitude and duration of the endocrine stress response (1–3). The feedback actions of GCs on hypothalamic CRF neurons can be both direct and indirect. For example, GCs, directly inhibit CRF synthesis and secretion by parvocellular neurons of the PVN. Indirectly, GCs modulate the activity of PVN neurons via descending pathways from the hippocampus, amygdala, and bed nucleus of the stria terminalis (BNST), or via pathways originating in the brainstem (3–5). Diverse molecular and cellular mechanisms may be involved in the actions of GCs on the central nervous system, including direct regulation of gene transcription, modulation of neuronal excitability, and rapid nongenomic actions (4–7).

Although the PVN CRF neurons are negatively regulated, GCs may stimulate other CRF expressing cells. For example, GCs up-regulate CRF expression in the central nucleus of the amygdala and BNST in rats (8–14). Furthermore, GCs increase CRF expression in syncytiotrophoblast cells of the placenta (15–18). The molecular mechanisms by which GCs differentially regulate CRF expression are poorly understood.

Very little is known about the regulation of CRF by GCs in nonmammalian vertebrates. Earlier, we reported that central CRF neurons of the frog *Xenopus laevis* are responsive to stressors, and that the distribution of CRF and glucocorticoid receptor-immunoreactivity (GR-ir) in the frog brain is evolutionarily conserved with mammals (19–21). We also identified putative GC response elements (GREs) in the proximal promoters of the frog CRF genes (21). In the present study, we asked whether CRF is differentially regulated by GCs in several regions of the frog brain, using analyses of CRF primary transcript [heteronuclear RNA (hnRNA)], mRNA (both analyzed by quantitative real-time PCR on microdissected brain sections), and protein [by immunohistochemistry (IHC)]. We also determined whether GCs can influence forskolin (Fsk)-induced transcriptional activity of the frog CRF promoters in transfected cells, or CRF promoter activity *in vivo* using somatic gene transfer into the frog brain. Finally, we examined whether GR can bind to the putative GRE sequences in the frog CRF promoters *in vitro*.

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Abbreviations: AP-1, Activating protein-1; BNST, bed nucleus of the stria terminalis; BW, body weight; CORT, corticosterone; CRE, cAMP response element; CRF, corticotropin-releasing factor; DEX, dexamethasone; Fsk, forskolin; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GR-ir, GR immunoreactivity; hnRNA, heteronuclear RNA; icv, intracerebroventricular; IHC, immunohistochemistry; ir, immunoreactivity; LSD, least significant difference; MeA, medial amygdala; MMTV, mouse mammary tumor virus promoter; MTP, metyrapone; POA, preoptic area; PVN, paraventricular nucleus; xGR, *X. laevis* glucocorticoid receptor protein.

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Materials and Methods

Animal husbandry

We purchased *X. laevis* juveniles from *Xenopus* I, Inc. (Dexter, MI), but generated tadpoles for study by spawning adults in our breeding colony at the University of Michigan. We reared tadpoles in dechlorinated tap water (20–22 C; 12-h light, 12-h dark cycles), and fed juvenile frogs and tadpoles Frog Brittle (Nasco, Fort Atkinson, WI) *ad libitum*. Developmental stages were assigned according to Nieuwkoop and Faber (22). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals at the University of Michigan.

Corticosterone (CORT) and metyrapone (MTP) treatment of juvenile X. laevis

We manipulated circulating corticosteroid concentrations of juvenile *X. laevis* by adding CORT or the corticosteroid synthesis inhibitor MTP (both from Sigma Chemical Co., St. Louis, MO) to the rearing water as described previously (23). The CORT and MTP were first dissolved in 100% ethanol, which was then added to the aquarium water; controls received ethanol vehicle, which was adjusted to a final concentration of 0.0005% in all tanks. Two treatment paradigms were used. For the short-term (6 h) treatment, juvenile frogs [body weight (BW) 0.8–1.3 g] were treated with CORT (500 nM) or MTP (100 μM) before being killed and tissue harvest for RNA extraction and analysis. We found that this dose of CORT caused an increase in plasma CORT comparable to that achieved during exposure to a handling/shaking stressor (from 1.5 ng/ml in the basal, untreated condition to 20–30 ng/ml stressed; also see Ref. 19), whereas the dose of MTP used decreased plasma CORT to below the detection limit of the RIA (data not shown; also see Ref. 19).

For the longer treatment, frogs were exposed to CORT (500 nM) for 4 d, MTP (100 μM) for 5 d, or MTP for 1 d, followed by MTP plus a replacement dose of CORT (50 nM) for 4 d before being killed and tissue harvest [plasma CORT concentration: control 1.23 $\pm$ 0.42; 500 nM CORT 28.82 $\pm$ 11.91; MTP 0.04 $\pm$ 0.03; MTP + 50 nM CORT 2.10 $\pm$ 2.08; compared with 4-h handling/shaking stressor 27.49 $\pm$ 16.33; see Ref. 23]. Animals showed no obvious adverse effects on overall health, BW, or behavior after treatment with CORT or MTP. Brains were fixed and stored at −80 C until cryosectioning and IHC.

RNA extraction and analysis

For analysis of CRF gene expression in the frog brain, we microdissected the anterior preoptic area (POA)/hypothalamus of individual juvenile frogs as described previously (21, 24). We isolated RNA using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and first-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamers following the manufacturer's instructions. We designed gene-specific TaqMan primer/probe sets that targeted the intronic regions of the *X. laevis* CRFa and CRFb genes to analyze expression of the primary transcripts [CRFa hnRNA and CRFb hnRNA; *X. laevis* possesses two CRF genes owing to its pseudotetraploidy (21)] and CRF mRNA [CRF mRNA; this primer/probe set could not distinguish between CRFa and CRFb mRNAs, owing to the high sequence similarity of the 5′ untranslated region and coding regions of the two frog CRF genes; Table 1 for primer and probe sequences]. Reactions were run using the Fast 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). For quantification and comparison of expression levels of the frog CRF hnRNAs and mRNA, we generated standard curves using known concentrations of plasmid DNA containing each of the frog CRF genomic clones (25). We normalized CRF gene expression to the *X. laevis* ribosomal protein L8 mRNA as previously described (21).

