CHAPTER 1

INTRODUCTION[1]

Overview

Developmental, physiological, and behavioral adjustments in response to environmental change are crucial for animal survival. In vertebrates, the neuroendocrine stress system, comprised of the hypothalamus, pituitary and adrenal/interrenal glands (HPA/HPI axis) plays a central role in adaptive stress responses. Corticotropin-releasing factor (CRF) is the primary hypothalamic neurohormone regulating the HPA/HPI axis. CRF also functions as a neurotransmitter/neuromodulator in the limbic system and brain stem to coordinate endocrine, behavioral, and autonomic responses to stressors. Glucocorticoids, the end products of the HPA/HPI axis, cause feedback regulation at multiple levels of the stress axis, exerting direct and indirect actions on CRF neurons. The spatial expression patterns of CRF, and stressor-dependent CRF gene activation in the central nervous system (CNS) are evolutionarily conserved. This suggests conservation of the gene regulatory mechanisms that underlie tissue-specific and stressor-dependent CRF expression. Comparative genomic analysis showed that the proximal promoter regions of vertebrate CRF genes are highly conserved. Several *cis* regulatory elements and *trans* acting factors have been implicated in stressor-dependent CRF gene activation, including cyclic AMP response element binding protein (CREB), activator

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protein 1 (AP-1/ Fos/Jun), and nerve growth factor induced gene B (NGFI-B). Glucocorticoids, acting through the glucocorticoid and mineralocorticoid receptors, either repress or promote CRF expression depending on physiological state and CNS region.

I. Introduction to the neuroendocrine stress system

"Life is stress and stress is life." -Hans Selye, MD

Animals must continuously adapt to intrinsic and external environmental changes that threaten to disrupt homeostasis. Allostasis, a term first introduced by Sterling and Eyer (Sterling and Eyer, 1981), refers to the maintenance of stability through change. McEwen and colleagues (McEwen and Stellar, 1993) later applied this concept to the body's response to stressors. The vertebrate stress response involves endocrine, behavioral, autonomic, immune, and metabolic adjustments that allow animals to adapt to challenges to homeostasis (Herman et al., 1996; Speert and Seasholtz, 2001). The perception of stressors involves multiple neural pathways that ultimately converge on peptidergic neurons located in the paraventricular nucleus (PVN) in mammals, or the anterior preoptic area (POA) in nonmammalian species. These neurons produce corticotropin-releasing factor (CRF), which is the primary neurohormone that activates the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis in response to a stressor. In addition to its hypophysiotropic role, CRF coordinates many aspects of the stress response such as behavioral and autonomic adjustments (see Aguilera, 1998; Ziegler and Herman, 2002 for reviews). In nonmammalian species, CRF and related peptides are potent thyrotropin (TSH) releasing factors where they regulate critical life history

transitions such as amphibian metamorphosis by activating the thyroid axis (see De Groef et al., 2006; Denver et al., 2002 for reviews).

Corticotropin releasing factor is a 41-amino acid peptide first isolated from sheep hypothalamus and named for its stimulatory actions on corticotropin release by the anterior pituitary gland (Vale et al., 1981). Since then, CRF has been isolated from diverse species representing each vertebrate class except reptiles (see Lovejoy and Balment, 1999 for review). In addition to its hypophysiotropic role, CRF is widely expressed in the brain and spinal cord in both mammalian and non-mammalian species (Calle et al., 2005; Cummings et al., 1983; Pepels et al., 2002; Richard et al., 2004; Swanson et al., 1983; Yao et al., 2004; Zupanc et al., 1999; see Boorse and Denver, 2006 for review) where it functions as a neurotransmitter/neuronmodulator to coordinate behavioral and autonomic responses to stress (Orozco-Cabal et al., 2006; see Lovejoy and Balment, 1999 for review). Corticotropin-releasing factor and related peptides are known to play important roles in satiety and food intake (Crespi and Denver, 2004; Crespi et al., 2004; see Mastorakos and Zapanti, 2004 for review) and also influence learning and memory consolidation (see Croiset et al., 2000; Fenoglio et al., 2006; Gulpinar and Yegen, 2004 for reviews). Components of the CRF signaling pathway, including CRF, urocortins, CRF receptors and CRF binding protein are also expressed in many peripheral tissues such as the adrenal gland, spleen, heart, lung, liver, thymus, pancreas, intestines, ovary, testis, and placenta (human and higher primates) (Boorse and Denver, 2006; Muglia et al., 1994; Petrusz et al., 1985; Suda et al., 1984). Corticotropin-releasing factor and related peptides likely influence most if not all physiological functions including nervous, endocrine, vascular, cardiovascular, skeletomuscular, immune and reproductive

systems (Boorse et al., 2006; see Boorse and Denver, 2006 for review). Dysregulation of CRF and the HPA axis have been implicated in pathogenesis of several human disorders such as anxiety and depression, eating disorders, inflammatory diseases, substance abuse, and preterm parturition (see Chrousos and Gold, 1992; Majzoub et al., 1999; Tsigos and Chrousos, 2002 for reviews).

The hypophysiotropic neurons of the PVN/POA exhibit the highest level of CRF expression in vertebrate brains, and play an essential role in HPA/HPI axis regulation. In tetrapod species, CRF neurons project axons to the median eminence where neurohormones are released into the pituitary portal circulation to regulate anterior pituitary function. In teleost fishes, the hypophysiotropic CRF neurons project to the proximal pars distalis where they release their contents in close proximity to corticotrope cells (reviewed by Lovejoy and Balment, 1999). These preoptic region neurons have received greatest attention with regard to factors that regulate expression of the CRF gene (Doyon et al., 2003; Yao et al., 2004). Other brain regions, in particular limbic (amygdala, bed nucleus of the stria terminalis) and hindbrain (locus coeruleus) areas also possess CRF neurons that respond to stressors and may be influenced by glucocorticoids (see Tsigos and Chrousos, 2002; Ziegler and Herman, 2002 for reviews). This introduction chapter focuses on the physiological and molecular regulation of CRF gene expression in the CNS by stressors and by glucocorticoids.

II. Physiological regulation of CRF gene expression in the central nervous system

1. Central distribution of CRF neurons and basal (unstressed) expression of the CRF gene

Corticotropin-releasing factor is expressed throughout the brain of mammals in regions that include the limbic system (hippocampus, amygdala, nucleus accumbens, and bed nucleus of the stria terminalis), hypothalamus, thalamus, cerebral cortex, cerebellum, and hindbrain (Cummings et al., 1983; Swanson et al., 1983). Similar distribution patterns of CRF neurons have also been described in nonmammalian species, which suggests that this expression pattern arose early in vertebrate evolution and has been maintained by strong selective forces (see Lovejoy and Balment, 1999; Yao et al., 2004). This distribution pattern also suggests that the gene regulatory sequences that determine tissue specific expression are evolutionarily conserved (discussed below).

The primary site of CRF synthesis in the mammalian hypothalamus is the parvocellular subdivision of the PVN. The homologous region in nonmammalian species is the anterior preoptic area (POA; preoptic nucleus). These neurons project axons to capillaries in the external zone of the median eminence, where CRF is released into the portal blood and subsequently transported to the anterior pituitary (see Aguilera, 1998 for review). Expression of CRF in the mammalian PVN is regulated by tonic input from several brain structures. There is a circadian rhythm in the expression of CRF mRNA and heteronuclear RNA (hnRNA) in the mammalian PVN (Kwak et al., 1993; Watts et al., 2004). The cyclic expression of the CRF gene is thought to be generated and maintained by activity in the suprachiasmatic nucleus (SCN) (Szafarczyk et al., 1979). Descending pathways from the hippocampus play an important role in maintaining the set point for

basal HPA activity through the communication of glucocorticoid (GC) negative feedback (Jacobson and Sapolsky, 1991; Meijer and de Kloet, 1998).

2. Stress-activation of the central CRF system and neuronal circuitry of stress integration

Upon exposure to a stressor, the HPA/HPI axis is rapidly activated. Corticotropin releasing factor is released from axonic terminals in the median eminence within seconds, and travels to the anterior pituitary where it activates CRF receptors to increase production of adrenocorticotropic hormone (ACTH). A rise in plasma ACTH leads to increased production of GCs by the adrenal cortex, and a rise in plasma GC concentration. Glucocorticoids exert diverse effects on target tissues to mobilize energy for the body to deal with the stressor. Plasma GCs also exert feedback at different levels of the HPA axis to modulate the stress response (see Sapolsky et al., 2000; Tsigos and Chrousos, 2002; Ziegler and Herman, 2002 for reviews).

Release of CRF from the dendritic terminals of PVN neurons into the hypophyseal portal blood is the key event that leads to activation of the HPA/HPI axis during the stress response. Increases in CRF mRNA, and in some cases hnRNA (a measure of CRF gene transcription) levels in these neurons have been documented after exposure of rats to different physical, physiological, and psychological stressors (e.g., foot shock, immobilization, forced swimming, ether stress, and endotoxin stimulation; Bartanusz et al., 1993b; Chen et al., 2001; Herman et al., 1989b; Hsu et al., 1998; Imaki et al., 1991; Kovács and Sawchenko, 1996a, b; Liu et al., 2001; Ma et al., 1997a, b; Rivest et al., 1995). Work from our laboratory in the frog *Xenopus laevis* showed that both CRF

immunoreactivity and CRF hnRNA exhibited rapid increases in the POA following exposure to a shaking/confinement stressor (Yao et al., 2007; Yao et al., 2004). Studies in the teleost fish (rainbow trout *Oncorhynchus mykiss* and tilapia *Oreochromis mossambicus*) also demonstrated that stressors elevated CRF mRNA in the POA (social stressor: Doyon et al., 2003; chasing or restraint stressor: Doyon et al., 2005) and increased concentrations of CRF in the systemic circulation (capture stressor: Pepels et al., 2004b); see also Flik et al., 2006. Taken together, findings in mammals, in *Xenopus* and in fishes show that stressor-dependent activation of CRF genes arose early in vertebrate evolution, and that exposure to stressors leads to a rapid increase in CRF gene transcription.

Hypothalamic CRF neurons receive dense projections from forebrain limbic sites, brain stem autonomic centers, and a number of local hypothalamic regions. Brain sites that do not have dense direct connections with the PVN such as the hippocampus, the amygdala, and the locus coeruleus likely influence PVN neurons indirectly via a relay mechanism (see Herman and Cullinan, 1997; Ziegler and Herman, 2002 for reviews). These neuronal connections play pivotal roles in the regulation of CRF gene activity under both basal and stressor-induced conditions.

Limbic structures are rapidly activated in response to stress, indicated by extensive induction of immediate early genes (IEGs; Cullinan et al., 1995; Honkaniemi et al., 1992) and increased CRF expression (Kalin et al., 1994; Merali et al., 1998; Yao et al., 2004). Damage to the hippocampus significantly increased CRF mRNA in the PVN of rats, and caused GC hypersecretion in Cynomolgus monkeys (Herman et al., 1989c; Sapolsky et al., 1991), showing that this brain structure exerts tonic repression on PVN CRF

expression. The hippocampus appears to have a stressor-dependent inhibitory input to the PVN, since stimulation of this region decreased HPA activity, while hippocampal lesions increased secretion of ACTH and GC in response to some stressors, but not others (see Herman and Cullinan, 1997; Jacobson and Sapolsky, 1991 for reviews). It is thought that the limbic system in mammals is more sensitive to psychological and multi-modal stressors requiring higher-order sensory processing (Herman and Cullinan, 1997).

Limbic structures such as the amygdala and the bed nucleus of the stria terminalis (BNST) seem to have the opposite influence on CRF neurons in the PVN compared with the hippocampus. The central nucleus of the amygdala plays a key role in mediating neuroendocrine as well as other aspects of the stress response. Electrical stimulation of this region caused secretion of CRF from the median eminence (Weidenfeld et al., 1997), whereas lesions of the amygdala decreased CRF mRNA in the PVN and CRF-like immunoreactivity in the median eminence in unstressed rats (Beaulieu et al., 1989; Prewitt and Herman, 1994), and blocked CRF induced behavioral responses and the HPA response to acoustic and photic stimulation (Liang et al., 1992). Injection of CRF into the amygdala enhanced inhibitory avoidance learning and decreased exploratory activity in rats (Liang and Lee, 1988), and antagonism of CRF receptors in the central amygdala attenuated foot shock-induced freezing behavior (Swiergiel et al., 1993). Thus, the amygdala circuit may be essential for relaying a stimulatory signal with certain types of stressors to the PVN CRF neurons (Gray, 1993; Herman and Cullinan, 1997).

Similar to the lesion studies of the amygdala, ablation of the BNST caused a significant decrease in CRF mRNA in the PVN in unstressed rats (Herman et al., 1994). Electrical stimulation of the medial and rostral BNST resulted in an increase in plasma

GC (Dunn, 1987), and lesions of the lateral BNST also attenuated conditioned fear-induced release of ACTH and GC (Gray et al., 1993). Furthermore, in response to a psychological stressor, CRF mRNA was increased in the limbic system (the central nucleus of the amygdala and the dorsolateral BNST) without a detectable increase in the hypothalamus (Makino et al., 1999), suggesting that the limbic stress pathway responds to certain types of stressors independent of HPA axis activation.

The brain stem is essential for autonomic responses to stressors. Sympathetic and parasympathetic systems of the autonomic nervous system regulate skeletal muscles and smooth muscles of the vasculature, heart, gut, and other organs to coordinate with other aspects of the stress response. The brain stem also mediates arousal, sensory processing, and responses to physiological stress (see Brunson et al., 2001; Tsigos and Chrousos, 2002; Ziegler and Herman, 2002 for reviews). Hemisection of the lower brainstem (including the locus coeruleus) decreased CRF mRNA in the ipsilateral PVN in unstressed rats (Kiss et al., 1996), suggesting basal CRF expression in the PVN is under tonic stimulatory control by brain stem structures. Lesions of the locus coeruleus caused a reduction in GC release after restraint stress, suggesting a stimulatory input from this site to the hypothalamic neuroendocrine cells (Ziegler et al., 1999).

Corticotropin-releasing factor also increases its own expression and that of CRF receptors, and thus might have autocrine or paracrine functions within a tissue. Both i.c.v. administration of CRF and exposure to acute immobilization rapidly induced expression of CRF and CRF receptor 1 mRNA in the PVN, suggesting autoregulation of the CRF gene in the stress response (Imaki et al., 1996; Luo et al., 1994). Pretreatment with a CRF receptor 1 antagonist significantly reduced stressor-induced ACTH secretion and *c-fos*

mRNA expression in the PVN (Imaki et al., 2001), suggesting that autoregulation of CRF contributes to stressor-dependent activation of the CRF gene, and perhaps other neurosecretory cells in the PVN.

In addition to CRF, other neuropeptides such as arginine vasopressin (AVP) and urocortins (CRF related peptides) may also be involved in stress activation of the HPA/HPI axis, but these pathways are beyond the scope of the current discussion (see Lovejoy and Balment, 1999 for review).

3. Feedback regulation of CRF expression by glucocorticoids

Glucocorticoids, the end effectors of the HPA/HPI axis, play critical roles in regulation of CRF expression and adrenal/interrenal activity. High concentrations of circulating GCs induced by stressors have an inhibitory effect on the synthesis and secretion of hypothalamic CRF, and the production and secretion of ACTH by the pituitary. Such negative feedback is a critical mechanism for terminating the stress response and restoring the system to homeostasis (see Makino et al., 2002a for review). In unstressed animals, CRF mRNA levels in the PVN exhibit a reverse correlation with plasma GC concentration (Kwak et al., 1992; Watts and Swanson, 1989). However, the circadian rhythm of CRF mRNA and heteronuclear RNA (hnRNA) levels in the PVN are maintained in adrenalectomized rats, indicating circulating GCs are not required for the cyclic expression of CRF (Kwak et al., 1993; Watts et al., 2004).

Two types of intracellular GC receptors are expressed in the brain: the high affinity type I receptor (also called the mineralocorticoid receptor; MR), and the lower affinity type II receptor (also called the glucocorticoid receptor; GR) (see Funder, 1997 for

review). The GR is expressed throughout the mammalian brain, with the highest level in the PVN, and in the the anterior pituitary. By contrast, the highest expression of MR in mammals is found in the hippocampus. Due to its high affinity for corticosterone, the MR expressed in the hippocampus is thought to mediate the feedback actions of GCs at the circadian peak, and to maintain the basal activity of the HPA axis (see De Kloet et al., 1998 for review). Following an acute stressor, the concentration of plasma GC is increased, and the low affinity receptor GR may play an essential role in mediating GC feedback directly on the PVN and the pituitary corticotropes to terminate the HPA stress response by negative feedback (De Kloet et al., 1998).

The predominant sites for inhibition of the HPA axis by the GR are the PVN and the pituitary corticotropes. It has been shown that activated GR suppresses expression of the CRF gene in the hypothalamus, and inhibits expression of pro-opiomelanocortin (POMC; a precursor protein of a variety of peptides including ACTH) in pituitary corticotropes (Dallman et al., 1992; see below for more discussion). The GR may also mediate feedback regulation by GCs in the limbic system including the hippocampus and amygdala, as well as in brain stem neurons such as the locus coeruleus. These neuronal groups have extensive direct and indirect connections with CRF neurons in the PVN, and activation of GR at these sites is involved in modulation of HPA activity in response to acute as well as chronic stress (De Kloet et al., 1998). In addition, acute stress caused increased GR expression in the PVN and pituitary (Mamalaki et al., 1992), while repeated stress or sustained administration of exogenous GCs down-regulated GR in the hippocampus and amygdala (Sapolsky et al., 1984), providing a secondary mechanism to modulate CRF expression and HPA activity.

Studies in rats showed that administration of the GC synthesis inhibitor metyrapone caused a rapid increase in CRF hnRNA in the PVN followed by an increase in CRF mRNA (Herman et al., 1992). Adrenalectomy (ADX) also increased CRF mRNA in the PVN, while high doses of dexamethasone decreased it (Beyer et al., 1988; Jingami et al., 1985). These results suggest that expression of the CRF gene is under tonic suppression by GCs in the unstressed condition. In addition, ADX augmented, but dexamethasone pretreatment inhibited CRF hnRNA and mRNA increases in the rat PVN following exposure to restraint or photic stimulation (Feldman and Weidenfeld, 2002; Imaki et al., 1995b).

However, physiological concentrations of GCs in intact animals have been shown to be essential for the initiation and sustenance of the stress response. In fact, in ADX rats levels of CRF hnRNA and mRNA in the PVN were decreased following stress stimulation (Tanimura and Watts, 1998). ADX rats supplemented with a low dose of GCs showed augmented PVN CRF mRNA levels following hypovolemia (Tanimura and Watts, 1998). In another study in rat, both CRF hnRNA and mRNA increased and remained elevated during sustained hypovolemia in adrenal intact animals (Tanimura and Watts, 2000). However, these investigators found that in ADX animals hypovolemia caused an earlier increase in CRF hnRNA in the PVN, which declined rapidly; no changes in CRF mRNA in the PVN were observed in the ADX rats. Furthermore, CRF peptide concentrations in the portal blood of ADX rats were decreased (Plotsky and Sawchenko, 1987), and stimulus-evoked CRF secretion from the mediobasal hypothalamus of transgenic mice with impaired GR was reduced compared to wild type (Dijkstra et al., 1998). Thus plasma GCs apparently exert a dual action on CRF

expression in the PVN: high concentrations of circulating GCs (exogenous or obtained during stress) inhibit CRF gene transcription in the PVN (negative feedback action), while lower levels of GCs may exert a permissive action on stressor-dependent gene activation and secretion, and the maintenance of CRF expression in the initial stages of the stress response (see Tanimura and Watts, 2001 for review).

Evidence for a role for the MR in negative feedback regulation of the HPA comes from studies of MR knockout mice. Compared with wildtype, the knockouts exhibited increased HPA activity as evidenced by elevated CRF mRNA levels in the PVN, and increased plasma ACTH and corticosterone concentrations (Gass et al., 2001). Thus GCs acting via the MR expressed in the limbic system are thought to exert a "tonic" inhibition on the CRF gene in the PVN, while GR likely mediates the "phasic" regulation of CRF in the stress response (Gass et al., 2001).

III. Molecular mechanisms of transcriptional regulation of CRF genes

The commonalities in the spatial, temporal and stressor-dependent expression of CRF among species suggest that the basic *cis* elements responsible for the regulation of CRF genes were present in the earliest vertebrates, and have been maintained by positive selection owing to the critical role that CRF plays in adaptive stress responses. We conducted a comparative genomic analysis of vertebrate CRF genes and identified several regions of strong sequence similarity (Fig. 1.1). Within the coding region the area of highest sequence similarity was in the mature peptide, while the cryptic peptide of the prohormone exhibited much lower conservation. There was virtually no sequence conservation in the introns among vertebrate classes, but surprisingly, the 3' untranslated

regions (3' UTR) exhibited several highly conserved stretches of nucleotide sequence. The functional significance of sequence conservation in the 3' UTR is not known, but could be related to conserved elements necessary for translational or transcriptional control. After the mature peptides, the proximal promoter regions shared the highest degree of sequence similarity; analysis of ~330 bp of upstream sequences showed 94% sequence similarity among human, rat, and ovine CRF genes (Vamvakopoulos et al., 1990), and ~72% similarity among frog (xCRFb gene) and mammalian genes.

Computer analysis of the proximal CRF promoters revealed several putative transcription regulatory elements, including two TATA boxes, CAAT boxes, a cAMP response element (CRE), several AP-1 protein (Fos/Jun) binding sites, several half-glucocorticoid response elements (GRE), and half-estrogen responsive elements (ERE) (Vamvakopoulos and Chrousos, 1994; Vamvakopoulos et al., 1990; Fig. 1.2). The majority of the *cis* elements within the proximal promoter region are highly conserved among vertebrate CRF genes (Yao et al., 2007; Fig. 1.2). The function of some of these putative regulatory elements in the human and rat CRF genes have been addressed using cell transfection and *in vitro* binding assays. A schematic diagram of several of these regulatory pathways is shown in Fig. 1.3. Below we address the functions of the major *cis* elements that have been characterized thus far, and discuss new data that provide evidence for their importance in mediating stressor-dependent gene activation *in vivo*.

1. Cyclic AMP response element binding protein

The activation of CRF promoter activity through the cAMP-dependent protein kinase A (PKA) pathway has been shown by cell transfection assay in a number of cell types:

mouse anterior pituitary cell AtT20 (Guardiola-Diaz et al., 1996; King et al., 2002; Malkoski et al., 1997; Rosen et al., 1992; Van et al., 1990), rat pheochromocytoma cell PC-12 (Guardiola-Diaz et al., 1994; Seasholtz et al., 1988), chicken macrophage HD11 (Van, 1993), neuroblastoma cell SK-N-MC, choriocaarccinoma cell JAR (Spengler et al., 1992), COS-7 cell (Vamvakopoulos and Chrousos, 1993b), and primary human placental cytotrophoblast cells (Cheng et al., 2000a) Also, upregulation of the endogenous CRF gene following activation of the PKA pathway has been shown in a primary fetal rat hypothalamic cell line (Emanuel et al., 1990).

Cyclic AMP response element binding protein (CREB) is a member of the basic leucine zipper (bZIP) family of transcription factors. This family also includes the cAMP response element modulatory protein (CREM) and the activating transcription factor 1 (ATF-1) (see Lonze and Ginty, 2002 for review). Members of this family form homo or heterodimers through their leucine zipper domains and bind to DNA. Transcriptional activity of CREB is regulated by phosphorylation at conserved serine residues, and several intracellular signaling pathways can induce phosphorylation of CREB, including the PKA, Ca^{2+}, and mitogen-activated protein kinases (MAPKs) (see Johannessen et al., 2004; Shaywitz and Greenberg, 1999 for reviews). Although phosphorylation did not alter CREB binding to DNA *in vitro* (Richards et al., 1996; Wu et al., 1998), phosphorylation has been shown to increase CREB DNA binding activity without changing the total CREB protein level *in vivo* (Hatalski and Baram, 1997; Hiroi et al., 2004; Whitehead and Carter, 1997; Wolfl et al., 1999). Phosphorylation of CREB also induces recruitment of the coactivators CBP and p300 to gene promoters (although the CRF promoter has not been studied in this regard) which promote gene activation

through their intrinsic histone acetyl transferase activity and interaction with the RNA polymerase II complex (see Johannessen et al., 2004; Mayr and Montminy, 2001 for reviews). A highly conserved cAMP response element (CRE) TGACGTCA is present in the human CRF gene promoter centered at –224 bp upstream of the transcription start site. This element can confer cAMP-responsiveness to a heterologous promoter, and mutation of this sequence abolished stimulation by 8-bromo-cAMP or forskolin (Seasholtz et al., 1988; Spengler et al., 1992). We have also shown that this element in the frog CRF gene promoters is responsible for PKA pathway-mediated gene activation *in vitro* and *in vivo* (Yao et al., 2007).

Several studies have shown that in the rat, CREB is rapidly phosphorylated in CRF neurons in response to various stressors, preceding elevation of CRF expression in those cells (Bilang-Bleuel et al., 2002; Chen et al., 2001; Kovács and Sawchenko, 1996b). We also showed in the frog that exposure to a shaking stressor increased phosphorylated CREB immunoreactivity in the POA by 20 min, and that pCREB immunoreactivity (CREB-ir) colocalizes with CRF-ir (Yao et al., 2007). Though indirect, these results support the view that activation of CREB could contribute to increased transcription of the CRF gene during the stress response.

The addition of 8-bromo-cAMP to perfused rat hypothalami caused a dose-dependent release of CRF (Suda et al., 1985; Turkelson, 1988). Microinjection of 8-bromo-cAMP into the PVN of rats increased CRF mRNA content, while preinjection of antisense oligodeoxyonucleotides to CREB blocked the increase in CRF mRNA caused by insulin-induced hypoglycemia (Itoi et al., 1996). We recently reported that mutation of the CRE site abolished PKA or and stressor-dependent activation of the frog CRF promoter in

transfected tadpole or frog brain (Yao et al., 2007). These data provide strong support that the PKA/CREB pathway, acting via the CRE site in the CRF promoter, plays a critical role in stress-induced CRF expression *in vivo*.

Among the other CREB family members, interest has focused recently on the inducible cAMP early repressor (ICER) which may be responsible for turning off stressor-dependent activation of the CRF gene. Consisting of only the DNA-binding domain and lacking the transactivation domain of CREM, ICER is able to dimerize with CREB and CREM and bind to a consensus CRE site, but does not activate transcription. Instead, the protein functions as a potent repressor of cAMP-induced transcription. Expression of ICER is induced by other CRE-binding proteins such as CREB or CREM binding to the ICER promoter, and it is the only member of the CREB family whose activity is regulated by its intracellular concentration rather than by phosphorylation state. Induction of ICER is thought to function as a feedback mechanism, turning down the activation induced by the initial phosphorylation of CREB or CREM (see Mioduszewska et al., 2003; Sassone-Corsi, 1995 for reviews). In unstressed rodents ICRE-immunoreactivity was found in the paraventricular nucleus in the hypothalamus (Conti et al., 2004; Kell et al., 2004). Restraint stress caused an increase in ICER mRNA and CREM recruitment to the CRF promoter in the PVN, which could be responsible for the decline in CRF mRNA following its initial induction by stress (Shepard et al., 2005). ICER was also increased in the hypothalamus following antidepressant treatment, and it is required for antidepressant treatment to reduce stress-induced elevations in plasma corticosterone in mice (Conti et al., 2004). In addition, stressors induced expression of ICER mRNA in both the pituitary and the adrenal gland (Della Fazia et al., 1998;

Mazzucchelli and Sassone-Corsi, 1999), suggesting upregulation of ICER may be an integral component of the regulation of the HPA axis, and perhaps a common mechanism for feedback regulation of stress-activated gene expression.

2. Activator protein 1 (Fos and Jun)

The protein kinase C (PKC) pathway, acting through the transcription factors Fos and Jun (originally termed activator protein 1; AP-1), is also implicated in the transactivation of the CRF gene. *c-fos* and *c-Jun* belong to the family of immediate early genes (IEGs) that were first identified based on their rapid and transient induction by growth factors (McMahon and Monroe, 1992; Robertson, 1992). The induction of *c-fos* (mRNA and/or Fos-ir) has been widely used as a marker for neuronal activation, and for mapping the stress-response circuitry in the CNS (Ceccatelli et al., 1989b; Emmert and Herman, 1999; see Hoffman et al., 1993; Senba and Ueyama, 1997 for reviews). Studies in rodents have shown that in response to a wide range of stressors *c-fos* mRNA increased in several stress-related structures including the PVN, central and medial amygdala, bed nucleus of the stria terminalis, hippocampus, and locus coeruleus (Ceccatelli et al., 1989b; Emmert and Herman, 1999; Imaki et al., 1992, 1993; Sawchenko et al., 1996; Watts and Sanchez-Watts, 2002). In agreement with findings in rodents, we also showed that Fos-ir was increased in the POA of the frog following exposure to a shaking stressor, and that Fos-ir was colocalized with CRF-ir (Yao et al., 2004). These results suggest that AP-1 proteins may play an evolutionarily conserved role in CRF gene regulation in response to stressors.

In COS-7 or HD11 cells transfected with a human CRF promoter-reporter construct, treatment with the phorbol ester TPA, which activates the PKC pathway, increased CRF promoter activity (Vamvakopoulos and Chrousos, 1993b; Van, 1993). Activation of the PKC pathway also increased expression of the endogenous CRF gene as measured by mRNA levels in the human hepatoma cell line NPLC, and in primary fetal rat hypothalamic cells (Adler et al., 1992; Emanuel et al., 1990; Rosen et al., 1992). The effect of TPA treatment on CRF promoter activity is cell type-dependent, and is not observed in transfected AtT 20 or PC-12 cells; nor did TPA upregulate endogenous CRF gene expression in primary cultures of rat amygdala (Kasckow et al., 2003a; Rosen et al., 1992; Van et al., 1990; A. F. Seasholtz, personal communication; and M. Yao and R.J. Denver, unpublished data). Treatment of NPLC cells with TPA increased CRF mRNA, which required *de novo* protein synthesis (Adler et al., 1992). In these cells TPA also increased CRF mRNA size owing to a 3-fold increase in the length of the poly(A) tail, which could potentially affect mRNA stability or translatability (Adler et al., 1992).

Several AP-1 binding sites are present in the 5' flanking region of vertebrate CRF genes (Vamvakopoulos and Chrousos, 1994; Fig. 1.2), although Fos/Jun binding at each of these sites has not been characterized. Two closely located AP-1 sites overlap with three putative GRE sites, and it was shown that both AP-1 proteins and GR can specifically bind to this region of the human CRF promoter (Malkoski and Dorin, 1999). We have also found that GR can bind to the homologous region of the frog CRF gene (M. Yao and R.J. Denver, unpublished data). Furthermore, mutational analysis suggests that this region is important both for AP-1-mediated stimulation and for GR-mediated repression of CRF promoter activity (Malkoski and Dorin, 1999). Composite AP-1/GRE

sites have been found in a number of genes, and opposing actions of AP-1 and GR proteins at these sites have been demonstrated (e.g., proliferin gene: Diamond et al., 1990; Miner and Yamamoto, 1992; α–fetoprotein gene: Zhang et al., 1991). Thus antagonism, or even cooperation, between Fos/Jun and GR (or MR) may be a common regulatory phenomenon. This hypothesis remains to be tested in the context of CRF gene regulation.

Despite the findings that *c-fos* is induced in CRF neurons by stressors *in vivo*, and that the PKC pathway can activate CRF gene transcription *in vitro*, other studies failed to find evidence for a role for this pathway. Itoi and colleagues (Itoi et al., 1996) observed no effect on PVN CRF mRNA levels following microinjection of TPA into the PVN of conscious rats; conversely, i.c.v. injection of antisense oligodeoxynucleotides directed against *c-fos* or *c-jun* mRNA did not affect activation of the CRF gene by insulin-induced hypoglycemia (Itoi et al., 1996). Other studies also demonstrated that there is a delayed increase in *c-fos* mRNA compared with CRF hnRNA in the PVN in response to a stressor (Kovács and Sawchenko, 1996a, b). Furthermore, blockade of protein synthesis in rats prevented induction of Fos, but had no effect on CREB phosphorylation or CRF hnRNA expression in the PVN during an acute stress response (Kovács et al., 1998). These findings have led to the conclusion that the early upregulation of CRF expression during the stress response does not depend on Fos, at least not in the PVN (Kovács et al., 1998; Kovács and Sawchenko, 1996a, b). Instead, rapid phosphorylation of CREB appears to be primarily responsible for the early induction of the CRF gene in the stress response. The PKC pathway effectors Fos/Jun may play a role in the delayed regulation of CRF expression in response to sustained or chronic stressors.

3. Corticosteroid receptors

Glucocorticoids play a critical role in the regulation of both basal and stressor-induced CRF gene expression. The actions of GCs on CRF expression vary with cell-type, physiological state and hormone dosage. Studies in rodents found that administration of corticosterone decreased CRF mRNA in the PVN, but increased CRF mRNA in the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Makino et al., 1994a, b), suggesting tissue-specific GC actions on CRF gene expression. It was also well demonstrated that GCs incrase CRF expression in the human planceta (Schulkin, 1999). Treatment of NPLC cells with dexamethasone (DEX), a potent agonist of the glucocorticoid receptor (GR), decreased endogenous CRF mRNA. Dexamethasone reduced basal activity of an 8 kb upstream human CRF gene promoter-reporter construct in transfected AtT20 cells (Adler et al., 1988; Rosen et al., 1992). Dexamethasone also significantly reduced PKA pathway-dependent activation of the human proximal CRF promoter in AtT20 cells, and TPA-induced CRF promoter activation in COS-7 and NPLC cells (Guardiola-Diaz et al., 1996; King et al., 2002; Malkoski et al., 1997; Rosen et al., 1992; Vamvakopoulos and Chrousos, 1993b; Van et al., 1990). By contrast, DEX enhanced PKA pathway-dependent induction of CRF mRNA in PC-12 cells and CRF promoter activity in transfected human placenta cells (Cheng et al., 2000b; Guardiola-Diaz et al., 1996); but, had no effect on basal or forskolin-induced CRF expression in primary amygdalar cultures (Kasckow et al., 1997).

The cellular targets, and the molecular mechanisms by which GCs regulate CRF gene expression are incompletely understood. As described above, the regulation of PVN CRF

expression is direct, likely involving GRE half sites present in the CRF promoter, and also indirect, involving a complex limbic circuit that includes the hippocampus and perhaps the amygdala (see Tsigos and Chrousos, 2002; Ziegler and Herman, 2002 for reviews). Glucocorticoids can also regulate CRF expression in these limbic structures, but the character of the regulation may differ from the PVN. The direct actions of GCs on the PVN or limbic structures in CRF gene regulation likely requires, in large part the expression in these cells of GR or MR (Cole et al., 1995; Gass et al., 2000; Tronche et al., 1999). However, there is evidence that rapid actions of GCs on the hippocampus and PVN are accomplished via mechanisms that do not depend on transcriptional activation by these receptors (i.e., nongenomic mechanisms; see Borski, 2000; Chen and Qiu, 1999; Norman et al., 2004 for reviews; discussed below).

The direct actions of GR on target genes are characterized by either DNA-binding dependent, or DNA binding independent mechanisms. Several regions of the human CRF gene have been found to be occupied by GR *in vitro* using DNase I protection assay (Guardiola-Diaz et al., 1996). One of these regions, the composite AP-1/GRE site, was shown to be important for the inhibition of 8-bromo-cAMP-induced activation of the CRF promoter by GCs in transfected cells (Malkoski and Dorin, 1999; Malkoski et al., 1997). We have found using electrophoretic mobility shift assay (EMSA) that GR binds to the homologous region of the frog CRF promoters (M. Yao and R.J. Denver, unpublished data). However, it is not yet clear in any species whether the direct regulation of CRF by GR is exerted through DNA-binding to these putative GRE sites.

Several lines of evidence suggest that the DNA-binding function of GR is critical for its transactivation function on certain genes (Reichardt et al., 1998; Scott et al., 1998). By

contrast, GR repression of AP-1 or NGFI-B-dependent transcription may not require direct interaction of GR with the target gene, but rather depends on protein-protein interaction (or "cross-talk") to inhibit their transactivation activity. In fact, GR was found to directly interact with several transcription factors including AP-1 (Fos/Jun), NGFI-B, CREB, and nuclear factor-κB (NF-κB) *in vitro* (De Bosscher et al., 1997; Diamond et al., 1990; Imai et al., 1993; Jonat et al., 1990; Martens et al., 2005; Yang-Yen et al., 1990), and a functional DNA-binding domain is not required for GR inhibition of AP-1 at the collagenase promoter (Heck et al., 1994; Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990), or of NGFI-B at the POMC promoter (Martens et al., 2005). In transgenic mice that express a DNA binding-defective GR mutant the cross-talk between GR and other transcription factors was not impaired (Reichardt et al., 1998).

In addition to the direct protein-protein interaction model, a competition mechanism has been proposed which postulates that GR antagonizes other transcription factors by competing for a limited amount of nuclear coactivators such as CBP, p300, and steroid receptor coactivator (SRC)-1 (Aarnisalo et al., 1998; Kamei et al., 1996; Sheppard et al., 1998). However, other studies have argued against this competition model by showing that repression of NF-κB by GR was not limited by intracellular concentrations of coactivators (De Bosscher et al., 2000; De Bosscher et al., 2001).

The GR may also interfere with target gene transcription indirectly by inhibiting activation of the c-Jun N-terminal kinase (JNK), and MAPKs such as the extracellular regulated kinase (ERK) and p38 (Caelles et al., 1997; Hirasawa et al., 1998; Lasa et al., 2001; Swantek et al., 1997). In cultured cells DEX increased expression of MAPK phosphatase MKP-1, an inhibitor of MAPKs (Kassel et al., 2001; Lasa et al., 2002).

Another mechanism by which GCs may regulate CRF gene expression is by modulating mRNA turnover. Treatment of rat hypothalamic explants with corticosterone significantly reduced CRF mRNA half-life, suggesting that in addition to their role as repressors of transcription, GCs may also down-regulate CRF expression by decreasing CRF mRNA stability (Ma et al., 2001).

Nongenomic mechanisms of GC signaling

In addition to the genomic mechanisms of GC actions, non-genomic actions that are mediated by G-protein coupled membrane receptors have also been shown (see Borski, 2000; Chen and Qiu, 1999; Norman et al., 2004 for reviews). At the level of the pituitary gland, GCs administered to rats intravenously inhibited CRF-induced ACTH secretion within 15 min, and this effect was not blocked by pretreatment with the GR antagonist, RU486 (Hinz and Hirschelmann, 2000). Similar rapid inhibition by GCs on CRF-induced ACTH secretion was observed with pituitary glands in perifusion culture (Widmaier and Dallman, 1984), and DEX rapidly inhibited CRF-induced cAMP accumulation and ACTH release by primary rat pituitary cells, an effect that could not be blocked by inhibition of protein synthesis (Bilezikjian and Vale, 1983; Iwasaki et al., 1997). At the hypothalamic level, DEX caused rapid suppression of basal CRF release from rat hypothalamic explants in perifusion culture (Suda et al., 1985).

In the hippocampus, corticosterone treatment rapidly increased synaptic potentiation in the CA1 area in mouse (Wiegert et al., 2006). Since hippocampal neurons project to the PVN and are thought to influence CRF neurons through a GABAergic inhibitory relay mechanism (Herman et al., 1995b; Herman et al., 2002), GCs could rapidly

modulate activity of PVN neurosecretary cells by regulating hippocampal output. Taken together, these studies highlight the potential for rapid, membrane mediated GC feedback on HPA activity.

4. Nerve Growth Factor Induced Gene B (NGFI-B)

Nerve Growth Factor Induced Gene B (also termed Nur77) is another IEG that is rapidly induced by exposure to stressors. NGFI-B and the related protein Nurr1 are orphan receptors belonging to the steroid/thyroid receptor superfamily (Hazel et al., 1988; Milbrandt, 1988). Expression of NGFI-B was rapidly induced in the PVN of rats following acute footshock (Rivest and Rivier, 1994) or capsaicin exposure (Honkaniemi et al., 1994), and in several brain regions following seizure (Watson and Milbrandt, 1989). Injection (i.c.v.) of CRF also caused an increase in NGFI-B mRNA in the PVN within 30 min, preceding the increase in CRF mRNA in the same region (Parkes et al., 1993). These results suggest that NGFI-B may be involved in the stressor-dependent activation of hypothalamic neurons.

The consensus NGFI-B binding site (NGFI-B response element; NBRE) comprises a 9 bp sequence AAAAGGTCA (Wilson et al., 1991). A NBRE with 1 bp difference from the consensus sequence is present in the rat CRF promoter (Wilson et al., 1991), but the functional significance of this element is unknown. Our comparative genomic analysis revealed a perfect NBRE in the *X. laevis* CRFb gene promoter, and a highly conserved NBRE with 1 bp substitution in the corresponding regions of the human, chimpanzee, mouse, and *X. laevis* CRFa genes (Yao et al., 2007). In the rat anterior pituitary cell line AtT20, treatment with CRF or forskolin rapidly increased nuclear DNA binding activity

of NGFI-B dimers (Maira et al., 2003). Similar treatments of AtT20 cells induced NGFI-B expression, and cotransfection with a NGFI-B expression vector resulted in a strong stimulation of the activity of the ovine CRF and POMC promoters in transfected AtT20 cells (Kovalovsky et al., 2002; Murphy and Conneely, 1997). Treatment of the mouse adrenocortical cell line Y1 with ACTH rapidly induced NGFI-B mRNA, and expression of recombinant NGFI-B significantly increased the promoter activity of the steroidogenic enzyme 21-hydroxylase gene in a dose dependent manner (Wilson et al., 1993). In addition, it has also been shown that NGFI-B antagonizes the actions of GR (Martens et al., 2005; Okabe et al., 1998; Phillips et al., 1997). Taken together, NGFI-B (and possibly other family members) may mediate stress-regulation of vertebrate CRF genes in the hypothalamus, the POMC gene in the pituitary corticotropes, and steroidogenic enzymes in the adrenal glands, and thus coordinate the activity of the HPA/HPI axis at multiple levels (Murphy and Conneely, 1997).

5. Other regulators of CRF expression

Other endocrine factors, in particular gonadal steroids, have been shown to interact with the CRF system to coordinate stress responses. For example, consensus estrogen response elements (EREs) were identified in the human CRF promoter, and shown to be capable of binding estrogen receptor (ER) and mediating estrogen's stimulatory effects on gene expression in CV-1 cells (Vamvakopoulos and Chrousos, 1993a). On the other hand, estrogen treatment of transfected human placental cells down-regulated basal and 8-bromo-cAMP-stimulated CRF promoter activity (Ni et al., 2004). Estrogen replacement of ovariectomized monkeys resulted in elevation of CRF mRNA in the PVN, an effect

that was abrogated by progesterone administration (Roy et al., 1999). These findings suggest that sex steroids may regulate CRF gene expression in a tissue specific manner, and this direct regulation may explain, in whole or in part the sexual dimorphism in basal CRF expression, and gender differences in the stress response.

The repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) is a zinc finger transcription factor initially thought to repress neuronal genes in nonneuronal cells by binding to the repressor element-1/neuron-restrictive silencing element (RE-1/NRSE) (Chong et al., 1995; Kraner et al., 1992; Mori et al., 1992; Schoenherr and Anderson, 1995). Later work showed a more complex regulation of gene expression by REST/NRSF, suggesting that this protein may have functions other than its role as a neuron restrictive silencing factor (Bessis et al., 1997; Kallunki et al., 1998; Palm et al., 1998). A highly conserved putative RE-1/NRSE is present in the intron of the CRF genes of human, rat, mouse, sheep, and *X. laevis* (Seth and Majzoub, 2001). We also identified a putative RE-1/NRSE site in the intron of the chicken CRF gene (Table 1.1). The REST/NRSF binds to the RE-1/NRSE site in the mouse/rat CRF gene and represses transcriptional activity in heterologous transfection assays (Seth and Majzoub, 2001). It is possible that REST/NRSF plays a role in the developmental and/or tissue-specific expression of CRF gene but this hypothesis has yet to be tested.

The POU-homeodomain protein Brn-2 has also been implicated in the regulation of the CRF gene in the hypothalamus. Brn-2 is required for the development of hypothalamic CRF neurons in that homozygous Brn-2 null mice lack CRF neurons (Schonemann et al., 1995). It binds to the CRF promoter *in vitro*, and increases CRF promoter activity in heterologous systems. It is not yet known whether this transcription

factor is involved in the stressor-dependent regulation of the CRF gene (see Burbach, 2002 for review).

Cytokines (interleukin-1, 2 and 6), GABA, and biogenic amines such as acetylcholine, serotonin, and norepinephrine have also been shown to modulate CRF expression *in vitro* and *in vivo* (see Itoi et al., 1998; Pisarska et al., 2001 for reviews). However, the intracellular signaling pathways induced by these factors, and the cellular processes that are initiated leading to the regulation of CRF expression have not been elucidated.

IV. Organization of the dissertation

My dissertation research focused on understanding the regulatory mechanisms of vertebrate CRF genes by taking a comparative/evolutionary approach. For this I used the South African clawed frog *Xenopus laevis* as a model system. My dissertation is comprised of four data chapters followed by a summary chapter. The major questions addressed and key experiments conducted for the studies described in each of the data chapters are discussed below.

The primary aim of the study described in chapter two was to map the expression of CRF in the CNS of the frog *X. laevis* by immunocytochemistry. I also investigated the stress responsiveness of CRF neurons in discrete brain regions.

In chapter three I investigated the conservation of the promoter elements among vertebrate CRF genes and focused on the role of the CRE site in mediating gene activation by the PKA pathway and in the stress response. I found that the CRF gene

structure and upstream sequences are highly conserved among vertebrates, and I identified putative transcription factor binding sites in the CRF proximal promoters.

In chapter four I describe the distribution of GR-ir throughout the CNS of *X. laevis*, and the regulation of GR by GCs. I show that the GR is widely expressed in the frog brain and anterior pituitary gland in a general pattern very similar to that of mammals. I found that GCs downregulated GR-ir in the POA, several limbic structures, and the anterior pituitary, suggesting that GCs may modulate the activity of the stress axis by regulating the level of their own receptors.

In chapter five I describe studies in which I investigated the feedback regulation of CRF neurons by GCs in *X. laevis*. I show that the expression of CRF-ir in the POA and several limbic structures is regulated by circulating GCs, and that the regulatory effects are cell type-specific. I also found that the proximal promoters of the frog CRF genes are responsive to inhibition of forskolin-induced activity by GCs.

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Table 1.1. Repressor element-1/neuron-restrictive silencing element (RE-1/NRSE) sequences found within the introns of CRF genes of human, mouse, rat, sheep, chicken, and *Xenopus laevis* (CRFa).

Consensus	TTCAGCACCACGGACAGCGCC
Human CRF	---------G----------
Mouse CRF	---------G---------T-
Rat CRF	---------G---------T-
Sheep CRF	--------T----------
Chicken CRF	---------G --------G-
Xenopus CRFa	----------AA

Highly conserved RE-1/NRSE sites are present in the introns of the human, mouse, rat, sheep, and *X. laevis* (gene a) CRF genes (from Seth and Majzoub, 2001). We also identified a putative RE-1/NRSE site in the intron of the chicken CRF gene. We could not locate a conserved RE-1/NRSE site in the intron of the *X. laevis* CRFb gene. The location of this site within the CRF introns is highly variable among species.

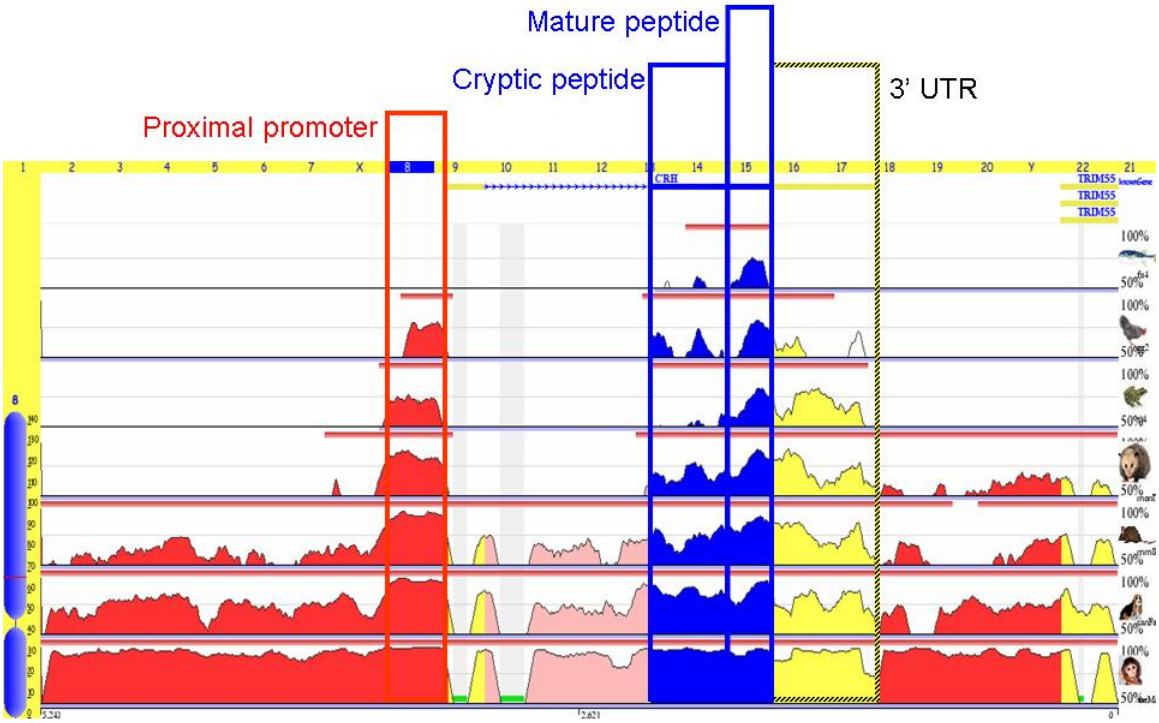

Figure 1.1. Alignment of vertebrate CRF genes. In descending order on figure: pufferfish (*Fugu rubripes)*, chicken (*Gallus domesticus*), frog (*Xenopus tropicalis*), opossum *(Didelphis virginiana),* mouse (*Mus musculus*), dog (*Canis domesticus*), and rhesus monkey (*Macaca mulatta*) genomes. The human genome is used as the base sequence. Peaks on the graph represent % identity between the aligned genomes at the specified positions. A vertical axis cut-off of 50% identity was used. Alignments were done using the Evolutionary Conserved Region (ECR) browser (Ovcharenko et al., 2004; http://ecrbrowser.dcode.org/). The most highly conserved regions are the mature peptide and the proximal promoter. The cryptic peptide is part of the prohormone that is cleaved to generate the mature peptide. Note that only limited 5' upstream sequence was available in the current ECR browser update for *X. tropicalis* and *Fugu rubripes* (the extent of sequence for each species used is indicated by the gray bars under each histogram.)

