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THE CORTICOTROPIN-RELEASING FACTOR FAMILY: PHYSIOLOGY OF THE STRESS RESPONSE

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Deussing JM, Chen A. The Corticotropin-Releasing Factor Family: Physiology of the Stress Response. *Physiol Rev* 98: 2225–2286, 2018. Published August 15, 2018; doi:10.1152/physrev.00042.2017.—The physiological stress response is responsible for the maintenance of homeostasis in the presence of real or perceived challenges. In this function, the brain activates adaptive responses that involve numerous neural circuits and effector molecules to adapt to the current and future demands. A maladaptive stress response has been linked to the etiology of a variety of disorders, such as anxiety and mood disorders, eating disorders, and the metabolic syndrome. The neuropeptide corticotropin-releasing factor (CRF) and its relatives, the urocortins 1–3, in concert with their receptors (CRFR1, CRFR2), have emerged as central components of the physiological stress response. This central peptidergic system impinges on a broad spectrum of physiological processes that are the basis for successful adaptation and concomitantly integrate autonomic, neuroendocrine, and behavioral stress responses. This review focuses on the physiology of CRF-related peptides and their cognate receptors with the aim of providing a comprehensive up-to-date overview of the field. We describe the major molecular features covering aspects of gene expression and regulation, structural properties, and molecular interactions, as well as mechanisms of signal transduction and their surveillance. In addition, we discuss the large body of published experimental studies focusing on state-of-the-art genetic approaches with high temporal and spatial precision, which collectively aimed to dissect the contribution of CRF-related ligands and receptors to different levels of the stress response. We discuss the controversies in the field and unravel knowledge gaps that might pave the way for future research directions and open up novel opportunities for therapeutic intervention.

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I. INTRODUCTION: WHAT IS STRESS?

In the postgenomic era, it has become increasingly evident that genetic factors alone are insufficient to explain the vast majority of complex neuropsychiatric disorders. Instead, disease risk is shaped by the interplay between genetic predisposition and environmental factors, which often manifest through epigenetic marks, i.e., chemical modifications of the DNA without changes in the nucleotide sequence. In this context, stress has emerged as the most prominent environmental factor causally involved in the etiology of psychiatric disorders (18, 114, 145, 223, 440). Accordingly, the field of stress research focuses on the comprehension of how the diversity of environmental factors and their immediate and long-term impact on the organism entail adaptive or maladaptive responses and ultimately disease. Not only in biomedical research, but also in general public use, "stress" is a popular term, and everybody has a sense of feeling what it means "to be stressed." However, a closer examination reveals that the general usage is burdened with ambiguities in such a way that the term "stress" is synonymously used to describe the cause, the process, as well as the response (406).

The origins of stress biology are classically ascribed to the French physiologist Claude Bernard who is considered as the founder of modern experimental physiology. Bernard conceptualized the idea that an organism has to preserve a constant internal milieu as a prerequisite for an autonomous life, independent of the external environment ("La fixité du milieu intérieur est la condition d'une vie libre et indépendante") (38). However, his visionary concept was ignored in his lifetime, and it took another 50 yr before it was revived and entered mainstream physiology (217). In particular, the American physiologist Walter B. Cannon advocated Bernard's ideas (82, 83). Cannon, a former student of Henry P. Bowditch, who had been trained in Paris by Bernard, pioneered stress research, and in the early 20th century defined what he called the "fight-or-flight" reaction

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(81). He integrated and extended the pursuit of a constant "milieu intérieur" into his concept of "homeostasis," which means "stability through constancy." Homeostasis describes the maintenance of several physiological variables, such as blood glucose and oxygen levels or body temperature, within narrow physiological ranges (83, 84). According to Cannon, any threat to homeostasis, including emotional distress, elicits the immediate activation of the adrenal medulla and the sympathetic nervous system. He considered these two synergistic effector systems as a functional unit, the sympathoadrenal (or sympathico-adrenal) system, which is critical for the preservation and restoration of homeostasis (83). He proposed that the catecholamine adrenaline (or epinephrine) would be the main effector of this unit, which he hypothesized was converted to excitatory and inhibitory sympathin (85, 86). Later it turned out that adrenaline is the key player of the adrenomedullary limb, while noradrenaline is the effector of the sympathoneuronal limb of the system's response (637) **(FIGURE 1)**.

While the sympatho-adrenomedullary system was central to Cannon's concept of homeostasis, the "father" of stress research, the Austrian-Canadian endocrinologist Hans Selye, emphasized the role of the pituitary-adrenocortical system and its main effector, glucocorticoids **(FIGURE 1)** (588). In 1936, Selye's remarkable Letter to the Editor was published in *Nature* in which he described the stereotypic triad of reactions of rats upon exposure to various noxious stimuli, i.e., a hypertrophy of the adrenal cortex, an atrophy of the thymus and lymph nodes as well as gastric erosions and

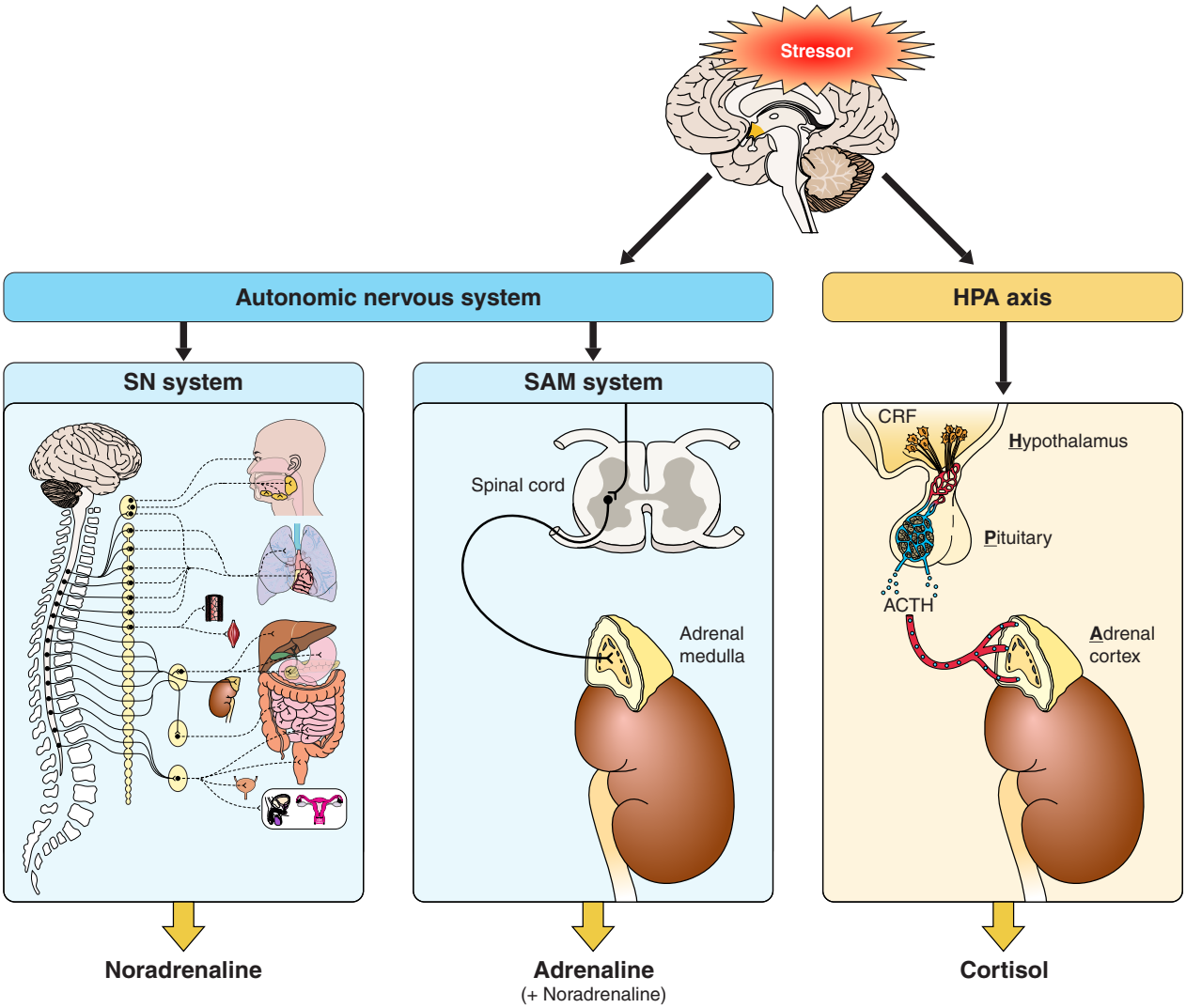

FIGURE 1. Effector systems of the stress response. A stressor elicits rapid activation of the autonomic nervous system with its sympathoneuronal (SN) and sympatho-adrenomedullary (SAM) limbs releasing their main effectors, noradrenaline and adrenaline, respectively. Activation of the hypothalamic-pituitary-adrenocortical (HPA) axis results in synthesis and release of its main effector, cortisol or corticosterone, in rodents. ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor.

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ulcers (174, 552). Based on these studies, Selye proposed the triphasic general adaptation syndrome (GAS), consisting of *1*) the initial alarm reaction (which can be regarded as equivalent to Cannon's fight-or-flight response); *2*) the stage of resistance involving adaptation to the stressor; and *3*) the stage of exhaustion, which in its extreme might lead to organismic death. Selye had used the term "stress" already in 1935 to describe "a state of non-specific tension in living matter, which manifests itself by tangible morphologic changes in various organs and particularly in the endocrine glands which are under anterior pituitary control" (554, 559). Originally, the term "stress" had been used in physics to describe the interaction between a deforming force and the resistance to it, which provokes a backlash that attempts to restore the unstressed state, as expressed in Hooke's law (269). However, it took until his first comprehensive monography that Selye systematically and steadily introduced the term "stress" in the context of the GAS (553). His numerous publications, including *The Stress of Life* (555) and *Stress Without Distress* (556), contributed to popularize the term also among the general public. Nowadays, "stress" is a commonly used term often associated with modern western lifestyle. However, a lot of confusion and misconception has been generated by its ambivalent usage. Its use lacks discrimination between the perceived stimulus causing the stress, the processing system, including the subjective experience of stress, and the physiological and/or behavioral responses activated to cope with those stimuli or the pathological consequences of overstimulation of the emergency response (351, 399, 527). Selye was aware of the ambiguity inherent to the term he had introduced. Therefore, he tried to clarify this confusion by defining "stress" as the nonspecific response of the body to any demand and clearly distinguished it from the "stressor," which he regarded as an agent or situation that produces stress at any time. He later also introduced the terms "eustress" and "distress" to differentiate between the adaptive and maladaptive consequences of the stress response (558). Accordingly, he considered the triphasic GAS as a chronologic response toward prolonged exposure to stressors (557). Central to Selye's model was the non-specificity of the response, almost a dogma which was later attenuated toward the view that any stressor would elicit the stereotypic non-specific stress syndrome only when it exceeds a certain threshold level (115, 399). However, the dogma of non-specificity was experimentally disproven by Pacak and colleagues (1998). Their work speaks against a uniform "stress syndrome" but rather favors stressor-specific central neurochemical and peripheral response signatures (453).

Cannon's concept of homeostasis, which builds on Bernard's idea of a constant "milieu intérieur," has for a long time dominated mainstream physiology. In this concept, stress is considered as any perceived threat to homeostasis (199). Stress is a condition in which expectations, whether genetically determined, established by prior learning, or inferred from given circumstances, do not match current or anticipated perceptions of the environment. This discrepancy between what is observed and what is expected or programmed elicits respective compensatory reactions. To accomplish compensatory reactions, the body possesses many homeostatic comparators, so-called homeostats, which can regulate effectors systems (198). However, homeostasis assumes for a given parameter a rather narrow window of constancy that directs adaptation of physiology. In reality, many parameters vary with the time of the day and year, as well as with responses to external or internal demands. Diurnal variations of body temperature, blood pressure, and heart rate, or changes in the cardiovascular and respiratory system during rest and activity are typical examples. This means the effort required to restore or maintain homeostasis differs, depending on the conditions the organism is facing in a given environment. This apparent deviation from pretended constancy was first taken into account by Sterling and Eyer (582) by introducing the term "allostasis," which literally means "stability through change." The concept of allostasis and the importance of changes in homeostatic requirements for the adaptation to stressors was further developed by Bruce McEwen and others and extended by the concept of "allostatic load" or "overload" (198, 405, 404). Mediators of allostasis, e.g., hormones of the hypothalamic-pituitary-adrenal (HPA) axis, catecholamines, and cytokines, are released on exposure to a stressor, promote adaptation, and thus are initially beneficial. However, chronically increased allostasis can lead to pathophysiology, which is described by the terms "allostatic load" or "allostatic overload." These terms refer to the wear and tear on the body and brain that results from either too much stress or from inefficient management of allostasis, for example, the failure to shut off the response efficiently, the absence of an adequate response, or lack of habituation to the recurrence of the same stressor and thus dampening of the allostatic response (404). Cumulative changes lead to allostatic overload, which in the long term conveys adverse physical or mental consequences. The allostasis concept considers the importance of changes in homeostatic requirements as a means to adaptation to stressors by emphasizing that parameters are variable, and that variation anticipates future demands. Allostasis involves mechanisms that change the controlled physiological variable by predicting what level will be needed to meet anticipated demands (324).

The concept of allostasis has been further refined in the reactive scope model, which presumes that hormonal, behavioral, and physiological mediators exist in four ranges. *1*) The homeostatic failure range does not allow sustained homeostasis. As a consequence an animal would die if its mediators were in this range. *2*) The predictive homeostasis range is located above homeostatic failure. Here the mediators vary daily and yearly, but always in response to highly predictable changes in their environment. *3*) In the reactive

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homeostasis range, the mediator must increase if unpredictable changes occur. This range is equal to the classic stress response, where the mediators help the animal to survive unpredictable changes. Together, the predictive and reactive homeostasis form the normal reactive scope of an animal. *4*) The range of homeostatic overload is entered if a mediator extends above the reactive homeostasis, thus provoking symptoms of chronic stress (527).

Despite the refinement of stress models, several conceptual difficulties in the field have been raised in recent years, including the inconsistency of scientific results across laboratories and stress models (325). In particular, the use of the term "stress" for a broad spectrum of conditions ranging from mildest challenges to severely aversive or traumatic conditions appears problematic. To overcome these controversies, Koolhaas and colleagues (325) have suggested more narrow definitions of the terms "stress" and "stressor," limiting them to conditions jeopardized by the lack of predictability and controllability, i.e., conditions in which an environmental demand exceeds the natural regulatory capacity of an organism. Similar doubts arise by the observation that stress responses to appetitive rewarding stimuli, such as sexual behavior or winning a social interaction, are indistinguishable from severe negative stimuli with respect to activation of the autonomic nervous system (ANS) or HPA axis (56, 326). Thus the key question is whether a given stimulus is perceived as a stressor, which ultimately results in a stress response. For instance, negative consequences on learning and memory in rats are only conveyed by a situation perceived as stressful, e.g., by exposure to a predator, but not by exposure to a receptive female, albeit the level of HPA axis activation was indistinguishable (664). These findings suggest that both the ANS and HPA axis are part of a general arousal system; however, Koolhaas and colleagues (325) point out that arousal and stress should not be used synonymously.

In general, the ANS and the HPA axis are regarded as the key players in the stress response. The immediate response to any threat to homeostasis is dominated by the rapid activation of the ANS, including its sympatho-adrenomedullary and sympathoneuronal limbs **(FIGURE 1)** (615). The mutual regulation of the sympathetic and parasympathetic arms warrants for the short half-life of this instantaneous reaction. Release of glucocorticoids by the major neuroendocrine stress response system, the HPA axis, peaks with a time lag (tens of minutes) after the encounter of a stressor **(FIGURE 1)**. Accordingly, the feedback regulation is more sluggish, and the ceasing of the neuroendocrine stress response lags significantly behind the ANS (136, 615). Beyond the ANS and the HPA axis, a plethora of molecules, i.e., neurotransmitters, steroid hormones, and neuropeptides, has been recognized to drive adaptive responses (292). The neuropeptide corticotropin-releasing factor (CRF), its related peptides, the urocortins and respective receptors, clearly stand out as a central peptidergic system capable of integrating a triad of physiological reactions toward environmental or homeostatic challenges. First, CRF was identified as the master regulator of the HPA axis localized to parvocellular hypothalamic neurons at the apex of the neuroendocrine cascade of stress hormones (619). Second, CRF has been demonstrated to directly modulate the rapid response of the ANS (68, 72). Third, CRF itself has been shown to elicit behaviors that are reminiscent of a typical stress response (155, 156, 357). To appreciate its prominent role in the stress response, this review will focus on CRF, related peptides, and receptors by providing a comprehensive overview of the body's key peptidergic stress response system.

II. CRF AND RELATED NEUROPEPTIDES

A. The Discovery of Corticotropin-Releasing Factor: A Historical View

The pioneering work by Bernard, Cannon, Selye, and others was the foundation for the evolution of the concept of neurosecretion and the ascent of a new field of research: neuroendocrinology (315). In the late 1930s, Selye had postulated:

> . . . [T]hrough some unknown pathway, the "first mediator" travels directly from the injured target area to the anterior pituitary. It notifies the latter that a condition of stress exists and thus induces it to discharge adrenocorticotropic hormone (ACTH).

Although it turned out that this first mediator is not identical to the one that induces the hormonal stress response resulting in the release of ACTH (554), Selye had somewhat anticipated what Geoffrey W. Harris later put forward as the concept of neurohumoral control of anterior pituitary secretion. Influenced by earlier work by Ernst and Berta Scharrer, Wolfgang Bargmann, and others, Harris was the first to demonstrate that the hypothalamic-hypophysial portal vasculature is required for the control of anterior pituitary activity (545). He postulated that nerve fibers of the hypothalamus release humoral substances into the capillaries of the primary plexus in the median eminence from where they reach the anterior pituitary and act on cells, which then release hormones into the general circulation (210, 228). At the time, this was a revolutionary, but also long disputed hypothesis, especially since Harris himself failed to isolate those postulated hypothalamic factors (151).

Selye's studies on the pituitary-adrenocortical system underlined its physiological, as well as clinical, relevance and attracted considerable interest. As Selye noted: "Identification of the first mediator appears to be one of the most fundamental tasks of future stress research" (557). The existence of this first mediator of the stress response promot-

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ing ACTH release from the anterior pituitary had already been demonstrated by two independent groups in 1955: Murray Saffran and Andrew V. Schally (535, 536) in Montreal, as well as Roger C. L. Guillemin, a former student of Selye, and Barry Rosenberg in Houston (219).

Schally and Saffran were the first to call this mediator corticotropin-releasing factor (CRF) (536). The nomenclature proposed by Schally used the term "factors" for the unknown hypothalamic substances yet to be isolated, which in his view should be renamed "hormones" as soon as they are identified and characterized (544). The term "hormone" is an adequate description of the activity of the hypothalamic releasing/inhibiting factors in the context of the HPA axis, but some of these neuropeptides, such as CRF, not only act solely as secretagogues, but have additional neuromodulatory or neuroregulatory properties in brain structures outside of the hypothalamus, a quality that is better represented by the more general term "factor." Despite attempts to unify the nomenclature by a committee for CRF receptors (CRFRs) of the International Union of Pharmacology, both terms, corticotropin-releasing factor (CRF) and corticotropin-releasing hormone (CRH), can be witnessed with similar frequencies. In particular, the notation "CRF_1" and "CRF_2" for the CRFRs are not in prevalent use so far (235). The fact that *CRH* or *Crh*, as well as *CRHR1*/*Crhr1* and *CRHR2*/*Crhr2*, are the official human and mouse gene symbols determined by the HUGO Gene Nomenclature Committee further contributes to the present lack of harmony in the nomenclature. For clarity reasons, in this review, we adhere to using the "factor"-based nomenclature.

In addition to its physiological importance, there were also very practical experimental reasons why CRF was the first hypothalamic factor to be detected and named. It was mainly because, of all hormones secreted by the anterior pituitary, only ACTH had a reliable method of measurement established. The first experimental demonstration of its existence led to intense but unavailing research efforts to identify CRF, but its biochemical nature remained unexplained for the next 3 decades. Nevertheless, the perseverance of the two teams gathered by Guillemin and Schally, largely driven by their scientific rivalry, ultimately led to the discovery of the first hypothalamic releasing and inhibiting factors, including the thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH), as well as the growth hormone inhibiting hormone (54, 75). For this major breakthrough in the field of neuroendocrinology, Schally and Guillemin, together with Rosalyn S. Yalow, were awarded with the Nobel Prize in Medicine or Physiology in 1977 (639).

The search for CRF stalled until the 1970s, when the quest for CRF isolation was resumed again (173). However, it took until 1981, 1 yr before Selye died, for the long-sought first mediator of the stress response to be isolated. Wyle W. Vale, a former postdoc of Guillemin, and his team at the Salk Institute succeeded in isolating the ACTH-releasing factor from the 490,000 ovine hypothalamic fragments (that had been previously used for the isolation of GnRH), which ultimately turned out to be CRF (173). With the isolation of the 41-amino acid peptide, which stimulates the release of ACTH in vitro and in vivo, a decades-lasting quest for CRF came to an end (316, 317, 619). Today we know that this was just the prelude to the discovery of an entire family of CRF-related peptides, specific receptors, and binding proteins, forming a complex system that is involved in a large array of physiological processes.

B. CRF-Related Genes and Proteins

In the decade following its first isolation, both the CRF cDNA (189, 291) and the gene were characterized in human, sheep, rat, and mouse (521, 522, 550, 565, 603). In humans, the *CRF* gene is located on chromosome 8 and in mice on chromosome 3. In all studied species, the gene contains two exons separated by a relatively short intron (600–800 bp). The second exon encodes the entire open frame. CRF is synthesized as a prepropeptide of 196 amino acids with an NH_2-terminal signal peptide of 24 amino acids. The proteolytic processing of the precursor at dibasic amino acids (lysine or arginine) is carried out by prohormone convertases. The mature neuropeptide only develops its full biological activity after amidation of the COOH-terminal Gly-Lys motif (619). In contrast to many other neuropeptides, no other bioactive peptide is encoded by the CRF precursor (268).

Independently, the CRF paralogues, sauvagine from the skin of the South American tree frog *Phyllomedusa sauvagei* (419, 420) and urotensin I from the urophysis of the teleost fish species *Cyprinus carpio* and *Catostomus commersoni*, were isolated (275, 353, 354, 388) **(FIGURE 2)**. These CRF-related peptides were shown to elicit similar biological effects as CRF (63). However, it was not until 1995 that the Vale group identified another CRF ortholog in mammals, which was named urocortin 1 (UCN1) due to its similarity to urotensin I (63% homology) and CRF (45% homology). Similar to CRF, synthetic UCN1 stimulates secretion of ACTH in vivo and in vitro (631). In 2001, two further members of the CRF family were identified, first named stresscopin and stresscopin-related peptide, which are now called urocortin 3 (UCN3) and urocortin 2 (UCN2), respectively (271, 359, 512) **(FIGURE 2)**.

The genome sequences of various species did not reveal any other CRF orthologs, indicating that the family of CRF-related peptides in vertebrates consists of four members: CRF, UCN1, UCN2, and UCN3 (91). An invertebrate homolog of CRF, the diuretic hormone 44 neuropeptide (DH_{44}) and respective receptors (DH_{44} receptor-1 and -2), has been characterized in *Drosophila* (87, 162, 241, 379,

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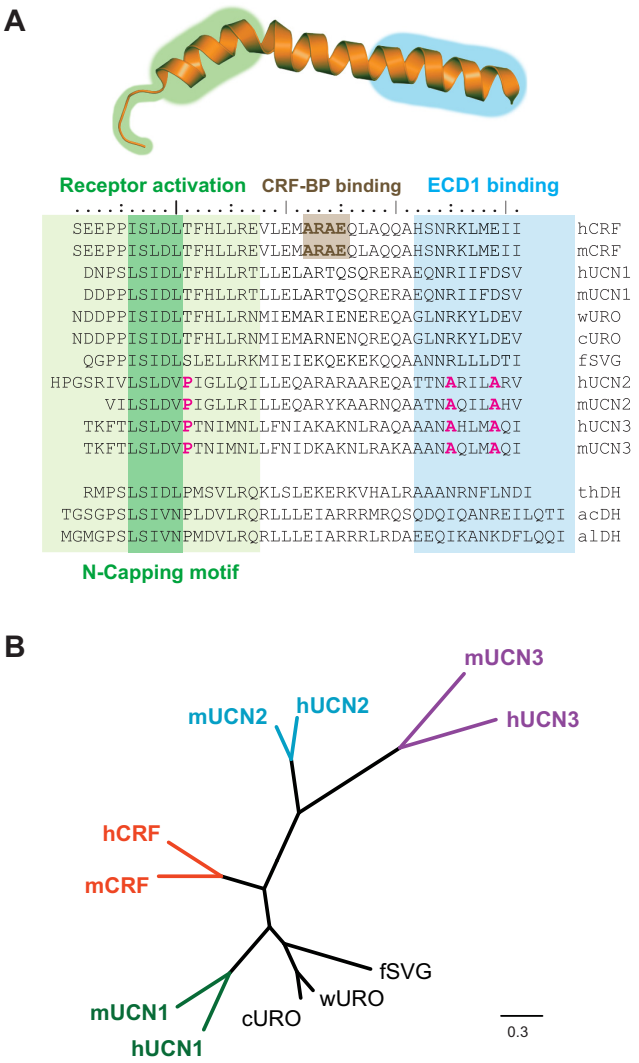

FIGURE 2. The family of corticotropin-releasing factor (CRF)-related neuropeptides. *A*: alignment of amino acid sequences of mature CRF-related peptides from vertebrates and of diuretic hormone (DH) from insects. The NH_2-terminal activating domain is highlighted in light green. The COOH-terminal domain binding to CRFRs' extracellular domain is highlighted in blue. The central linker domain is essential for CRF-binding protein (BP) binding. The CRF-specific Ala-Arg-Ala-Glu motif, which conveys CRF-BP binding, is depicted in brown. The N-capping motif is highlighted in dark green. Amino acid residues conferring CRF receptor type 2 (CRFR2)-specificity are indicated in pink. A three-dimensional structure of CRF is depicted *above* the alignment. *B:* phylogenetic tree of the four CRF paralogs in vertebrates. The phylogenetic tree was generated using *Phylogeny.fr* (140). ac, *Periplanata Americana*; al, *Locusta migratoria*; c, *Cyprinus carpio*; f, *Phyllomedusa sauvageii*; ECD1, extracellular domain; h, *Homo sapiens*; m, *Mus musculus*; SVG, sauvagine; th, *Manduca sexta*; UCN, urocortin; URO, urotensin; w, *Castostomus commersonii*.