IHC and morphometric analysis

We conducted IHC on frog brain as described previously using highly specific, affinity purified IgGs (15–20 ng IgG/μl) raised in rabbits against synthetic *X. laevis* CRF or a synthetic peptide corresponding to a unique region of the *X. laevis* GR (19, 23). Single IHC for CRF immunoreactivity (ir) or GR-ir was conducted using the Vectastain elite ABC (rabbit) and Vector VIP kits (both from Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's protocols. We used double-labeling fluorescence IHC to determine colocalization of CRF-ir and GR-ir in selected brain regions following the method that we described previously for the colocalization of c-*fos* and CRF in the frog brain (19). Double-labeling experiments were analyzed by confocal microscopy using a Zeiss laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with optical sections of 1-μm thickness captured through the z-axis.

We quantified CRF-ir in discrete brain regions using MetaMorph software (version 6.2r4; Molecular Devices, Sunnyvale, CA). We processed all samples simultaneously under identical conditions. Three sections that contained the POA, or amygdala plus BNST, were analyzed for each animal. All sections were carefully matched for anatomical level and digital images captured at $\times$200 magnification for morphometric analysis. Image analysis was conducted in a blinded manner. The brain regions were isolated using a handmade frame that covered the area of interest. For quantification of CRF-ir, the total area of positive staining particles above a standard density threshold in the selected area was counted automatically, and the mean density for each animal was calculated as the total positive staining area on multiple sections, divided by the total selected area (19).

Cell culture and transient transfection

We maintained monolayer cultures of PC-12 cells in high-glucose DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% steroid-stripped bovine calf serum, 5% steroid-stripped equine serum (both sera from HyClone, Logan, UT), and antibiotics in a humidified atmosphere of 5% CO_2 at 37 C. The sera were stripped of steroid hormones by mixing 25 ml serum with 50 mg charcoal [coated with 5-mg Dextran T 70 (Amersham Biosciences Inc., Piscataway, NJ) in PBS] and shaking at room temperature for 5 h, followed by centrifugation at 1000 $\times$ *g* for 10 min. The supernatant was mixed again with 50 mg charcoal-Dextran and shaken at room temperature overnight before a final centrifugation at 30,000 $\times$ *g* for 20 min at 4 C.

We conducted transient transfections in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) as described by Yao *et al*. (21). Cells were plated at a density of 4×10^5 cells per well in 24-well plates 24 h before transfection. Each well received 800 ng reporter plasmid containing the proximal promoter regions of the two frog CRF genes driving luciferase (pGL3-xCRFa533 or pGL3-xCRFb576) (21) plus 10 ng pRL-null plasmid (control for transfection efficiency; Promega Corp., Madison, WI). Twenty-four hours after transfection, the medium was changed to fresh medium with or without regulators. A 25-mM stock of Fsk (Sigma-Aldrich) was prepared in dimethylsulfoxide and added to wells to a final concentration of 25 μM. CORT or dexamethasone (DEX) (Sigma-Aldrich) was first dissolved in 100% ethanol before addition to the wells 1 h before the addition of Fsk (*i.e.* 7 h before cell harvest). Cells in the control group received the same concentration (0.001%) of dimethylsulfoxide and ethanol vehicles as the Fsk and steroid-treated cells.

We harvested cells at different times and measured luciferase activity using a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. All treatments were done in triplicate, and each experiment was repeated at least three times. Firefly luciferase activities (the reporter) were normalized to *Renilla* luciferase activities in each cell lysate, and the values are expressed as relative luciferase activity.

TABLE 1. Sequences of primers and probes used for real-time quantitative PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan probe (5′–3′)
CRFa hnRNA	GACACAAACCCACTTTTATGTTGTTGAA	CTTGCTCAGGTGCCAATAATACC	6FAM-TTGTCAAAACTGGGAATTACAT-MGB
CRFb hnRNA	TGACACTTCACAATATGCACACAGT	CATTCAACAAGTTCTGCACATCAGT	6FAM-CAAGGACTCATAAAGCATTTG-MGB
CRF mRNA	CATTTCCCTGGATCTGACTTTTCAC	TTGCTCAGCTCTTGCCATTTCTA	6FAM-TTGCTCCGTGAAGTCT-MGB
rpL8 mRNA	TTTGCTGAAAGAAATGGCTACATC	CACGGCCTGGATCATGGA	VIC-AGGGTATTGTGAAAGACA-MGB

Electroporation-mediated gene transfer

To test whether GCs can regulate the frog CRFb promoter *in vivo*, we transfected tadpole brain with the pGL3-xCRFb576 reporter plasmid using bulk electroporation-mediated gene transfer as described previously (21) and treated animals with CORT. *X. laevis* tadpoles (Nieuwkoop and Faber stage 49–51) were anesthetized by immersion in 0.002% benzocaine and given intracerebroventricular (icv) microinjections of a DNA solution containing 1 μg/μl of the reporter plasmid pGL3-xCRFb576, 50 ng/μl pRL-null plasmid, 400 ng/μl pEGFP-N1 plasmid (Clontech, Palo Alto, CA; to monitor transfection efficiency), and 0.02% fastgreen dye. Immediately after the injection, we placed a pair of platinum electrodes over the skull and delivered five pulses of 30 V each. We reversed the polarity and repeated the current delivery. Animals were screened for high enhanced green fluorescence protein expression in the region surrounding the third ventricle 5 d after transfection using a Leica MZFLIII fluorescent stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). We next administered icv injections of CORT (100 nl injection of 500 nM CORT; ~20 pg/g BW) or vehicle (saline with 0.0048% ethanol) and waited 6 h before harvesting brains. This dose of CORT was chosen based on the known stress level of plasma CORT obtained in the frog [~50 nM (19, 23)] and a rough estimation of the dilution that would occur within the brain ventricles. We analyzed luciferase activity in tadpole brain homogenates using the Dual-Luciferase Reporter Assay System. We processed each brain separately in a microcentrifuge tube with a hand homogenizer in 20 μl passive lysis buffer, incubated on ice for 30 min with vortexing every 10 min, and then analyzed 10 μl of each homogenate in the dual-luciferase assay.