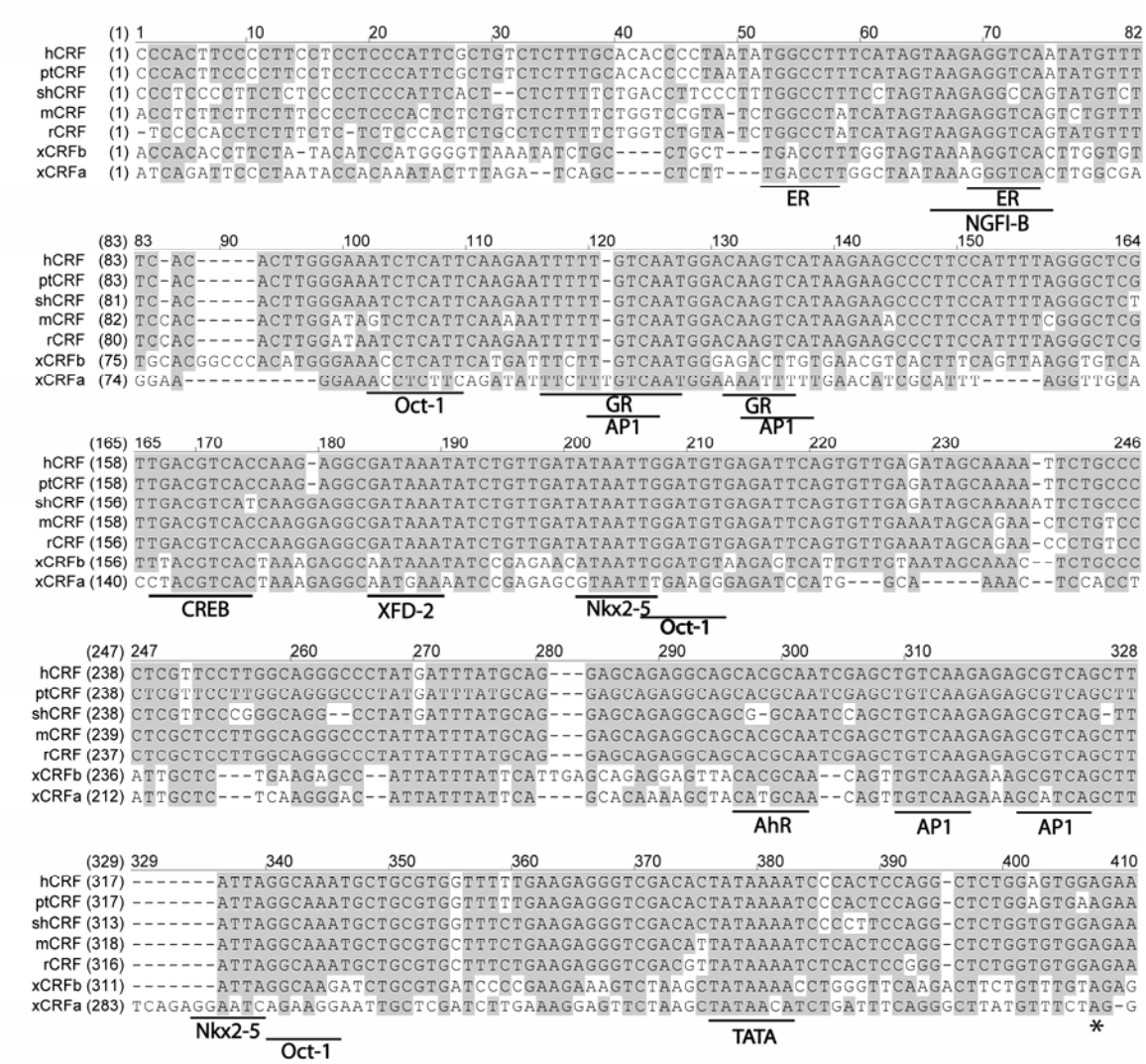

Figure 1.2. ClustalW alignment of the proximal promoter regions of human (h), chimp (pt), sheep (sh), mouse (m), rat (r), and *Xenopus laevis* (x; genes a and b) CRF genes. Sequences that are identical among CRF genes are shadowed. The asterisk indicates the predicted transcriptional start sites for each gene. Putative binding sites of transcription factors are indicated. NGFI-B: nerve growth factor induced gene B; ER: estrogen receptor; Oct-1, octomer biding factor 1; GR, glucocotricoid receptor; AP1, activation protein 1 (Jun/ Fos); CREB, cyclic AMP response element binding protein; XFD2, *Xenopus* forkhead domain factor 2; Nkx 2-5, cardiac-specific homobox protein; AhR, aryl hydrocarbon receptor. (Modified from Yao et al., 2007).

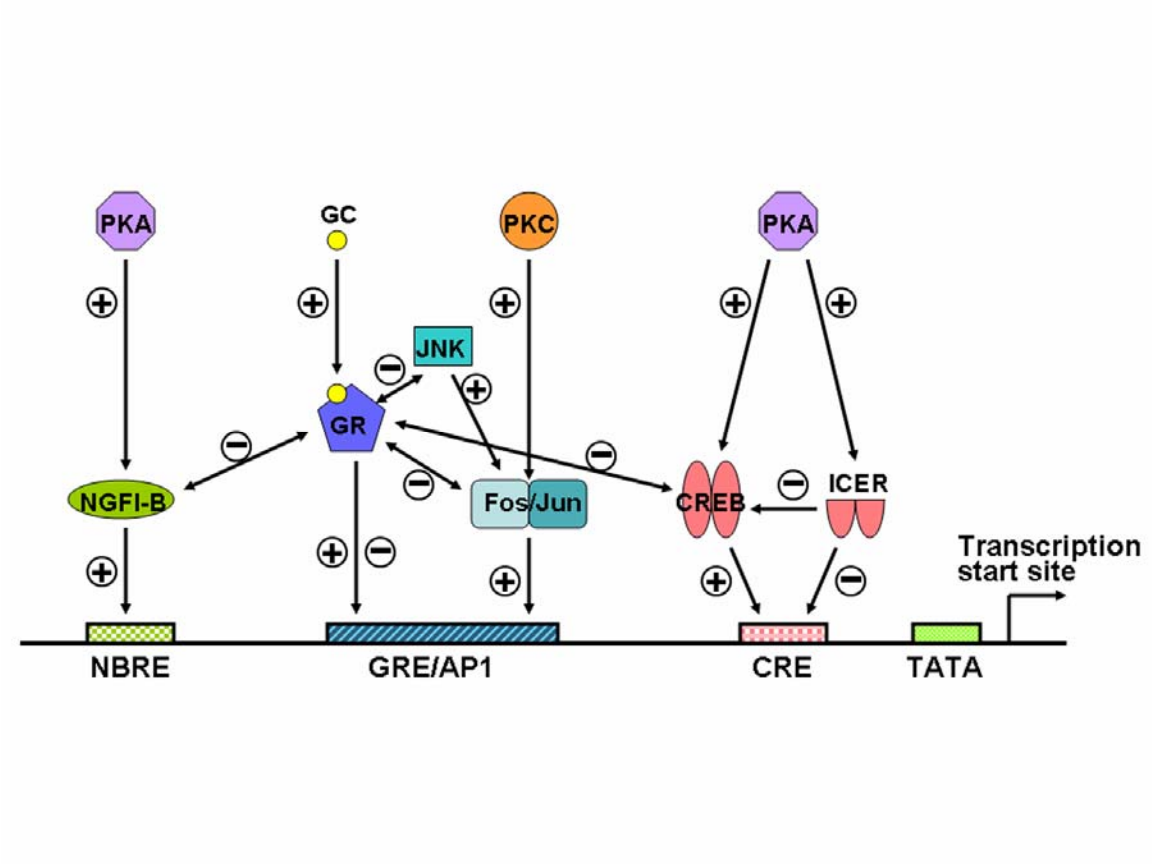

Figure 1.3. Schematic representation of the major pathways involved in the regulation of CRF gene transcription at the proximal promoter. PKA: protein kinase A; PKC: protein kinase C; GC: glucocorticoid; GR: glucocorticoid receptor; GRE/AP1: composite glucocorticoid response element and AP1 (Fos/Jun) binding sites; NGFI-B: nerve growth factor-induced gene B; NBRE: NGFI-B-response element; CRE: cyclic AMP-response element; CREB: CRE binding protein; ICER: inducible cAMP early repressor; JNK: Jun N-terminal kinase. TATA box and transcription start site are indicated. Positive and negative regulation are indicated as + and – respectively.

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CHAPTER 2

DISTRIBUTION AND ACUTE STRESSOR-INDUCED ACTIVATION OF CORTICOTROPIN-RELEASING FACTOR NEURONS IN THE CNS OF *XENOPUS LAEVIS*[2]

Abstract

In mammals, corticotropin-releasing factor (CRF) and related peptides are known to play essential roles in the regulation of neuroendocrine, autonomic and behavioral responses to physical and emotional stress. In nonmammalian species, CRF-like peptides are hypothesized to play similar neuroendocrine and neurocrine roles. However, there is relatively little detailed information on the distribution of CRF neurons in the central nervous system (CNS) of nonmammalian vertebrates, and there are currently no comparative data on stress-induced changes in CRF neuronal physiology. We used a specific, affinity-purified antibody raised against synthetic *Xenopus laevis* CRF to map the distribution of CRF in the CNS of juvenile South African clawed frog. We then analyzed stress-induced changes in CRF immunoreactivity (CRF-ir) throughout the CNS. We found that CRF-positive cell bodies and fibers are widely distributed throughout the brain and rostral spinal cord of juvenile *X. laevis*. Strong CRF-ir was found in cell bodies and fibers in the anterior preoptic area (POA, an area homologous to the mammalian

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paraventricular nucleus) and the external zone of the median eminence. Specific CRF-ir cell bodies and fibers were also identified in the septum, pallium, and striatum in the telencephalon; the amygdala, bed nucleus of the stria terminalis (BNST), and various hypothalamic and thalamic nuclei in the diencephalon; the tectum, torus semicirculus, and tegmental nuclei of the mesencephalon; the cerebellum and locus coeruleus in the rhombencephalon; and the ventral horn of the rostral spinal cord.

To determine if exposure to an acute physical stressor alters CRF neuronal physiology, we exposed juvenile frogs to shaking/handling and conducted morphometric analysis. Plasma corticosterone was significantly elevated by 30 minutes after exposure to the stressor and continued to increase up to 6 hr. Morphometric analysis of CRF-ir after 4 hr of stress showed a significant increase in CRF-ir in parvocellular neurons of the anterior preoptic area, the medial amygdala (MeA), and the bed nucleus of the stria terminalis, but not in other brain regions. The stress-induced increase in CRF-ir in the POA was associated with increased Fos-like immunoreactivity (Fos-LI), and confocal microscopy showed that CRF-ir colocalized with Fos-LI in a subset of Fos-LI-positive neurons. Our results support the view that the basic pattern of CNS CRF expression arose early in vertebrate evolution and lend further support to earlier studies suggesting that amphibians may be a transitional species for descending CRF-ergic pathways. Furthermore, CRF neurons in the frog brain exhibit changes in response to a physical stressor that parallel those seen in mammals, and thus are likely to play an active role in mediating neuroendocrine, behavioral and autonomic stress responses.

Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid neuropeptide first isolated by Vale and colleagues from the ovine hypothalamus (Spiess et al., 1981), has since been isolated and characterized in representatives of most vertebrate classes (Lovejoy and Balment, 1999). Subsequent to its isolation, it was discovered that CRF is a member of a family of related peptides in vertebrates that includes the fish urotensins-I, frog sauvagine and the urocortin/stresscopin peptides (Lovejoy and Balment, 1999). Corticotropin-releasing factor is generally regarded as the primary hypothalamic neurohormone within the hypothalamic-pituitary-adrenal (HPA) axis responsible for mediating neuroendocrine responses to stress. In addition to its role as a hypophysiotropin, CRF also functions as a neurotransmitter/neuromodulator, mediating autonomic and behavioral responses to stress (for review, see Dautzenberg and Hauger, 2002; Lovejoy and Balment, 1999). However, these conclusions are based largely on studies in mammals, with little comparative data available in nonmammalian species.

The mature CRF peptide is highly conserved among vertebrates, especially among the tetrapods (Lovejoy and Balment, 1999). There are two closely related CRF genes in *Xenopus laevis* (designated xCRFa and xCRFb) due to the pseudotetraploid nature of its genome (Stenzel-Poore et al., 1992). The mature peptides encoded by the two genes are identical in their deduced primary amino acid sequences. The amino acid sequences of the CRFs from human and rat are identical, and differ from the frog peptide in only three positions (Thompson et al., 1987; Vale et al., 1981). Among the few amphibian species in which CRF has been cloned, the mature peptides of *Spea hammondii* (Genbank accession #AY262255) and *Phyllomedusa sauvageii* (Genbank accession #AY596828) are identical

to *X. laevis* (Genbank accession #S50096), while the CRF of *Rana catesbeiana* differs from the other anuran peptides in only the first amino acid residue (Genbank accession #AB161633).

In mammals CRF is widely distributed throughout the CNS, but a circumscribed group of parvocellular neurosecretory neurons localized to the paraventricular nucleus of the hypothalamus (PVN) comprise the major hypophysiotropic neurons of the hypothalamic-pituitary-adrenal (HPA) axis. The PVN neurons are the principal source of CRF delivered to the external zone of the median eminence, where CRF is released into the hypophyseal portal system and subsequently stimulates the release of ACTH from the anterior pituitary (for review, see Owens and Nemeroff, 1991; Sawchenko and Swanson, 1989).

Earlier studies of the distribution of CRF-like immunoreactivity in amphibians depended on the use of antisera raised against mammalian CRFs. CRF-immunoreactivity (CRF-ir) has been shown in somata and fibers in the brain and pituitary of several amphibian species (Urodela: *Pleurodeles waltlii*, *Ambystoma mexicaum*: Olivereau et al., 1987, and *Triturus cristatus*: Fasolo et al., 1984; Anura: *Rana ridibunda*: Gonzalez and Lederis, 1988; Olivereau et al., 1987, *Rana catesbeiana*: Gonzalez and Lederis, 1988, and *X. laevis*: Ogawa et al., 1995; Olivereau et al., 1987). In these studies the greatest intensity of CRF-like immunoreactivity was found in the preoptic nucleus (homolog of the mammalian PVN) and in the external zone of the median eminence. Close morphological examination of CRF projections in ranid frogs revealed that the CRF neurons of the ventral preoptic nucleus project caudally and terminate in the external zone of the median eminence (Carr and Norris, 1990; Gonzalez and Lederis, 1988; Tonon et al., 1985).

Most studies in amphibians have focused on the hypophysiotropic CRF neurons and little attention has been directed towards other CRF systems in the brain and spinal cord. Using heterologous antisera Bhargava and Rao (Bhargava and Rao, 1993) reported CRF-ir in the mesencephalon (optic tectum and interpeduncular nucleus) of tiger frogs and Olivereau and colleagues (Olivereau et al., 1987) reported CRF-ir in the cerebellum and interpeduncular nucleus of two ranid and two urodele species. In rats the CRF neurons of the PVN (particularly the parvocellular neurons) have been shown to be stress-responsive. For example, CRF gene expression (mRNA, heteronuclear RNA [hnRNA] or CRF-ir) increased in the PVN following exposure to different stressor paradigms (Bartanusz et al., 1993b; Bruijnzeel et al., 2001; Hand et al., 2002; Herman et al., 1989b; Hsu et al., 1998; Imaki et al., 1991; Imaki et al., 1996; Imaki et al., 1995a; Kovács and Sawchenko, 1996a; Liu et al., 2001; Ma et al., 1997a). However, these neurons respond differently to different kinds of stressors, and decreases in CRF gene expression (mRNA or CRF-ir) in these cells as well as no change have been reported (Imaki et al., 2001; Kay-Nishiyama and Watts, 1999; Ma et al., 1997a; Makino et al., 1999). Similar studies have yet to be conducted in species other than rats.

In the present study we used homologous, affinity purified antibodies generated against synthetic *X. laevis* CRF (xCRF) to map the distribution of CRF-ir somata and fibers throughout the CNS of juvenile *X. laevis*. We then used morphometric analysis to analyze stress-induced changes in CRF neuronal physiology throughout the frog brain. Our results support and extend previous studies of CRF-ir in the frog brain by showing, in addition to forebrain and mesencephalic locations previously reported (Bhargava and Rao, 1993; Fasolo et al., 1984; Gonzalez and Lederis, 1988; Ogawa et al., 1995;

Olivereau et al., 1987; Verhaert et al., 1984), strong CRF expression in the cerebellum, rhombencephalon and rostral spinal cord of frogs. We also present evidence for stress-activation of CRF neurons in the anterior preoptic area (POA), the medial amygdala (MeA), and the bed nucleus of the stria terminalis (BNST) in *X. laevis*. We also show that the immediate early gene product Fos (Fos-like immunoreactivity; Fos-LI) increased in the POA following exposure to the stressor and Fos-LI was.colocalized with CRF-ir in a subset of Fos-LI-positive neurons.

Materials and Methods

Animals and tissue preparation

Juvenile *X. laevis* (2.5-5.0g body weight) were derived from an in-house breeding stock (population originally derived from Xenopus I, Dexter, MI). Animals were fed beef liver and maintained at 22-23°C on a 12L:12D photoperiod. For immunohistochemical mapping of CRF neurons and processes, animals were anesthetized by immersion in 0.01% benzocaine and given intracerebroventricular injections of colchicine (7.5ng/μl in 0.06% saline and 1% methyl green, 150-200 nl/injection). Frogs were euthanized 42 hours after injection and the skulls were fixed overnight in 4% paraformaldehyde at 4°C.

The brains were dissected and post-fixed for one hour, followed by immersion in 30% sucrose/PBS at 4°C overnight. Following the protection with sucrose, brains were snap-frozen and stored at –80°C until sectioning. Prior to sectioning brains were embedded using M-1 embedding matrix (Shandon Lipshaw, Inc., Pittsburgh, PA). Brains were cryo-sectioned transversely at 12 μm and stored at –80°C until analysis. Eight brains were analyzed for CRF-ir distribution in the frog CNS.

Antibody purification

We identified CRF-positive neurons and processes using a polyclonal rabbit antiserum raised against synthetic *X. laevis* CRF (xCRF) conjugated to human alpha globulins (Boorse and Denver, 2004; Denver, 1997). The IgG fraction of the xCRF antiserum was first affinity purified using an Affi-Gel Protein A purification column and a column containing xCRF conjugated to Affi-Gel 10 (Bio-Rad Laboratories, CA). The construction of the xCRF-Affi-Gel 10 matrix was modified to adjust for the low isoelectric point of xCRF (pI=5.05) following the manufacturer's specifications. The affinity column was prepared by coupling 1mg of synthetic xCRF dissolved in 1 ml coupling buffer (0.1M MOPS pH=7.5, 80mM $CaCl_2$) to 0.5ml washed Affi-Gel 10 matrix. The reaction included 100,000 cpm [125I]-xCRF (prepared by the iodogen method; Denver, 1997) to allow for determination of coupling efficiency (which was 86%).

We recently isolated cDNA clones for *X. laevis* UCN1 (Genbank accession #AY596827) and UCN3 (Genbank accession #AY596826; G.C. Boorse and R.J. Denver, unpublished) and we have synthesized the peptides based on their deduced amino acid sequences. The deduced amino acid sequence of *X. laevis* UCN1 (xUCN1) is 72.5% similar to rodent UCN1. *X. laevis* UCN3 (xUCN3) shares 87.5% sequence similarity with rodent UCN3. We determined the crossreactivities of our xCRF antiserum with xUCN1 and xUCN3 by radioimmunoassay (see also Boorse and Denver, 2004 for crossreactivity analysis with heterologous CRF-like peptides). The xUCN1 showed approximately 3% crossreaction, while UCN3 did not crossreact with the anti-xCRF serum (G.C. Boorse

and R.J. Denver, unpublished). We used these peptides to preabsorb our anti-xCRF IgG prior to immunohistochemistry (see below).

Immunohistochemistry

Immunohistochemistry (IHC) was used to localize xCRF-ir and Fos-like immunoreactivity (Fos-LI) neurons in the brain and rostral spinal cord. The rabbit polyclonal c-Fos antiserum that we used (sc-253, Santa Cruz Biotechnology) was raised against an epitope that shares 88% sequence similarity with *Xenopus* c-Fos (communication with Santa Cruz Biotechnology; the sequence of the epitope is proprietary) and has been used successfully for detection of Fos-LI in *X. laevis* (Ubink et al., 1997). The specificity of our affinity-purified anti-xCRF IgG was confirmed by pre-absorption of the primary antibody with synthetic xCRF, xUCN1 or xUCN3 (50 μg/ml) overnight at 4°C prior to IHC.

We used two methods to detect CRF-ir, immunofluorescence or horseradish peroxidase/diaminobenzidine (HRP/DAB). For immunofluorescent detection of xCRF-ir or Fos-LI the sections were incubated in the primary antiserum (1:15 dilution for the anti-xCRF IgG, and 1:4000 dilution for anti-Fos serum in PBS with 2% NGS) for 20 h at 20°C or 4°C. For HRP/DAB the tissue sections were first incubated in 0.3% hydrogen peroxide (in distilled water) for 30 min to quench endogenous peroxidase activity, followed by 30 min incubation in 2% normal goat serum (NGS) in PBS. We used a Vectastain elite ABC kit (rabbit) and Vector VIP kit (both from Vector Laboratories, Inc., Burlingame, CA) for HRP/DAB staining following the manufacturer's protocols. The micrographs shown in the figures are derived from HRP/DAB staining (except for the

dual immunofluorescence shown in Fig. 9), but both methods gave identical results with regard to the distribution of CRF-ir cells. However, the immunofluorescence method allowed for better visualization of neuronal processes, and thus CRF-ir fibers depicted in Figs. 2 and 3 are based on analysis by immunofluorescence.

Because antisera to both xCRF and c-Fos were generated in rabbits we conducted dual immunofluorescence histochemistry according to the methods of Negoescu and colleagues (Negoescu et al., 1994). This method depends on sequential incubation with primary antisera and the use of a fluorescein (FITC)-conjugated Fab fragment as the secondary antibody for the first primary antibody (in our case the anti-xCRF). Briefly, cryosections were incubated in blocking buffer (10% normal goat serum in TBS) for 1 hr at room temperature and then incubated overnight at 4°C with the affinity purified anti-xCRF IgG diluted 1:15 in blocking buffer. Sections were washed three times with TBS and then incubated overnight at 4°C with a fluorescein (FITC)-conjugated Fab fragment of goat anti-rabbit IgG(H+L) (diluted 1:100 ; Jackson ImmunoResearch, PA). After rinsing, the sections were incubated with the anti-Fos serum (1:4000) for 3 hr at r.t., followed by extensive washes and then incubation with a Cy3-conjugated polyconal goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch) for 2 hr at r.t.. After rinsing, the sections were nuclear-counterstained with DAPI (200 ng/ml; Sigma D9542) for 30 min, then cover-slipped with Prolong Anti-Fade (Molecular Probes, OR). To control for crossreaction of the second secondary antibody with the first primary antibody we conducted interference control staining for the use of Fab fragment as described by Negoescu and colleagues (Negoescu et al., 1994). Briefly, the dual immunofluorescence procedures were conducted by substitution of non-immune serum for the second primary

antiserum (i.e. rabbit anti-Fos serum). No staining by the second secondary antiserum (i.e. Cy3-conjugated goat anti-rabbit IgG) was observed, demonstrating that the Fab fragment of goat anti-rabbit IgG saturated all the immunoreactive sites of the first primary antibody (i.e. rabbit anti-xCRF IgG), and no crossreaction occurred between the second secondary antiserum and the first primary antibody. Single immunofluorescence histochemistry for xCRF or c-Fos was also conducted for comparison.

Routine fluorescence microscopy was conducted using an Olympus U-TBI90 microscope and digital images captured using a Retiga 1300R camera. Sections were also analyzed by confocal microscopy (Zeiss laser scanning confocal microscope). Optical sections (1 μm thickness) were captured through the Z-axis to determine if CRF-ir and Fos-LI were colocalized.

Shaking stressor

Two days prior to the start of the experiment, juvenile frogs (body weight 5-8 g) were randomly assigned to treatment groups and placed in tanks surrounded by opaque barriers to block visual stimuli. On the day of the experiment, the "stressed" group was placed into 32 oz white polypropylene containers, with 3 frogs in 250ml well water. The containers were placed on an orbital shaker and shaken continuously at 100 rpm for various times. The shaking intensity was just enough to require constant spatial adjustment by the frogs but not enough to cause physical damage (Glennemeier and Denver, 2002a). The frogs were euthanized by submersion in 0.05% benzocaine. The unstressed (nonhandled) group was euthanized at the same time. For plasma corticosterone analysis frogs were euthanized at 0, 30 minutes, 3 hr and 6 hr after the initiation of the shaking stressor (n=10/time point). For immunohistochemistry, frogs

were euthanized at 0 time and then 4 hr after the initiation of the shaking stressor (n=6/time point). All frogs were weighed, blood was collected immediately into prechilled capillary tubes, and heads were fixed in cold 4% paraformaldehyde.

Plasma corticosterone radioimmunoassay (RIA)

Corticosterone was measured in frog plasma using methods described by Licht and colleagues (Licht et al., 1983). Briefly, plasma was extracted using diethyl ether and the RIA was conducted using a corticosterone antiserum obtained from Esoterix Endocrinology (San Diego, CA). All samples were measured in a single assay, and the intra-assay coefficient of variation was 10%. The effect of exposure to the shaking stressor on plasma corticosterone content was analyzed using one-way ANOVA of shaking time versus corticosterone content.

Morphometric analysis of CRF immunoreactivity

CRF immunoreactivity was quantified on brain sections using MetaMorph software (v 6.2r4). All sections were processed simultaneously under identical conditions. Three to five sections containing each brain region of interest were analyzed for each animal. All sections were carefully matched for anatomical level, and digital images were captured at 100X or 200X magnification for densitometric analysis. A random procedure was carried out throughout the image analysis. The brain regions were analyzed using a hand-made frame covering the complete area of interest, and the total area of the positive staining particles above a standard density threshold in the selected area was counted automatically. The mean density for each animal was calculated as the total positive

staining area on the multiple sections divided by the total selected area. The effects of the shaking stressor on CRF-ir mean density in the brain regions analyzed was analyzed by Student's unpaired *t*-test ($p<0.05$) using the SPSS statistical package v11.5 for Windows.

Results

Distribution of CRF immunoreactivity in juvenile *X. laevis* brain and spinal cord

We found CRF-ir cell bodies and fibers to be widely distributed throughout the CNS of juvenile *X. laevis.* Alternating sections exhibited robust staining in the same neuroanatomical regions. Furthermore, preabsorption of the affinity-purified antibodies with synthetic xCRF completely abolished all staining, while preabsorption with synthetic xUCN1 or xUCN3 did not alter staining, thus demonstrating the specificity of the antibodies (Fig. 2.1). Well-organized groups of cells and fibers were found in regions of the telencephalon, diencephalon, mesencephalon, rhombencephalon and rostral spinal cord. Contrary to earlier reports that used heterologous antisera (Tonon et al., 1985; Verburg-van Kemenade et al., 1987) we did not observe CRF-ir in the pituitary gland. A schematic representation of the distribution of CRF positive cell bodies and fibers throughout the *X. laevis* brain and rostral spinal cord is shown in Figs. 2.2 and 2.3.

Telencephalon The most rostral sites of CRF-ir were localized to small somata in the mitral layer of the olfactory bulb, the medial septum, medial, dorsal and lateral pallium, and striatum. Large somata with CRF-ir axons were found in the nucleus accumbens and less frequently in the lateral pallium (Fig. 2.2 A-C; see also Fig. 2.4 A-D).

Diencephalon The largest group of CRF-ir cells was found in both the magnocellular and parvocellular divisions of the POA (Fig. 2.2 D, E; see also Fig. 2.4 F and Fig. 2.7). Immunoreactive somata were predominantly found in the subependymal zones with axons projecting medially, sending projections towards the third ventricle, and also laterally. Sparse but large immunoreactive somata were seen in the ependymal zones with axons projecting medially to the ventricle. In caudal regions of the POA, immunoreactive somata were also found in a more dorsal position with axons projecting ventrolaterally.

Scattered, smaller CRF-ir cells and fibers were also localized to the amygdala and the bed nucleus of the stria terminalis (Fig. 2.2 D; see also Fig. 2.4 E, G; Fig. 2.7). Well-organized groups of smaller cells were also observed in various hypothalamic and thalamic nuclei (Fig. 2.2 D-F, see also Fig. 2.4 H, I; Fig. 2.5 A). In the thalamus, CRF-ir was observed in well-organized bands of cells consistent with the neuroanatomical organization and localization of the habenular, anterior thalamic, ventromedial thalamic and ventrolateral thalamic nuclei. The ventral thalamus and posterior tubercle contained larger somata, localized to ventricular regions with axons projecting ventrolaterally and dorsolaterally, respectively. Strong immunoreactive fiber staining was present in the external capsule of the median eminence (Fig. 2.3 H; see also Fig. 2.5 B). There was an absence of CRF-ir cells in all pituitary lobes.

Mesencephalon Relatively weak but specific staining of smaller cells was observed in the oculomotor nuclei, tectal regions, the torus semicirculus (homolog of the mammalian inferior colliculi), and various tegmental nuclei (Fig. 2.3 H, I; see also Fig.2.5 C, D).

Rhombencephalon Robust CRF-ir was localized to the locus coeruleus (Fig. 2.3 J; see also Fig. 2.5 E) and the cerebellum (primarily the Purkinje cell layer, Fig. 2.3 K; see also Fig. 2.5 F). Smaller immunoreactive cells were localized to the reticular nuclei, and the nuclei of cranial nerves V (trigeminal motor), VIIId/v (nucleus of the lateral line), IX (glossopharyngeal) and X (vagal) (Fig. 2.3 K-M; see also Fig. 5 G).

Rostral Spinal Cord CRF-ir was restricted to the ventral horn of the rostral spinal cord. Robust staining was present in the large pyramidal-shaped lateral motor neurons (Fig. 2.3 N). Less intense CRF-ir was observed in smaller cells of the medial and dorsal regions of the ventral horn. Caudal regions of the spinal cord were not examined.

Effects of shaking stressor on CRF-ir in the CNS

Exposure of juvenile frogs to shaking stress resulted in the rapid activation of the hypothalamic-pituitary interrenal (HPI) axis as evidenced by a 3.6-fold increase in plasma corticosterone by 30 minutes and a 4.3-fold increase by 3 hr (Fig. 2.6); plasma corticosterone continued to increase by 20-fold up to 6 hr.

We analyzed an intermediate time point (4 hr) for changes in CRF-ir in the frog CNS. The shaking stressor produced a robust increase in the intensity of CRF-ir staining and the number of CRF-ir positive neurons in the POA (Fig. 2.7). In stressed animals greater intensity of CRF staining was observed in both the ependymal and subependymal zones compared with unstressed controls. Densitometric analysis revealed that shaking stress significantly increased CRF-ir count density in the POA (ependymal and subependymal

zones analyzed together; 177% of control, n = 6/treatment, P = 0.0001; Fig. 2.7). Exposure to shaking stressor also significantly increased CRF-ir in the medial amygdala (MeA, 134% of control, n = 5-6/treatment, P = 0.028) and in the bed nucleus of the stria terminalis (BNST, 151% of control, n = 5-6/treatment, P = 0.003) at the level of POA (Fig. 2.7).

The shaking stressor did not alter CRF-ir in the median eminence (n = 5/treatment, P = 0.8238). No changes in CRF-ir were observed in the nucleus accumbens (Acc; n = 5/treatment, P = 0.235), the locus ceoruleus (LC; n = 5-6/treatment, P = 0.365), or the cerebellum (Cb; n = 5-6/treatment, P =0.168).

Effects of shaking stress on Fos-LI

As previously described by Ubink and colleagues (Ubink et al., 1997), we found Fos-LI to be exclusively nuclear with varying intensity from cell to cell. In the CNS of unstressed frogs, Fos-LI positive nuclei were occasionally observed in the internal granule cell layer, striatum, nucleus accumbens, lateral pallium, and ventral hypothalamic nucleus. No Fos-LI positive neurons were seen in the POA in unstressed frogs, nor were they detected in other regions of the hypothalamus, amygdala, BNST, thalamus, pituitary, optic tectum, cerebellum, or spinal cord. Following exposure to 4 hr shaking stressor, we observed an increase in Fos-LI positive cells throughout the brain. In addition to more Fos-LI-positive cells in the same regions as in the unstressed frog brains, detectable Fos-LI expanded to the lateral septum, medial pallium, lateral and medial amygdala, BNST, POA, various thalamic nuclei, suprachiasmatic nucleus, posterior tuberculum, optic

tectum, torus semicircularis, and tegmental nuclei. By contrast, we observed no Fos-LI-positive cells in the cerebellum or the LC following exposure to the shaking stressor. We quantified the robust increase in the number of Fos-LI positive cells in the POA, which were mainly localized to the rostro-ventral region (n= 5-6/treatment, P=0.002; Fig. 2.8). The total number of Fos-LI-positive cells in the POA differed significantly between individuals, ranging from 14 to 150 per animal.

Dual immunofluorescent, confocal microscopy for CRF and Fos in the POA showed that these two proteins were colocalized in a subset of Fos-LI-positive neurons (Fig. 2.9). However, not all CRF-positive cells exhibited Fos-LI. We were unable to precisely quantitate the numbers of Fos-LI, CRF-ir and CRF-ir+Fos-LI positive cells in this experiment.

Discussion

We have mapped the distribution of CRF-positive somata and fibers in the brain and rostral spinal cord of *X. laevis* using a homologous and highly specific affinity-purified antiserum. We also show for the first time stressor-induced changes in the physiology of discrete populations of CRF neurons in the CNS of a non-mammalian species. The distribution of CRF-ir within the hypothalamus of *X. laevis* reported here is in principal agreement with the distribution of CRF-ir described in other amphibian species in which antisera to mammalian CRFs were used (Bhargava and Rao, 1993; Carr and Norris, 1990; Gonzalez and Lederis, 1988; Olivereau et al., 1987; Verhaert et al., 1984). Our findings of abundant CRF somata in the POA and CRF-ir in the external zone of the ME, and the

existence of fibers projecting from the POA to the ME is consistent with previous studies and further supports a role for CRF as a hypophysiotropin in amphibians. Absence of CRF-ir cells in all pituitary lobes in our study is consistent with previous findings in other amphibian species (*Rana tigrina*: Bhargava and Rao, 1993, *Rana castenbeiana*: Carr and Norris, 1990; Gonzalez and Lederis, 1988) but contrary to previous reports in *X. laevis* in which heterologous antisera were used (Tonon et al., 1985; Verburg-van Kemenade et al., 1987). However, recent data suggest that the majority of CRF-like immunoreactivity in the *X. laevis* pituitary neural lobe is derived from UCN1 (Calle et al., 2005).

Few studies have examined in detail the CRF-ir in locations outside of the hypothalamic/pituitary region in amphibians. Our data provide the first detailed distribution map of CRF-ir in the telecephalon, diencephalon, mesencephalon, rhombencephalon, and rostral spinal cord in an amphibian species. The distribution of CRF-ir in the telecephalon of *X. laevis* is consistent with previous results obtained in *Rana tigrina* and *Rana ridibunda* (Bhargava and Rao, 1993; Tonon et al., 1985). We also identified CRF-ir positive cells and fibers in the amygdala, which is in agreement with previous reports in frogs (Bhargava and Rao, 1993; Gonzalez and Lederis, 1988), reptiles (Avalos et al., 1993; Mancera et al., 1991; Silveira et al., 2001), birds (Richard et al., 2004; Yamada and Mikami, 1985), and mammals (Merchenthaler et al., 1982). In the mesencephalon of other amphibian species, CRF-ir was reportedly restricted to the interpeduncular nucleus and tectal regions (Bhargava and Rao, 1993; Olivereau et al., 1987). By contrast, in *X. laevis* we observed expanded mesencephalic sites of CRF-ir, including the oculomotor nuclei, tectal regions, the torus semicirculus (homolog of the mammalian inferior colliculi), and various tegmental nuclei. Consistent with our study,

the oculomotor complex has been shown to express CRF-ir in reptiles (Mancera et al., 1991; Silveira et al., 2001) and birds (Bons et al., 1988), while in an amphibian, this structure was recently shown to express UCN-like-ir (*Rana esculenta*, Kozicz et al., 2002; using a heterologous antiserum). In the rhombencephalon, we identified robust CRF-ir in the locus coeruleus and the cerebellum (primarily the Purkinje cell layer). While this pattern of CRF-ir has not been described in other amphibian species, CRF is widely expressed in the cerebellum (all three layers) and in the locus coeruleus in mammals (Morin et al., 1999; Richard et al., 2004; Swanson et al., 1983). In support of our findings in *X. laevis*, the torus semicirculus and tectal regions are also regions of CRF-ir in other vertebrates (reptiles: Avalos et al., 1993; birds: Richard et al., 2004; mammals: Merchenthaler, 1984). Reticular locations and cranial nerves have not previously been shown to be CRF immunoreactive in amphibian species; however, a number of other vertebrate species including reptiles (Mancera et al., 1991; Silveira et al., 2001) birds (Bons et al., 1988; Jozsa et al., 1984; Richard et al., 2004) and mammals (Merchenthaler et al., 1982; Morin et al., 1999; Swanson et al., 1983) exhibit CRF-ir in these areas. Ours is the first study to report CRF-ir in the spinal cord of amphibians. It should be noted that it is not clear whether previous studies in amphibians investigated rostral spinal cord levels for CRF-ir. Spinal cord CRF-ir has been described in mammals; but, unlike frogs, it was localized to the dorsal horn (Merchenthaler, 1984).

The wide distribution of CRF in the frog CNS is similar to that reported in mammals, and further supports a role for CRF as a neurotransmitter/neuromodulator in addition to its role as a neurohormone. The existence of CRF-ir in the subependymal zones of the telecephalon, in the amygdala, the cerebellum, locus coeruleus, various cranial nerve

nuclei, and the spinal cord in both mammals (Merchenthaler et al., 1982; Shioda et al., 1985; Swanson et al., 1983) and the frog suggests that the pattern of central CRF expression and CRF neuronal circuitry arose early in vertebrate evolution (see also Lovejoy and Balment, 1999). Furthermore, our study describes for the first time the presence of descending CRF-ergic pathways in a non-amniote species. Lovejoy and Balment (Lovejoy and Balment, 1999) suggested that amphibians could represent a transitional species with respect to descending CRF-ergic pathways and our results lend support to this idea.

It is now well established that the mammalian PVN (homologous to the amphibian POA) is the principal site of neuroendocrine responses to stress (Ziegler and Herman, 2002). Early studies in an amphibian (*Bufo melanosticus*) found an elevation in plasma corticosteroids after electrical stimulation of the POA, ventral hypothalamus, or the median eminence, but not after stimulation of other brain regions (Roy, 1969/71). Also, lesioning of the ventral region of the POA in *X. laevis* tadpoles caused the pituitary corticotropes to regress (Notenboom et al., 1976). In the present study we investigated the effects of a physical stressor, handling/shaking on CRF neuronal physiology in *X. laevis*. Exposure to the shaking stressor for 4 hr caused a robust increase in both the CRF-ir staining intensity and the number of cells stained in the POA, compared with unstressed controls (Fig. 2.7). We also observed significant increases in CRF-ir in the MeA and BNST, which suggests that these structures are involved in mediating the stress response. By contrast, we found no changes in CRF-ir in the median eminence, nucleus accumbens, locus coeruleus, or cerebellum. The robust increase in CRF-ir in the POA and the parallel elevation of plasma corticosterone supports the view that these are the primary

hypophysiotropic CRF neurons regulating the activity of the hypothalamic-pituitary-interrenal axis in *X. laevis*.

Our findings that CRF-ir was increased in the MeA and in the BNST at the level of POA in frogs represents the first report that CRF neurons resident in these CNS structures are responsive to stress. Studies of neuronal connections of the amygdala in mammals reveal extensive connections with cortical and subcortical regions (for review, see McDonald, 1998; Sah et al., 2003). Electrical stimulation of the amygdala of rats elicited autonomic, endocrine, and behavioral responses similar to those induced by exposure to stressors (Gelsema et al., 1989; Rosen and Davis, 1988, 1990). In mammals, the central nucleus of the amygdala (CeA) is involved with fear/anxiety-related behaviors, and the MeA has been shown to play a role in cardiovascular responses to restraint stress and the control of hemodynamics (Cullinan et al., 1995; Gray, 1991; Kubo et al., 2004; Rosen and Davis, 1988, 1990). The BNST is believed to play an important role in stress regulation of the HPA axis by relaying to the PVN from the amygdala and the hippocampus (Herman and Cullinan, 1997; Schulkin et al., 1998).

The functions of the different compartments of the amygdala and the BNST in nonmammalian species are poorly understood. We followed the anatomical definition of these nuclei suggested by Marin and colleagues (Marín et al., 1998c) for the frog. Among the compartments of the amygdaloid complex in *X. laevis*, the CeA was not easily identified in our analyses. Thus, the MeA region that we analyzed could include a small portion of the dorsal CeA, since there is no anatomical marker to clearly separate the two parts. Nonetheless, our results clearly show that the MeA and the BNST are involved in

stress-circuitry in the amphibian CNS as in mammals, suggesting that their stress-related functions were established prior to the divergence of the amphibian and amniote lineages.

A critical component of the integrated physiological response to a variety of stressors is the locus coeruleus-norepinephrine, (LC-NE)/autonomic nervous system located in the brainstem (Mason, 1968, McCarty, 1983). The LC-NE/autonomic system activates the mesocortical and mesolimbic dopaminergic system, as well as the amygdala/hippocampal complex during stress (Deutch et al., 1990; Deutch et al., 1986; Gray, 1991; Roth et al., 1988). Furthermore, activation of the LC can increase hypothalamic CRF secretion (Calogero et al., 1988; Day et al., 1985; Kannan et al., 1987; Plotsky, 1987; Tanaka et al., 1985). CRF has been proposed to function as a neurotransmitter to initiate autonomic and behavioral responses via activation of the LC-NE system during stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991). We identified CRF-ir in the hindbrain in *X. laevis*, but we did not observe changes in CRF-ir in the LC or in the cerebellum following exposure to the shaking stressor. It is possible that we missed earlier or later changes in CRF-ir in these regions since we examined only a single time point in the current study. It is also possible that other types of stressor not analyzed in our study might preferentially activate hindbrain CRF neurons. For example, Boorse and Denver (Boorse and Denver, 2003) found that exposure of Western spadefoot toad tadpoles to simulated pond drying increased hindbrain CRF content (as measured by tissue extraction and RIA). Further study is required to understand the role that extra-hypothalamic CRF neurons play in stress responses in amphibians.

The immediate early gene *c-fos* has been widely used as a maker of neuronal activation, especially of stress-response circuitry (Ceccatelli et al., 1989b; Emmert and

Herman, 1999). Studies in rodents show that *c-fos* mRNA and/or Fos-ir is increased in stress-related structures in the brain in response to a variety of stressors. For example, *c-fos* mRNA expression was increased in the MeA and lateral septum (ls) following exposure to two acute stressors (immobilization and electrical foot-shock), in the MeA, ls, and the suprachiasmatic nucleus (SC) following exposure to a novel stressor (open field), and was induced in the CeA and the lateral nucleus of the BNST by a systemic stressor (injection of cytokines; Day and Akil, 1996; Emmert and Herman, 1999; Imaki et al., 1993; Sawchenko et al., 1996). Our observation that Fos-LI appeared in the MeA, BNST, ls, and SC following exposure to 4 hr shaking stressor is in agreement with these data, suggesting that much of the stress circuitry is conserved among tetrapods. One structure that is invariably activated by stress in rodents is the PVN. In accordance with these data, our results show that the number of Fos-LI-positive cells is elevated in the POA in the frog *X. laevis* following exposure to shaking stress.

The robust increase in Fos-LI and its colocalization with CRF-ir in the frog POA, similar to that observed in the mammalian PVN (Imaki et al., 1992; Watts and Sanchez-Watts, 2002), suggests that the induction of Fos and CRF by stress are functionally linked. For example, both mammalian and frog CRF genes possess AP-1 sites to which Fos-Jun heterodimers can bind (Yao et al., 2002), and there is evidence that Fos can regulate CRF gene expression in rodents (Imaki et al., 2001; Timofeeva et al., 2003; Watts and Sanchez-Watts, 2002).

In conclusion, our findings in the frog *X. laevis* show that the basic pattern of CRF expression in the CNS arose early in vertebrate evolution. Our data also lend further support to earlier studies that suggested that amphibians may be a transitional species for

descending CRF-ergic pathways. We found that CRF neurons in the frog brain exhibit changes in response to physical stress that parallel those seen in mammals, and thus are likely to play important roles in the mediation of neuroendocrine, behavioral and autonomic responses to stress. Stressor activation of the POA, MeA and BNST in the frog suggests that the stress-related functions of these CNS structures were established prior to the divergence of the amphibian and amniote lineages.

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Table 2.1. Abbreviations.

A, anterior thalamic nucleus
Acc, nucleus accumbens
BNST, bed nucleus of the stria terminalis
Cb, cerebellum
CeA, central amygdala
DB, nucleus of the diagonal band of Broca
dp, dorsal pallium
Hd, dorsal habenular nucleus
Hv, ventral habenular nucleus
igl, internal granule cell layer
Ip, interpeduncular nucleus
Is, nucleus isthmi
gl, glomerular layer
L, lateral thalamic nucleus, pars anterior
LA, lateral amygdala
Lc, locus coeruleus
lmf, lateral motor field of spinal grey
lp, lateral pallium
Lpv, lateral thalamic nucleus, pars posteroventralis
ls, lateral septum
lv, lateral ventricle
ME, median eminence
MeA, medial amygdala
ml, mitral layer
mp, medial pallium
ms, medial septum
NPv, nucleus of the paraventricular organ
nII, cranial nerve II
nV, nervus trigeminus
nIX, nervus glossopharyngeus
nX, nervus vagus
P, posterior thalamic nucleus
pd, pars distalis
pi, pars intermedia
pn, pars nervosa
POA, preoptic area
Ra, raphe nucleus
Rm, nucleus reticularis medius
SC, suprachiasmatic nucleus
sol, solitary nucleus
Str, striatum
tect, optic tectum
tegm, mesencephalic tegmentum
Tn, tegmental nuclei
Tor, torus semicircularis
TP, posterior tuberculum
VH, ventral hypothalamic nucleus
VLs, superficial ventral nucleus
VM, ventromedial thalamic nucleus
Vm, nucleus motorius nervi trigemini
Vpr, nucleus sensorius principalis nervi trigemini
IXm, nucleus motorius nervi glossopharyngei
Xm, nucleus motorius nervi vagi

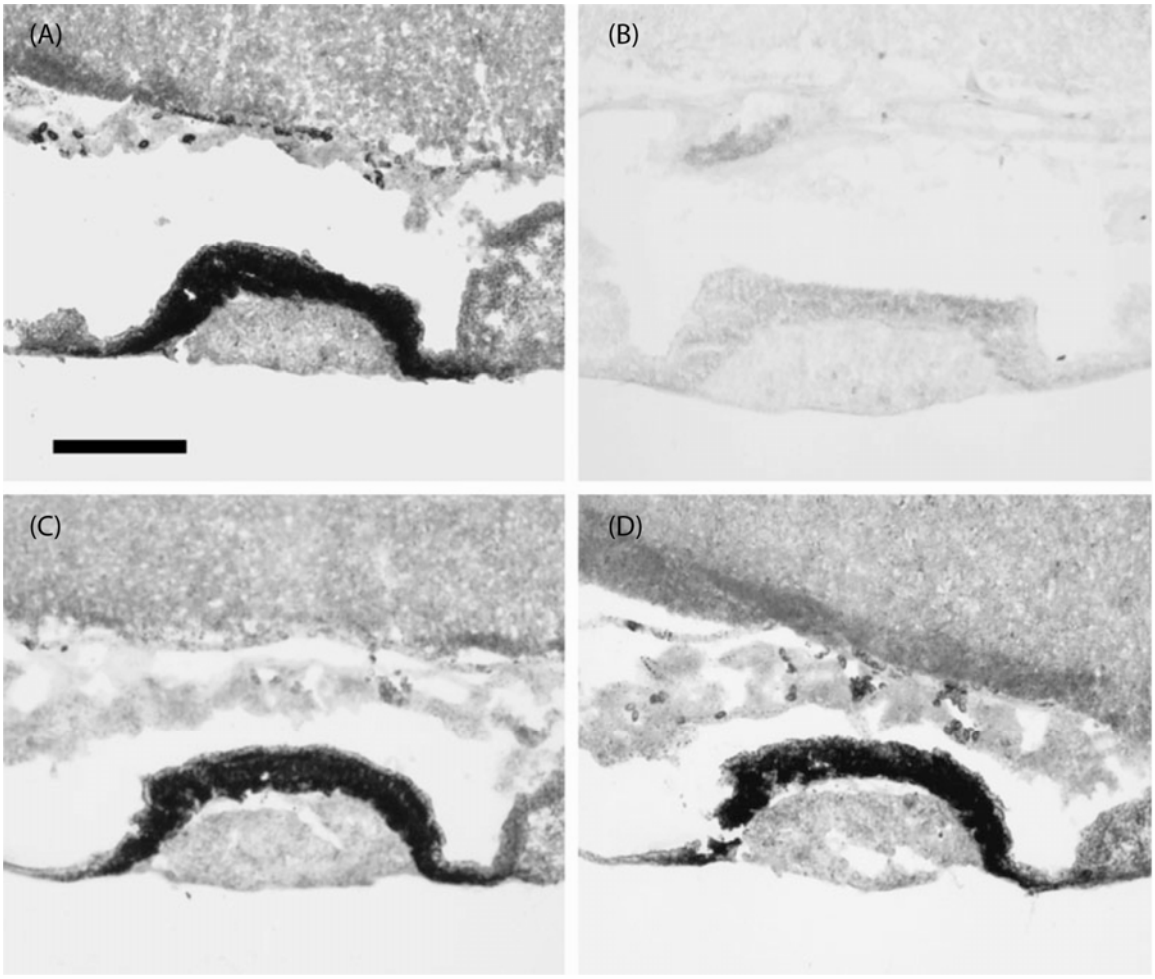

Figure 2.1. Photomicrographs of transverse sections through the brain of a juvenile *X. laevis* at the level of the median eminence showing the specificity of the affinity-purified anti-xCRF IgG. Dorsal is up in the photomicrographs in this and all other figures. Adjacent transverse sections were incubated with affinity-purified anti-xCRF IgG (A) or with anti-xCRF IgG that had been preabsorbed with 50 μg/ml of synthetic xCRF (B), xUCN1 (C) or xUCN3 (D). Bar = 200 um.

