Neurobiology of Stress: Bridging the Genotype-Phenotype Gap

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Abstract

The biological response to stress is concerned with the maintenance of homeostasis in the presence of real or perceived challenges. This process requires numerous adaptive responses involving changes in the central nervous and neuroendocrine systems. When a situation is perceived as stressful, the brain activates many neuronal circuits linking centers involved in sensory, motor, autonomic, neuroendocrine, cognitive, and emotional functions in order to adapt to the demand. However, the details of the pathways by which the brain translates stressful stimuli into the final, integrated biological response are presently incompletely understood. Nevertheless, it is clear that dysregulation of these physiological responses to stress can have severe psychological and physiological consequences, and there is much evidence to suggest that inappropriate regulation, disproportional intensity, or chronic and/or irreversible activation of the stress response is linked to the etiology and pathophysiology of anxiety, depression and metabolic disorders.

Understanding the neurobiology of stress by focusing on the brain circuits and genes, which are associated with, or altered by, the stress response will provide important insights into the brain mechanisms by which stress affects psychological and physiological disorders. We are employing integrated molecular, biochemical, physiological and behavioral methods, focusing on the generation of mice genetic models as an *in vivo* tool, in order to study the central pathways and molecular mechanisms mediating the stress response. Defining the contributions of known and novel gene products to the maintenance of stress-linked homeostasis may improve our ability to design therapeutic interventions for, and thus manage, stress-related disorders.

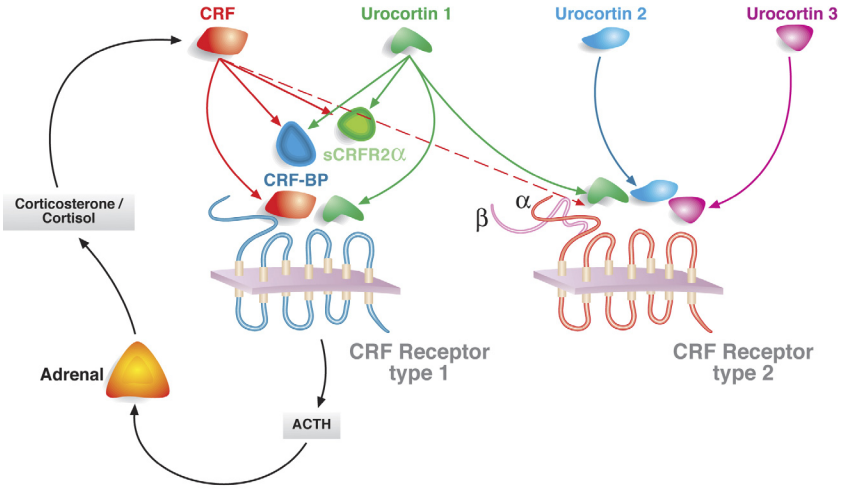

Fig. 1 *Schematic representation of the mammalian corticotropin releasing factor (CRF)/ Urocortin family of peptides, receptors and binding proteins. Colored arrows indicate relative receptor affinities.*