EMSA

We conducted EMSA using *in vitro*-synthesized *X. laevis* GR protein produced by coupled transcription-translation using the p6xGR vector (26) following the manufacturer's instructions (TNT system; Promega). Nuclear extracts were prepared from the POA/diencephalon region of juvenile frog brains following methods described previously (21).

We prepared DNA probes for EMSA by annealing complementary oligonucleotides (Table 2) and end labeling using the large Klenow fragment of DNA polymerase (Promega) and ^{32}P-labeled dCTP (Perkin-Elmer, Inc. Boston, MA). The mouse mammary tumor virus promoter (MMTV) GRE probe was described by Malkoski and Dorin (27), the collagenase AP1 probe by Jonat *et al.* (28), and the human CRE probe by Yao *et al.* (21). EMSAs were conducted following the methods of Dignam *et al.* (29) with minor modifications. *In vitro* produced GR (1 μl of 50 μl TNT reaction) was incubated with 20,000 cpm ^{32}P-labeled double-stranded oligonucleotides and 1.4 μg double-stranded poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.8), 1 mM dithiothreitol, 0.1% IGEPAL CA-630, 50 mM KCl, and 20% glycerol. The reaction continued at room temperature for 40 min before fractionation by 6% nondenaturing PAGE in 0.25 × Tris-borate EDTA buffer. The gel was dried and analyzed by autoradiography. Competitive displacement was conducted by addition of various concentrations of radioinert specific or nonspecific oligonucleotides during the incubation.

Statistical analysis

We analyzed data by the unpaired Student's *t* test or by one-way ANOVA, followed by Fisher's least significant difference (LSD) multiple comparisons tests using the SYSTAT v10 software (SPSS, Inc., Chicago, IL). The data were $\log_{10}$-transformed when the variances were heterogeneous (Bartlett's test). The values presented are mean + SEM, and $P < 0.05$ was considered statistically significant.

TABLE 2. Sequences of oligonucleotides used for EMSA (upper strands only)

MMTV GRE	GTTGGGTTACAAACTGTTCTAACCA
AP-1	AGCTTGATGAGTCAGCCGGATC
xCRFaGRE	TTTCTTTGTCAATGGAAAATTTTTGAACA
xCRFbGRE	TTTCTTGTCAATGGGAGACTTGTGAACG
hCRFCRE	GGCTCGTTGACGTCACCAAGAGGC

Results

GCs negatively regulate CRF primary transcript and mRNA in the frog POA/hypothalamus

We treated juvenile *X. laevis* with CORT (500 nM added to the rearing water) or MTP (100 μM added to the rearing water) for 6 h before tissue harvest, and analyzed CRF hnRNA and mRNA in the POA/hypothalamus by real-time quantitative PCR. Treatment with CORT for 6 h caused the levels of CRFb hnRNA and CRF mRNA to decrease significantly in the POA/hypothalamus ($P = 0.034$ and $P = 0.025$ *vs.* control group, respectively; n = 5 per treatment; Fig. 1, B and C), whereas no significant difference was observed in the level of CRFa hnRNA (Fig. 1A). By contrast, MTP treatment caused a significant increase in the level of CRFb hnRNA ($P = 0.027$ *vs.* control group; n = 5 per treatment) and a trend toward increase in the level of CRFa hnRNA ($P = 0.050$ *vs.* control group; n = 5 per treatment). No change was seen in the level of CRF mRNA with MTP treatment.

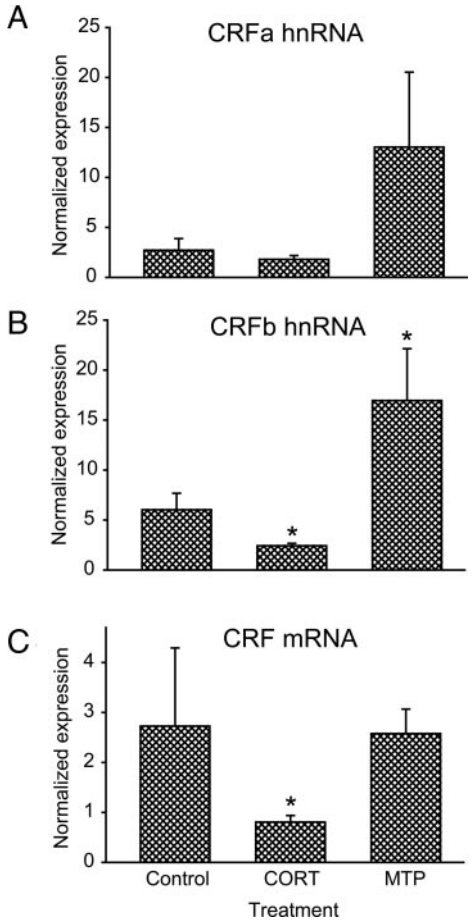

FIG. 1. GCs negatively regulate CRF gene expression in the anterior POA/hypothalamus of juvenile *X. laevis*. Analysis of CRFa hnRNA (A), CRFb hnRNA (B), and CRF mRNA (C) in microdissected frog POA/hypothalamus by real-time quantitative PCR. Frogs were untreated (control), or exposed to 500 nM CORT or 100 μM MTP for 6 h added to the aquarium water. Data presented are the mean + SEM. A significant difference from controls is denoted by an *asterisk* (*) for $P < 0.05$ (n = 5 per treatment).

