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Molecular Genetic Approaches to the Neuropharmacology of Corticotropin-Releasing Factor

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This article describes the integration of neurobiological and molecular genetic approaches in the study of the function of the neuropeptide corticotropin-releasing factor (CRF). CRF is a particularly relevant subject for targeted genetic mutation because of its hypothesized critical role in hormonal and behavioral responses to stressors, its potential role in psychopathology, and the limited neuropharmacological agents available to increase or decrease function in this system. We review the strategy of targeted genetic mutation in neuropharmacology using research directed at understanding the function of CRF and related neuropeptides through molecular genetic manipulation of the levels of the endogenous agonist, its different receptors, and the binding protein. Genetically engineered approaches to functional analysis cross-validate experiments to promote the neurobiological elucidation of complex systems and provide critical information about constituent differences that contribute to individual differences in brain function.

The generation of genetically engineered animals has become a broadly used method in the past 15 years, used first by immunologists and endocrinologists. The behavioral phenotypes of these mutants often were missed because their discovery and detection would have required sophisticated measures which were unavailable at the time. We are now amid a turning point where the behavioral phenotyping of mutant animals has come to the foreground even when the molecular geneticist has manipulated genes that are not themselves expressed in the brain. This chapter describes the purposeful meeting of neurobiology and molecular genetics in studying one of the best known neuropeptide modulators of behavior, the brain peptide corticotropin-releasing factor (CRF). CRF is a large neuropeptide and its production can be eminently regulated by methods of molecular genetics, while medicinal chemists have thus far failed to make really effective, systemically active, high-affinity selective agonists and antagonists (even if the latter are forthcoming). The CRF receptors 1 and 2 (CRF_1 and CRF_2), are large seven transmembrane G-protein-coupled receptors which are ideal targets for molecular genetic studies. The CRF system also contains a large, high affinity gene product CRF binding protein (BP) that affects levels of the free ligand. CRF-BP can be genetically modified with respect to its levels and its affinity for CRF.

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Genetic Mutation Strategies

Classic approaches to understanding the function of a signal substance or neuronal projection in neuropharmacology include manipulation of neurotransmitter function by the administration of direct or indirect agonists, or antagonists or chemically selective lesions. In addition, neuropharmacology has employed direct measurement of extracellular levels of neurotransmitters during functional states using *in vivo* microdialysis or *in vivo* voltametry. Genetic manipulations historically involved selective breeding or breeding/cross-breeding of inbred strains for genetic analysis. The explosion of research in molecular biology led to the development of novel approaches, largely in mice, of functional strategies employing the manipulation of specific genes. Such genetic manipulations have different goals, including understanding gene function (inducible and tissue-specific are desirable), mimicry of human chronic conditions (classic knockout or transgenic are desirable), and understanding the role of molecular pathways and secondary gene expression changes (inducible overexpressing is desirable). *Transgenic* mice have an extra gene introduced into their germline. An additional copy of a normal gene is inserted into the genome of the mouse to examine the effects of overexpression of the product of that gene. Alternatively, a new gene, not normally found in the mouse, can be added, such as a gene associated with a specific pathology in humans. *Knockout* mice have a gene inactivated by homologous recombination. A knockout mouse deficient in both alleles of a gene is *homozygous* for the deletion and is termed a *null* mutation (-/-). A mouse which is deficient in only one of the two alleles for the gene is termed a *heterozygote* (+/-). *Wildtype* controls are animals bred through the same breeding strategies involving mice that received the transgene injected into the fertilized egg (transgenics) or a targeted gene construct injected into the genome via embryonic stem cells (knockout) but lacking the mutation on either allele of the gene in question.

The purpose of this article is to review the strategy of targeted genetic mutation in neuropharmacology using as an example the research directed at understanding the function of CRF and related peptides in the brain. CRF is a particularly germane subject for targeted genetic mutation because of 1) its hypothesized critical role in hormonal and behavioral responses to stressors, 2) its potential role in psychopathology, and 3) the limited neuropharmacological agents available to increase or decrease function in this system (Figure 1).

Transgenic Approaches

Transgenic approaches involve the introduction of foreign genes into the germline with the successful expression of the inserted gene (Crawley, 2000; Gold, 1996). This requires ultimately that a functional gene will undergo the necessary transcriptional and translational processing to produce a gene product. The introduction of a foreign gene requires the construction of a transgene. The transgene is made up of complementary DNA (cDNA) or a complete genomic sequence (native DNA) and a carefully selected promoter sequence that allows the initiation of transcription to occur at the coding region of the inserted gene. The transgene DNA fragment is microinjected into the nuclei of the oocytes of donor mice. The

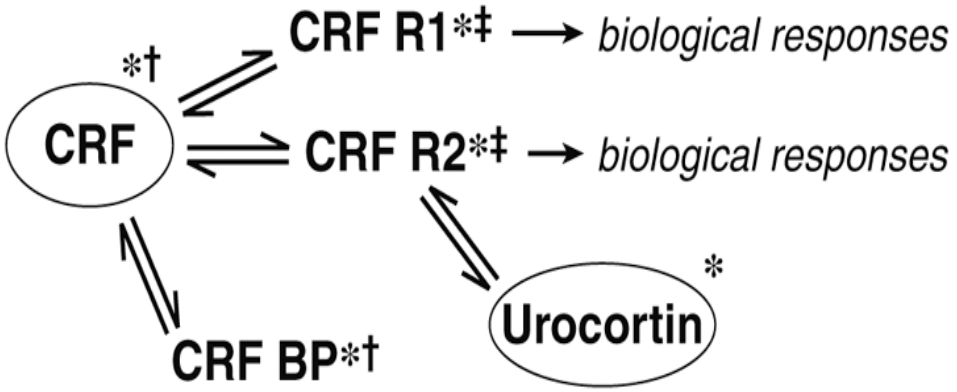

Figure 1. Schematic of presently available manipulations of the CRF system. * knock-out. + overexpression. ‡ non-peptide, systemically acting antagonist available.

microinjected oocytes then are implanted into the oviducts of normal, healthy, pseudopregnant females. The fusion gene product becomes integrated into the genome randomly, although they may be targeted to a particular cell type using cell-specific promoters. Progeny are screened by analysis of their DNA. The mouse that develops from each microinjected egg is termed the founder of the mutant line. The DNA integration site in each founder will be different; however, all transgenic mice that descend from a single founder will share the same transgene locus. Heterozygotes then are mated and the resultant offspring are homozygous transgenics, heterozygotes, and wildtype littermate controls.