380). There was probably already a CRF-like gene in the first Metazoan, before the formation of the Chordata. The four members of the CRF family are thought to have emerged as a result of two repeated genome duplications. However, the characterization of a series of CRF-like genes in invertebrates also leaves open the possibility that the first chordates already had two CRF-like genes, and that these were duplicated only once (379, 381). UCN2 and UCN3 form a distinct branch in the CRF family, which probably originated at a similar time as the CRF/UCN1 branch (91, 168). In contrast, sauvagine in the frog and urotensin I in the bonefish are thought to have arisen through additional gene duplication events within this lineage **(FIGURE 2)**.

The genes of the CRF-related family members are distributed over different chromosomes and appear with a uniform gene structure, i.e., they comprise two exons, with the second exon encoding the prepropeptide (381). Similar to CRF, the other family members are also synthesized as precursors that comprise a signal peptide that is essential for secretion and an NH_2-terminal propeptide, whose cleavage leads to an active neuropeptide. The proteolytic processing at conserved recognition sequences and COOH-terminal amidation are required for generation of a bioactive peptide (91, 268). In the case of human UCN2, it is, therefore, not entirely clear whether a biologically active peptide is generated, since the consensus sequence for the COOH-terminal processing and amidation is missing from the prepro-UCN2 (235) **(FIGURE 3)**. In vertebrates, only four distinct CRF-related paralogues exist. However, in recent years, the teneurin COOH-terminal associated peptides (TCAPs) have been shown to harbor some structural homology to the CRF family, although their overall sequence identity is <20% (377, 493). TCAPs are present at the COOH-terminus of each of the four teneurins, type II transmembrane (TM) proteins present in the central nervous system (CNS), from where they potentially can be cleaved and released as bioactive peptides. Synthetic TCAPs have been shown to act in a CRF-receptor-independent manner, inhibiting stress-associated behaviors in rodents (113).

C. Central and Peripheral Ligand Expression

The detection of neuropeptides by immunohistochemistry is inherently difficult due to the fact that neuropeptides following synthesis are rapidly sorted into large, dense core vesicles (LDCV) for transportation to their site of action. On route, they mature through specific proteolytic processing and COOH-terminal amidation (268, 624). Therefore, it is difficult to detect CRF or UCNs as proteins at the soma of neurosecretory cells without prior disruption of vesicular transport, e.g., by inhibition of microtubule polymerization via colchicine. The currently most reliable detection with high spatial resolution is on the mRNA level via in situ hybridization. Due to the limited access to human brain tissue, the central expression of CRF-related neuropeptides, research is dominated by findings from rodent studies, albeit a few human studies exist (182, 592). This trend has been intensified by the ascent of novel mouse genetic tools providing access to neuronal populations expressing spe-

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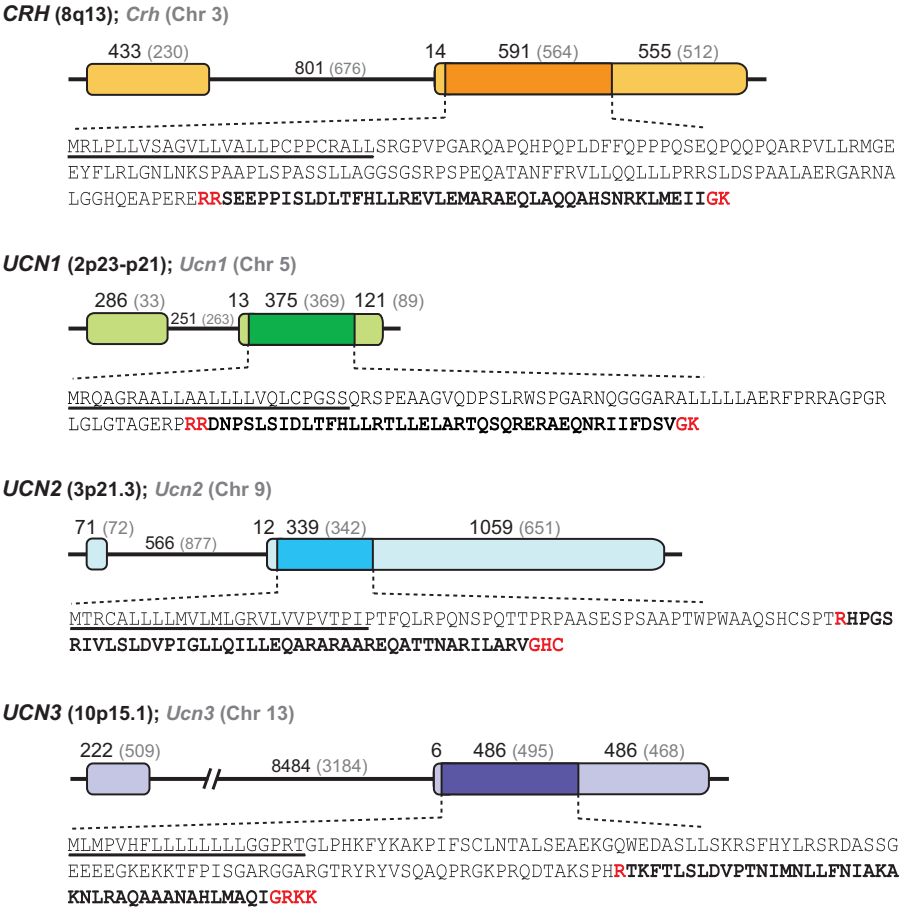

FIGURE 3. Genes and proteins of the CRF family members. Genes of CRF-related peptides possess a uniform appearance, consisting of two exons separated by an intron of variable size. Exon 1 encodes the 5′ untranslated region. Exon 2 harbors the entire open reading frame depicted as darker colored box. *Above* each gene the official human and murine gene symbols and chromosomal localizations are indicated. Numbers *above* the exons refer to the size of exons and introns or to translated vs. untranslated regions of exon 2 (in bp). Gray numbers in parentheses provide detailed intron/exon sizes for the respective mouse gene. The complete polypeptide sequence is depicted *below* each gene structure. The signal peptide is underlined, and amino acid residues at sites of proteolytic processing are indicated in red. The mature biologically active peptide is indicated in bold.

cific neuropeptides, i.e., mice, and more recently also rats, expressing either a reporter gene (6, 282, 322) or a Cre recombinase (333, 398, 541, 562, 596) driven by promoter elements of respective CRF-related peptide genes. These mouse lines allow direct visualization of the reporter or, alternatively, activation of a Cre-dependent reporter, either by breeding to a respective mouse line or by stereotaxic injection of Cre-inducible viral constructs, such as adeno-associated viruses (AAVs). The sensitivity of the Cre-recombinase approach exceeds all other approaches, since even a low level of gene or Cre expression is capable to activate strong reporter gene expression. However, this approach will also report legacy expression in a fate-mapping manner, i.e., once activated, a reporter will be continuously expressed, even if the CRF-related peptide was only transiently expressed. In addition, particular caution is required using reporter or Cre lines to establish to what extent endogenous expression is faithfully recapitulated (110, 143).

Of the CRF-related neuropeptides, CRF shows the most widespread expression in the brain (126, 410, 537, 543). In the hypothalamus, CRF is strongly expressed in the paraventricular nucleus (PVN), where it regulates the activity of the HPA axis. In addition, CRF is expressed in the olfactory bulb, shell of the nucleus accumbens, the entire neocortex, piriform cortex, scattered interneurons of the hippocampus, the central amygdala (CeA; mainly the centrolateral division), the interstitial nucleus of the posterior limb of the anterior commissure, and different divisions of the bed nucleus of the stria terminalis (BNST). In the brain stem, CRF is expressed in Barrington's nucleus, in the laterodorsal tegmental nucleus, in the nucleus parabrachialis, and very strongly in the brain stem inferior olive (IO) **(FIGURE 4)** (6, 282, 322, 596). Recently, Cre recombinase-driven reporter gene expression has been utilized to systematically analyze the brainwide distribution of CRF neurons (465). Up until now, the identity of CRF neurons throughout the CNS has only partially been revealed. In the hippocampus, CRF is exclusively expressed in a population of γ-aminobutyric acid (GABA)-ergic interneurons that innervate CA1 and CA3 pyramidal neurons. Most hippocampal CRF neurons show the appearance of basket cells and coexpress parvalbumin (PV) but neither calbindin nor cholecystokinin (CCK) (106). In the neocortex, CRF neurons appear either as small basket cells, descending basket cells, or double bouquet cells. These cells are also GABAergic but show more diversity with respect to colocalization of chemical markers. The majority of CRF neurons coexpress vasoactive intestinal polypeptide or somatostatin (SOM) in a layer-dependent manner. In contrast to the hippocampus,

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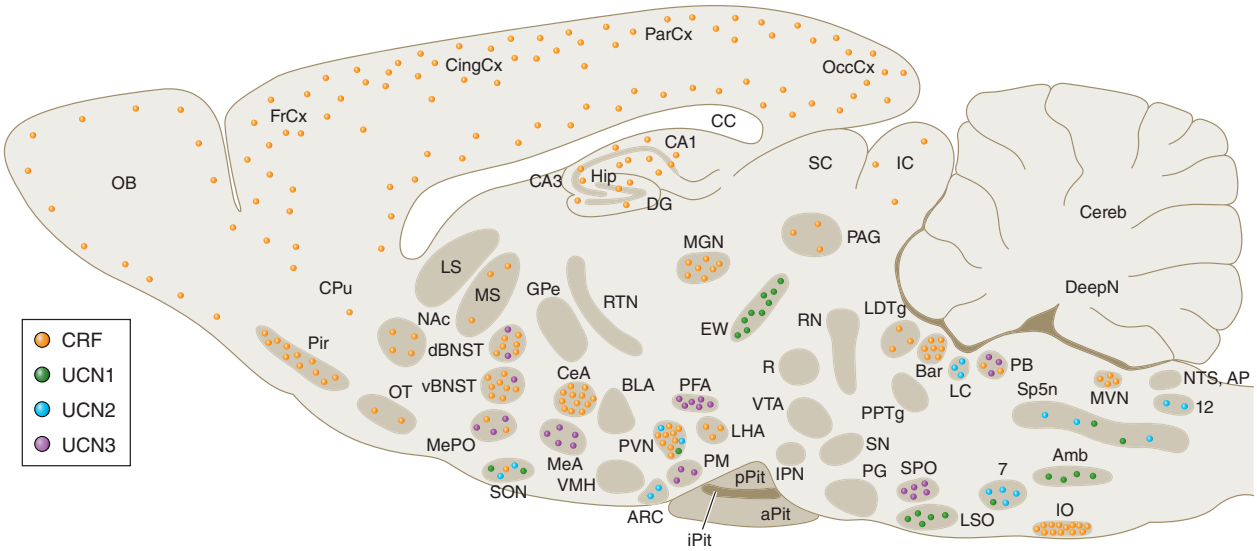

FIGURE 4. Distribution of mRNA expression of corticotropin-releasing factor (CRF)-related peptides in the rodent brain. Three-dimensional expression patterns of CRF-related peptide were collapsed onto a single sagittal brain section. Depicted are well-documented sites of high to moderate expression. Sites of expression are indicated by colored dots: CRF (orange), urocortin (UCN) 1 (green), UCN2 (light blue), UCN3 (purple). 7, Facial nerve; 12, hypoglossal nerve; Amb, ambiguous nucleus; AP, area postrema; arc, arcuate nucleus; Bar, Barrington's nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis (d, dorsal aspect; v, ventral aspect); CA1, cornu ammonis subfield 1; CA3, cornu ammonis subfield 3; CC, corpus callosum; CeA, central amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CPu, caudate putamen; DeepN, deep nucleus of cerebellum; DG, dentate gyrus; EW, Edinger Westphal nucleus; FrCx, frontal cortex; GPe, external globus pallidus; Hip, hippocampus; IC, inferior colliculus; IO, inferior olive; IPN, interpeduncular nucleus; LC, locus coeruleus; LH, lateral hypothalamus; LS, lateral septum; LSO, lateral superior olive; LTDg, laterodorsal tegmental nucleus; MeA, medial amygdala; MePO, median preoptic area; MGN, medial geniculate nucleus; MS, medial septum; MVN, medial vestibular nucleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OccCx, occipital cortex; OT, olfactory tubercle; PAG, periaqueductal gray; ParCx, parietal cortex; PB, parabrachial nucleus; PFA, perifornical area; PG, pontine gray; Pir, piriform cortex; Pit, pituitary (p, lobe, anterior lobe, intermediate, posterior lobe); PM, premammillary nucleus; PPTg, pedunculo-pontine tegmental nucleus; PVN, paraventricular nucleus of the hypothalamus; R, red nucleus; RN, raphe nuclei; RTB, reticular thalamic nucleus; SC, superior colliculus; SN, substantia nigra; Sp5n, spinal trigeminal nucleus; SPO, superior paraolivary nucleus; VMH, ventromedial hypothalamus, VTA, ventral tegmental area.

neocortical CRF neurons are largely PV negative. While only few CRF neurons coexpress CCK or calretinin (337). In the periphery, a striking differential expression pattern has been observed in humans and rodents. While the human placenta shows increased levels of CRF expression during gestation, neither rat nor mouse placenta express any CRF (391, 487). CRF and all other family members are expressed throughout different cell types of the gastrointestinal (GI) tract (73). CRF neurons in the eye are either GABAergic displaced amacrine cells or, to a smaller extent, non-GABAergic retinal ganglion cells (678). Interestingly, CRFR1 has also been localized to retinal ganglion cells (339). In addition, the cochlea was shown to harbor a complete system of CRF-related peptides and receptors (204).

UCN1 shows a much more restricted distribution and is expressed mainly in the Edinger-Westphal nucleus. In addition, scattered UCN1-expressing cells are found in the supraoptic nucleus and lateral superior olive **(FIGURE 4)**. The lateral septum (LS) is one major projection site of UCN1 neurons (331, 421). Also expression in the GI tract, e.g., in the enteric system and in the colonic mucosa, has been reported (225, 427).

The highest levels of UCN2 expression have been detected in skeletal muscle and skin (94). The spatial distribution of UCN2 expression in the brain is still a matter of debate, since the first description of its expression in the rat brain has not been reproduced in mice (512, 595). Of note, UCN2 knockout mice in which the open reading frame of UCN2 was replaced by a β-galactosidase reporter gene did not reveal any reporter gene activity (62, 144). This may suggest that UCN2 expression under basal conditions is close to the detection limit. In the initial study, UCN2 expression had been demonstrated by mRNA in situ hybridization in the PVN, supraoptic nucleus, arcuate nucleus, locus coeruleus (LC), and some motor nuclei of the brain stem (trigeminal, facial, hypoglossal), as well as of the spinal ventral horn **(FIGURE 4)**. In addition, expression in the meninges has been reported (512). All other studies demonstrating UCN2 expression in the brain used RT-PCR, which does not provide high spatial resolution (94, 97). In the periphery, UCN2 has

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been detected in cardiomyocytes of the heart, where it acts in an autocrine/paracrine fashion (362).

UCN3 is expressed in the small intestine, where it has been located to cells in the intestinal crypts (e.g., enterochromaffin cells) and goblet cells (144, 218). Moreover, UCN3 is strongly expressed in pancreatic β-cells (144, 360). In the meantime, UCN3 is accepted as one of the earliest specific markers for differentiation of pancreatic β-cells (51, 626, 627). In the brain, the most abundant of the CRFR2-specific ligands is found in the median preoptic area (MePO), the medial amygdala (MeA), the BNST, the rostral perifornical area of the hypothalamus, the ventral premammilary nucleus, the nucleus parabrachialis, and the superior olivary nucleus **(FIGURE 4)** (144, 359). UCN3-positive fibers have been shown to colocalize with sites of CRFR2 expression, suggesting that UCN3 is the main endogenous ligand of CRFR2 (361).

III. CRF RECEPTORS AND BINDING PROTEINS

The biological activity of CRF is mediated through two heptahelical G protein-coupled receptors (GPCRs). The first CRFR to be cloned and functionally characterized was the CRFR type 1 (CRFR1) from human, mouse, and rat (92, 102, 468, 636). Only 2 yr later, another CRFR was identified, the CRFR type 2 (312, 466). In addition, a third CRFR was found in the catfish *Ameiurus nebulosus* (9), which appears to be species specific, because no similar receptor could be identified in the genomes of other vertebrates. The CRFRs belong to the family B (secretin-like hormone receptors, subfamily B1) of GPCRs **(FIGURE 5)**. Hallmarks of the family B are a relatively large NH_2-terminal extracellular domain (ECD) and large peptide ligands (48, 88, 185, 226, 349). The homology of CRFR1 between different species is in general >80%. Human CRFR1 shares 70% homology with human CRFR2 (237). The greatest homology between both receptors is observed in the intracellular loops (ICLs) and the COOH-terminal intracellular domain (ICD) [ICL1, ICL2, ICD: 90% identical; ICL3 (G protein-binding domain) 100%] and in the TM helices (85% identical). The NH_2-terminal ECD and the extracellular loops (ECLs) are far less conserved (60%).

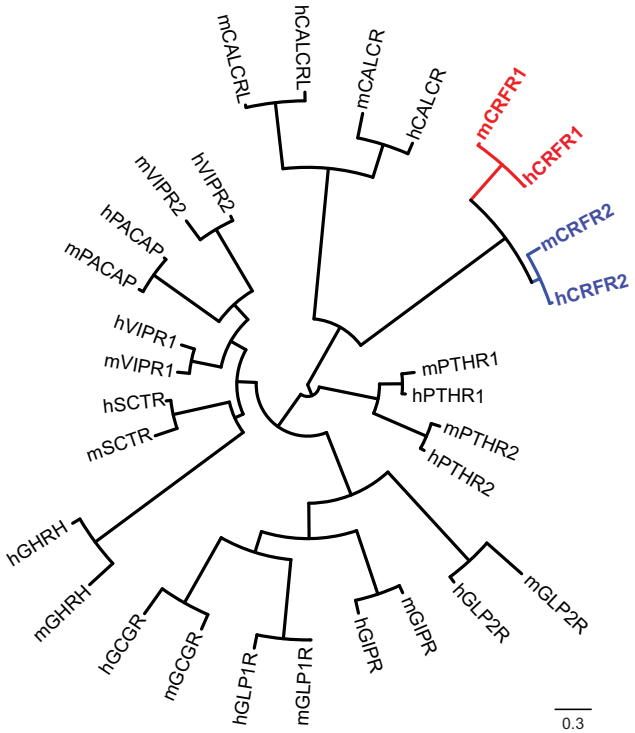

FIGURE 5. Corticotropin-releasing factor (CRF) receptors belong to the family B of secretin-like G protein-coupled receptors (GPCRs). Phylogenetic tree of the 15 family members of human and mouse class B secretin-like GPCRs is shown. The seven transmembrane regions from transmembrane (TM) I to the end of TMVII were used to generate a phylogenetic tree using *Phylogeny.fr* (140). CALCR, calcitonin receptor; CRFR, corticotropin-releasing factor receptor; GCGR, glucagon receptor; GHRHR, growth hormone-releasing hormone receptor; GIPR, gastric inhibitory polypeptide receptor; GLPR, glucagon-like peptide receptor; h, *Homo sapiens*; m: *Mus musculus*; PACAP, pituitary adenylyl cyclase-activating protein; PTHR, parathyroid hormone receptor; SCTR, secretin receptor; VIPR, vasoactive intestinal peptide receptor.

A. CRF Receptor Type 1 (CRFR1)

The human *CRFR1* gene was mapped to chromosome 17 (482, 622) and extends over ~50 kb. To date, 14 exons have been characterized, which deviates from the mouse and rat genes that only have 13 exons (77, 609). The mouse gene is located on chromosome 11 in a region of conserved synteny (77). In humans, eight splice variants of the *CRFR1* gene have been identified primarily in the skin, placenta, or endometrium **(FIGURE 6)** (250, 679). Only the isoform CRFR1β, also known as proCRFR1, is encoded by all 14 exons (600). In all other isoforms, exon 6 is spliced out. The splice variants can be divided into *1*) full-length receptors-α and -β, *2*) COOH-terminal truncations, or *3*) truncations of the ECD, and *4*) isoforms with a shortened 7-TM domain (TMD) or soluble receptors where the TMD is absent **(FIGURE 6)**. Alternative splicing is a widespread mechanism among GPCRs, particularly in the secretin family, to regulate signal transduction (397) and thus contributes to the differential cell- and tissue-type-specific response of a receptor to agonists or antagonists (250, 477). The CRFR1α variant plays a dominant role as a fully functional receptor, the activity of which is regulated by the other variants. This regulation can be achieved either by the fact that an increased generation of the other splice variants regulates the availability of the CRFR1α or through dimerization/oligomerization of CRFR1α with these variants, which can change the function of the receptor, e.g., ligand binding, intracellular localization and activity (397). Furthermore,

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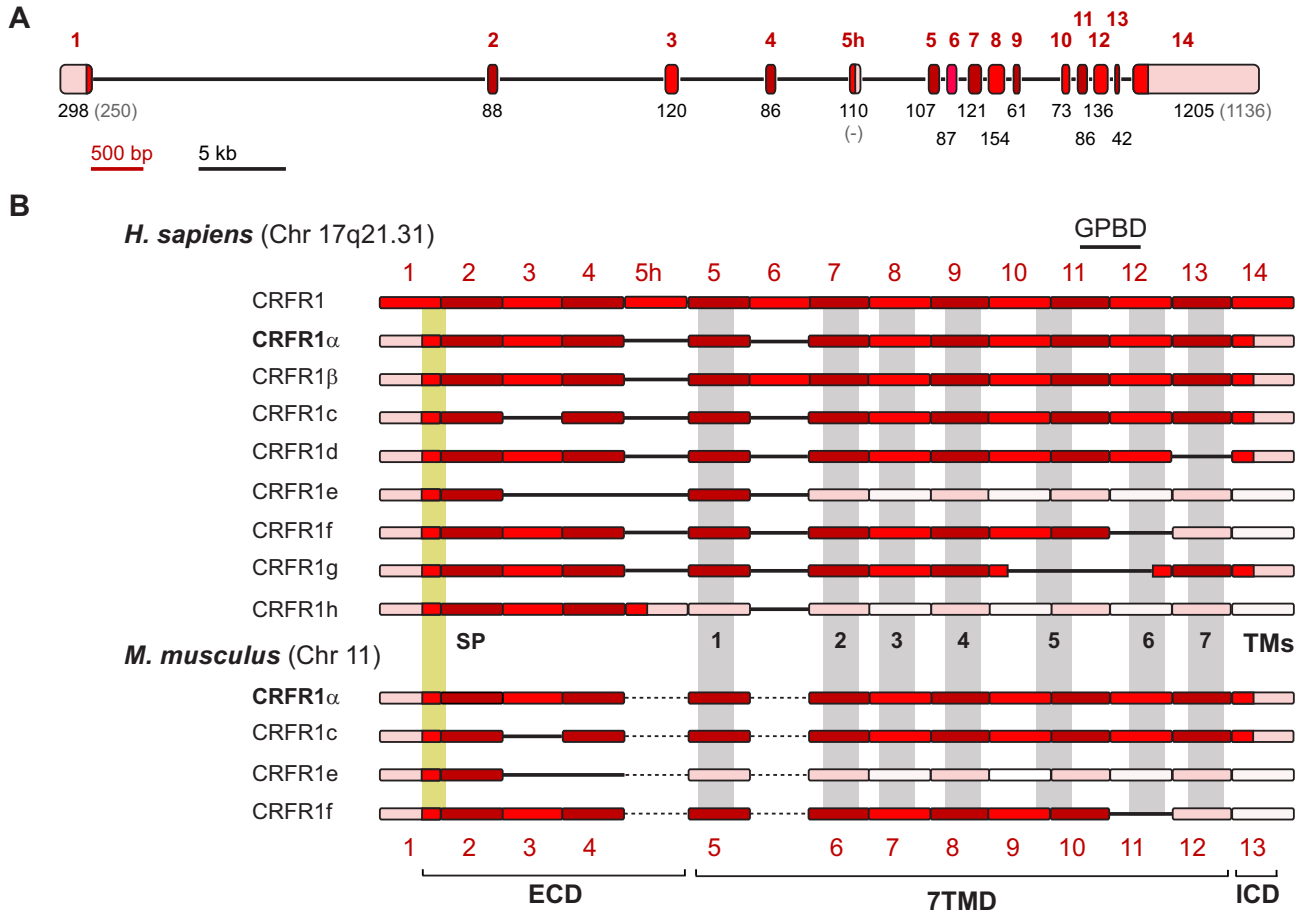

FIGURE 6. The *CRFR1* gene and isoforms generated by alternative splicing. *A*: schematic representation of the human *CRFR1* gene structure. The size of exons (in bp) is depicted *below* the exons. Deviating sizes of murine exon sizes are additionally indicated in parentheses and gray. For better visualization, different scales were used for exons (red) and introns (black lines). *B*: human and murine *CRFR1* splice variants. Exon segments encoding the signal peptide are highlighted by a yellow vertical bar. Exon segments coding for transmembrane helices (TMs 1–7) are indicated as numbered vertical gray bars. Exons or exon segments in red indicate translated segments, whereas pink segments indicate 5′ or 3′ untranslated regions. 7TMD, seven transmembrane domain; ECD, extracellular domain; GPBD, G protein coupling domain; ICD, intracellular domain; SP, signal peptide.

soluble isoforms can function in a way similar to the CRF binding protein (CRF-BP) and regulate the bioavailability of the CRF-related ligands, acting as so-called "decoy" receptors (96).