GCs differentially regulate CRF-ir in the frog POA vs. *the amygdala and BNST*

Manipulation of corticosteroids in juvenile *X. laevis* caused significant changes in CRF-ir in the POA (ANOVA: $F_{(3, 16)} = 21.780$; $P < 0.001$; n = 5 per treatment). Treatment with CORT for 4 d caused a marked decrease in CRF-ir, whereas treatment with 110 μM MTP for 5 d significantly increased CRF-ir in this region (Fig. 2A). Simultaneous treatment with MTP plus CORT (50 nM) reversed the increase in CRF-ir caused by MTP.

Manipulation of corticosteroids also caused significant changes in CRF-ir in the medial amygdala (MeA) and BNST, but the direction of change was opposite of that observed in the POA (ANOVA for CRF-ir in the MeA: $F_{(3, 16)} = 17.982$, $P < 0.001$; in the BNST: $F_{(3, 16)} = 7.394$, $P = 0.003$; n = 5 per treatment for both regions). Treatment with CORT increased, whereas MTP decreased CRF-ir in the MeA; the decrease caused by MTP was reversed by simultaneous treatment with 50 nM CORT (Fig. 2B). In the BNST, CORT increased CRF-ir, but MTP had no effect (Fig. 2C).

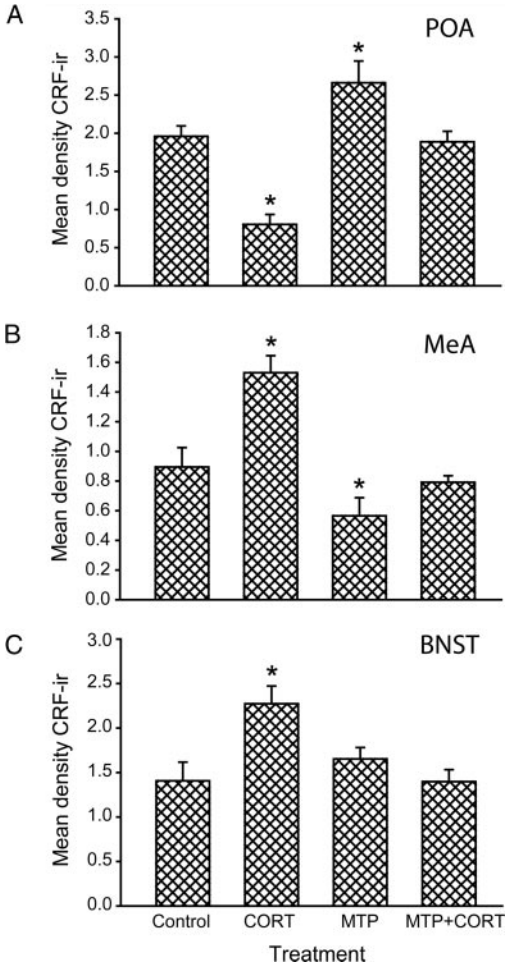

FIG. 2. Effects of corticosteroid manipulations on CRF-ir in the anterior POA (A), MeA (B), and BNST (C) of juvenile *X. laevis*. Frogs were untreated (control), or exposed to 500 nM CORT for 4 d, 110 μM MTP for 5 d, or 110 μM MTP for 1 d, followed by 110 μM MTP plus 50 nM CORT for 4 d (CORT + MTP). Data presented are the mean + SEM. The *asterisk* (*) indicates a significant difference compared with the control ($P < 0.05$; n = 5 per treatment).

CRF and GR-ir colocalize in cells of the frog brain

Using dual-fluorescence IHC and confocal microscopy, we found CRF-ir and GR-ir to be colocalized in cells of the POA in juvenile frog brain (Fig. 3). We did not analyze colocalization in the MeA or the BNST; however, we have described GR-ir in both of these regions (23). We also found colocalization of CRF and GR in the medial pallium, which is a brain region homologous to the mammalian hippocampus (data not shown; we did not observe changes in CRF-ir in this region after corticosteroid manipulation). Over 90% of CRF-ir positive cells in the POA expressed GR-ir, and over 90% of GR-ir cells in the POA also expressed CRF-ir.

GCs suppress frog CRF promoter activity in transfected PC-12 cells

Earlier, we discovered that the frog CRF promoters possess regions that appear homologous to a composite GRE/

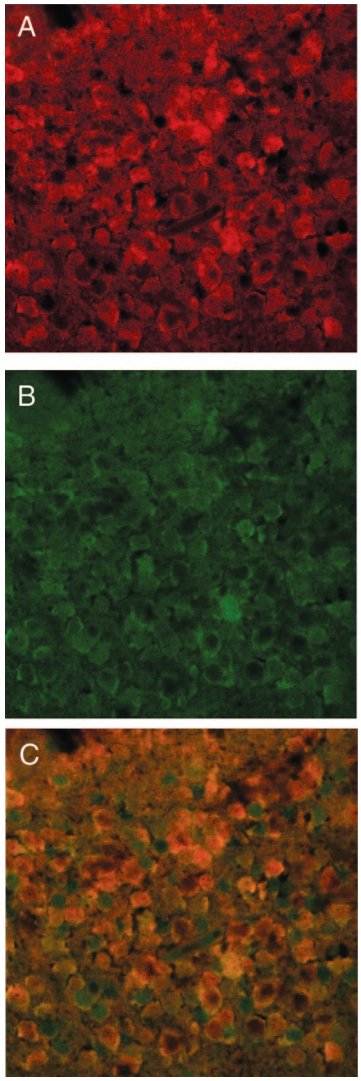

FIG. 3. Colocalization of CRF-ir (A; *red*) and GR-ir (B; *green*) in the frog anterior POA. CRF-ir was observed primarily in the cytoplasm, whereas GR-ir was seen in both the cytoplasm and the nucleus. C, The merged image shows colocalization of the CRF-ir and GR-ir. Magnification, ×200.