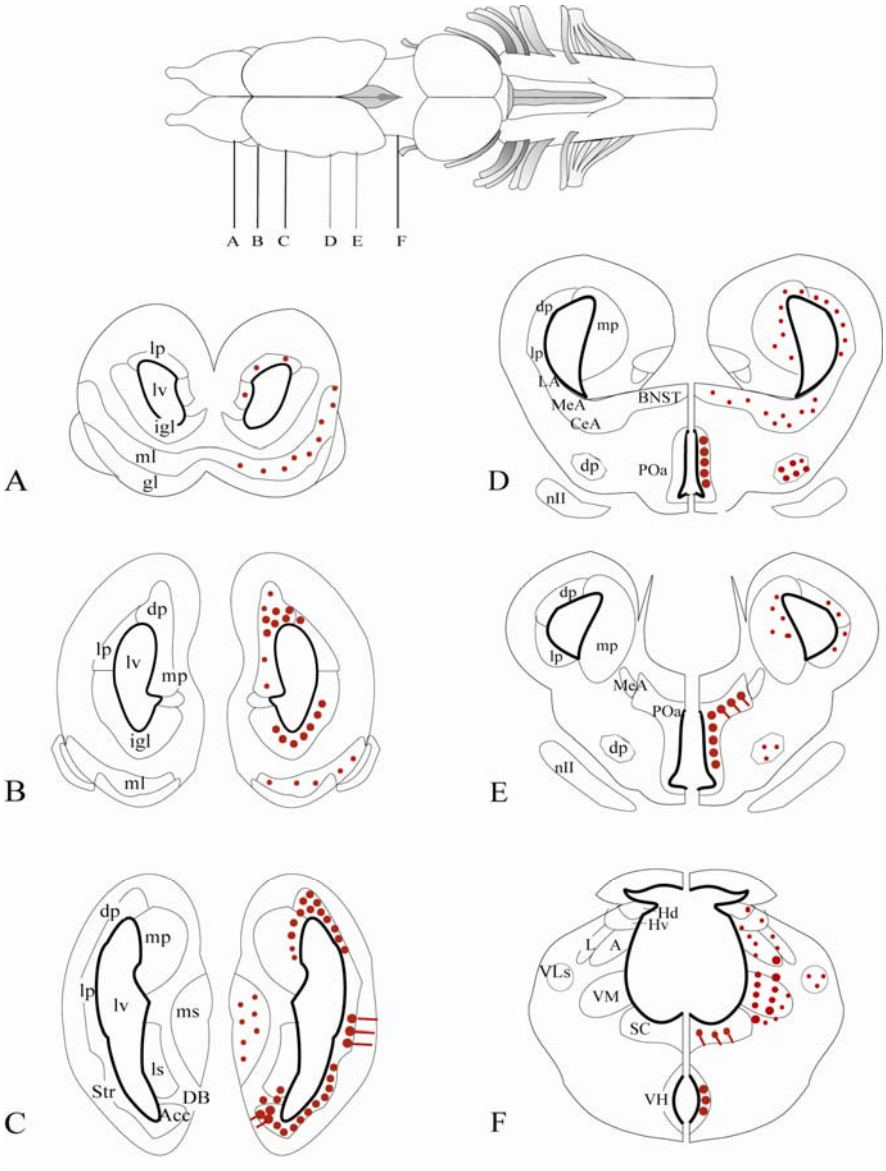

Figure 2.2. Schematic coronal illustration of CRF-ir distribution in forebrain regions of juvenile *X. laevis*. The drawing at the top of the figure shows a dorsal view of the whole brain. xCRF-ir in coronal sections (16 μm) of juvenile *X. laevis* brain. Letters correspond to the rostro-caudal location of sections as depicted in the whole brain drawing. Large circles represent large, robust CRF-ir, small circles represent smaller cells with less reactive CRF-ir, and lines represent CRF-ir fibers. Robust CRF-ir was observed in the lateral pallium, lp; striatum, Str; nucleus accumbens, Acc; anterior preoptic area, POA; and ventral hypothalamus, VH. See Table 2.1 for a complete description of abbreviations. The anatomical drawings are from Tuinhof and colleagues (Tuinhof et al., 1998) with modification of basal ganglia subdivisions according to Marin and colleagues (Marín et al., 1998c).

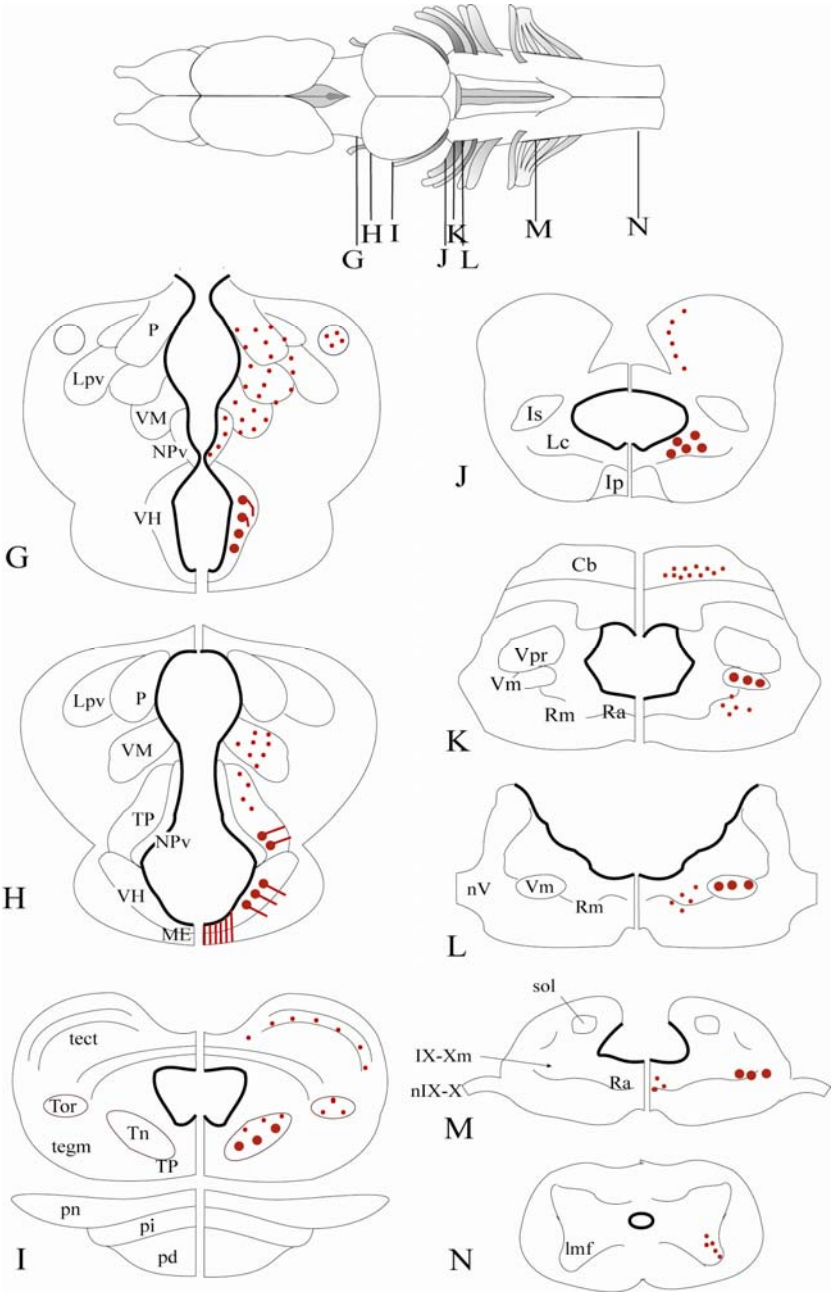

Figure 2.3. Schematic coronal illustration of CRF-ir distribution in hindbrain and rostral spinal cord regions of juvenile *X. laevis*. The drawing at the top of the figure shows a dorsal view of the whole brain. Letters correspond to the rostro-caudal location of sections as depicted in the whole brain drawing. Large circles represent large, robust CRF-ir, small circles represent smaller cells with less reactive xCRF-ir, and lines represent CRF-ir fibers. Robust CRF-ir was observed in the ventral thalamus, VH; posterior tuberacle, TP; median eminence, ME; cerebellum, Cb; locus coeruleus, LC; and lateral motor neurons of the spinal cord, lmn. See Table 2.1 for a complete description of abbreviations.

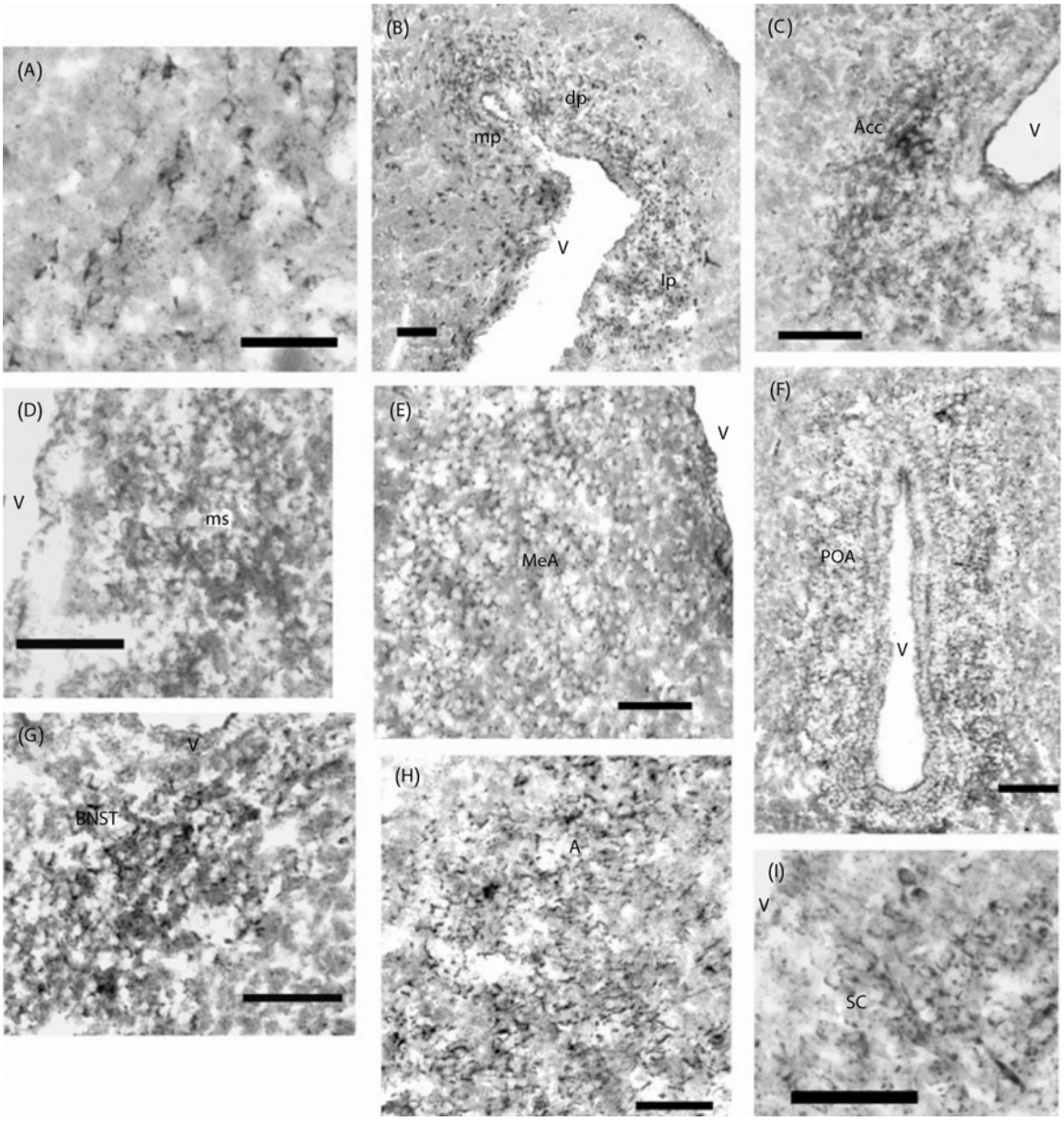

Figure 2.4. Photomicrographs of transverse sections through the forebrain and part of the midbrain of juvenile *X. laevis* showing the distribution of CRF-ir positive somata and fibers. **A:** Mitral layer of the olfactory bulb (ml). **B:** Pallium. **C:** Nucleus accumbens (Acc). **D:** Medial septum (ms). **E:** Medial amygdala (MeA). **F**: Anterior preoptic area (POA). **G:** Bed nucleus of the stria terminalis (BNST). **H:** Anterior thalamic nucleus (A). **I:** Suprachiasmatic nucleus (SC). See Table 2.1 for a complete description of abbreviations. Bars = 75 um. V - ventricle.

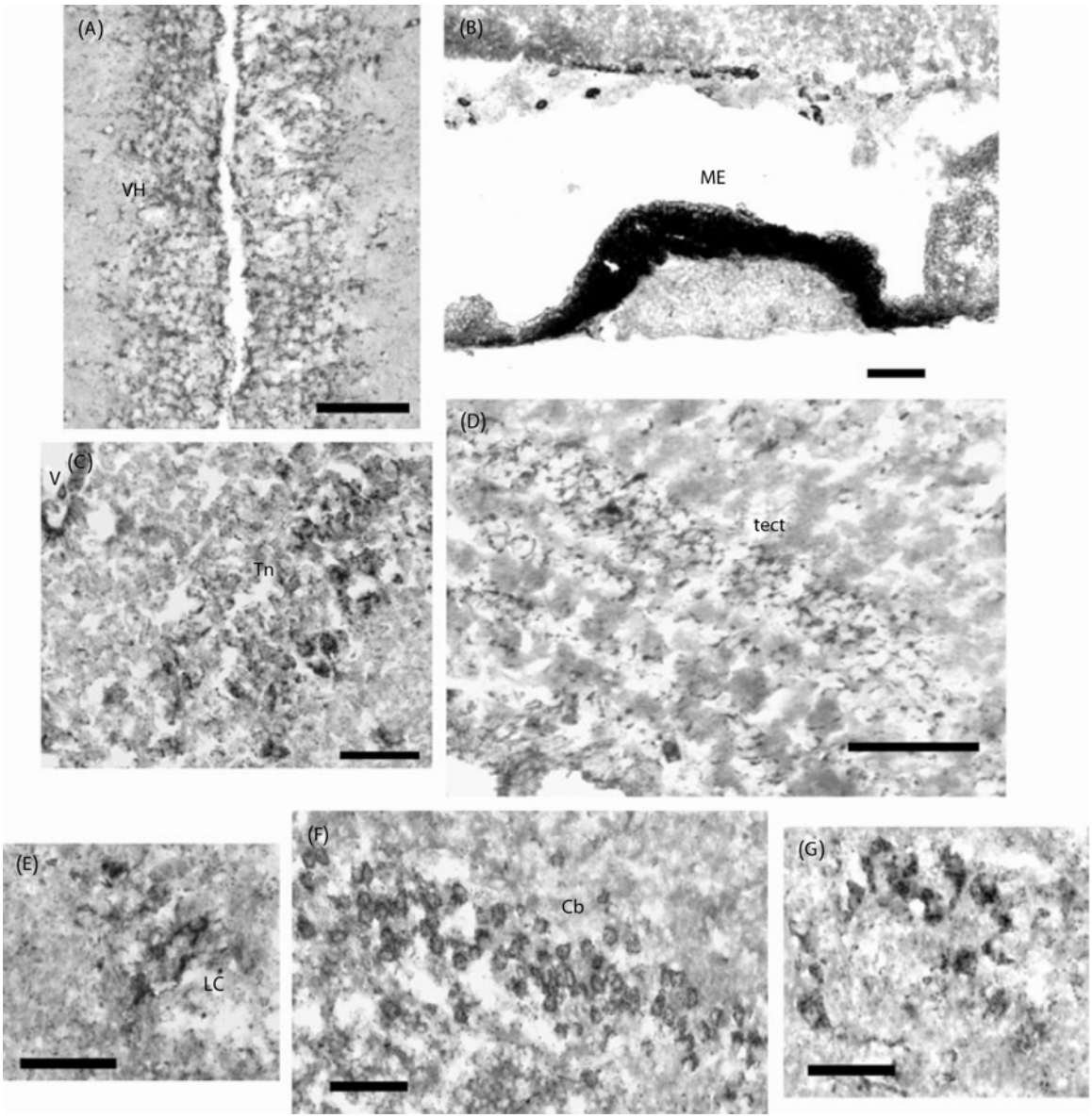

Figure 2.5. Photomicrographs of transverse sections through part of the midbrain and the hindbrain of juvenile *X. laevis* showing the distribution of CRF-ir positive somata and fibers. **A:** Ventral hypothalamic nucleus (VH). **B:** Medium eminence (ME). **C:** Tegmental nuclei (Tn). **D:** Optic tectum (tect). **E:** Locus coeruleus (LC). **F**: Cerebellum (Cb). **G:** Moter nucleus of the trigeminal nerve (Vm). See Table 2.1 for a complete description of abbreviations. Bars = 75 um. V - ventricle.

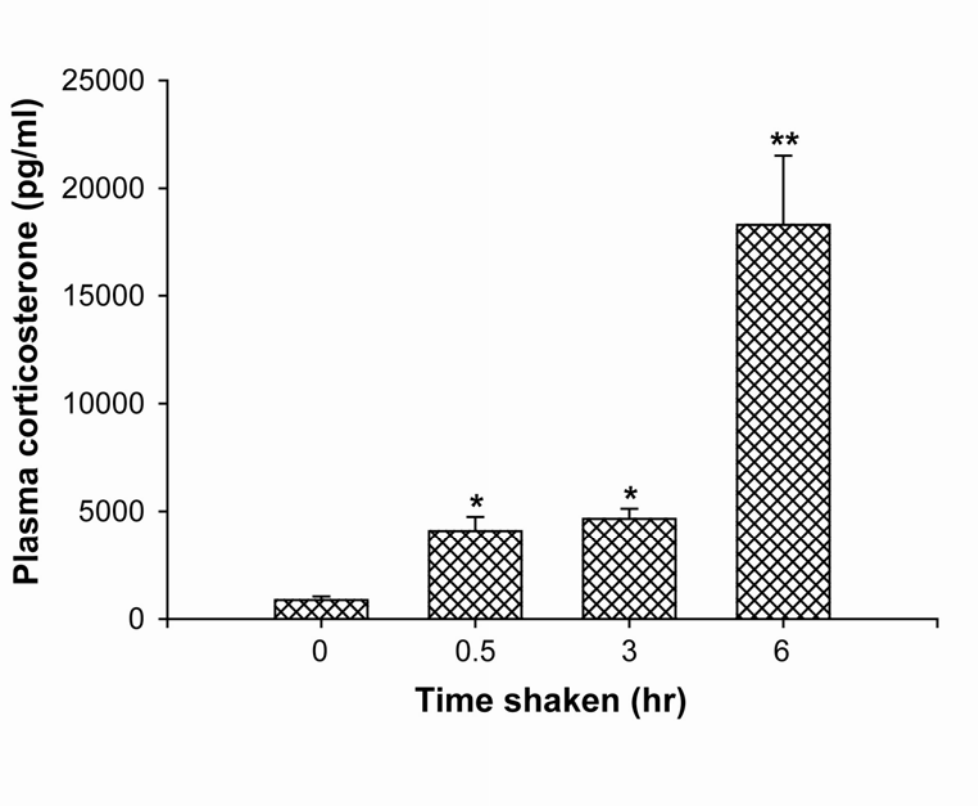

Figure 2.6. Plasma corticosterone concentrations of *X. laevis* juveniles exposed to shaking stress for various times (n = 10/ time point). Data presented are the mean + SEM. Significant differences from unstressed controls are denoted by * for $P < 0.05$ and ** for $P < 0.01$.

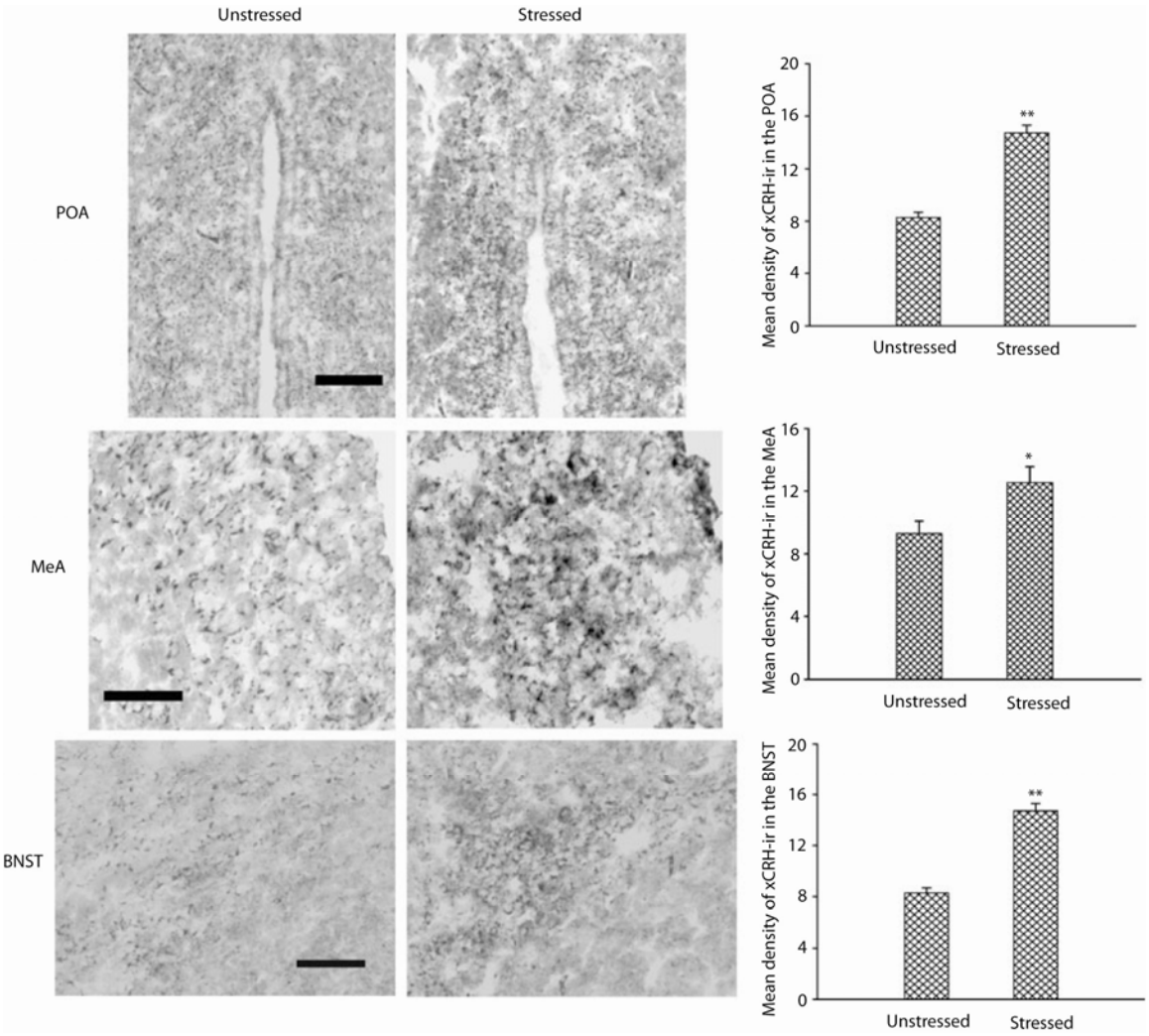

Figure 2.7. Effects of shaking stress on CRF-ir in the anterior preoptic area (POA), medial amygdala (MeA), and bed nucleus of the stria terminalis (BNST) of juvenile *X. laevis*. Micrographs are of representative transverse sections (12 μm) in the same anatomical plane of the POA. Representative sections of unstressed animal brain are shown on the left, and those of stressed brain are shown on the right. Bar = 150 um in the micrographs of the POA, and 75 um in the micrographs of the MeA and BNST. The graphs show densitometric analyses of CRF-ir in the POA, MeA, and BST of juvenile *X. laevis* following exposure to 4 hr shaking stress. CRF-ir mean density was significantly increased in the POA, MeA, and BNST in stressed *X. laevis* compared with unstressed controls. Data presented are the mean + SEM. Significant differences from unstressed control are denoted by * for $P < 0.05$ and ** for $P < 0.01$.

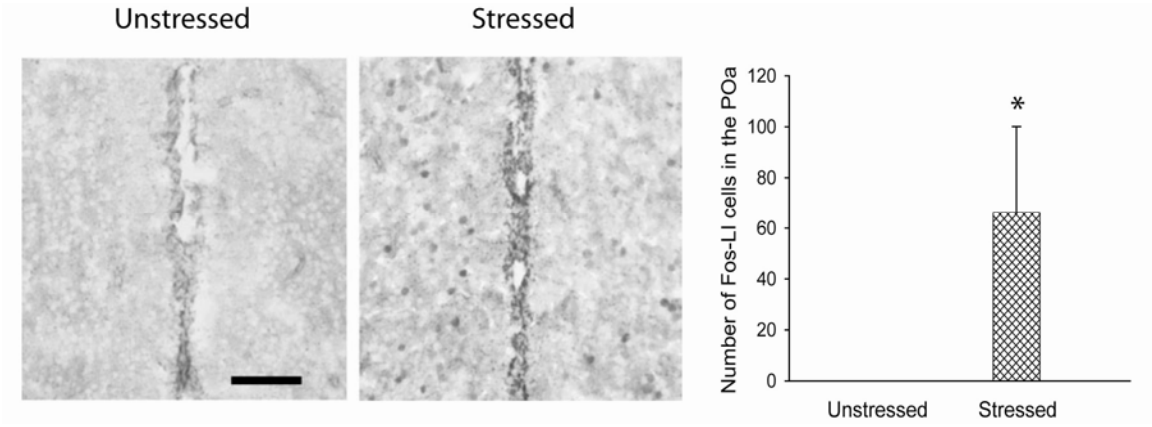

Figure 2.8. Changes in Fos-LI in the anterior preoptic area (POA) in control (A) and shaking-stressed (B) juvenile *X. laevis*. Bar = 75 um. The graph shows that the total number of Fos-LI positive cells was significantly increased in the POA in stressed *X. laevis* compared with unstressed controls. Data presented are the mean + SEM. A significant difference from unstressed controls is denoted by * for $P < 0.01$.

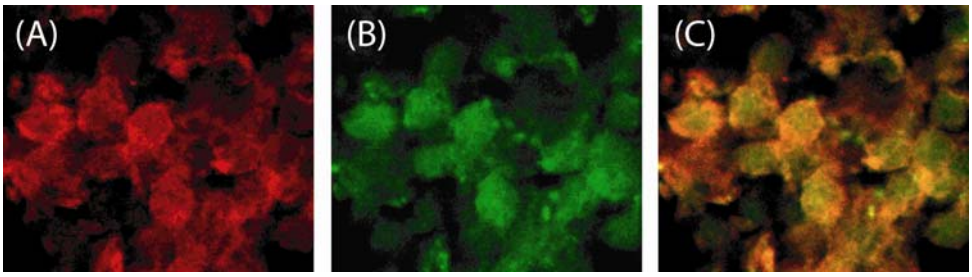

Figure 2.9. Colocalization of CRF-ir and Fos-LI in the anterior preoptic area (POA). Immunofluorescence confocal microscopy was conducted on double-labeled transverse sections through the POA of frogs exposed to 4 hr shaking stressor. The same field of the POA was scanned to reveal CRF-ir (A, red) and Fos-LI (B, green). CRF-ir was mostly located in the cytoplasm of the cells, while Fos-LI was restricted to the nuclei. The merged image of CRF-ir and Fos-LI (C) shows colocalization of CRF-ir and Fos-LI in a subset of neurons in the POA. The ability to clearly distinguish cytoplasmic from nuclear staining is limited by the small size of the cells.

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CHAPTER 3

STRUCTURAL AND FUNCTIONAL CONSERVATION OF VERTEBRATE CORTICOTROPIN-RELEASING FACTOR GENES: EVIDENCE FOR A CRITICAL ROLE FOR A CONSERVED CYCLIC AMP RESPONSE ELEMENT[3]

Abstract

Corticotropin releasing factor (CRF) plays a central role in neuroendocrine, autonomic, immune, and behavioral responses to stressors. We analyzed the proximal promoters of two *Xenopus laevis* CRF genes and found them to be remarkably conserved with mammalian CRF genes. We found several conserved *cis* elements in the frog CRF genes including a cyclic AMP response element (CRE), activator protein 1 binding sites (AP1s), and glucocorticoid response elements (GREs). Exposure to a physical stressor caused a rapid elevation in phosphorylated CRE binding protein (pCREB; 20 min) and CRF (1 h) in the anterior preoptic area of juvenile frogs. CREB bound to the putative frog CREs *in vitro*, which was disrupted by point mutations introduced into the CRE. The frog proximal CRF promoters supported basal transcription in transfection assays, and forskolin caused robust transcriptional activation. Mutagenesis of the CRE or overexpression of a dominant-negative CREB reduced forskolin-induced promoter activation. Using electroporation-mediated gene transfer in tadpole brain, we show that

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the proximal CRF promoters support cyclic AMP or stressor-dependent transcription *in vivo*, which was abolished by mutation of the CRE. Using chromatin immunoprecipitation we found that CREB associated with the proximal frog CRF promoter *in vivo* in a stressor-dependent manner. These data provide strong support for the hypothesis that stressor-induced CRF gene activation *in vivo* depends on CREB binding to the CRE in the promoter. Our findings show that the basic regulatory elements of the CRF gene responsible for stressor-induced activation arose early in vertebrate evolution and have been maintained by strong positive selection.

Introduction

In vertebrates, exposure to physical or emotional stressors leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The central regulator of the HPA axis is corticotropin-releasing factor (CRF), which acts on the pituitary to stimulate adrenocorticotropin (ACTH) secretion leading to increases in plasma glucocorticoids that influence a wide array of physiological, immune, and behavioral responses. Besides the hypophysiotropic function of hypothalamic CRF, the peptide is widely expressed in the brain where it functions as a neurotransmitter/neuromodulator influencing stress-related behaviors and sympathetic output (Aguilera, 1998; Tsigos and Chrousos, 2002).

Transcription of the CRF gene in mammals is rapidly induced following exposure to physical stressors and most current knowledge of CRF gene regulation comes from studies in rodents. The gene structure and nucleotide sequences of the coding and upstream promoter regions are highly conserved among mammalian CRF genes. All consist of two exons and a single intron of approximately 400-800 base pairs (human

801bp, rat 687bp, ovine 745bp). Although the protein coding sequences of mammalian CRF genes are very similar (~90% sequence similarity among human, rat and ovine), the highest degree of sequence similarity was found among the 330 base pair long proximal segment of the 5' flanking region (94% similarity between human and ovine, Vamvakopoulos and Chrousos, 1994; also 94% similarity between human and rat and 90% similarity between ovine and rat, M. Yao unpublished data), suggesting its functional importance in transcriptional regulation. This segment contains a number of putative transcription factor binding sites highly conserved among mammalian CRF genes. Using cell transfection assays it was shown that activity of the human CRF gene can be influenced by protein kinase A (PKA, acting via the cAMP-response element, CRE), protein kinase C (PKC, acting via the activator protein 1 [AP1] sites), and glucocorticoid receptor (GR, acting via the glucocorticoid response element, GRE) pathways (Guardiola-Diaz et al., 1994; Guardiola-Diaz et al., 1996; Kasckow et al., 2003a; King et al., 2002; Liu et al., 2006; Malkoski and Dorin, 1999; Malkoski et al., 1997; Nikodemova et al., 2003; Seasholtz et al., 1988; Spengler et al., 1992; Van, 1993; also see Kasckow et al., 2003b for review). *In vivo* studies in rodents showed a correlation of increased phosphorylated CRE-binding protein (pCREB) with CRF gene activation in several brain regions (as measured by heteronuclear RNA expression; hnRNA; Chen et al., 2001; Kovács and Sawchenko, 1996a, b; Shepard et al., 2005). Similar results were found with the AP1 protein c-Fos, although the kinetics of activation of c-Fos (as measured by mRNA) were delayed compared with pCREB. This has led some to question whether c-Fos is involved in the rapid stressor-induced activation of the CRF gene (Kovács and Sawchenko, 1996a, b). Although these studies suggest that these

transcription factor binding sites are important for CRF transcription, direct evidence for a role for these *cis* elements in stressor-induced transcriptional activation *in vivo* is lacking.

Limited comparative studies of CRF gene expression in non-mammalian species suggest that the gene regulatory elements responsible for tissue-specific and stressor-dependent gene activation may have arisen early, and have been conserved through vertebrate evolution. Some of the most detailed structural and functional analyses of the CRF signaling pathway in a nonmammalian species have been conducted in the South African clawed frog *Xenopus laevis*. *Xenopus laevis* possesses two CRF genes, designated xCRFa and xCRFb, owing to its pseudotetraploid genome (Stenzel-Poore et al., 1992). The two genes code for mature peptides with identical amino acid sequences. We showed previously that the distribution of CRF neurons in the frog brain is highly conserved when compared with mammals (Yao et al., 2004). Furthermore, we showed that CRF neurons are strongly activated in discrete brain regions in the frog brain in response to an acute physical stressor (Yao et al., 2004). These regions included the parvocellular neurons of the anterior preoptic area (POA; a region homologous to the mammalian paraventricular nucleus; PVN), the medial amygdala, and the bed nucleus of the stria terminalis; CRF neurons in each of these brain regions are similarly activated by exposure to physical stressors in mammals (Bartanusz et al., 1993a; Cullinan et al., 1995; Gray, 1991; Hand et al., 2002; Herman and Cullinan, 1997; Hsu et al., 1998; Kubo et al., 2004; Liu et al., 2001; Ma et al., 1997a; Rosen and Davis, 1988, 1990; Schulkin et al., 1998).

In the present study we analyzed the gene structures of two CRF genes of *X. laevis*, and compared the regulatory regions of the frog genes with mammalian CRF genes. We found that the gene organization and sequences of the regulatory regions of the frog CRF genes are highly conserved with mammals. Sequence analysis revealed conserved binding sites for CREB, AP1, GR and nerve growth factor induced gene B (NGFI-B) in the proximal promoters of the frog genes. Other, as yet uncharacterized regions of strong sequence similarity among frog and mammal CRF genes suggest an evolutionarily conserved role in gene regulation. We showed that CREB was rapidly phosphorylated in the frog POA following exposure to a shaking/handling stressor, which preceeded an increase in CRF immunoreactivity in the same cell populations. We tested the functionality of the putative CREs present in the frog CRF gene promoters using both *in vitro* and *in vivo* approaches. Our data show that this element supports activation of the promoter by the cAMP pathway and is specifically bound by CREB protein *in vitro.* Furthermore, we found that CREB associates with the proximal promoters of the frog CRF genes, and the CRE is required for stressor-dependent gene activation *in vivo*.

Materials and Methods

Animal husbandry

Xenopus laevis tadpoles and juveniles were obtained by in-house breeding or were purchased from Xenopus I (Dexter, Michigan). Tadpoles were raised in dechlorinated tap water (20-22 ^{o}C; 12L:12D) and fed Frog Brittle (Nasco, Fort Atkinson, WI) *ad libitum*. Developmental stages were assigned according to Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994). For electroporation-mediated gene transfer (see below) tadpoles were

anesthetized in 0.002% benzocaine. 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 (UCCUA).

RNA Isolation, RT-PCR, and RT-quantitative PCR Analysis

We used RT-PCR to determine if both CRF genes are expressed in *X. laevis* brain. Total RNA was isolated from the tadpole brain using the TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA). First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamers following the manufacturer's instructions. We designed gene specific PCR primers that spanned the predicted intronic regions of both frog CRF genes (see Supplementary Table 3.1 for primer sequences). The RT-PCR products were isolated by agarose gel electrophoresis and their sequences determined by direct DNA sequencing.

We also used RT-quantitative PCR (RT-qPCR) to determine the levels of expression of both CRF genes in the brain or the POA/hypothalamic region of unstressed and stressed frogs (see below). We designed gene specific Taqman primer/probe sets for detecting the primary transcripts (hnRNA) and mature RNA (mRNA) of both frog CRF genes (see Supplementary Table 3.2 for primer and probe sequences). Reactions were run using the Fast 7500 Real-Time PCR System (Applied Biosystems, CA). For analysis of expression of CRF hnRNAs and mRNA in unstressed and stressed animals, we used a relative quantitation method and expressed the concentrations of the RT-qPCR products in arbitrary units. For quantification and comparison of expression levels of the two frog CRF genes, we generated standard curves using known concentrations of plasmid DNA containing each of the frog CRF genomic clones.

Sequencing of *X. laevis* genomic clones and comparative genomic analysis

We designed primers based on the published sequences of the coding regions of the two *X. laevis* CRF genes (Stenzel-Poore et al., 1992) to sequence their upstream regions using the isolated genomic clones as templates. DNA sequence alignment analysis was conducted using AlignX of Vector NTI Suite 5.5 software. The promoter sequences of human (AC090195), chimp (LOC464215), sheep (M22853), mouse (AC141209), and rat (M54987) CRF genes were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). Transcription factor binding sites within the proximal promoters of the CRF genes were predicted using the on-line program Match™ using a library of mononucleotide weight matrices from TRANSFAC® 6.0 (www.gene-regulation.com) with both core similarity and matrix similarity of the transcription factor binding sites set higher than 0.85.

Shaking/handling stressor

The shaking/handling stressor paradigm that we used was described previously (Glennemeier and Denver, 2002a, Yao et al., 2004). Briefly, for immunohistochemical (IHC) analysis, three juvenile frogs (body weight 5-8 g) were placed into 32 oz white polypropylene containers with 250ml water. The containers were placed on an orbital shaker and shaken continuously at 100 rpm for various times. Controls were left undisturbed in holding tanks until sacrifice. The frogs were rapidly euthanized by submersion in 0.05% benzocaine and tissues collected. Frogs were euthanized at 0, 20 min, 1 h and 2h after the initiation of the stressor (n=3/time point), and the heads were

fixed in cold 4% paraformaldehyde. Brains were dissected, post-fixed, and submerged in 30% sucrose before snap-freezing and transverse cryo-sectioning at 12 μm.

Immunohistochemistry and morphometric analysis

We used IHC to analyze CRF immunoreactive (CRF-ir) and phospho CREB immunoreactive (pCREB-ir) neurons in the frog brain as described previously (Yao et al., 2004). The rabbit polyclonal antiserum to pCREB that we used was raised against an epitope of mouse CREB that is identical to the sequence of *Xenopus* CREB (Cat# 06-519, Upstate Biochemicals, Lake Placid, NY). We used a highly specific affinity-purified anti-xCRF IgG described previously (Yao et al., 2004). Single IHC for pCREB-ir and CRF-ir were conducted using the Vectastain elite ABC (rabbit) and Vector VIP kits (both from Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's protocols (antibodies: 1:500 dilution of rabbit polyclonal anti-phospho-CREB; 15-20 ng/μl of affinity purified polyclonal rabbit IgG raised against synthetic frog CRF; see Yao et al., 2004). We used double labeling fluorescence immunohistochemistry to determine colocalization of pCREB-ir and CRF-ir following a method that we used previously to colocalize CRF and c-Fos (Yao et al., 2004). Double labeling experiments were analyzed by confocal microscopy (using a Zeiss laser scanning confocal microscope) with optical sections of 1 μm thickness captured through the Z-axis.

We quantified CRF-ir and pCREB-ir in discrete brain regions using MetaMorph software (v 6.2r4). For each antibody we processed all samples simultaneously under identical conditions. Three sections that contained the anterior preoptic regions were analyzed for each animal. All sections were carefully matched for anatomical level, and

digital images were captured at 200X magnification for morphometric analysis. Image analysis was conducted in a blinded manner. The brain regions were isolated using a hand-made frame that covered the area of interest. For quantification of pCREB-ir (exclusively nuclear staining), the total number of positive nuclei in the areas of interest were counted automatically, and averages of the three sections were calculated for each animal. For quantification of CRF-ir, the total area of the 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 the multiple sections, divided by the total selected area (see Yao et al., 2004). The effects of the shaking/handling stressor on the numbers of pCREB-ir positive cells and CRF-ir mean density in the brain regions studied were analyzed by Student's unpaired *t*-test ($p < 0.05$) using the SPSS statistical package v11.5 for Windows.

Plasmid constructs

We constructed a *X. laevis* CREB expression vector by isolating a cDNA for the entire coding region of the frog CREB gene by RT-PCR using total RNA isolated from juvenile frog brain. The primers used to amplify the *X. laevis* CREB were (*Hind*III and *BamH*I sites are shown in lower case letters): 5'-cccaagcttGTGTTACATGGTGGGGAAG-3' and 5'-cgcggatccGCCTCCTAATCAGATTTGTGG-3'. We directionally cloned the frog CREB (xCREB) cDNA into the pSP64 Poly(A) vector (Promega Corp., Madison, WI) to produce pSP64A-xCREB. The orientation and sequence of the construct was confirmed by direct DNA sequencing.

All plasmid reporter constructs were generated using the parent plasmid pGL3-basic (Promega), which contains a modified coding region for firefly luciferase and lacks eukaryotic promoter and enhancer sequences. The numbering system used to describe the frog gene promoter fragments for analysis is based on the translation start sites ATG (+1); the transcription start sites are estimates based on sequence alignment with mammalian CRF genes (Fig. 3.1). We chose the ATGs as the basis for our numbering since many genes utilize alternative transcription start sites, and the transcription start sites of the frog CRF genes have not yet been determined. We generated the xCRF promoter constructs by first PCR amplifying DNA fragments using the xCRF genomic clones as templates (Stenzel-Poore et al., 1992) and oligonucleotide primers harboring *Kpn*I (5') and *Hind*III (3') restriction sites for directional cloning. The purified PCR products were double digested with *Kpn*I and *Hind*III (Promega) and ligated into pGL3-basic in a forward orientation to produce pGL3-xCRFa533 and pGL3- xCRFb576. The pGL3-xCRFa533 contains 533 bp of a xCRFa genomic fragment (-1327 to -795), including 396 bp of 5' flanking sequence and 137 bp of untranslated region. The pGL3-xCRFb576 contains 576 bp of a xCRFb genomic fragment (-1079 to -504), including 452 bp of 5' flanking sequence and 124 bp of untranslated region. Although the 5' and 3' limits of the two promoter fragments overlap, the sizes of the fragments are not identical due to deletions and insertions in the duplicated genes. We conducted oligonucleotide-directed mutagenesis of the CREs present in the promoter constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Sequences of all constructs were confirmed by direct DNA sequencing.

Cell culture and transient transfection

Monolayer cultures of PC-12 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 5% bovine calf serum, 5% equine serum (both from HyClone, UT), and antibiotics in a humidified atmosphere of 5% CO_2 at 37^{o}C. Monolayer cultures of a *X. laevis* tail myoblast cell line (XLT-15; these cells express CRF; Boorse et al., 2006; Yaoita and Nakajima, 1997) were maintained in Leibovitz's L15 medium (Invitrogen; diluted 1:1.5 for amphibian cells) supplemented with 10% thyroid hormone-stripped fetal bovine serum (Gibco BRL, Grand Island, NY) and antibiotics and cultured under a humidified atmosphere of 5% CO2 at 25^{o}C. Thyroid hormone removal from fetal bovine serum was conducted following the protocol of Samuels; Samuels et al., 1979).

Transient transfections were conducted in triplicate in 24-well plates (Falcon, MA). The pRL-null plasmid (Promega), which contains a promoterless *Renilla* luciferase gene, was cotransfected as an internal control for transfection efficiency. PC-12 cells were plated at a density of 4 x 10^5 cells/well and XLT-15 cells at a density of 6 x 10^4 cells/well in 24 well plates twenty four hours before transfection. For PC-12 cells we conducted transfections using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Each well received 800 ng of reporter plasmid plus 10 ng of pRL-null plasmid (Promega). For transfection of XLT-15 cells we used FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. Each well received 100 ng of reporter plasmid plus 2 ng of pRL-null. Twenty four hours after transfection the medium was changed to fresh medium with or without 25 μM forskolin (Sigma; stock was dissolved in DMSO and added to the medium

at 1:1000 dilution). The control group received the same concentration of DMSO as the forskolin-treated group. We found that XLT-15 cells, unlike PC-12 cells, required pre- and simultaneous treatment with the phosphodiesterase inhibitor IBMX (500 nM for 1h pretreatment prior to the addition of forskolin) in order to observe an increase in promoter activity, suggesting that the frog cells express higher phosphodiesterase activity; e.g., see Nikodemova et al., 2003.

Cells were harvested at different times and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega). All cell transfection experiments were conducted with 3 replicate wells per treatment and each experiment was repeated at least three times. The firefly luciferase activities were normalized to the *Renilla* luciferase activities in each cell lysate and the values are expressed as relative luciferase activity.

Electrophoretic mobility shift assay

We conducted electrophoretic mobility shift assay (EMSA) using *in vitro* synthesized *X. laevis* cyclic AMP binding protein (xCREB) or nuclear extracts of juvenile frog brain. We produced recombinant xCREB protein by coupled *in vitro* transcription-translation with the pSP64A-xCREB vector following the manufacturer's instructions (TNT system; Promega).

For nuclear extracts we microdissected the preoptic area/diencephalon region from the brains of juvenile frogs, homogenized the tissues in a hypotonic buffer (10 mM HEPES, 10 mM KCl, 1mM DTT, and protease inhibitors), incubated on ice for 30 m, then pelleted by centrifugation at 300 x g for 10 m. After homogenization, 0.1 total

volume of sucrose restore buffer (containing 6.75% sucrose, 50 mM HEPES, 10 mM KCl, 1 mM DTT, and protease inhibitors) was added, followed by centrifugation. We resuspended the pellet in a nuclear extraction buffer (containing 50 mM HEPES, 10 mM KCl, 1 mM DTT, and protease inhibitors) and incubated it on ice for 40 min with vortexing every 10 min. After centrifugation at 100,000 x g for 1 h at 4 ˚C, the nuclear extract (supernatant) was removed and stored at -80 ˚C. We determined the total protein concentrations of each sample using a standard protein assay (BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL).

DNA probes were prepared for EMSA by annealing complementary oligonucleotides. End-labeling was conducted using the large Klenow fragment of DNA polymerase (Promega) and 32P labeled dCTP (Perkin Elmer), and the products were purified over Sephadex G50 columns. Sequences of the oligonucleotides are shown in Table 3.1.

Electrophoretic mobility shift assays were conducted following the methods of Dignam; Dignam et al., 1983) with minor modifications. *In vitro* produced proteins (1 μl of 50ul TNT reaction) or 1 μl (0.8 μg total protein/μl) of brain nuclear extract were incubated with 20,000 cpm double-stranded ^{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 (DTT), 0.1% IGEPAL CA-630, 50 mM KCl, and 20% glycerol. The reaction continued at room temperature for 40 min before fractionation by 6% non-denaturing PAGE in 0.25 x TBE buffer. The gel was dried and analyzed by autoradiography. Competition was conducted by addition of 100 nM of unlabled specific or nonspecific oligonucleiotides (~100-fold excess) during the incubation. To identify nuclear proteins present in supershifted complexes we added varying amounts of rabbit

anti-CREB serum (Rockland Immunochemicals Inc. Gilbertsville, PA), anti-phosphoCREB serum (Cat#06-519, Upstate), or normal rabbit serum (NRS) to the EMSA reaction immediately before adding the oligonucleotide probe.

Chromatin immunoprecipitation assay

We prepared chromatin from juvenile frog brains and conducted chromatin immunoprecipitation assay (ChIP) as described by Sachs and Shi (Sachs and Shi, 2000). For each replicate chromatin preparation we pooled brain sections containing the POA plus hypothalamus from 10 juvenile frogs (BW 1-2 g; n=3-4 per treatment). We homogenized the tissue in nuclear extraction buffer (0.5% Triton X-100, 10 mM Tris-HCl, 3 mM $CaCl_2$, 0.25 M sucrose, 1 mM DTT, and protease inhibitor cocktail) and incubated in 37% formaldehyde for 15 min. Nuclei were then pelleted and resuspended in nuclear extraction buffer, followed by homogenization and filtration through a 100 μm cell strainer (Falcon, MA). After centrifugation, we resuspended the pellet in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated with five 10-second pulses at an intensity of five using a Fisherbrand Sonic Dimembrator 100 and pelleted at 20000 x g for 10 min. We then measured the DNA concentration in the supernatant by spectrophotometry, diluted to 100 ng/μl with SDS lysis buffer and stored the chromatin samples frozen at -80 ^{o}C until assay.

We conducted ChIP assays with 15 μg chromatin using the Upstate Biochemicals ChIP assay kit following the manufacturer's instructions. After pre-clearing with protein A agarose/salmon sperm DNA slurry we incubated chromatin with primary antibodies overnight at 4 ^{o}C. We used 5 μl of anti-CREB (Rockland Immunochemicals Inc.,

Gilbertsville, PA; based on Moreno et al., 1999), anti-acetyl-Histone H4 (Cat# 06-598, Upstate) or normal rabbit serum in the immunoprecipitations. Immune complexes were precipitated with protein A agarose/salmon sperm DNA, washed, and reverse-crosslinked in 0.2 M NaCl overnight at 65 ^{o}C. After incubation in 40 μg/ml Proteinase K (in 10 mM EDTA, 40 mM Tris-HCl, pH 7.4) for 1 h at 45 ^{o}C the DNA was purified using the High Pure PCR Product Purification Kit (Roche) and analyzed by quantitative PCR (qPCR).

Quantitative PCR

For quantitative PCR (qPCR) analysis of ChIP assays we developed Taqman assays to target the xCRFb core promoter containing the CRE and the coding region (CDS) of CRF genes (the assay recognized both genes and served as a negative control in the ChIP assay; see Supplementary Table 3.3 for primer and probe sequences.) Reactions were run using the Fast 7500 Real-Time PCR System (Applied Biosystems, CA). We applied a relative quantitation method using pooled chromatin as the standard for each primer/probe set. ChIP values for anti-CREB or anti-acetyl-Histone H4 were normalized first to the respective input, followed by subtraction of the background signal for ChIP with NRS for each sample.

Electroporation-mediated gene transfer

To test the functionality of promoter elements *in vivo* we conducted bulk electroporation-mediated gene transfer into tadpole brain as described by Haas and colleagues (Haas et al., 2001) with minor modifications. *X. laevis* tadpoles (NF stage 49-51) were anesthetized by immersion in 0.002% benzocaine prior to i.c.v. microinjection

of 92 nl DNA solution. Each DNA solution contained 1 μg/μl of the reporter plasmid, 50 ng/μl of pRL-null plasmid, 400 ng/μl of 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. We reversed the polarity and repeated the current delivery. Animals were allowed to recover and screened for high EGFP expression five days after the procedure using a Leica MZFLIII fluorescent stereomicroscope.

To test the functionality and stress responsiveness of the frog CRF promoters *in vivo* we administered i.c.v. injections of ovine CRF (oCRF; 20 ng/g body weight) or saline vehicle and waited 6 h before harvesting brains. In a second experiment we exposed juvenile frogs to shaking/handling stressor as described above. Animals were euthanized and brains dissected at 0 and 6 h after initiation of the stressor.

We analyzed luciferase activity in tadpole brain homogenates using the Dual-Luciferase Reporter Assay System (Promega). We processed each brain separately in a Dounce homogenizer in 20 μl of 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.

Statistical analysis

Statistically significant differences were determined using unpaired Student's t test or one-way ANOVA followed by Fisher's LSD multiple comparisons tests. Data were log10-transformed when the variances were found to be heterogeneous. The values are presented as mean + SEM, and $p < 0.05$ was considered statistically significant.

Results

Expression and organization of the *X. laevis* CRF genes

Using RT-PCR we found that both CRF genes are expressed in *X. laevis* brain; the identities of the PCR products were confirmed by DNA sequencing. With RT-quantitative PCR (RT-qPCR) we found that xCRFb hnRNA is approximately 10 times more abundant than xCRFa hnRNA when analyzed in whole brain or just in the POA/hypothalamic region (data not shown).

We sequenced the entire 5' flanking regions represented within genomic clones of two *X. laevis* CRF genes, which include ~ 4.5 kb of CRFa and 1.3 kb of CRFb upstream sequence from the respective ATG translation start sites (Stenzel-Poore et al., 1992; Genbank accession nos. DQ865136 and DQ865137). Alignment of partial cDNA sequences (obtained by sequencing of PCR products and cDNA library screening; data not shown) with the genomic sequences revealed that the two frog CRF genes, like mammalian CRF genes, possess two exons and a single intron. We aligned the upstream regions of the frog and human CRF genes, which allowed us to assign provisional transcription start sites for the frog genes. Based on these criteria, the xCRFa gene is composed of a first exon of 141 bp, followed by an intron of 697 bp, and the beginning of the protein coding region (AUG codon) is 93 bp downstream of the start of the second exon. The xCRFb gene is composed of a first exon of 162 bp, an intron of 410 bp, and the AUG codon is 55 bp downstream of the start of the second exon. The highest degree of sequence similarity between the two frog CRF genes was found in the coding regions, with 95% sequence identity. The first exon of the two *X. laevis* CRF genes that contain

most of the 5' untranslated regions share 70% sequence identity, while their introns are 65% identical.