B. CRF Receptor Type 2 (CRFR2)

The 16 exons of the human *CRFR2* gene that have been identified to date extend over ~50 kb on chromosome 7. In the mouse, the *CRFR2* gene is located on chromosome 6 (358). The *CRFR2* gene shows alternative splicing predominantly at its 5′ end, which may underlie its distinct physiological role compared with CRFR1. Amphibians possess the CRFR2α variant only (89), whereas primates and rodents have both CRFR2α (312) and CRFR2β (618). Thus far, the CRHR2γ variant has been identified in humans only (328). However, in mouse, a soluble variant of the CRFR2 was identified, which has not been found in other species (99). Whether this variant is also acting as "decoy" receptor, by regulating the availability of CRF-receptor ligands similar to CRF-BP, requires further investigations. It appears that the soluble variant is efficiently translated but not necessarily secreted (170). All of the CRFR2 splice variants arise through utilization of 5′ localized alternative first exons, which are spliced to a common second exon. These CRFR2 isoforms have identical 7-TMDs and COOH-terminal domains **(FIGURE 7)**. In addition, a large number of alternative splicing variants were detected to arise from the alternative 5′ exons, which, however, partially encoded for strongly truncated nonfunctional receptors (89, 416). Another splice variant of the CRFR2 was identified in mouse,

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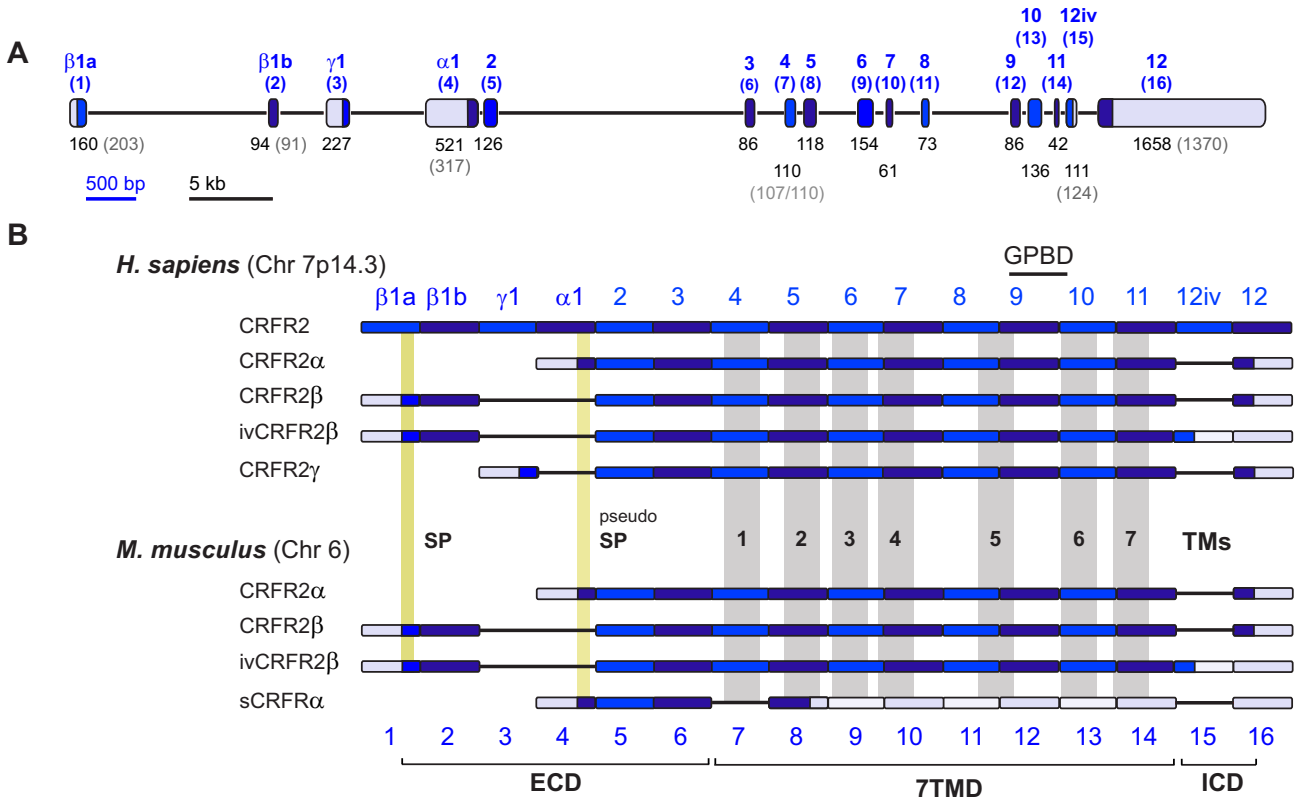

FIGURE 7. The *CRFR2* gene and isoforms generated by alternative splicing. *A*: schematic representation of the human *CRFR2* gene structure. The size of exons (in bp) is depicted *below* the exons. Deviating sizes of murine exon sizes are additionally indicated in parentheses and gray. For better visualization, different scales were used for exons (blue) and introns (black lines). *B*: human and murine *CRFR2* splice variants. Exon segments coding for transmembrane helices (TMs 1–7) are indicated as numbered vertical gray bars. Exons or exon segments in blue indicate translated segments, whereas light blue segments indicate 5′ or 3′ untranslated regions. 7TMD, seven transmembrane domain; ECD, extracellular domain; GPBD, G protein coupling domain; ICD, intracellular domain; SP, signal peptide.

which presumably also occurs in humans, since the identified exon is highly conserved. This insertion variant (ivCRFR2) is characterized by the use of an alternative last exon, resulting in a different COOH-terminal cytoplasmic domain **(FIGURE 7)** (589). The CRFR2 variants are expressed in a tissue-specific, but also species-specific, manner. In humans, CRFR2β and CRHR2γ are expressed in neurons of the brain, whereas CRFR2α is expressed in the periphery, such as in the heart, skeletal muscle, and skin (328, 618). In contrast, in the mouse, the splice variants are expressed in reverse, i.e., CRFR2α in CNS neurons and CRFR2β in the periphery (96, 382). In addition, human CRFR2α and murine CRFR2β are expressed in the choroid plexus, which produces cerebrospinal fluid (CSF) (505).

C. CRF Binding Protein (CRF-BP)

The biological activity of CRF-related peptides is mediated via CRFR1 and CRFR2. In addition, the CRF-BP regulates the availability of CRF by its capability to sequester the peptide. The human *CRF-BP* gene is located on the long arm of chromosome 5 (623) and extends over 16 kb and includes seven exons (35). The CRF-BP was isolated and characterized before the identification of the CRFRs. This discovery was directly related to the observation that the normally very low CRF concentration in the peripheral circulation (370, 371, 585) dramatically increases during pregnancy in humans (196, 542). The CRF is produced directly by the placenta. At the end of gestation, plasma CRF concentrations are reached that typically elicit the secretion of ACTH in the pituitary portal system (175, 479). Paradoxically, maternal ACTH and cortisol levels remain largely unchanged during pregnancy (502). The isolation of the 37-kDa CRF-BP from the blood plasma ultimately provided an explanation for the unchanged ACTH and cortisol levels. The CRF-BP binds CRF, thereby neutralizing its biological activity and preventing unwanted activation of the HPA axis (34, 371, 451, 484). In contrast to humans, the rodent placenta produces no CRF. However, CRF-BP is not only present in the plasma and pituitary gland, but is also

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widespread in the CNS, where it regulates the biological availability of CRF and UCN1 but not UCN2 or UCN3 in humans and rodents (31, 359, 485, 631). In addition, CRF-BP also seems to facilitate some of the actions of CRF/UCN1 in a CRFR2-dependent manner (616, 647). The alternative functional roles, as an autocrine/paracrine modulator of local CRF/UCN1 effects compared with the role as a regulator of receptor function at synapses, might depend on the observed differential cell-type-specific ultrastructural localization of CRF-BP (475).

D. Central and Peripheral Receptor Expression

Similar to the analysis of ligand expression, the comprehension of the distribution of CRFRs has been hampered by some difficulties with respect to antibody sensitivity and specificity (504), which might explain some of the observed discrepancies between mRNA and protein expression (107). Nevertheless, the obvious mismatch of mRNA expression when comparing ligand and receptor distribution can only be solved on the protein level. Another valuable method of receptor detection is based on highly specific radioligands; however, these do not provide the same spatial resolution as mRNA in situ hybridization or immunohistochemistry.

Since their initial discovery, several studies have focused on the central and peripheral expression of CRFRs, particularly in rat or mouse (90, 107, 382, 486). Despite both being rodents, there are several differences in expression patterns between mouse and rat. For example, CRFR1 is highly expressed in the nucleus incertus of the rat, but only lowly expressed in the corresponding nucleus of the mouse (630). This is of relevance, considering that the nucleus incertus has gained considerable attention as a brain stem nucleus involved in the stress response, arousal, and memory. In the rat nucleus incertus, CRFR1 is coexpressed with relaxin-3 (340, 387, 534). Similarly, significant differences in distribution of CRFRs between rodents and primates have been reported (329, 538). In the periphery, CRFR1 is found in the skin, GI tract, lymphatic tissues, and testis (19, 73, 403, 461, 574, 613). High expression is also found in the anterior and intermediate lobes of the pituitary, where CRFR1 stimulation triggers secretion of ACTH into the circulation. The most complete analysis of mRNA expression of CRFR1 and CRFR2 in the brain has been provided by the group of Paul E. Sawchenko [van Pett et al. (630)]. This detailed study was recently complemented on the protein level using the high-affinity radioligand PD-sauvagine (473, 593). CRFR expression in the CNS is dominated by CRFR1, which is expressed throughout the brain with moderate to high levels of expression in olfactory regions (including olfactory bulb, olfactory tubercle, and piriform cortex), throughout cortical structures (with highest level in cortical layer IV), hippocampus (CA1, CA2, and polymorph layer of dentate gyrus), subregions of the BNST, basal ganglia [including globus pallidus, ventral tegmental area (VTA), and substantia nigra], reticular thalamic nucleus, red nucleus, geniculate nucleus, reticular thalamic nucleus, hypothalamic nuclei (e.g., arcuate nucleus), periaqueductal gray (PAG), pedunculopontine nucleus, cerebellum, and multiple brain stem nuclei **(FIGURE 8)** (630). In addition, two reporter mouse lines have been generated (293, 339) and repeatedly used to address CRFR1 expression in the brain (192, 193, 209, 249, 500, 530, 590, 617, 655).

The CRFR2 is strongly expressed in skeletal muscle and heart (cardiomyocytes), as well as in smooth muscle cells of the vascular system (304, 344). In addition, expression has been reported in the pancreas (274). This peripheral expression pattern of CRFR2 and its ligands UCN2/3 is interesting in the light of the receptor's role in metabolic function (342). In the CNS, strongest CRFR2 expression is found in the LS, ventromedial hypothalamus, mammillary nucleus, as well as in the raphe nucleus (90, 382, 383, 630). In the raphe nucleus, CRFR2 is mainly found in the dorsal and median aspect, where it is predominantly expressed in serotonergic neurons (132, 385).

Although experimentally not tested, it appears that coexpression of both CRFRs is relatively limited. Recently, the first Cre driver lines for CRFR1 (193, 540) and CRFR2 (8, 245, 562) have been reported, which provide access to CRFR-expressing neurons to express reporters or other effectors to manipulate their properties.

CRF-BP shows differential expression in primates and rodents. In primates, CRF-BP has been detected in the liver, placenta, anterior pituitary, and brain, whereas rodents show only expression in pituitary and brain (307, 484, 660). CRF-BP is expressed in the anterior pituitary, where it is mainly localized to ACTH expressing corticotropes (485). In addition, CRF-BP is dimorphically expressed in male and female mice. Compared with males, CRF-BP shows a stronger upregulation by stress in females and is additionally expressed in pituitary gonadotropes and lactotropes. In the latter, CRF-BP expression is directly regulated by estrogen (577, 584). In corticotropes, CRF-BP was detected in secondary lysosomes and multivesicular bodies, but not in secretory granules. This speaks against corelease with ACTH, but rather suggests a role in processing and degradation of CRF or ligand receptor complexes, a process that might be involved in restoration of HPA axis homeostasis after stress (475). Accordingly, mice overexpressing CRF-BP in the anterior pituitary show a compensatory upregulation of CRF in the PVN to maintain normal HPA axis function (76). In the CNS, CRF-BP is expressed throughout the cerebral cortex, where it is, for instance, expressed in a specific class of interneurons also expressing the oxytocin receptor (363). In the prefrontal cortex, CRF-BP is ex-

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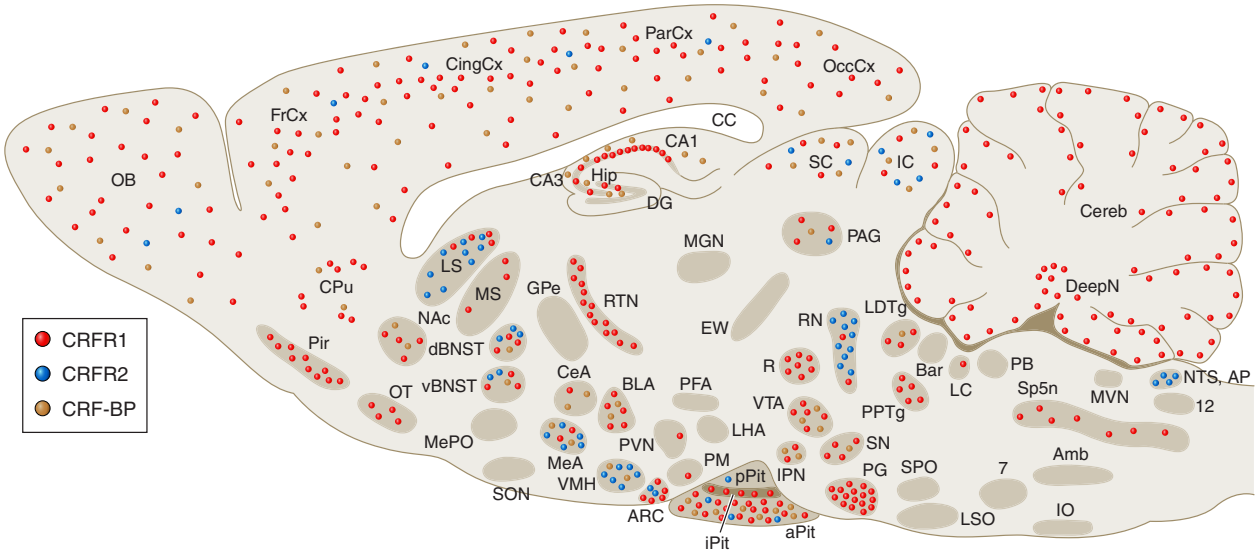

FIGURE 8. Distribution of mRNA expression of corticotropin-releasing factor (CRF)-related pepCRFR1, CRF receptor 2 (CRFR2), and CRF-binding protein (BP) in the rodent brain. Three-dimensional expression patterns of CRFRs and CRF-BP were collapsed onto a single sagittal brain section. Depicted are well-documented sites of high to moderate expression. Sites of expression are indicated by colored squares: CRFR1 (red), CRFR2 (dark blue), CRF-BP (brown). 7, Facial nerve; 12, hypoglossal nerve; Amb, ambiguous nucleus; AP, area postrema; arc, arcuate nucleus; Bar, Barrington's nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis (d, dorsal aspect; v, ventral aspect); CA1, cornu ammonis subfield 1; CA3, cornu ammonis subfield 3; CC, corpus callosum; CeA, central amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CPu, caudate putamen; DeepN, deep nucleus of cerebellum; DG, dentate gyrus; EW, Edinger Westphal nucleus; FrCx, frontal cortex; GPe, external globus pallidus; Hip, hippocampus; IC, inferior colliculus; IO, inferior olive; IPN, interpeduncular nucleus; LC, locus coeruleus; LH, lateral hypothalamus; LS, lateral septum; LSO, lateral superior olive; LTDg, laterodorsal tegmental nucleus; MeA, medial amygdala; MePO, median preoptic area; MGN, medial geniculate nucleus; MS, medial septum; MVN, medial vestibular nucleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OccCx, occipital cortex; OT, olfactory tubercle; PAG, periaqueductal gray; ParCx, parietal cortex; PB, parabrachial nucleus; PFA, perifornical area; PG, pontine gray; Pir, piriform cortex; Pit, pituitary (p, lobe, anterior lobe, intermediate, posterior lobe); PM, premammillary nucleus; PPTg, pedunculopontine tegmental nucleus; PVN, paraventricular nucleus of the hypothalamus; R, red nucleus; RN, raphe nuclei; RTB, reticular thalamic nucleus; SC, superior colliculus; SN, substantia nigra; Sp5n, spinal trigeminal nucleus; SPO, superior paraolivary nucleus; VMH, ventromedial hypothalamus, VTA, ventral tegmental area.

pressed in GABAergic neurons predominantly coexpressing SOM, whereas the colocalization with PV- and CCK-expressing neurons is only minor (308). Moreover, CRF-BP is found in the olfactory bulb, the hippocampus (particularly in cells in the stratum oriens), and all subnuclei of the amygdala. Expression in the hypothalamus is rather limited (307, 485). In the VTA, CRF-BP is confined to a subset of dopaminergic neurons **(FIGURE 8)** (648). Considering the function of CRF-BP as a protein sequestering CRF, it is interesting that there is only limited coexpression of CRF-BP on the cellular level. However, colocalization of CRF terminals and CRF-BP-expressing cells has been detected. Along this line, direct coexpression might not be required due to the secretory capacity of the CRF-BP glycoprotein (660).

E. Ligand-Receptor Interaction

The activity or availability of CRF-related peptides, CRFRs and CRF-BPs, is primarily controlled at the transcriptional level (74, 96, 97, 121, 169, 295, 433, 458, 669), which is further subjected to dynamic regulation by epigenetic mechanisms (100, 167, 415, 568, 583, 625, 658). In addition, mRNA processing and alternative splicing are common ways of regulating class B GPCR availability (188). On the protein level, the activity of the CRF system is regulated by the direct interaction of CRF-related ligands with their receptors and binding proteins. While CRF has a relatively higher affinity for the CRFR1, UCN1 binds with similar affinity to CRFR1 and CRFR2. In contrast, UCN2 and UCN3 are selective ligands of CRFR2 **(FIGURE 9)**. Activation of CRFR1 by UCN2 has only been reported at very high, i.e., nonphysiological concentrations (359).

On the receptor site it is the large NH_2-terminal ECD that predominantly determines ligand specificity, but also the ECLs and TMDs are crucially involved in the interaction **(FIGURE 10)** (201, 202, 472). Studies on a soluble variant of the ECD of CRFR1 have identified disulfide bonds between Cys^{30} and Cys^{54}, Cys^{44}, and Cys^{87}, and between Cys^{68} and

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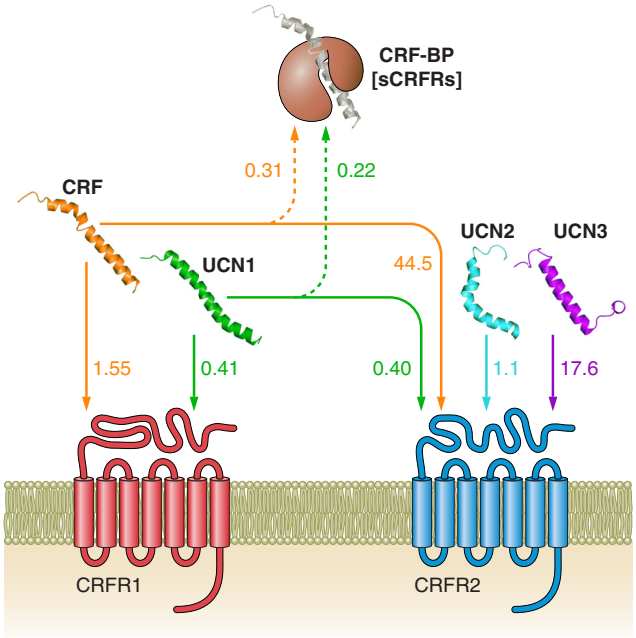

FIGURE 9. Interactions of corticotropin-releasing factor (CRF)-related peptide ligands with their receptors and binding proteins. The neuropeptides CRF and urocortin (UCN) 1 can bind to CRF receptor type 1 (CRFR1) and CRFR2. UCN2 and UCN3 are exclusive ligands of CRFR2. CRF-binding protein (BP) is able to sequester CRF and UCN1 and thereby controls their availability for receptor activation. To what extent soluble variants of CRF receptors (sCRFRs) bind CRF-related peptide ligands requires further investigation. Ligand-receptor affinities are indicated next to the arrows. Values are averaged K_i (nM) values collected from different studies (129, 131, 150, 164, 359, 631).

Cys^{102} (259, 469, 491). These disulfide bridges are also found in the CRFR2, as well as in the NH_2-terminal domains of all other members of the B1 subfamily (208, 467). A further disulfide bridge is thought to occur between the first two ECLs, since the cysteines Cys^{188} and Cys^{258} are conserved in all class B GPCRs (181, 301, 349). Furthermore, a conserved Sushi domain or short consensus repeat (SCR) domain was identified by nuclear magnetic resonance-based structural analyzes in CRFR2β. The SCR/Sushi domain is characterized by the three disulfide bridges mentioned above and includes a stabilizing internal salt-bridge (CRFR2: Asp^{65}-Arg^{101}; CRFR1: Asp^{49}-Arg^{85}). SCR/Sushi domains are present in complement and adhesion proteins, where they promote protein-protein interactions as so-called complement control protein modules (201, 470, 471).

Cross-linking studies and mutagenesis of amino acid residues in the NH_2-terminal domain have identified different regions essential for ligand binding. These include in particular amino acid residues 43–50 and 76–84. In addition, the amino acid segment in the immediate vicinity of the first TM plays an important role in ligand receptor interaction acting as a hinge between the ECD and TMD **(FIGURE 10)** (203, 250, 662). Interactions with the ECLs are also important for ligand binding affinity. In the J-domain, two regions in the first ECL have an important function: amino acids 175–178, as well as Arg^{189} (CRFR2, His^{189}), located between ECL1 and TM3. Further important regions are located at the junction of ECL2 to TM5-Val^{266}-Tyr^{267}-Thr^{268}, as well as at the junction of ECL3 to TM7-Tyr^{346} and Asn^{348}. The latter is important for the binding of CRF and UCN1, whereas Asp^{254} is particularly important for the binding of sauvagine to ECL2 (366, 367).

Finally, glycosylation of CRFR1, and presumably also CRFR2, plays another important role in ligand binding. Glycosylation of at least three of the four highly conserved potential glycosylation sites, Asn^{38}, Asn^{45}, Asn^{78}, and Asn^{90}, is a prerequisite for the functionality of CRFR1 (15). Asn^{90} and Asn^{98} appear to be particularly important for glycosylation. A lack of glycosylation at these two amino acids leads to reduced cell surface expression. Although G protein coupling is unaffected, the efficiency of CRF-induced cyclic adenosine monophosphate (cAMP) production and extracellular signal regulated kinase (ERK)1/2 phosphorylation appears to be adversely affected (224).