AP1 site that had been described previously in the human CRF gene and shown to bind GR (21, 27). Therefore, we tested frog CRF promoter plasmids that we constructed previously (pGL3-xCRFa533 or pGL3-xCRFb576; 21) for responsiveness to GCs in transient transfection assays using PC-12 cells. Treatment with DEX (100 μM) had no effect on basal promoter activity in PC-12 cells. Fsk (25 μM) strongly increased frog CRF promoter activity, as found previously (21), and simultaneous treatment with DEX significantly reduced Fsk-induced activation of both promoter constructs ($P = 0.001$ for both *vs.* Fsk treatment; Fig. 4A).

The dose of DEX used (100 μM) was high, and we reasoned that if 25 μM Fsk caused a maximal promoter response, we may not be able to detect effects of DEX at lower doses. Therefore, we first conducted a dose response to Fsk using pGL3-xCRFb576, which showed that the minimum effective dose of Fsk was 50 nM ($P = 0.013$ *vs.* control), and maximal promoter activity was reached at 2.5 μM Fsk (Fig. 4B). We next examined activity of the pGL3-xCRFb576 construct in the presence of a half-maximal dose of Fsk (200 nM) plus different concentrations DEX. In this experiment the minimum effective inhibitory dose of DEX on Fsk-induced promoter activity was found to be 1 μM ($P = 0.026$ *vs.* Fsk alone), and the 10-μM dose further decreased Fsk-induced activity to 43% of Fsk alone ($P < 0.001$ *vs.* Fsk alone; Fig. 4C). The 1-μM dose of DEX used here is comparable to that found to suppress the human CRF promoter in transient transfection assays (30, 31).

GCs suppress frog CRF promoter activity in transfected tadpole brain in vivo

To determine if GCs can influence CRF promoter activity *in vivo*, we used electroporation-mediated gene transfer to transfect into tadpole brain pGL3-xCRFb576. We then injected tadpoles icv with saline vehicle or CORT (~20 pg/g BW) and harvested brains 6 h later for dual luciferase assay. Injection of CORT significantly decreased luciferase activity in brains from animals transfected with pGL3-xCRFb576 compared with no injection or vehicle (saline with 0.0048% ethanol)-injected controls ($P = 0.041$ *vs.* no injection control; n = 5 per treatment). The same treatment did not alter luciferase activity in animals transfected with empty vector. Vehicle injection did not alter the expression of either construct compared with the respective no-injection controls (Fig. 5).

GR binds to the composite activating protein-1 (AP-1)/GRE sites in the frog CRF promoters in vitro

Earlier, using computer analysis, we described composite AP-1/GRE sites in the *X. laevis* CRF genes that exhibit high degrees of sequence similarity to a region of the human CRF promoter that has been shown to bind GR, and is hypothesized to mediate GC-dependent transrepression (Fig. 6A) (21, 27). Therefore, we tested whether this region of the frog promoters can bind GR *in vitro*. Shifted protein-DNA complexes of similar mobility were formed in EMSA on the GREs from both frog CRF genes ("aGRE" and "bGRE," from CRFa and CRFb genes, respectively) using *in vitro* expressed frog GR (Fig. 6B). A similar-sized complex was formed with the concensus MMTV (included as a positive control). We also observed low but detectable binding of frog GR to a probe