Structural analysis of the 5' regulatory regions of *X. laevis* CRF genes

An earlier study by Vamvakopoulos and colleagues revealed a high degree of sequence similarity (94%) among the first 334 bases upstream of the transcription start sites in the human and ovine CRF genes; but, the sequence similarity dropped to 72% approximately 0.6 kb upstream of this region (Vamvakopoulos et al., 1990; we compared human and rat and found 94% similarity in the proximal 334 bases, and 67% ~0.6 kb upstream; M. Yao, unpublished). Since all of the characterized transcription factor binding sites in the mammalian CRF promoters are located within the first 330 bp of 5' flanking sequence, we focused on these regions of the frog CRF genes for our initial promoter analyses.

Comparison of the 5' proximal sequences of the two frog CRF genes (325-345 bp upstream of the transcription start sites) revealed ~75% sequence similarity. Within the first ~340-360 bp of the 5' flanking region the human CRF gene shares 63% and 72% sequence similarity with the xCRFa and xCRFb genes, respectively (Fig. 3.1). A search for transcription factor binding sites in the 5' flanking regions of the frog genes revealed a putative cAMP response element (CRE), a NGFI-B response element (NBRE), several AP1 sites, and half-glucocorticoid response elements (GRE). All of these elements and their flanking sequences in the frog share very high degrees of similarity with those identified in human and rat CRF genes (Fig. 3.1; Malkoski and Dorin, 1999; Seasholtz et al., 1988; Spengler et al., 1992).

Exposure to a physical stressor activates transcription of the CRF gene and increases CRF and pCREB immunoreactivity in the frog POA

We exposed juvenile frogs to shaking/handling stressor for 1 h and analyzed expression of CRF genes in the POA/hypothalamic region by RT-qPCR. We designed specific Taqman assays that targeted the intronic regions of the xCRFa and xCRFb genes to analyze expression of the primary transcripts (hnRNA). The 5' UTR and coding regions of the mRNAs of the two frog CRF genes are highly conserved; thus, the single CRF mRNA Taqman assay detected mature transcripts for both genes. We found that the hnRNA of xCRFb increased significantly ($p = 0.02$) after 1 h exposure to shaking/handling (Fig. 3.2A), However, we saw no change in xCRFa hnRNA (data not shown). Also, there was no significant change in CRF mRNA after 1 h exposure to the stressor (Fig. 3.2A).

We then exposed juvenile frogs to shaking/handling stressor for 20 min, 1 h, and 2 h and analyzed CRF-ir and pCREB-ir throughout the brain. In all cases the subcellular location of CRF-ir was predominantly cytosolic, while pCREB-ir was exclusively nuclear. In unstressed frogs, pCREB-ir positive neurons were found throughout the brain (data not shown). Exposure to shaking/handling stressor caused a significant increase in CRF-ir in the POA by 1 h that was maintained through 2 h (1 h, $p = 0.037$; 2 h, $p = 0.013$. n = 3/treatment; Fig. 3.2B-C). The stressor produced a rapid (by 20 min) increase in the number of pCREB-ir nuclei in the POA, which was maintained through 2 h (Fig. 3.3A). Morphometric analysis revealed that exposure to the stressor produced significant

increases in the number of pCREB-ir cells in the POA at all time points examined (20 min, $p = 0.047$; 1 h, $p = 0.026$; 2 h, $p = 0.014$. n = 3/treatment; Fig. 3.3B).

Using dual immunofluorescence detection coupled with confocal microscopy we found that in the POA, the majority of the CRF-ir positive cells are also pCREB-ir positive, while not all pCREB-ir positive cells express CRF-ir (data not shown).

The proximal frog CRF promoters support basal and forskolin-induced transcription in transient transfection assays

We constructed promoter-reporter constructs designated pGL3-xCRFa533 and pGL3-xCRFb576 that contain the 5' proximal sequences of the frog CRFa and CRFb genes, respectively (see Materials and Methods). Both pGL3-xCRFa533 and pGL3-xCRFb576 supported basal transcription in transfected PC-12 cells that was 2-3 times that of the parent vector pGL3-basic ($p < 0.05$). Treatment with 25 μM forskolin, an activator of adenylyl cyclase, for 6 h increased luciferase activity by an average of 10-fold for pGL3-xCRFa533 and by an average of 43-fold for pGL3-xCRFb576. Luciferase activity in pGL3-basic-transfected cells did not change following treatment with forskolin (Fig. 3.4A).

We next conducted a time course experiment to determine an appropriate treatment duration to further analyze forskolin-induced activation of the frog CRF promoters. We focused on the pGL3-xCRFb576 construct which gave a greater response than the pGL3-xCRFa533 at the one time point tested (6 h). Forskolin caused statistically significant promoter activation by 1 h ($p < 0.05$), which continued to increase up to 5 h and was maintained through 7 h (Fig. 3.4B). By 24 h the luciferase activity remained elevated

above untreated, transfected cells and was comparable to the forskolin-induced response at 2 h. Luciferase activity of pGL3-xCRFb576 transfected cells cultured in the absence of forskolin did not change over the 24 h period. Based on these data we chose 6 h as a routine time point for subsequent analyses. This timepoint has also been used in transfection experiments with mammalian CRF gene promoter constructs in PC-12 cells (Guardiola-Diaz et al., 1994; Seasholtz et al., 1988).

To determine if forskolin-dependent activation of the frog CRFb gene was mediated by the predicted CRE, we introduced two point mutations into the CRE octomer present in the xCRFb core promoter (changed from TTACGTCA to TTAAGTCT) to create pGL3-xCRFb[CREmut]. Similar point mutations have been shown to eliminate forskolin-responsiveness of the human CRF promoter in cell transfection (Hyman et al., 1988). This construct exhibited 63% less basal, and 86% less forskolin-induced luciferase activities when compared with the wildtype promoter construct ($p < 0.05$ for both comparisons, Fig. 3.4C).

These results show that the CRE identified in the xCRFb promoter is functional and mediates gene regulation by the cAMP pathway, presumably through binding of CREB. However, we observed a residual forskolin-induced activation in the pGL3-xCRFb[CREmut] transfected cells. We found similar residual forskolin-dependent responses when we replaced the entire CRE octomer with an unrelated sequence (AGCTAGCT; data not shown) suggesting that it is not due to low affinity binding of CREB at this site (see also EMSA results below). We hypothesized that this activity could be due to: (1) a second (or more) CRE or CRE-like element in the CRF promoter, or (2) activation of the cAMP pathway inducing expression of other transcription factors

such as c-Fos or NGFI-B. We conducted a time course experiment with forskolin treatment of cells transfected with the pGL3- xCRFb576 or pGL3-xCRFb[CREmut] constructs. Although the magnitude was much lower, the time course of activation of pGL3-xCRFb[CREmut] paralleled that of pGL3-xCRFb576 with significant elevation ($p = 0.002$) over the no treatment control by 1.5 h (Fig. 3.4D; also see Supplemental Data Fig. 3.1 for analysis of the fold increase caused by forskolin treatment of both promoter constructs). The early kinetics are consistent with activation mediated by CREB, although we cannot rule out the involvement of other transcription factors, particularly at later time points.

We next used a dominant negative approach to further investigate a role for CREB in forskolin-induced CRF promoter activity. We cotransfected PC-12 cells with pGL3-xCRFa533 or pGL3-xCRFb576, and a vector to express a dominant-negative mutant form of CREB (ACREB; Ahn et al., 1998; Fig. 3.4C). Cotransfection of ACREB reduced basal promoter activity by 74% (pGL3-xCRFa533) and 84% (pGL3-xCRFb576), and completely blocked forskolin-induced activation of both constructs. Furthermore, ACREB reduced basal activity by 25% and abolished forskolin-induced activity of the pGL3-xCRFb[CREmut]. These data suggest the existence of a second, perhaps lower affinity CRE-like site in the xCRFb promoter.

As described above, we found that activation of the xCRFb promoter by forskolin was 2-3-fold greater than for the xCRFa. We thus sought to test whether this difference in responsiveness was due to the composition of the major CRE sites present in the two core promoters. The xCRFa CRE differs from xCRFb, and thus the consensus CRE octomer in that it has a 5' T→C substitution. We first introduced point mutations to convert the

xCRFa CRE into the xCRFb CRE (changed from cCTACGTCA to tTTACGTCA) thus creating pGL3-CRFa[CREb]. We also mutated the same two nucleotides in the xCRFb CRE to convert it to the xCRFa CRE (changed from tTTACGTCA to cCTACGTCA) thus creating pGL3-CRFb[CREa]. We found that converting the CRE of the 'a' gene to that of the 'b' gene (pGL3-xCRFa[CREb]) increased basal promoter activity by 1.7-fold ($p < 0.001$) and forskolin-induced promoter activity by 1.8-fold ($p = 0.002$) compared to that of the pGL3-CRFa533 construct (Fig. 3.5A; also see Supplemental Data Fig. 3.2 for analysis of the fold increase caused by forskolin treatment). Forskolin-induced activation of pGL3-xCRFa[CREb] was of similar magnitude to that of pGL3-xCRFb576. By contrast, converting the CRE of the 'b' gene to that of the 'a' gene (pGL3-CRFb[CREa]) had no effect on promoter activity, resulting in basal and forskolin-induced promoter activities similar to those of the native promoter construct pGL3-xCRFb576. Thus, the composition of the CRE elements only partly determines the differences in forskolin-induced activity of the two promoters, and other promoter elements also contribute to the responsiveness to forskolin treatment.

PC-12 cells have been used to study mammalian CRF promoter function because they have a functional cAMP pathway, and we found that the frog CRF promoters were functional in these cells (and also in NIH3T3 cells; data not shown). To test if our CRF promoter constructs were functional in frog cells we used a *X. laevis* tadpole tail myoblast-derived cell line, XLT-15 (Yaoita and Nakajima, 1997). This cell line expresses CRF and also CRF type 1 receptor (Boorse et al., 2006). We found that both pGL3-xCRFa533 and pGL3-xCRFb576 exhibited significantly higher basal activity in XLT-15 cells compared with PC-12 cells (Fig. 3.5B). Treatment of XLT-15 cells with forskolin

plus IBMX produced an ~2-fold activation of both promoters; there were no differences in the promoter activities among the 'a' and the 'b' genes in XLT-15 cells. Basal activity of the xCRFb construct was significantly reduced by mutation of the CRE (pGL3-xCRFb[CREmut]; $p < 0.001$; compared with basal activity of pGL3-xCRFb). By contrast to its residual activity in PC-12 cells, we found no increase in promoter activity of the pGL3-xCRFb[CREmut] following forskolin treatment in XLT-15 cells.

Interestingly, we found that changing the CRE in the xCRFa to CREb resulted in a higher induction by forskolin than either of the native promoters (pGL3-xCRFa533 or pGL3-xCRFb576; Fig. 3.5B). Similar to the results in transfected PC-12 cells, basal and forskolin-induced activities of pGL3-xCRFb[CREa] in XLT-15 cells are similar to those of pGL3-xCRFb576 (Fig. 3.5B).

CREB binds to the putative CREs present in the frog CRF promoters

Supershifted protein-DNA complexes of similar mobility were formed in EMSA on the CREs from both frog CRF genes using either *in vitro* expressed recombinant *X. laevis* CREB (rxCREB) or frog brain nuclear extract (Fig. 3.6A). A similar sized complex was formed with the CRE from the human CRF promoter (hCRE), which contains a palindromic consensus CRE octomer (included as a positive control). We observed little or no binding of rxCREB, and no binding of proteins in the nuclear extract to the mutated CRE from the 'b' gene (xCRFb[CREmut]; possessing the same point mutations in the CRE as in the pGL3-xCRFb[CREmut] used in transfection) or to the xCRFb[CREdel] probe (Fig. 3.6A). The specificity of CREB binding to the human and *X. laevis* CRE probes was confirmed by complete displacement of radiolabeled probes by the addition

of radioinert oligonucleotides (100 nM) but not by the addition of xCRFb[CREmut] or xCRFb[CREdel] (Fig. 3.6B).

We confirmed the identity of proteins present in frog brain nuclear extract that formed a complex on the CRE probes by antibody supershift. The addition of antiserums to CREB or pCREB to the EMSA reactions supershifted the protein-DNA complexes, thus demonstrating that the major band formed in the EMSA contained CREB or CREB-like proteins (Fig. 3.6C).

CREB associates with proximal frog CRF promoter *in vivo*

We used chromatin immunoprecipitation (ChIP) assay combined with quantitative PCR to determine whether CREB associates with the frog CRF promoters *in vivo*. We prepared chromatin from brain sections of juvenile frogs that included the POA and hypothalamus, and conducted ChIP assays for CREB and acetylated histone H4. Nonimmune normal rabbit serum was used as a negative control. Our results show that the ChIP signal for CREB at the CRE region of the xCRFb proximal promoter was significantly higher than at the CDS of the xCRF genes ($p = 0.02$; analysis of the CDS was included as a negative control; Fig. 3.7A). We also observed significantly higher acetylated histone H4 at the CRE region compared with the CDS of the xCRF genes ($p < 0.001$; Fig. 3.7B).

Following exposure to shaking/handling stressor for 1 h we observed significantly greater association of CREB at the CRE region of the xCRFb promoter ($p = 0.026$). There were no changes in CREB association in the CDS of the CRF genes following

exposure to the stressor (Fig. 3.7C), nor at the proximal promoter of the xCRFa gene (data not shown).

Analysis of CRF promoter activity in transfected *X. laevis* brain *in vivo*

To test the functionality of the frog CRF promoter-luciferase reporter constructs *in vivo* we transfected brains of NF stage 50 tadpoles by electroporation-mediated (EM) gene transfer. We used two approaches to control for transfection efficiency. First, we cotransfected the pEGFP-N1 vector and screened tadpoles for EGFP expression five days later. We examined the animals under a fluorescent stereomicroscope and selected those with high and comparable EGFP fluorescence in the region of the third ventricle (~75% of electroporated tadpoles; an example is shown in Fig. 3.8A). Second, we cotransfected a promoterless Renilla luciferase vector, which allowed us to conduct a dual luciferase assay on tissue homogenates from transfected animals. We thus normalized the firefly luciferase activity (which was under the regulation of the CRF promoters) to the Renilla luciferase.

We analyzed basal and induced expression of the xCRFa533, xCRFb576, and xCRFb[CREmut] constructs in tadpole brain (Fig. 3.8B), but only the xCRFb576 construct in juvenile frog brain (Fig. 3.8C; animals were transfected as tadpoles, then grown to the juvenile frog stage). In the tadpole, the basal activity of pGL3-xCRFb576 was indistinguishable from the parent vector pGL3basic, while activity of pGL3-xCRFa533 was significantly higher than pGL3-basic and pGL3-xCRFb576 (Fig. 3.8B; $p < 0.001$). By contrast, activity of the pGL3-xCRFb[CREmut] was significantly lower compared with pGL3-basic ($p = 0.011$). To test for inducible activity of the frog CRF

promoter constructs *in vivo*, we injected ovine CRF i.c.v. (oCRF, 20 ng/g BW) and sacrificed animals 6 h later for dual luciferase assay. oCRF binds to and activates the frog type 1 CRF receptor resulting in elevations of intracellular cAMP and thus activation of the cAMP pathway (Dautzenberg et al., 1997). We chose to use oCRF because it does not bind to the frog CRF binding protein and thus its activity would not be abrogated by such an interaction (Valverde et al., 2001). We found that oCRF caused a significant increase in luciferase activity in brain homogenates from pGL3-xCRFb576 transfected animals compared with no injection or saline-injected controls ($p = 0.002$). The same treatment did not alter the activity of pGL3-basic, pGL3-xCRFa533, or pGL3-xCRFb[CREmut]. Saline injection did not alter the expression of any of the constructs compared with the respective no injection controls. We also subjected the transfected tadpoles to shaking/handling stressor for 6 h, but observed no significant changes in luciferase activity with any of the constructs compared with the no handling controls (Fig. 3.8B).

We raised a subset of transfected tadpoles through metamorphosis and analyzed promoter activity in juvenile frogs. Luciferase activity in brain homogenates of transfected animals was detectable at the juvenile stage, although it was significantly lower compared with activity in the tadpole stage. We first injected oCRF i.c.v. and measured luciferase activity 6 h later. Similar to the results in transfected tadpoles, oCRF caused a significant increase in luciferase activity in pGL3-xCRFb576 transfected frogs compared with no injection ($p = 0.033$) or saline-injected controls ($p = 0.005$); the same treatment did not alter activity in pGL3-basic transfected animals. Injection of saline did not alter the expression of any of the constructs compared with the no injection controls (Fig. 3.8C).

We also subjected frogs transfected with pGL3-xCRFb576 to shaking/handling stressor for 6 h and, by contrast to the transfected tadpoles, we observed significantly increased luciferase activity compared with no handling controls ($p = 0.03$; Fig. 3.8C; i.c.v. oCRF was included as a positive control in the experiment). We observed no change in pGL3-basic activity following exposure to the stressor.

Discussion

We discovered that the gene organization and proximal promoter elements of frog CRF genes are remarkably conserved with mammalian CRF genes, two tetrapod lineages that diverged over 200 million years ago. This high degree of conservation argues that the structural elements responsible for the spatial and physiological regulation of CRF genes arose early in vertebrate evolution, and have been maintained by strong positive selection owing to the pivotal role that CRF plays in physiological and behavioral adaptation. Furthermore, our findings provide strong support for the hypothesis that stressor-induced activation of vertebrate CRF genes *in vivo* is dependent upon CREB binding to the CRE located in the proximal promoter region.

Earlier we found that the distribution of CRF neurons in the brain of *X. laevis* was highly conserved with that of mammals, and that CRF-ir was strongly increased in the anterior preoptic area (homologous to the mammalian paraventricular nucleus), the medial amygdala, and the bed nucleus of the stria terminalis following exposure to shaking/handling stressor (Yao et al., 2004). The similarity of the central distribution of frog CRF neurons to mammals, and their stressor-dependent activation suggested the existence of conserved gene regulatory mechanisms. Mammalian CRF genes exhibit a

very high degree of conservation (94%) within the proximal promoter regions (first 330 bp flanking sequence; Vamvakopoulos et al., 1990; M. Yao unpublished data). We found that the frog CRF promoters (~340 bp flanking sequence) are also very highly conserved with mammalian genes, exhibiting 63% (xCRFa) and 72% (xCRFb) similarity with the corresponding region of the human CRF gene.

The overall sequence similarity between the frog CRFa and CRFb genes within the ~340 bp upstream of the transcription start site is 75%, which is comparable to the sequence similarity between either of the frog genes and the human gene. Thus, the duplicate frog CRF genes have been diverging at a faster rate than CRF genes among mammalian species. The proximal promoter region of xCRFb is more highly conserved with mammalian genes than xCRFa, suggesting that the 'a' gene may be diverging at a faster rate than the 'b' gene. This divergence may explain the low responsiveness of the xCRFa promoter to forskolin treatment in transfected cells, and its lack of activation by i.c.v. CRF injection in transfected tadpole brains. Furthermore, data from both semi-quantitative and quantitative RT-PCR analysis of hnRNA suggest that the xCRFb gene is expressed at a higher level than the xCRFa gene in the frog brain (Yao and Denver, unpublished data). This may mean that the 'a' gene is becoming a pseudogene. Alternatively, the 'a' gene may be acquiring a novel functional role based on the modification of its regulatory regions. The conservation of the coding regions of the two frog CRF genes suggests that there is selection to maintain the structure of the mature CRF peptides, but divergent evolution in the regulatory regions of the two genes.

Computer analysis of the frog genes revealed several putative transcription factor binding sites in the proximal promoter region that are conserved with mammalian genes.

These included consensus CRE, NBRE, AP1, and GRE sites which have been implicated in CRF gene regulation in mammals. We also have evidence that the AP1 and GRE sites in the frog CRF promoters are functional (M. Yao and R.J. Denver, unpublished data). However, in the present study we focused on the role of the putative CRE sites, and we provide several lines of evidence that support the functionality and essential nature of the CREs in the frog genes for gene activation by the cAMP pathway *in vivo*.

Several studies in mammals have shown that CREB is rapidly phosphorylated and thus activated in CRF neurons in response to various stressors, preceding an elevation of CRF expression in the same cells (Bilang-Bleuel et al., 2002; Chen et al., 2001; Kovács and Sawchenko, 1996a). We found that in juvenile frogs, pCREB-ir was significantly increased in the POA after 20 min exposure to shaking/handling stressor, which is consistent with the early kinetics of pCREB accumulation observed in mammals. To our knowledge, this is the first demonstration of stressor-dependent CREB activation in the brain of a nonmammalian vertebrate. In the frog as in the mammal we observed a delay in the increase of CRF-ir in the same cells that were immunopositive for pCREB, occurring 1 h after exposure to the stressor. The early appearance of pCREB in CRF neurons of the POA show that pCREB is present in these neurons and thus could mediate stressor-dependent activation of frog CRF genes *in vivo*.

We used transient transfection assays in PC-12 cells to determine if the frog CRF promoters support basal transcription, and whether the putative CREs present in the genes are functional. Both frog CRF promoters exhibited basal activities that were 2-3-fold that of the empty vector control. The activity increased following treatment with forskolin by 10 fold for xCRFa, and 43 fold for xCRFb. We also determined basal and

forskolin-induced CRF promoter activity in a frog cell line, the myoblast-derived XLT-15 which expresses CRF (Boorse et al., 2006). Both promoter constructs showed much higher basal activity in XLT-15 than in PC-12 cells (15-19 times the basal activity of the empty vector). We found similar activation of both promoters by forskolin in the frog cells, although to a lesser degree than in PC-12 cells (about 2-fold). These results show that the frog CRF promoters support basal transcriptional activity and activation by the cAMP pathway.

Specific roles for the putative CREs in the frog CRF genes are supported by mutagenesis experiments, where we found that mutation of the CRE in the xCRFb promoter significantly reduced both basal and forskolin-induced promoter activity in PC-12 cells, and completely eliminated forskolin induction in XLT-15 cells. Expression of a dominant-negative CREB strongly reduced basal, and eliminated forskolin-induced activity. In further support of the frog CREs being bona fide CREB binding sites, we showed by EMSA that recombinant *Xenopus* CREB bound both CREs *in vitro*. The specificity of this binding was demonstrated by displacement with radioinert CRE probes, and by the failure of the protein to bind to the mutant CREb. Using antibodies to both CREB and pCREB we showed that the protein-DNA complex formed with frog brain nuclear extract and the CREb probe in EMSA contained predominantly CREB. These data strongly support the hypothesis that CREB binds to the identified CREs in the frog genes and plays an important role in regulating both basal and cAMP-dependent CRF promoter activity. The mutagenesis and dominant negative CREB experiments suggest that the identified CREs mediate the majority, if not all, of the cAMP-dependent activity.

The frog CRFa promoter exhibited consistently lower forskolin-induced activity than the CRFb promoter in PC-12 cells, both in terms of the absolute level of luciferase and the fold increase above basal caused by forskolin (see Fig. 3.4A). We hypothesized that this difference was due to sequence variation among the CREs of the two genes, and thus tested this by exchanging their CREs through site directed mutagenesis. Mutating the CRE of the 'a' gene into the CREb elevated the absolute level of both basal and forskolin-induced activity in PC-12 cells compared with the native xCRFa promoter (see Fig. 3.5A). However, the fold increase was not significantly changed from the native CRFa promoter (see Supplemental Data Fig. 3.2). By contrast, swapping the CREb into the 'a' gene did not alter the absolute magnitude of basal or forskolin-induced activity or the fold increase. These data suggest that the structure of the CRE can influence the absolute level of both basal and cAMP-dependent promoter activity. However, it is clear that other, as yet uncharacterized elements in the core promoters of the two genes also contribute to the differences in activity.

By contrast to PC-12 cells, in the frog cell line XLT-15 we found that replacing the CRE of the 'a' gene with the CREb resulted in higher forskolin-induced activity than both native promoters. On the other hand, changing the CRE of the 'b' gene to that of the 'a' gene did not affect xCRFb promoter activity in XLT-15 cells. Taken together, our data suggest that the core sequence of the CRE consensus site (octomer) is crucial to the basal activity and the responsiveness of the promoters to cAMP pathway activation, while other promoter elements also play a significant role in the functionality of the element. Furthermore, promoter activity is dependent on the cell type in which the gene is

expressed, which likely reflects cell-specific expression of phosphodiesterase, second messenger molecules, transcription factors, etc.

To determine if CREB occupies the proximal frog CRF promoters *in vivo* we conducted ChIP assay on chromatin prepared from diencephalon (POA/hypothalamus) of juvenile frogs. This assay confirmed that CREB associated with the CRE region of a frog CRF gene *in vivo* (only the xCRFb gene was analyzed), as has been shown for the CRF gene in the central nervous system of the rat (Shepard et al., 2005). This result is in agreement with a genome-wide analysis conducted on human and rat cell lines which suggested that CREB occupies a majority of CRE sites located near transcription start sites (Impey et al., 2004; Zhang et al., 2005). Following cAMP activation CREB is phosphorylated at a conserved serine (Ser-133), which leads to recruitment of the coactivator CREB-binding protein (CBP)/p300 which has histone acetyltransferase activity and thus promotes transcriptional activation of target genes (Cardinaux et al., 2000; Du et al., 2000; Goodman and Smolik, 2000; Mayr and Montminy, 2001). Consistent with this model, we found that the xCRFb proximal promoter had significantly higher acetylated histone H4 than the CDS region. We also found increased CREB association with the xCRFb proximal promoter following exposure to shaking/handling stressor, suggesting that the recruitment of CREB is enhanced by exposure to a stressor. Increased association of CREB with the CRF promoter following activation of the cAMP pathway has been observed in cultured cells (Ngo et al., 2002; Wolfl et al., 1999; Zhang et al., 2004). Shepard and colleagues (Shepard et al., 2005) used ChIP assay to show that the pCREB signal at the CRF promoter in rat brain increased following exposure to a stressor.

The findings discussed above support the view that the CREs present in the proximal promoters of vertebrate CRF genes mediate stressor-dependent activation through CREB binding. In the current study, we used EM gene transfer into frog brain to test whether the CRE is required for CRF gene activation in an *in vivo* context. *In vivo* EM gene transfer has been used for different purposes including the mapping and study of *cis*-regulatory elements (Haas et al., 2001; Itasaki et al., 1999; Muramatsu et al., 1997; Osumi and Inoue, 2001; Swartz et al., 2001; Yasugi and Nakamura, 2000). We used bulk EM gene transfer to transfect a majority of cells surrounding the third ventricle of the tadpole brain. This method transfects diverse cell types in the brain, and thus we do not know if the measured luciferase activity reflects CRF neuronal activity per se. However, this approach provides a means for testing whether the CRF promoter constructs, which are functional in cultured cells, can support transcription in cells of a live animal.

Using EM gene transfer we found that activity of the 'b' promoter (pGL3-xCRFb576) was increased significantly in brains of transfected tadpoles by i.c.v. injection of CRF. This showed that the promoter can be activated *in vivo* by an agent that induces cAMP pathway activation. Mutation of the CRE significantly reduced basal activity, and abolished promoter activation caused by CRF. We subjected transfected tadpoles to shaking/handling stressor but detected no changes in the activities of any of the constructs. However, when raised to the juvenile frog stage we found that the activity of pGL3-xCRFb576 was significantly increased both by injection of CRF and by shaking/handling stressor. This suggests that the ability of a stressor to activate the CRF promoter may be dependent on the developmental stage, which requires further study. Taken together, our findings with EM gene transfer *in vivo* support *in vitro* cell culture

experiments that showed that the CRE in the CRF proximal promoter plays an essential role in both basal and induced gene activity.

Interestingly, although the activity of pGL3-xCRFa533 was significantly higher than pGL3-xCRFb576 *in vivo*, the activity of this construct was not altered by i.c.v. injection of CRF. This finding supports that there are functionally important differences between the regulatory elements of the duplicated genes, and that the xCRFa gene may not be inducible *in vivo*.

In conclusion, we provide evidence for an essential role for the CRE present in vertebrate CRF genes for stressor-dependent gene activation. The gene organization and proximal *cis* regulatory elements of CRF genes are highly conserved among frogs and mammals, suggesting that these features of CRF genes were present in the earliest land dwelling vertebrates. Similar responsiveness of fish CRF genes to stressors (Bernier and Craig, 2005; Doyon et al., 2003; Doyon et al., 2005; Pepels et al., 2004a) support the view that these regulatory elements arose before the evolution of the tetrapods, and have been maintained by strong positive selection owing to the essential nature of the physiological systems controlled by CRF.

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Table 3.1. Sequences of oligonucleotide probes used in EMSA.

DNA probe	Sequence
hCRFCRE	GGCTCGTTGACGTCACCAAGAGGC
xCRFaCRE	**GTTGCACCTACGTCACTAAAGAGGC**
xCRFbCRE	GTGTCATTTACGTCACTAAAGAGGC
xCRFb[CREmut]	GTGTCATTTA**A**GTC**T**CTAAAGAGGC
xCRFb[CREdel]	GTGTCAT**AGCTAGCT**CTAAAGAGGC

Only upper strands are shown (5' → 3'). The CRE octomers or the corresponding sequences in the mutated forms are underlined, and the mutated bases are shown in bold.

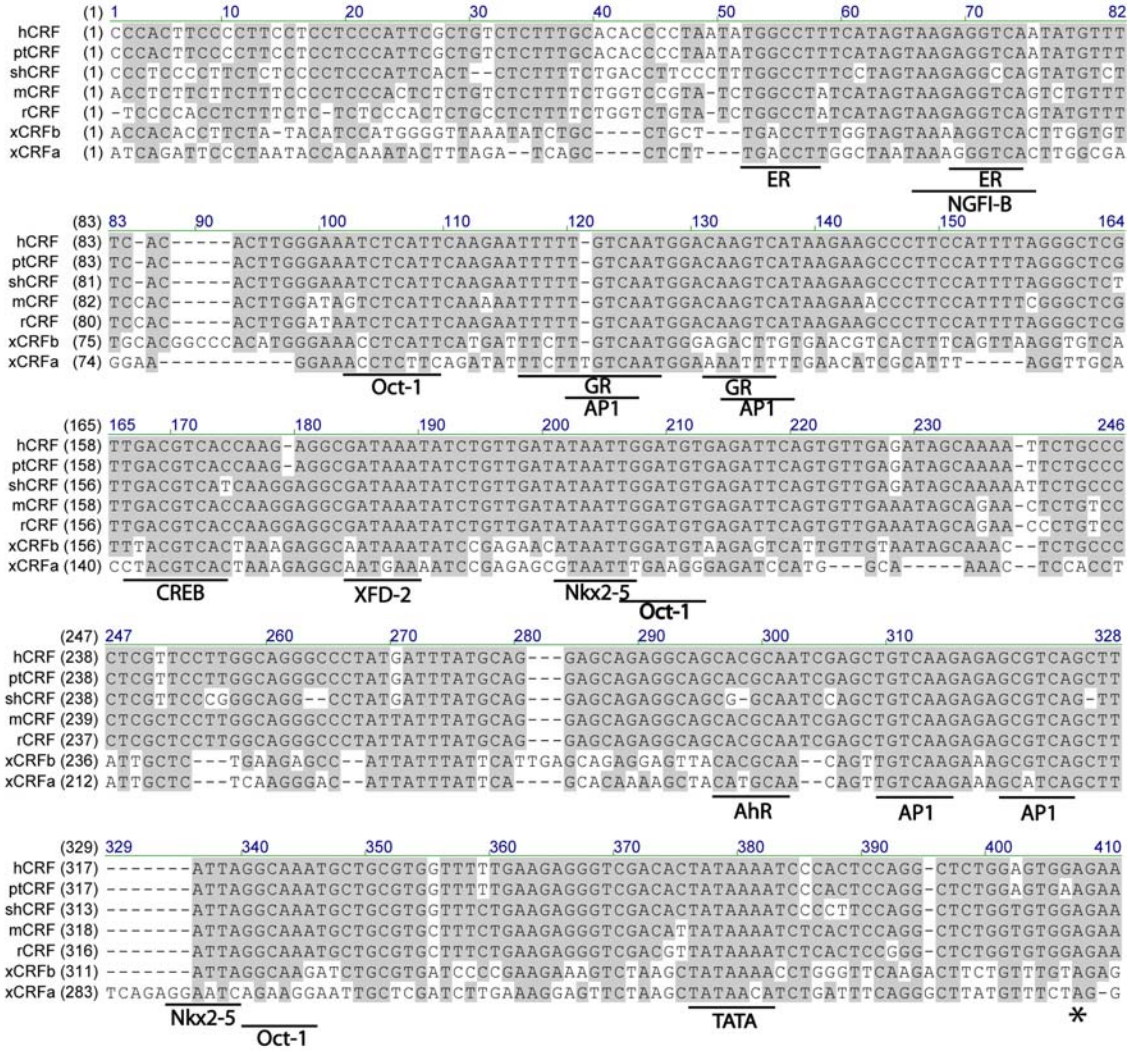


Figure 3.1. ClustalW alignment of 5' promoter regions of human (h), chimp (pt), sheep (sh), mouse (m), rat (r), and *Xenopus* CRF (x) genes. Identical sequences among the CRF genes are shadowed. The asterisk indicates transcription start sites. Putative binding sites for transcription factors predicted by the on-line program Match™ using a library of mononucleotide weight matrices from TRANSFAC® 6.0 (www.gene-regulation.com) are indicated. ER: estrogen receptor; NGFI-B: nerve growth factor induced gene B; Oct-1, octamer binding transcription factor 1; GR, glucocorticoid receptor; AP1, activator protein 1 (Jun/Fos); CREB, CRE binding protein; XFD2, *Xenopus* forkhead domain factor 2; Nkx 2-5, cardiac-specific homobox protein; AhR, aryl hydrocarbon receptor; TATA: TATA box.

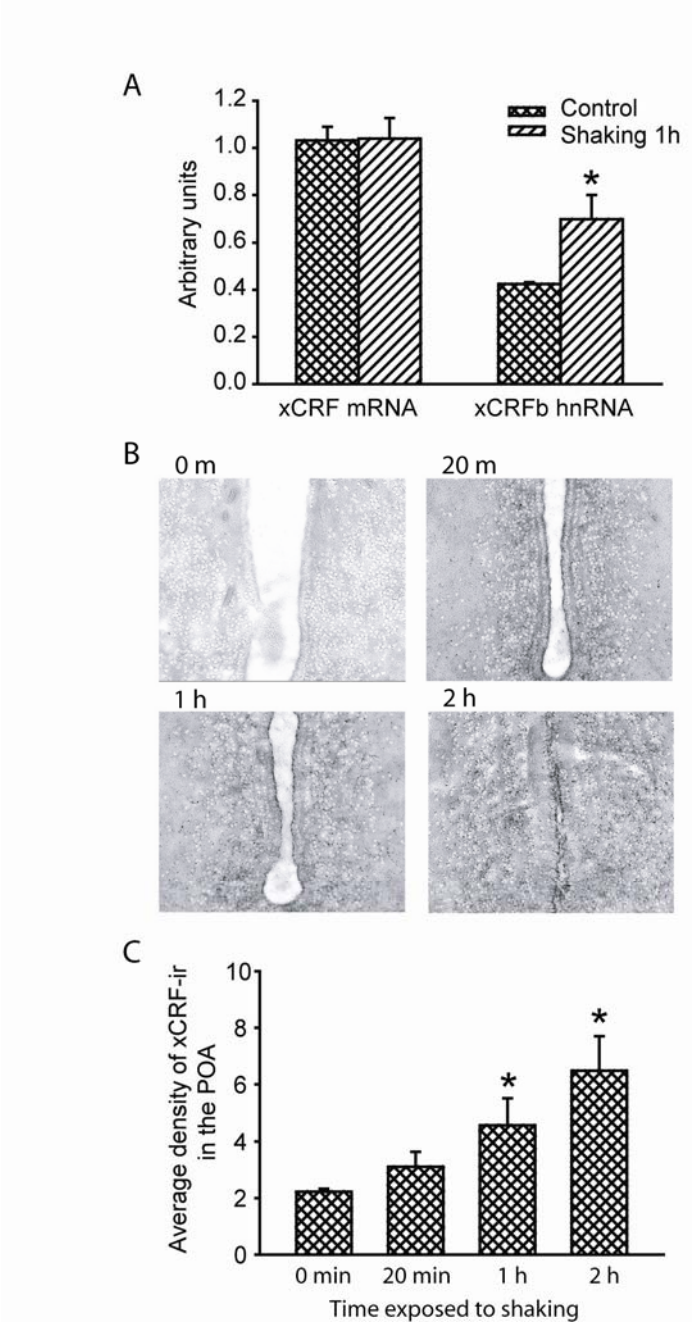


Figure 3.2. Effects of shaking stressor on CRF expression in the anterior preoptic area (POA) of juvenile *X. laevis*. (A) Analysis of CRF mRNA and xCRFb hnRNA in the POA/hypothalamus of juvenile frogs by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Frogs were either left undisturbed (unstressed) or exposed to 1 h shaking/handling stressor. (B) Changes in CRF immunoreactivity (CRF-ir) in the POA of juvenile *X. laevis* at 0, 20 min, 1 h, and 2 h after exposure to shaking/handling stressor. (C) Densitometric analysis shows the mean density of CRF-ir was significantly increased in the POA at 1 h and 2 h in stressed *X. laevis* compared with animals at 0 time (unstressed controls) and 20 min. Data presented are the mean + SEM. A significant difference from unstressed controls is denoted by * for $p < 0.05$.

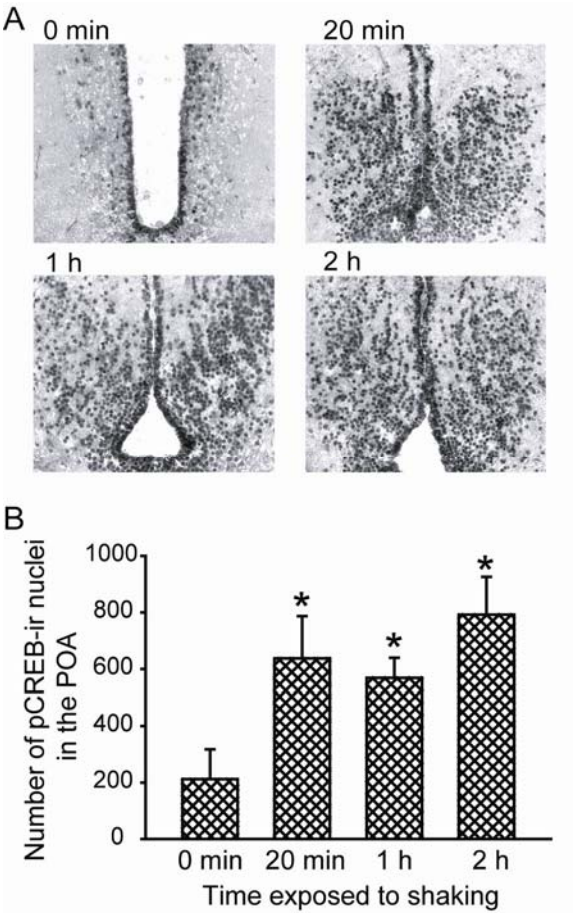
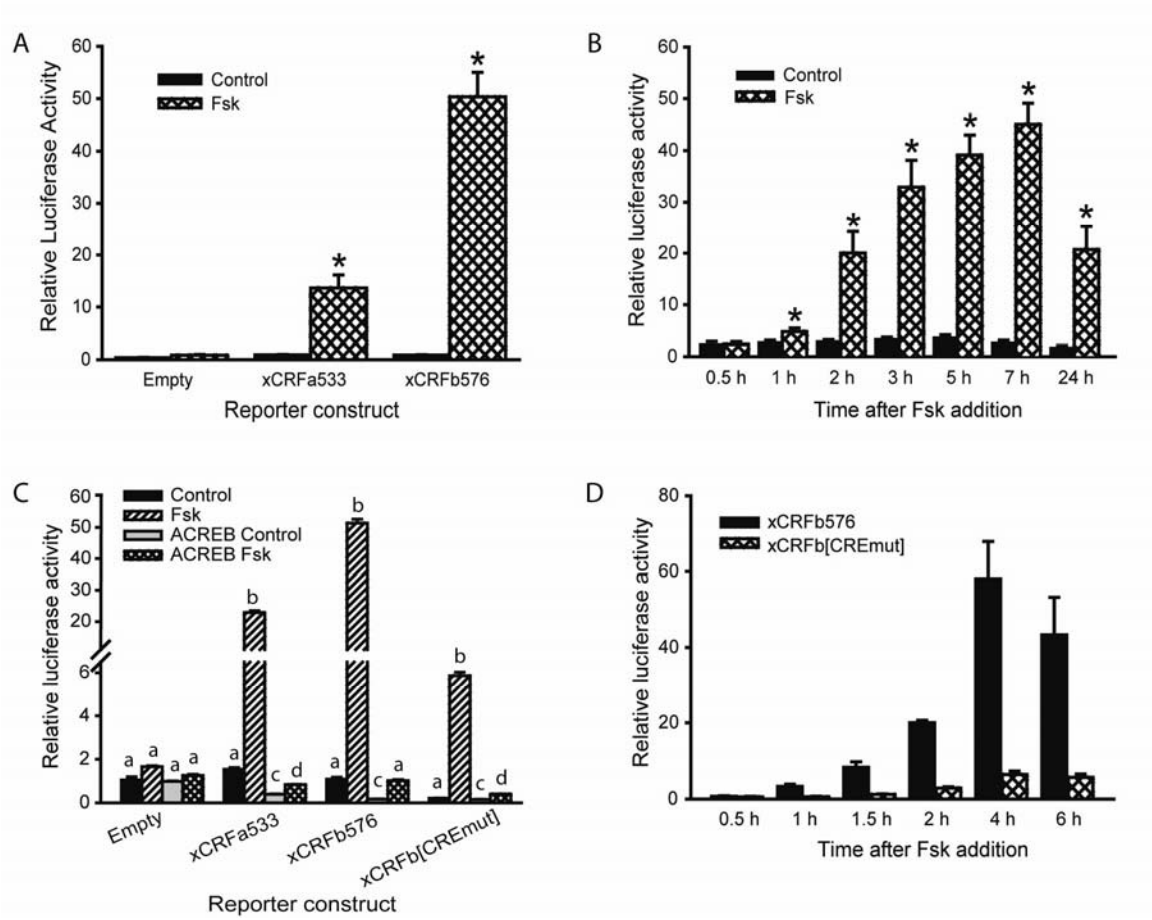


Figure 3.3. Effects of shaking stressor on phosphorylated CREB immunoreactivity (pCREB-ir) in the anterior preoptic area (POA) of juvenile *X. laevis*. (A) Changes in pCREB-ir in the POA of juvenile *X. laevis* exposed to shaking/handling stressor. (B) The total number of pCREB-ir positive nuclei in the POA was significantly increased at 20 min, 1 h, and 2 h in stressed *X. laevis* compared with unstressed controls. Data presented are the mean + SEM. Significant differences from unstressed controls are denoted by * for $p < 0.05$.

Figure 3.4. Regulation of the frog CRF promoters by activation of the PKA/CREB pathway in transiently transfected PC-12 cells. (A) Activation of frog CRF promoter-reporter constructs by 6 h treatment with forskolin (Fsk; 25 μM) in transiently transfected PC-12 cells. PC-12 cells were transfected with empty vector pGL3-basic (Empty), pGL3-xCRFa533 (xCRFa533; 533 bp fragment of the xCRFa promoter) or pGL3-xCRFb576 (xCRFb576; 576 bp fragment of the xCRFb promoter) plus pRL-null to normalize for transfection efficiency (dual luciferase assay; see Materials and Methods). The experiment was repeated five times in triplicate, and data (mean + SEM) of a representative experiment are shown. The asterisk indicates a significant difference compared with the respective control (Student's *t* tests, $p < 0.05$). (B) Time course of activition of pGL3-xCRFb576 by forskolin in transiently transfected PC-12 cells. The construct pGL3-xCRFb[CREmut] (xCRFb[CREmut]) contains the same promoter fragment as the pGL3-xCRFb576 except with two point mutations introduced into the CRE (from TTACGTCA to TTAAGTCT). Transfected cells were treated with or without forskolin (25 μM) for various times. Bars represent means + SEM of two independent experiments conducted in triplicate. Asterisks indicate significant differences between Fsk treated and control cells (Student's *t* tests, $p < 0.05$). (C) Effect of point mutations introduced into the putative CRE of the xCRFb promoter and cotransfection of a vector expressing ACREB, a mutant form of CREB with dominant negative activity, on basal activity and the response to forskolin (25 μM; 6 h) in transiently transfected PC-12 cells. The experiment was repeated three times in triplicate, and data (mean + SEM) of one representative experiment are shown. Letters indicate significant differences among treatment means within each reporter construct ($p < 0.05$). (D) Effect of point mutations in the putative CRE of the xCRFb promoter on the time course of forskolin (25 μM)-induced activation in transfected PC-12 cells.


A
Relative Luciferase Activity
Control
Fsk
Empty
xCRFa533
xCRFb576
Reporter construct
B
Relative luciferase activity
Control
Fsk
0.5 h
1 h
2 h
3 h
5 h
7 h
24 h
Time after Fsk addition
C
Relative luciferase activity
Control
Fsk
ACREB Control
ACREB Fsk
Empty
xCRFa533
xCRFb576
xCRFb[CREmut]
Reporter construct
D
Relative luciferase activity
xCRFb576
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0.5 h
1 h
1.5 h
2 h
4 h
6 h
Time after Fsk addition

Figure 3.4

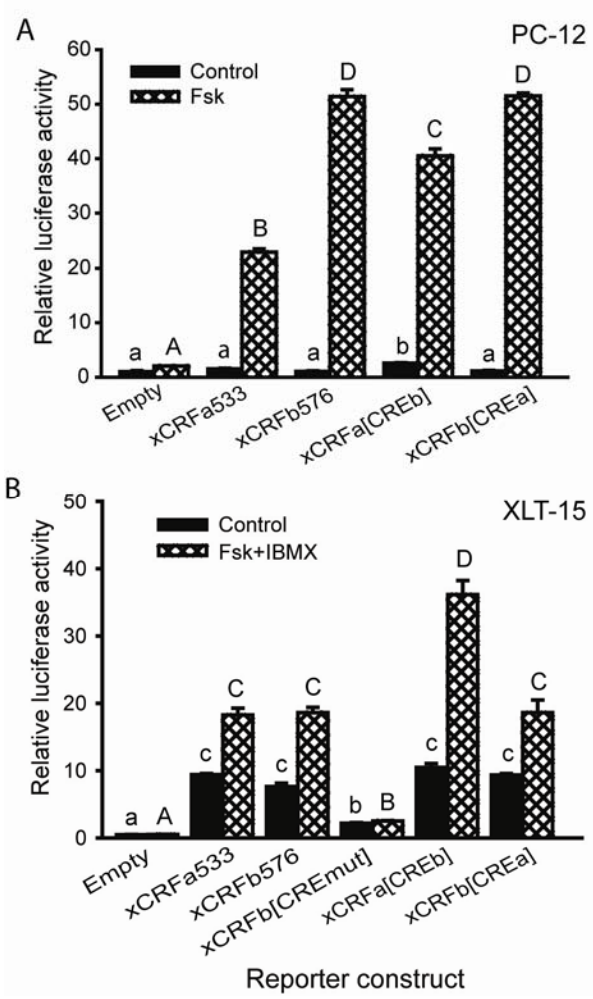

Figure 3.5. Effects of exchanging the CREs present in the xCRFa and xCRFb genes on promoter activity in transiently transfected PC-12 and XLT-15 cells. (A) Effects of exchanging the CREs present in the xCRFa and xCRFb genes on basal and forskolin-dependent (Fsk; 25 μM; 6 h) promoter activity in transiently transfected PC-12 cells. The experiment was repeated three times in triplicate, and data from one representative experiment are shown (mean + SEM). Letters indicate significant differences among constructs within a treatment (control or forskolin; ANOVA F (9,20)= 1239.5, Fisher's LSD multiple comparisons tests, $p < 0.001$). (B) Basal and forskolin-induced activity (Fsk 25 μM + IBMX 500 nM; 24 h) of frog native and CRE mutant CRF promoter constructs in transiently transfected *X. laevis* tadpole tail myoblast-derived (XLT-15) cells. Shown are the means + SEM and letters indicate significant differences among constructs within a treatment (control or Fsk+IBMX; ANOVA F (13,28)= 188.7, Fisher's LSD multiple comparisons tests, $p < 0.001$). Each experiment was repeated three times in triplicate, and data from one representative experiment are shown. All reporter constructs were constructed using the pGL3-basic vector.

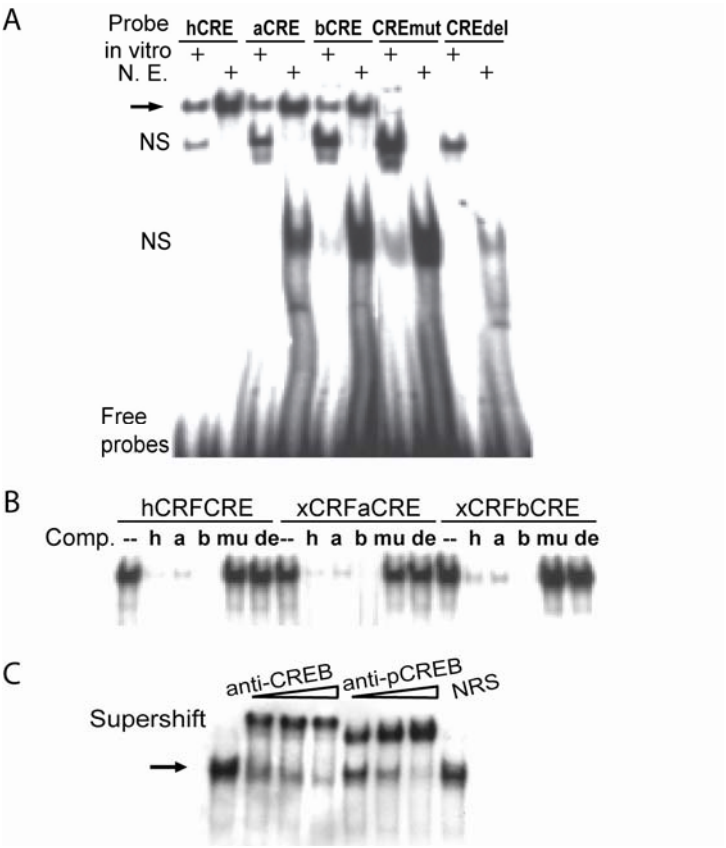

Figure 3.6. Binding of CREB to the CREs in the CRF promoters as analyzed by electrophoretic mobility shift assay (EMSA) (A) Binding of recombinant *X. laevis* CREB (rxCREB) or nuclear extracts derived from the anterior preoptic area (POA)/hypothalamus of the juvenile frog brain (see Materials and Methods) to ^{32}P-labeled CRE probes as analyzed by EMSA. Sequences of the probes are given in Table 3.1. Lane 1, 3, 5, 7, 9 incubated with rxCREB; lane 2, 4, 6, 8, 10, incubated with brain nuclear extract (N.E.). The major band is indicated by an arrow. NS, nonspecific bands. (B) Cross-competition of CREB binding to ^{32}P-labeled CRE probes by radioinert oligonucleotides (Comp. = competitor). The ^{32}P-labeled hCRFCRE, xCRFaCRE or xCRFbCRE probes were incubated with brain nuclear extract in the presence or absence of 100 nM of radioinert competitor oligonucleotides: --, no competitor; h, hCRFCRE; a, xCRFaCRE; b, xCRFbCRE; mu, xCRFb[CREmut]; de, xCRFb[CREdel]. (C) Antibody super-shift EMSA using antiserum to CREB (anti-CREB), phosphorylated CREB (anti-pCREB) or normal rabbit serum (NRS). The ^{32}P-labeled xCRFbCRE probe was incubated with frog brain nuclear extract in the presence or absence of varying amounts of serum (1, 2, and 4 µl of anti-CREB; 0.25, 0.5, and 1 µl of anti-pCREB; 10 µg of NRS IgG). The specific CREB-DNA complex is indicated by the arrow.