The regional cell type distribution of the transgene in the brain depends on the promoter employed, the strength of the employed promoter, as well as the site of chromosomal integration. More directed overexpression can be produced by using tissue-specific promoters that will allow only expression in certain cell types and perhaps in certain brain areas. For example, targeting to neurons can be accomplished by the use of neuron-specific enolase, and targeting to astrocytes can be accomplished using glial fibrillary acidic protein (Campbell & Gold, 1996). The experimental objective and nature of the protein dictates the choice of which promoter construct to use. Even with the use of cell-specific promoters, the site of integration will vary between different founder mice. This variation may lead to expression of the transgene from zero to very high levels or even may result in the disruption of an endogenous gene by incorporation into its coding region or by incorporation into regions that exert regulatory control over the activity of the endogenous gene. While these results of the random integration process may be viewed as problematic, phenotypic analysis of independent transgenic lines for a given transgene (the recommended approach) actually can be advantageous. For example, transgene dose-effect relationships can be established. In a related vein, low-expressing lines may permit the examination of expression of highly active or biologically potent molecules, whereas high expression would be toxic or lethal. Examination of different transgenic lines also is important for confirming that a phenotype is due to

transgene expression and not due to some other non-specific mechanism such as integration site-induced mutation or toxicity toward the transgene-expressing cells.

Overexpression of a gene product under a heterologous promoter will direct the gene product to a specific set of cells. In the case of neuropeptide hormones such as CRF, several specific issues arise with overexpression. When rat CRF cDNA is overexpressed instead of mouse CRF cDNA then one may or may not have expressed an alternative ligand. For example, it has been shown that rat and mouse CRF differ slightly (Seasholtz et al., 1991). Therefore, one has to ask the following questions: (1) Does the agonist prefer any of the receptor subtypes in particular? (2) Does it act as a full agonist? (3) Is it degraded at the same rate as mouse CRF?

Another important aspect with regard to CRF is the choice of the promoter. Generally, neuronal forebrain expression can be driven by neuronal-specific enolase or by a platelet-derived growth factor-ß prohibitor. To direct CRF expression only into catecholaminergic cells, one could use tyrosine hydroxylase promoter (and into cholinergic cells, chloramphenicol acetyl transferase promoter). A question that remains, however, is whether these cells can process the preprohormone gene product proteolytically to produce mature CRF, and whether they store and release CRF in large, dense core vesicles. Overexpression, when used carefully, thus gives a biological tool for targeted agonist presentation in a neuropeptide system.

Gene Targeting Mutations

With targeted gene mutation a specific gene is deleted (or inactivated or up- or downregulated) to produce a knockout mouse. To accomplish this, specified changes in the nucleotide sequence are introduced into a coding region of the chosen gene often through insertion of a piece of foreign DNA into a portion of an exon critical in the expression of the gene. Ultimately, the goal is to have this new mutated portion of the gene replace the endogenous gene in an embryonic stem (ES) cell through a process called *homologous recombination* (Figure 2). This is a rare event whereby regions of identical DNA sequence line up next to each other, are cut, and then spliced at the cut ends. In order to be able to select cells that have incorporated the targeted gene construct into the genome, marker genes are added to the construct. An antibiotic-resistant gene such as a neomycin resistance tag gene (*neoR*) is inserted onto the targeted gene. This *neoR* marker gene conveys resistance to the antibiotic drug neomycin; therefore, any ES cell that has incorporated the mutation into its genome will survive exposure to neomycin. To ensure that the gene has been incorporated by homologous recombination (replacing the endogenous gene as opposed to random integration), a second marker (typically the herpes simplex virus thymidine kinase [HSV-*tk*] gene) is placed just downstream of the region of homology. If the vector is inserted by homologous recombination then the HSV-*tk* gene will be excluded. The HSV-*tk* marker gene conveys sensitivity to the antiviral drug ganciclovir; therefore, ES cells that have incorporated the mutation in a manner that excludes this marker gene will survive exposure to ganciclovir. ES cells positive for *neoR* and negative for HSV-*tk* are selected as candidates likely to contain the mutated gene in their genome.

Historically, ES cells have been derived from the 129 inbred mouse strain because they are likely to remain viable throughout the process of gene transfer.