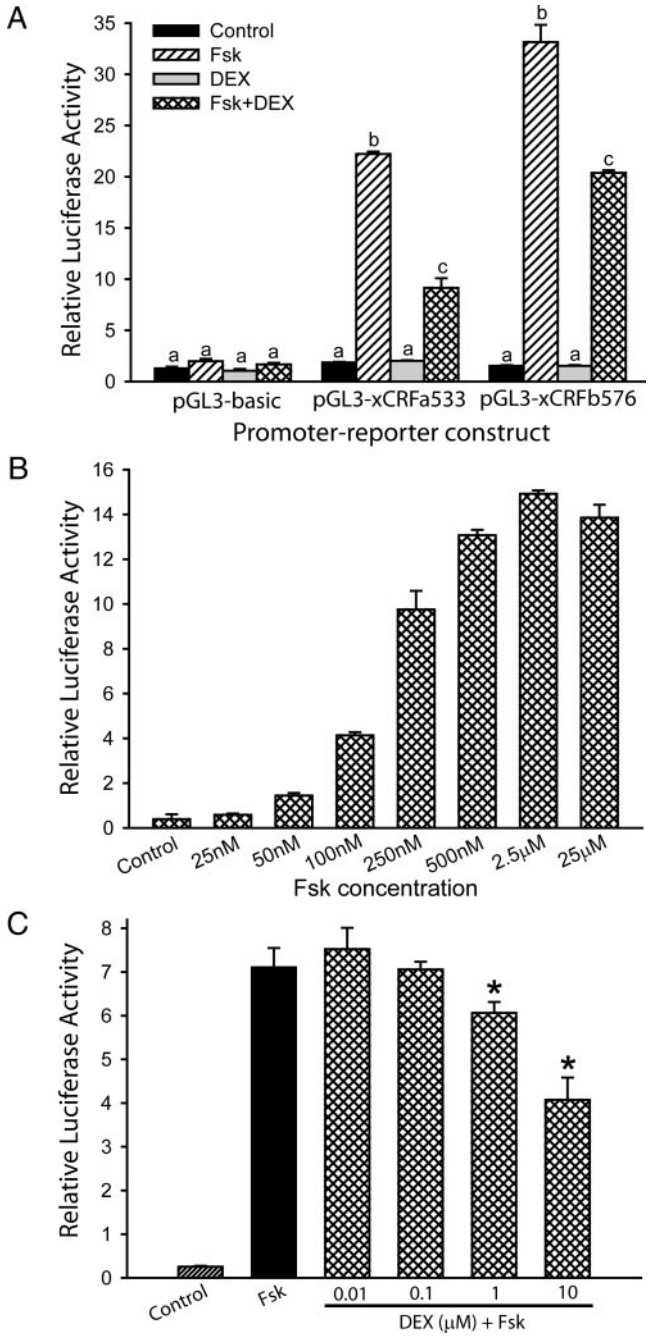

FIG. 4. GCs negatively regulate Fsk-induced activity of the frog CRF promoters in transiently transfected PC-12 cells. A, Effects of DEX on frog CRF promoter activity. PC-12 cells were transfected with pGL3-basic (empty vector), pGL3-xCRFa533, or pGL3-xCRFb576 plus pRL-null to normalize for transfection efficiency. Cells were then cultured with or without Fsk (25 μM) and DEX (100 μM) for 6 h before harvest and dual luciferase assay (see *Materials and Methods*). *Letters* indicate significant differences among treatments within a promoter-reporter construct (Fisher's LSD $P < 0.05$). B, Dose-dependent activation of the frog CRFb proximal promoter by Fsk in transiently transfected PC-12 cells. Cells transfected with pGL3-xCRFb576 were treated with increasing concentrations of Fsk for 6 h. C, Dose-dependent repression of Fsk-dependent activation of the frog CRFb proximal promoter by DEX. The *asterisk* indicates a significant difference between the Fsk and CORT or DEX-treated and Fsk-treated cells (Student's *t* tests, $P < 0.05$). For the experiments shown in the figure, each treatment was conducted in triplicate, and the experiment was repeated two to three times. The means + SEM of representative experiments are shown.

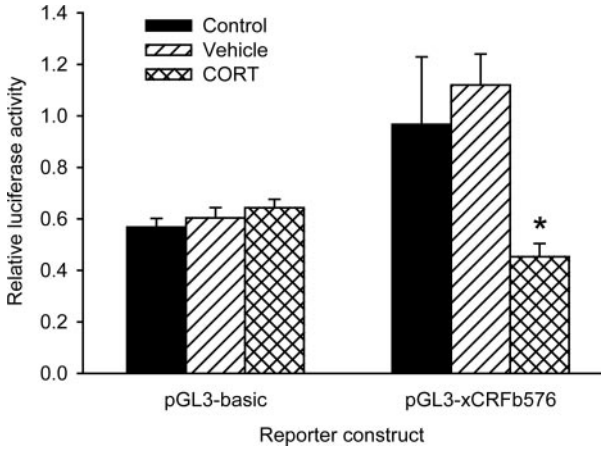

FIG. 5. GC suppresses CRF promoter activity in transfected tadpole brain *in vivo*. Tadpole brains were transfected by electroporation-mediated gene transfer with pGL3-basic (empty vector) or pGL3-xCRFb576 plus pRL-null to control for transfection efficiency (see *Materials and Methods*). One week later tadpoles received icv injections of either vehicle or CORT (20 pg/g BW). Animals were killed 6 h after injection and brains collected for dual luciferase assay. The *asterisk* indicates a significant difference from control within a promoter-reporter construct (Fisher's LSD $P < 0.05$; n = 6–8 per treatment).

containing the AP-1 binding site of the collagenase promoter. By contrast, no shifted complexes were seen on the CRE from the human CRF gene (included as a negative control). Using frog brain nuclear extracts, a complex was formed with the CRE probe, thus showing that the probe was capable of being bound by proteins present in frog brain. All the complexes formed with GR could be displaced by 100-nM (about 100-fold excess) radioinert oligonucleotides that corresponded to the radiolabeled probes (Fig. 6B).

The specificity of GR binding to the human and *X. laevis* GRE probes was examined by competition assay using radioinert oligonucleotides. The complex formed on the xCRFbGRE was greatly reduced by the addition of 10-nM radioinert oligonucleotides (~10-fold excess of the radiolabeled probes), and completely displaced by 30- and 100-nM radioinert oligonucleotides. The Ki (the concentration of the competitor at which binding is half-maximal binding) was calculated to be approximately 5 nM. Addition of 100-nM radioinert MMTV GRE oligonucleotide also disrupted the GR-xCRFbGRE complex by more than half. Addition of 100-nM radioinert collagenase AP-1 oligonucleotide slightly reduced the GR-xCRFbGRE complex, and no displacement was observed by the addition of the radioinert CRE oligonucleotide (Fig. 6C; data not shown).

Discussion

Here, we report for the first time in a nonmammalian species that CRF expression is differentially regulated by GCs in discrete regions of the central nervous system. The regulation of CRF expression in mammals is cell-type specific, being inhibited by GCs in the hypothalamus/POA, but stimulated in limbic structures. We also show that the frog proximal promoters are negatively regulated by GCs, and a composite AP-1/GRE site implicated in the negative regulation of the human CRF gene is structurally and functionally conserved in the frog genes.