Extensive biochemical studies have identified the amino acid residues that are primarily responsible for the CRFR2 selectivity of UCN2 and UCN3. For example, Pro^{11} (numbering according to mature human CRF) is only found in CRFR2-specific peptide ligands (285, 401). Other important amino acid residues are Ala^{35} and Ala^{39} **(FIGURE 2A)**. Introduced into the nonselective peptide ligands CRF and UCN1, they confer increased affinity for CRFR2. Conversely, their substitution in UCN2 reduces its affinity for CRFR2, while it increases the affinity for CRFR1 (401, 455). Similar to other GPCRs of the B1 subfamily, the binding of CRF and related peptide ligands to the CRFRs follows a two-domain model that takes into account the modular structure of these ligands **(FIGURE 11)** (251, 254). The mature biologically active peptide can be divided into three functional units: *1*) the NH_2-terminal domain (amino acids 1–16), which is essential for agonistic receptor binding and activation (213, 445); *2*) the central binding domain (amino acids 17–31), which contains the binding site for CRF-BP with the Ala^{22}-Arg^{23}-Ala^{24}-Glu^{25} motif (284); and *3*) the COOH-terminal domain (amino acids 32–41), which is responsible for binding to the receptor's ECD and thus is responsible for the affinity and specificity of the ligand **(FIGURE 2A)** (200, 250).

In aqueous solution, peptide ligands show a rather variable, not clearly defined, random-coil conformation. It is only on binding that the formation of an α-helix occurs (444, 579). As a first step of receptor activation, the COOH-terminal domain of the ligand binds to the receptor's ECD, which is the main binding site on the receptor. This positions the NH_2-terminal signal transduction domain of the ligand in

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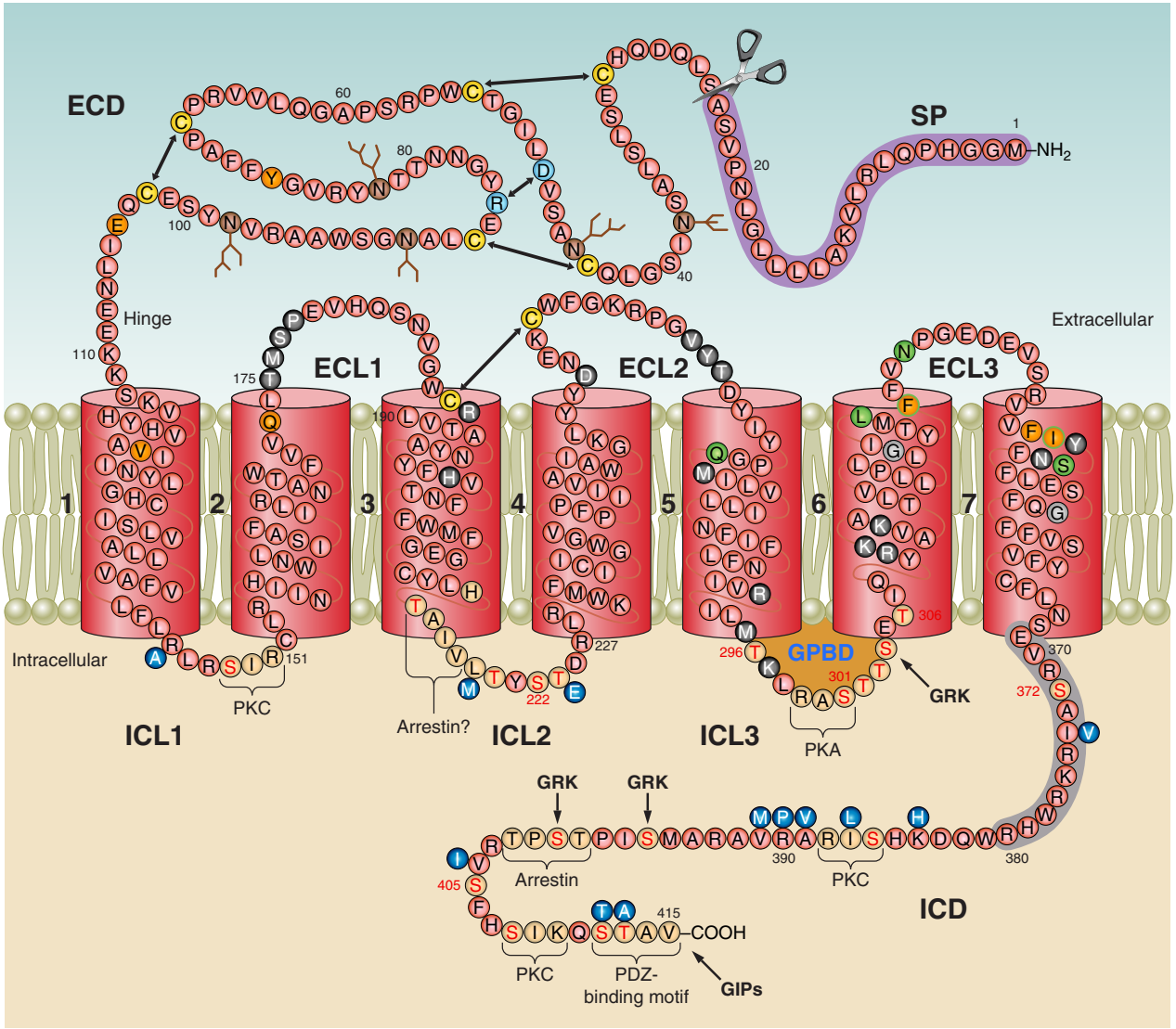

FIGURE 10. Topography of the human corticotropin-releasing factor (CRF) receptor type 1 (CRFR1). Snake plot of the human CRFR1α. The transmembrane (TM) domains are delineated according to the CRFR1 crystal structure (261). In the extracellular domain, the NH_2-terminal signal peptide (purple background) and its cleavage site are indicated. The extracellular portion of the CRFR1 comprises eight cysteine residues forming four disulfide bonds indicated in yellow (Cys^{30}-Cys^{54}, Cys^{44}-Cys^{87}, Cys^{68}-Cys^{102}, Cys^{188}-Cys^{258}). Amino acid residues forming the salt bridge of the short consensus repeats (SCR)/SUSHI domain are indicated in blue (Asp^{49}-Arg^{85}). Potential glycosylation sites are indicated by symbolic glycosylation trees (Asn^{38}, Asn^{45}, Asn^{78}, Asn^{90}, Asn^{98}). Amino acid residues in black and brown are highlighted in the text with regards to their role in ligand and G protein interaction. Amino acid residues in orange and green have been identified by photo-cross-linking to directly interact with CRF and urocortin (UCN) 1, respectively (551). The intracellular domain (ICD) is highly conserved between CRFR1 and CRFR2. Respective amino acid substitutions in CRFR2 are indicated as blue amino acid residues depicted adjacent to the CRFR1 polypeptide sequence. Of note, the COOH-terminal PDZ-binding motif is absent in CRFR2. Substitutions in the extracellular domain are not depicted for clarity reasons. The portion of the ICD forming *helix 8* is indicated by a gray background. In the ICD, confirmed and hypothetical binding sites for arrestins are depicted. Similarly, sequence motifs amenable to phosphorylation by protein kinase A (PKA), protein kinase C (PKC), or G protein-coupled receptor kinase (GRK) are indicated in beige. Potentially phosphorylated serine or threonine residues are shown in red on a beige background. ECD, extracellular domain; GIPs, GPCR-interacting proteins; GPBD, G protein binding domain; ICD, intracellular domain; ICL, intracellular loop; SP, signal peptide; TM, transmembrane helix.

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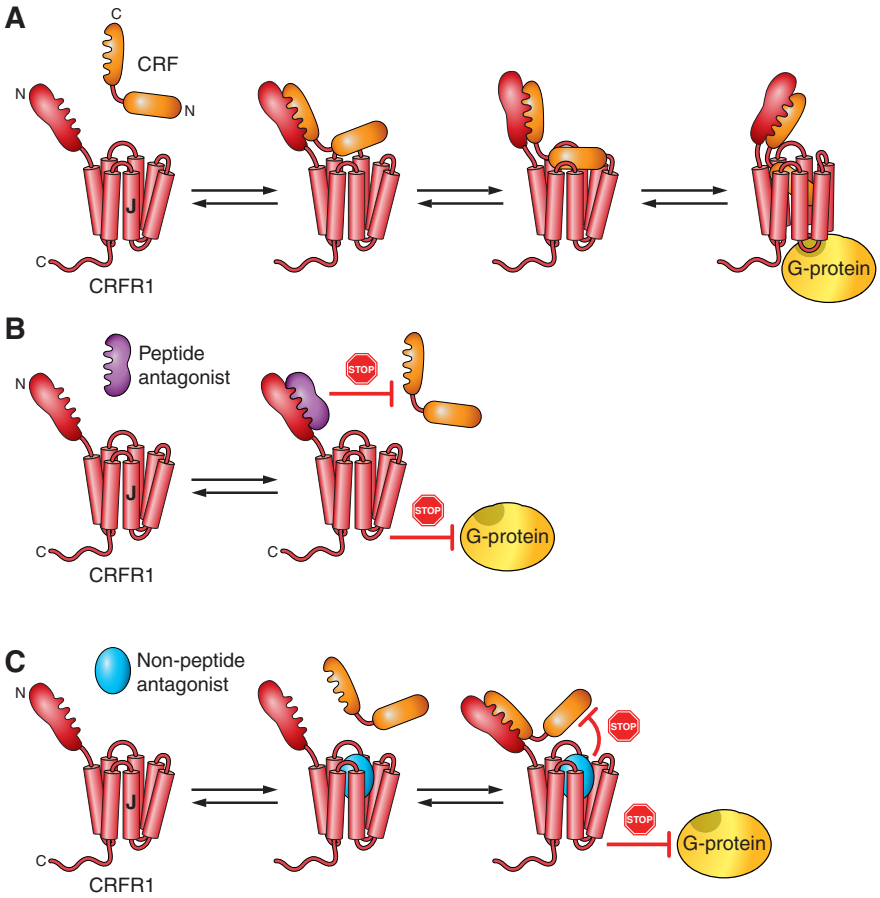

FIGURE 11. Interaction of corticotropin-releasing factor (CRF) and different categories of antagonist with CRF receptor type 1 (CRFR1). *A*: The COOH-terminal (C) region of CRF binds to the NH_2-terminal (N) extracellular domain (ECD) of CRFR1. This interaction increases the local concentration of the NH_2-terminal CRF region in the vicinity of the J-domain, facilitating the weak interaction. Binding of CRF to the J-domain promotes a conformational change and the receptors' interaction with a G protein. This interaction further stabilizes the CRF-CRFR1 interaction. *B*: binding of a peptide antagonist that lacks an NH_2-terminal activating domain. The peptide binds to the ECD of CRFR1 but cannot bind the J-domain to induce conformational changes required for G protein interaction. *C*: binding of a nonpeptide antagonist within the J-domain causes a conformational change that blocks ligand binding to the J-domain, preventing G protein binding. CRF binding to the ECD of CRFR1 is not blocked.

the immediate vicinity of the juxtamembrane region (J-domain) of the receptor **(FIGURE 11*A*)** (16, 255, 256, 445). All peptide ligands of the B1 subfamily of GPCRs show a highly conserved helical N-capping motif, which stabilizes the NH_2-terminal helices by a specific local complex. This process defines the orientation of the ligand's backbone and the specific spatial positioning of the side chains, which ultimately determines the pharmacological properties. The CRF-related peptides have a relatively longer NH_2-terminus compared with other B1-family ligands, but also show an N-capping motif in the NH_2-terminal domain. In the case of CRF, amino acids 6–10 of the NH_2-terminus comprise this motif: [Ile/Leu/Met]^6-Ser-[Ile/Leu]-Asp-Leu^{10} **(FIGURE 2*A*)** (260, 444, 460).

The NH_2- and COOH-terminal domains of these ligands are functionally largely independent of one another (43). This property has been used to generate a constitutively active CRFR1 by the direct fusion of the NH_2-terminal CRF domain to the NH_2-terminus of CRFR1 (445). In the case of CRFR1, CRF binds to the ECD (amino acids 1–118) with moderate affinity. The interaction with the J-domain stabilizes this binding minimally (twofold), whereas the binding is stabilized greatly when the interaction with the J-domain is succeeded by G protein binding, (>1,000-fold) (37, 250). The binding of G proteins stabilizes CRFR1 conformation and increases ligand affinity by an allosteric effect **(FIGURE 11*A*)** (256). Interestingly, this has not been observed to the same extent for CRFR2 (474). The CRFR1β variant, which carries an insertion of 29-amino acids in the first ICL, shows reduced G protein coupling and thus reduced ligand affinity. The J-domain ultimately determines the G protein coupling sensitivity of the receptor. The change in conformation, resulting from this coupling, changes in turn the receptor's affinity for the ligand. Different conformations of the J-domain result in the coupling to different G proteins (37). Like other GPCRs, CRFRs bind to heterotrimeric GDP/GTP-bound G proteins, resulting in an exchange of GDP (inactive) to GTP (active state). When GTP is bound to the Gα-subunit, the Gβ/γ dimer dissociates, and, via the Gα-subunit, various signal transduction pathways are activated. Which pathways are activated largely depends on the nature of the G protein.

Antagonists, e.g., astressin, which lack the NH_2-terminal domain, bind to the ECD with high-affinity blocking agonist binding. Due to the absence of the NH_2-terminus, G protein coupling is prevented and, consequently, also the activation of signal transduction **(FIGURE 11*B*)** (37, 520). In contrast, COOH-terminally truncated ligands can activate

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the receptor but bind with only low affinity (255, 256, 467). Different agonists and antagonists lead to the formation of different receptor conformations, which, in turn, differentially regulate receptor internalization. While CRF leads to rapid internalization of the CRFR1 involving phosphorylation and recruitment of β-arrestin 2, astressin-mediated internalization is much slower and independent of both clathrin-coated pits and caveolae (474).

Small molecule antagonists bind almost exclusively to the J-domain by allosteric interaction, which partially inhibits peptide ligand binding **(FIGURE 11*C*)** (254, 255, 674). These studies suggest that the currently available antagonists based on small molecules are allosteric inhibitors rather than competitive CRFR1 antagonists. In particular, two amino acid residues located in the TM3 (His^{199}) and TM5 (Met^{276}) of CRFR1 seem to play an important role for the affinity of nonpeptide ligands. The substitution of these amino acid residues with the corresponding residues of CRFR2 (Val^{199} and Ile^{276}) results in a reduction in the affinity of the CRFR1-specific antagonist NBI27914 by a factor of 40–200 (252, 366, 367).

More detailed insights into the structure of CRFR1 and its interaction with a small-molecule antagonist have been provided by the crystal structure of a core CRFR1 comprising TM1–TM7 (261). The crystal structure shows that some of the amino acids important for ligand binding are not located at the extracellular end of the TM helices or in the ECLs, as originally assumed, but are located much deeper within the membrane. Along these lines, the binding site for small-molecule antagonists is located unexpectedly deep in the cytoplasmic half of the CRFR1, at some distance from the orthosteric binding site (152, 261). It appears that the ligands are present in a conformation that increases flexibility of the first few amino acids, thus allowing the ligand to extend deeper into the binding pocket between the TM helices (261). Additional features identified in the crystal structure are the fact that the NH_2-terminal helical turns of TM1 extend into the extracellular space and an unusually long helix 8 (Asn^{367}-Arg^{380}) connected to TM7 (125).

The interaction of CRFR1 with its peptide ligands CRF and UCN1 was revealed in high resolution using photo-cross-linking in living cells, confirming previous sites of interaction, but also revealing previously unrecognized sites (118, 551). In the same study, chemical pairwise cross-linking identified residue pairs of close proximity between the COOH-terminus of CRF and the receptor's ECD (CRF-CRFR1; Ser^{33}-Tyr^{73}), hinge region (Ala^{31}-Glu^{104}), and TM1 (Val^{18}-Val^{120}), as well as between the NH_2-terminus of CRF and the receptor's TM2 (Leu^{15}-Gln^{173}), TM6 (His^{13}-Phe^{330}), and TM7 (His^{13}-Phe^{344}, Glu^{17}-Ile^{345}). CRF binds the ECD of the CRFR1 via its α-helical COOH-terminal portion (Ile^{41}-Val^{18}). The peptide enters the receptor's TMD in the groove formed by TM helices 1, 2, and 7. It undergoes a conformational change forming a short loop (Arg^{16}-Leu^{14}) and an α-helical segment (His^{13}-Ser^{7}). The peptide has contact to all TMs, except TM4. The NH_2-terminus (Ile^{6}-Ser^{1}) exits the binding pocket between TMD helices 5 and 6. The binding of CRF or a CRF-based peptide antagonist (CRF_{12-41}) results in different receptor confirmations: agonist-bound "wide" vs. antagonist-bound "compact" conformation. These different receptor states are generated by bending of the helical TMs 6 and 7, which involves ECL3/ECD, as well as flexible glycine hinges in TM6 (Gly^{324}) and TM7 (Gly^{356}). These glycine residues are fully conserved among class B GPCRs, suggesting that the stabilization of a specific conformation of these TM helices on agonist binding could represent a general mechanism for the activation of class B GPCRs and might, for example, entail the opening of the G protein binding pocket (551). The recently solved cryo-EM structure of the calcitonin receptor in complex with a trimeric G_s protein demonstrated that ligand binding results in an outward movement of the extracellular ends of TM helices 6 and 7 that is accompanied by an outward movement of the intracellular end of TM helix 6, allowing the interaction with $G\alpha_s$ (365).

IV. CRF SYSTEM-RELATED SIGNAL TRANSDUCTION

A. G Protein Coupling

Ligand-induced binding of G proteins stabilizes the receptor's conformation, thereby promoting increased ligand affinity, and determines the activation of various downstream signal transduction pathways (251). Early studies demonstrated that stimulation of CRFRs in cultures of primary pituitary cells leads to activation of G_s and elicits a cAMP response (414). Most physiological functions of CRF/CRFR1 have been assigned to G_s coupling. However, as shown experimentally, binding to other G proteins also occurs. For example, CRF/CRFR effects in testis-derived Leydig cells and membranes derived from the feto-placental unit are predominantly mediated via $G\alpha_q$ or $G\alpha_o$, although $G\alpha_s$ is present in both tissues (303, 614). Overexpression experiments in heterologous systems confirm that both CRFR1 and CRFR2 primarily activate the $G\alpha_s$ adenylyl cyclase/cAMP signaling pathway. However, both receptors are highly promiscuous and, in principle, can activate any $G\alpha$-subunit, although with different potency: $G\alpha_s \geq G\alpha_o > G\alpha_{q/11} > G\alpha_{i1/2} > G\alpha_z$ (206, 207, 250, 348). There are indications that an intact conformation of the COOH-terminal ICD of the CRFR1 is important for interaction with G proteins; however, the structures or sequences that determine the binding of the receptor to G proteins are still not completely understood (206). The diversity of the GPCRs suggests that structural motifs, in the form of conformation or charge, and, to a lesser extent, the primary peptide sequence play a role in G protein interaction (180). It has been shown that the ICL3 plays a central role in G protein bind-

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ing (272, 476). In particular, basic and aliphatic amino acids in the juxtamembrane regions of ICL3 that form α-helical structures are important for G protein activation (612). For CRFR1, amino acids in the region of the transitions TM5/ICL3 (Arg^{292}-Met^{295}) and ICL3/TM6 (Lys^{311}-Lys^{314}) are shown to be important for an active conformation of CRFR1 and thus directly involved in the specific recognition of G proteins following agonist binding **(FIGURE 10)**. Correspondingly, the simultaneous substitution of these proximal and distal motifs in ICL3 by alanine residues leads to reduced ligand binding and disturbed signal transduction. Interestingly, however, the substitution of only one of the two motifs leads to increased adenylyl cyclase activation; simultaneously, activation of inositol trisphosphate (IP_3) and ERK1/2 is diminished. Furthermore, residues Arg^{292}, Lys^{297}, Arg^{310}, Lys^{311}, and Lys^{314} in ICL3 are important for the activation of adenylyl cyclase **(FIGURE 10)**, which is partly explained by a reduction in the inhibition of adenylyl cyclase by G_i proteins. Thus, these highly conserved hydrophobic microdomains within ICL3 coordinate the CRFR1-mediated ERK1/2 signal transduction primarily by activation of G_i and G_q proteins (490).

Intracellular mechanisms are another important aspect in the interaction between CRFRs and G proteins since they enable the receptor to couple to different G proteins and thus activate different signaling cascades. It is known from other GPCRs that phosphorylation by intracellular protein kinases plays an important role in this context. In primary cells derived from pregnant myometrium, UCN1, but not the CRF, activates the mitogen-activated protein (MAP) kinase signaling pathways via CRFR1 and stimulates G_q proteins to activate the IP_3-protein kinase C (PKC) signaling pathway (207). It was shown that activation of the adenylyl cyclase protein kinase A (PKA) signaling pathway inhibits UCN1-induced activation of the MAP kinase ERK1/2. This inhibition involved a phosphorylation of CRFR1 at Ser^{301} in the ICL3, which results in selective inhibition of CRFR1 binding to $G\alpha_q$ (457). Similarly, the additional peptide sequence in the first ICL of the CRFR1 variant β conveys reduced G protein binding (432).

As described above, it is assumed that peptide ligands induce conformational changes in the J-domain, which can then, for example, bind either to $G\alpha_s$ or $G\alpha_i$ **(FIGURE 12)**. There are indications that the coupling of CRFR1 to G_s and G_i takes place via two distinct J-domain conformations. These conformations are antagonized competitively (G_s) or allosterically (G_i) by nonpeptide antagonists (37). Evidence for the existence of distinct receptor conformations, which result in ligand-specific signal transduction, has already been demonstrated at the level of G protein coupling. By substituting single amino acids in UCN1 at *positions 6–15*, it was possible to create ligands that activate G_s but could no longer activate G_i. These peptide analogs, which are simultaneously agonists and antagonists, activate different G proteins by the same receptor, suggesting that there are different active conformations, depending on the G protein bound (42).

In vivo, the mechanisms of coupling of CRFRs to G proteins are still largely unexplored, mainly owing to the complexity of tissues. A further indication of the complexity of G protein coupling is the observation that CRFRs in the hippocampus of C57BL/6J mice activate $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_i$, whereas they just activate $G\alpha_{q/11}$ in Balb/c mice. This might be due to the different configuration with G proteins in the two mouse lines (50). Recently, in a mouse model for epileptic seizures, switching of G protein coupling of the CRFR1 could be detected. As a consequence of epileptic seizures, a switchover of coupling from $G\alpha_{q/11}$ to $G\alpha_s$ occurs in the piriform cortex. This is primarily explained by a reduced availability of the regulator of G protein signaling type 2 (RGS2), which has been reported to inhibit $G\alpha_s$-dependent signaling. This altered coupling was also confirmed in RGS2 knockout mice (434).

Taken together, experiments applying cell lines with endogenous expression or heterologous (over)expression, as well as studies using primary cells, mainly from pituitary, pregnant myometrium, or feto-placental tissue, have revealed the intricacy of G protein coupling to CRFRs. Key factors determining G protein coupling are as follows: *1*) the cellular equipment with G proteins, *2*) intrinsic properties of receptors and their binding domains, *3*) changes in receptor conformation induced by binding of different ligands and *4*) modulation of receptor conformation by different kinases and phosphatases. Finally, other proteins, e.g., RGS proteins, might be involved in this process. However, we are just beginning to understand the mechanisms underlying the preference for coupling to a specific G protein (180).

B. Signaling Pathways

CRFRs have the competence to couple to different G proteins, enabling them to activate a broad spectrum of downstream signaling pathways. The activation is dependent on the cellular configuration, which provides the respective signaling machinery and molecules. Moreover, it is assumed that simultaneous activation of different signaling pathways can occur, which in turn can interact with one another, be it in a synergistic or inhibitory manner (14, 205, 238).