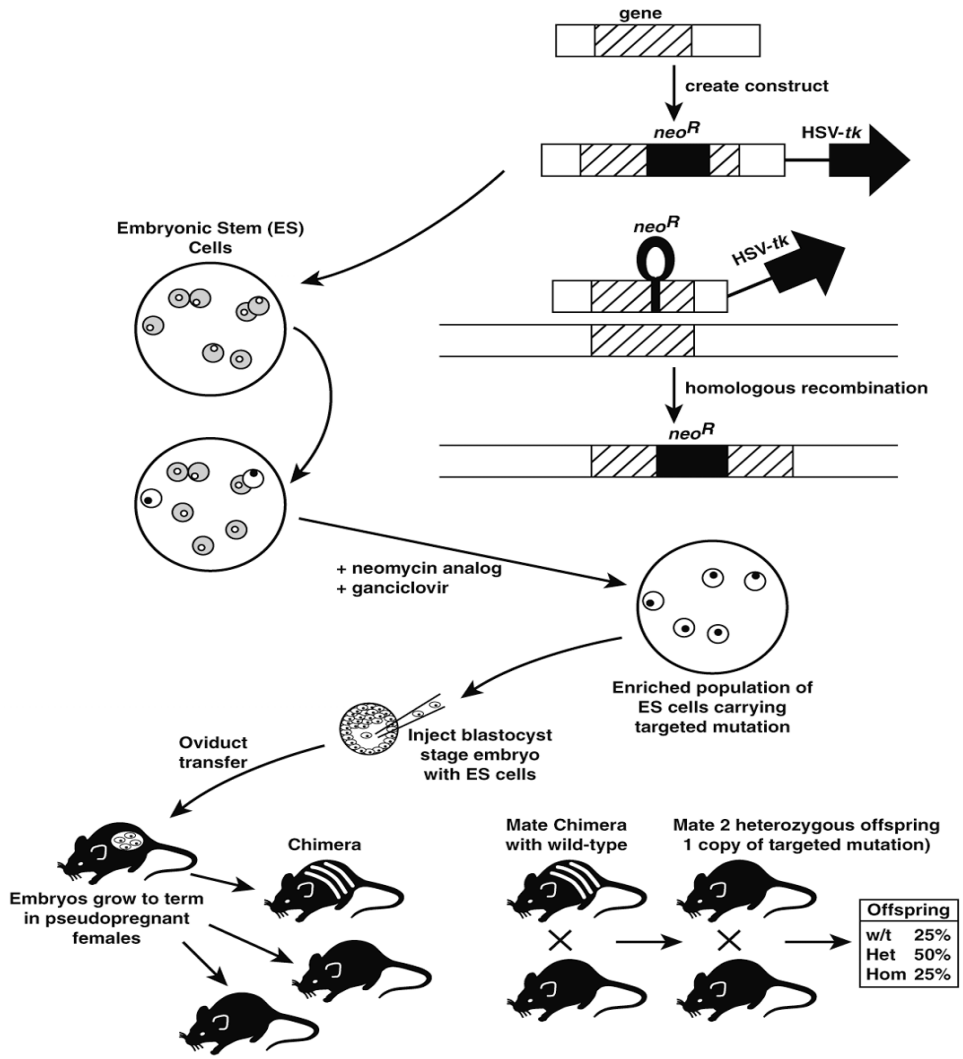

Figure 2. Generation of knockout mouse mutants by targeted gene replacement. A targeting vector is constructed in which a neomycin resistance (*neoR*) gene is inserted into a protein coding region of the gene of interest. Attached to one end of the targeting vector is a thymidine kinase (*tk*) gene from a herpes simplex virus that will serve as a negative selectable marker. The vectors are introduced into embryonic stem (ES) cells where they line up with the chromosomes allowing exchange of identical regions of the chromosomes and the targeting vector, termed homologous recombination. Homologous recombination results in chromosomes that possess the targeted insertion, and thus are resistant to neomycin analogs. Also associated with homologous recombination is a loss of the terminal *tk* gene, thus eliminating the susceptibility to ganciclovir. Taking advantage of these positive and negative selectable markers by treatment of the ES cells with a neomycin analog and ganciclovir eliminates those cells that do not possess a *neoR* gene and those that retain the *tk* gene. The ES cell population is thus enriched for cells carrying the targeted mutation. These cells then are injected into a blastocyst stage embryo derived from a second mouse strain. Chimeric male mice bearing cells from both mouse strains then are bred with wildtype mice. The heterozygous offspring, possessing one copy of the mutated gene, are mated. The genotype of the progeny is determined by analysis of tail DNA to be either wildtype (w/t), heterozygous (Het) or homozygous (Hom) for the targeted mutation. From Gold (1996). Copyright by Lippincott Williams & Wilkins, reprinted by permission.

The ES cells are transfected with the vector usually by electroporation, whereby the passage of electrical current makes transient pores in the cell membrane through which the construct enters and then diffuses through the cytoplasm to the nucleus. As mentioned above, a very small percentage of ES cells incorporate the vector and an even smaller percentage incorporate the vector in the correct position in the genome (through homologous recombination). At this time the cells are exposed to neomycin and ganciclovir, and survivors are harvested.

Candidate ES cells are grown into colonies in tissue culture. The cells are microinjected into the inner cavity of the blastocyst (usually of the C57BL/6 mouse strain) and then are implanted into pseudopregnant female recipients (again, usually of the C57BL/6 mouse strain). If both the blastocyst and the ES cells grow successfully, a chimeric pup results which is comprised of cells from these two genetic sources (e.g., the 129 embryonic stem cells and the C57BL/6 blastula cells). Because the 129 and C57BL/6 mouse strains have different coat colors (grayish brown and black), successful chimeras typically will have coats with patches of both colors. Further breeding is used to determine whether the targeted gene construct has been incorporated into germline gametes, making the mutation heritable. Positive heterozygous F1 (or first filial generation) offspring are mated with each other to produce the F2 generation, which should be comprised of mice homozygous for the mutation (25%), homozygous for the wildtype gene (25%), and heterozygous for the mutation (50%). Progeny are screened for evidence of the targeted mutation using genotyping of tail DNA. Various breeding strategies can be employed to obtain mice for experimentation. Using a similar approach, a gene can be added (for example, under the control of the endogenous regulatory sequences of a different gene). This manipulation has been termed a "knockin" strategy.

Two important issues regarding both the transgenic and targeted gene mutation approaches are relevant to this review. The first involves the issue of the background genetic makeup of the mouse carrying the mutation. Complex interactions occur between the background gene alleles and the targeted locus, and it has been shown that the phenotypic expression of several mutations is widely divergent when the same mutation is maintained on different strain backgrounds (Crawley, 2000). Most knockout mice are produced on a mixed C57BL/6 x 129 genetic background. As discussed later in this review, it is likely that the choice of a different substrain of 129 mice for ES cells may explain at least a portion of the differences found between CRF receptor knockout mice produced by different laboratories. One approach being used by investigators wishing to standardize the knockout procedure is to backcross the mutation onto a pure mouse strain background, producing a congenic strain. Wildtype and knockout mice would be identical except for the mutation, and presumably knockout mice created by independent groups would be more comparable. The C57BL/6 mouse seems to be the most popular choice for creating congenic strains, but it is clear that examining the effects of a mutation on more than one background strain will be important to definitively assess the effects of deleting or adding a particular gene product.