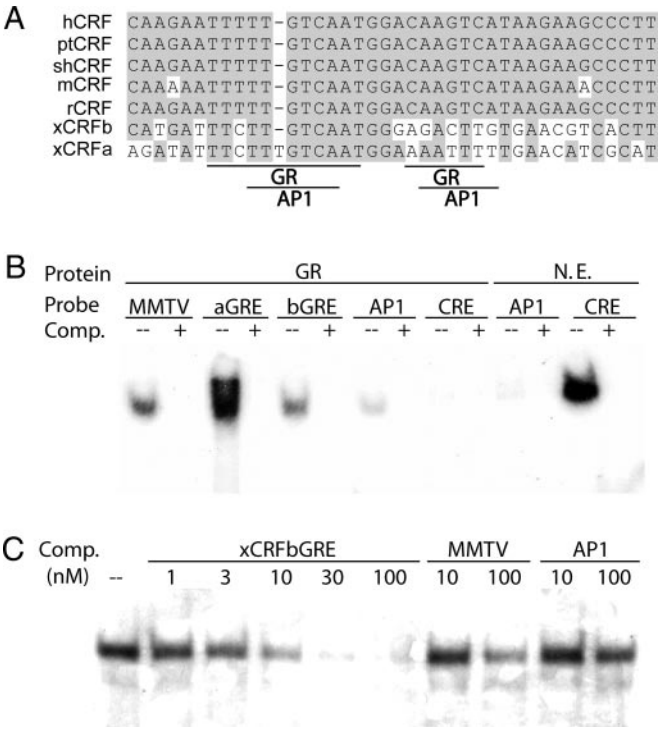

FIG. 6. Binding of frog GR to the AP-1/GRE sites in the frog CRF promoters analyzed by EMSA. A, ClustalW alignment of the putative GRE-containing sequences of the proximal promoter regions of human (h), chimp (pt), sheep (sh), mouse (m), rat (r), and *Xenopus* (x) CRF genes. Identical sequences among the CRF genes are *shadowed*. Putative GRE and AP-1 sites predicted by the online program Match using a library of mononucleotide weight matrices from TRANSFAC 6.0 (www.gene-regulation.com) are indicated. B, Binding of frog GR to ^{32}P-labeled oligonucleotides as analyzed by EMSA. Sequences of the oligonucleotides are given in Table 2. *Lanes 1*, *3*, *5*, *7*, *9*, *11*, and *13* received radiolabeled oligonucleotides only; *lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14* received radiolabeled oligonucleotides plus 100-nM radioinert oligonucleotides of the same sequences. C, Cross-competition of GR binding to ^{32}P-labeled xCRFbGRE oligonucleotides by radioinert oligonucleotides. The ^{32}P-labeled xCRFbCRE oligonucleotides were incubated with *in vitro* synthesized frog GR in the presence of increasing concentrations of radioinert competitor (Comp) oligonucleotides. Concentrations of the competitor oligonucleotides are indicated (in nM). –, No competitor; N.E., nuclear extract.

GC negative feedback on hypophysiotropic CRF neurons

We found that CRF expression is negatively regulated by GCs in the frog POA. GC treatment decreased, whereas inhibition of corticosteroid synthesis increased the levels of CRF primary transcript (hnRNA) and peptide in the POA. The level of CRF mRNA was decreased by 6-h GC treatment but was not altered by MTP. Previously, we found that CRF mRNA does not change after exposure to shaking/handling stressor, although the hnRNA and peptide increase (21). GCs are well known to exert negative feedback on CRF expression in parvocellular neurons of the PVN of mammals, and this action is considered an important means for modulating the hypothalamo-pituitary-adrenal axis (1, 5, 32). Similarly, negative regulation of CRF expression has been reported in several teleost fishes (33–37). Together, the findings support the hypothesis that GC negative feedback on hypophysiotropic CRF neurons was established before the divergence of

the actinopterygian and sarcopterygian fishes, and has been evolutionarily conserved.

A direct, negative action of GCs on hypophysiotropic CRF neurons is supported by our finding that CRF and GR colocalize in cells of the frog POA. In the rat, CRF and GR were found to colocalize in parvocellular neurons of the PVN (38–40). There is also evidence for indirect GC negative feedback on PVN neurons in mammals via a descending pathway originating in the hippocampus (3–5). High expression of GR is observed in cells of the mammalian hippocampus, which send inhibitory projections to the PVN (3, 41–43). Although we have not determined if such an indirect pathway is also present in frogs, it is noteworthy that the frog medial pallium, which is a brain region homologous to the mammalian hippocampus, expresses high levels of GR (23). Like the mammalian hippocampus, the frog medial pallium expresses CRF, and we found that GR was also expressed in these cells (data not shown).

Evolutionarily conserved negative GC regulation of the frog CRF promoters

Using transient transfection assays in PC-12 cells, we found that cAMP-dependent activation of the frog CRF proximal promoters was repressed by the GC agonist DEX. DEX decreased CRF promoter activity, CRF mRNA expression, or CRF secretion in several mammalian cell lines or primary hypothalamic cells (30, 44–48). As with the human CRF promoter, DEX did not alter basal activity of the frog CRF promoters but significantly reduced Fsk-induced activity. GCs decreased mammalian CRF gene activation caused by cAMP analogs, phorbol esters, or cytokines (30, 45, 46, 49–52). Together, these findings show that GCs can directly influence, acting via the GR, transcription of the CRF gene, and that such regulation is phylogenetically ancient.

Malkoski and Dorin (27) identified a negative GRE in the proximal promoter of the human CRF gene that overlaps with binding sites for the AP-1 nucleoproteins *fos* and *jun*. GCs repress AP-1 stimulated activity mediated by the composite AP-1/GRE site, and also repress, via the AP-1/GRE, activation mediated by the cAMP response element (CRE) located in the proximal CRF promoter (27, 49). The two frog CRF genes possess CREs that are structurally and functionally homologous to the human (21). By computer analysis we also identified regions of the frog CRF genes that are homologous to the AP-1/GRE site present in the human (21).