As already explained, CRFRs couple primarily to G_s proteins activating the adenylyl cyclase cAMP signal pathway. $G\alpha_s$ binds to adenylyl cyclase, thereby catalyzing the formation of the second-messenger cAMP from ATP with the elimination of pyrophosphate (597). Elevated intracellular cAMP and/or Ca^{2+} levels lead to activation of intracellular kinases, such as PKA or calcium/calmodulin-dependent kinase II (CaMKII). cAMP binding to the regulatory subunit

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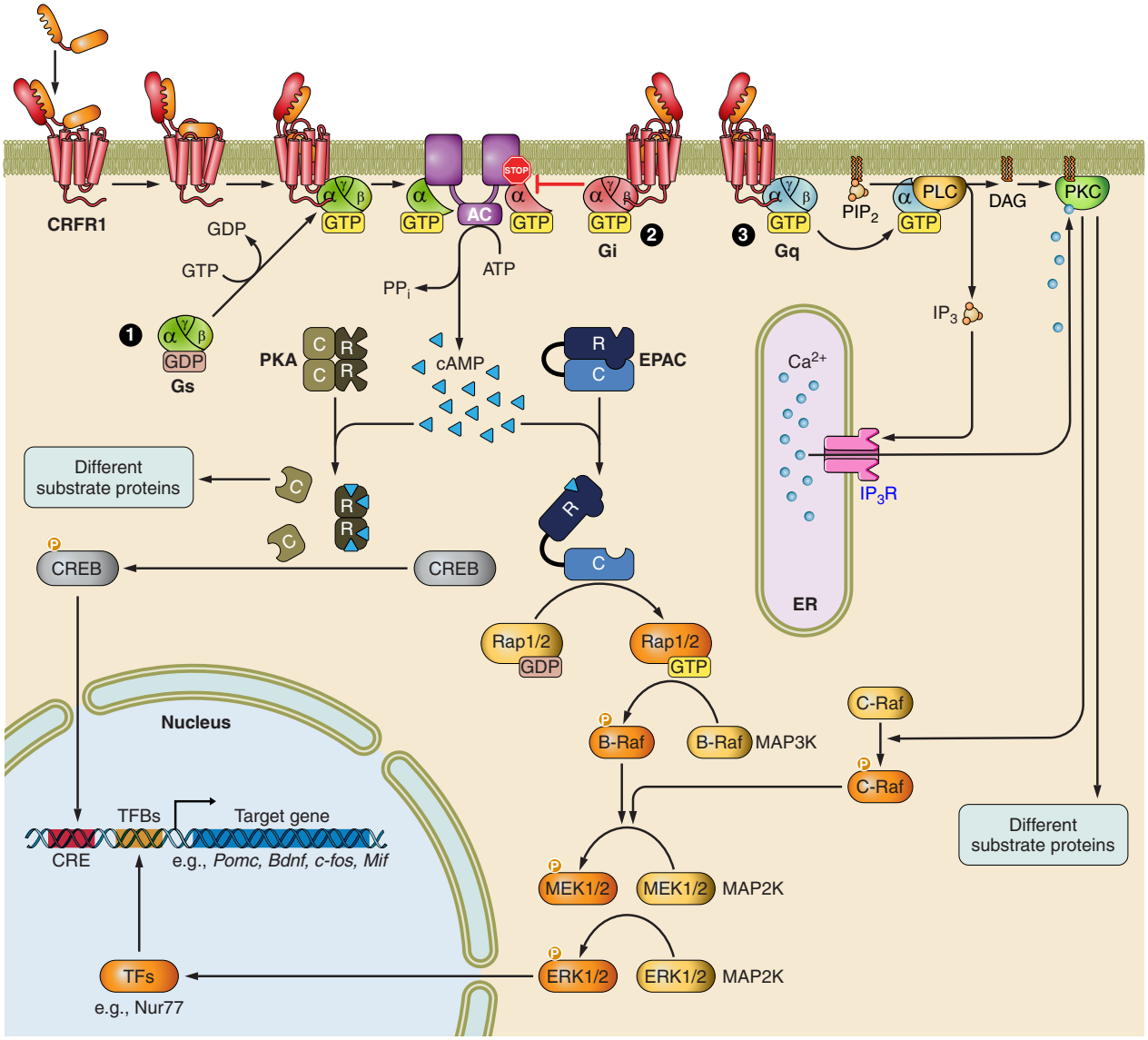

FIGURE 12. Corticotropin-releasing factor receptor type 1 (CRFR1)-dependent signaling pathways. Binding of a peptide ligand increases the affinity of CRFR1 for G proteins. The activation of downstream signaling pathways depends on G protein binding (*1*). Binding of G_s activates membrane-bound adenylyl cyclase (AC) and synthesis of cAMP, which activates protein kinase A (PKA). PKA phosphorylates different substrate proteins, including CREB (Ca^{2+}/cAMP response-element binding protein), which regulates expression of different target genes in the nucleus, such as *Pomc*. In parallel, cAMP activates EPAC (exchange proteins directly activated by cAMP), which in turn activates a MAP kinase cascade, leading ultimately to phosphorylation of ERK1/2. ERK1/2 activates different transcription factors (TFs), including Nur77 in the nucleus (*2*). In contrast, binding of G_i blocks the activity of adenylyl cyclases (*3*). Alternatively, the binding of G_q activates phospholipase C (PLC) and the processing of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates IP_3 receptors, mobilizing Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER). Ca^{2+}, together with DAG, activate protein kinase C (PKC), which phosphorylates various substrate proteins and activates the MAP kinase cascade and ultimately ERK1/2. IP_3R, inositol trisphosphate receptor; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase.

of PKA releases the catalytic subunit, which in turn phosphorylates target proteins in the cytosol or nucleus. One important target protein of general relevance is the transcription factor CREB (Ca^{2+}/cAMP response-element binding protein). CRF-induced CREB phosphorylation leads to its binding to cAMP response elements (CREs) in promoter sequences and transcription of target genes such as *c-fos* (59) or the macrophage migration inhibitory factor (640)

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(FIGURE 12). However, the activation of downstream genes can also be affected indirectly by other mechanisms. For example, the expression of proopiomelanocortin (POMC) in the pituitary cell line AtT-20 is regulated by the nuclear transcription factors Nurr1/Nur77, whose expression is in turn induced via CRFR1 and the downstream cAMP-PKA signaling pathway (429, 430). Here, the CREB phosphorylation plays no role; instead the activation of PKA via CRF and cAMP stimulates two parallel signal transduction pathways (330). One signaling pathway is calcium dependent and involves the CaMKII. The other is calcium independent. In addition, CRF binding to CRFR1 activates the MAP kinase signaling pathway, which involves the small G proteins Rap1 and B-Raf, as well as the MAP kinases MEK and ERK1/2. This signal cascade leads to phosphorylation and activation of Nur77 (FIGURE 12). Ultimately, these different signaling pathways regulate the expression of POMC, from which ACTH is liberated by proteolytic processing (330). In AtT-20 cells, both PKA- and PKC-dependent signaling pathways are involved in regulation of CREB activity through its phosphorylation. In contrast, in A7r5 cells, which express only CRFR2, CREB phosphorylation involves only the PKA pathway. CRF increases phosphorylation of ERK1/2 in AtT-20 cells, while UCN1 reduces its phosphorylation in A7r5 cells in a PKC-dependent manner, a finding that is in contrast to other studies (61). Taken together, these findings suggest that phosphorylation of CREB and ERK1/2 are differentially regulated by the two CRFRs (294).

Besides the classical adenylyl cyclase cAMP-PKA signaling pathway, MAP kinase-dependent signal transduction mechanisms are particularly well understood. Here, one must discriminate between results involving heterologous (over)expression of the CRFRs in cell lines and primary cells and results originating from cell lines with endogenous receptor expression (61). The two different approaches are believed to be an explanation for the partially contradictory results of different studies.

In the pheochromocytoma cell line PC12, CRF activates the MAP kinase p38 via CRFR1. This is a prerequisite for activation of Fas ligand expression, as well as the induction of apoptosis. In parallel, CRF can activate ERK1/2 rapidly and transiently in PC12 cells; however, this signaling pathway is not associated with apoptotic processes (142). Furthermore, CRF in PC12 cells differentially regulates the phosphorylation of PKC isoforms. While CRF induces the rapid phosphorylation of PKC-α and PKC-β and increases their concentration in the cell nucleus, it inhibits the phosphorylation of PKC-δ and PKC-θ, which remain predominantly in the cytoplasm. The blockade of conventional PKC phosphorylation inhibits CRF-induced intracellular Ca^{2+} mobilization and expression of Fas ligand as well as CRF-induced apoptosis (141).

Both CRFR1 (via CRF and UCN1) and CRFR2 (via UCN1, UCN2, and UCN3) are able to activate the ERK1/2 (61). Activation of ERK1/2 via CRFRs was systematically tested in stable CRFR1- or CRFR2-expressing Chinese hamster ovary (CHO) cells. In addition, cell lines with endogenous CRFR expression were analyzed, including CATHa (murine cholinergic cells: $CRFR1^+$, $CRFR2^+$), AtT-20 (murine anterior pituitary corticotrope cells: $CRFR1^+$, $CRFR2^-$), and A7r5 (rat smooth muscle cells: $CRFR1^-$, $CRFR2^+$). These studies show that the activation of ERK1/2 by CRF-related ligands correlates with the cell type used and the stimulated receptor. Surprisingly, CRF does not induce ERK1/2 activation in stably transfected CHO cells via CRFR2, suggesting differences in the agonist-induced conformation and the associated coupling to different signal transduction mechanisms (61, 238). However, the effect of CRF on the maximal ERK1/2 activation is stronger than that of UCN1. For both receptors, the activation was shown to depend on phosphatidylinositol-3-OH (PI_3) kinase, MAP kinase 1, and phospholipase C (PLC). Moreover, UCN1-dependent activation plays a central role in Raf-1 kinase, tyrosine kinases, and intracellular Ca^{2+} signaling. In endogenous cells, this UCN1-dependent activation is also dependent on G_i and G_o proteins (61). However, the classical activation of adenylyl cyclase via G proteins may also play a role, since it can contribute to the activation of Rab1, B-Raf, and MEK via PKA, and as a result of which ERK1/2 can also be activated. Accordingly, UCN1-mediated activation of ERK1/2 via PKA has been shown in some cell types, such as hippocampal neurons (462). In contrast, other studies using CHO cells stably transfected with CRFRs have demonstrated that activation of ERK1/2 is completely independent of PKA (531). Here, the ERK1/2 activation occurs instead via PI_3 kinase, PI-specific PLC, and the Ca^{2+}-sensitive proline-rich tyrosine kinase, Pyk-2. In the myometrium in which both different CRFR1 splice variants and CRFR2 are expressed, only UCN1, but not CRF, activates the MEK1-ERK1/2-MAP kinase signaling pathway (207).

A further signaling pathway leading to the activation of ERK1/2 involves cAMP-dependent mechanisms mediated by exchange proteins directly activated by cAMP (EPACs; FIGURE 12) (629). EPAC2 is a guanine nucleotide exchange factor, which is directly activated by cAMP and is also known as Rap guanine nucleotide exchange factor 4 (58). In the pituitary or corresponding cell lines (e.g., AtT-20), CRF-dependent activation of ERK1/2 among others activated the expression of POMC. This regulation is cAMP dependent, whereas ERK1/2 activation is independent of PKA. Other studies show that ERK1/2 activation in AtT-20 cells with endogenous CRFR1 is independent of PKA, PKC, or PI_3 kinase (294). In contrast, CREB activation/phosphorylation is regulated by PKA. In particular, EPAC2 appears to be important for ERK1/2 activation in AtT-20 cells. Along this line, the downregulation of EPAC2 by short

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interfering RNA inhibits CRF-induced ERK1/2 phosphorylation (629). The same was observed in the differentiation of noradrenergic (NA) neurons. Here, CRF has a synergistic effect on brain-derived neurotrophic factor-induced differentiation. This effect is mediated by CRF-dependent activation of ERK1/2, which is also dependent on EPAC (608).

The CRF/CRFR1-induced activation of ERK1/2 has a biphasic kinetic, i.e., in the early phase signal transduction depends on cAMP and B-Raf, whereas the late phase depends on CRFR1 internalization and β-arrestin 2. Both phases are Ca^{2+} dependent and are influenced by CaMKII (55). Recently, it has been shown that CRFR1 is able to activate the atypical soluble adenylyl cyclase (sAC) besides the classical TM adenylyl cyclases. Both adenylyl cyclases are relevant for the cAMP production and required for the acute activation of ERK1/2 immediately following CRFR1 activation. However, for the sustained and prolonged signaling following receptor internalization, sAC is used as an alternative source of cAMP production, which is essential for sustained ERK1/2 activation and endosome-based signaling **(FIGURE 13)** (278). In addition, CRF effects on growth arrest and neurite outgrowth of a hippocampal neuronal cell line depend directly on sAC activation, but not on ERK1/2 activation (277).

Stimulation of both CRFR1 and CRFR2 leads to Ca^{2+} mobilization from intracellular Ca^{2+} stores of HEK-293 cells. This can be completely blocked by inhibition of PLC. Activation of PLC and the PLC-dependent IP_3 production via G_q was also demonstrated in Leydig cells and in the placenta (303, 614). Intracellular Ca^{2+} mobilization occurs primarily through activation of IP_3 receptors, whereas neither PKA nor ryanodine receptors seem to play a role in Ca^{2+} release into the cytoplasm from the sarcoplasmic reticulum (221). Activation of PLC involves primarily G_q, but also G_i **(FIGURE 12)**. In addition, $G\alpha_s$ can also mobilize intracellular calcium stores via EPAC (299), and EPAC activates the PLC-ε isoform via the small GTPase Rap-1 and converges with the G_q/G_i signaling pathway onto Ca^{2+} mobilization (221). In contrast, CRF stimulation in the neuroblastoma cell line SK-N-MC does not lead to Ca^{2+} mobilization. Interestingly, these cells lack two important molecules that play a role in the context of CRF-dependent calcium mobilization, the PLC-ε and EPAC2 (130).

Conversely, ex vivo recordings from brain slices revealed that Ca^{2+} release from intracellular stores of CRFR-expressing midbrain dopaminergic neurons can be positively modulated by CRF via adenylyl cyclase and PKA, with both ryanodine and IP_3 receptors appearing to play a role here. The increase in cytoplasmic Ca^{2+} potentiates Ca^{2+}-sensitive potassium channels, which are normally activated by action potentials or G_q-coupled glutamate or acetylcholine receptors. Therefore, CRF can influence G_q-coupled regulation of excitability and plasticity in dopamine-producing neurons (514). In vivo, CRF selectively activates ERK1/2 in specific regions of the brain via CRFR1, suggesting that this signaling pathway plays an important role in stress adaptation as a function of cellular context and configuration (503).

Taken together, the results mainly obtained from heterologous expression and cell lines possessing endogenous CRFRs and to a lesser extent also from in vivo studies revealed that the activation of CRFR downstream signaling pathways largely depends on the cellular context and requirements. The promiscuity of CRFRs toward the entire range of G proteins initially enables activation of a broad spectrum of signaling pathways. The initiation of downstream signaling pathways is determined by coupling to a specific G protein and further defined by the cellular availability of signaling components. The net effect of CRFR activation is a consequence of concurrent activation of different signaling pathways and their mutual interference.

C. Regulation of Desensitization

In general, GPCR signaling pathways are regulated by processes of homologous and heterologous desensitization. These processes are primarily controlled by phosphorylation of the receptor. For desensitization, phosphorylation occurs primarily via GPCR kinases (GRKs), but can also be influenced by PKA and PKC **(FIGURE 13)** (172, 250, 336, 528). GRK inhibition leads to a significantly reduced desensitization in the retinoblastoma cell line Y-79 (601). Besides GRK3, other studies emphasize the importance of membrane-bound GRK6 (263, 601). In addition, GRK2 and GRK5 have been shown to reduce the recruitment of β-arrestin 2 to CRFR1 and receptor desensitization (263). These findings are consistent with earlier studies that showed that GRK3 and GRK6 are the major isoforms interacting with CRFR1. The nonmembrane-bound isoform GRK3 also requires Gβγ-subunits and β-arrestin for recruitment at the receptor. Furthermore, Thr^{399} appears to be important in the COOH-terminus of CRFR1 for GRK-induced receptor phosphorylation and desensitization. The substitution of this threonine residue with an alanine residue significantly reduces CRF-induced receptor phosphorylation and desensitization (601). It is possible that Thr^{399} is preferentially phosphorylated by GRK3, activating the Thr^{399}-Ser^{400}-Pro^{401}-Thr^{402} motif for β-arrestin binding **(FIGURE 10)** (448). Since GRKs do not have a consensus motif for phosphorylation, it is quite possible that other serine/threonine residues besides the Thr^{399} play a role in this process. In addition, it has to be mentioned that receptor phosphorylation does not appear to be an absolute prerequisite for desensitization.

In general, phosphorylation leads to recruitment of arrestins, which in turn results in the decoupling of the G protein and receptor, and finally initiates receptor endocytosis and

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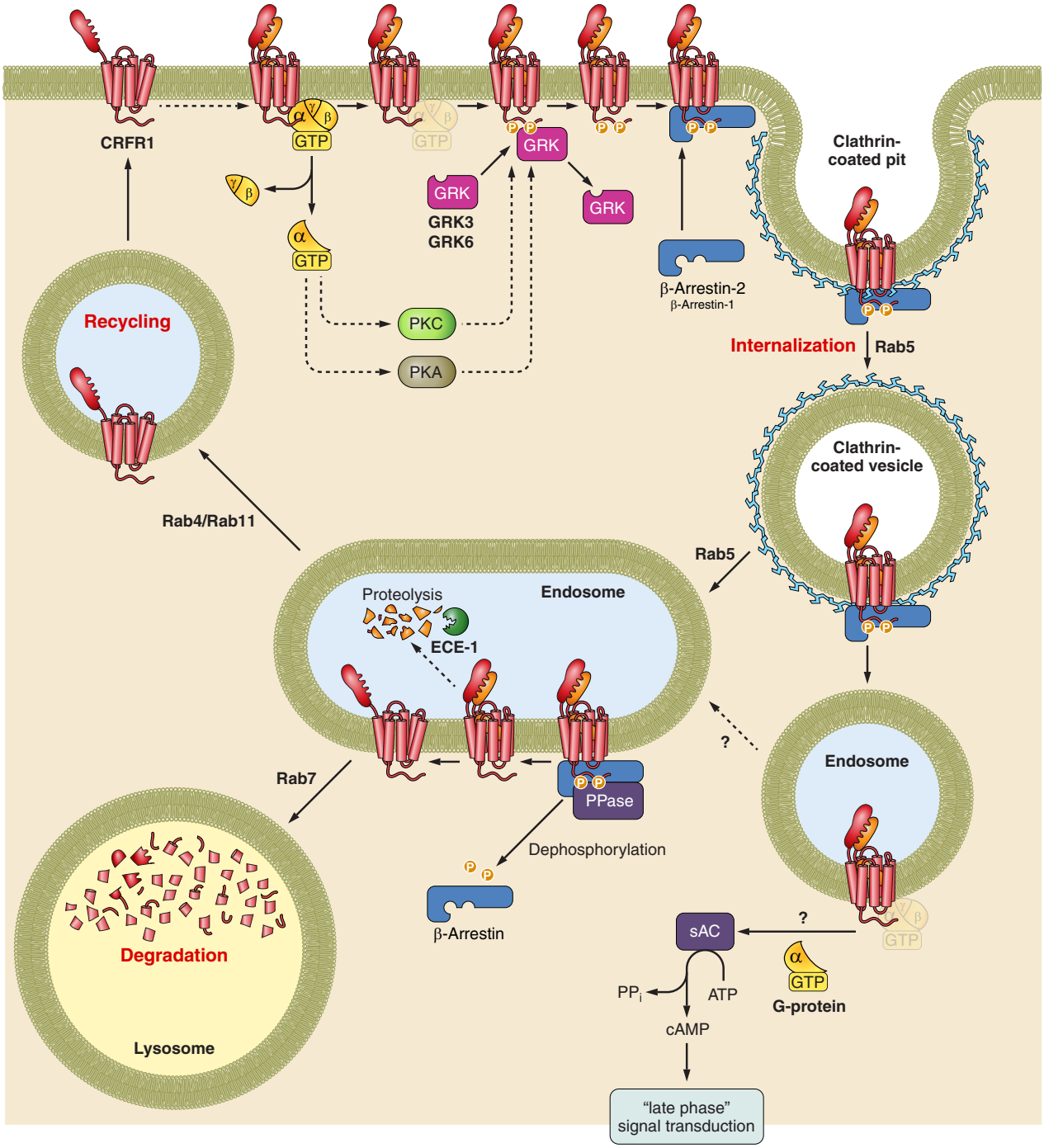

FIGURE 13. Mechanisms of desensitization exemplified by corticotropin-releasing factor receptor type 1 (CRFR1). Receptor desensitization is initiated by G protein coupling and receptor phosphorylation by G protein-coupled receptor kinases (GRKs), protein kinase A (PKA), or protein kinase C (PKC), resulting in recruitment of β-arrestins. β-Arrestins act as adaptor proteins binding to components of the endocytosis machinery, such as clathrin and β-adaptin (not shown for better visualization) to initiate internalization via clathrin-coated pits. The CRFR1 is sorted to the endosomal compartment, where the peptide ligand is degraded, e.g., by endothelin-converting enzyme-1 (ECE-1), and phosphorylation of the receptor occurs. Subsequent degradation in the lysosome leads to permanent desensitization. Alternatively, resensitization can occur by CRFR1 recycling to the cell membrane. In the endosomal compartment, stimulation of soluble adenylyl cyclase (sAC) provides late-phase signal transduction.

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internalization **(FIGURE 13)** (148, 186, 234, 238, 239, 386, 446, 528, 601). For CRFRs, these processes have been extensively studied in recent years (237, 238). After agonist activation, CRFR1 has a preference toward recruiting β-arrestin 2 compared with β-arrestin 1 (263, 448). It was also shown that none of the β-arrestin isoforms are internalized together with CRFR1, which suggests that CRFR1 is in fact a "class A" GPCR. Only cortical neurons have been found to induce internalization of β-arrestin 2, however, mainly in CRFR1-negative vesicles (263). Class A GPCRs are characterized by comparatively fast recycling and resensitization (386). The categorization as a class A GPCR corresponds to the observed resensitization of the CRFR within 1–2 h of desensitization in HEK-293 cells (258, 263, 601). However, conditions under which resensitization occurs after 24 h were observed also, which better corresponds with CRFR1 being a "class B" GPCR (234, 528). Furthermore, it has been shown that β-arrestin binding can also take place via phosphorylation-independent mechanisms (501).

CRFR1 has various serine and/or theronine residues in its intracellular COOH-terminal domain and third ICL, which represent potential GRK phosphorylation sites. Truncation of the receptor at Ser^{386} leads to an almost complete blockade of agonist-induced phosphorylation of the receptor, but only a partially reduced recruitment of β-arrestin 2. Mutagenesis of a serine-threonine cluster in the third ICL reduced phosphorylation, but did not lead to any alteration in β-arrestin 2 binding. The difference in the extent of the phosphorylation between the two mutant receptor variants suggests hierarchical phosphorylation, i.e., that it is carried out first at the COOH-terminus and only thereafter in the ICL3. When phosphorylation is blocked at the COOH-terminus, phosphorylation at ICL3 cannot occur either. The same mechanism was shown for the dopamine D1 receptor (309). Combining the truncation of the COOH-terminus with a mutated ICL3 led to complete loss of ligand-dependent phosphorylation, but did not completely prevent β-arrestin 2 recruitment. Surprisingly, this modified receptor also showed no limitations with respect to membrane localization and receptor-induced cAMP production (448). Similar observations were made in experiments in which all serine and theronine residues of the ICL3 and Ser^{372} were substituted with alanine residues, and the COOH-terminus was truncated at Lys^{384}. This CRFR1 variant was only limited in β-arrestin 1 binding, without any impairment of receptor internalization (501). This behavior suggests that, besides the serine and threonine residues in the ICL3, as well as in the intracellular COOH-terminus, there are other receptor structures that are capable of a phosphorylation-independent interaction with β-arrestin following receptor activation. One possibility is that the ICL2, whose proximal part (His^{214}, Ala^{216}-Ile^{217}-Val^{218}-Leu^{219}) is similar to parts of the ICL2 of GPCRs of the rhodopsin family, may show structurally determined phosphorylation-independent arrestin binding **(FIGURE 10)** (395). Alternatively, other amino acids, such as a lysine residue in the third ICL (Lys^{382}) or an asparagine residue in the third TM helix (Asp^{289}), have been shown to be important for the interaction with β-arrestin in the parathyroid hormone receptor, another member of the B1 subfamily of GPCRs (635).

Receptor-bound β-arrestins terminate the interaction of the receptor with its G protein and simultaneously functions as an adapter protein that binds to components of clathrin-dependent endocytic machinery, including clathrins and β_2-adaptin (AP-2). Therefore, internalization occurs via clathrin-coated vesicles and dynamin-dependent endocytosis (117, 336, 386, 489). Internalization of CRFRs occurs predominantly via clathrin-coated vesicles, which can be inhibited by hypertonic sucrose, K^+ depletion, or dominant-negative dynamin mutants, components of the trafficking machinery (263, 448, 501). It has also been suggested that receptor internalization can occur via caveolae. For the CRFR1, this pathway does not seem to play a role, as shown by experiments with the cholesterol-depleting and caveolae-inhibiting agent filipin III, as well as with a dominant-negative variant of caveolin 1 (474, 546). Interestingly, clathrin-coated vesicles are internalized after CRF stimulation, but not after treatment with the antagonist astressin. Astressin is truncated at the NH_2-terminus. Thus activation of the receptor cannot take place; instead, competitive inhibition of agonists occurs. In addition it was shown that parts of the J-domain play a role in astressin binding and receptor internalization. Astressin, unlike CRF, does not induce receptor phosphorylation or recruitment of β-arrestin. Nevertheless, internalization is still observed to a limited extent, but the route is unclear because it is dependent on neither clathrin-coated vesicles nor caveolae (474). After internalization, many GPCRs are downregulated by being sorted into lysosomes for degradation or being sequestered in intracellular compartments, representing a receptor pool that, on demand, can be redirected to the membrane **(FIGURE 13)**. In addition to their classical roles in GPCR desensitization, β-arrestins are also critically involved in receptor endocytosis, ubiquitylation, and signaling events (566). Therefore, it is highly likely that the well-established diversity of β-arrestin-dependent signaling is also accessible to the CRFRs.