Another issue related to the genetic strategies discussed herein is that these mutations are constitutively expressed. In other words, the genetically engineered changes are present throughout the life of the animal, and expression follows the particular characteristics of the promoter used. There has been considerable argument regarding the possible induction of compensatory pathways or optimization of

redundant mechanisms in the conventional mutation strategies. All currently available CRF system mutants fall into this category. Two major systems currently are being used that allow spatiotemporal control of the expression of proteins or gene mutations (Jaisser, 2000) and these will likely be used in further investigations of CRF function. To use the tetracycline-inducible and Cre/*lox* systems it is necessary to generate two sets of mutant mice. One mouse line is produced that expresses the activator (tetracycline-controlled transactivator [tTA], reverse tTA or Cre recombinase) under the control of a selected tissue-specific promoter. Another mouse line is produced that expresses the transgene or modified gene which has been constructed to be under the control of either the target sequence for the tTA/rtTA transactivators or is flanked by *lox*P sequences. When these mice are mated the transgene or desired gene alteration can be controlled in offspring by the addition of doxycycline to the food or drinking water (tetracycline-inducible system) or exposure to non-naturally occurring steroids (Cre/*lox* system).

Corticotropin-Releasing Factor

The neuropeptide corticotropin-releasing factor (CRF) has a central role in the response of the body to stressors. Stressors are various external and internal challenges to the body and brain, and the construct of *stress* may represent the pathological continuum of overactivation of the normal activational (arousal) or emotional systems of the body (Hennessy & Levine, 1979). Historically, a state of stress has been defined biologically by various physiological changes that include activation of the pituitary-adrenal axis and release of glucocorticoids into the bloodstream, and activation of the sympathetic nervous system. The activation of the hypothalamic pituitary-adrenal axis by stress long has been hypothesized to involve the action of CRF liberated from the hypothalamus into the pituitary portal system of the median eminence to trigger the release of adrenocorticotropic hormone (ACTH) from the pituitary. CRF, via a neurotropic action in the pons and brainstem, also activates the sympathetic nervous system (Fisher, 1993; Valentino et al., 1993). Another major component of the response to bodily demands or challenges to homeostasis (e.g., stressors) are behavioral changes that may be at least initially adaptive, and these changes most likely involve extrahypothalamic CRF systems (Koob et al., 1993). Evidence demonstrating a neurotropic role for CRF in the central nervous system outside the pituitary-adrenal axis suggests a means for mediating behavioral responses to stressors and a contribution to the behavioral state of stress in addition to the classic activation of adrenal steroids and the sympathetic nervous system.

Substantial neurobiological evidence supports the hypothesis of a neurotropic role for CRF in the central nervous system. Immunoreactivity for CRF has been localized in the central nervous system both in the hypothalamus and in extrahypothalamic structures (Bloom et al., 1982; Swanson et al., 1983). CRF-stained cells and fibers are found in high concentrations in the central nucleus of the amygdala, parabrachial area and the substantia innominata, bed nucleus of the stria terminalis, locus coeruleus, and olfactory bulb.

Urocortin, another CRF-related neuropeptide that occurs in the mammalian brain, was discovered in 1995 (Vaughan et al., 1995) and is structurally and pharmacologically similar to members of the CRF family of peptides (including CRF,

sauvagine [a frog peptide], and urotensin I). Substantial neurobiological evidence points to a neurotropic role for urocortin in the central nervous system. High levels of urocortin mRNA have been observed in the Edinger-Westphal nucleus and the lateral superior olive, and urocortin mRNA has been detected in several somatomotor nuclei, a subset of magnocellular neurons in the supraoptic nucleus and, caudally, in the lateral hypothalamus. Projections of urocortin immunoreactivity are observed throughout the brain and spinal cord, the lateral septum, the supraoptic nucleus and paraventricular nucleus, the central and periaqueductal gray, and the Edinger-Westphal nucleus (Kozicz et al., 1998). In contrast to CRF, levels of urocortin immunoreactivity are not high in the median eminence, suggesting that urocortin is not an important endocrine factor in the regulation of ACTH or ß-endorphin release (Kozicz et al., 1998).

More recently, two additional urocortin-like peptides have been identified in the mammalian brain. Mouse urocortin II (mUcnII) is a 38 amino acid neuropeptide related to the CRF family. Human urocortin II shares a 76% amino acid identity with mUcnII and is proposed to be the human analog of mUcnII (Reyes et al., 2001). Interestingly, both mouse and human Ucn II are 1000-fold more selective in binding to the CRF2 receptor compared to the CRF1 receptor, and equipotent at binding to the CRF2 receptor. A third urocortin-like peptide, urocortin III, also was characterized in mouse and human and also is a 38 amino acid peptide (Lewis et al., 2001). In addition, stresscopin-related peptide and stresscopin have been characterized by another group and appear to be virtually identical to Ucn II and Ucn III, respectively (Hsu & Hsieh, 2001).

Multiple CRF receptors have been cloned, and to date there are two major receptor types, CRF1 and CRF2. Both are seven transmembrane domain-containing G-protein-coupled plasma membrane-bound receptors, and the distributions of CRF neurons, the CRF binding sites, and *in situ* hybridization of CRF receptor mRNA (Perrin et al., 1993) show good correspondence (De Souza, 1987). CRF1 receptors are expressed mainly in the medial septum, pituitary, cortex, cerebellum, hindbrain, and olfactory bulb, whereas CRF2 receptors are found in the lateral septum, ventral medial hypothalamus, and choroid plexus (Chalmers et al., 1995; Perrin et al., 1995). CRF and urocortin both bind with high affinity to the CRF1 receptor, but only urocortin and other urocortin-related peptides bind with high affinity to the CRF2 receptor leading to the hypothesis that urocortin may be an endogenous ligand for the CRF2 receptor (Vaughan et al., 1995). The CRF binding protein represents a third protein other than CRF1 and CRF2 that has high affinity for CRF and thus may affect functional levels of this signal substance (Seasholtz et al., 2001).