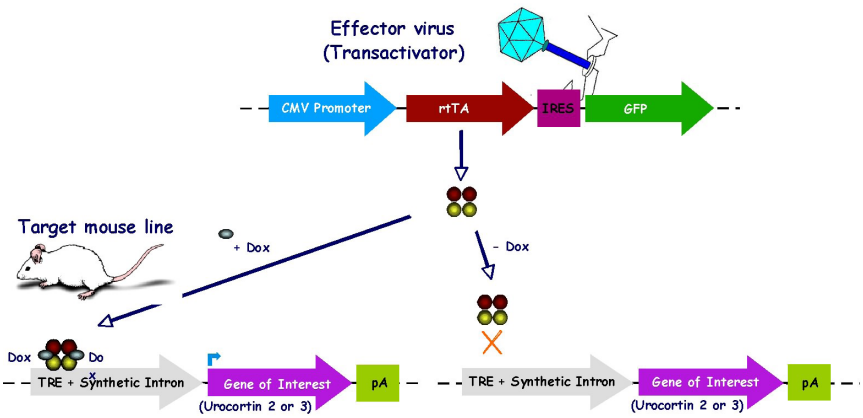

Fig. 2 *Lentiviral-based system for site-specific and inducible over-expression of Urocortin 2 or Urocortin 3 using the Tet-On system in transgenic mice. rtTA, reverse chimeric transactivator; TRE, tetracycline responsive element; DOX, doxycycline.*

Scientific background

The biological system that has been most closely linked to the stress response in mammals is the neuroendocrine limbic-hypotalamic-pituitary-adrenal (LHPA) axis. Perception of physical or psychological stress by an organism is followed by a series of events, which result in changes in emotional and cognitive functions, modulation of autonomic activities and the secretion of glucocorticoids from the adrenal cortex. Both activation and termination of the behavioral, autonomic and adrenocortical stress responses are critical for adaptation and survival. The neuropeptide corticotropin releasing factor (CRF), expressed and secreted from the parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus, represent the final common path for the integration of the neuroendocrine stress response in the brain. CRF and, to a lesser extent, arginine vasopressin playing an important and well-established role in the regulation of the HPA axis