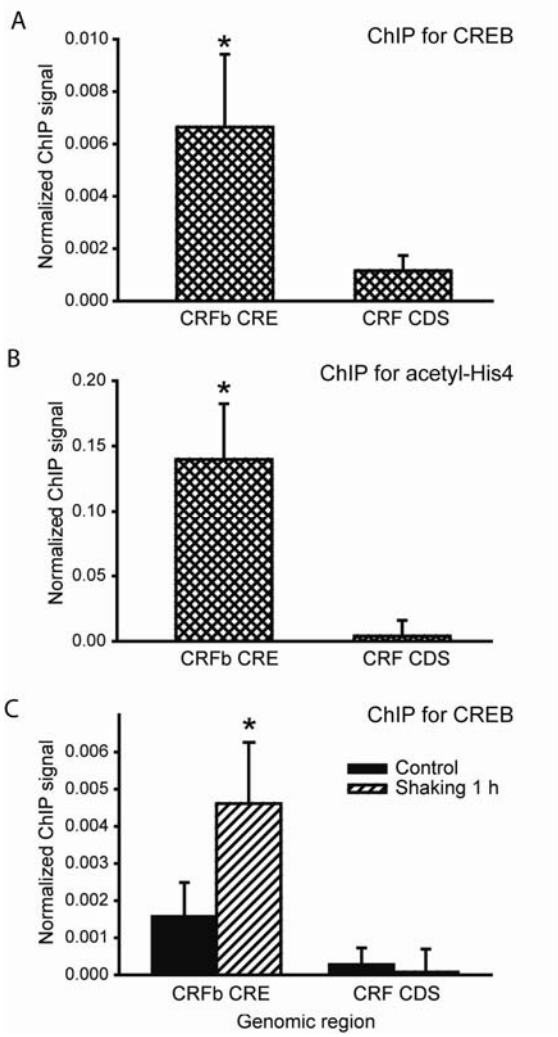

Figure 3.7. Association of CREB or acetylated histone H4 with the CRE region of the CRFb proximal promoter as analyzed by chromatin immunoprecipitation (ChIP) assay. (A) ChIP assays for CREB or (B) acetylated histone H4 on chromatin obtained from brain of unstressed frogs. (C) ChIP assay for CREB on chromatin obtained from brain of frogs exposed to shaking stressor for 1 h. The genomic regions analyzed were the proximal promoter of xCRFb targeting the CRE (CRFb CRE), the coding regions of xCRF genes (CRF CDS; assay did not distinguish the two genes; considered a negative control). Ten hypothalamic sections derived from juvenile frogs were pooled for each sample, and three to four replicate pools were analyzed for each treatment. Data are expressed as normalized ChIP signal. That is, the values for each sample obtained by ChIP with anti-CREB or anti-acetyl-Histone H4 were first divided by the respective input values, followed by subtraction of the values obtained by ChIP using NRS (background signal). Shown are means + SEM. Derived values were $\log_{10}$ transformed before ANOVA followed by Fisher's LSD multiple comparisons tests. Asterisks indicate significant difference from the coding regions of xCRF (CRF CDS; panel A, B) or unstressed control (panel C) ($p < 0.05$).

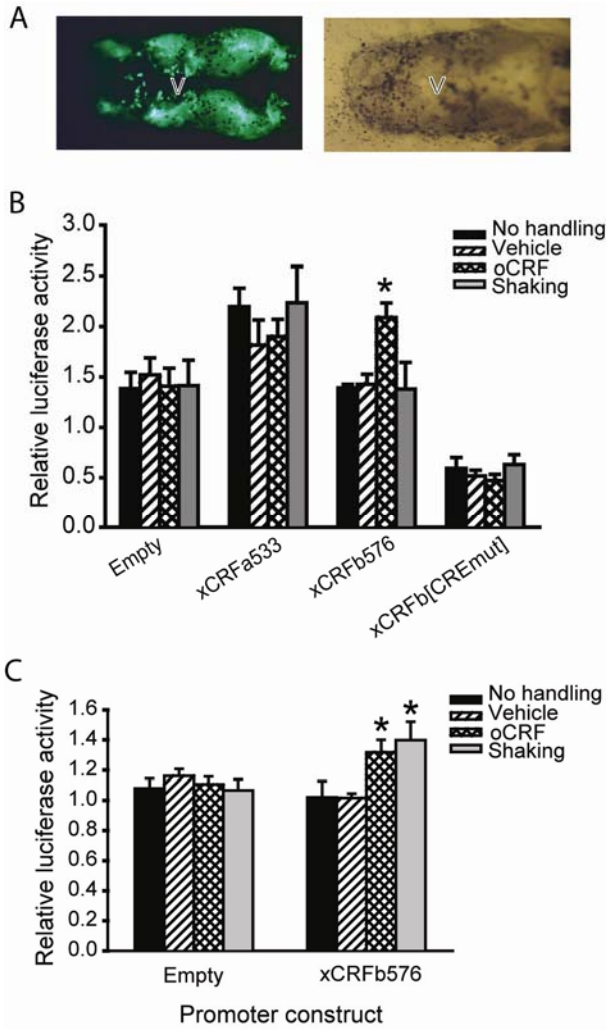

Figure 3.8. Effects of injection of ovine CRF (oCRF) or shaking stressor on the activity of CRF promoter constructs transfected into *X. laevis* brain. (A) Fluorescent (left) and light (right) microscopic images of the brain of a tadpole transfected with pEGFP-N1 plasmid by electroporation. V: third ventricle. (B) Effects of i.c.v. injection of ovine CRF (oCRF) or exposure to shaking/handling stressor on the activity of CRF promoter constructs transfected into tadpole brain by electroporation mediated (EM) gene transfer. Tadpoles were electroporated with the indicated constructs plus pRL-null to control for transfection efficiency and 1-2 weeks later given i.c.v. injections of either saline or oCRF (20 ng/g B.W.), or exposed to shaking/handling stressor. Animals were sacrificed 6 h after injection or initiation of the shaking/handling stressor and brains collected for dual luciferase assay. (C) Effects of i.c.v. injection of oCRF (20 ng/g B.W.) or exposure to shaking/handling stressor on the activity of CRF promoter constructs in brains of juvenile frogs (B.W. 0.6~2.5 g). Plasmids were transfected into premetamorphic tadpole brain by EM gene transfer and the animals were raised to 2-3 weeks post-metamorphosis. All animals were sacrificed 6 h after i.c.v. injection or initiation of the shaking stressor. Each experiment was repeated twice, and data from a representative experiment are shown as the mean + SEM. Sample sizes are 8 to 19 in (B) and 6 to 8 in (C) for each treatment group. Asterisks indicate significant differences compared with the respective no injection/no handling controls within a construct ($p < 0.05$).

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CHAPTER 4

DISTRIBUTION AND CORTICOSTEROID REGULATION OF THE GLUCOCORTICOID RECEPTOR IN THE CENTRAL NERVOUS SYSTEM OF *XENOPUS LAEVIS*

Abstract

Glucocorticoids (GCs) play pivotal roles in regulating metabolism, immune function, and development and are the effectors of endocrine stress response in vertebrates. A majority of GC-dependent actions appear to be mediated by the glucocorticoid receptor (GR). Virtually nothing is known about the distribution of GR expression in the central nervous system, the regulation of GR by GCs, or the role of GR in the stress response in nonmammalian tetrapods. We used an affinity-purified antibody raised against *Xenopus laevis* GR to map its distribution in the brain of the frog by immunocytochemistry. We found GR immunoreactive (GR-ir) cells distributed throughout the frog brain. The highest densities of GR-ir were identified in the medial pallium (mp; homolog of the mammalian hippocampus), accumbens, anterior preoptic area (POA; homolog of the mammalian paraventricular nucleus), the Purkinje cell layer of the cerebellum and the rostral anterior pituitary gland (location of corticotropes). Lower but distinct GR-ir was observed in the internal granule cell layer of the olfactory bulbs, dorsal and lateral pallium, striatum, various subfields of the amygdala, bed nucleus of the stria terminalis (BNST), optic tectum, various tegmental nuclei, locus coeruleus, Raphe nuclei, reticular

nuclei, and the nuclei of the trigeminal motor nerves. To determine if circulating corticosteroids influence GR-ir in the brain and pituitary, we exposed juvenile frogs to corticosterone (CORT) or the corticosteroid synthesis inhibitor metyrapone (MTP) in their rearing water for five days. Treatment with CORT caused significant decreases in GR-ir in the POA, mp, medial amygdala, BNST, and the rostral anterior pituitary. Treatment with MTP also resulted in significant reductions in GR-ir in the POA, mp, medial amygdala, and BNST, but not in the rostral anterior pituitary. Replacement with a low dose of CORT in MTP-treated animals reversed these effects. Thus, both chronically increased or decreased circulating corticosteroids reduce GR-ir in the frog brain and pituitary. Our results show that the central distribution of GR-ir is highly conserved among vertebrates, and that expression of GR in the frog brain and pituitary is under the control of circulating corticosteroids in a manner similar to that observed in mammals. Glucocorticoid-dependent changes in GR expression may represent an important mechanism for regulating tissue sensitivity to GCs.

Introduction

Glucocorticoids (GCs) regulate a diversity of metabolic, immune, memory, and behavioral functions (Chrousos, 1998; Chrousos and Gold, 1992). The production of GCs by adrenocortical cells is controlled by pituitary adrenocorticotropic hormone (ACTH) whose synthesis and secretion is regulated by hypothalamic corticotropin-releasing factor (CRF) and arginine vasopressin (AVP). Circulating GCs increase rapidly following exposure to internal or external stressors and serve to coordinate physiological and behavioral adjustments that maintain stability. Elevated plasma GCs exert negative

feedback at multiple levels of the hypothalamo-pituitary-adrenal (HPA) axis to prevent continued activation (Makino et al., 2002a).

Two kinds of corticosteroid receptors were originally identified in mammals based on their binding affinities: the high affinity type I receptor (also called the mineralocorticoid receptor; MR; NR3C2) and the lower affinity type II receptor (also called the glucocorticoid receptor; GR; NR3C1). In mammals, evidence suggests that the MR functions to maintain basal expression and the circadian rhythm of circulating GCs, while most GC-dependent physiological changes that occur in response to stressors, and feedback regulation by GCs are mediated by the GR (Bamberger et al., 1996; De Kloet et al., 1998; Wei et al., 2004).

The GR and MR belong to the nuclear hormone receptor superfamily. Phylogenetic analysis suggests that these two receptors arose by a gene duplication event in the gnathastome lineage (Bridgham et al., 2006; Thornton, 2001). These modular proteins consist of an N-terminal transactivation domain, a highly conserved DNA-binding domain, and a C-terminal ligand-binding domain that is also involved in transactivation (Duma et al., 2006). The trafficking of GR within the cell has been studied intensively (Pratt and Toft, 1997). In the absence of ligand, the GR is located in the cytosol associated with heat shock proteins and immunphilins (referred to as the 'foldosome'; Pratt and Toft, 1997). The major function of the foldosome is to stabilize GR and maintain it in an inactive yet ligand binding conformation (Pratt and Toft, 1997). Ligand binding causes disassociation of the protein complex and translocation of GR into the nucleus. In the nucleus, GR may enhance or repress transcriptional activity of its target

genes by direct binding to glucocorticoid response elements (GREs) or by protein-protein interactions with other transcription factors and cofactors (Kumar and Thompson, 2005).

Full-length or partial cDNA sequences for the GR have been isolated from nonmammalian species including birds (chicken, Kwok et al., 2007; zebra finch [*Taeniopygia guttata*], Hodgson et al., 2007), fishes (the rainbow trout [*Onchorhynchus mykiss*], Ducouret et al., 1995; tilapia [*Oreochromis mossambicus*], Tagawa, 1997 #642}; Japanese flounder, Tokuda et al., 1999; fathead minnow [*Pimephales promelas*], Filby and Tyler, 2007; and the frog *Xenopus laevis*, Gao et al., 1994) and have been found to be highly conserved. These genes were isolated and named based on their sequence similarity to the mammalian GR (Gao et al., 1994; Thornton, 2001). The primary amino acid sequences of the DNA binding domains of *Xenopus* and human GR are identical, while the hormone binding domains share 86% similarity. The N-terminal domains of frog and mammalian GR proteins are less conserved, showing 51% similarity at the amino acid level (Gao et al., 1994). The *Xenopus* GR, when expressed in COS cells, was capable of mediating hormone-dependent transactivation of a GRE-containing promoter to a similar extent as the rat GR (Gao et al., 1994). These data suggest that the structure and function of GR are evolutionarily conserved among vertebrate species.

Glucocorticoid receptor protein and mRNA are widely distributed in the CNS of rodents (Ahima and Harlan, 1990; Morimoto et al., 1996). The highest levels of GR-immunoreactivity (GR-ir) and mRNA are found in the cerebral cortex, olfactory pyramidal layers, hippocampal formation, paraventricular nucleus (PVN), medial and central nuclei of the amygdala, granule layer of the cerebellar cortex, and the locus coeruleus (Ahima and Harlan, 1990; Morimoto et al., 1996).

Very limited studies have been conducted on the central distribution of GR in non-mammalian species. To our knowledge, nothing is known about GR distribution in amphibians or reptiles. Except for one report on GR-like immunoreactivity in the Japanese quail (Kovács et al., 1989), most studies on central distribution of GR in non-mammalian vertebrates have been conducted on teleost fishes. In the rainbow trout (*Oncorhynchus mykiss*), among the many brain regions where GR-ir was observed (detected using antibodies generated against the N-terminus of the rainbow trout GR fused with GST), the highest densities were found in the ventral telencephalon (homolog of the mammalian amygdala), preoptic region (homolog of the mammalian paraventricular nucleus), mediobasal hypothalamus, and the optic tectum, and lower densities of GR were seen in the internal cell layer of the olfactory bulb and lateral regions of the dorsal telencephalon (homolog of the mammalian hippocampus) (Teitsma et al., 1998). Using antibodies to mammalian GR, Carruth and colleagues (Carruth et al., 2000) reported a similar distribution pattern of GR immunoreactivity in the brain of kokanee salmon (*Oncorhynchus nerka kennerlyi*). The generally conserved central distribution of GR in fish and mammals suggests that expression and function of GR in the CNS might be evolutionarily conserved.

Among the GR-expressing brain regions of the rodent brain, the hippocampus and the paraventricular nucleus (PVN) have been intensively investigated due to their important roles in regulating activity of the HPA axis (De Kloet et al., 1998; Herman et al., 2005; Makino et al., 2002a). The hippocampus expresses the highest concentration of GR in the brain, and it exerts a tonic inhibitory influence on neurosecretory neurons in the PVN to down-regulate the activity of the HPA axis (Herman et al., 2005). The PVN neurons also

express high levels of GR and thus are direct targets for circulating GCs. Multiple lines of evidence support that GR mediates the negative feedback actions of circulating GCs on the HPA axis in both the hippocampus and the PVN (Makino et al., 2002a).

The expression of GR is also regulated by its ligands, GCs. Autoregulation of GR has been studied *in vitro* and *in vivo* in the rat. In primary cells, tissue explant cultures, and in a wide variety of cell lines, treatment with GCs was found to reduce expression of both GR mRNA and protein and the activity of the GR gene promoter (Bellingham et al., 1992; Burnstein et al., 1991; Dong et al., 1988; Meyer and Schmidt, 1995; Okret et al., 1986; Silva et al., 1994; Vedeckis et al., 1989). In the rat, administration of GCs caused decreases in expression of GR mRNA and/or protein in the hippocampus and cortex (Ghosh et al., 2000; Hügin-Flores et al., 2004; Spencer et al., 2000), whereas short-term (within one week) adrenalectomy (removal of endogenous corticosteroids) resulted in an elevation of GR mRNA in the hippocampus and PVN (Chao et al., 1998; Han et al., 2007; Holmes et al., 1995; Hügin-Flores et al., 2004; Reul et al., 1989). These results suggest that GCs inhibit transcriptional activity of the GR gene in these brain regions.

In the current study we analyzed the detailed distribution of GR-ir in the brain and the pituitary gland of the frog *Xenopus laevis*. We found that the basic pattern of GR-ir distribution in the brain is highly conserved among vertebrate species. We also found that GR-ir in specific brain regions was modulated by circulating corticosteroids. Thus we show the first detailed map of GR-ir distribution in the CNS of a non-mammalian tetrapod, and provide evidence that the expression of GR in the CNS and its regulation by corticosteriods are phylogenetically ancient features that have been maintained by natural selection.

Materials and Methods

Animal husbandry

Xenopus laevis juveniles were purchased from Xenopus I (Dexter, Michigan). Tadpoles were raised in dechlorinated tap water (20-22 ^{o}C; 12L:12D) and fed Frog Brittle (Nasco, Fort Atkinson, WI) *ad libitum*. Developmental stages were assigned according to Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994). 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 (UCCUA).

Production and purification of polyclonal antiserum to *Xenopus laevis* GR

We generated polyclonal antiserums to *X. laevis* GR in rabbits (Lampire Biological, Downingtown, PA, USA) using a synthetic peptide corresponding to amino acids 8-25 of frog GR conjugated to keyhole limpet hemocyanin. The antiserum was affinity purified first over an Affi-Gel Protein A column, followed by affinity purification over a peptide affinity column of the *X. laevis* GR antigenic peptide conjugated to Ultralink iodoacetyl matrix (Pierce Biotechnology, Inc., Rockfold, IL, USA) following the manufacturer's instructions. The concentration of the purified IgG was determined using the BCA Protein Assay (Pierce).

Western blotting analysis

We conducted Western blotting analysis using *in vitro* synthesized *X. laevis* GR protein or whole cell extract of XTC-2 cells (*X. laevis* carcass cell; Pudney et al., 1973).

We produced GR protein using the p6xGR vector (Gao et al., 1994) and the TNT system (Promega Corp., Madison, WI, USA) following the manufacturer's protocols. To determine the quality of the synthesized protein, we included ^{35}S-methionine in the *in vitro* transcription-translation reaction and analyzed the reaction on a 10% SDS polyacrylamide gel followed by autoradiography.

Protein samples were boiled in SDS sample buffer (containing 0.5% SDS, 0.15 mM Tris base, 2.5% glycerol, and 0.0125% Bromophenol blue) for 5 min, and then separated on a 10% SDS polyacrylamide gel. The proteins were electophoretically transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA, USA) using a Trans-Blot SD transfer cell (Bio-Rad). Membranes were blocked in phosphate-buffered saline with 0.05% Tween-20 (PBST), 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), and 10% normal goat serum overnight at 4 ^{o}C, then incubated with affinity-purified rabbit anti-*X. laevis* GR IgG (0.5 μg/ml in PBST with 5% BSA) overnight at 4 ^{o}C. Following washes in PBST immunopositive bands were visualized by the ECL Western blotting analysis system (Amersham, GE Healthcare, Buckinghamshire, UK).

Cell culture and glucocorticoid treatment

Monolayer cultures of a *X. laevis* kidney cell line A6 were maintained in Leibovitz's L15 medium (Invitrogen Corp., Carlsbad, CA, USA; diluted 1:1.5 for amphibian cells) supplemented with 10% steroid hormone-stripped fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and antibiotics. Steroid hormone removal from fetal bovine serum was conducted by mixing 25 ml serum with 50 mg charcoal (coated with 5 mg

Dextran T 70 [Pharmarcia] in phosphate-buffered saline [PBS]), and shaking at room temperature for 5 h followed by centrifugation at 1000 x 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 x g for 20 min at 4°C.

The A6 cells were plated on glass slides and cultured under a humidified atmosphere of 5% CO2 at 25 ^{o}C. Cells were incubated with 100 nM dexamethasone (Sigma D1756), 100 nM corticosterone (Sigma C2505), or vehicle (ethanol) in the normal growth medium for 1.5 h, then cells were fixed in 4% paraformaldehyde and processed for immunohistochemical analysis for GR (see below).

Immunohistochemistry (IHC)

We used IHC following methods described previously (Yao et al., 2004) to analyze the distribution of GR immunoreactivity (GR-ir) in the *X. laevis* brain. Single IHC for GR-ir was conducted using the Vectastain elite ABC (rabbit) and Vector VIP kits (both from Vector Laboratories, Inc., Burlingame, CA, USA) following the manufacturer's instructions (0.5 μg/ml of affinity purified polyclonal rabbit anti-GR IgG). The specificity of the affinity-purified anti-GR IgG was tested by preabsorption with the antigenic GR peptide, or with unrelated peptides corresponding to three regions of the frog MR. The MR peptides correspond to amino acid positions 6-45, 32-49, and 160-177 of the *X. laevis* MR protein and share no sequence similarity with the antigenic GR peptide. The antibodies were incubated with peptides (10 ng/μl) overnight at 4 °C before immunohistochemistry.

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

For each treatment group, six juvenile frogs (body weight 20-31 g) were placed into 5 liters of water in 30x20x20cm tanks and the tanks were shielded to minimize disturbance by investigators. Corticosterone (CORT; Sigma C2505) and metyrapone (MTP; Sigma 856525) were first dissolved in ethanol and added to the aquarium water to the appropriate final concentrations. The vehicle ethanol was adjusted to a final concentration of 0.0005% in all tanks including the controls. The CORT-treated group received 500 nM CORT in the aquarium water for 4 days (treatment started on day 1 and frogs were euthanized on day 5). This dose of CORT produced a stress level plasma CORT concentration (see Results; Fig. 4.7). The water was changed on day 3, and CORT was added to the water daily. The nominal concentration of CORT in the aquarium water decreases by 24 hr following addition (M. Yao unpublished data); we therefore added the hormone to the tank without water change on days 2 and 4. This served to minimize disturbance of the animals. The MTP-treated group was treated for 5 days with 110 μM MTP (treatment started on day 1 and frogs were euthanized on day 6). This dose of MTP was chosen based on Glennemeier and Denver (Glennemeier and Denver, 2002). Metyrapone was added on day 1 and day 3 at the time of water change. For the MTP+CORT treatment, frogs were treated with 110 μM MTP for one day, followed by MTP and a replacement dose of 50 nM CORT for 4 days. This dose of CORT produced a plasma CORT concentration similar to basal, unstressed levels in MTP-treated animals (see Results, Fig. 4.7). The MTP and CORT were added to the aquarium water in the same manner as the single drug treatments described above. All frogs were rapidly euthanized by submersion in 0.05% benzocaine between 1300 and 1500h and tissues

were collected. Blood was collected and plasma separated for CORT radioimmunoassay (see below). The heads were fixed in cold 4% paraformaldehyde overnight. Brains were dissected, post-fixed, and submerged in 30% sucrose before snap-freezing and transverse cryo-sectioning at 10 μm.

Shaking/handling stressor

Juvenile *X. laevis* were subjected to a shaking/handling stressor as previously described (Yao et al., 2004). Briefly, frogs were placed into 32 oz white polypropylene containers, with 2-3 frogs in 250ml well water. The containers were placed on an orbital shaker and shaken continuously at 100 rpm for 6 h. The frogs were euthanized by immersion in 0.05% benzocaine, and blood was collected for CORT RIA.

Plasma corticosterone radioimmunoassay (RIA)

Corticosterone was measured in frog plasma using methods described by Licht et al. (Licht et al., 1983). Briefly, plasma was extracted using diethyl ether and the RIA was conducted using a corticosterone antiserum (MP Biomedicals, Orangeburg, NY, USA). All samples were measured in a single assay, and intra-assay coefficient of variation was 10%. The effects of CORT or MTP treatment on plasma CORT concentrations were analyzed using one-way ANOVA followed by Fisher's multiple comparisons test using the SYSTAT v10 statistical software (SPSS Inc., Chicago, IL, USA). The values are presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

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Morphometric analysis of GR immunoreactivity

We quantified GR-ir in discrete brain regions using MetaMorph software (v 6.2r4). We processed all samples simultaneously under identical conditions. Three to five coronal sections that contained the anterior preoptic area (POA) or medial pallium (mp) were analyzed for each animal. All sections were carefully matched for anatomical level, and digital images were captured at 100X magnification for morphometric analysis. Image analysis was conducted in a blinded manner. The brain regions were isolated using a hand-made frame that covered the area of interest. The total area of the 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 the multiple sections, divided by the total selected area (see Yao et al., 2007). The effects of CORT or MTP treatment on the GR-ir mean density in the brain regions studied were analyzed by Student's unpaired t-test or one-way ANOVA followed by Fisher's LSD multiple comparisons tests using the SYSTAT v10 software (SPSS Inc., Chicago, IL, USA). The values are presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Results

Production of polyclonal antiserum to *X. laevis* GR

We generated antisera in rabbits against a short peptide conjugated to keyhole limpet hemocyanin that is located in the N-terminal region of *X. laevis* GR (amino acid residues 8 to 25). Bioinformatic analysis showed that this peptide corresponds to an antigenic region of the protein that is unique to the frog GR. This region shows no sequence

similarity to other known nuclear hormone receptors in the frog (compared with *X. laevis* mineralocorticoid receptor, androgen receptor, estrogen receptor, progesterone receptor, thyroid receptor α, and thyroid receptor β; M. Yao, unpublished data), and we did not find any sequences in other known proteins by BLAST search of the NCBI database. To further enhance the specificity of the reagent, we conducted peptide affinity purification of the antiserum (see Materials and Methods).

Verification of the specificity of the affinity-purified *X. laevis* GR antibody

The specificity of the affinity purified *X. laevis* GR antibody was first examined by Western blotting analysis on *in vitro* expressed frog GR. A single protein of the expected size (96 kD) was synthesized in the *in vitro* expression reaction (Fig. 4.1A) and this band was recognized by Western blotting; no immunoreactive bands were recognized in the unprogrammed TNT reticulocyte lysate (Fig. 4.1B).

We conducted immunocyctochemistry (ICC) on A6 cells (*X. laevis* kidney cells that express GR; Claire et al., 1989; Watlington et al., 1982) using the same affinity purified antibody, and found that immunoreactivity was in the cytoplasm and nucleus of the cells (Fig. 4.2A, left panel). Preabsorption of the antibody with the antigenic GR peptide eliminated immunostaining (Fig. 4.2A, right panel). Exposure to the GR agonist dexamethasone (DEX; 100 nM) for one and one half hours caused the GR-ir to concentrate in the nucleus (Fig. 4.2B). Exposure to corticosterone (CORT; 100 nM) also caused translocation of GR to the nucleus in A6 cells (data not shown).

The unique sequence of the antigenic GR peptide, the results of the above analyses, together with the specificity of the antibody in immunohistochemical studies and the

highly conserved patterns of expression of the GR-ir in the frog brain compared with other vertebrates (see below) all support that our antibody specifically recognizes full length frog GR.

Distribution of GR immunoreactivity in the brain of juvenile *X. laevis*

Using the *X. laevis* GR-specific antibody, we found that GR-ir cells were widely distributed throughout the brain and pituitary of juvenile *X. laevis*. Preabsorption of the affinity-purified antibodies with the GR antigenic peptide abolished the signal, while preabsorption with unrelated peptides did not alter the staining (Fig. 4.3).

Discrete groups of GR-ir positive cells were seen in the telencephalon, diencephalon, mesencephalon, and rhombencephalon. We identified the highest densities of GR-ir in the medial pallium, accumbens, anterior preoptic area (POA), the Purkinje cell layer of the cerebellum, and the rostral pars distalis. Slightly lower but still strong immunoreactivity was observed in the internal granule layer of the olfactory bulbs, dorsal and lateral pallium, striatum, various subfields of the amygdala, bed nucleus of the stria terminalis (BNST), optic tectum, various tegmental nuclei, locus coeruleus, Raphe nuclei, reticular nuclei, and the nuclei of trigeminal motor nerves. Weak and scattered immunoreactivity was seen throughout the brain, in particular in many of the periventricular structures surrounding the lateral, third, and fourth ventricles.

We also identified GR-ir cells in the pituitary gland. Among the immunoreacitive cells identified in the brain and pituitary gland of intact frogs, those cells in the parvocellular preoptic area and the rostral pars distalis of the anterior pituitary (location of corticotropes) exhibited the highest density of GR-ir. The GR-ir in the rostal pars

distalis was primarily nuclear; however, it is difficult to determine if the GR-ir is primarily nuclear in the cells of the parvocellular preoptic area due to their small size and relatively large nuclei. However, in most GR-ir cells in other regions of the brain, the immunoreactivity was localized in both the nucleus and the cytoplasm, and in some regions the immunostaining was higher in the cytoplasm than in the nucleus. A schematic representation of the distribution of GR-ir positive cells in the brain and pituitary gland of *X. laevis* is shown in Fig. 4.4.

Telencephalon The most rostral sites of GR-ir were found in small cells in the lateral pallium and internal granule cell layer of the olfactory bulbs. In the more caudal portions of the telencephalon, many small GR-ir cells were localized in the dorsal pallium, striatum, and lateral septum. Large GR-ir cells were found in the medial pallium and accumbens (Fig. 4.4C; see also Fig. 4.5C,D). The immunoreactivity was seen in both the nuclei and cytoplasm in most of these GR-ir positive cells.

Diencephalon Small GR-ir cells were seen in the lateral, medial, and central amygdala, and bed nucleus of stria terminalis (Fig. 4.4D; see also Fig. 4.5E,F) The highest density of GR-ir positive cells was localized in both the parvocellular and magnocellular divisions of the POA. In rostral regions of the POA, immunostaining was mostly found in the parvocellular cells in the periventricular zones of the third ventricle, and fewer GR-ir cells were found in the subventricular zones (Fig. 4.4D; see also Fig. 4.5G). We were unable to determine whether the immunoreactivity was mostly nuclear or cytosolic due to the small cell size and relatively large nuclei of these neurons. In caudal portions of the

POA, strong immunoreactive cells were observed in the more dorsal magnocellular regions (Fig. 4.4E; see also Fig. 4.5H). Compared with subcellular localization of GR-ir in the rostral POA, immunostaining in these cells was found in both the nuclei and cytoplasm. A number of scattered small GR-ir cells were seen in the hypothalamus and thalamus, including the ventral habenlar, anterior thalamic, ventromedial thalamic, ventrolateral thalamic, posterior thalamic, and ventral hypothalamic nuclei (Fig. 4.4F, G; see also Fig. 4.5I). Larger GR-ir cells were found in the suprachiasmatic nucleus (Fig. 4.4F; see also Fig. 4.6A). The immunoreactivity was distributed in both the nucleus and cytoplasm in most of these hypothalamic and thalamic GR-ir positive cells. We also found strong GR-ir in the fibers of the median eminence. The immunoreactivity was mostly located in the internal zone and much less in the external zone (Fig. 4.6E). Fibers in the external zone terminate on blood vessels within the median eminence, while those in the internal zone project to the pars nervosa.

Mesencephalon Well-organized small GR-ir cells were also seen in various layers of the optic tectum. High concentrations of GR-ir staining were found in the torus semicircularis, posterior tuberculum regions, and tegmental nuclei (Fig. 4.4H; see also Fig. 4.6B). The immunoreactivity was found in both the nuclei and cytoplasm of these GR-ir cells.

Rhombencephalon Larger immunoreactive cells were observed in the cerebellum especially in the Purkinje cell layer (Fig. 4.4J; see also Fig. 4.6C). Small GR-ir positive cells were found in the locus coeruleus, Raphe nuclei, reticular nuclei, and the nuclei of

trigeminal motor nerves (Fig. 4.4I, J; see also Fig. 4.6D). Subcellular distribution of GR-ir in these cells was also both nuclear and cytoplasmic.

Pituitary Dense GR-ir positive cells were found in rostral portions of the pars distalis which contains a high density of corticotropes (Campantico et al., 1985). These cells exhibited very high levels of GR-ir in their nuclei. Many fewer GR-ir cells were seen in the caudal regions of the pars distalis. Very weak and scattered GR-ir signal was also seen in the pars intermedia layer, though to a much lesser extent. No GR-ir was observed in the pars nervosa layer (Fig. 4.4H; see also Fig. 4.6E).

Effects of corticosteroid manipulation on GR-ir in the CNS and pituitary gland

To determine if circulating GCs alter GR-ir we treated juvenile *X. laevis* with CORT, MTP or MTP+CORT for 5 days. Exposure to MTP (110 μM in the rearing water) caused the plasma CORT to drop below the detection limit by by the RIA (Fig. 4.7). Exposure of juvenile *X. laevis* to 50 nM CORT did not significantly change the plasma CORT concentration, whereas treatment with 500 nM CORT for 4 days significantly increased the plasma CORT concentration by about 22-fold, which was similar to the plasma CORT concentration following exposure to a shaking/handling stressor for 6 h(see Materials and Methods). Exposure to MTP (110 μM) for 1 day then MTP in combination with a replacement dose of 50 nM CORT for 4 days resulted in plasma CORT concentrations comparable to those of the control animals (Fig. 4.7).

We analyzed GR-ir in discrete CNS regions and the pituitary gland of untreated and corticosteroid manipulated juvenile frogs. Treatment with 500 nM CORT caused a robust

reduction in the number of GR-ir positive cells and GR-ir density to almost undetectable levels in the POA (n = 5-6/treatment, $p < 0.001$ vs. control; Fig. 4.8). Treatment with MTP also caused a marked decrease in the number of GR-ir cells and GR-ir density in the POA (n = 5-6/treatment, $p = 0.004$ vs. control), but some GR immunostaining remained in neurons of the periventricular POA region (Fig. 4.8). GR-ir in the POA of animals treated with MTP and a replacement dose of CORT (50 nM) was not significantly different from controls (Fig. 4.8).

Treatment with 500 nM CORT almost eliminated GR-ir signal in the medial pallium (mp; homolog of the mammalian hippocampus). Analysis of the density of GR-ir in the mp revealed that it was significantly decreased following 500 nM CORT treatment (n = 5-6/treatment, $p < 0.001$ vs. control), and a decrease in GR-ir density was also seen following MTP treatment, though to a lesser extent (n = 5-6/treatment, $p < 0.001$ vs. control). Treatment with MTP plus a replacement dose of CORT (50 nM) restored GR-ir density in the mp to control levels (Fig. 4.9A).

In the medial amygdala (MeA) and BNST, treatment with 500 nM CORT caused a significant decrease in GR-ir density (n = 5-6/treatment, $p = 0.009$ and 0.003, respectively). Treatment with MTP also produced a decrease in GR-ir in these regions (n = 5-6/treatment, $p = 0.001$ and 0.002, compared with the controls respectively). Animals treated with MTP plus a replacement dose of CORT (50 nM) had GR-ir densities in the MeA and BNST similar to controls (Fig. 4.9B,C).

In the rostral portion of the anterior pituitary, treatment with 500 nM CORT caused a remarkable reduction in GR-ir signal (n = 5-6/treatment, $p = 0.001$ vs. control). However,

treatment with MTP alone or in combination with 50 nM CORT did not significantly affect GR-ir density in this region (Fig. 4.10).

Discussion

Here we present the first detailed study in a nonmammalian tetrapod of the distribution of GR-ir in the brain and pituitary gland, and the regulation of GR by circulating corticosteroids. We found GR-ir positive cells distributed throughout all parts of the brain from the olfactory bulbs to the brain stem. In all frogs analyzed, the highest densities of GR-ir were consistently found in the medial pallium (homolog of the mammalian hippocampus) and the nucleus accumbens in the telencephalon, the anterior preoptic area (POA), the Purkinje cell layer of the cerebellum and the rostral pars distalis. Slightly lower but strong immunoreactivity was observed in the internal granule layer of the olfactory bulbs, dorsal and lateral pallium, striatum, subfields of the amygdala, bed nucleus of the stria terminalis (BNST), optic tectum, various tegmental nuclei, locus coeruleus, Raphe nuclei, reticular nuclei, and the nuclei of the trigeminal motor nerves. The wide distribution of GR-ir in the CNS is consistent with studies in mammals, a bird, and the teleost fishes; expression of GR in all of the regions of the frog brain mentioned above has been reported for homologous brain regions of the rat (Ahima and Harlan, 1990; Morimoto et al., 1996) and Japanese quail (Kovács et al., 1989), and most of these structures in fishes (Carruth et al., 2000; Teitsma et al., 1998). The wide-spread strong GR-ir signal in forebrain structures is consistent with the our RT-qPCR analysis of the frog brain regions which showed that GR mRNA levels are higher in the telencephalon

and preoptic area compared with other regions such as the olfactory bulbs, optic tectum, hindbrain, and spinal cord (F. Hu, unpublished data).

High levels of GR-ir in nuclei of the parvocellular division of the POA in the frog brain (homolog of the PVN of mammals) is in agreement with studies conducted in the rat and in fishes (Carruth et al., 2000; Teitsma et al., 1998). Neurons in this region of the brain are neurosecretory, synthesizing a variety of neuropeptides including corticotropin-releasing factor (CRF), thyrotopin releasing hormone (TRH), neurotensin, enkephalin, cholecystokinin, vasoactive intestinal polypeptide (VIP) and somatostatin, and GR-ir was found in each parvocellular neuron that expressed these peptides (Ceccatelli et al., 1989). We and others have also shown that this group of neurons expresses a high density of CRF, CRF receptor type 1 (CRF1), TRH, TRH receptors, vasotocin (AVT), mesotocin, Neuropeptide Y (NPY), and GnRH in frogs (Bidaud et al., 2004; Calle et al., 2006; Daniello et al., 1996; DiMatteo et al., 1996; González et al., 1995; Yao et al., 2004). Colocalization of GR and the neuropeptides suggests that GR might directly regulate their expression.

A significant difference between GR-ir distribution in the frog, bird or mammal is that a high density of GR-ir was found in the magnocellular division of the POA of the frog, whereas no GR-ir was detected in the magnocellular division of the PVN in intact rats or Japanese quail (Kovács et al., 1989). The presence of GR-ir in this region is consistent with the presence of GR-ir in the internal zone of the median eminence of the frog since the magnocellular neurons project to the internal zone of the median eminence. Neurons in this region express CRF, urocortin I and arginine vasopressin (AVP; or arginine vasotocin [AVT] in non-mammalian vertebrates; Acher, 1996; Calle et al.,

2005). Strong GR-ir in these cells was also found in rainbow trout and kokanee salmon (Carruth et al., 2000; Teitsma et al., 1998). The finding of high basal GR expression in the magnocellular POA in the frog and in fishes, but not in the homologous regions of a bird or mammals could be a reflection of the aquatic vs. terrestrial lifestyle of these species. Although rats have undetectable GR in the magnocellular PVN neurons, Berghorn and colleagues (Berghorn et al., 1995) found that exposure to osmotic stress induced GR expression in AVP neurons in this region. Also, studies in transfected cells found direct inhibition of AVP gene promoter activity by dexamethasone (Iwasaki et al., 1997). Thus, while AVP directly stimulates pituitary ACTH secretion in mammals and is subject to negative feedback by GCs, these results also suggest that GR could play a role in osmoregulation by influencing AVP secretion (Papanek and Raff, 1994).

The expression of GR mRNA and protein in limbic structures is well documented in mammals (Ahima and Harlan, 1990; Morimoto et al., 1996). In the mammalian hippocampus, the CA1 and CA2 pyramidal cell layers, and the dentate gyrus all express high levels of GR mRNA and GR-ir (Ahima and Harlan, 1990; Morimoto et al., 1996). Strong expression of GR mRNA and immunoreactivity were also seen in all subfields of the amygdala and bed nucleus of the stria terminalis (BNST) (Ahima and Harlan, 1990; Morimoto et al., 1996). The amphibian and fish homolog of the hippocampus (medial pallium) is much less differentiated than in mammals. Also, while the subfields of the amygdala are biochemically distinct in frogs and fish, they are more difficult to distinguish morphologically than those of mammals. Nonetheless, we observed dense GR-ir positive cells in the frog mp, the MeA and the BNST. Similar GR distribution was observed in fishes (Carruth et al., 2000; Teitsma et al., 1998). The only study on GR

distribution in the bird brain did not find GR-ir in the hippocampus or the archistriatum whereas GR-ir was observed in the lateral septum (Kovács et al., 1989). The investigators did not report if GR-ir was found in other limbic structures (i.e. the amygdala or BNST) in the bird brain. Conserved patterns of GR expression in limbic structures in fishes, frog, and mammals suggest that regulation of these regions by GCs may be phylogenetically ancient and evolutionarily conserved.

We found strong GR-ir in cell nuclei of the rostral pars distalis in the frog. High density of GR-ir in this region was also reported in mammals and fishes (Ahima and Harlan, 1990; Carruth et al., 2000; Morimoto et al., 1996; Teitsma et al., 1998). The anterior pituitary endocrine cells synthesize and secrete a variety of hormones including the adrenocorticotropic hormone (ACTH), growth hormone (GH), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL). Colocalization of GR-ir with these hormones within the anterior pituitary has been documented (Kononen et al., 1993; Ozawa et al., 1999). The hypothalamic-pituitary axis orchestrates functions of many physiological systems, and the pituitary is a major site for GC feed back regulation during stress (Herman et al., 2003). Circulating GCs may directly regulate the production and release of the hormones by the anterior pituitary cells and modulate various physiological pathways.

We also found weak and scattered GR-ir staining in the neurointermediate lobe of the pituitary gland. Studies of GR distribution in intact rats and the rainbow trout failed to detect GR-ir in this region, although dense GR mRNA signal in this lobe has been reported in the rat (Ahima and Harlan, 1990; Morimoto et al., 1996; Ozawa et al., 1999; Teitsma et al., 1998). A separate study has found GC (dexamethasone) binding activity in

the anterior and the neurointermediate pituitary lobes in the rat (Sheppard et al., 1993). It has been hypothesized that GR expression in these cells is inhibited by the neural input from the hypothalamus, since removal of hypothalamic influence (by lesions in the basal hypothalamus or the pituitary stalk) resulted in the appearance of GR-ir in the neuronintermediate lobe in the rat pituitary (Antakly et al., 1987; Antakly et al., 1985; Seger et al., 1988). The presence of GR in the neurointermediate lobe of the pituitary gland in the frog suggests that these cells are responsive to GCs. It is known that these cells in *X. laevis* express POMC, the precursor protein of several peptides including ACTH and αMSH (Dotman et al., 1996; Loh et al., 1985; Tuinhof et al., 1998). These observations suggest that expression of the POMC gene in the intermediate lobe may be subject to regulation by circulating GCs. The lack of GR-ir in the posterior pituitary (neural lobe) in the frog is in agreement with studies in the rainbow trout (Teitsma et al., 1998), whereas GR-ir has been found in this portion of the pituitary in the rat (Kononen et al., 1993; Ozawa et al., 1999). Interesting, we observed GR-ir in the magnocellular division of the POA and the internal zone of the median eminence. The magnocellular neurons project to the pars nervosa through the internal zone of the median eminence. Further investigations are required to understand the differences in GR-ir expression in these regions.

In most studies of GR subcellular localization in the brains of intact (unstressed) rats, bird and fishes, GR-ir was almost always observed in the nucleus, with very low to no cytoplasmic immunoreactivity (Ahima and Harlan, 1990; Kovács et al., 1989; Morimoto et al., 1996; Teitsma et al., 1998). Following adrenalectomy in rats, GR-ir in most brain cells was found to be cytoplasmic and the nucleus was devoid of immunoreactivity (Fuxe

et al., 1987; Han et al., 2005; Usuku et al., 2005). By contrast, in the intact frog we found that except for the rostral pars distalis where GR-ir was predominantly nuclear, GR-ir in most other regions of the frog brain was seen in both the nucleus and cytoplasm, and in some regions the density was higher in the cytoplasm than in the nucleus. Possible reasons for these observations could be species differences in circulating GCs or trafficking and translocation of the GR, or the developmental stage of the frog (young juvenile) that we studied. We used juvenile frogs that were sexually differentiated yet reproductively immature to avoid potential confounding influences of sex steroids on GR, whereas in most of the studies in rats and fish above, adult animals were used. A study in kokanee salmon demonstrated that GR-ir was predominantly cytoplasmic in many brain regions in sexually immature fish but nuclear in spawning fish. This translocation corresponds with the increased plasma cortisol concentrations during sexual maturation of the fish (Carruth et al., 2000). It would be interesting to further investigate the correlation of circulating GC concentration with subcellular localization of GR during development in the frog and other species. Furthermore, the circadian cycle and stress can affect circulating GCs and regulate subcellular localization of GR in the brain. Elevation of circulating GC concentrations during the dark phase or after stress induced a marked nuclear translocation of GR and increased GRE binding in the rat brain (Kitchener et al., 2004). Thus, subcellular localization of GR in the brain is influenced by many factors including the developmental stage, circadian rhythms, and exposure to stressors.

We analyzed changes in GR-ir in various brain structures and the pituitary gland in response to an elevation or removal of circulating corticosteroids. We observed

significantly decreased GR-ir in the POA, medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), and anterior pituitary in CORT treated frogs. Treatment with MTP also resulted in decreased GR-ir in the POA, MeA, and BNST, but did not affect GR-ir in the anterior pituitary. These results suggest that GR-ir is regulated by GCs in discrete regions of the brain and pituitary, and the GC regulation is cell type-specific. Our observations in the frog are in general agreement with studies conducted in mammals. In the rat, administration of high doses of GCs for days to weeks resulted in decreased GR in the hippocampus, amygdala, and cortex measured by receptor binding activity or Western blotting (Sapolsky et al., 1984; Spencer et al., 2000). On the other hand, short-term adrenalectomy (one to five days) in rats caused significant increases in total GR protein in the hippocampus, hypothalamus/PVN, and cortex measured by Western blotting (Kalman and Spencer, 2002; O'donnell et al., 1995; Spencer et al., 2000). These results suggest that expression of GR in these brain regions is inhibited by GCs. It should be noted that differential subcellular localization of GR could influence its detection using different methodologies. For the same time period (within one week) following adrenoectomy in rats, marked decreases in GR-ir were observed in many brain areas including the hippocampus, amygdala, PVN, arcuate nucleus, and cerebral cortex (Hu et al., 1997b; Rosenfeld et al., 1988; Visser et al., 1996). Thus, the decreased GR-ir in adrenalectomized animals measured by immunohistochemical methods may be a consequence of diffuse protein distribution within the cells rather than a reduction in the total protein. Furthermore, longer-term adrenalectomy (≥1 week) in rats resulted in loss of detectable GR-ir in the forebrain, cortex, and hippocampus (Han et al., 2005; Hu et al., 1997a; Visser et al., 1996). These data support the notion that although high

concentrations of GCs inhibit of the GR gene expression, the presence of GCs is essential for the maintenance of basal expression of the GR gene.

Autoregulation of GR by its ligands has been studied *in vitro* and *in vivo* in the rat. Administration of GCs to rats caused decreases in expression of GR mRNA and/or protein in the hippocampus and cortex (Ghosh et al., 2000; Hügin-Flores et al., 2004; Spencer et al., 2000). Inhibition of GR mRNA expression by GCs has also been observed in cell and tissue culture studies. Treatment with GCs significantly reduced expression of both GR mRNA and protein or activity of the GR gene promoter in transfected cells (Bellingham et al., 1992; Burnstein et al., 1991; Dong et al., 1988; Meyer and Schmidt, 1995; Okret et al., 1986; Silva et al., 1994; Vedeckis et al., 1989). Different mechanisms of this down-regulation of GR by its own ligand have been suggested, which include transcriptional inhibition (decreased transcription; Dong et al., 1988; Okret et al., 1991; Rosewicz et al., 1988), post-transcription regulation (decreased mRNA stability or translatability; Burnstein et al., 1991; Meyer and Schmidt, 1995; Okret et al., 1986; Vedeckis et al., 1989), and post-translational regulation (decreased protein stability and/or increased protein degradation; Dong et al., 1988; Okret et al., 1991; Webster et al., 1997).

Short-term (within one week) adrenalectomy caused increases in GR mRNA in the hippocampus, while GR mRNA in the PVN was increased or not affected. Furthermore, the adrenalectomy-induced changes of GR mRNA were abolished with GC supplementation, and administration of high dose of GCs resulted in decreases in GR mRNA in these regions (Chao et al., 1998; Han et al., 2007; Holmes et al., 1995; Hügin-Flores et al., 2004; Reul et al., 1989). These results support that transcriptional activity of

the GR gene in these brain regions is down-regulated by circulating corticosteroids. We do not know if the observed decreases in GR-ir in specific brain regions in MTP-treated frogs were due to diffuse protein localization in the cell, or decreased expression of the gene, or both. Analysis of GR mRNA levels (by RT-qPCR) in the forebrain (including the telencephalon and diencephalon) of MTP-treated frogs did not show differences compared with the control (F. Hu, unpublished data), which suggests that transcriptional activity of the GR gene in the frog forebrain may not be affected by changes in the circulating corticosteroids. However, changes in GR mRNA in small populations of neurons may not be detected by this method. Further studies are necessary to decipher the mechanism of the observed changes in GR-ir in MTP-treated animals.

Autoregulation of GR by circulating GCs is hypothesized to be an important mechanism for regulating the responsiveness of the HPA axis during chronic stress. In rats exposed to repeated or chronic stressors, GR mRNA expression was down-regulated in the hippocampus, frontparietal cortex, PVN, and LC (Gomez et al., 1996; Herman et al., 1995; Makino et al., 1995, 2002b; Nishimura et al., 2004). Decreases in GR expression in these brain regions during chronic stress may indicate a reduced inhibition of the HPA activity by elevated circulating GCs, which could contribute to maintaining the sensitivity of the tissue to GCs and sustaining the responsiveness of the HPA axis to further stimulation (Makino et al., 2002a; Makino et al., 1995).

In conclusion, our results show that the general patterns of central expression of GR are highly conserved among vertebrates. Thus GR is likely to play a similar role in mediating the actions of corticosteroids on the frog brain as in mammals. Expression of GR-ir in the frog brain areas involved in stress response (POA, mp, MeA, BNST) and the

anterior pituitary is regulated by circulating corticosteroids. These findings suggest that the ligand-dependent regulation of GR in specific brain regions may be an evolutionarily conserved mechanism for modulating the responsiveness of the stress axis in vertebrates.

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Table 4.1. Abbreviations.