We used electroporation-mediated gene transfer into tadpole brain (21) to test whether the proximal frog CRF promoter could be regulated by GCs *in vivo*. As in transfection assays with cultured cells, treatment with GCs significantly reduced CRF promoter activity in transfected tadpole brain, suggesting that this region of the frog CRF gene possesses a negative GRE that is functional *in vivo*. We did not artificially activate the PKA pathway *in vivo*, but our earlier work suggests that a significant level of cAMP-dependent CRF expression occurs in the frog brain under basal or unstressed conditions in the laboratory (20, 53). To our knowledge, this is the first demonstration that the proximal promoter of a vertebrate CRF gene possesses a DNA element(s) that can function in negative regulation of the gene *in vivo*.

As observed for the human AP-1/GRE site, we found specific binding of full-length *X. laevis* GR to the homologous sites present in the proximal frog CRF promoters. This binding was of high affinity, occurring in the low nanomolar range (Ki ~5 nM), suggesting that the AP-1/GRE sites located in the frog CRF genes are *bona fide* binding sites for GR. However, whether this site functions *in vivo* in the frog to mediate GC repression of CRF requires further study.

In mammals, stressor-dependent activation of CRF gene transcription in the PVN as measured by *in situ* hybridization of CRF hnRNA is rapid but transient (54, 55). We observed a similar, rapid increase in CRF hnRNA in the frog POA measured by quantitative real-time PCR (21), although we did not conduct a detailed analysis of the time course in the decay of the response. Shepard *et al.* (31) provided evidence for a GC-independent mechanism that limits CRF gene transcription, at least in the short-term after exposure to an acute stressor. They found that CRF hnRNA was rapidly increased by restraint up to 30 min but returned to basal levels by 90 min in both intact and adrenalectomized rats. They also showed that the expression of the repressor isoform of CRE modulator, the inducible cAMP early repressor, was elevated in the rat PVN after exposure to the stressor in a GC-independent manner, and CRE modulator proteins were recruited to the CRF promoter after 3-h restraint. They suggested that, whereas GCs likely play a role in limiting the sensitivity and magnitude of CRF transcriptional responses, the up-regulation of inducible cAMP early repressor may play an important role in repressing CRF transcription immediately after an acute stressor.

GC positive feedback on limbic CRF neurons

Although GCs are well known to exert negative feedback on PVN CRF neurons in mammals, and we have now shown similar regulation in an amphibian, several groups have shown that GCs have opposite effects on CRF expression in the amygdala and BNST (8–13). We found a similar relationship in the frog, in which CRF-ir was increased by treatment with CORT in the MeA and BNST. This is the first demonstration of differential regulation of CRF expression by GCs in the brain of a nonmammalian species. The positive regulation of CRF by GCs could be, at least in part, direct on CRF expressing neurons because CRF and GR were found to colocalize in these cells in the rat brain (38–40). In the frog brain, CRF and GR are both expressed in the MeA and BNST (19, 23), although we have not yet verified by confocal fluorescence microscopy that they are expressed in the same cells.

Positive regulation of the CRF gene by GCs has been described in placental syncytiotrophoblast cells (15, 56), in which increasing GCs of fetal origin up-regulate placental CRF, which is thought to be responsible for initiating parturition (17, 18, 57). However, the mechanism by which GCs positively regulate CRF gene expression is poorly understood. King *et al.* (49) showed that removal of the AP-1/GRE site confers positive regulation by GCs on the human CRF promoter in transient transfection assays in AtT20 cells. They also found that this positive regulation was mediated via the CRE, and a region located between −213 and −99 bp, which also may mediate cAMP-dependent activation [perhaps in-

volving a caudal type homeobox response element (49)]. They proposed that nuclear proteins that act at or near the CRE, and are expressed in a tissue-specific manner, determine whether GC-dependent inhibition occurs (58). However, the molecular mechanisms for transrepression or transactivation of the CRF gene by GCs have not yet been elucidated.

Evolutionarily conserved structure and function of the limbic system in tetrapods

In mammals, the amygdala and BNST play central roles in the expression of fear and anxiety-related behaviors (43, 59–62). These limbic structures have extensive connections (direct and/or indirect) with the telencephalon, hypothalamus, thalamus, and brainstem, and are known to influence neuroendocrine and autonomic functions (43, 62–64). It is well established in rodents that CRF neurons in the amygdala and BNST are activated in response to fear/anxiety provoking stressors (65–71). The BNST composes the main relay between the amygdala and hypothalamus, and is the major direct nonhypothalamic input to the parvocellular PVN (72, 73). The CRF-expressing pathways in the amygdala and BNST may be involved in relaying stress input to the hypothalamus and facilitating CRF release from the PVN (70).

Very little is known about the function and regulation of limbic structures in nonmammalian species. The anatomical definition of these brain nuclei in the frog was based on the expression of neurotransmitters, enzymes, and other neural molecules (74). To our knowledge, no studies have addressed whether the limbic systems in amphibians or other nonmammalian vertebrates are functionally homologous to their counterparts in mammals. Our work provides several lines of evidence that suggest evolutionary conservation in the function of the limbic system between amphibians and mammals. CRF is expressed in the amygdala, BNST, and medial pallium of the frog, and its expression and that of the immediate early gene c-*fos* are increased after exposure to a stressor (19). The GR is expressed in the frog amygdala, BNST, and medial pallium (23), and GCs increased CRF in the amygdala and BNST (this study). Together, our findings are in agreement with those of Laberge *et al*. (75) who argue against the view that large changes occurred in the functional organization of the amygdala in the amphibian-reptilian transition (76). Thus, the basic functions of limbic structures in the stress response and the nature of the feedback regulation by GCs likely occurred before the divergence of the amphibian and amniote lineages, and may be common features in tetrapods.

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