After ligand binding, CRFR1 in HEK-293 cells and primary cortical neurons is initially sorted into Rab5-positive early endosomes (529). This is followed by rapid recycling of Rab4-positive endosomes (263). Other studies have shown a slower recycling through Rab11-positive endosomes, which may be explained by the different cellular systems and agonist concentrations that had been used **(FIGURE 13)** (229). A prerequisite for receptor recycling is the dephosphorylation of the receptor by phosphatases (318), as well as the separation of the ligand from the receptor, which can be achieved, for example, by metalloendopeptidases, such as the endothelin-converting enzyme-1 (ECE-1). In the case

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of CRFR1, differential degradation by ECE-1 protects the receptor from overactivation (229). In addition to the desensitization primarily mediated by GRKs, GPCR desensitization can occur via phosphorylation of serine or threonine residues in the intracellular consensus PKA motif. As described, signal transduction via CRFR1 is dominated by coupling to G_s and the activation of the adenylyl cyclase cAMP-PKA signaling pathway. Ser^{301} is part of the typical PKA phosphorylation site, and its phosphorylation has an influence on the activation of the MAP kinase ERK1/2 without significantly influencing desensitization (457, 601). PKA was originally thought to play a dominant role in CRFR1 desensitization (102). However, neither maximal PKA stimulation nor inhibition has a significant effect on CRFR1-phosphorylation or desensitization (128, 239). Similarly, there are studies demonstrating that pharmacological inhibition of PKA or PKC does not lead to any alteration in receptor internalization, indicating it may be independent of the second-messenger kinases, PKA and PKC (501). In addition, neither pharmacological inhibition of PKA nor its activation changes the desensitization with respect to CRF-induced cAMP accumulation (128).

CRFR1 has a series of serine/threonine residues representing potential PKC phosphorylation sites Ser^{146}, Ser^{222}, Ser^{386}, and Ser^{408} **(FIGURE 10)**. The substitution of Ser^{408} by an alanine residue makes CRFR1 susceptible to PKC-induced desensitization and β-arrestin-independent internalization. It is possible that Ser^{408} is the primary target of PKC-induced phosphorylation, which in turn inhibits CRFR1 desensitization (600). It has been shown that PKC plays a particular role in desensitization of the CRFR1β variant, which has reduced agonist binding and signal transduction (666). The two potential PKC phosphorylation sites (Ser^{386} and Ser^{408}) in the intracellular COOH-terminal domain seem to be involved in desensitization processes (236). However, it is also quite possible that PKC-mediated desensitization takes place via heterologous interactions with other G_q-coupled GPCRs. For example, the activation of oxytocin receptors has been shown to induce a heterologous desensitization of CRF-stimulated cAMP production (206). Alternatively, homologous desensitization mechanisms may play a role in which the agonist-activated CRFR couples itself to G_q and induces phosphorylation via PKC.

CRFR1 trafficking has also been studied in vivo in the LC using electron microscopy (509). Local application of CRF, but also swim-induced stress, causes CRFR1 internalization into early endosomes, allowing for receptor degradation or recycling, thereby regulating the sensitivity of the LC to CRF (510, 511). Stress seems to differentially affect CRFR1 internalization in male and female rats. In females, CRFR1 association with β-arrestin 2 is impaired, which might explain the reduced internalization compared with males. The resulting increase in CRFR1 signaling renders female LC $CRFR1^+$ more sensitive even to low levels of CRF and thus less adaptable to stress levels of CRF (24).

In summary, CRFR desensitization is initiated by receptor phosphorylation through GRKs, PKA, or PKC, entailing β-arrestin recruitment and receptor endocytosis via clathrin-coated vesicles. The receptors' fate is either sorting to lysosomal degradation or rerouting to recycling, i.e., resensitization, via the endosomal compartment involving ligand degradation and receptor dephosphorylation. Recently, mechanisms of delayed receptor signaling in the endosomal compartment have emerged as another regulatory level **(FIGURE 13)**.

D. Interacting Proteins

GPCRs do not only interact with G proteins and β-arrestins, but also with many other proteins that can influence the receptor's properties and the resulting signal transduction. In the past few years, a variety of GPCR-interacting proteins (GIPs) have been identified for the CRFRs. A recently identified CRFR-interacting protein is the CRF-BP, which regulates CRF-system activity by sequestering CRF and UCN1. CRF-BP has also been shown to potentiate CRF and UCN1 effects through CRFR2 (616, 647). In addition, to its well-known function, CRF-BP has been demonstrated to physically interact with CRFR2α but not CRFR2β. This interaction is relevant for the control of CRFR2α trafficking from intracellular stores to the cell surface (572). Other potential interactors for CRFRs are receptor activity modifying proteins (RAMPs) (29, 240). The coexpression of CRFR1 and RAMP2 mutually enhances their cell surface expression and modulates G protein coupling of CRFR1. While binding to G_s remained unaffected, coupling to $G_{i/o/t/z}$, $G_{q/11}$, and $G_{12/13}$ was enhanced. In addition, the CRFR1-RAMP2 complex promoted increased intracellular Ca^{2+} levels in response to CRF or UCN1 stimulation (665). An unexpected interaction has been reported between CRFR1 and γ-secretase via β-arrestin binding motifs. CRFR1 and γ-secretase are colocalized in lipid rafts. CRF treatment increases γ-secretase activity and accumulation in lipid rafts (459). This interaction might be another functional link between stress effects and the pathophysiology of Alzheimer's disease, in addition to the findings describing the effects on tau phosphorylation and β-amyloid peptide (Aβ) accumulation (80, 300, 516). To date, the majority of identified GIPs preferentially interact with the intracellular COOH-terminus of GPCRs. These interactions have a direct influence, for example, sorting into specific cell compartments, the interaction with different effectors, and the allosteric regulation of receptor functions (52). Many GPCRs have a motif at their outermost COOH-terminus, which is capable of interacting with so-called PDZ domains (named after the first proteins in which they were identified: PSD-95/Disk Large/ZO-1) (53, 159). In the case of CRFR1, a potential PDZ binding motif was postulated for the first

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time as part of a sequence comparison with PDZ binding motifs in the COOH-terminus of *N*-methyl-D-aspartic acid (NMDA) receptor subunits (327). The CRFR1 COOH-terminal sequence S^{412}-T^{413}-A^{414}-V^{415} is a typical class I PDZ-binding motif of the consensus sequence X_{P3}-S/T_{P2}-X_{P1}-Φ_{P0}, where Φ stands for 1 of the hydrophobic amino acids Val, Ile, or Leu (227).

Molecules interacting with the COOH-terminus of CRFR1 were systematically identified using overlay assays testing the interaction of glutathione *S*-transferase fusion protein corresponding to the CRFR1 COOH-terminus against 96 individual PDZ domains (160). In addition, a yeast two-hybrid approach was used to identify interaction partners of the COOH-terminus in a brain-specific cDNA library. Besides the well-known interactor β-arrestin, this approach also identified numerous proteins possessing PDZ domains (36). With both methods, particularly members of the family of membrane-associated guanylate kinases were identified. These include postsynaptic density protein 95 (PSD-95), PSD-93, synapse-associated protein 102 (SAP102), and SAP97, as well as the inverted membrane-associated guanylate kinases 1 (MAGI-1), MAGI-2, and MAGI-3. In addition, PDZ domains of the proteins cystic fibrosis TM conductance regulator-associated ligand (CAL; also known as Golgi-associated PDZ and coiled-coil motif-containing protein, GOPC), multi-PDZ domain protein 1 (MUPP1), protein tyrosine phosphatase non-receptor type 13 (PTPN13), and PDZ domain-containing 1 (PDZK1, also known as Na^+/H^+ exchange regulatory cofactor 3, NHERF3) were identified as potential interaction partners. The fact that other PDZ domains of related proteins, such as NHERF1, NHERF2, or NHERF4, did not interact with the COOH-terminus of CRFR1 supports the specificity of the applied methods (160).

These potential interaction partners and their influence on CRFR1 function have been intensively investigated in recent years with respect to receptor localization, CRF-induced internalization, and signal transduction via the MAP kinases ERK1/2 and the second messenger cAMP. For PSD-95, PSD-93, SAP102, SAP97, MAGI-2, and CAL, heterologous expression and coimmunoprecipitation were able to demonstrate that protein interaction indeed occurs via the COOH-terminal PDZ-binding motif of CRFR1 (36, 158, 160, 224, 643). At the endogenous level, however, this could only be demonstrated for SAP97 and PSD-95. In addition, the PDZ domains that interact with the PDZ binding motif of the CRFR1 were specified (36). It was shown that stimulation of CRFR1 does not lead to any change in the interaction of the receptor with the respective PDZ proteins. In contrast, the subcellular localization of CRFR1 and PDZ proteins is influenced by the mutual interaction, albeit the deletion of the PDZ-binding motif in primary differentiated neurons did not lead to a clear change in the subcellular localization of the receptor (36). CRF-mediated receptor internalization appears to be dependent on an intact PDZ binding motif (158, 160). However, there are also studies that were not able to confirm this observation (224, 643). The effects on ERK1/2 activation depend specifically on the respective interaction partner, whereas the sole loss of the S-T-A-V motif does not have a serious effect on the MAP kinase signaling pathway. The same is true for CRF-induced cAMP generation, which is largely independent of the PDZ binding motif and unaffected by the interacting PDZ proteins, i.e., by their overexpression or knockdown.

The recruitment of SAP97 to CRFR1 and thus to the plasma membrane depends directly on an intact PDZ binding motif (160). Overexpression of SAP97 results in reduced CRF-induced receptor internalization, whereas a reduction in endogenous SAP97 expression enhances internalization as a function of an intact S-T-A-V motif. The inhibition of internalization is, however, only partial, and the remaining CRFR1 internalization occurs together with SAP97 in endocytic vesicles. In contrast, the interaction with SAP97 does not seem to have an effect on cAMP production, nor does SAP97 overexpression have an effect on CRF-induced ERK1/2 phosphorylation. Only the reduction of endogenous SAP97 expression by a short hairpin RNA (shRNA) results in reduced ERK1/2 phosphorylation in CRFR1-overexpressing HEK-293 cells. A similar effect on ERK1/2 phosphorylation was demonstrated by reducing SAP97 expression in endogenous CRFR1-expressing AtT-20 cells. Surprisingly, however, the effect on ERK1/2 phosphorylation was independent of the PDZ binding motif, which is further underlined by the fact that SAP97 has the same effect on CRFR2, although its COOH-terminus (T_{P3}-A_{P2}-A_{P1}-V_{P0}) lacks a functional PDZ binding motif (160).

In the case of the PDZK1, the interaction with the CRFR1 is reduced after deletion of the PDZ-binding motif, but is not completely abolished. PDZK1 has no effect on the endocytosis/internalization of CRFR1 (643). In contrast, PDZK1 overexpression leads to enhanced CRF-dependent ERK1/2 phosphorylation, which is strongly dependent on the COOH-terminal PDZ binding motif. Conversely, reduced PDZK1 expression results in decreased ERK1/2 phosphorylation. If the CRFR2 is equipped with the S-T-A-V motif of the CRFR1, it develops the ability to bind PDZK1 and also shows increased ERK1/2 phosphorylation after CRF stimulation (643).

The interaction of CAL with the CRFR1 depends entirely on the PDZ binding motif. Joint overexpression results in a colocalization and accumulation of both proteins in the Golgi complex. Therefore, the reduced endocytosis caused by CAL overexpression could also be a consequence of the retention in the Golgi complex. In contrast to SAP97, CAL regulates CRFR1-dependent ERK1/2 signal transduction negatively. In addition, CAL appears to affect CRFR1 gly-

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cosylation as a function of an intact PDZ binding motif. In particular, reduced glycosylation at Asn^{90} and Asn^{98} is correlated with overexpression of CAL, which is associated with reduced localization in the plasma membrane and a slightly reduced efficiency in CRF-induced cAMP production (224).

The interaction of CRHR1 with PSD-95 was revealed in the mouse cortex. PSD-95, however, has no effect on CRF-induced ERK1/2 phosphorylation. PSD-95 overexpression inhibits CRF-induced internalization in a PDZ-binding motif-dependent manner by inhibiting the recruitment of β-arrestin 2. However, neither the overexpression of PSD-95 nor the knockdown of endogenous PSD-95 had an effect on CRF-mediated activation of ERK1/2 or cAMP production (158).

It has to be taken into account that GIPs have so far been investigated almost exclusively in heterologous systems by overexpression of the respective interaction partner. It cannot be ruled out that overexpression of CRFR1 with the respective PDZ protein can also lead to nonphysiological interactions (573). In addition, overexpression could overwhelm the intracellular sorting machinery of the cell, leading to an inaccurate localization of the respective proteins. In addition, it needs to be clarified whether interactors are coexpressed with CRFR1, as this is one of the basic prerequisites for physical interaction. PSD-95, PSD-93, SAP102, SAP97, and MAGI-2 are coexpressed with the CRFR1 in the brain of the mouse, enabling an interaction (36). However, for instance PDZK1 is predominantly found in epithelial cells of the kidneys and the GI tract, but hardly in the brain, making an interaction questionable (643).

However, the interaction with GIPs not only affects the receptor itself, but can also influence the interaction with other GPCRs. It has already been shown that previous activation of CRFR1 leads to sensitization of serotonin (5-HT) receptor 5-HT_{2A}, so that its stimulation results in increased inositol phosphate production (389). In vivo, local CRF application in the prefrontal cortex resulted in increased anxiety in mice given the 5-HT receptor agonist 2,5-dimethoxy-4-iodoamphetamine. The CRF/CRFR1-mediated effects were dependent on increased internalization and faster receptor recycling. This resulted in an increased presence of 5-HT_{2A} receptors on the cell surface. The interaction is dependent on the presence of an intact PDZ-binding motif in both receptors (389). However, it is still unclear which PDZ domain protein is responsible, as neither SAP97 nor PSD-95 seem to mediate this effect (158, 161). Similarly, an indirect interaction of CRFR1 with the G protein-coupled estrogen receptor 1 (GPER1 or GPR30) was demonstrated. This interaction was promoted by PSD-95 and depended on the presence of intact PDZ-binding motifs in both receptors (4). The functional significance of this heteromerization remains unknown.

GPCRs were originally thought to act as monomers, but accumulating experimental evidence indicates that homo- and heteromerization is a common phenomenon, which in some cases has been shown to play an important functional role (298, 523). First indications for dimerization of CRFR1, which was independent of receptor activation, were obtained by fluorescence resonance energy transfer studies (332). It has been shown that CRFR1 exists in an equilibrium state of monomers and dimers that is already established in the endoplasmic reticulum (598). In addition, the formation of homo-oligomers of CRFR1 and CRFR2, respectively, has been reported. These appear also to have a rather fixed stoichiometry, which is independent of agonist stimulation, and in line with previous work suggesting that the oligomeric state of class B GPCRs is not subject to regulation by agonist occupancy (413). Other studies report that CRFR2α occurs mainly as a monomer that depends on its unique uncleaved pseudosignal peptide (533, 547, 599). Recently, the heteromerization of CRFR1 with CRFR2β has been demonstrated in HEK-293 cells coexpressing both CRFRs and in vivo in mouse pancreas, where both receptors are endogenously coexpressed. Coimmunoprecipitation and mass spectrometry showed that CRFR1 and CRFR2β form heteromeric complexes with actin. These complexes influenced the transport of CRF2β to the cell surface but had no effect on membrane availability of CRFR1. Stimulation of cells coexpressing both CRFRs with CRF and UCN2 resulted in twofold increase in peak Ca^{2+} responses, whereas stimulation with UCN1 was ineffective (230). Moreover, heteromerization of CRFR1 with class A GPCRs, such as the vasopressin receptors V1b (428, 670), its nonmammalian vertebrate homolog the vasotocin receptor 2 (VT2R) (412), or the orexin receptor 1 (OX1R) have been demonstrated (435). In these cases, the heteromerization has been shown to promote synergist effects of CRF and vasopressin, e.g., on cAMP production or potentiation of secretory processes, such as the release of catecholamines and corticosterone, respectively (412, 428). In turn, heterooligomers involving CRFR1 and OX1R mediate a negative cross-talk between CRF and orexin-A, which is influenced by cocaine and plays a role in dendritic dopamine release in the VTA (435). The interaction between GPCRs is presumably mediated by their TMDs, as seen in the case of VT2R. Here the TM4 was identified as the possible interface for dimerization between VT2R and CRFR1 (411).

In conclusion, the ability of CRFRs to form homo- and heteromeric complexes and to associate with different GIPs provides the means to achieve maximal diversification of function and mechanisms of regulation. The interaction with different GIPs is the basis for recruitment of CRFRs to different signaling complexes and concomitant localization to signaling microdomains, which are crucial units underlying cellular communication.

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E. Neuromodulation and Structural Plasticity

CRF has a dual mode of action: on the one hand it possesses all features of a classical hypothalamic releasing hormone or so-called secretagogue, and on the other hand it acts as a neuromodulator in the context of neuronal communication.

Intracerebroventricular (ICV) application of CRF at a low dose (0.0015–0.15 nM) causes changes in behavior suggestive of increased arousal, which can be detected, for example, by electroencephalogram (EEG) changes. Higher doses (1.5–3.75 nM) administered over a longer period cause epileptiform activity in the amygdala and hippocampus, similar to electrical "kindling" of the amygdala (165). Similarly, ICV infusion of CRF causes epileptic seizures in young rats (27, 28). In this context, CRF may also play a role in the development of febrile convulsions. The net effect of CRF is a preferential amplification of incoming excitatory signals that are strong enough to exceed the threshold for triggering an action potential (262). In addition to these excitatory properties, an inhibition of neurons in the thalamus and LS could also be detected after iontophoretic application of CRF (163).

Historically, CRF had been classified as a neurotransmitter and neuromodulator. However, the classification as a neurotransmitter has been dismissed due to the fact that only very high CRF concentrations (from ~1 mM) lead to a direct depolarization of neurons. Neurotransmitters in the narrower sense denote endogenous neuroactive substances, which, when released from nerve endings, lead to activation of synaptic membrane receptors, triggering a change in the membrane potential within a few milliseconds. These include substances that activate ionotropic receptors, e.g., GABA, glutamate, or acetylcholine (450). Physiological CRF concentrations in the nanomolar range (up to 250 nM) do not lead to a direct change in the membrane potential. In this concentration range, however, CRF increases the frequency of spontaneous action potential discharges in hippocampal pyramidal neurons and reduces the afterhyperpolarization following spontaneous bursts of spikes or spike trains evoked by depolarizing current pulses (5, 233). Of note, correct processing and amidation at the COOH-terminus of the neuropeptide is essential for these activities. Afterhyperpolarization in hippocampal pyramidal cells is a consequence of the activation of calcium-dependent potassium channels. CRF blocks the afterhyperpolarization by a mechanism secondary to calcium influx, possibly by direct antagonism of the potassium flow or by inhibiting the interaction of calcium and potassium currents. It was shown that CRF's effect is mediated at least by slow afterhyperpolarization, presumably by direct inhibition of slow Ca^{2+}-activated K^+ current in the hippocampal CA1 region. At the molecular level, this occurs mainly due to the activation of the cAMP-dependent PKA (233, 334). It has been speculated that the inhibition of the afterhyperpolarization leads to increased firing by a type of intrinsic disinhibition (5).

CRF has a similar effect in the dentate gyrus, where it was shown that it is responsible for long-term enhancement of synaptic efficacy, which is reflected by an increase of amplitude and slope of population excitatory postsynaptic potentials (650) that partially depends on a protein de novo synthesis (649). In agreement with these results, it was shown that direct application of CRF in the hippocampus increases long-term potentiation of population spikes similar to acute stress, which leads to a long-lasting increase of neuronal excitability and at the same time improves context-dependent fear conditioning. These effects could be blocked by CRFR antagonists or protein kinase inhibition (49). Interestingly, differences in the used mouse strains are observed. In C57BL/6N mice, the increased neuronal activity in the hippocampus is mediated via PKA, whereas in BALB/c mice the effects are mediated via PKC (50). Comparable results were reported by Wang and colleagues (649, 650), who observed a long-lasting improvement in the synaptic efficacy in the hippocampus of rats following CRF application, which was also cAMP dependent.

The characteristics of CRF in the CNS are typical features of a neuromodulator. Neuromodulators (e.g., biogenic amines, opiates, and neuropeptides) are endogenous substances that alter the action of a neurotransmitter, either by enhancing or repressing the primary alteration of the membrane potential by the neurotransmitter. A neuromodulator has a modulating activity as it changes the membrane potential or the electrical excitability in a location where the receptor and neurotransmitter are found. This neuromodulatory activity can lead to direct modification of neurotransmitter receptors (e.g., phosphorylation) or to a change in the number of receptors at the neuronal membrane. In general, neuropeptides can act on the postsynaptic level as well as on the presynaptic level, where they directly regulate neurotransmitter release (624). Receptors of neuromodulators are usually metabotropic receptors, as opposed to the ionotropic receptors for neurotransmitters. Accordingly, the speed with which a neuromodulator affects a response is in the range of several hundred to 1,000 ms (450).

In addition to the well-studied role of CRF in the hippocampus, a number of independent studies have investigated the effects of CRF and related ligands, as well as their receptors, by means of electrophysiological methods in various amygdalar nuclei and in the LS, especially in the context of cocaine effects (187, 191, 335, 372, 373, 449, 450, 481). In addition, there are extensive studies on the role of CRF in NA (LC) and dopaminergic nuclei (VTA). ICV but also local application of CRF leads to an increased discharge rate in the LC (620) and increases the firing rate of NA neurons in a dose-depending manner. These effects are at

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least partially CRFR1 dependent and cAMP mediated (288) and are associated with an increased release of norepinephrine in corresponding target areas (575).

The direct application of CRF in the VTA stimulates dopamine-dependent addictive behavior (646) and locomotor activity (297). CRF directly increases firing of dopaminergic neurons via a PKC-mediated amplification of the hyperpolarization-activated unselective cation current (I_h) (645). CRF amplifies NMDA receptor currents in dopaminergic neurons of the VTA (616). Stress stimulates the release of CRF into the VTA (646), but also stimulates the firing rate of dopaminergic neurons, thereby increasing dopamine release in dopaminergic target regions, such as the prefrontal cortex (1, 604).

In summary, the role of CRF as a synaptic regulator is complex and strongly dependent on the synaptic/cellular context. CRF can influence a wide array of ionic currents, ranging from Ca^{2+}-activated K^+ currents responsible for afterhyperpolarization (5, 497), inwardly rectifying K^+ currents (346), calcium currents (671), to I_h (494). Effects of CRF activate or inactivate intracellular signaling pathways that can either enhance or attenuate the effects of neurotransmitters (e.g., glutamate, GABA) or neuromodulators (e.g., noradrenaline, dopamine, 5-HT).

Recently, experimental evidence has been provided for an endogenous CRF tone within the hippocampus as a mechanism to modulate synaptic transmission. It has been shown that constitutive release of CRF within the hippocampus enhances excitatory drive onto CA3 pyramidal cells while having no significant effect on inhibitory transmission. Accordingly, antagonist-based inhibition of CRFR1 localized on dendrites of pyramidal neurons results in reduced intrinsic excitability (prolongation of I_{AHP}) and impaired integration of synaptic input. CRF may influence glutamatergic vesicle release probability from presynaptic terminals by calcium-dependent or -independent mechanisms. Accordingly, CRFR1 has been localized at presynaptic sites, which is in agreement with previous observations at other brain sites, e.g., the amygdala (220).

The electrophysiological properties of CRF have some correlates on the level of structural plasticity, which have been best studied in the hippocampus. In the hippocampus, CRF is primarily expressed in PV-expressing basket cells (106, 667, 668). These neurons innervate the pyramidal layer, mainly contacting the soma and axon initial segment of pyramidal neurons. However, CRFR1 is localized at some distance on dendritic spines of pyramidal neurons positioned in the stratum radiatum (106). Currently, there is no evidence for dendritic CRF release indicated by the exclusive vesicular storage in axon terminals (105). Moreover, inhibitory GABAergic PV^+ neurons do not synapse on dendritic spines, but typically make inhibitory synapses on dendritic shafts. Collectively, this mismatch suggests that CRF is released perisomatically to reach CRFR1 on dendritic spines in the stratum radiatum in a volume transmission manner (2, 112). Alternatively, CRF might activate receptors localized at the soma and axon initial segment. Upon stress, excitatory CRF is rapidly released to act on synaptic plasticity thereby acutely promoting learning and memory. CRF has profound effects on structural plasticity related to synaptic pruning and concomitant shaping of the dendritic tree, which depends on the CRF levels and duration of exposure (103). Exposure of organotypic cultures to CRF leads to blunted complexity of dendritic trees. In contrast, treatment with a CRFR1 antagonist leads to enlarged dendritic trees, which have also been observed in constitutive CRFR1 knockout mice (104), but not in forebrain-specific knockout mice, suggesting an additional involvement of corticosterone (652). High levels of CRF lead particularly to retraction of a subset of thin spines that are essential for learning and memory-associated plasticity as they are transformed into mushroom spines in the process of memory formation (108). The retraction of spines seems to depend on the GTPase RhoA, which plays an important role in regulation of actin polymerization (109), as well as on NMDA receptors and the downstream enzyme calpain (7). Besides the hippocampus, the olfactory bulb has been identified as a brain structure, where local CRF neurons play an important role in circuit plasticity, synapse formation, and integration of new neurons. Here, CRF neurons provide extensive presynaptic inputs onto new neurons, thereby promoting and stabilizing synapses, which facilitates the integration in the existing neural circuits of the olfactory bulb (192, 193).