A, anterior thalamic nucleus
Acc, nucleus accumbens
BNST, bed nucleus of the stria terminalis
Cb, cerebellum
CeA, central amygdala
dp, dorsal pallium
Hd, dorsal habenular nucleus
Hv, ventral habenular nucleus
igl, internal granule cell layer
Is, nucleus isthmi
L, lateral thalamic nucleus
LA, lateral amygdala
Lc, locus coeruleus
lp, lateral pallium
LPv, lateral thalamic nucleus
ls, lateral septum
lv, lateral ventricle
ME, median eminence
MeA, medial amygdala
ml, mitral layer
mp, medial pallium
ms, medial septum
nII, cranial nerve II
P, posterior thalamic nucleus
pd, pars distalis
pi, pars intermedia
pn, pars nervosa
POA, preoptic area
Ra, raphe nucleus
Ri, inferior reticular nucleus
Rm, nucleus reticularis medius
SC, suprachiasmatic nucleus
Str, striatum
tect, optic tectum
tegm, mesencephalic tectum
Tn, tegmental nuclei
TP, posterior tuberculum
VH, ventral hypothalamic nucleus
VLs, superficial ventral nucleus
VM, ventromedial thalamic nucleus
Vm, nucleus motorius nervi trigemini
Vpr, nucleus sensorius principalis nervi trigemini

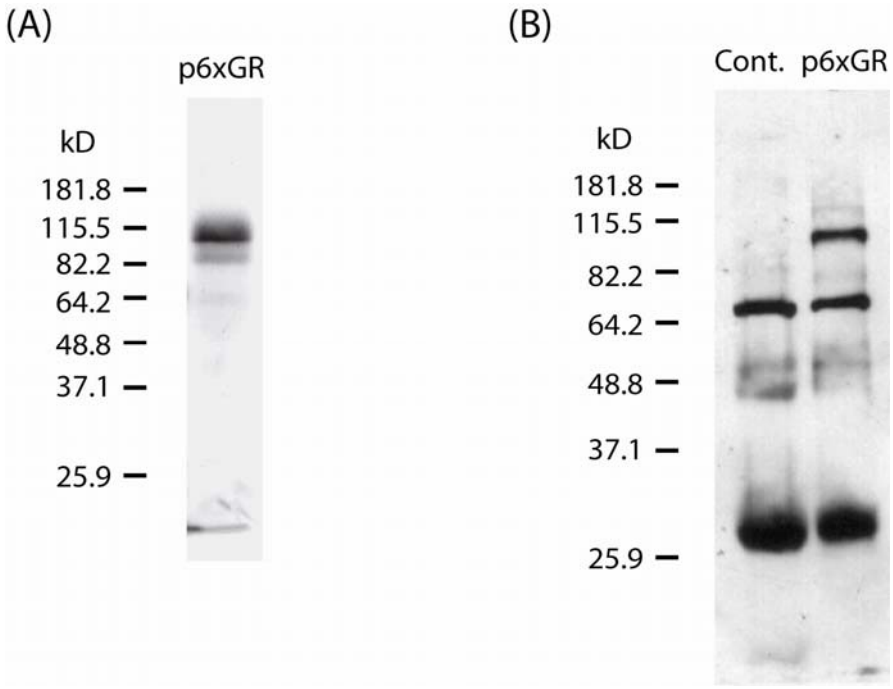

Figure 4.1. The affinity purified GR IgG recognizes *in vitro* synthesized *Xenopus laevis* GR. (A) A major product of ~ 96 kD is synthesized in the *in vitro* transcription/translation reaction. ^{35}S-methionine is included in the *in vitro* transcription-translation reaction and the reaction is analyzed on a 10% SDS polyacrylamide gel followed by autoradiography. (B) A protein of ~ 96 kD is detected by Western blotting in the *in vitro* transcription/translation reaction from the p6xGR vector, which is not present in the control (no vector) reaction.

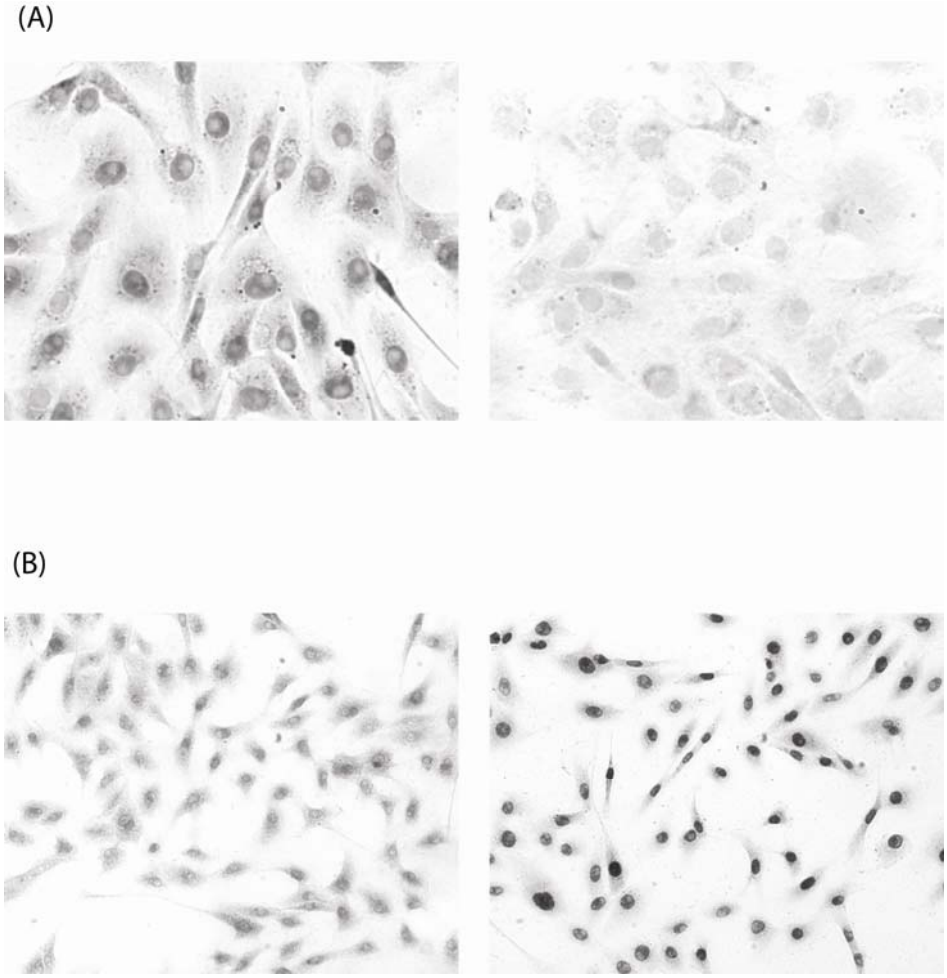

Figure 4.2. The affinity-purified GR IgG recognizes a protein that shows nuclear translocation in response to dexamethasone treatment in A6 cells. (A) A6 cells were incubated with the affinity-purified antibody (left) or with the antibody that had been preabsorbed with 50 μg/ml of the antigenic GR peptide (right). (B) Immunosignal in A6 cells before (left) and after (right) 1.5 h treatment of dexamethasone (DEX; 100 nM).

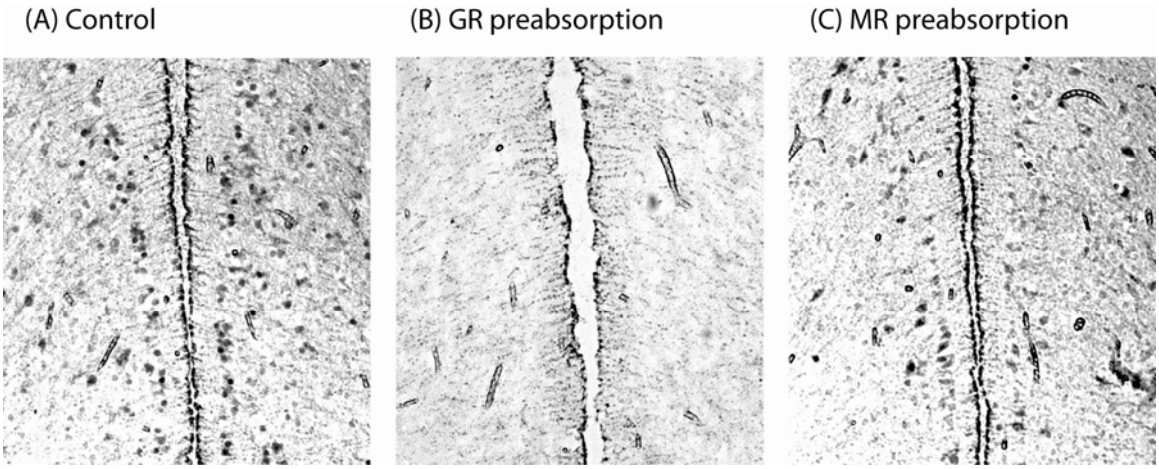

Figure 4.3. Photomicrographs of transverse sections through the anterior preoptic area (POA) of the brain of a juvenile *Xenopus laevis* showing the specificity of the affinity-purified anti-GR IgG. Dorsal is up in this and all other figures of photomicrographs of the brain. Adjacent sections were incubated with affinity-purified anti-GR IgG (A) or with anti-GR IgG that had been preabsorbed with 50 μg/ml of the antigenic GR peptide (B) or the antigenic MR peptide (C).

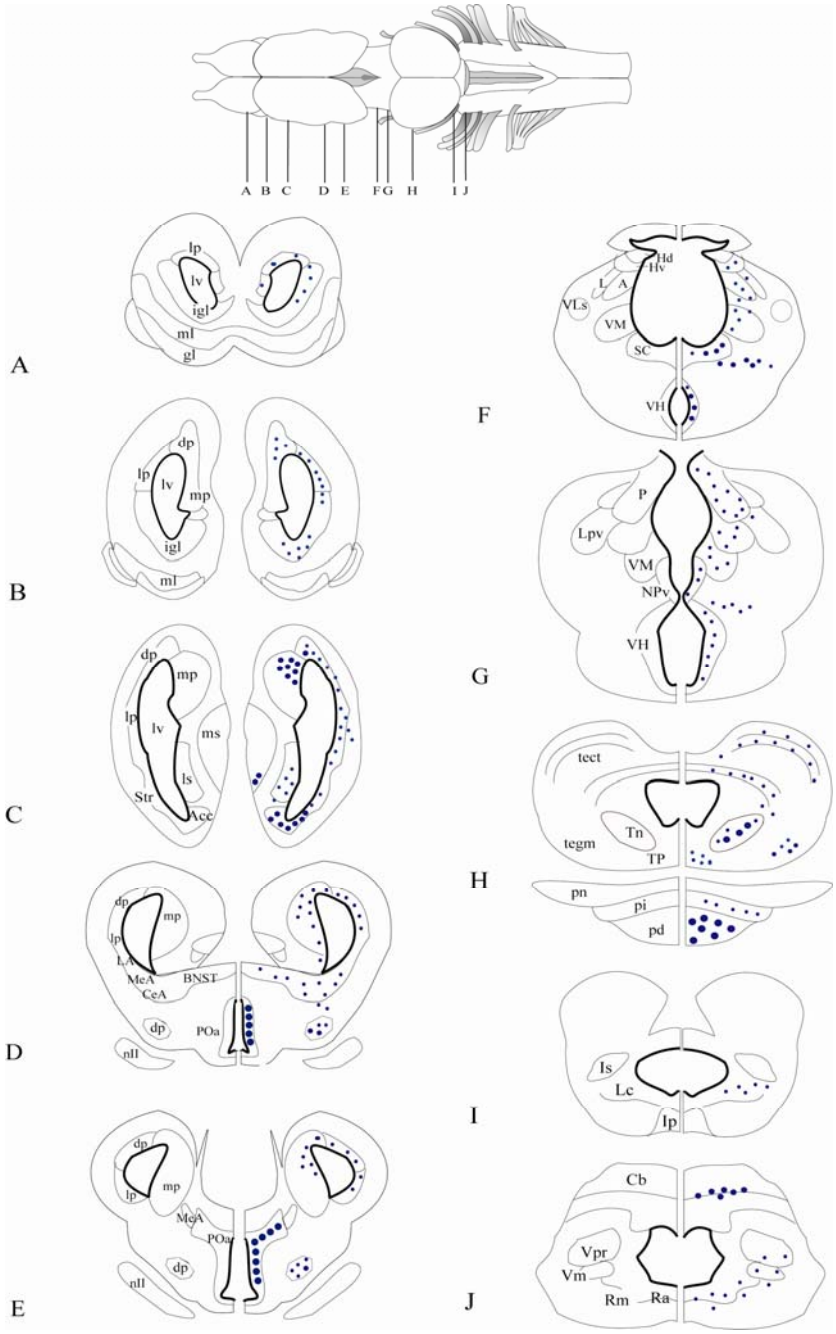

Figure 4.4. Schematic coronal illustration of glucocorticoid receptor (GR)-immunoreactivity (ir) distribution in the brain of juvenile *Xenopus laevis*. The drawing at the top of the figure shows a dorsal view of the whole brain. Letters correspond to the rostro-caudal location of sections as depicted in the whole brain drawing. Large circles represent large, robust GR-ir, and small circles represent smaller, less dense GR-ir. See Table 4.1 for abbreviations. The anatomical drawings are from Tuinof *et al.* (Tuinhof et al., 1998) with modifications of basal ganglia subdivisions according to Marin *et al.* (Marín et al., 1998).

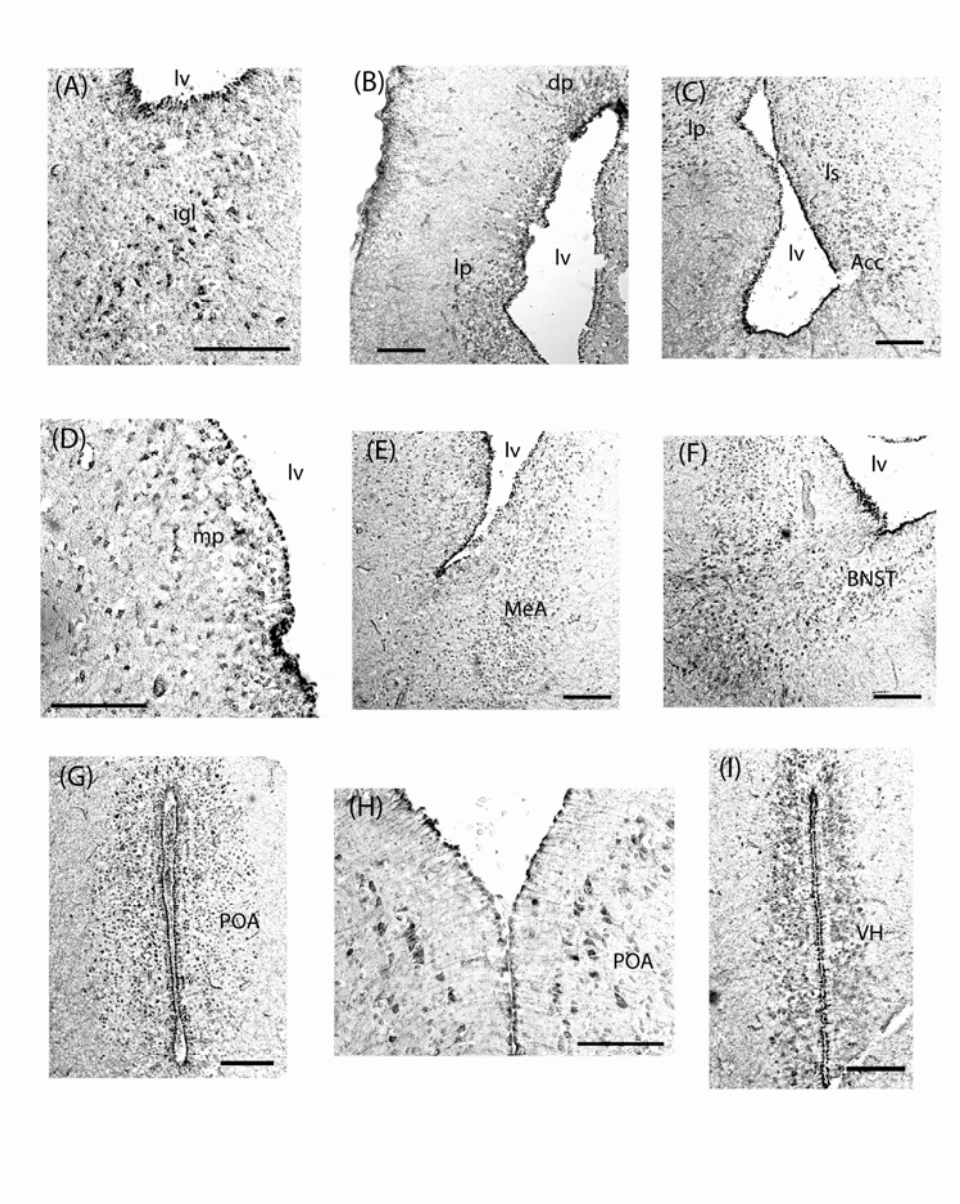

Figure 4.5. Photomicrographs of transverse sections through the forebrain and part of the midbrain of juvenile *Xenopus laevis* showing the distribution of glucocorticoid receptor (GR)-immunoreactive (ir) cells. (A) Internal granule cell layer of the olfactory bulb (igl). (B) Dorsal pallium (dp) and lateral pallium (lp). (C) Nucleus accumbens (Acc) and lateral septum (ls). (D) Medial pallium (mp). (E) Medial amygdala (MeA). (F) Bed nucleus of the stria terminalis (BNST). (G) Anterior preoptic area (POA). (H) Posterior preoptic area (POA). (I) Ventral hypothalamic nucleus (VH). See Table 4.1 for a complete description of abbreviations. Scale bars = 120 μm. lv, lateral ventricle.

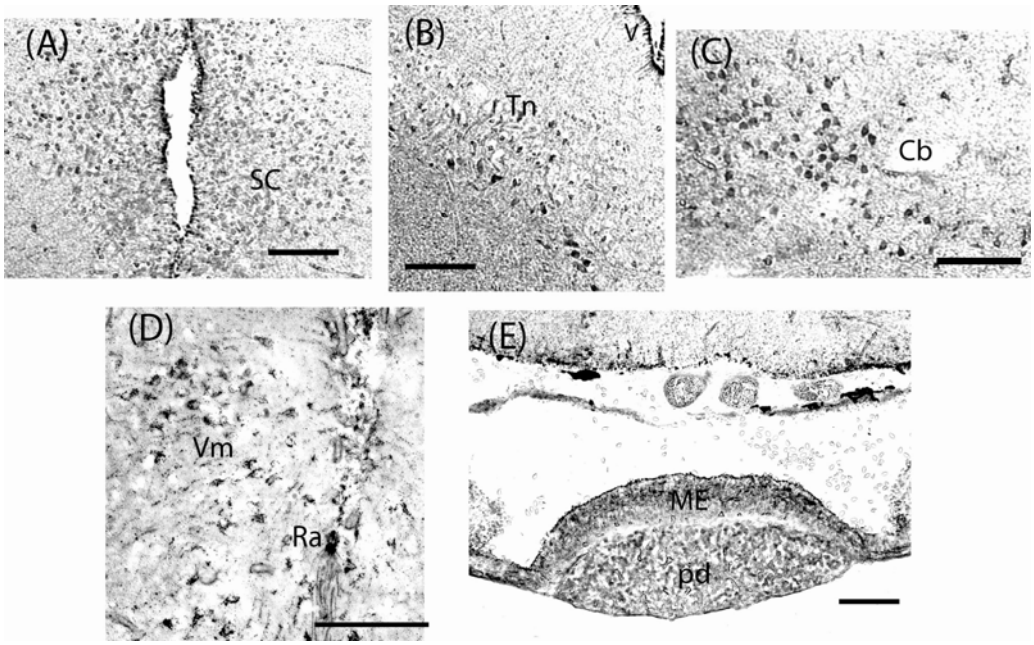

Figure 4.6. Photomicrographs of transverse sections through the midbrain and the hindbrain of juvenile *Xenopus laevis* showing the distribution of glucocorticoid receptor (GR)-immunoreactive (ir) cells. (A) Suprachiasmatic nucleus (SC). (B) Tegmental nuclei (Tn). (C) Cerebellum (Cb). (D) Motor nucleus of the trigeminal nerve (Vm) and Raphe nucleus (Ra). (E) Anterior pars distalis layer of the pituitary gland (pd). See Table 4.1 for a complete description of abbreviations. Scale bars = 120 μm. V, ventricle.

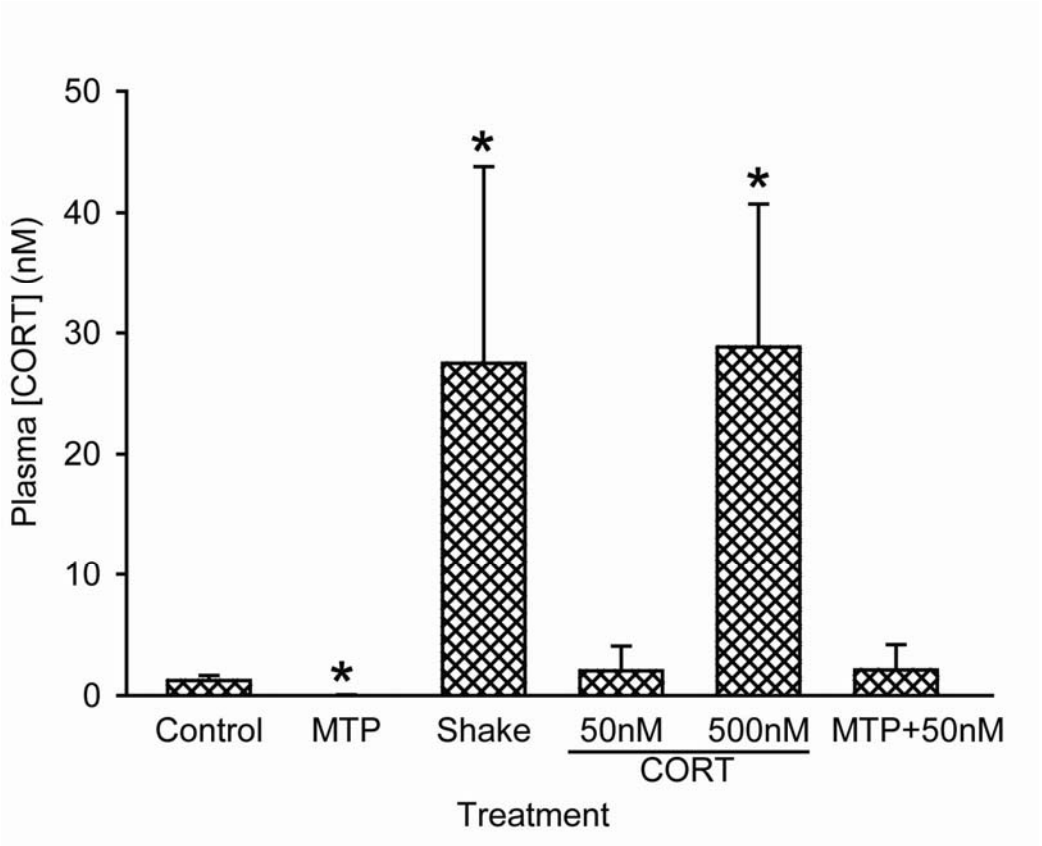

Figure 4.7. Plasma corticosterone concentrations of *Xenopus laevis* juveniles following various treatments. MTP, 110 μM of metyrapone for 5 d; Shake, 6 h of shaking/handling; CORT, 50 nM or 500 nM of corticosterone for 4 d. MTP+50 nM, 110 μM metyrapone for 1 d followed by metyrapone and a replacement dose of 50 nM corticosterone for 4 d. Data presented are the mean + SEM. Significant differences from control are indicated (n = 5-6/treatment, * $p < 0.05$).

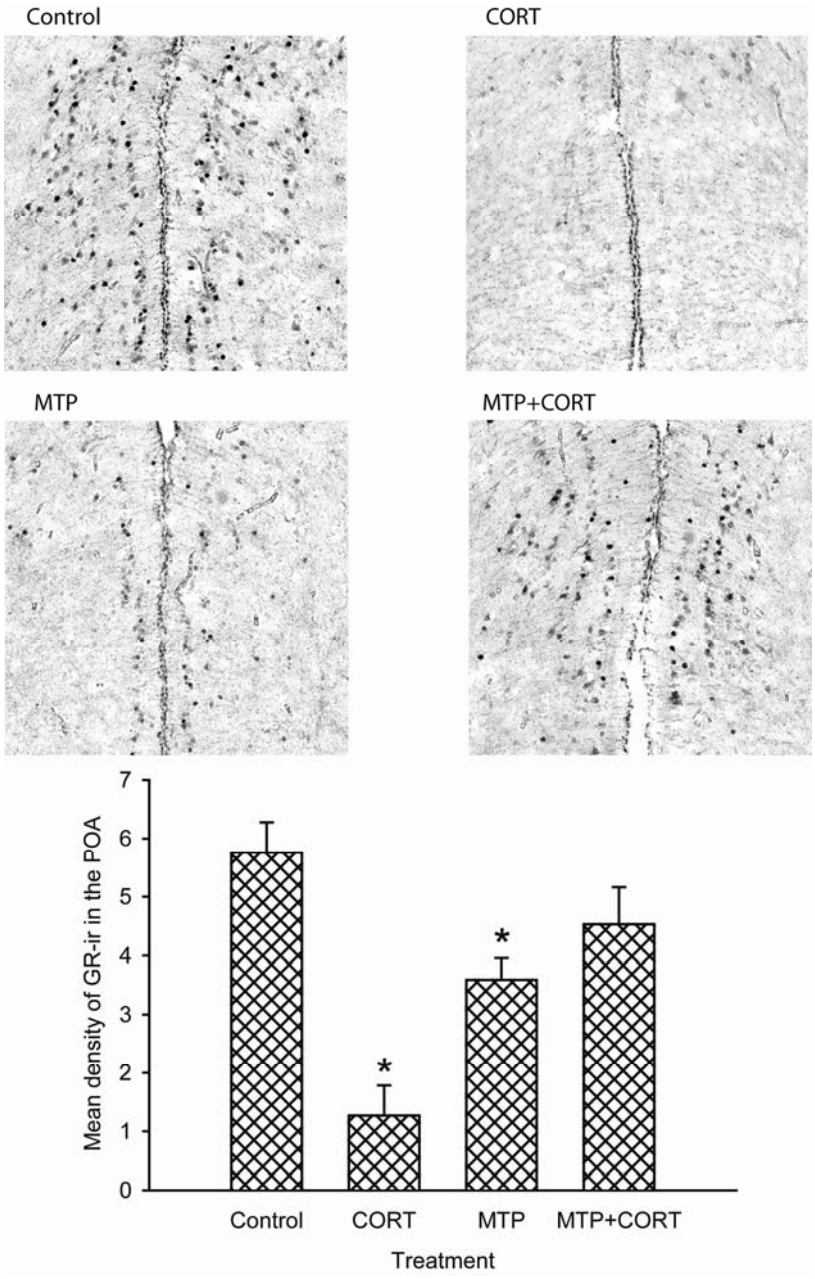

Figure 4.8. Effects of corticosterone manipulation on glucocorticoid receptor (GR)-immunoreactivity (ir) in the anterior preoptic area (POA) of juvenile *Xenopus laevis*. (A) Photomicrographs are of representative transverse sections in the same anatomical plane of the POA of juvenile *X. laevis* following corticosterone manipulation. (B) Densitometric analyses of GR-ir in the POA of juvenile *X. laevis* following corticosterone manipulation. CORT, 500 nM of corticosterone for 4 d; MTP, 110 μM of metyrapone for 5 d; MTP+CORT, 110 μM metyrapone for 1 d followed by metyrapone plus 50 nM of corticosterone for 4 d. Data presented are the mean + SEM. Significant differences from control are indicated (n = 5-6/treatment, * $p < 0.05$).

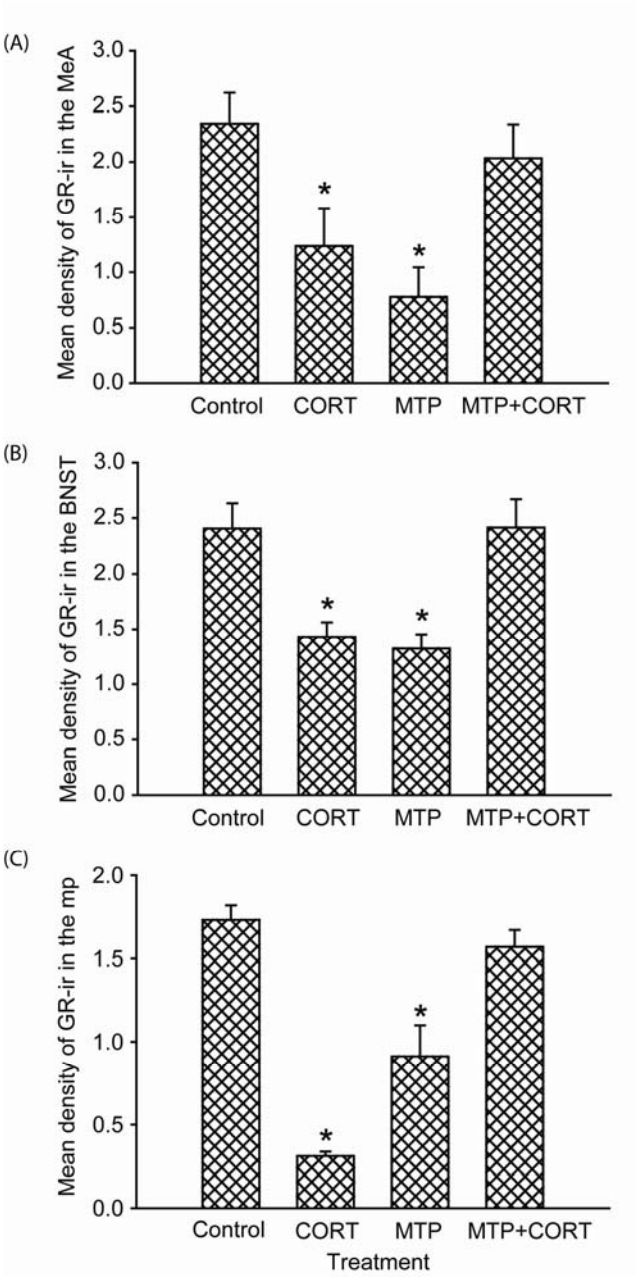

Figure 4.9. Effects of corticosterone manipulation on glucocorticoid receptor (GR)-immunoreactivity (ir) in (A) the medial pallium (mp), (B) the medial amygdala (MeA), and (C) the bed nucleus of stria terminalis (BNST) of juvenile *Xenopus laevis*. Results of densitometric analyses of GR-ir in each of the brain regions following the treatments are shown. CORT, 500 nM of corticosterone for 4 d; MTP, 110 μM of metyrapone for 5 d; MTP+CORT, 110 μM metyrapone for 1 d followed by metyrapone plus 50 nM of corticosterone for 4 d. Data presented are the mean + SEM. Significant differences from control are indicated (n = 5-6/treatment, * $p < 0.05$).

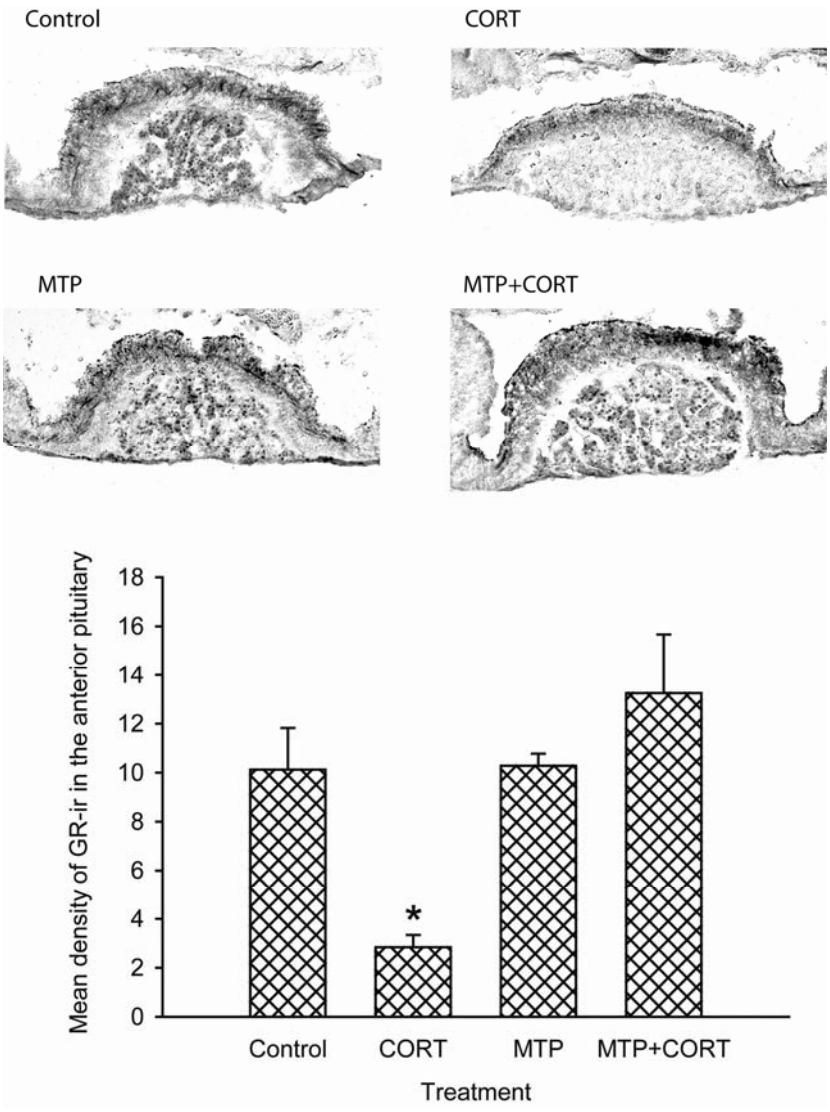

Figure 4.10. Effects of corticosterone manipulation on glucocorticoid receptor (GR)-immunoreactivity (ir) in the rostral portion of anterior pituitary of juvenile *Xenopus laevis*. (A) Photomicrographs are of representative transverse sections through the rostral anterior pituitary of juvenile *X. laevis* following corticosterone manipulation. (B) Densitometric analyses of GR-ir in the rostral anterior pituitary of juvenile *X. laevis* following corticosterone manipulation. CORT, 500 nM of corticosterone for 4 d; MTP, 110 μM of metyrapone for 5 d; MTP+CORT, 110 μM metyrapone for 1 d followed by metyrapone plus 50 nM of corticosterone for 4 d. Data presented are the mean + SEM. Significant differences from control are indicated (n = 5-6/treatment, * $p < 0.05$).

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CHAPTER 5

DIFFERENTIAL REGULATION OF CORTICOTROPIN-RELEASING FACTOR (CRF) GENE EXPRESSION BY GLUCOCORTICOIDS IN THE BRAIN OF *XENOPUS LAEVIS*

Abstract

Corticotropin-releasing factor (CRF) is the key neurohormone controlling the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) stress axis in vertebrates. Glucocorticoids (GCs), the end effectors of the stress response, exert feedback regulation on the expression and secretion of CRF, and modulate the activity of the HPA axis in mammals. However, little is known about feedback regulation of CRF gene expression by GCs in non-mammalian vertebrates. Immunohistochemical analysis showed that treatment with corticosterone for 4 d decreased while treatment with the corticosteroid synthesis inhibitor metyrapone (MTP) increased CRF immunoreactivity (-ir) in the preoptic area (POA; homolog of the mammalian paraventricular nucleus [PVN]). By contrast, treatment with corticosterone increased CRF-ir in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST), while treatment with MTP decreased CRF-ir in the MeA. We also found that CRF-ir and glucocorticoid receptor (GR)-ir were colocalized in cells in the POA and medial pallium (homolog of the mammalian hippocampus). Using RT-quantitative PCR, we found that short term corticosterone treatment (6 h) decreased while MTP increased CRF gene transcription in the forebrain

of *Xenopus laevis*. In transient transfection assays in PC-12 cells treatment with dexamethasone decreased forskolin-induced activation of the frog CRF promoters. Treatment with corticosterone also reduced basal CRF promoter activity in transfected tadpole brains. Using electrophoretic mobility shift assay, we show that GR specifically binds to a conserved, composite GRE/AP1 site located in the proximal promoters of the frog CRF genes. Our findings suggest that the mechanisms and cell-type specificity of GC-dependent regulation of the CRF gene are conserved among vertebrates.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is the principal pathway that mediates physiological responses to stressors in mammals. Activation of this pathway by internal or external stressors results in increased expression and secretion of corticotropin-releasing factor (CRF) and other neuropeptides by the hypothalamus. These neuropeptides (mainly CRF and arginine vasopressin [AVP]) subsequently increase the release of adrenocorticotropin (ACTH) from the pituitary, which ultimately leads to elevated glucocorticoid (GC) secretion from the adrenal cortex into the general circulation. In addition to the diverse actions on a variety of target tissues, GCs also exert feedback regulation on the expression and secretion of CRF and ACTH to modulate the activity of the HPA axis and prevent continued activation of the stress response (Dallman et al., 1992; Makino et al., 2002; Whitnall, 1993).

The negative feedback actions of GCs on hypothalamic CRF neurons are both direct and indirect. For example, GCs exert GR-mediated inhibition of CRF synthesis and secretion by the parvocellular neurons in the paraventricular nucleus (PVN), or modulate

the activity of these neurons indirectly via the hippocampus, the amygdala and bed nucleus of the stria terminalis (BNST), or the brainstem pathways (De Kloet et al., 1998; Makino et al., 2002). Neurons of these extra hypothalamic regions project to the CRF neurons in the PVN and send excitatory or inhibitory signals. These neurons also express GR and GC action on these neurons can positively or negatively regulate the CRF expression in the PVN (Makino et al., 2002).

Multiple mechanisms are involved in the feedback regulation of corticosteroids on the HPA axis, which include direct regulation of gene transcription, modulation of neuronal excitability, and fast non-genomic actions (De Kloet et al., 1998; Schulkin et al., 1998). Although in general, elevated circulating GCs following a stress stimulation inhibit the overall activity of the HPA axis, the effects of corticosteroids on CRF expression differ depending on the cell type. Several groups reported that GCs down-regulate CRF expression in the PVN, while up-regulating it in the amygdala and bed nucleus of the stria terminalis in rats (Makino et al., 1994a, b; Shepard et al., 2000; Swanson and Simmons, 1989; Watts and Sanchez-Watts, 1995). Furthermore, GCs are known to increase CRF expression in the placenta (Majzoub and Karalis, 1999; Power and Schulkin, 2006). The molecular mechanisms for the differential regulation of CRF by GCs in different tissues are not understood. It is likely that cell type-specific expression of signaling molecules, transcription factors and other co-factors may be responsible for the different effects of corticosteroids.

Two intracellular receptors for corticosteroids have been identified in vertebrates: the type I or mineralocorticoid receptor (MR), and the type II or glucocorticoid receptor (GR). The central distribution and binding characteristics of the two receptors differ

significantly. In the rat, GR is widely expressed throughout the brain, with higher levels in the hippocampus and the PVN. Expression of MR is more restricted with the highest levels in the hippocampus (measured by in situ hybridization and immunohistochemistry; Ahima et al., 1991; Cintra et al., 1994; Han et al., 2005; Herman et al., 1989a; Morimoto et al., 1996). The binding affinity of MR for glucocorticoids is ten times higher than that of GR. At basal levels of circulating GCs, MR binding sites in the brain are saturated, whereas GR binding sites become occupied as plasma GC concentrations rise such as at the circadian peak or during the stress response (Reul et al., 1987). Thus it is hypothesized that the majority of actions of GCs in the stress response are mediated through activation of GR, while MR functions primarily in the basal and circadian regulation by GCs (De Kloet et al., 1998).

Very little is known about the regulation of CRF by corticosteroids in non-mammalian species. Studies in teleost fishes suggest that GCs decrease CRF expression in the telencephalon-hypothalamic area (Bernier et al., 2004; Bernier et al., 1999; Olivereau and Olivereau, 1988).We have previously described the distribution of CRF and GR immunoreactivity in the brain and pituitary gland of *Xenopus laevis*, and showed that the basic expression patterns are highly conserved with mammals (Yao et al., 2004; Chapter 4). Using a comparative genomic approach, we also identified putative glucocorticoid response elements (GREs) located in the proximal promoters of the frog CRF genes (Yao et al., 2007). Here we show that expression of CRF protein analyzed by immunohistochemistry, and hnRNA (the primary transcripts) and mRNA analyzed by RT-quantitative PCR in discrete regions of the frog brain are regulated by circulating corticosteroids. Glucocorticoids inhibited forskolin-induced transcriptional activation of

the frog CRF promoters in transfected PC-12 cells. Furthermore, administration of corticosteroids reduced basal expression of a CRF promoter reporter construct transfected into tadpole brain. We also show that GR specifically binds *in vitro* to a conserved, composite GRE/AP1 site located in the proximal promoters of the frog CRF genes. Our findings provide further evidence for the evolutionary conservation of the regulatory mechanisms of the vertebrate neuroendocrine stress system, and show that the frog is a valuable vertebrate model system for understanding the physiology and molecular mechanisms of gene regulation within the HPA axis.

Materials and Methods

Animal husbandry

Xenopus laevis juveniles were purchased from Xenopus I (Dexter, Michigan). Tadpoles were raised in dechlorinated tap water (20-22 ^{o}C; 12 light:12 dark) and fed Frog Brittle (Nasco, Fort Atkinson, WI) *ad libitum*. Developmental stages were assigned according to Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994). 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 (UCCUA).

Corticosterone and metyrapone treatment of juvenile *X. laevis*

The procedures for of corticosterone (CORT) or metyrapone (MTP) treatment of juvenile *X. laevis* were described previously (see Chapter 4). For the short-term (6 h) treatment, juvenile frogs (B.W. 0.8-1.3 g) were treated with CORT (500 nM; Sigma C2505) or MTP (100 μM; Sigma 856525). The CORT and MTP were first dissolved in ethanol and then

added to the aquarium water to the appropriate concentrations. The vehicle ethanol was adjusted to a final concentration of 0.0005% in all tanks including the controls. Animals were put into the tanks and brains were collected 6 h later for RNA extraction.

The long-term (4-5 d) treatments of juvenile frogs with CORT, MTP, or both were described previously (see Chapter 4). Blood was collected and plasma separated for CORT radioimmunoassay (RIA; see below). Brains were fixed and cryo-sectioned at 10 μm for immunohistochemical analysis.

Corticosterone radioimmunoassay (RIA)

Corticosterone was measured in frog plasma using methods described by Licht and colleagues (Licht et al., 1983). Briefly, plasma was extracted using diethyl ether and the RIA was conducted using a corticosterone antiserum obtained from MP Biomedicals (Orangeburg, NY). All samples were measured in a single assay, and the intra-assay coefficient of variation was 10%. The effect of corticosterone manipulation on plasma corticosterone content was analyzed using one-way ANOVA.

Immunohistochemistry (IHC) and morphometric analysis

We used IHC to analyze GR immunoreactive (GR-ir) and CRF immunoreactive (CRF-ir) neurons in the frog brain as described previously (Yao et al., 2004). We used a highly specific affinity-purified anti-xCRF IgG and anti-xGR IgG described previously (see Chapter 2 and 4). Single IHC for GR-ir and CRF-ir were conducted using the Vectastain elite ABC (rabbit) and Vector VIP kits (both from Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's protocols (antibodies: 15-20 ng/μl and 0.5

ng/μl of affinity purified polyclonal rabbit IgG raised against synthetic frog CRF and GR peptide, respectively). We used double labeling fluorescence IHC to determine colocalization of GR-ir and CRF-ir following a method that we used previously to colocalize c-Fos and CRF (Yao et al., 2004). Double labeling experiments were analyzed by confocal microscopy (using a Zeiss laser scanning confocal microscope) with optical sections of 1 μm thickness captured through the Z-axis.

We quantified CRF-ir in discrete brain regions using MetaMorph software (v 6.2r4). We processed all samples simultaneously under identical conditions. Three sections that contained the anterior preoptic regions were analyzed for each animal. All sections were carefully matched for anatomical level, and digital images were captured at 200X magnification for morphometric analysis. Image analysis was conducted in a blinded manner. The brain regions were isolated using a hand-made frame that covered the area of interest. For quantification of CRF-ir, the total area of the 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 the multiple sections, divided by the total selected area (see Yao et al., 2004).

RT-quantitative PCR (RT-qPCR)

For RT-qPCR analysis of CRF gene expression in the brain, we microdissected the preoptic area (POA)/hypothalamus of individual juvenile frogs and isolated total RNA using the TRIZOL Reagent (Invitrogen Corp. Carlsbad, CA). 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 for detecting the primary transcripts (xCRFa hnRNA and xCRFb hnRNA) and mature RNA (xCRF mRNA) of the frog CRF genes (the xCRF mRNA primer/probe set detects both xCRFa and xCRFb mRNA; see Table 5.1 for primer and probe sequences). Reactions were run using the Fast 7500 Real-Time PCR System (Applied Biosystems, 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. To standardize RT input in each qPCR, we also generated standard curves of *X. laevis* ribosomal protein L8 mRNA (this gene is a house-keeping gene and not regulated by corticosterone; Shi and Liang, 1994) using serial dilutions of a RT product, and quantitated expression levels of L8 mRNA in each RT sample of the POA/hypothalamus. Expression levels of CRF hnRNAs and mRNA are presented as relative values normalized to L8 mRNA levels in each sample.

Cell culture and transient transfection

Monolayer cultures of PC-12 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 5% steroid stripped bovine calf serum, 5% steroid stripped equine serum (both sera from HyClone, UT) and antibiotics in a humidified atmosphere of 5% CO_2 at 37^{o}C. Steroid hormone removal was conducted by mixing 25 ml serum with 50 mg charcoal (coated with 5 mg Dextran T 70 [Pharmarcia] in phosphate-buffered saline [PBS]), and shaking at room temperature for 5 h followed by centrifugation at 1000 x 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 x g for 20 min at 4°C.

Transient transfections were conducted in triplicate in 24-well plates (Falcon, MA). The pRL-null plasmid (Promega, Madison, WI), which contains a promoterless *Renilla* luciferase gene, was cotransfected as an internal control for transfection efficiency. PC-12 cells were plated at a density of 4 x 10^5 cells/well in 24 well plates twenty four hours before transfection. We conducted transfections using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Each well received 800 ng of reporter plasmid plus 10 ng of pRL-null plasmid (Promega). Twenty four hours after transfection the medium was changed to fresh medium with or without regulators. For treatment with forskolin (Sigma), the stock was 25 mM dissolved in DMSO and added to the medium at appropriate dilutions. For treatment with corticosterone or dexamethasone (both from Sigma) in combination with forskolin, the stocks were dissolved in ethanol and added to the medium 1 h before addition of forskolin (i.e. 7 h before cell harvest). The control group received the same concentrations of vehicle as the regulator-treated groups.

Cells were harvested at different times and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega). All cell transfection experiments were conducted with 3 replicate wells per treatment and each experiment was repeated at least three times. The firefly luciferase activities were normalized to the *Renilla* luciferase activities in each cell lysate and the values are expressed as relative luciferase activity.

Electroporation-mediated gene transfer

To test the functionality of promoter elements *in vivo* we conducted bulk electroporation-mediated gene transfer into tadpole brain as described previously (Yao et al., 2007). *X. laevis* tadpoles (NF stage 49-51) were anesthetized by immersion in 0.002% benzocaine prior to i.c.v. microinjection of 92 nl DNA solution. Each DNA solution contained 1 μg/μl of the reporter plasmid, 50 ng/μl of pRL-null plasmid, 400 ng/μl of 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. We reversed the polarity and repeated the current delivery. Animals were allowed to recover and screened for high EGFP expression five days after the procedure using a Leica MZFLIII fluorescent stereomicroscope.

To test the responsiveness of the frog CRF promoters to corticosteroids *in vivo* we administered i.c.v. injections of CORT (100 nl injection of 500 nM CORT; ~ 20 pg/g body weight) 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 in the frog (~ 50 nM; see Chapter 4) 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 (Promega). We processed each brain separately in a microcentrifuge tube with a hand homogenizer in 20 μl of 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.

Electrophoretic mobility shift assay

We conducted electrophoretic mobility shift assay (EMSA) using *in vitro* synthesized *X. laevis* GR protein (xGR). We produced recombinant xGR protein by coupled *in vitro* transcription-translation with the p6xGR vector (Gao et al., 1994) following the manufacturer's instructions (TNT system; Promega). We prepared nuclear extracts from the preoptic area/diencephalon region of the juvenile frog brains following methods described previously (Yao et al., 2007).

DNA probes were prepared for EMSA by annealing complementary oligonucleotides. End-labeling was conducted using the large Klenow fragment of DNA polymerase (Promega) and ^{32}P labeled dCTP (Perkin Elmer, Inc. Boston, MA), and the products were purified over Sephadex G50 columns. Sequences of the oligonucleotides are shown in Table 5.2. The MMTVGRE probe was described by Malkoski and Dorin (Malkoski and Dorin, 1999), and the collagenase AP1 probe was described by Jonat et al. (Jonat et al., 1990).

Electrophoretic mobility shift assays were conducted following the methods of Dignam; Dignam et al., 1983) with minor modifications. *In vitro* produced GR proteins (1 μl of 50ul TNT reaction) were 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 (DTT), 0.1% IGEPAL CA-630, 50 mM KCl, and 20% glycerol. The reaction continued at room temperature for 40 min before fractionation by 6% non-denaturing PAGE in 0.25 x TBE buffer. The gel was dried and analyzed by autoradiography. Competition was conducted by addition of various

concentrations of unlabled specific or nonspecific oligonucleiotides during the incubation.

Statistical analysis

Statistically significant differences were determined by unpaired Student's t-test or one-way ANOVA followed by Fisher's LSD multiple comparisons tests using the SYSTAT v10 software (SPSS Inc., Chicago, IL, USA). Data were log10-transformed when the variances were found to be heterogeneous. The values are presented as mean + SEM, and $p < 0.05$ was considered statistically significant.

Results

Effects of corticosteroids manipulation on CRF immunoreactivity in the brain of *X. laevis*

Exposure of juvenile *X. laevis* to 500 nM CORT in the rearing water for 4 d caused a marked decrease in CRF-ir in the anterior preoptic area (POA), and treatment with 110 μM metyrapone (MTP) for 5 d resulted in an increase in CRF-ir in this region (Fig. 5.1). Densitometric analysis of the mean densities of CRF-ir in the POA confirmed that the observed differences were statistically significant (ANOVA: $F_{(3, 16)} = 21.780$, $p < 0.001$, n = 5/ treatment). Treatment with both MTP and a replacement dose (50 nM) of CORT did not change CRF-ir in the POA compared with control (Fig. 5.2A). We showed previously that this dose of CORT produced a plasma CORT concentration similar to basal, unstressed levels in MTP-treated frogs (see Chapter 4). Densitometric analyses of CRF-ir in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) at the level

of the anterior POA showed significant increases following the same CORT treatment (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/ treatment for both regions). Treatment with MTP caused a decrease in CRF-ir in the MeA, while no effect was observed in CRF-ir in the BNST. Treatment with MTP and CORT did not cause significant differences in either the MeA or the BNST (Fig. 5.2B,C).

Colocalization of CRF and GR immunoreactivity in the brain of *X. laevis*

Using dual immunohistochemistry and confocal microscopy, we showed that CRF-ir and GR-ir were colocalized in the cells of the POA and medial pallium in the normal juvenile frog brain (Fig. 5.3). Over 90% of CRF-ir positive cells in these regions expressed GR-ir, and over 90% of GR-ir cells in these regions also expressed CRF-ir.

Effects of corticosteroid manipulation on CRF hnRNA and mRNA in the frog brain

We subjected juvenile *X. laevis* to treatment with corticosterone (CORT; 500 nM in rearing water) or a corticosteroid synthesis inhibitor metyrapone (MTP; 100 μM in rearing water) for 6 h and analyzed expression of CRF hnRNA and mRNA in the POA/hypothalamus of the brain by RT-quantitative PCR (RT-qPCR). The dose of CORT chosen was shown previously to increase plasma CORT to stress levels but remained within the physiological range. The dose of MTP was shown previously to decrease plasma CORT to below the detection limit of the RIA (see Chapter 4). We designed specific Taqman assays that targeted the intronic regions of the *X. laevis* CRFa and CRFb genes to analyze expression of the primary transcripts (hnRNA). The 5' UTR and coding regions of the mRNAs of the two frog CRF genes are highly conserved; thus, our CRF

mRNA Taqman assay detected mature transcripts of both genes. We found that following treatment with CORT, the levels of CRFb hnRNA and CRF mRNA were significantly decreased in this region of the brain ($p = 0.034$ and $p = 0.025$ vs. control group, respectively; n = 5/ treatment), whereas no significant difference was observed in the level of CRFa hnRNA (Fig. 5.4).