Behavioral Effects of CRF and Urocortin

Administration of CRF and urocortin into the central nervous system intracerebroventricularly, or intracerebrally at specific brain sites, produces a wide variety of behavioral effects that initially led to the hypothesis that these peptides were involved in behavioral responses to stressors. The behavioral pharmacological profile resulting from exogenous administration of these neuropeptides depends on the baseline state of arousal and stress of the animal (Table 1; for reviews see Aldenhoff et al., 1983; Baldwin et al., 1990; Dunn & Berridge, 1990).

Table 1
Behavioral Effects of Centrally Administered CRF Agonist and Antagonist Peptides.

CRF Receptor Agonist	Paradigm	CRF Receptor Antagonist
• Suppresses exploration of unfamiliar environment	Elevated plus-maze	• Reverses stress-, drug-, and genotypically induced suppression of exploration
• Facilitates startle	Acoustic startle	• Blocks fear-potentiated startle
• Induces conditioned fear	Conditioned emotional response	• Blocks acquisition of conditioned emotional response
• Enhances stress-induced freezing	Cued electric shock	• Attenuates stress-induced freezing
• Decreases food intake	Deprivation-induced eating	• Reverses stress- and drug-induced anorexia
• Produces aversion	Taste/Place conditioning	• Weakens drug-induced place aversion
• Enhances sensitization	Amphetamine stereotypy	• Attenuates stress-induced sensitization
• Enhances defensive burying	Shock-probe	• Reduces defensive burying

In nonstressed animals under low arousal conditions, CRF administered intracerebroventricularly produces dose-dependent behavioral activation that includes increases in locomotor activity, rearing and grooming when rats are tested in a familiar environment (Dunn & Berridge, 1990; Koob et al., 1984; Sherman & Kalin, 1987; Sutton et al., 1982). This activation is not observed following systemic administration of CRF and is not blocked by hypophysectomy or pretreatment with dexamethasone, suggesting that this effect of CRF is mediated by actions in the central nervous system independent of the pituitary-adrenal axis (Britton et al., 1986; Eaves et al., 1985). Urocortin has less activating effects than CRF except at higher doses, and both urocortin II and III appear to have little or no behavioral-activating effects in the rat (Valdez et al., in press).

The arousal and activating effects of CRF extend to a facilitation of measures of learning and memory, enhancing retention at low doses and impairing performance at higher doses (Koob & Bloom, 1985). Recent studies have shown that the CRF-induced facilitation of memory may be mediated by CRF_1 receptors (Radulovic et al., 1999). Injection of CRF into the hippocampus enhanced learning and this was blocked by a CRF_1, but not a CRF_2, receptor antagonist. Urocortin also is similarly potent to CRF in its memory-modulating effects, facilitating performance in tests of learning and memory, such as passive avoidance and the Morris water maze, at low doses, but decreasing performance at high doses (Zorrilla et al., 2002).

The profile of the behavioral effects of exogenously administered CRF and urocortin changes when animals are exposed to a more stressful environment to reflect an enhanced behavioral response to stressors. The same intracerebroventricular doses that produce marked behavioral activation in a familiar environment produce behavioral suppression in a novel, presumably stressful environment. Rodents pretreated with CRF show decreases in behavior in an open-field (Sutton et al., 1982; Takahashi et al., 1989; with or without food: Britton et al., 1982), decreased exploration in a multi-compartment chamber (Berridge & Dunn, 1986), and decreased exploration in an elevated plus-maze (Baldwin et al., 1991). Evidence that this anx-

iogenic-like effect is mediated by CRF_1 receptors has been obtained by differential antistress effects of CRF_1 antisense oligonucleotides versus CRF_2 antisense oligonucleotides (Liebsch et al., 1999). Urocortin also shares the anxiogenic-like properties of CRF, a putative CRF1-mediated effect, as shown by behavior in several paradigms, including the open-field, the elevated plus-maze, and the light-dark transfer test (Jones et al., 1998; Moreau et al., 1997), although in the rat urocortin II and III do not (Valdez et al., 2002; Zorrilla et al., 2001). The behavioral suppression observed in these exploration tests is supported by other studies showing that CRF and urocortin have "anxiogenic-like" or stress-like effects. CRF enhances the acoustic startle response (Swerdlow et al., 1986), increases conditioned fear in a conditioned suppression test (Cole & Koob, 1988), and enhances stress-induced freezing behavior (Sherman & Kalin, 1988). CRF administration produces decreases in food intake (Arase et al., 1988; De Pedro et al., 1993; Krahn et al., 1986; Rosenthal & Morley, 1989), decreases in alcohol intake (Bell et al., 1998), decreases in sexual behavior (Sirinathsinghji, 1986; Sirinathsinghji et al., 1983), and increases in defensive burying in habituated rats (Diamant et al., 1992; see Table 1). The stress-like effects of CRF clearly have aversive properties in that CRF at high doses can produce both taste aversions and place aversions (Cador et al., 1992; Heinrichs et al., 1991). Thus, exogenously administered CRF and urocortin produce behavioral activation, enhance behavioral responses to stress, and produce a behavioral state that is aversive and resembles a state of stress (for detailed reviews, see Aldenhoff et al., 1983; Dunn & Berridge, 1990; Koob et al., 1993).

Exogenous urocortin administered intracerebroventricularly elicits many of the same behavioral effects as other members of the CRF peptide family. However, urocortin more potently reduces food intake than CRF under both fasted and ad libitum feeding conditions in mice, rats, and sheep (Contarino et al., 1999; Parkes et al., 1997; Spina et al., 1996). This has led to the hypothesis that the CRF2 receptor is involved in the anorectic effects of CRF-related compounds, a hypothesis supported by some preliminary antisense studies (Smagin et al., 1998). Urocortin also suppresses food intake following peripheral administration in mice (Asakawa et al., 1999). Similar profiles, but with even less activation and anxiogenic-like effects, have been observed with urocortin II and III (Valdez et al., 2002; Zorrilla et al., 2001).