V. HPA AXIS REGULATION

The discovery of CRF is inseparably connected to its role controlling the neuroendocrine limb of the stress response, the HPA axis (619, 661). In the PVN, CRF is almost exclusively expressed in parvocellular neurons with neurosecretory properties. These neurons are located at the top of the HPA axis and integrate environmental inputs to convey a secretory signal to anterior pituitary corticotropes (247, 567). CRF neurons are a specific population of parvocellular neurons (i.e., ~4,000 in the rat) that express the glutamatergic marker VGLUT2 (127). Similar results were obtained from single-cell sequencing approaches, which confirmed that CRF is expressed in GABAergic neurons in most structures of the hypothalamus, except the PVN, where CRF neurons are glutamatergic (525, 526). Identities of CRF neurons in the PVN have also been revealed using CRF-Cre mice, showing that CRF neurons are a unique population of neurons only rarely coexpressing other peptides, such as oxytocin. There is no overlap with TRH or SOM, and under baseline conditions only 5% of CRF neurons coexpress arginine vasopressin (AVP) (644). This is at first sight unexpected, considering the well-

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known synergistic effect of AVP, i.e., corelease of CRF and AVP potentiate the ACTH release (194, 517, 518). Similarly, only the simultaneous inhibition of AVP and CRFRs leads to a complete insensitivity of the HPA axis toward any type of stressor (499). However, it has been shown that AVP expression is significantly upregulated in CRF neurons under conditions of low circulating corticosterone levels, for example following adrenalectomy or in CRFR1 knockout mice (314, 425).

CRF synthesized in PVN neurons is stored in LDCV of axon terminals in the external zone of the median eminence. Whether CRF is stored in an amyloid-like cross-β-sheet-rich conformation, as suggested by in vitro studies, is not fully clear (390). CRF is released upon stress, but also during the regular circadian cycle, into the portal vasculature and is transported to the adenohypophysis. The release of neuropeptides such as CRF depends on the assembly of a soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor complex, which allows for priming, docking, and fusion of LDCVs with the membrane at the site of release (456). Recently, CRF mRNA was found to be coexpressed in the PVN with secretagogin (SCGN). SCGN is an EF-hand Ca^{2+}-binding protein highly expressed in neuroendocrine cells of the pituitary, adrenal gland, and pancreas, but also in the PVN (424, 641). SCGN comprises the basic properties required to orchestrate the release of neuropeptide-containing LDCVs. On the protein level, SCGN is strongly colocalized with CRF in PVN axon terminals within the median eminence. Accordingly, SCGN is capable of modulating CRF release, as demonstrated by knockdown experiments. Downregulation of SCGN in vivo in the PVN leads to reduced ACTH release upon a noxious stimuli (524, 525).

CRF is released into the portal vasculature from where it reaches corticotropes of the anterior and intermediate lobe. The local activation of CRFR1 on corticotropes of the anterior pituitary triggers the release of ACTH into the systemic circulation. In the adrenal cortex, ACTH binds to the ACTH receptor (also known as melanocortin receptor type 2) and stimulates the synthesis and release of glucocorticoids from the zona fasciculate, cortisol in primates and corticosterone in rodents (247). Glucocorticoids are well recognized as the main effectors of the neuroendocrine stress axis, orchestrating the restoration of homeostasis and adaptation to a given stressor (136, 246). To this end, they play a major role in regulating energy metabolism by promoting gluconeogenesis in the liver, proteolysis and amino acid mobilization in muscle, and lipolysis in fat, which ultimately mobilize energy stores (134).

CRF neurons also control the circadian secretion of ACTH and glucocorticoids with a peak at the onset of wakefulness and a trough 12 h later (247). Moreover, ACTH is released in a typically pulsatile pattern that is overlaid by ultradian oscillations generated on the pituitary level (532, 642). The HPA axis activity is under tight control of CRF on the hypothalamic level and CRFR1 on the pituitary level. Along this line, CRF as well as CRFR1 knockout mice show severely reduced glucocorticoid production and no HPA axis response to stressful challenges (422, 576, 605). This finding clearly suggests that no other CRF family member or other ACTH secretagogue is able to fully compensate the loss of CRF or CRFR1. A partial compensation by AVP has been reported for CRFR1 knockout mice, as these mice still possess low ACTH and corticosterone levels. However, the diurnal activity of the HPA axis and its response to stressful stimuli is disrupted (425). In addition, mutant mice revealed CRF/CRFR1-independent mechanisms of HPA axis regulation. For instance, female CRF knockout mice show a residual stress reactivity of their HPA axis (422). Particularly, under conditions of immune challenge, corticosterone is released independent of CRF/CRFR1. In the absence of CRF, HPA axis activation is mainly driven by interleukin-6 activating the HPA axis via interleukin-6 receptors present on corticotropes as well as adrenocortical cells (41, 610, 632). In contrast to CRF inactivation, CRF overexpression regularly results in signs of a Cushing's syndrome-like phenotype as a consequence of elevated circulating ACTH and corticosterone levels (139, 149, 321, 580, 634). Mice conditionally overexpressing CRF in the CNS showed normal nonstressed HPA axis parameters due to an adaptive downregulation of endogenous CRF. However, under stressful conditions, these mice show a higher reactivity of the HPA axis involving hyperactivity of catecholaminergic circuits (384).

From the other members of the CRF family, UCN1 has the capability to activate CRFR1 and thus to stimulate the HPA axis. However, the restricted expression precludes a direct involvement in HPA axis regulation. Accordingly, UCN1 knockout mice show no alterations of HPA axis activity (633, 651). Impaired neuroendocrine adaptation has only been reported in UCN1 knockout mice following severe stressful challenges (672). No alterations of HPA axis function were observed in mice deficient for UCN2 or UCN3, neither as single, double, or triple knockout, in combination with UCN1 (144, 441, 443). The only deviation is female UCN2 knockout mice, which have been reported to show estrogen-dependent alterations in circadian HPA axis activity (98). Direct expression of CRFR2 in HPA axis components is limited (90, 382, 630), and accordingly acute stimulation of CRFR2 has only minor impact on its activity (463, 464, 519). However, the inactivation of CRFR2 in mice revealed a significant role of CRFR2 in HPA axis regulation that might be connected to feedback regulation on the level of the BNST. CRFR2 knockout mice show a more rapid HPA axis activation and aberrant, i.e., prolonged recovery of the neuroendocrine stress response (20, 122). The neuroendocrine consequences of simultaneous disruption

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of CRFR1 and CRFR2 is superimposed by the dominant CRFR1-related HPA axis phenotype (21, 488).

The activity of the HPA axis is tightly regulated to maintain glucocorticoid levels within tolerable ranges (246, 247). The detrimental consequences of chronically elevated glucocorticoid levels can be observed in patients with Cushing's disease. These patients suffer from multiple morbidities, including substantial mental health problems (561). Glucocorticoids, the main effectors of the HPA axis, play an important role in negative feedback inhibition on all levels of the HPA axis, including parvocellular CRF neurons at the apex of the stress hormone cascade (431). Their effects are mediated via nuclear receptors: the mineralocorticoid (MRs) and glucocorticoid receptors (GRs). These act as ligand-activated transcription factors regulating the activity of target genes (136). Although the CRF promoter does not contain canonical glucocorticoid response elements, both inhibitory and stimulating effects of glucocorticoids on CRF expression have been demonstrated (3). Of note, these effects can be brain region specific, e.g., glucocorticoids inhibit CRF expression in the PVN, but activate its transcription in the CeA (231, 232, 392). In the PVN, the GR and possibly also the MR play an important role for the glucocorticoid-mediated negative feedback regulation of the HPA axis. However, CRF-positive neurons represent only a subset of all GR-expressing PVN neurons. GRs in parvocellular PVN neurons are supposed to inhibit neuropeptide expression directly in CRF neurons. In addition, an indirect regulation of CRF neurons via local CRF-negative but GR-expressing PVN neurons is possible. With current mouse genetic tools, it will be possible in the future to address the exact role of the GR (and MR) exclusively present in CRF-positive neurons of the PVN. The PVN receives on the one hand direct inputs from visceral afferents, brain stem nuclei, and circumventricular organs to react to immediate homeostatic challenges. On the other hand, the PVN receives indirect inputs that convey different responses to challenges predicted by innate programs, even in the absence of any immediate physiological perturbations (247, 676). Moreover, clear evidence has been demonstrated for direct control of CRF neurons in the PVN transmitted via multisynaptic inputs from BNST, hypothalamic, and brain stem nuclei. PVN excitation and inhibition is gated by local circuit neurons, which consist of stress-responsive GABAergic neurons projecting to the PVN (248). Besides the feedback regulation via glucocorticoids and GR, an involvement of the CRFR1 has been postulated. Mice with a forebrain-specific deletion of CRFR1 show a delayed HPA axis recovery (426). This particular feedback deficit was not observed in any other conditional CRFR1 knockout mouse line. Therefore, it can only be speculated that this HPA axis phenotype originates from the postnatal deletion of CRFR1 or from the difference in the respective spatial patterns of CRFR1 deletion (504). In general, the expression of CRFR1 in parvocellular neurons of the murine PVN is limited (339, 630). This is in contrast to rats, in which CRFR1 in the PVN is readily detectable, in particular following stressful challenges (276, 393). Only recently, CRFR1 reporter mice allowed the identification of a unique population of CRFR1-positive neurons in the PVN (293). These GABAergic neurons do not coexpress any typical PVN markers, such as CRF, AVP, or oxytocin, but they are localized in close proximity to CRF neurons, and these appear to make direct synaptic contacts with CRFR1-positive PVN neurons (500). Expression of CRFR1 in these neurons is positively regulated by glucocorticoids and probably directly controlled by the GR. Inactivation of CRFR1 in the hypothalamus using Sim1-Cre mice resulted in significantly decreased basal plasma corticosterone levels and decreased anxiety-like behavior 10 days after subjecting the animals to chronic social defeat stress. Chronic stress resulted in the specific recruitment of CRFR1 neurons in the PVN, which are under the control of a positive feedback regulation, i.e., high glucocorticoid levels upregulate CRFR1 expression. This population of CRFR1 expressing PVN neurons represents an important component of the HPA axis regulation, particularly in preparing the organism for successive exposure to stressful stimuli (500).

VI. BEHAVIORAL STRESS RESPONSE

The dominant role of CRF and its type 1 receptor in the context of HPA axis regulation is comparably well understood. This is in contrast to our comprehension of the CRF system's role in coordinating complex behaviors and autonomic functions in response to stressful stimuli. Soon after its discovery, it was demonstrated that central (e.g., ICV) application of CRF is capable of provoking reactions that share signs of behavioral and physiological stress responses (157). CRF elicits behavioral activation similar to increased arousal (78, 586). It enhances sympathoadrenal outflow, which entails reactions similar to Cannon's fight-or-flight response or a state of stress, including enhanced heart rate, mean arterial blood pressure, plasma glucose levels, and oxygen consumption (69, 70, 71, 155, 176). Behavioral activation occurs independent of HPA axis activity and is primarily seen in a familiar environment, while CRF suppresses exploration in an unfamiliar potentially stressful environment. In general, central application of CRF was shown to promote primarily anxiogenic effects, as demonstrated in different behavioral tasks, such as the elevated plus maze, acoustic startle response, and social interaction test (39, 65, 66, 242, 564, 587). Since those early days, the CRF system has attracted enormous scientific interest, which is reflected by the multitude of studies applying pharmacological administration of CRF-related peptides and CRFR antagonists either ICV or in a brain region-specific manner (244). These studies have provided valuable insights into the role of CRF-related peptides and their receptors modulating emotional states and behavioral responses to stress (23). However, it has also been recognized that the

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application of an agonist/antagonist is accompanied by some technical limitations that might be responsible for observed ambiguities. For instance, it is relatively difficult to control the magnitude of the brain area to which an injected substance gains access, since this depends on the local diffusion properties of the injected tissue but also on the bioavailability of the utilized substance. In addition, there are no means by which a compound can discriminate between receptors localized on different types of neurons, e.g., excitatory vs. inhibitory, or between receptors localized on pre- vs. postsynaptic sites. Moreover, the dosage of applied substances might give rise to variable outcomes, taking into account the frequently observed inverted U-shaped dose-response curves of GPCRs and lack of specificity at higher dosages. Finally, it has been shown that some of currently available antagonists are not able to reliably discriminate between CRFR1 and CRFR2 (682). The advent of gene-targeting technologies in the mouse was a major leap forward for all biomedical research fields, including research on the CRF/CRFR system (143). The recently implemented combination of mouse genetic tools with specific manipulations using viral vectors has opened up further perspectives to address specific research questions. In the following subsections we will review key findings from studies using mouse genetic tools, as well as viral approaches focusing on emotional states related to anxiety and depression.

A. Genetic Mouse Models

A wealth of findings contributed to shape our perception of the CRF/CRFR system as a dualistic unit regulating adaptation to environmental challenges by two oppositely acting limbs of the stress response, i.e., on the one hand the stress-initiating/anxiogenic CRFR1-CRF system, and on the other hand the stress recovery-related/anxiolytic CRFR2-UCNs system (135). However, in recent years, more refined methods have uncovered novel findings that make a revision of this dualistic model inevitable (287). Ample evidence from conditional knockout mice and viral approaches advocate replacing the dualistic view with a perspective that takes into account region- and cell-type-specific actions of CRFRs, which depend on their spatial distribution, but also on the timing and duration of activation (244).

Targeted downregulation using antisense oligodeoxynucleotides provided the first evidence that loss of CRF function has anxiolytic effects (570). The generation of knockout mice allowed researchers to selectively address CRF's role in modulating behavioral stress responses (422, 423). However, despite an overt HPA axis phenotype, CRF-deficient mice do not show a clear behavioral phenotype under standard housing conditions or following various stressors. Even more surprisingly, selective CRFR1 antagonists are still able to attenuate stress-induced behaviors in CRF knockout mice suggesting that stress-induced behaviors require CRFR1 but not CRF (659). Different hypothetical explanations have been suggested, including *1*) the existence of other yet unknown CRFR1 ligands, *2*) compensatory mechanisms activated due to early development inactivation, e.g., involving UCN1, *3*) CRF being mainly relevant under conditions of severe or chronic stress, *4*) intrinsic CRFR1 activity, which does not require activation by CRF, or *5*) lack of CRFR1 antagonist specificity. Recently, the first conditional CRF knockout mice were described (675). Selective inactivation of CRF in the hypothalamus based on Sim1-Cre mice resulted in a 60–70% reduction of CRF expression in the PVN, but with only a 30% reduction in the entire hypothalamus. The deletion of CRF resulted in severely impaired HPA axis function, reflected by reduced diurnals ACTH and corticosterone plasma levels. In addition, a markedly attenuated stress response of the HPA axis was observed; however, it was less severe compared with constitutive CRF knockout mice (422). Surprisingly, CRF knockout mice showed a significant reduction in anxiety-related behavior measured in different tests (open field, dark/light box, elevated plus maze, and hole board test), essentially phenocopying CRFR1 knockout mice. A substitution of corticosterone levels did not rescue the attenuated anxiety, suggesting that the phenotype is independent of the disturbed HPA axis function. Anterograde tracing revealed that PVN CRF^+ neurons project to many brain areas besides the median eminence, including nuclei of the amygdala, BNST, nucleus accumbens, hypothalamus, midbrain, and brain stem. The loss of CRF input to these structures might account for the observed anxiolytic effect of selective CRF deletion in the PVN (675). This is an interesting finding, suggesting that the absence of an anxiety phenotype in constitutive CRF knockout mice can be explained by compensatory mechanisms, which kick in when CRF is deleted very early in development but not at later stages when Sim1-Cre starts to become active. Nevertheless, several questions remain to be investigated in the future. For instance, the less pronounced HPA axis dysfunction suggests a partial loss of CRF expression in the PVN, which might be confined to a behaviorally relevant subset of PVN CRF^+ neurons. Moreover, it would be of interest to gain more insights into the loss of CRF expression in the hypothalamic structures also targeted by the Sim1-Cre outside of the PVN.

Similar to pharmacological studies, genetic mouse models of CRF excess show signs of chronic stresslike activation (149), including decreased floating in the Porsolt forced swim test, i.e., enhanced active stress coping behavior (384, 628, 634) and anxiogenic-like effects (177, 321, 504, 581, 607). There is some variability in the expression of behavioral phenotypes. For example, while many studies showed decreased floating in the forced swim test, others observed increased floating or no alterations (215, 321). These differences can be attributed to the respective genetic designs on which these mouse models of CRF excess are based

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promoting mouse line-specific CRF overexpression in different structures and time points (138, 350). In addition, the development of a Cushing's syndrome-like phenotype as a result of chronic HPA axis activity might also interfere with the observed phenotypes (139, 216, 580). More refined approaches of CRF overexpression have been developed based on viral vectors, which allow selective targeting of specific brain structures, as detailed in the next section.

CRFR antagonists are able to attenuate or reverse effects of various stressors, suggesting that CRF itself is a mediator of the stress response (40, 67, 157, 242). Also in CRF overexpressing mice, CRFR1 antagonists are able to revert the CRF-induced phenotypes (310, 384, 581). Based on antagonist (222, 683) and antisense oligonucleotide approaches (369, 571), the stress-inducing and anxiogenic effects of CRF were attributed to the CRFR1. This was further substantiated by different CRFR1 knockout mouse models. CRFR1 deficiency has a strong anxiolytic effect, as demonstrated in multiple behavioral tasks, e.g., open field, elevated plus maze, and dark-light box tests (119, 576, 605). This anxiety phenotype is independent of CRFR1 in the pituitary controlling HPA axis function, as demonstrated by forebrain-specific CRFR1 knockout mice, which spare the CRFR1 expression domain in the anterior pituitary (426).

However, CRFR1 plays not only a role in controlling acute stress-induced behaviors, but has also been shown to modulate the negative consequences of early life stress and stress in adulthood on cognition and structural plasticity. Early life stress induced by a well-established paradigm of limited nesting and bedding material (513) has profound consequences in mice reflected by impaired spatial learning and memory. These cognitive deficits are associated with disrupted long-term potentiation and changes in structural plasticity, i.e., reduced number of spines in the hippocampal CA3 region (283, 654). Similar negative effects on cognitive performance were observed in mice overexpressing CRF in forebrain principal neurons. The involvement of CRFR1 in these negative stress effects was demonstrated by treating the stressed animals with a CRFR1 antagonist, which was able to prevent some of the stress effects (283). Similarly, mice lacking CRFR1 in forebrain principal neuron were largely protected from severe effects of early life stress. This is surprising, considering that the inactivation of CRFR1 is controlled by the Camk2a-Cre, which leads to a postnatal CRFR1 inactivation only after completion of the stress paradigm conducted at postnatal *days 2–9* (654). In addition, the stress-induced increase in anxiety-related behavior seen in wild-type mice was also partly prevented by postnatal inactivation of CRFR1 in forebrain principal neurons (653). Analyses at the molecular level revealed that activation of CRF/CRFR1 signaling is able to downregulate the nectin-3/afidin complex, thereby mediating the stress effects on spatial memory and structural plasticity (655). Chronic social defeat stress in adulthood also downregulates nectin-3 in the hippocampus, alters structural plasticity in the CA3, and impairs spatial memory, effects that are abolished in forebrain principal neuron-specific CRFR1 knockout mice (652). Importantly, these findings are independent of glucocorticoid effects, since the HPA axis is not affected in forebrain-specific CRFR1 knockout mice. Chronic stress has also been implicated in the pathophysiology of neurodegenerative disorders (638). Although numerically outnumbered by studies addressing mood and anxiety disorders, CRF has also been implicated in neurodegenerative disease. An early study demonstrated that displacement of CRF from the CRF-BP might have therapeutic potential in Alzheimer's disease (32, 33). Chronic and acute stress are capable of increasing Aβ levels in the interstitial fluid. This effect can be mimicked by exogenous CRF, but not by corticosterone. In contrast, inhibition of endogenous CRFRs or neuronal activity blocked the effects of acute stress on Aβ, suggesting that the CRF system conveys stress effects that might directly affect the pathogenesis of Alzheimer's disease (300). The CRF system also has a direct influence on the other hallmark of Alzheimer's disease, i.e., tau phosphorylation. Induction of tau phosphorylation and aggregation is inhibited by pharmacological or genetic blockade of CRFR1, whereas CRF overexpression exaggerates tau phosphorylation and aggregation. In particular, studies of Alzheimer's disease mouse models with a CRFR1 deficiency or treated with CRFR1 antagonists support CRFR1 as a promising target for the treatment of Alzheimer's disease (79, 80, 515, 516).

Collectively, these results suggest that the CRFR/CRF system plays an equally important role besides glucocorticoids and their receptors with respect to controlling structural plasticity and aspects of learning and memory (136). In addition, it is likely that CRF and glucocorticoids act in concert. Along this line, a recent study confirmed synergistic effects of CRF and corticosterone on stress-induced memory impairment (111).

CRFR1 is the dominantly expressed CRFR in the brain. As a consequence of its broad expression, CRFR1 is expressed in an array of different neurons specified by their neurotransmitter identity. CRFR1 expression in forebrain glutamatergic neurons was confined to structures such as the cerebral cortex, hippocampus, and basolateral amygdala. Expression in inhibitory GABAergic neurons was detected in the olfactory bulb, globus pallidus, and reticular thalamic nucleus. Strong colocalization with dopaminergic markers was observed in the VTA and substantia nigra pars compacta of the midbrain. In contrast, only a few CRFR1 neurons in the raphe nuclei were identified as serotonergic (504). Floxed CRFR1 mice and cell-type-specific Cre drivers provided access to address the impact of CRFR1 in these individual neuronal circuits on emotional states. The selective inactivation of CRFR1 in previously identified neuro-

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nal cell types unveiled a bidirectional control of anxiety-related behavior via CRFR1. CRFR1 in forebrain glutamatergic neurons has anxiogenic effects, whereas CRFR1 in midbrain dopaminergic neurons shows anxiolytic properties. Additionally, these conditional CRFR1 knockout mice revealed that CRFR1 is able to directly modulate glutamatergic and dopaminergic neurotransmission (504). These findings provide a novel perspective with respect to the potential involvement of the CRF system in the pathophysiology of stress-related disorders, suggesting a disbalance of CRFR1-controlled anxiogenic glutamatergic and anxiolytic dopaminergic circuits. The strong regional and cell-type-specific effects of the CRF/CRFR system (244) are further imposed upon by environmental factors and individual stress experience. This has, for instance, been shown in a place preference paradigm in which stress can switch appetitive effects of CRF toward aversive behaviors (356).

Our understanding of the physiological implications of CRFR2 and its interaction with peptide agonists has advanced significantly over the last few years, as witnessed by an increasing number of studies targeting UCNs and CRFR2 in mice. UCN1 was inactivated independently by three different groups; however, increased anxiety was only observed in one mouse line (633) and not in the others (651, 672). The altered startle response of knockout mice was causally linked to hearing deficits (633, 651). UCN2 inactivation affects diurnal plasma ACTH and corticosterone levels in female mice, resulting in reduced immobility in the forced swim test, while anxiety-related behavior is unaffected (98). Independently generated male UCN2 knockout mice also showed no alterations in anxiety, but had reduced aggressive behavior (62). UCN3-deficient mice were largely inconspicuous, except they presented an improved social discrimination memory, which was confirmed in CRFR2 knockout mice (144). This was the first indication that CRFR2 and its ligand UCN3 play an important role in the ethological domain of social behavior, which is in agreement with its expression in brain region relevant for processing social olfactory cues. In a recent study, UCN3 and CRFR2 knockout mice showed abnormally low preference for novel conspecifics (562). This behavior particularly depends on CRFR2 in the MeA. Knockdown of CRFR2 in the MeA recapitulates the reduced social preference for novel conspecifics. In contrast, local application of UCN3, as well as optogenetic activation of MeA UCN3$^+$ neurons, increases preference for novel mice. Chemogenetic inhibition of MeA UCN3$^+$ neurons elicits prosocial behavior in group-housed mice (562). These findings establish the CRFR2/UCN3 system as a peptidergic system controlling social behavior besides the classical "social hormones" oxytocin and AVP. This is also interesting in light of the recently demonstrated cross-talk between CRF and oxytocin signaling in the medial prefrontal cortex involving CRF-BP (363).