Following 6 h of MTP treatment, the level of CRFb hnRNA was significantly increased in the POA/hypothalamus ($p = 0.027$ vs. control group; n = 5/ treatment). The level of CRFa hnRNA was on the verge of significant increase from the control ($p = 0.050$ vs. control group; n = 5/ treatment). No change was observed in the level of CRF mRNA (Fig. 5.4).

Effects of glucocorticoids on CRF promoter activity in transfection assays

We constructed promoter-reporter constructs for the two frog CRF genes designated pGL3-xCRFa533 and pGL3-xCRFb576 that contain ~ 500 bp of their 5' proximal sequences (see Yao et al., 2007). We found in the frog CRF genes a homologous GRE/AP1 region that was described in the human CRF gene (Malkoski and Dorin, 1999; Yao et al., 2007). In a first experiment, treatment of PC 12 cells transfected with pGL3-xCRFa533 or pGL3-xCRFb576 with dexamethasone (DEX; 100 μM) did not affect the basal activity of either promoter construct. Forskolin (Fsk; 25 μM), an activator of the adenylyl cyclase, strongly stimulated the promoter activity of both constructs, and DEX significantly reduced Fsk-induced activity of both constructs ($p = 0.001$ for both vs. Fsk treatment) (Fig. 5.5A).

We reasoned that if 25 μM Fsk was causing a maximal response we may not detect an effect of GCs at lower doses. To determine the appropriate dose of Fsk for the study of GC regulation of the CRF gene, we examined the activity of the pGL3-xCRFb576 construct in response to a series of concentrations of Fsk. We found that the activity of the construct was significantly increased in response to 50 nM Fsk in the culture medium ($p = 0.013$ vs. control). The activity continued to increase with increasing concentrations of forskolin, and reached the maximum levels in the presence of 2.5 μM Fsk (Fig. 5.5B). We decided in the following cell transfection assays to use 200 nM Fsk, which would cause approximately a half maximal induction of the promoter activity.

We examined activity of the pGL3-xCRFb576 construct in the presence of Fsk (200 nM) and various concentrations of CORT or DEX. Up to 10 μM of CORT did not significantly affect Fsk-induced activity of the construct, whereas treatment with 100 μM of CORT reduced Fsk-induced activity by 48% ($p < 0.001$ vs. Fsk treatment). Treatment with DEX significantly reduced Fsk-induced promoter activity when the concentration of DEX reached 1 μM ($p = 0.026$ vs. Fsk treatment), and decreased Fsk-induced activity by 43% at a concentration of 10 μM ($p < 0.001$ vs. Fsk treatment) (Fig. 5.5C)

Effects of corticosterone on CRF promoter activity in transfected *X. laevis* brain *in vivo*

To test the effects of corticosteroids on transcriptional activity of the CRF gene *in vivo*, we transfected brains of NF stage 50 *X. laevis* tadpoles by the method of electroporation-mediated gene transfer. We cotransfected the pEGFP-N1 vector with the reporter constructs and screened tadpoles for high and comparable EGFP fluorescence in

the region of the third ventricle 5 d later. We have shown that this method is efficient for transfecting large numbers of cells in the brain (Yao et al., 2007). We also cotransfected a promoterless *Renilla* luciferase vector, and the firefly luciferase activity of the reporter constructs was normalized to the *Renilla* luciferase and presented as relative luciferase activity.

We focused on regulation of the pGL3-xCRFb576 construct since it has consistently shown greater responsiveness to the regulators in our studies (Yao et al., 2007). To test for responsiveness of the construct *in vivo*, we injected corticosterone i.c.v. (CORT; ~ 20 pg/g B.W.) and sacrificed animals 6 h later for dual luciferase assay. We found that CORT caused a significant decrease in luciferase activity in brain homogenates from pGL3-xCRFb576 transfected animals compared with no injection or vehicle (saline with 0.0048% ethanol)-injected controls ($p = 0.041$ vs. no injection control; n = 5/ treatment). The same treatment did not alter the activity of pGL3-basic. Vehicle injection did not alter the expression of either construct compared with the respective no injection controls (Fig. 5.6).

Binding of GR to the putative GREs in the *X. laevis* CRF promoters

Sequence analysis of the proximal promoters of the *X. laevis* CRF genes revealed putative binding sites for GR (GREs; Fig. 5.7A; Yao et al., 2007). The regions containing the putative GRE sequences in the CRF promoters are highly conserved among tetrapods, and several GREs have been identified in this region of the human CRF gene. These GREs overlap with AP1 sites to form a composite AP1/GRE site (Malkoski and Dorin, 1999).

Shifted protein-DNA complexes of similar mobility were formed in EMSA on the GREs from both frog CRF genes ("aGRE" and "bGRE", from xCRFa and xCRFb genes, respectively) using *in vitro* expressed recombinant *X. laevis* GR (rxGR) (Fig. 5.7B). A similar sized complex was formed with the GRE from the mouse mammary tumor virus (MMTV) promoter ("MMTV"), which contains a classical consensus GRE (included as a positive control). Since the GRE-containing regions of the CRF promoters also contain putative binding sites for AP1 protein, we tested binding of GR to the AP1 sequence in EMSA. Slight binding was observed of the GR protein to the AP1 binding site in the collagenase promoter ("AP1"). No shifted complex was seen on the CRE from the human CRF gene ("CRE"; included as a negative control), and using nuclear extracts of the frog brain a complex was formed with the CRE oligonucleotides, demonstrating that the probe was capable of binding by a protein present in the extracts. All the complexes formed with GR could be displaced by 100 nM (about 100-fold excess) of radioinert oligonucleotides of the same type (Fig. 5.7B).

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 of radioinert oligonucleotides (approximately 10-fold excess of the radiolabled probes), and completely displaced by 30 and 100 nM of radioinert oligonucleotides. The Ki (the concentration of the competitor at which binding is half maximal binding) is approximately 5 nM. 100 nM of MMTVGRE oligonucleotides also disrupted the GR-xCRFbGRE complex by more than half. Addition of 100 nM of radioinert colleganase AP1 oligonucleotides slightly reduced the GR-

xCRFbGRE complex, and no displacement was observed by the addition of the CRE oligonucleotides (Fig. 5.7C and data not shown).

Discussion

We have previously shown that the basic distribution pattern of CRF immunoreactivity (-ir) in the central nervous system of *Xenopus laevis* is evolutionarily highly conserved, and that discrete groups of CRF neurons were activated in response to a stressor in a similar manner to that observed in mammals (Yao et al., 2004). In the present study, we report that CRF expression is regulated by corticosteroids in the frog, and corticosteroids have differential effects on CRF-ir in the anterior preoptic area (POA) and the limbic structure the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST). This is the first detailed study showing that glucocorticoids (GC) can differentially regulate CRF expression in a brain cell type-dependent manner in a non-mammalian species.

It has been shown that in the rat GCs down-regulate CRF mRNA in the paraventricluar nucleus (PVN; homolog of the POA in non-mammals), while they up-regulate it in the amygdala and BNST (Makino et al., 1994a, b; Shepard et al., 2000; Swanson and Simmons, 1989; Watts and Sanchez-Watts, 1995). Adrenalectomy in the rat resulted in increased CRF mRNA in the PVN, and decreased or no effect on CRF mRNA in the central amygdala or the BNST (Beyer et al., 1988; Makino et al., 1994b). Studies in teleost fishes showed that treatment with cortisol reduced CRF-like immunoreactivity in the POA of eels (*Anguilla anguilla*) (Olivereau and Olivereau, 1988), whereas treatment with MTP had the opposite effect in eels (Olivereau and Olivereau, 1990) and

goldfish (Fryer and Boudreault-Chateauvert, 1981). However, nothing is known about glucocorticoid regulation of CRF expression in the amygdala or BNST in non-mammalian species. In the present report we showed that CRF-ir in the MeA and BNST in the frog brain was upregulated by corticosteroids, an effect opposite to that in the POA. This provides the first evidence for differential regulation of CORT on CRF in different brain regions in a non-mammalian species.

Both the amygdala and the BNST are limbic structures involved in expression of fear and anxiety in mammals (Herman et al., 2005; Schulkin et al., 1998; Schulkin et al., 1994). Studies in the rat showed that stimulation of the amygdala and BNST increased activity of the HPA axis and potentiated the fear response, but lesions had the opposite effect (for reviews see Schulkin et al., 1998; Yao and Denver, 2007). Little is known about the function and regulation of these structures in non-mammals. The anatomical definition of these nuclei in the frog brain was determined based on expression of neurotransmitters, enzymes, and other neural molecules (Marín et al., 1998c). We have previously demonstrated that CRF-ir was increased in both the medial amygdala (MeA) and BNST as well as in the POA of frogs exposed to a shaking/handling stressor (Yao et al., 2004), suggesting a conserved neuronal circuitry of stress activation in the CNS of vertebrates. The mechanisms involved in the cell-specific regulation of the CRF gene by GCs are poorly understood. Studies using primary cell cultures or cell lines raised some hypotheses (discussed below), but further investigations are necessary to validate the relevance of these studies *in vivo*. In the current study, we showed that the corticosteroid regulation of CRF-ir in these regions is identical to mammals, thus providing strong

support for the notion that the function and regulation of these limbic structures evolved before the separation of the amphibian and amniote lineages.

In further support of the direct regulation of CRF by GCs, we found that CRF-ir and GR-ir colocalized in ells in the POA and medial pallium (mp; homologue of the mammalian hippocampus). The CRF-ir and GR-ir were colocalized in neurons of the parvocellular division of the PVN, central and medial amygdala, and the BNST of the rat brain (Ceccatelli et al., 1989a; Cintra et al., 1987; Cintra et al., 1991). We did not analyze the colocalization of the two proteins in the amygdala, BNST, or other hypothalamic regions. However, since CRF and GR are both expressed in these regions in the frog brain (Yao et al., 2004; Chapter 4), it is likely that they also colocalize in these regions. The colocalization of CRF and GR in the cells of the stress-related brain regions suggest that glucocorticoids may directly modulate CRF gene expression via the GR in response to stress.

In order to study the short-term effects of GCs on CRF expression, we treated juvenile *X. laevis* with CORT or MTP for 6 h. We found that 6 h treatment with 500 nM CORT caused a significant decrease of CRFb hnRNA and CRF mRNA in the forebrain (this includes the telencephalon and diencephalon). The decrease in CRFb hnRNA suggests that the negative regulation of the CRF gene occurred, at least in part, at the level of gene transcription. In mammals GCs are well known to exert negative feedback on CRF expression in the PVN (for review see De Kloet et al., 1998). Studies in teleost fishes also showed that GCs can negatively regulate CRF expression in the POA region. Using quantitative real time PCR Bernier and colleagues (Bernier et al., 2004; Bernier et al., 1999) showed that treating goldfish with cortisol decreased CRF mRNA levels in the

telencephalon-preoptic region, but had no effect in other brain areas. Furthermore, treatment with MTP increased CRF mRNA levels in the hypothalamic region of white sucker as analyzed by Northern blotting (Morley et al., 1991). Taken together, these data show that the negative feedback regulation of CRF in the PVN/POA by GCs was established early in vertebrate evolution.

Despite the significant changes in CRFb hnRNA and CRF mRNA in the forebrain in response to GC manipulation, treatment with CORT or MTP had no effect on CRFa hnRNA. The two *X. laevis* CRF genes exhibit differences in their 5' regulatory regions, and the CRFb gene has consistently shown higher responsiveness to positive regulators in transfected cells and to stress stimulation *in vivo* (Yao et al., 2007). These results suggest that the CRFa gene may not be responsive to CORT regulation, and that transcriptional control of CRF mRNA may be mostly contributed by regulation of the CRFb gene. Despite the significant increase in CRFb hnRNA following treatment with MTP, we did not detect changes in CRF mRNA. This could be due to the timepoint studied, and CRF mRNA may increase with a longer treatment of MTP. Alternatively, the turn-over rate of CRF mRNA could also be increased by the treatment which results in the lack of change in the total mRNA.

We showed that treatment with MTP for 6 h caused a significant increase in CRFb hnRNA in the forebrain of juvenile frogs. Rapid regulation of CRF transcription by MTP has been described previously. Herman and colleges (Herman et al., 1992) found significant increases in CRF hnRNA and CRF mRNA in the rat PVN 30 min and 60 min post-injection of MTP, respectively, compared with vehicle-injected controls. This treatment with MTP completely blocked the elevation in plasma CORT caused by the

handling/injection stressor (while the vehicle-injection group showed a significant increase in plasma CORT). It is not clear how treatment with MTP modulated CRF expression without detectable changes in plasma CORT. The hypothalamic CRF neurons may be very sensitive to changes in plasma CORT concentrations and a small decrease in CORT may cause significant activation of the CRF gene. Since in our study the CRFa hnRNA and CRF mRNA in the same brain RNA samples did not show any significant changes, the observed increase in CRFb hnRNA is likely to be due specifically to the removal of CORT and not because of a nonspecific action of the drug.

The forebrain regions include a variety of cell types, and although the microdissection technique can isolate the major parts of the frog brain, it cannot separate smaller structures. Thus, this method would not detect gene expression changes that may occur in only a subset of CRF expressing neurons within the tissue section. Nonetheless, the RT-qPCR results show that CRF gene transcription in the frog brain is influenced by circulating corticosteroids.

We used transient transfection assays in PC-12 cells to determine if the CRF promoters were regulated by GCs. Glucocorticoids did not significantly alter basal activity of the frog CRF promoters. However, GC treatment greatly reduced forskolin-stimulated promoter activity. This observation is consistent with a study that showed that DEX inhibited cAMP and cytokine-stimulated human CRF promoter activity in transfected PC-12 cells (Wagner et al., 2006). However, an earlier study of the human CRF promoter reported that DEX increased Fsk-induced promoter activity in PC-12 cells (Guardiola-Diaz et al., 1996). The doses of DEX are similar in these studies (0.5 ~ 1 μM). We speculate that the discrepancy between these studies could be due to the different

promoter fragments in the constructs and/or treatment durations (varying from 6 h to 48 h).

The results of our cell transfection assays are also consistent with studies of the human CRF promoter in transfected mammalian cells which generally showed no effects of GCs on the basal promoter activities, but significant decreases in activity induced by forskolin, cyclic AMP analogs, or phorbol esters (Guardiola-Diaz et al., 1996; King et al., 2002; Malkoski et al., 1997; Rosen et al., 1992; Vamvakopoulos and Chrousos, 1993b; Van et al., 1990). These results also suggest that GCs could inhibit CRF promoter activity by interfering with the PKA or PKC pathways. These actions may not require DNA binding by the GR. A number of studies have demonstrated protein-protein interaction (or "cross-talk") between GR and a number of transcription factors, co-factors, and signaling molecules including AP1, CREB, NGFI-B, NF-κB, CBP, p300, JNK, ERK, p38, etc. (for review see Yao and Denver, 2007).

In vitro studies using transfected cell lines have shown that the effects of GCs on CRF expression are cell-type dependent. Glucocorticoid (dexamethasone) treatment decreased CRF promoter activity, CRF mRNA expression, or CRF secrtion in a variety of cells including AtT-20 cells (Adler et al., 1988; Guardiola-Diaz et al., 1996; Malkoski et al., 1997; Van et al., 1990), NPLC-KC cells (Parkes et al., 1993), and rat hypothalamic cells (Hu et al., 1992). By contrast to the *in vivo* studies that showed increased CRF expression in the amygdala in response to GC administration, DEX did not alter CRF expression in primary rat amygdalar cultures (Kasckow et al., 1997). However, DEX induced CRF expression in human placenta cells (Cheng et al., 2000b; Robinson et al., 1988). It has been suggested that cell-specific usage of CRF promoter elements could

contribute to the differential regulation of the CRF gene. This in turn may be due to the different combinations of transcription factors, cofactors, and signaling pathways expressed in different cell types (King and Nicholson, 2007).

To test whether the frog CRF proximal promoters possess functional, negative GREs, we used electroporation-mediated gene transfer to introduce a CRF promoter-reporter construct into the tapole brain (Yao et al., 2007). Treatment with CORT significantly reduced CRFb promoter activity in transfected tadpole brains, suggesting the promoter fragment contains functional element(s) responsive to CORT regulation. This suggests that CRF gene transcription is under the transcriptional regulation of CORT in the *X. laevis* brain *in vivo*.

Computer analysis of the proximal promoter regions of the *X. laevis* CRF genes identified a conserved GRE/AP1 site that was previously shown to bind GR in the human CRF gene (Yao et al., 2007; Malkoski and Dorin, 1999; Malkoski et al., 1997). Both GR (DNA binding domain) and AP1 proteins (Jun/Fos) bound to this region in the human CRF promoter, and the binding could be disrupted by specific mutations in the sequence (Malkoski and Dorin, 1999). We also showed specific binding of the full-length *X. laevis* GR protein to the GREs-containing regions in the frog CRFa and CRFb promoters. The *X. laevis* GR seemed to bind to the frog GREs with a higher affinity than to the classical GRE sequence (the MMTV GRE), since the complex formed with the CRFb GRE probe was displaced more efficiently by the CRFb GRE oligonucleotides than the MMTV GRE oligonucleotides. The Ki of displacement by the CRFb GRE oligonucleotides is around 5 nM. The low nanomolor Ki suggests that the binding of GR to this site is specific, and the putative GRE/AP1 in the CRFb promoter could be a bona fide binding site for GR *in*

vivo. These results suggest that GR could regulate transcriptional activity by directly interacting with the CRF promoter. However, as discussed above, other modes of action by GR that do not require DNA-binding can also be involved in modulating CRF expression in different cell types.

It is interesting that while the CRFa promoter was responsive to CORT inhibition in the presence of forskolin, and GR bound to the putative GREs in the CRFa promoter, expression of this gene (measured by RT-qPCR) was not significantly influenced by 6 h of CORT or metyrapone treatment, unlike that of the CRFb. The different responsiveness of the gene in *in vitro* and *in vivo* assays might be explained by the many more intracellular and intercellular factors implicated in the brain tissue compared with a homogenous cell line or the *in vitro* binding assay. Similar discrepancies between *in vitro* and *in vivo* assays have been observed with regard to the responsiveness of CRFa to regulation by the PKA pathway (Yao et al., 2007). As for PKA pathway activation of the two genes (Yao et al., 2007), this study further supports that there are functional differences between the two duplicated CRF genes, and that the CRFa gene may not be responsive to CORT regulation *in vivo*.

Our results show GCs can influence the levels of CRF-ir and expression of CRF mRNA in the POA, which suggests that GCs exert negative feedback regulation of these cells as in mammals. In mammals there is evidence for both direct and indirect effects of GCs on hypothalamic CRF neurons (De Kloet et al., 1998; Makino et al., 2002). Our cell transfection assays and *in vitro* binding experiments provide support for direct regulation of the frog CRF genes by GCs. On the other hand, an important site for indirect negative feedback on neurosecretory CRF neurons in mammals is via descending inhibitory

pathways that originate in the hippocampus (Makino et al., 2002; Herman et al., 2005). The mammalian hippocampus expresses high levels of GR, and our finding that the homologous structure in the frog, the medial pallium, also expresses GR suggests the hypothesis that this indirect pathway could have been present prior to the evolution of the amniotes. This hypothesis awaits a direct test.

In conclusion, our discoveries in the frog *Xenopus laevis* support that the basic mechanisms and cell specificity of GC regulation of the CRF gene arose early in evolution. Similarities between GC regulation of the CRF gene in the POA/PVN, amygdala, and BNST in the frog and mammals suggest that the functions of stress-related brain structures were established before the divergence of the amphibian and amniote lineages and maintained due to the essential roles of the stress response system.

Acknowledgements

We thank Dr. Olivier Destrée for providing the p6xGR vector and Dr. Jesse Hay for providing PC-12 cells.

Table 5.1. Sequences of primers and probes used in RT-qPCR.

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3'; 5' FAM or VIC-labeled)
xCRFa hnRNA	GACACAAACCCACTT TTATGTTGTTGAA	CTTGCTCAGGTGCCA ATAATACC	TTGTCAAAACTGGG AATTACAT
xCRFb hnRNA	TGACACTTCACAATA TGCACACAGT	CATTCAACAAGTTCT GCACATCAGT	CAAGGACTCATAAA GCATTTG
xCRF mRNA	CATTTCCCTGGATCT GACTTTTCAC	TTGCTCAGCTCTTGC CATTTCTA	TTGCTCCGTGAAGT CT
xL8 mRNA	TTT GCT GAA AGA AAT GGC TAC ATC	CAC GGC CTG GAT CAT GGA	AGG GTA TTG TGA AAG ACA

All the CRF probes are FAM labeled, and the xL8 probe is VIC labeled.

Table 5.2. Sequences of oligonucleotide probes used in EMSA.

MMTVGRE	GTTGGGTTACAAACTGTTCTAACCA
AP1	AGCTTGATGAGTCAGCCGGATC
xCRFaGRE	TTTCTTTGTCAATGGAAAATTTTTGAACA
xCRFbGRE	TTTCTTGTCAATGGGAGACTTGTGAACG
hCRFCRE	GGCTCGTTGACGTCACCAAGAGGC

Upper strands are shown for each probe (5'→3').

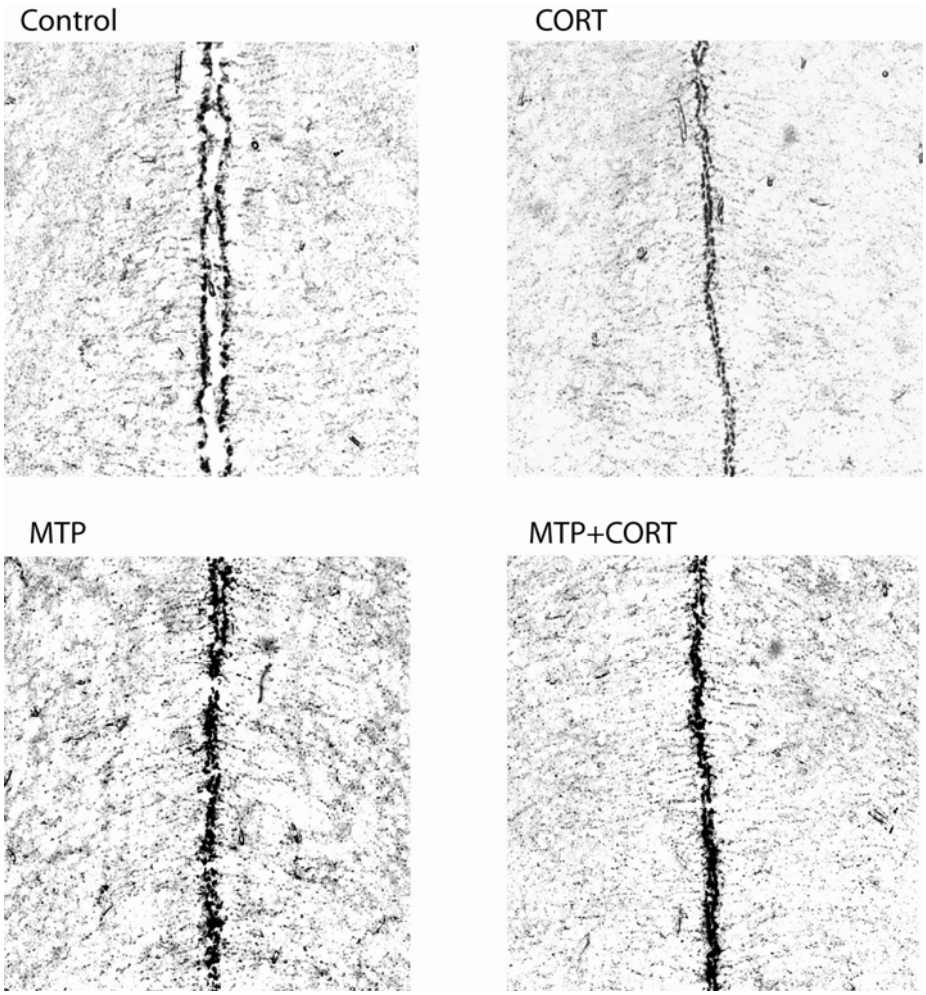

Figure 5.1. Effects of corticosterone manipulation on CRF immunoreactivity (ir) in the anterior preoptic area (POA) of juvenile *Xenopus laevis*. Micrographs are of representative transverse sections in the same anatomical planes. Frogs were untreated (Control), or exposed to 500 nM of corticosterone for 4 d (CORT), or 110 μM metyrapone (MTP) for 5 d, or 110 μM metyrapone for 1 d followed by 110 μM metyrapone and 50 nM of corticosterone for 4 d (CORT + MTP).

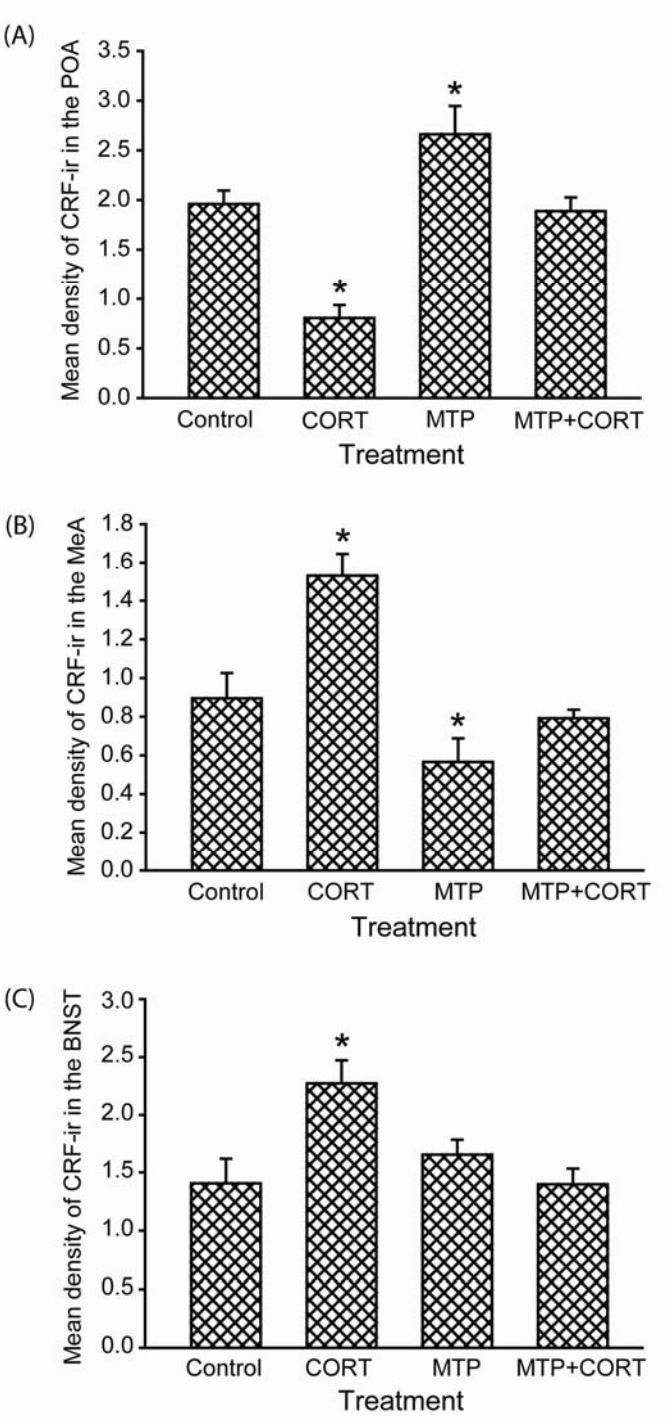

Figure 5.2. Effects of corticosterone manipulation of CRF immunoreactivity (ir) in the (A) the anterior preoptic area (POA), (B) the medial amygdala (MeA), and (C) the bed nucleus of stria terminalis (BNST) revealed by densitometric analyses. Treatments are the same as in Fig. 5.2. Data presented are the mean + SEM. * indicates a significant difference compared with the control ($p < 0.05$; n = 5/ treatment).

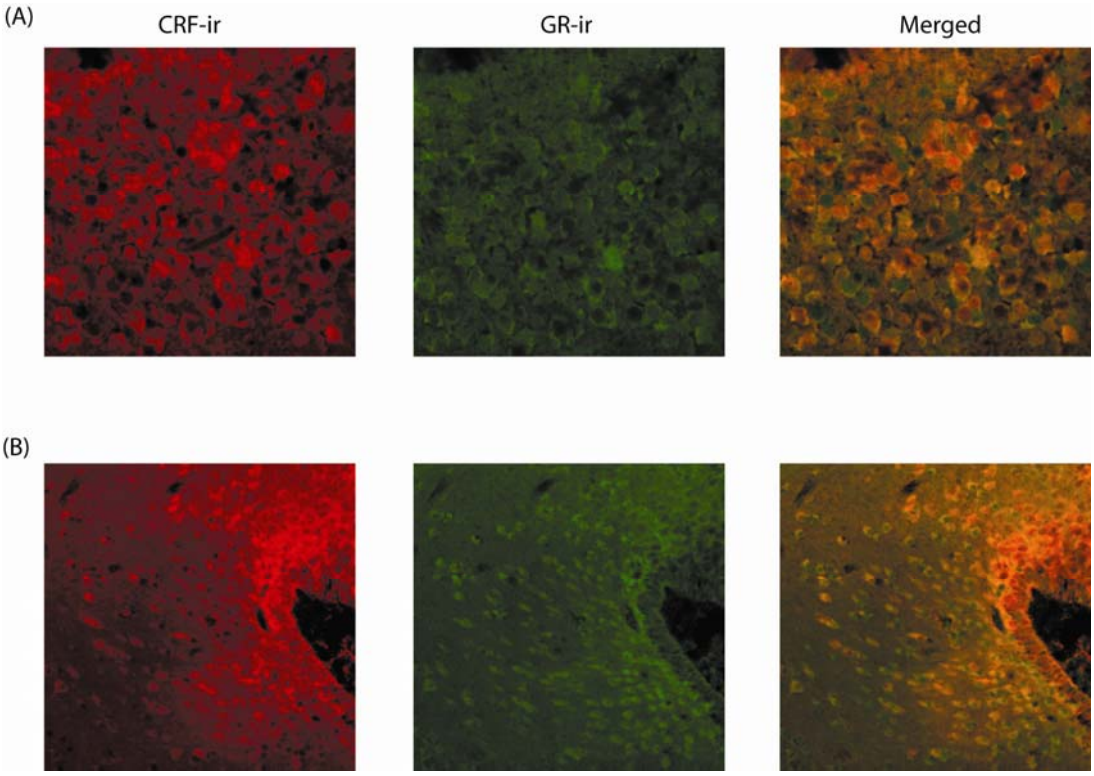

Figure 5.3. Colocalization of CRF immunoreactivity (ir) and GR immunoreactivity (ir) in (A) the anterior preoptic area (POA), and (B) the medial pallium (mp). The same field of the regions were scanned to reveal CRF-ir (red) and GR-ir (green). CRF-ir was mostly localized in the cytoplasm of the cells, while GR-ir was localized in both the cytoplasm and nucleus. The merged images of CRF-ir and GR-ir show colocalization of the two proteins in the neurons in the POA and mp. The micrographs of the POA were taken at 200x magnification. The micrographs of the mp were taken at 100x magnification.

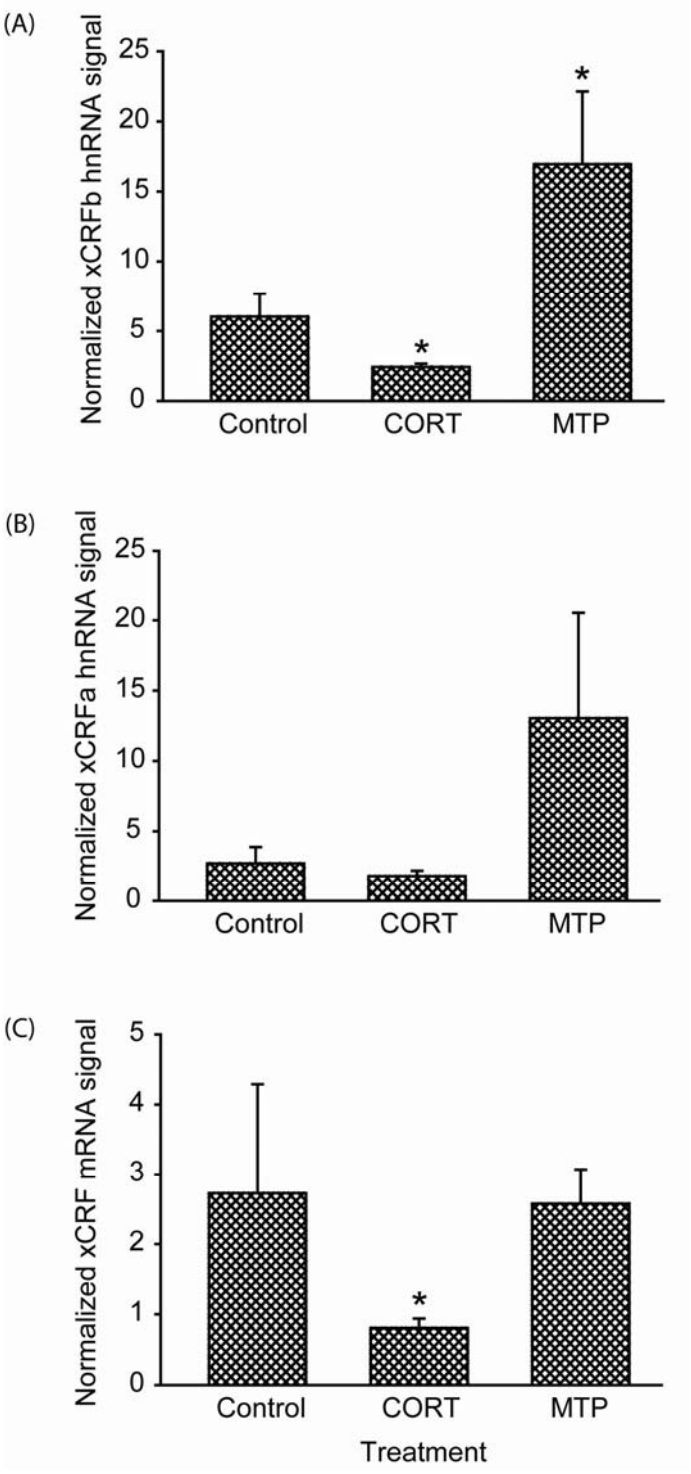

Figure 5.4. Analysis of (A) xCRFb hnRNA, (B) xCRFa hnRNA, and (C) CRF mRNA in the anterior preoptic area (POA)/hypothalamus of juvenile *Xenopus laevis* by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Frogs were untreated (Control), or exposed to 500 nM of corticosterone (CORT) or 100 μM metyrapone (MTP) for 6 h. Data presented are the mean + SEM. A significant difference from unstressed controls is denoted by * for $p < 0.05$; n = 5/ treatment.

Figure 5.5. Effects of treatment with glucocorticoids on the activity of the frog CRF promoters in transiently transfected PC-12 cells. (A) Effects of treatment with dexamethasone (DEX) on frog CRF promoter-reporter constructs in transiently transfected PC-12 cells. PC-12 cells were transfected with empty vector pGL3-basic (Empty), pGL3-xCRFa533 (xCRFa533; 533 bp fragment of the xCRFa promoter) or pGL3-xCRFb576 (xCRFb576; 576 bp fragment of the xCRFb promoter) plus pRL-null to normalize for transfection efficiency (dual luciferase assay; see Materials and Methods). Dexamethasone (DEX), 100 μM; Forskolin (Fsk), 25 μM. The experiment was repeated three times in triplicate, and data (mean + SEM) of a representative experiment are shown. * indicates a significant difference compared with the respective control, and # indicates a significant difference compared with the respective Fsk treatment (Student's *t* tests, $p < 0.05$). (B) Dose-dependent activation of pGL3-xCRFb576 by forskolin in transiently transfected PC-12 cells. Transfected cells were treated with or without various concentrations of forskolin for 6 h. The experiment was repeated twice in triplicate, and data (mean + SEM) of one representative experiment are shown. * indicates a significant difference between Fsk treated and control cells (Student's *t* tests, $p < 0.05$). (C) Dose effects of corticosterone (CORT) or dexamethasone (DEX) on forskolin (Fsk)-induced activity of pGL3-xCRFb576 in transiently transfected PC-12 cells. The experiment was repeated twice in triplicate, and data (mean + SEM) of one representative experiment are shown. * indicates a significant difference between the Fsk and CORT or DEX treated and Fsk-treated cells (Student's *t* tests, $p < 0.05$).

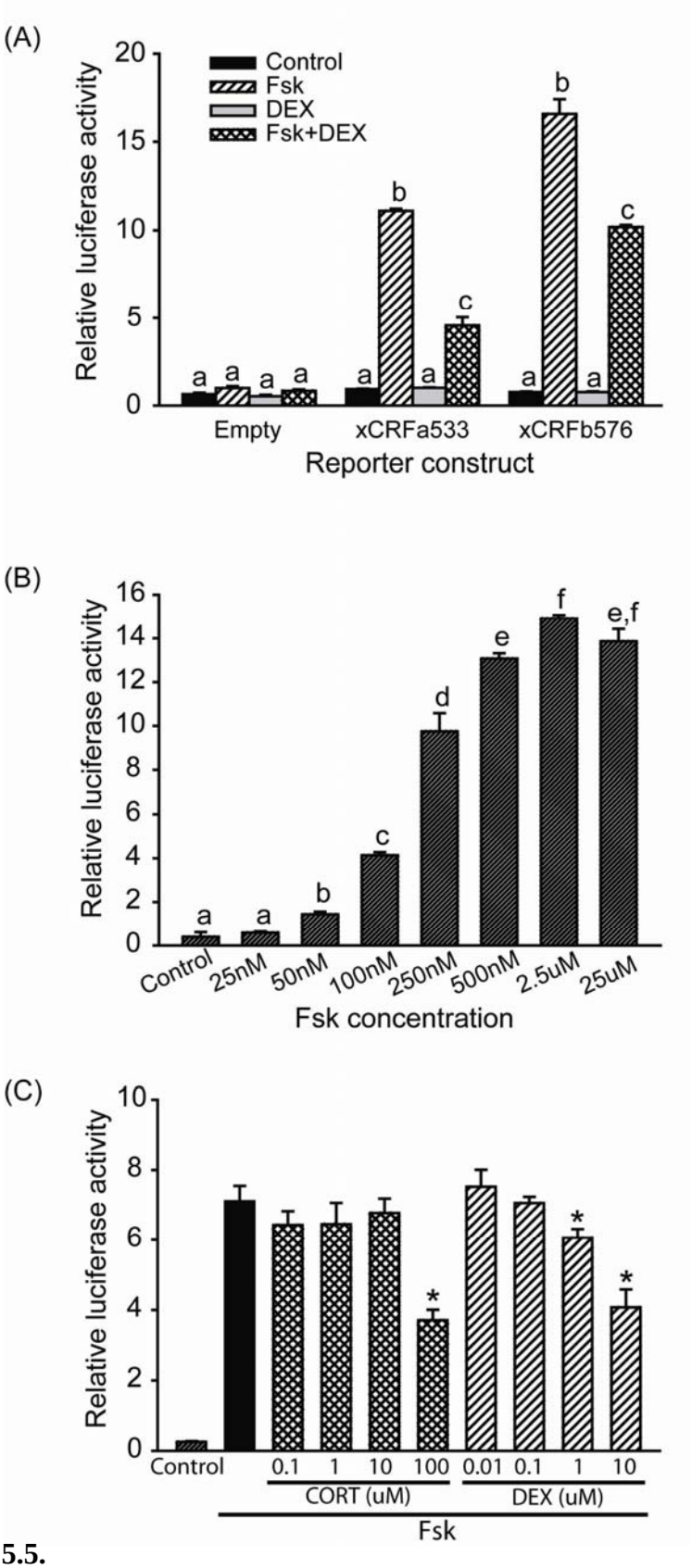
(A)
Relative luciferase activity
Control
Fsk
DEX
Fsk+DEX
Empty
xCRFa533
xCRFb576
Reporter construct
(B)
Relative luciferase activity
Control
25nM
50nM
100nM
250nM
500nM
2.5uM
25uM
Fsk concentration
(C)
Relative luciferase activity
Control
0.1
1
10
100
0.01
0.1
1
10
CORT (uM)
DEX (uM)
Fsk

Figure 5.5.

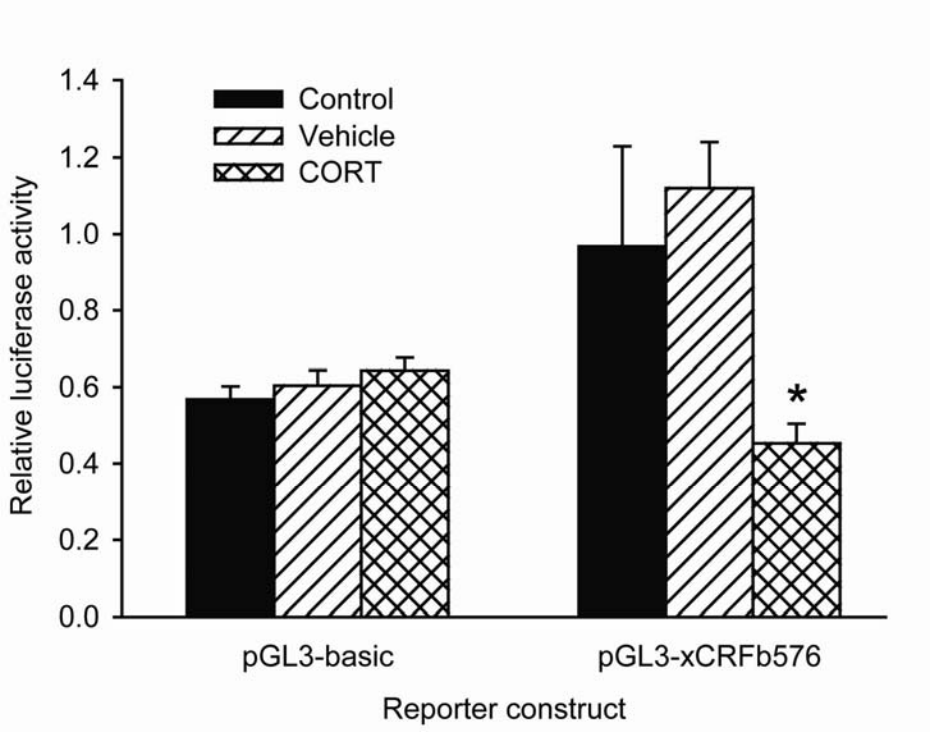

Figure 5.6. Effects of i.c.v. injection of corticosterone (CORT) on the activity of CRFb promoter construct transfected into tadpole brain by electroporation mediated (EM) gene transfer. Tadpoles were electroporated with the indicated constructs plus pRL-null to control for transfection efficiency (see Materials and Methods) and 1-2 weeks later given i.c.v. injections of either vehicle or CORT (20 pg/g B.W.). Animals were sacrificed 6 h after injection and brains collected for dual luciferase assay.

Figure 5.7. Binding of recombinant *X. laevis* GR (rxGR) to the GRE sites in the CRF promoters analyzed by electrophoretic mobility shift assay (EMSA). (A) ClustalW algnment of the putative glucocorticoid response elements (GREs)-containing sequences of 5' promoter regions of human (h), chimp (pt), sheep (sh), mouse (m), rat (r), and *Xenopus* CRF (x) genes. Identical sequences among the CRF genes are shadowed. Putative GRE and AP1 sites predicted by the on-line program Match™ using a library of mononucleotide weight matrices from TRANSFAC® 6.0 (www.gene-regulation.com) are indicated. Modified from Yao and Denver, 2007. (B) Binding of recombinant *X. laevis* GR (rxGR) to ^{32}P-labeled probes as analyzed by electrophoretic mobility shift assay (EMSA). Sequences of the probes are given in Table 5.2. Lane 1, 3, 5, 7, 9, 11, 13 incubated with radiolabled probes; lane 2, 4, 6, 8, 10, 12, 14 incubated with radiolabled probes (Comp. = competitor) and 100 nM of radioinert probes of the same sequences. (C) Cross-competition of GR binding to ^{32}P-labeled xCRFbGRE probes by radioinert oligonucleotides (Comp. = competitor). The ^{32}P-labeled xCRFbCRE probes were incubated with rxGR in the presence of various concentrations of radioinert competitor oligonucleotides. Concentrations of the competitor probes are indicated (in nM). --, no competitor.

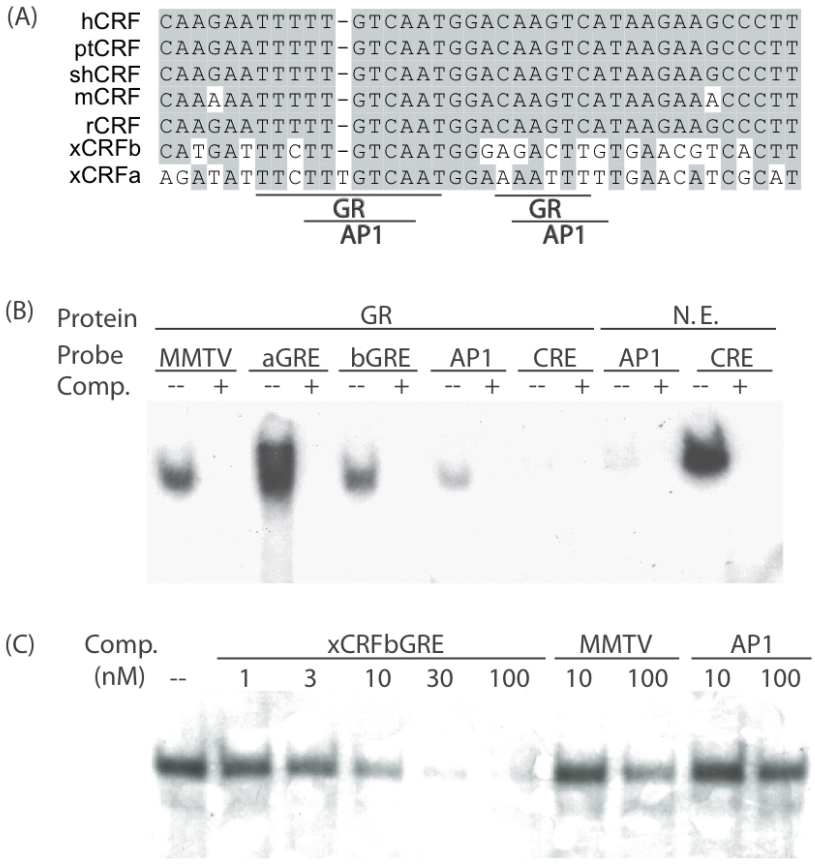
(A)
hCRF CAAGAATTTTT-GTCAATGGACAAGTCATAAGAAGCCCTT
ptCRF CAAGAATTTTT-GTCAATGGACAAGTCATAAGAAGCCCTT
shCRF CAAGAATTTTT-GTCAATGGACAAGTCATAAGAAGCCCTT
mCRF CAAAAATTTTT-GTCAATGGACAAGTCATAAGAAACCCTT
rCRF CAAGAATTTTT-GTCAATGGACAAGTCATAAGAAGCCCTT
xCRFb CATGATTTCTT-GTCAATGGGAGACTTGTGAACGTCACTT
xCRFa AGATATTTCTTTGTCAATGGAAAATTTTTGAACATCGCAT
GR
AP1
GR
AP1
(B)
Protein
GR
N.E.
Probe
MMTV
aGRE
bGRE
AP1
CRE
AP1
CRE
Comp.
-- + -- + -- + -- + -- + -- + -- +
(C)
Comp.
(nM)
--
xCRFbGRE
1 3 10 30 100
MMTV
10 100
AP1
10 100

Figure 5.7.

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CHAPTER 6

CONCLUSIONS

Corticotropin-releasing factor (CRF) plays a central role in modulating the activity of the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis, and in coordinating behavioral and autonomic aspects of the stress response in vertebrates. Since the initial isolation of CRF by Vale and colleagues (Vale et al., 1981), the mechanisms of endocrine, neural, and molecular regulation of the CRF gene in the stress response have been studied intensively but only in mammals. The CRF gene is rapidly activated in discrete regions of the central nervous system (CNS) in response to a wide variety of stressors, and circulating glucocorticoids (GCs) play an important role in feedback regulation of the CRF gene following stress activation. A number of neurotransmitters have been found to be involved in the regulation of the CRF neurons. Several intracellular molecular pathways functioning in transcriptional regulation of the CRF gene have been investigated *in vitro* and *in vivo* in rodent models (see Chapter 1).

Despite the extensive literature concerning the regulatory mechanisms of the CRF gene in mammals (rodents in particular), very little is known about the function and regulation of the CRF system in non-mammalian vertebrates. This lack of comparative data hinders our understanding of how the CRF system evolved and its adaptive significance in vertebrates. My dissertation research has characterized the expression and

regulation of the key components of the CRF system in the South African clawed frog *Xenopus laevis*, and showed that the cell-specific expression within the CNS, stress responsiveness, feedback regulation, and molecular regulatory mechanisms of CRF genes are highly conserved among the amphibian and mammals. My discoveries suggest that the basic regulatory mechanisms and neuronal circuits of the CRF system arose before the divergence of the amphibian and amniote lineages, and have been conserved by strong positive selection. Therefore, the frog is a relevant vertebrate model for the study of the ontogeny and function of the stress axis in tetrapods. In addition, with the wide variety of tools available in the frog such as the near complete genome sequence database, electroporation-mediated gene transfer, and germ-line transgenesis, this species provides a great model system for studies of the general endocrine system and physiological regulation in vertebrates.

Stress activation of the CRF gene

Regulation of the CRF gene in the stress response has long been studied in rodents. In general, CRF expression (hnRNA, mRNA and/or immunoreactivity) is increased in the rodent parvocellular paraventricular nucleus (PVN; the primary site for neuroendocrine regulation of the HPA axis) as well as selected other brain regions by physical, psychological, and physiological stressors (for reviews see Brunson et al., 2001; Makino et al., 2002). Upregulation of the CRF gene in these brain regions is usually preceded by stimulation of the immediate early genes (IEGs) such as *c-fos* or activation of the IEG proteins such as CREB (by phosphorylation) (Bilang-Bleuel et al., 2002; Emmert and Herman, 1999; Kovács and Sawchenko, 1996; Sawchenko et al., 1996; see Chapter 1),

suggesting that these proteins may be involved in transcriptional regulation of the CRF gene.

My dissertation study presents the first evidence for stress-activation of CRF neurons in the CNS of a non-mammalian vertebrate. I first carefully mapped CRF expression in the CNS of juvenile *X. laevis*, and found that the basic patterns of CRF immunoreactivity (-ir) are highly conserved in the frog brain compared with mammals. Using a shaking/handling stressor, I produced robust increases in CRF-ir in the anterior preoptic area (POA; homolog of the mammalian PVN), medial amygdala (MeA), and bed nucleus of the stria terminalis (BNST) in the brain of juvenile *X. laevis* (see Chapter 2). I also observed increases in Fos-ir in these and other brain regions following exposure to the stressor (see Chapter 2). The stress-activation of the CRF neurons in the frog brain was rapid; significant increases in CRF-ir in the POA were be detected by 1 h exposure to the stressor. Activation of CREB in the POA was even more rapid. By 20 min exposure to the stressor, significantly higher numbers of pCREB-ir positive neurons were found in this region (see Chapter 3).

These findings show that the CRF neurons respond rapidly to stressors in a cell-specific manner in the frog as in mammals. Studies in teleot fish also showed that stressors increased CRF mRNA in the POA (see Chapter 1). Together these results support the hypothesis that the tissue-specific expression and stress regulation of the CRF system was established early in the evolution of the tetrapods. My results also show that the frog is sensitive to environmental stressors and is an appropriate model organism for studying the vertebrate neuroendocrine stress system. Since the embryos of the frog develop externally (as free-living tadpoles), we can readily expose them to various

environmental conditions at different developmental stages and analyze the effects on the neuroendocrine stress system. Studies in this species will further our understanding of the function and evolution of the stress system in vertebrates.