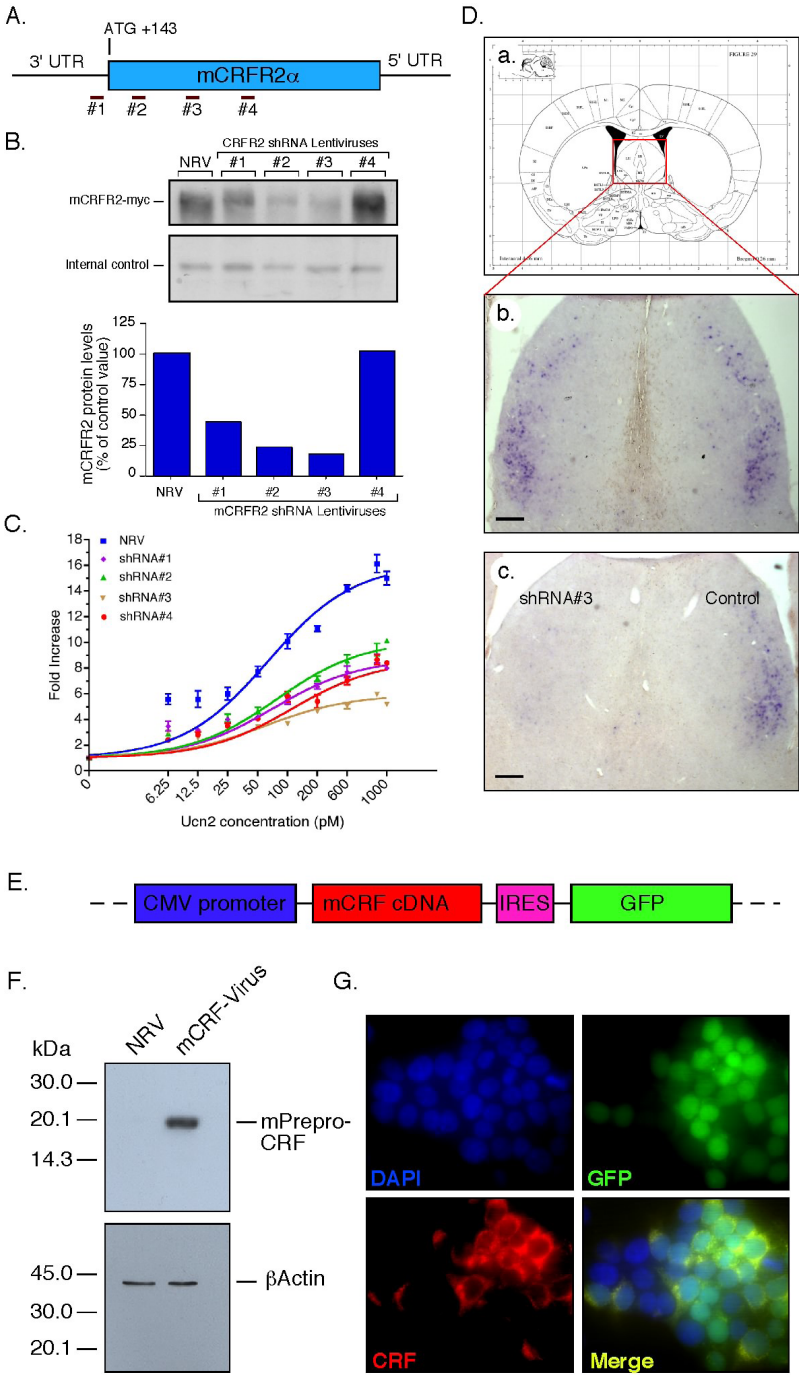

Fig. 3 *Construct design and in vitro and in vivo validation of lentiviral constructs for CRFR2 knockdown and CRF over-expression. A and E, Construct design and diagrammatic representation of mouse CRFR2 (A) and CRF (E) lentiviral constructs. B and F, In vitro validation of mouse CRFR2-siRNA (B) and CRF over-expression (F) lentiviral constructs. Confirmation of silencing of mouse CRFR2 (B) or over-expression of mouse CRF (F) by western blot analysis. C, Inhibition of cAMP signaling mediated by CRFR2 activation in cells infected with or without siCRFR2 lentiviruses. D, Site-specific delivery of siCRFR2 into the lateral septum. Schematic representation of site of delivery (Da); Representative in situ hybridization images of coronal brain sections of control (Db) or injected (Dc) mouse brain. G, HEK293T cells infected with mCRF over-expressing lentiviruses and confirmation of CRF over-expression performed using immunocytochemistry.*

under basal and stress conditions. In addition to its hypophysiotropic action, CRF is proposed to integrate the autonomic, metabolic and behavioral responses to stressors. CRF and its receptors are implicated in the control of arousal, anxiety, cognitive functions and appetite, however, the brain circuits and downstream signals responsible for these CRF-linked stress related responses are less understood. Dysregulation of the stress response can have severe psychological and physiological consequences. Chronic hyperactivation of the CRF system has been linked to stress-related emotional disorders such as anxiety, anorexia nervosa and depression. In addition to CRF, the mammalian CRF-peptide family contains Urocortin 1, and the more recently identified peptides, Urocortin 2 and Urocortin 3 (figure 1). The effects of CRF-related peptides are mediated through activation of two known receptors, CRF receptor type 1 (CRFR1) and CRFR2 (figure 1). CRFR1 mRNA is widely expressed in mammalian brain and pituitary, with high levels in the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulb. CRFR2 has two apparent membrane bound splice variants, which results in two putative receptor proteins of 411 and 431 amino acids (CRFR2α and CRFR2β, respectively) (figure 1). In rodents, CRFR2α is predominantly expressed in the brain in a discrete pattern with highest densities in the lateral septal nucleus, bed nucleus of the stria terminalis, ventromedial hypothalamic nucleus, olfactory bulb, mesencephalic raphe nuclei and medial amygdala. The CRFR2β splice form is expressed primarily in peripheral tissues, with the highest levels of expression in the skeletal muscle and heart, the choroids plexus of the brain and the gastrointestinal tract. Receptor binding and intracellular cAMP accumulation studies have demonstrated that CRFR1 and CRFR2 differ pharmacologically. CRF has relatively lower affinity for CRFR2 compared to its affinity for CRFR1, Urocortin 1 has equal affinities for both receptors, and Urocortin 2 and Urocortin 3 appear to be selective

for CRFR2 (figure 1). The existence of the CRF-binding protein (CRF-BP) and the recently identified soluble splice variant of CRFR2α (sCRFR2α), both of which bind CRF and Urocortin 1 with high affinity, add a further level of complexity to the control of the action of these ligands (figure 1).