Behavioral Effects of CRF Receptor Antagonists

The demonstration of antistress actions of CRF receptor antagonists provides more compelling evidence for a role of endogenous CRF-like neuropeptides in behavioral responses to stressors. Most behavioral studies have used analogs and fragments of CRF that are large peptides and have to be injected intracerebroventricularly. Recent years have seen the development of a few nonpeptide-type, low molecular weight, systemically active CRF1 and CRF1/2 antagonists which will contribute to the pharmacological characterization of CRF1 and CRF2 receptor function (De Souza, 1987). Competitive peptide-type CRF receptor antagonists, such as α-helical CRF_{9-41} and [D-Phe12, $Nle_{21,38}$, Cα-MeLeu37] CRF_{12-41} (D-Phe CRF_{12-41}) (Curtis et al., 1994; Vale et al., 1981) have high affinity for both the CRF1 and CRF2 receptors (Vaughan et al., 1995). These peptide-type antagonists injected intracerebroventricularly reverse the attenuation of feeding induced by CRF and

restraint stress in rats (Krahn et al., 1986), and attenuate stress-induced fighting in rats (Tazi et al., 1987), suggesting that both the suppression and activation in behavior associated with stressors may involve endogenous CRF systems. In mice, α-helical CRF_{9-41} reversed the suppression in exploratory behavior produced by restraint stress (Berridge & Dunn, 1987), and in rats produced a more rapid emergence from a small dark enclosure into a large open-field and more exploration of the unfamiliar open-field (Takahashi et al., 1989). Subsequent studies have shown that CRF receptor antagonists are very effective in reversing the decrease in exploration of the open arms of an elevated plus-maze caused by exposure to a variety of stressors including restraint stress, swim stress, ethanol withdrawal and social stress (Heinrichs et al., 1992, 1994; Menzaghi et al., 1994). CRF antagonists inhibited stress-induced freezing behavior in rats (Kalin et al., 1988) and fear potentiation of the acoustic startle response (Swerdlow et al., 1989), and attenuated the acquisition of the conditioned suppression test (Cole et al., 1987). Other effects of CRF receptor antagonists consistent with an antistress effect include a reduction in defensive burying (Korte et al., 1994), reversal of the decrease in food intake produced by 17-ß-estradiol (Dagnault et al., 1993), and reversal of the "anxiogenic-like" effects of cholecystokinin in rats (Biro et al., 1993). However, CRF receptor antagonists are ineffective in certain operant tests such as the Geller-Seifter conflict test (Koob and Britton, unpublished observations) where the behavioral situations involve a highly trained response to punishment that already has been learned.

Corticotropin-Releasing Factor Transgenics

Studies from animal models using administration of CRF, urocortin, and CRF receptor antagonists have provided evidence that overactivity of CRF-containing neurons may be involved in behavioral responses to stressors and, by extrapolation to the human condition, a number of psychiatric disorders involving a high level of stress, including anxiety and affective disorders. A strength of the molecular genetic approach is the ability to produce a model of chronic CRF overactivation. A transgenic mouse model of CRF overproduction was developed where a CRF transgene was composed of a rat genomic CRF gene and 3' and 5' substitutions (Stenzel-Poore et al., 1994). Male CRF transgenic mice produced on a C57BL/6 x SJL genetic strain background were the subjects for the two studies discussed below.

Neuroendocrine Dysfunction

CRF transgenic mice develop a phenotype like Cushing's syndrome due to excess glucocorticoid production (Stenzel-Poore et al., 1992). These transgenics produced rat CRF that is recognized by mouse CRF_1 and CRF_2 receptors in quantities that represent a substantial overoccupancy of CRF receptors in the mice in the absence of stress. These mice showed endocrine abnormalities involving the hypothalamic-pituitary-adrenal axis, including elevated plasma levels of ACTH and glucocorticoids. They also developed physical changes similar to those of patients with Cushing's syndrome, including excess abdominal fat accumulation, muscle atrophy, thin skin, and alopecia. These mice show that chronic production of CRF resulting

in elevated ACTH and glucocorticoids leads to the development of Cushing's syndrome.

Behavioral Phenotype

CRF transgenic mice also showed enhanced responsiveness to novelty and an "anxiogenic-like" response on the elevated plus-maze. Locomotor activity in a novel environment was decreased in male transgenic mice compared to male littermate controls, and CRF-treated mice showed a greater suppression of locomotor activity after social defeat than controls. In the elevated plus-maze the CRF transgenic mice showed significantly less percent time on the open arms of the maze than controls (Table 2). These behavioral effects were reversed by intracerebroventricular administration of the CRF peptide-type antagonist α-helical CRF_{9-41}, providing evidence that the phenotype was due to overexpression of CRF. These nonselective transgenic mice show elevated signals for CRF mRNA in nearly all areas of expression shared by control animals (Stenzel-Poore et al., 1992) and some that were not detectable in control mice. These included some hypothalamic areas, habenula, dentate gyrus, dorsal subiculum, and cerebellum.

Mice with the same transgenic background also were tested for their learning and memory capacities using a forced alternation T-maze task and the Morris water maze task (Heinrichs et al., 1996). In the T-maze testing the control mice reached a criterion of 70% correct after 5 days of trials while the performance of the transgenic subjects was still random. In the Morris water maze task, control subjects reached the submerged platform significantly faster after 3 days of trials, while the transgenic mice did not improve over the same period. The benzodiazepine anxiolytic chlordiazepoxide administered prior to acquisition reversed the impairment of the transgenic mice in the water maze. No impairment was observed in transgenic mice when the platform was visible. These results show that the CRF transgenic mice exhibit a learning deficit without sensory or motor-related impairments and that this deficit can be reversed by anxiolytic pretreatment. These results suggest that the hyperemotionality associated with constitutive overabundance of CRF may interfere with learned behavior.