Feedback regulation of CRF expression by glucocorticoids

Feedback regulation by GCs has been recognized as the most important mechanism of negative regulation of the CRF gene in mammals. The GC-dependent regulation of the hypothalamic CRF neurons can be direct or indirect. In mammals, GCs can exert GR-mediated inhibition of CRF synthesis and secretion by CRF neurons in the PVN, or modulate their activity indirectly via limbic (i.e., hippocampus, amygdala and bed nucleus of the stria terminalis) or brainstem structures. Neurons of these regions and send excitatory or inhibitory projections to PVN CRF neurons. These neurons also express GR, and thus GC action on these cells can positively or negatively regulate CRF expression in the PVN. It is hypothesized that regulation of hypothalamic CRF neurons by multiple brain pathways is an integrative mechanism for controlling the HPA response to stressors of different nature (De Kloet et al., 1998; Schulkin et al., 1998 Makino et al., 2002).

In order to understand the feedback regulation of CRF expression in *X. laevis*, and to study the evolution and conservation of the GC-dependent regulatory pathway, I first analyzed the distribution of GR-ir in the CNS and pituitary gland of the juvenile frog. My research shows that the general patterns of central expression of GR are highly conserved between the frog and mammals. Similar to the studies in rodents, I also observed that expression of GR-ir in the frog brain areas involved in the stress response (POA, mp,

MeA, and BNST) and the anterior pituitary is regulated by circulating GCs. These findings suggest that GR is likely to play a similar role in mediating the actions of GCs in the frog brain as in mammals, and the ligand-dependent regulation of GR in specific brain regions may be an evolutionarily conserved mechanism for modulating the responsiveness of the stress axis in vertebrates.

I exposed juvenile frogs to GCs or a corticosteroid synthesis inhibitor to manipulate the plasma GC concentration, and analyzed the expression of CRF RNA and protein in the brain. I show the first evidence that CRF expression is regulated by GCs in the frog, and GCs have differential effects on CRF-ir in the POA and the limbic structure MeA and BNST. My discoveries in the frog *X. laevis* support the hypothesis that the basic mechanisms and cell specificity of GC regulation of the CRF gene arose early in evolution. Similarities between GC regulation of the CRF gene in the POA/PVN, amygdala, and BNST in the frog and mammals suggest that the functions of stress-related brain structures were established early in evolution and maintained due to the essential roles of the stress response system. Our observations in *X. laevis* of activation of CRF neurons in the MeA and BNST by exposure to shaking stressor (see Chapter 2), and regulation of GR-ir and CRF-ir in these limbic structures by GCs (see Chapter 4and 5) suggest that the limbic structures are an integrated component of the stress response neural circuitry, and GCs may exert feedback regulation on these neurons to affect the CRF neurons in the frog POA. Stimulation, lesion, or implants of GCs in limbic structures of rodents showed that these brain regions exert substantial regulation of CRF neurons in the PVN and HPA activity (see Herman et al., 2005 for review), thus

supporting the role of the limbic system in mediating GC feedback regulation of the stress axis.

Glucocorticoids may also directly regulate CRF expression through interaction of GR with the CRF gene. I explored the molecular mechanism for direct GC regulation of CRF expression. I showed that the proximal promoters of the frog CRF genes are responsive to GC inhibition (under the condition of forskolin-activation). The proximal promoters contain putative GRE sites that are highly conserved among vertebrate CRF genes, and I show that GR specifically binds to the GRE sites *in vitro*. This suggests that GCs may regulate CRF transcription by inducing GR binding to the promoter. Previous studies in rodents showed that GR may regulate gene expression through DNA binding-depend or -independent mechanisms in different cell or tissue types (Reichardt et al., 1998). Further investigations are necessary for understanding the molecular mechanisms of GC feedback regulation of CRF expression in various brain regions (see Future directions).

Conserved regulatory elements in the CRF promoters

Using a comparative genomics approach and bioinformatic analysis, I identified putative transcription factor binding sites in the proximal promoters of the *X. laevis* CRF genes. I first focused on the putative cyclic AMP response element (CRE; the binding site for CREB). My study showed that it is a functional binding site for CREB *in vitro* and *in vivo*, and this sequence mediates cAMP-dependent and stressor-induced activation of the CRF promoters (see Chapter 3). I also investigated the functionality of the putative glucocorticoid response elements (GREs) in the proximal promoters of the frog CRF genes. I found that GR specifically binds to the GREs in the *X. laevis* CRF promoters,

and the proximal promoters of the CRF genes are responsive to glucocorticoid treatment *in vitro* and *in vivo* (see Chapter 5). These results strongly support that the functionality of the CRF promoter elements are conserved between the frog and mammals, and comparative genomic analysis is a powerful tool for identifying functionally conserved gene sequences.

The sequence comparison among the vertebrate CRF genes reveals that sequence similarity is not uniform throughout the promoter regions. The proximal promoters (~ 340 bp upstream of the transcription start sites) share the highest sequence similarity among the mammalian CRF genes, and the similarity decreases with increased distance (going upstream) from the transcription start sites (see Chapter 3). Within the proximal promoters there is also variation in sequence similarity. Highly conserved fragments are clearly separated by less conserved sequences in the promoters. This suggests that certain sequences have been maintained in the CRF promoter due to their critical function in regulating gene transcription.

A number of putative transcription factor binding sites were identified in the computer-based analysis of the proximal promoters of the frog CRF genes. I have only explored the functionality of the CRE and GREs, but have not examined the other sites, including the binding sites for AP1 proteins, NGFI-B, and ER. Most of these putative sites have not been studied in mammalian systems, and virtually nothing is known about their functionality. Furthermore, several sequences in the proximal promoters of the frog CRF genes show extremely high conservation across the vertebrate species where no putative transcription factor binding sites have been identified in my analysis. I hypothesize that these could be of importance for basal expression, tissue specificity,

developmental control, or stress-regulation of CRF gene transcription. It will be interesting to test the functionality of the putative binding sites and the highly conserved sequences in the regulation of gene expression both *in vitro* (in cell transfection assays) and *in vivo* (in electroporation-mediated gene transfer and transgenic animals; see Future directions).

Conserved functions of the limbic system and implications for evolution of the vertebrate brain

One significant discovery of my dissertation research is the conserved function of the limbic system in response to stress and glucocorticoid treatments among tetrapods. In mammals, the limbic structures (for example, the hippocampus and the amygdaloid complex) play pivotal roles in memory formation, processing of complex stressors, and expression of fear/anxiety (Charney et al., 1998; Morgane et al., 2005). The limbic structures have extensive connections (directly and/or indirectly) with a number of brain regions in the telencephalon, hypothalamus and thalamus, and brainstem, and regulate neuroendocrine and autonomic functions of the CNS (Gray, 1991; Herman et al., 2005; Morgane et al., 2005; Ongur and Price, 2000). It has been well established in rodents that similar to the PVN, the CRF neurons in the subfields of the amygdala (the central, medial, and/or lateral amygdala) are activated in response to fear/anxiety-provoking stressors (Becker et al., 2007; Bruijnzeel et al., 2001; Merali et al., 1998; Rotllant et al., 2007). The bed nucleus of the stria terminalis (BNST), which composes the main connection between the amygdala and the hypothalamus and the major direct non-hypothalamic input to the parvocellular PVN (Cunningham et al., 1990; Sawchenko and

Swanson, 1983), has also been shown to be responsive to fear-related stressors (Casada and Dafny, 1991; Gray et al., 1993; Makino et al., 1999). 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 (Gray et al., 1993). Another intriguing observation is that high levels of circulating GCs inhibit CRF expression in the PVN, while they stimulate it in the amygdala and BNST (Makino et al., 1994a, b; Makino et al., 2002), suggesting cell-specific effects of GCs and differential regulations of the CRF gene in these brain regions in the stress response.

The limbic structures in non-mammalian vertebrates (fish, amphibians, reptiles, and birds) are poorly defined. Nomenclatures based on cell morphology or topographical localization compared with the mammalian brains often lead to inconsistency and confusion. With advances in techniques such as tract-tracing and immunohistochemical methods, in combination with knowledge on brain development in these species, many of these limbic regions were redefined and characterized for their neuronal connections and expression of specific biochemical markers. It was found that the basal forebrain in frogs is highly organized and shares common features with its counterparts in mammals (Marín et al., 1998a, b; Marín et al., 1998c; Moreno and Gonzalez, 2005). However, to my knowledge there are no studies that have shown that the limbic systems in amphibians or other non-mammalian vertebrates are functionally homologous to their counterparts in mammals.

My dissertation research provides several lines of evidence that support the conserved functions of the limbic system in the frog compared with mammals: (1) The neurons in the amygdala, BNST, and medial pallium (mp; homolog of the mammalian

hippocampus) of the frog brain express CRF, and CRF-ir was increased in response to a stressor in the MeA and BNST (see Chapter 2). (2) The immediate early gene *c-fos* was activated in the limbic structures including the MeA, BNST, and mp following exposure to a stressor (see Chapter 2). (3) The neurons in the frog amygdala, BNST, and mp express GR (see Chapter 4). (4) The GR-ir was decreased in the frog MeA and BNST by high concentrations of circulating GCs (see Chapter 4). (5) The CRF-ir was increased in the frog MeA and BNST by high concentrations of circulating GCs (see Chapter 5). All these observations in the frog are generally in agreement with the studies in rodents (see Discussions of the Chapters). This supports the hypothesis that a functionally homologous limbic neurocircuitry was present in the earliest tetrapods, thus arguing against the view that large changes occurred in functional organization of the amygdala in the amphibian-reptilian transition (Bruce and Neary, 1995). Overall, my results suggest that functions of the limbic structures in stress response and feedback regulation by GCs arose before the divergence of the amphibian and amniote lineages and are common features in tetrapods. Further studies are required in other non-mammalian vertebrates to know if the limbic system is also functionally conserved in fishes (fishes have anatomically defined limbic structures; Bradford, 1995; Wong, 1997), and how the functions of the limbic structures have evolved in birds and reptiles.

Future directions

Studies in rodents have shown that activation of the stress neurocircuitry is stressor-specific, and different types of stressors evoke responses in different groups of brain nuclei. For example, physiological or "systemic" stressors activate brain stem pathways,

while psychological or "processive" stressors tend to stimulate the limbic pathways (for reviews see Herman and Cullinan, 1997; Herman et al., 2003). It is important to understand how differential neuronal responses to different types of stressors have evolved. Using *Xenopus laevis* as a model system, we can readily study the regulation of the HPI axis and activation of neurocircuitry by various types of external and internal stressors. These studies will provide information on the function and evolution of the vertebrate stress response system.

The highly conserved patterns of central distribution and stress activation of CRF expression in the frog suggest that the regulatory elements for tissue-specific expression and stress activation of the CRF genes are conserved between the frog and mammals. My dissertation study explored the functions of the CRE and GRE sequences in the promoters of the *X. laevis* CRF genes (see Chapter 3 and 5). However, little is known about the function of other putative regulatory elements (for example, the binding sites for the AP-1 and NGFI-B; see Chapter 1 and 3) identified in our study, nor about the nature of the regulatory elements involved in developmental regulation and tissue-specific expression of CRF. I used electroporation-mediated gene transfer to study the CRF promoter elements in tadpole brain. While this somatic gene transfer technique allows for rapid analysis of the functionality of specific gene regulatory sequences *in vivo* germ-line transgenesis is a better approach for analyzing *cis* elements that govern developmental and cell-specific gene regulation. Germ line transgenesis is now used routinely in frogs to generate transgenic animals. Future studies using transgenic animals harboring reporter constructs driven by fragments of the frog CRF gene would allow for analysis of important *cis* elements. The use of fluorescent reporter proteins will allow for

live-imaging of gene expression in the tadpole brain. My immunohistochemical analyses of the central distribution of CRF in *X. laevis* has set the stage for these experiments. Comparative genomic analysis coupled with a high throughput transgenic assay allows for the identification of gene regulatory elements responsible for spatial and temporal control of gene expression (Burch, 2005; Pennacchio et al., 2006; Tümpel et al., 2002). Transgenesis with bacterial artificial chromosomes (BACs) is a powerful tool for analyzing large fragments of DNA (up to 300 kb) in mice and in frogs (Giraldo and Montoliu, 2001; Ishii et al., 2004).

The human CRF gene contains a composite GRE/AP1 site in the proximal promoter (Malkoski and Dorin, 1999). It has been shown in several gene promoters that interaction of GR with AP1 family members at their composite binding sites is an important mechanism for conferring cell-specific regulation. Yamamoto and colleagues (Diamond et al., 1990; Miner and Yamamoto, 1992) have shown in a serious of elegantly designed experiments that GCs can stimulate or repress the transcriptional activity of the proliferin gene through a composite GRE/AP1 site in the promoter in different cell lines. They further found that the different concentrations and combinations of the AP1 family proteins in these cell lines can determine whether the GC regulation of gene transcription is positive or negative, and manipulating intracellular levels of corticosteroid receptors (the GR and MR) also profoundly influences the regulatory effects by GCs. *In vivo*, GCs exert the opposite regulation on CRF expression in the POA vs. the amygdala and BNST (see Chapter 5). The differences in the cell-specific expression of the transcription factors (the AP1 proteins in particular) and the corticosteroid receptors (the GR and MR) might explain the different effects of GCs on CRF regulation in these cells. To address this

question, we can first test if GCs have the same regulatory effects on the CRF promoter as on the proliferin gene promoter in the cell lines that Yamamoto and colleagues have characterized. If the CRF promoter in these cells is regulated by GCs in the same manners as the proliferin promoter, then it strongly supports that the composite GRE/AP1 sites in these genes are similarly regulated by GCs, and the cell type-dependent GC regulation may be due to the differences in the cell-specific expression of the AP1 proteins. If the CRF promoter responds to GCs differently from the proliferin promoter, it might be due to the differences in the GRE/AP1 sites of the two genes, or in the other regulatory elements of the promoter regions. To test if the composite sites are solely responsible for the different GC regulation, we can exchange the GRE/AP1 sites in the two gene promoters and study their regulation by GCs in the cell lines. If the GRE/AP1 sites alone determine the regulation by GCs, replacing the site in the CRF promoter with that in the proliferin promoter will confer the CRF regulation by GCs in the same manner as the proliferin gene in the cells (and vise versa). If exchanging the GRE/AP1 sites do not affect the promoter activity regulated by GCs, further investigations on other components of the two promoters will be required to identify the cause for the different regulation.

Multiple modes of action of GR have been proposed and tested by *in vitro* assays. In general, the genomic actions of GR can be either DNA binding-dependent or -independent. The DNA binding dependent mechanisms involve direct binding of GR to the promoter of the target gene, thus negatively regulating the transcriptional acitivity. This could involve the recruitment of coactivators or corepressors to activate or repress the gene, or the interference with other transcription factors. The DNA binding-

independent actions of GR can be divided into mechanisms that involve direct protein-protein interaction with other transcription factors, competiton with other transcription factors for the cofactors, or modulation of the activity of other transcription factors (for review see Karin, 1998). Although other investigators and I showed that GR binds to the putative GRE sites in the CRF promoters (Malkoski and Dorin, 1999; see Chapter 5), it is not known whether activated GR down-regulates CRF expression in the POA or upregulates it in the amygdala or BNST by directing interacting with the gene promoter. In transgenic mice expressing GR defective in dimerization and DNA binding (GR^{dim}), CRF-ir in the median eminence was not significantly different from the wild type controls (Reichardt et al., 1998). However these investigators did not report the comparison of CRF-ir in the PVN between the mutant and wild type animals. If the negative regulation of hypothalamic CRF by GR is DNA binding-dependent, we may observe increased CRF expression in the PVN of the GR^{dim} mice. However, if CRF expression in the PVN of the GR^{dim} mice is not different from wild type, it will still not be possible to conclude that GR action in these neurons is DNA binding-independent since CRF expression in the PVN is regulated indirectly by descending limbic pathways (see above) and other molecular pathways that could exhibit compensatory changes. The loss of DNA binding-dependent inhibition of the CRF gene by GR could be compensated by indirect regulation through neural pathways in which GR action is DNA binding-independent.

Although a large body of data shows that GCs can negatively regulated CRF expression, and a composite GRE/AP1 site has been identified in the CRF gene of both mammals and frog, to date there has been no direct test of the hypothesis that GR binds to

or associates with the CRF gene promoter *in vivo*. To begin to address this question we can use ChIP assay on chromatin extracted from the PVN of rodents or the POA of the frog. Since the GR could associate with the DNA directly or indirectly through other proteins, we would need to use a bifunctional protein crosslinking agent such as DSP in the ChIP experiments. The immunoprecipitated DNA will be analyzed by quantitative PCR (qPCR) using primer/probe sets spanning the putative GRE/AP1 site. If the result shows that more GR is associated with this region than other regions (such as the coding sequences that do not contain a GRE), we can then design other primer/probe sets in the flanking sequences of the CRF promoters to find additional GREs using ChIP assay. Follow-up experiments could be done with the chromatin from the PVN of the GR^{dim} mice to see if GR association with the DNA is affected by the DNA-binding mutation. Cells lines derived from different regions of the brain (such as the hippocampal and amygdalar cell lines) could also be used to study cell-specific association of GR with the DNA.

Concluding remarks

The vertebrate stress axis plays a critical role in the stress response. However, little is know about the evolution of the vertebrate stress system. My thesis research has shown that the nucleotide sequences, distribution, function, and regulation of the frog CRF gene are similar to mammals. The similarity among the structure and function of the stress system in the fish, frog, and mammals suggests that these features were possessed by the common ancestor of these modern vertebrates, and have been conserved through millions years of evolution by positive selection. My study covered a wide variety of disciplines

including the neuronanatomy, physiology, and molecular biology of the stress system, which is first of its kind in a nonmammalian vertebrate.

The highly conserved nature of the stress response system supports its essential roles in maintaining the homeostasis and fitness of an organism. Stress is inevitable part of life, and proper stress response is critical for human health. The fast pace and increased competition and work load of the modern life style have posed new challenges to the human stress response system. A better understanding of the function and regulation of the vertebrate stress system will be valuable for unwinding the pathogenesis of many stress-related disorders.

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Appendix A

Supplemental Table 1.

Oligonucleotides used for reverse transcriptase-PCR detection of primary xCRFa and xCRFb transcripts.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
xCRFa hnRNA	GTGCATTTAATCATATAGCC	GAGAGATGAGAGAGATTTAGAG
xCRFb hnRNA	GCAGCGGAAATGGTAGTGA	AAAGGGAAATTGGGGAGAA

Appendix B

Supplemental Table 2.

Oligonucleotides and fluorescent-labeled probes (Taqman) used for reverse transcriptase quantitative PCR (RTqPCR) for xCRFa hnRNA, xCRFb hnRNA, and xCRF mRNA (recognize transcripts of both a and b genes).

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3'; 5' FAM-labeled)
xCRFa hnRNA	GACACAAACCCACTTTTATGTTGTTGAA	CTTGCTCAGGTGCCAATAATACC	TTGTCAAAACTGGGAATTACAT
xCRFb hnRNA	TGACACTTCACAATATGCACACAGT	CATTCAACAAGTTCTGCACATCAGT	CAAGGACTCATAAAGCATTTG
xCRF mRNA	CATTTCCCTGGATCTGACTTTTCAC	TTGCTCAGCTCTTGCCATTTCTA	TTGCTCCGTGAAGTCT

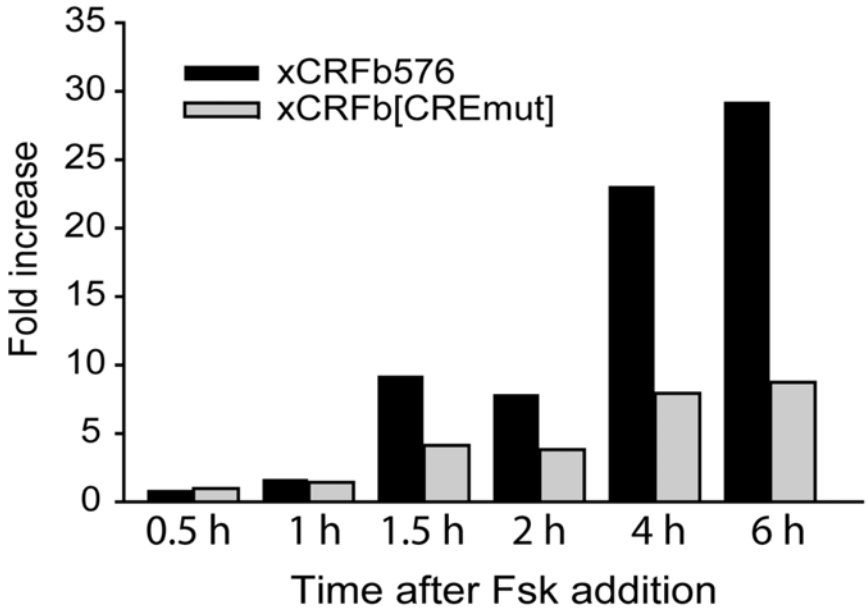
Appendix C

Supplemental Table 3.

Oligonucleotides and fluorescent-labeled probes (Taqman) used in chromatin immunoprecipitation (ChIP) assays for quantitative PCR analysis of xCRFb core promoter (CRFb CRE) and xCRF coding region (CRF CDS; recognize both a and b genes):

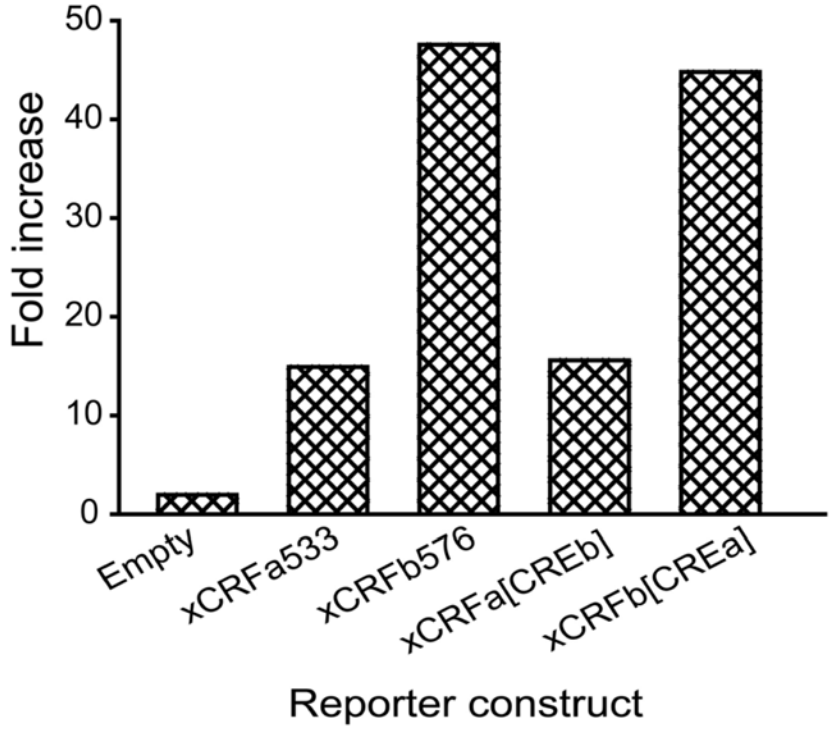
	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3'; 5' FAM-labeled)
CRFb CRE	GTCAATGGGAGAC TTGTGAACGT	GTTCTCGGATATTTA TTGCCTCTTTAGTGA	CAGTTAAGGT GTCATTTACG
CRF CDS	CATTTCCCTGGATC TGACTTTTCAC	TTGCTCAGCTCTTGC CATTTCTA	TTGCTCCGTG AAGTCT

Appendix D



Supplemental Figure 1
Time course of fold-increase of pGL3-xCRFb576 and pGL3-xCRFb[CREmut] by forskolin in transiently transfected PC-12 cells. Transfected cells were treated with or without forskolin (Fsk; 25 μM) for various times. Controls (no treatment) of both constructs showed no significant difference at any time point examined.

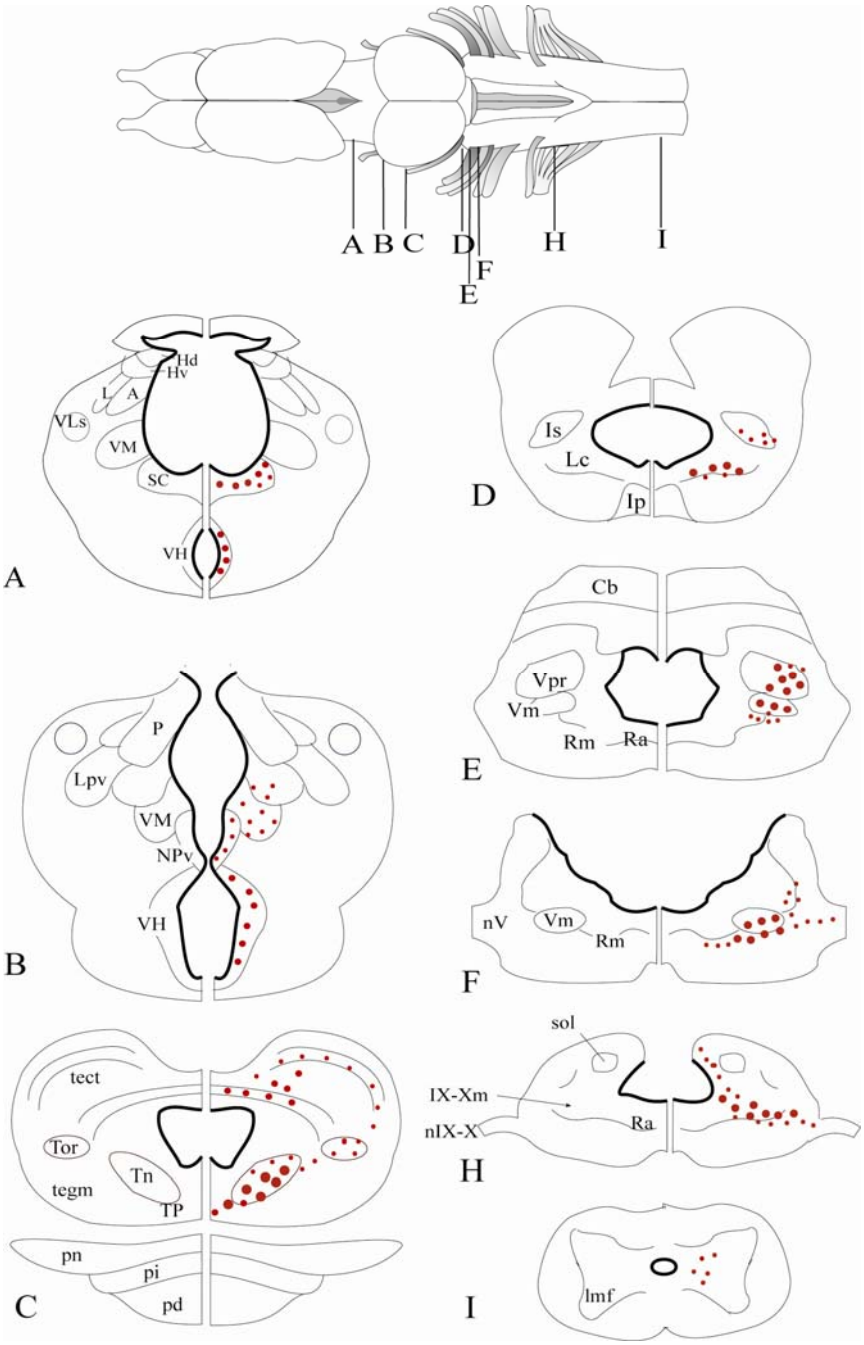
Appendix E



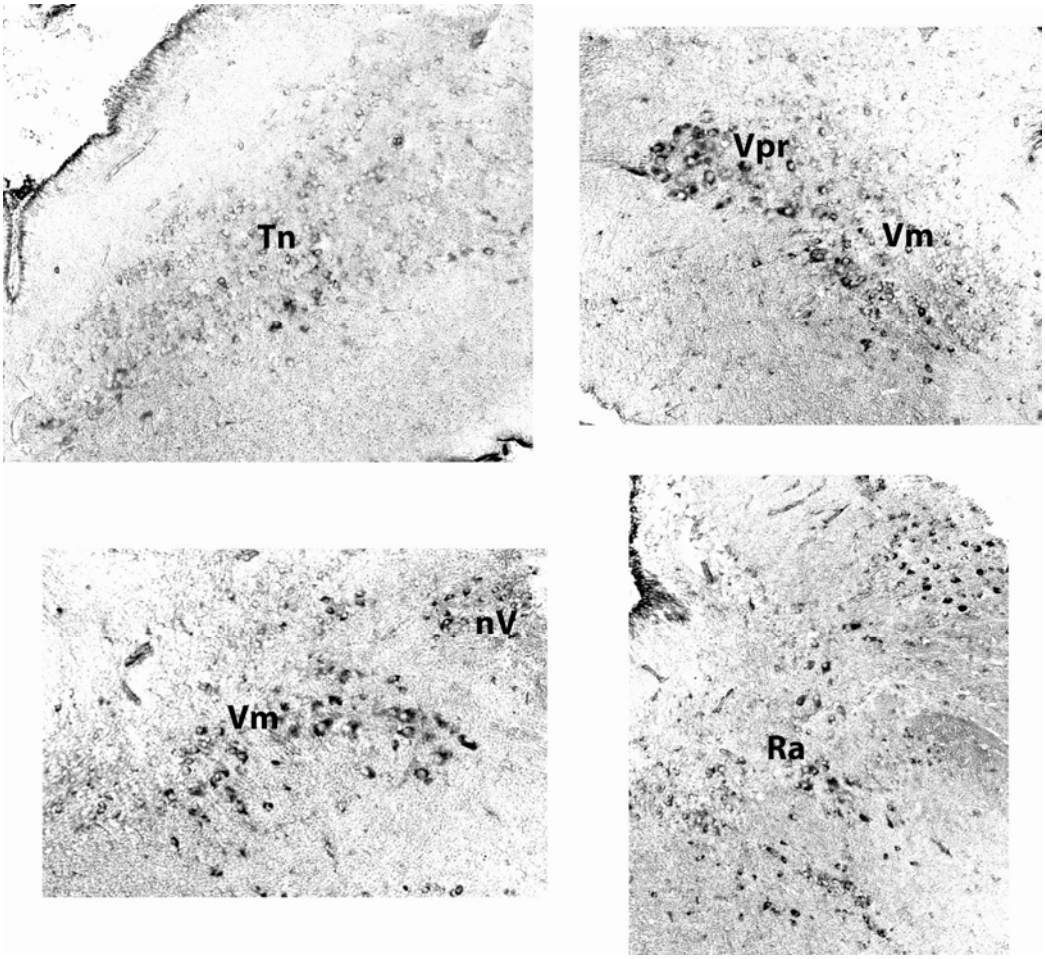
Supplemental Figure 2
Effects of exchanging the CREs present in the xCRFa and xCRFb genes on the fold-increase of the promoter activity induced by forskolin (Fsk; 25 μM) in transiently transfected PC-12 cells. All reporter constructs were constructed using the pGL3-basic vector. The experiment was repeated three times in triplicate with similar results, and data from one representative experiment are shown.

Appendix F

Schematic coronal illustration of mineralocorticoid receptor (MR)-immunoreactivity (ir) distribution in the brain of juvenile *Xenopus laevis.* Large red circles represent large MR-ir positive cells, and small red circles represent smaller MR-ir positive cells. See Table 2.1 for a complete description of abbreviations.

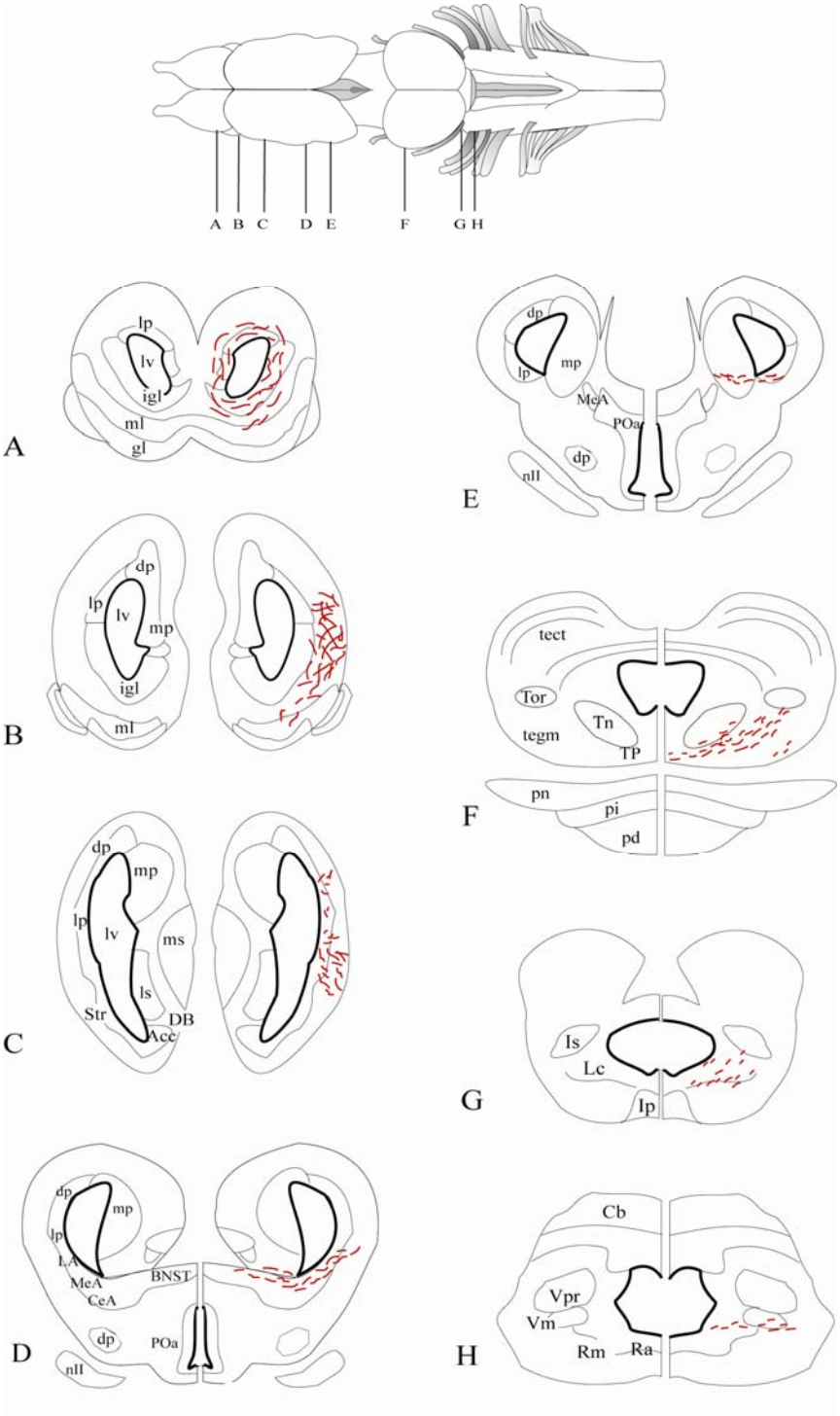


Photomicrographs at 200x magnification of transverse sections through the forebrain and part of the midbrain of juvenile *X. laevis* showing the distribution of MR-ir positive cells. The immunohistochemistry was conducted using a Vectastain elite ABC kit (rabbit) and Vector VIP kit (both from Vector Laboratories, Inc., Burlingame, CA) for HRP/DAB staining following the manufacturer's protocols. The affinity-purified anti-*Xenopus* MR antiserum was diluted 1:50 for incubation of the sections. See Table 2.1 for a complete description of abbreviations.

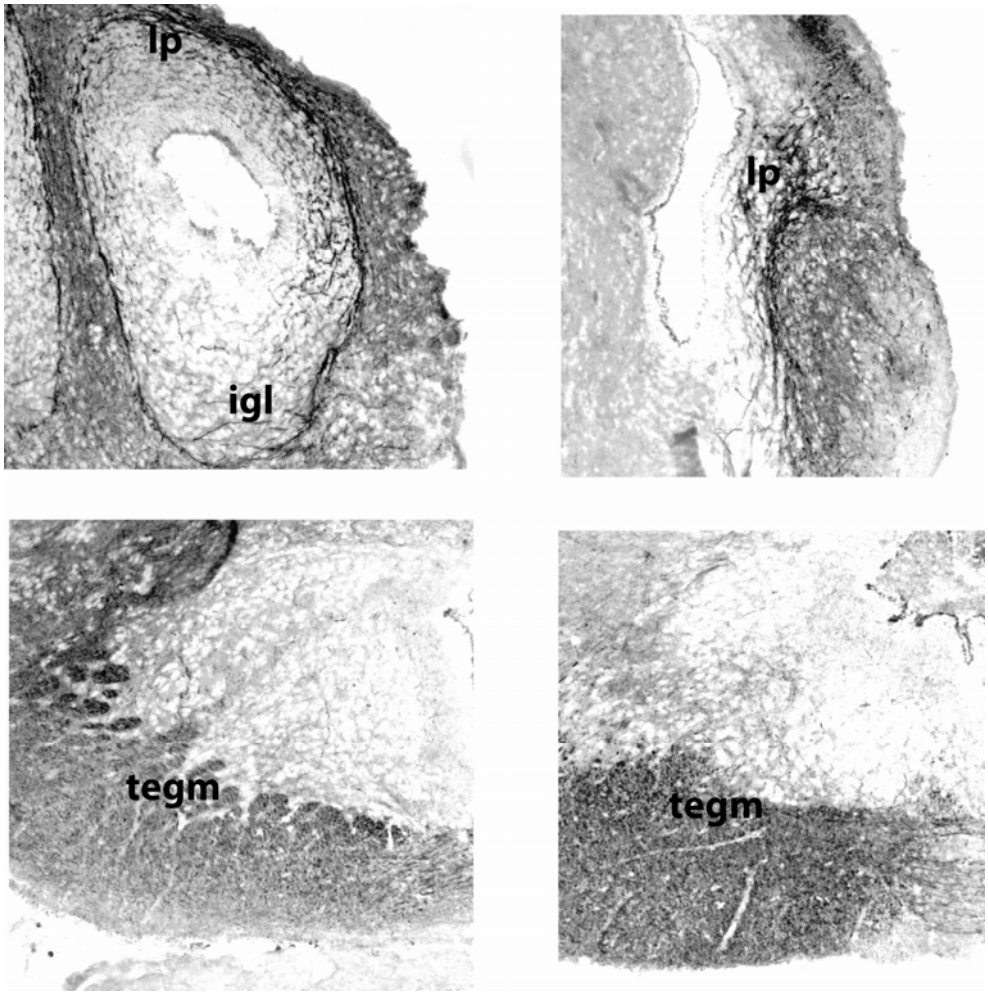


Appendix G

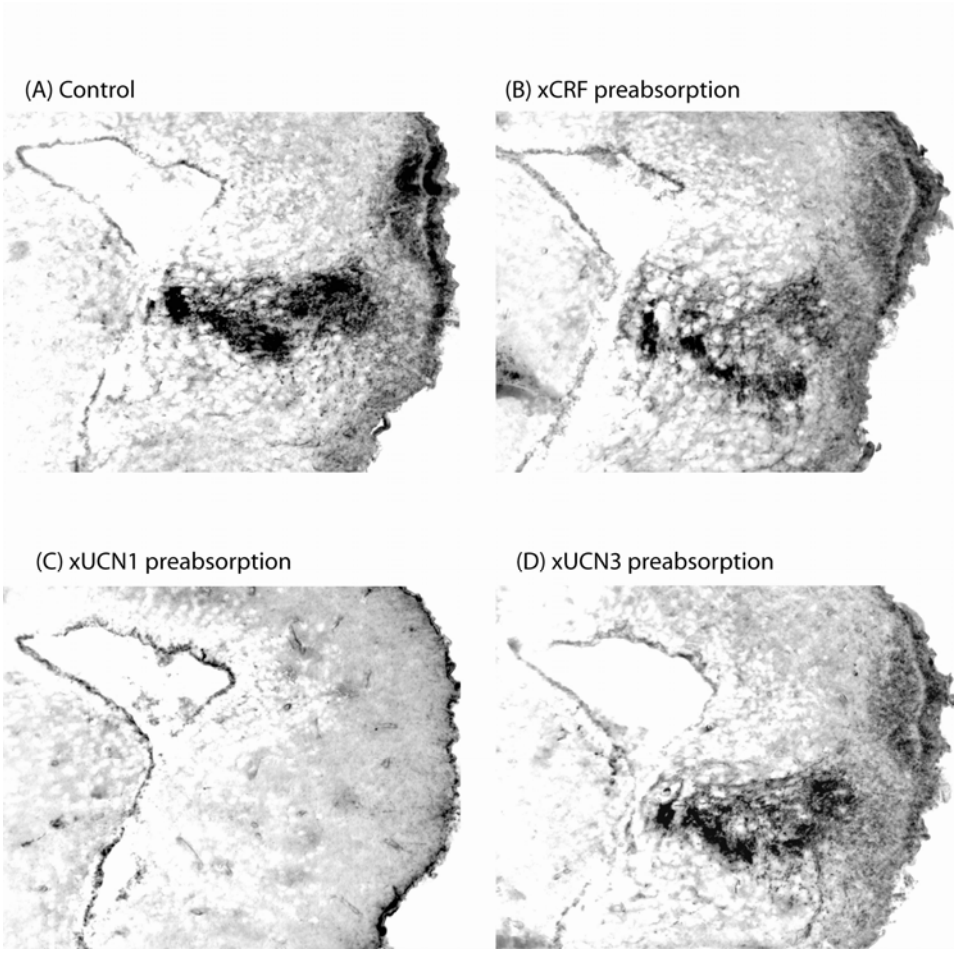
Schematic coronal illustration of urocortin 1 (UCN1)-immunoreactivity (ir) distribution in the brain of juvenile *Xenopus laevis.* Lines represent UCN1-ir fibers. See Table 2.1 for a complete description of abbreviations.



Photomicrographs at 200x magnification of transverse sections through the forebrain and part of the midbrain of juvenile *X. laevis* showing the distribution of UCN1-ir fibers. The immunohistochemistry was conducted using a Vectastain elite ABC kit (rabbit) and Vector VIP kit (both from Vector Laboratories, Inc., Burlingame, CA) for HRP/DAB staining following the manufacturer's protocols. The affinity-purified anti-*Xenopus* UCN1 antiserum was diluted 1:300 for incubation of the sections. See Table 2.1 for a complete description of abbreviations.

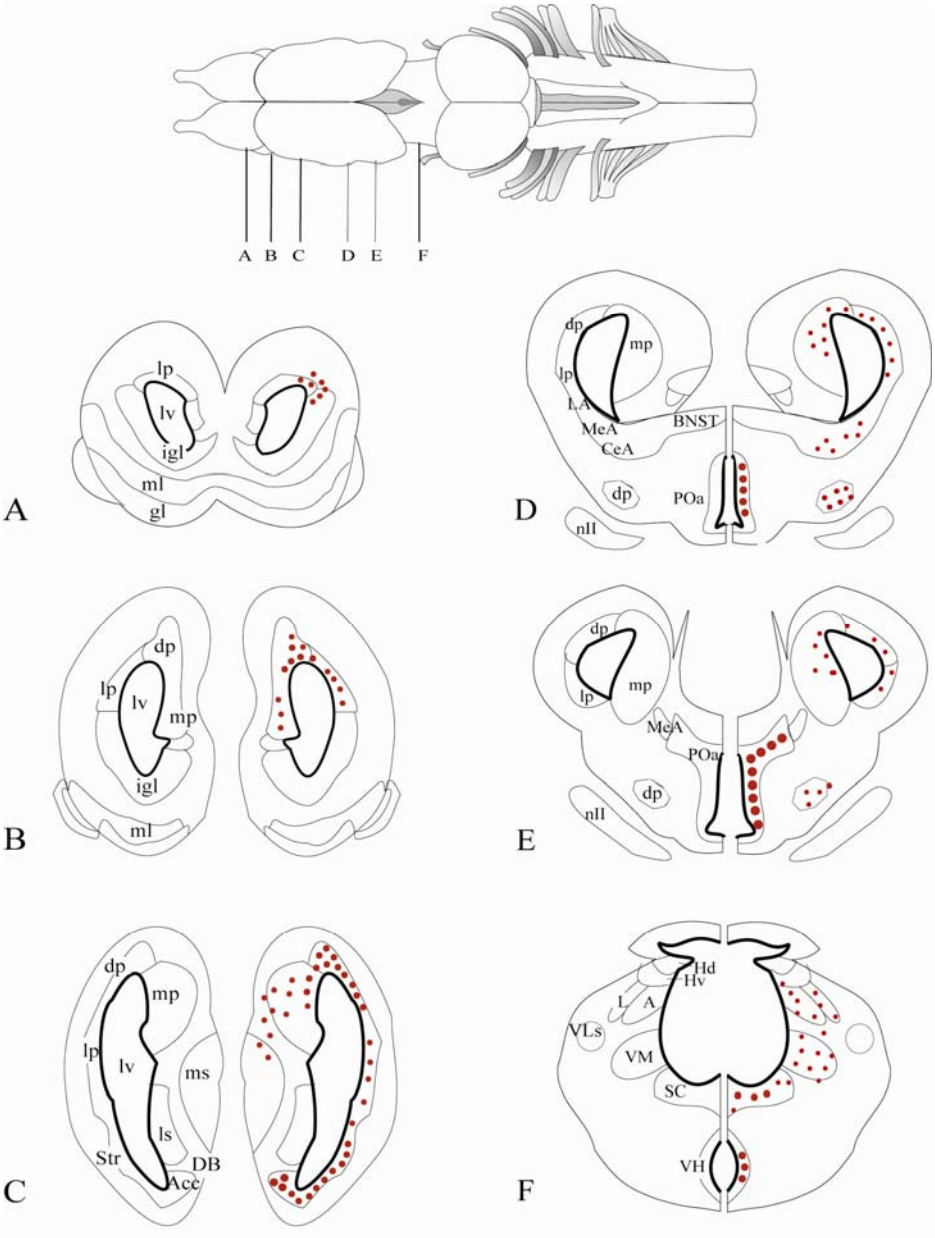


Photomicrographs (200x magnification) of transverse sections through the brain of a juvenile *X. laevis* at the level of the lateral pallium showing the specificity of the affinity-purified anti-UCN1 IgG. Dorsal is up in the photomicrographs in this and all other figures. Adjacent transverse sections were incubated with affinity-purified anti-*Xenopus* UCN1 IgG (A) or with anti-*Xenopus* UCN1 IgG that had been preabsorbed with 50 µg/ml of synthetic xCRF (B), xUCN1 (C) or xUCN3 (D).

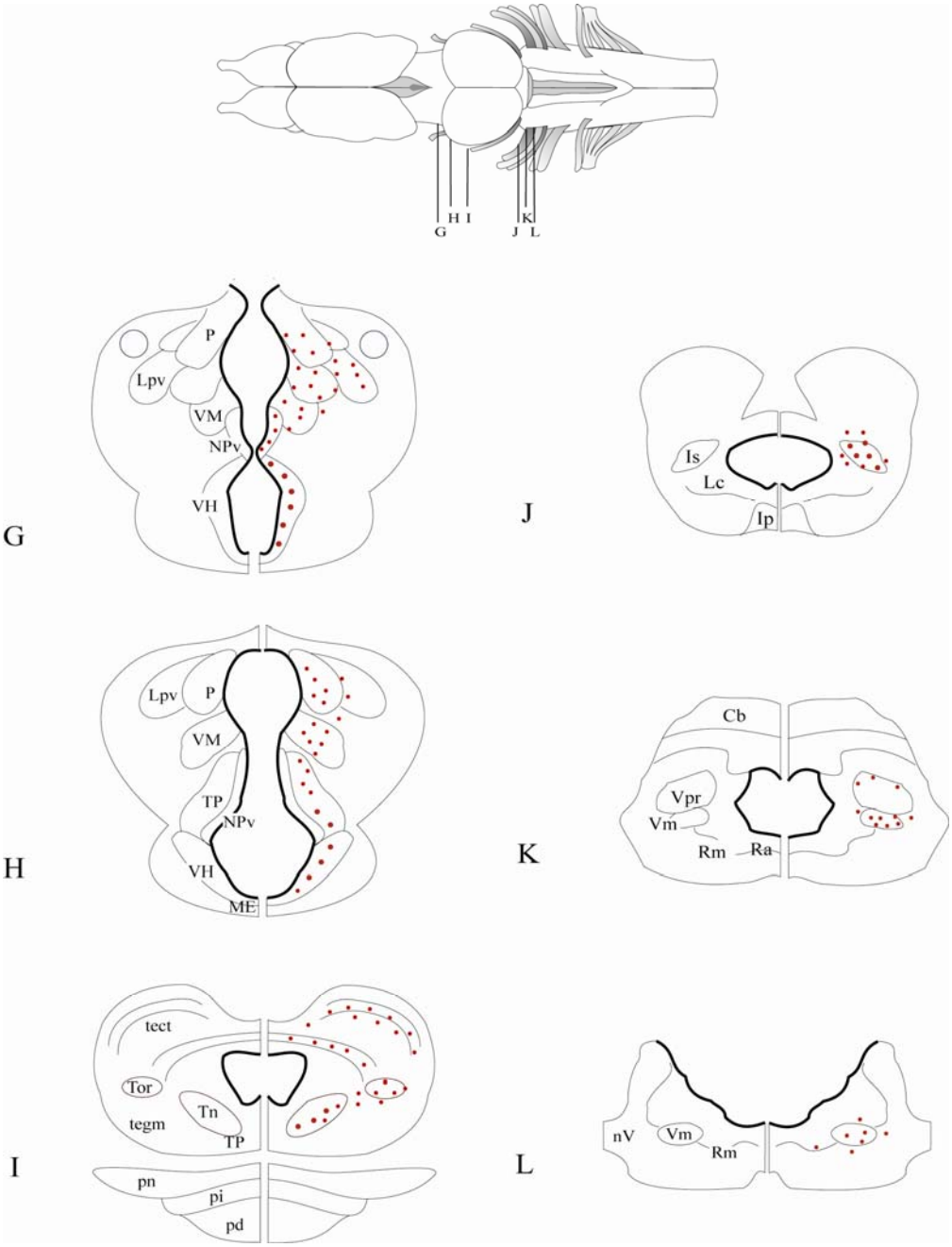


Appendix H

Schematic coronal illustration of urocortin 3 (UCN3)-immunoreactivity (ir) distribution in the brain of juvenile *Xenopus laevis* (I). Large red circles represent large UCN3-ir positive cells, and small red circles represent smaller UCN3-ir positive cells. See Table 2.1 for a complete description of abbreviations.



Schematic coronal illustration of urocortin 3 (UCN3)-immunoreactivity (ir) distribution in the brain of juvenile *Xenopus laevis* (II).



Photomicrographs of transverse sections through the forebrain and part of the midbrain of juvenile *X. laevis* showing the distribution of UCN3-ir fibers. **A**, **D**, **E**: 100x magnification; **B**, **C**: 200x magnification; **F**: 400x magnification. The immunohistochemistry was conducted using a Vectastain elite ABC kit (rabbit) and Vector VIP kit (both from Vector Laboratories, Inc., Burlingame, CA) for HRP/DAB staining following the manufacturer's protocols. The affinity-purified anti-*Xenopus* UCN3 antiserum was diluted 1:5 for incubation of the sections. See Table 2.1 for a complete description of abbreviations.