Research objectives & expected significance

The long-term goal of our research is to elucidate the pathways by which stress is perceived, processed, and transduced into neuroendocrine and behavioral responses. We are studying the CRF/Urocortin family of peptides and receptors as the research model system and examining the hypothesis that this family plays important roles in the modulation of neuroendocrine and behavioral responses to challenge. Using genetic manipulation *in vivo* we are currently generating different transgenic mice and brain nuclei specific knockdown or over-expressing mice models. Molecular, biochemical and morphological analysis of these mice will be followed by stress-linked physiological and behavioral studies. Combining the data obtained from these studies with a biochemical neuroanatomy analysis and DNA and microRNA expression array studies will provide a better understanding of the specific circuitry and genes that involved in initiation or termination the stress response.

The CRF/Urocortin family of ligands and cognate receptors is of seminal importance to three overlapping fields: neuroendocrinology, stress biology and regulatory peptides. Specifying the contributions of the CRF/Urocortin family of ligands and receptors to the maintenance of homeostasis and to stress-linked allostasis may improve our ability to design therapeutic interventions and thus manage affective and other stress-related disorders.

Methodology

We are using integrated molecular, biochemical, physiological and behavioral methods, focusing on the generation of mice models as an *in vivo* tool, in order to study the physiological roles of CRF/Urocortin neuropeptides, acting at their specific receptors, in coordinating the neuroendocrine and behavioral responses to stress. Genetic manipulation of CRF/Urocortin receptors and ligands expression in the whole animal context will permit us to examine their role in both behavioral and physiological functions. In order to explore the physiological roles of CRFR1 and CRFR2-dependent pathways in the maintenance of homeostasis and stress-linked allostasis, we are manipulating both the levels and the site of expression of different family members within key CNS regions and are studying the specific neuronal circuits mediating their effects by generating the following models and subsequently analyzing their neuroendocrine and behavioral stress-related phenotypes:

- Inducible over-expression of the CRF/Urocortin neuropeptide family, within specific endogenously expressing brain nuclei, using the Tet-On system in transgenic mice (figure 2).
- Site-specific knockdown of CRF/Urocortin family of neuropeptides and receptors, using small interfering RNA expressing lentiviruses, in adult mice (for example see figure 3A-D).
- Site-specific over-expression of CRF/Urocortin family of neuropeptides and receptors, using lentiviruses in adult mice (for example see figure 3E-G).
- "Rescue studies" - Delivery of neuropeptides and receptors expressing lentiviruses to specific brain nuclei of the respective-null mice (available in our laboratory).
- The use of Light-gated ion channels to study the neural substrates of stress in adult mice.

In parallel for generating and phenotyping the above mice models; we are using biochemical neuroanatomy studies to explore the involvement of CRF/Urocortin pathways in monoaminergic circuitries. Furthermore, we are exploring the molecular mechanisms of central CRF receptor subtypes, express in distinct brain nuclei, by identifying genes and pathways downstream of the CRF receptors. We are dissecting specific brain nuclei from different mice models or following specific stress-related paradigm and determine the gene expression and microRNA profiles using microarray technology and bioinformatics analysis. Confirmation of candidate genes or microRNAs, using real-time PCR, in-situ hybridization, immunohistochemistry or western blot, is followed by *in vivo* functional analysis using mouse genetics and/or direct siRNA-lentiviral approach.