Corticotropin-Releasing Factor Knockouts

CRF_1 Receptor

CRF_1 receptor-deficient knockout mice strains (CRF_1 -/-) were generated by homologous recombination in embryonic stem cells as described above (Smith et al., 1998). A targeting vector was constructed from a 129 mouse strain genomic library in which the portion of the CRF_1 receptor gene encoding the last 12 amino acids of the first extracellular domain was replaced with a neomycin resistance gene cassette. Cells from embryonic stem cell positive clones were injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric males were outcrossed to C57BL/6 females. The offspring from homozygote crosses died within 48 hr after birth due to pronounced lung dysplasia requiring heterozygote breeding to produce homozygous mice. Male mice homozygous for the deletion of the CRF1 receptor displayed low plasma corticosterone concentrations due to marked agenesis of the

zona fasciculata region of the adrenal gland, and CRF (-/-) mice failed to exhibit the characteristic hormonal response to stress due to disruption of the hypothalamic-pituitary-adrenal axis.

Table 2
Neuroendocrine and behavioral effects of genetic alterations of CRF signaling.

	Tg OE CRF	**CRF1 knockout**	**CRF2 knockout**	**CRF knockout**	**CRF BP knockout**
HPA axis	↑	↓	↑	↓	---
Elevated plus-maze	↑	↓	↑		↑
Open field		↓	↑	---	
Learning/memory	↓	↓			
Basal feeding		---	---		
Basal weight gain		---	---		

Note. Dashed lines (---) indicate no effect. Blank spaces indicate measures not yet tested.

Mice with the CRF1 null mutation also showed less of an anxiogenic-like response in the elevated plus-maze as well as in the light/dark transfer test (Smith et al., 1998). In the elevated plus-maze, the mutant mice spent more time in the open arms and scored more entries in the open arms, with or without corticosterone replacement. In the light/dark transfer test, the mutant mice spent more time in the open-field and more time in the open-field per exit. In a subsequent study with the same null mutant mice, CRF1 knockout mice showed increased exploratory behavior in both the elevated plus-maze and the light/dark transfer test. The CRF1 knockout mice also were impaired in a spatial recognition memory task. Here, wild-type mice made more visits to and spent more time on the novel arms of a Y-maze apparatus as opposed to the familiar arms. The CRF1 deficient mice showed no increase in the level of exploration of the novel arms.

CRF1 receptor deficient mice also showed no locomotor response to intracerebroventricular administration of CRF (Contarino et al., 2000). However, CRF decreased appetite in both the CRF1 null mutants and the wildtype animals, suggesting that CRF1 receptors may be critical for the locomotor activation associated with CRF, but CRF2 receptors may be more involved in the appetite-suppressing effects of CRF. These results are consistent with the results with non-selective CRF antagonists and the results with selective CRF1 antagonists, and support the hypothesis that the CRF1 receptor mediates the anxiogenic-like effects of CRF.

CRF2 Receptor

CRF2 receptor-deficient knockout mice strains (CRF2 -/-) were generated by homologous recombination in embryonic stem cells as described above (Bale et al., 2000). A targeting vector was constructed from a 129/Sv-Ter mouse strain genomic library in which the portion of CRF2 encoding one-half of the fifth trans-

membrane domain through the end of the seventh transmembrane domain was replaced with a neomycin resistance gene cassette. Cells from embryonic stem cell positive clones were injected into C57BL/6 blastocysts to generate chimeric mice. Germline transmission of the disrupted allele was confirmed by Southern-blot analysis. Chimeric males were outcrossed to C57BL/6 females, and heterozygous mice were produced on a mixed C57BL/6 x 129 genetic background and maintained via heterozygote breeding.

The CRF2 mutant mice were hypersensitive to stress and showed a greater ACTH response following restraint stress compared to wildtype mice. The null mutant mice also showed increased anxiety-like behavior as measured by decreased open-arm time in an elevated plus-maze and decreased exploration in the inner squares of an open-field. The CRF2 mutant mice had normal basal feeding and weight gain but decreased food intake following food deprivation. One hypothesis to explain these results is that removal of the CRF2 receptor produced alterations in gene expression of other components of the CRF signaling pathway. Supporting this hypothesis was evidence of increased urocortin mRNA in the Edinger-Westphal nucleus and increased CRF in the central nucleus of the amygdala.

Two important notes should be made regarding the phenotype of the CRF2 knockout mice. First, the increased anxiety-like behavior observed in the CRF2 knockout mice was observable because the wildtype mice showed less of an anxiety-like effect than wildtype mice tested in the CRF1 experiments. The CRF2 wildtypes showed 10-20% time on the open-arms; the CRF1 wildtypes showed less than 5% time on the open arms. This difference was affected by group-housing the CRF2 wildtypes and knockouts and single-housing the CRF1 wildtypes and knockouts. Second, two additional studies were published on CRF2 knockout mice at the same time as the Bale et al. (2000) study. Some of the observations show a different phenotype and probably reflect differences in the targeting vector and/or background strain used to produce the knockout lines (Table 3). For example, Coste et al. (2000) showed that the initial hypothalamic pituitary adrenal response to stress was normal in CRF2 knockout mice, but the CRF2 knockout mice showed an early termination of the ACTH release to a stressor. Moreover, stress-coping behaviors in these mice were reduced (less time in the center of an open-field and less grooming). Furthermore, the feeding suppression produced by urocortin was not sustained in these CRF knockout mice. For the Coste et al. (2000) CRF2 knockout mice, the targeting vector was a 10.4 kb segment of CRF2 from the 129/Sv strain. ES cell clones were injected into blastocysts of C57BL/6 mice and the chimeras were backcrossed to C57BL/6 mice (3 generations). Kishimoto et al. (2000) showed that CRF2 male knockout mice, but not CRF female knockout mice, showed anxiety-like behavior in several animal models of anxiety, including the elevated plus-maze and the light/dark transfer test. No alterations in the HPA axis or feeding responses were observed. For the Kishimoto et al. knockout, the targeting vector was a transcript from the CRF2 transmembrane region IV to VII from a 129/Sv mouse genomic library. Breeding involved littermates from the F1 intercross between 129/Sv1 and C57BL/6 and the experiments were performed on F2-F4 hybrids.

CRF Binding Protein Knockout Mice

A CRF binding protein that is unrelated to CRF receptors has been characterized in human plasma and the rodent and human pituitary and brain (Behan et al., 1989; Orth & Mount, 1987). Both CRF and urocortin bind to CRF-BP, and a high affinity ligand for the CRF-BP would be hypothesized to increase available CRF or urocortin available for binding CRF receptors in the brain. Studies in rats using a CRF analog with high affinity for the CRF binding protein (hCRF6-33) have shown that when this analog is injected into the brain it decreases weight gain in genetically obese rats (Heinrichs et al., 1996) and can produce facilitation of learning and memory (Heinrichs et al., 1997).

Knockout mice with inactivation of the CRF binding protein show increased anxiety-like responses in the elevated plus-maze and defensive withdrawal tests (Karolyi et al., 1999), and mice overexpressing CRF-BP show some decreased anxiety-like behavior (Burrows et al., 1998). CRF-BP transgenic mice that overexpress CRF-BP throughout the brain and body show weight gain over wildtype controls (Lovejoy et al., 1998).

Table 3
Phenotypic comparison of different CRF_2 knockout mice.

Reference	Targeting Vector	Blastocyst Strain	Breeding	Phenotype		
				anxiety	HPA	feeding
Bale et al. (2000)	50% transmembrane regions V-VII 129/Sv-Ter strain	C57BL/6	Heterozygote F3-F4	↑	↑	---
Coste et al. (2000)	transmembrane regions III and IV 129/Sv strain	C57BL/6	backcrossed to C57BL/6 3 generations	↑	---	↓ (urocortin)
Kishimoto et al. (2000)	transmembrane regions IV-VII 129/Sv strain		heterozygote F2-F4	↑	---	---

CRF knockout mice

CRF knockout mice have been generated by use of a targeting vector that replaces the entire pre-proCRF coding region from the 129Sv strain with a neomycin resistance cassette. This target vector was introduced into ES cells and produced clones that had one copy of the endogenous CRF gene replaced with the CRF null allele (Muglia et al., 1997). Injection of the targeted ES cells into wildtype blastocysts yielded a germline transmission of the mutant CRF allele. Heterozygote

breeding was used to generate homozygous CRF-deficient (CRF knockout) mice (Muglia et al., 1997).

The phenotype of these mice includes a blunted plasma corticosterone response to restraint and ether stress that was greater in male knockout mice (Jacobson et al., 2000; Muglia et al., 2001). Also blunted, but not eliminated, glucocorticoid secretion was observed in knockout mice to prolonged restraint stress and severe physical stressors. CRF knockout mice showed little or no circadian modulation of corticosterone secretion (Muglia et al., 1997). However, these mice failed to show any changes in anxiety-related behavior in a number of animal models of anxiety. These included negative results in the multicompartment chamber and conditioned fear paradigms (Weninger et al., 1999). CRF knockout mice also showed normal basal food intake and normal decreases in food intake to stressors (Swiergiel & Dunn, 1999). These results, combined with studies showing that combined CRF_1 and CRF_2 receptor antagonists are effective in both wildtype and CRF knockout mice (Weninger et al., 1999), suggest that another, perhaps unidentified CRF-like molecule modulates anxiety-like responses in the absence of CRF itself (Muglia et al., 2001).

Summary and Conclusions

One of the major advantages of the molecular genetic approach is that the manipulation of the independent variable is virtually absolute. Removal of the gene responsible for expression of a given protein usually is complete in the knockout procedure and is not subject to the vagaries of binding affinities for different receptor subtypes of antagonist drugs. Similar agonist specificity can be assumed for the transgene overexpression of transgenic mice. With tissue-specific promoters, particular parts of the brain can be targeted and functional differences can be explored. Another advantage of the molecular genetic approach is that the changes in genetic expression outlined above are constitutive. Thus, these manipulations provide excellent animal models for longterm chronic inactivation or overactivation of specific elements of neuropharmacological systems. For example, in the CRF domain, the phenotype of CRF overexpression in specific brain areas may provide clues to certain anxiety and depression psychopathologies in the human condition.

The disadvantages of the molecular genetics approach are to some extent the mirror image of its advantages. Because the manipulation is constitutive, there is time for adaptation and plasticity to occur that in fact may produce the phenotype. The results with the CRF_2 knockouts may reflect this type of compensation. The phenotype of increased responsiveness to stressors and anxiety-like responses may reflect more the compensatory increase in CRF and urocortin activity with intact CRF_1 receptors rather than the loss of function of the CRF_2 receptors. However, the development of selective antagonists for a given receptor in the context of such genetic models allows not only confirmation of the selectivity of the antagonist but also insights into the hypothesized compensatory responses observed. Clearly, the combination of classic neuropharmacology and the molecular genetic approach will provide a powerful means of scientific study that each approach in isolation cannot convey.

It is important to remember that one of the great early frustrations of molecular genetics came with CRF deficient mice (CRF -/-) that were expected to have

a broadly deranged hypothalamic-pituitary-adrenal axis and stress tolerance but lacked this phenotype, which showed that behavioral adaptation during development is powerful. We will see the generation of increasingly more conditional knockouts and more knockout/knockin-type genetically engineered mouse strains. The conditional knockouts will bring the genetically engineered animal experiment closer to classical neuropharmacology in as much that the change in a ligand or receptor concentration will be temporary, thereby sparing the whole life of the animal; the time of appearance of the change is determined by the experimentation. Knockout/knockin utilization will permit the neurobiological study of behavioral manifestations of the expression of a "diseased gene" that replaces in the knockin the healthy knockout gene without the confounding effects of the presence of the product of the healthy gene.

The time and effort spent on genetically engineered approaches compares to the difficulty medicinal chemists experience in generating high affinity, high selectivity, organ- and tissue-specific ligands. Medicinal chemical and genetically engineered approaches are here to stay and are used to cross-validate experiments to promote the neurobiological elucidation of complex systems.

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