Urocortins: Emerging metabolic and energy homeostasis perspectives

Maintaining energy homeostasis in the presence of diverse challenges, such as starvation, exercise or high fat diet, requires numerous adaptive responses in both central and peripheral tissues. Recent studies have clearly demonstrated that Urocortin 2 and Urocortin 3, acting through their specific type 2 CRF receptor, can serve as autocrine and/or paracrine regulators of glucose homeostasis by modulating insulin sensitivity in skeletal muscle or by regulating glucose-induced insulin secretion in the beta cells of the pancreas, respectively (figure 4). The anatomical distribution of Urocortin 2, Urocortin 3 and CRF2 within peripheral and central tissues key to regulation of energy homeostasis, together with the robust metabolic phenotypes of mice deficient for these factors leave them poised as major new players in this field (figure 4). The CRF/Urocortin family of peptides and receptors are not only structurally and pharmacologically related, but share additional common denominators, such as their regulation by glucocorticoids, supporting the concept that they are likely to operate in concert as a single functional system. Further insights into the detailed physiology of this system will be aided by the generation of tissue-specific Urocortin 2, Urocortin 3 and CRFR2 transgenic animal models for manipulation of expression levels of the receptor or ligands. Study of such models will facilitate our understanding of the specific roles of central or peripheral CRFR2 in modulating metabolic functions. Likewise, understanding of the detailed regulation of central and peripheral CRFR2 and Urocortins under

different physiological conditions (basal or challenged) will also contribute to elucidating the cellular and molecular mechanisms mediating their effects. The novel functions for CRFR2 and its ligands Urocortin 2 and Urocrtin-3 as local regulators of glucose uptake not only add to out current understanding of the physiology of energy metabolism, but are of potential interest as therapeutic targets for the management of type 2 diabetes and other metabolic disorders.

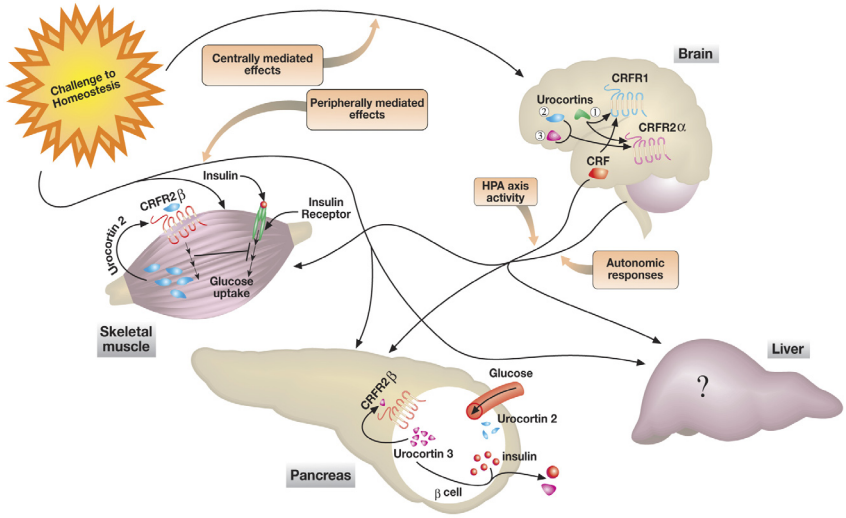

Figure 4. *Schematic representation summarizing the proposed roles of central and peripheral CRF/Urocortin peptides and receptors in modulating glucose homeostasis. Following stressful stimuli, glucocorticoid exposure resulting from HPA axis activation by hypothalamic CRF, and changes in autonomic activity will modulate skeletal muscle, pancreatic and hepatic functions. CRF and Urocortins, acting via both type 1 and type 2 CRF receptors in the brain, will modulate food intake and glucose homeostasis. Peripherally, Urocortin 2 produced in skeletal muscle and acting locally at CRFR2 will regulate glucose uptake in skeletal muscle by inhibiting insulin signaling. Urocortin 3, produced by the pancreatic β-cells, regulates high glucose-induced insulin secretion.*

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