CRFR1-counteracting effects have been ascribed to the CRFR2 based on the accelerated HPA axis response and delayed feedback regulation observed in CRFR2 knockout lines (20, 122, 313). In addition, CRFR2 knockout mouse lines show increased anxiety-related behavior, although these findings were not as consistent when comparing the three independently generated mouse lines. Only one group reported increased anxiety-related behavior in male and female knockout mice (20), whereas another group reported increased anxiety only in male mice (313), and the third line did not reveal any changes in anxiety-related behavior (122). These discrepancies might be explained by differences in the genetic background of the different mouse lines, different targeting strategies affecting splice variants differently, or simply by differences in housing and testing conditions, which might have affected behavior in anxiety tasks. The latter is supported by the observation that CRFR2 knockout mice showed increased anxiety 24 h after exposure to restraint stress. In contrast, no effect was seen immediately after acute restraint stress or following a chronic variable mild stress paradigm (281). In agreement with these findings, mice lacking all three ligands of the CRFR2, i.e., UCNs1–3, showed similarly increased anxiety when tested 24 h following acute restraint stress (443). In contrast, double knockout mice lacking UCN1 and UCN2 show decreased anxiety already under basal conditions. Unfortunately, no results following stressor exposure have been reported (441). Mice with chronic activation of CRFR2 by ubiquitously overexpressing UCN3 showed an anxiety-like state with an attenuated response to stress (442). Later, two slightly contradictory studies emphasize the importance of the spatial component of inactivation or overexpression, which might trigger compensatory mechanisms. The behavioral changes following modulation of CRFR2 activity were consistently accompanied by alterations in the 5-HT system, which is related to the high abundance of CRFR2 in midbrain raphe nuclei (281, 441–443). The 5-HT system plays an important role in the pathophysiology of depression, which, to some extent, can be interrogated in rodents using the classical antidepressant screening paradigm: the forced swim test. Behavioral changes in the forced swim test are used to predict the efficacy of a compound to enhance synaptic levels of biogenic monoamines such as 5-HT (124). In the forced swim test, CRFR2 knockout mice show consistently increased immobility (22, 606). Accordingly, central application of UCN2 or UCN3 has the opposite effect and decreases immobility (594). Specific activation of CRFR1 in CRFR2 knockout mice also decreases immobility (606), which is in line with reduced immobility seen after stress-induced activation of CRFR1 (384).

The comprehensive analysis of energy expenditure and metabolic function of mouse lines related to the CRF/CRFR system has revealed a particularly strong contribution of CRFR2 and its ligands (95, 286, 342, 343), but also of

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CRFR1 (345), to metabolic homeostasis, which cannot be further discussed in the context of this review.

The CRF-BP has the capacity to sequester CRF and UCN1, thus suggesting an important role in regulating the availability of both peptides. Constitutive CRF-BP knockout mice show signs of increased anxiety, which might be related to increased levels of free CRF (302). Transgenic mice overexpressing CRF-BP under the control of the glycoprotein hormone α-subunit promoter show increased levels of CRF-BP in anterior pituitary gonadotropes and thyrotropes, which is thought to bind CRF released from the median eminence. CRF-BP transgenic mice show mild signs of decreased anxiety, but also increased locomotion, which might influence the behavioral phenotype. Additionally, it was not investigated in detail to what extent the occasionally detected expression in the brain might have contributed to the phenotype (76). In another mouse line overexpressing CRF-BP more ubiquitously, by using the metallothionein I promoter, no alterations of anxiety-related behavior have been reported (378). The CRF-BP has been slightly neglected in the past, but recent findings demonstrating that the interaction of CRF-BP modulates the activity of CRFRs should reattract considerable interest into CRF-BP research (363, 572, 616, 647).

Taken together, genetic mouse models specifically targeting CRFRs, CRF-BPs, and CRF-related peptides have established the regional and cell type specificity of CRF system components. The reactivity of the CRF system is further influenced by previous exposure to environmental factors and individual experience on the organismic level. This complicates and challenges a coherent interpretation of CRF system physiology, in particular with respect to emotional states and behavior. A future challenge is to decode this complexity by interrogating the underlying structural and functional mechanisms.

B. Viral Approaches

In addition to gene targeting in mice, an increasing number of studies applied viral-mediated overexpression or knockdown of CRF system components to address region-specific effects. An elegant approach to mimic global CRF hyperactivity was achieved by combining the Tet-On system with a lentiviral approach. To direct CRF overexpression into the ventricular space, a reverse tetracycline transactivator was expressed under the control of the choroid plexus-specific murine CRFR2β promoter. Coinjection with a viral vector possessing CRF under the control of a tetracycline-responsive element resulted in inducible overexpression, which promoted increased anxiety-like behavior in mice (505). To target specific brain regions, predominantly lentiviral but also AAV-based vectors have been applied by stereotactic delivery. Local virus-assisted overexpression or knockdown has been utilized in mice, rats, and nonhuman primates.

A first out of a series of studies conducted by different groups showed that CRF overexpression in the CeA resulted in increased anxiety and increased floating, interpreted as despairlike behavior measured in the acoustic startle and forced swim test, respectively (306). In another study, chronic (4 mo) CRF overexpression in the CeA attenuated stress-induced anxiety, but did not alter behavior in the forced swim test (506). In contrast, short-term (3 days) CRF overexpression in the same brain structure augmented stress-induced anxiety-related behavior, but similarly had no effect in the forced swim test or in the closely related tail suspension test. However, CRF knockdown in the CeA strongly attenuated stress-induced anxiety (507). Interestingly, overexpression in the lateral aspect of the BNST also elicited increased floating in the forced swim test, whereas it did not affect anxiety-related behavior (506).

In an attempt to specifically target CRF neurons in the rat, Flandreau and colleagues (178) employed a 3.0-kb fragment of the CRF promoter to drive CRF expression from a lentiviral vector. CRF overexpression in the amygdala resulted in increased anxiety, as observed in some parameters of the elevated plus maze and in the defensive withdrawal test; however, overexpression had no effect in the open field, sucrose preference, or forced swim test. This inconsistency might be related to the duration of overexpression at the time point of testing. Along these lines, HPA axis hyperactivity in overexpressing rats was only observed after 10 wk of overexpression, but not after 6 wk (178). When a CMV promoter was used for CRF overexpression in the CeA of female rats, the increased anxiety was manifested as decreased social interaction and avoidance of open arms in the elevated plus maze. Surprisingly, this phenotype only occurred with the onset of puberty but not before. In addition, CRF overexpression resulted in advanced puberty and disturbed reproductive cycles in female rats (364). The prolonged AAV-mediated overexpression of CRF in the CeA resulted in adaptive changes of CRFR1 and CRFR2 expression, which might be causal for the observed attenuation of the dysphoric-like state associated with a model of nicotine withdrawal (492).

Lentiviral overexpression of CRF in the BNST of rats, using the CRF promoter fragment, did not affect anxiety-related behavior, but altered conditioned anxiety, suggesting that CRF might contribute to inappropriate regulation of emotional memories. It appeared that CRF overexpression had an effect on the expression of CRFRs: CRFR1 was downregulated in the BNST, while CRFR2 was upregulated in the dorsal raphe nucleus. This compensatory change might to some extent underlie the observed changes in conditioned anxiety (569). Of note, rats susceptible to a model of psychological trauma induced by exposure to predator-associated cues also showed a marked regulation of CRFRs, i.e., a downregulation of CRFR2 in the posterointermediate

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part of the medial division of the BNST. Lentivirus-mediated overexpression of CRFR2 in this particular nucleus was able to improve posttraumatic stress disorder (PTSD)-like symptoms of susceptible rats (166). Interestingly, mice subjected to a different PTSD-like animal model showed a robust upregulation of CRFR2 in the BNST. Accordingly, lentivirus-mediated knockdown of CRFR2 resulted in reduced susceptibility to develop PTSD-like symptoms (352). CRFR2 is highly expressed in the ventromedial nucleus of the hypothalamus, one of the major control regions of feeding and energy metabolism. Knockdown of CRFR2 in the ventromedial nucleus of the hypothalamus of mice recapitulated the phenotype of UCN3 knockout mice, promoting weight gain due to accumulation of white adipose tissue (93). Along these lines, using a lentivirus-based, tetracycline-inducible system to overexpress UCN3 in the perifornical area significantly increased metabolic rate, while it decreased insulin sensitivity. This metabolic phenotype was accompanied by increased anxiety-like behavior, positioning UCN3 as a neuromodulator linking stress-induced anxiety and energy homeostasis (343).

Lentivirus-mediated knockdown of CRFR1 in the basolateral amygdala consistently results in decreased anxiety-like behavior, which mimics the effect of CRFR1 upregulation seen with exposure to an enriched environment (591). In contrast, mice with a knockdown of CRFR1 in the external globus pallidus, a site of strong CRFR1 expression, showed increased anxiety, suggesting anxiolytic properties of CRFR1 in this brain structure comparable to those of CRFR1 in dopaminergic neurons of the VTA (504, 590). In the VTA, a local population of dopaminergic neurons upregulating CRF expression during chronic nicotine exposure has been identified. The specific lentivirus-mediated downregulation of CRF in dopaminergic VTA neurons prevents the aversive effects of nicotine withdrawal by limiting the effect of nicotine on GABAergic input to dopaminergic neurons (212). In addition, these CRF neurons project to the neighboring intermediate region of the interpeduncular nucleus. Thus VTA-specific knockdown of CRF also prevented interpeduncular nucleus activation during nicotine withdrawal, which involves CRFR1 upregulation and modulation of glutamatergic input from the medial habenula (677). Another study targeting VTA CRFR1 shows that the knockdown of CRFR1 in the VTA is able to attenuate stress-induced cocaine seeking (101).

In the brain stem, CRF is highly expressed in calbindin-expressing neurons of the IO. CRF-positive neurons of the IO project directly to the cerebellar nuclei, exciting glutamatergic nuclei in the interpositus nucleus via CRFR1 and CRFR2. Using lentivirus-mediated knockdown of CRF in the IO induces ataxia-like motor abnormalities, revealing a previously unknown role of CRF in cerebellar motor coordination (656).

Besides using overexpression and shRNA-mediated knockdown strategies, the stereotactic application of Cre-expressing viruses offers another possibility to selectively target a gene of interest in a given brain region. For instance, the local AAV-Cre-mediated inactivation of CRFR1 in the prefrontal cortex of floxed mice revealed that CRFR1 activation is causally involved in acute stress-induced executive dysfunction, as was assessed in tests of temporal order memory and reversal learning. These results suggest that CRFR1 inhibition could represent an intervention strategy for stress-induced cognitive dysfunction (617).

Taken together, the complexity of the CRF/CRFR system is beginning to be revealed, particularly owing to local virus-assisted CRF overexpression studies. It seems likely that the significant degree of variability of behavioral CRF effects between the different studies largely depends on the temporal and spatial pattern of expression. Another factor is the fidelity of overexpression, i.e., the question of to what extent the overexpression is matching endogenous expression. The CRF promoter fragment might have provided some specificity, but the Cre line based on the same construct clearly raises some doubts (110). In the meantime, well-characterized and highly specific CRF Cre drivers are available that provide the means to overcome this obstacle by using Cre-dependent viral vectors (138). In case of the shRNA-based knockdown strategies, the efficacy of downregulation of a gene of interest has to be controlled carefully. In this regard, the combination of Cre-expressing viral vectors with floxed mice represents an alternative strategy with high efficiency (617).

Viral vectors open up the possibility to target the CRF/CRFR system in other species besides rodents. A first study using AAV vectors in nonhuman primates investigated the consequences of CRF overexpression in the dorsal amygdala of rhesus monkeys (296). The overexpression covering the lateral and medial divisions of the CeA elicited an increase in anxious temperament in the monkeys. Anxious temperament is a well-established traitlike disposition that has been identified as a childhood risk factor for development of psychiatric pathologies, such as anxiety disorders and depression later in life (183). In the brains of these overexpressing rhesus monkeys, changes in the local metabolism of the dorsal amygdala were observed but also changes in distant structures known to be part of the network related to anxious temperament that are affected in human anxiety disorder, such as the orbital proisocortex/anterior insular cortices and the hippocampus. In addition, alterations in functional and structural connectivity were observed. For example, structural changes in the medial thalamus might link the dorsal amygdala to metabolic changes in orbital proisocortex/anterior insular cortices. This is a study of particular importance, as it demonstrates the feasibility to test hypotheses generated in rodent studies and takes into account the differences between primates

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and rodents. Moreover, it allows researchers to assess in vivo parameters of brain function and structure that can also be used in human neuropsychiatric patients (323).

C. Neuronal Circuits

The establishment of Cre driver mouse lines, which provide access to genetically defined neuronal population, opens up new avenues to control the activity of neurons and circuits using optogenetic or chemogenetic tools. Particularly recent findings in the CRF field merit a closer inspection as they significantly deepen our understanding of CRF- and CRFR-specific circuits modulating the stress response and anxiety-related behavior.

A first study focusing on CRF system-related circuits employed the observation that CRFR2 expression is confined to a subset of neurons in the LS providing a cellular entry point to investigate the role of LS circuits in stress-induced anxiety (8). Using a BAC transgenic CRFR2-Cre line enabled optogenetic stimulation of GABAergic LS CRFR2$^+$ neurons, which elicited enhanced stress-induced anxiety. Transient activation causes a persistent but reversible state of anxiety that required a particular stimulation frequency (15 Hz). In contrast, optogenetic inhibition of CRFR2$^+$ neurons suppresses the expression of stress-induced anxiety. LS CRFR2$^+$ neurons predominantly innervate the anterior hypothalamic area (AHA), forming inhibitory synapses with target neurons. In accordance with experiments in the LS, optogenetic stimulation or inhibition of CRFR2$^+$ efferents in the AHA produced similar anxiogenic or anxiolytic effects (8). While LS CRFR2$^+$ efferents spare the PVN, it was shown that CRFR2$^+$ neurons inhibit neurons in the AHA, which then innervate the PVN. This disynaptic disinhibitory connection was experimentally verified by the observation that optogenetic stimulation of LS CRFR2$^+$ neurons is able to increase plasma corticosterone levels, whereas inhibition attenuates the stress-induced release of corticosterone (8).

Using a different BAC transgenic CRFR2-Cre line, the properties of a population of GABAergic CRFR2$^+$ neurons in the posterior BNST (pBNST) were investigated (245). These CRFR2$^+$ neurons show dense projections to hypothalamic nuclei, the MeA, paraventricular thalamic nucleus, LS, and brain stem nuclei. In contrast to LS CRFR2$^+$ neurons, optogenetic activation of pBNST CRFR2$^+$ neurons reduces anxiety. In addition, photostimulation of pBNST CRFR2$^+$ neurons contributes to stress recovery by attenuating the neuroendocrine stress response, ameliorating stress-induced anxiety, and mitigating the memory of the stressful event. In contrast, optogenetic inhibition of pBNST CRFR2$^+$ neurons had the opposite effect, increasing stress-induced anxiety and enhancing fear memory. In addition, optogenetic inhibition delayed the neuroendocrine stress recovery. These findings suggest that the activity of pBNST CRFR2$^+$ neurons contributes to coping with stressful situations and recovery from acute stress exposure. Accordingly, subjecting mice to a protocol of trauma-induced PTSD revealed that, by increasing the activity of pBNST CRFR2$^+$ neurons while triggering the PTSD-like symptoms, one is able to reduce the susceptibility to develop PTSD-like symptoms (245).

Another prominent example for the biological relevance of CRF-specific circuits is related to the control of micturition, i.e., release of urine, which is crucial to regulate water balance and expel waste, but also plays an important role in the context of social communication. The central control of micturition is executed by the pontine micturition center (PMC), also known as Barrington's nucleus. Of all the extrahypothalamic brain regions, this nucleus presents with the highest CRF expression. CRF$^+$ neurons comprise one-half of the PMC neuronal population. These neurons are almost exclusively glutamatergic projection neurons and innervate as descending command neurons in spinal cord preganglionic bladder motoneurons (270). Using a well-established CRF-Cre knock-in mouse line (596) allowed the optogenetical activation PMC CRF$^+$ neurons and inducement of bladder contraction while chemogenetic inhibition decreased micturition. Vice versa bladder contraction and micturition correlated with activation of PMC CRF$^+$ neurons as measured by Ca^{2+} influx using a genetically encoded calcium sensor. Of note, AAV-based and Ef1α promoter-driven CRF overexpression resulted in attenuated micturition pressure, which was also reflected by an increase in bladder-to-body weight ratio (407). Among the multiple pro- and anti-micturition inputs converging on PMC CFR$^+$ neurons, the MePO was demonstrated to provide inhibitory input. Inhibition of MePO neurons resulted in a disinhibition of PMC CRF$^+$ neurons accompanied by reduced number of urine marks and equalization of social rank-dependent differences in micturition behavior between dominant and subordinate males (270).

The hypothalamic POA is also involved in sleep generation. Among other neuropeptide-expressing neurons in the POA, CRF$^+$ neurons were identified as sleep-promoting neurons. Tuberomammillary nucleus-projecting CRF$^+$ neurons significantly decreased wakefulness and increased rapid eye movement (REM) and non-REM sleep upon photostimulation. Conversely, pharmocogenetic inhibition of POA CRF$^+$ neurons promoted increased wakefulness (116).

Besides Barrington's nucleus, CRF is expressed at high levels in the CeA, where the properties of CRF neurons have been interrogated in the context of amygdalar fear circuits. The CeA is composed of GABAergic neurons that can be distinguished by their expression of SOM, PKC-δ, and CRF. CRF$^+$ neurons are predominantly located in the rostral aspect of the lateral CeA (CeA_L), which is devoid of SOM$^+$ and PKC-δ^+ neurons. But also in more caudal as-

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pects the coexpression with SOM and PKC-δ is limited, indicating that CeA_L CRF^+ neurons represent a unique population of GABAergic CeA_L cells (540). Recently, Fadok and colleagues (171) developed a model that allows one to assess a switch between conditioned freezing and flight behavior. CRF^+ neurons have been shown to mediate conditioned flight responses, whereas SOM^+ neurons initiate passive freezing behavior. The balance between conditioned flight and freezing is regulated by local inhibitory connections between CRF^+ and SOM^+ neurons. Inhibition of CRF^+ neurons prevented conditioned flight behavior, whereas inhibition of PKC-δ^+ and SOM^+ neurons had no effect, suggesting the CRF^+ neurons are crucial for expression of conditioned flight. Accordingly, CRF^+ neurons are activated during flight, but not freezing, which is in opposition to SOM^+ neurons, which are activated during freezing, but inhibited during flight. Selective activation of CRF^+ neurons during tone exposure decreases freezing and leads to more active behavior in the conditioning context. Of note, these effects are only observed in conditioned but not in naive mice. While both SOM^+ and PKC-δ^+ neurons receive inhibitory input from CRF^+ cells, significantly more SOM^+ cells receive inhibitory input from CRF^+ cells, suggesting reciprocal inhibitory synaptic connections that allow the rapid switching between different behavioral strategies. Moreover CRF^+ and SOM^+ neurons receive differential input from presynaptic regions, which is crucial for the processes of action selection. The integration of signals necessary to orchestrate flight depends on CRF^+ target regions, such as the hypothalamus and the PAG (171).

In a similar approach, Sanford and colleagues (540) described CRF^+ neurons in the CeA that are required for discriminative fear but are dispensable for generalized fear. CeA_L CRF^+ neurons undergo threat-specific changes in plasticity and selectively respond to thereat-predictive cues. Foot shock-conditioned mice showed increased AMPA/NMDA excitatory postsynaptic currents due to increased enhancement of AMPA currents compared with control mice receiving only cue presentation but no shock. Selective silencing of CRF^+ neurons by expression of tetanus toxin light chain resulted in reduced freezing following low intensity of the unconditioned stimulus (US; 0.3 mA). However, at higher US intensities (0.5 mA), silencing of CRF neurons did not affect the conditioned fear response. Interestingly, the general inactivation of synaptic release in CRF^+ neurons had the same phenotypic consequences as the disruption of CRF itself, suggesting that CRF plays a major role in CRF^+ neuron function. Conditional knockout mice selectively lacking CRF in the CeA showed reduced freezing to the conditioned stimulus (CS^+) relative to control animals; however, only if they were conditioned with a low-intensity US. At higher intensities, no difference between control and knockout mice was observed. Chemogenetic inactivation of CRF^+ neurons was only effective when applied during the acquisition phase, but not during expression of conditioned threat, suggesting that CRF^+ neurons are specialized for fear acquisition. Moreover, CRFR1-Cre knock-in mice revealed local CRFR1 neurons in the rostral CeA, where no PKC-δ or SOM is expressed. In more medial and caudal regions, higher overlap with SOM than with PKC-δ was detected. Inhibition of CRFR1 with antalarmin impaired conditioned fear acquisition. Taken together, these findings support a general role for CeA_L CRF^+ neurons and CRF itself in conditioned threat learning and discrimination (540).

Besides local projections, CeA CRF neurons also project to distant sites, e.g., in the BNST, PAG, or LC. LC-NA neurons play an important role in a broad range of physiological functions, including arousal and stress reactivity. Increased tonic activity of the LC-NA system is necessary and sufficient for stress-induced anxiety and place aversion. In response to stress, tonic activity of the LC-NA system increases (621). Acute restraint stress induces anxiety-like behavior, which can be suppressed by selective chemogenetic inhibition of LC-NA neurons. In contrast, optogenetic activation of LC-NA neurons has an acute anxiogenic effect by itself without prior stress exposure. CeA CRF^+ neurons send dense projections to the LC. Optogenetic activation of these CRF^+ terminals increases tonic firing of LC neurons, which entails place aversion and anxiogenic behavior. Taking into account that changing the activity of a given neuron is not necessarily synonymous to changing the levels of a neuropeptide or the activity of a receptor, this study demonstrated that local CRFR1 antagonist infusion into the LC was able to revert the effects of photostimulation of CeA CRF^+ neurons (402).

In the BNST, two populations of CRF^+ neurons were identified that are activated by 5-HT originating from the dorsal raphe nucleus. About 70% of dorsal and ~40% of ventral BNST CRF^+ neurons express the 5-HT receptor 2C (5-$HT_{2C}R$). These neurons are depolarized by 5-HT but do not project to the VTA. The second population of CRF^+ neurons projects to the VTA and is hyperpolarized by 5-HT. Since these CRF^+ neurons are 5-$HT_{2C}R$ negative, another 5-HT receptor seems to promote this effect. Around 60% of these CRF^+ neurons project either to the VTA or the lateral hypothalamus, whereas 20–30% show parallel projections to both structures. The 5-$HT_{2C}R$-expressing CRF^+ neurons project locally and inhibit the anxiolytic GABAergic VTA- and LH-projecting neurons and thus act anxiogenic (289). This provides a possible mechanism to previously observed aversive actions of 5-HT signaling in the BNST and thus might explain acute aversive effects of selective serotonin reuptake inhibitors, which could potentially be attenuated by a CRFR1 antagonist (394).

Parvocellular CRF^+ neurons of the PVN innervate the median eminence to control the HPA axis, but it has been observed that these neurons also send projections to other

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hypothalamic areas, as well as structures outside the hypothalamus relevant for the control of emotional states (675). A recent study demonstrated that PVN CRF$^+$ neurons are able to orchestrate complex behaviors independent of their role in HPA axis control (190). Using extensive phenotyping, a broad array of different natural behaviors of mice was quantified. After stress, these behaviors are not generally changed, but their organization changes, including the time allocated to each behavior. Stress shifts behaviors toward exploratory behaviors (rearing, walking) immediately following the stressor and increases grooming behavior. Optogenetic silencing of PVN CRF$^+$ neurons had a profound effect on the behavior by decreasing the time spent grooming, while the time spent rearing and walking increased without changing the temporal organization of the behaviors. In contrast, photoactivation of the same neurons resulted in more self-directed, internally focused behaviors, particularly expressed by increased grooming. This effect on grooming was confined to a population of neurons in the lateral hypothalamus, which is innervated by axon collaterals of PVN CRF$^+$ neuron efferents to the median eminence. The general adaptation of the behaviors to the environmental context was overlaid by activation of PVN CRF$^+$ neurons, resulting in behaviors not aligned to the context (190). Collectively, these cell-type-specific manipulations unveil a previously unrecognized role of PVN CRF$^+$ neurons in the context of complex behaviors. Recent studies involving selective inactivation of CRF in PVN neurons further support a role of the neuropeptide itself in controlling emotional states and social behavior (167, 675).

The optogenetic and chemogenetic manipulation of CRF- and CRFR-specific circuits has revealed their importance in different aspects of behavioral physiology. However, it has to be noted that the interference on a circuit level is not necessarily synonymous with the action of a peptide ligand or its receptor. Activation or inhibition on the circuit level might entail the induction or blockade of neuropeptide release, but will simultaneously affect other effectors, e.g., neuropeptides and neurotransmitters, which should be taken into account in the interpretation of findings.

VII. THERAPEUTIC POTENTIAL

The CRF hypothesis of depression was launched based on the wealth of clinical findings and postmortem studies and propose CRFR1 as a promising target for the development of next-generation antidepressants and anxiolytics (12, 265, 657). Many patients suffering from major depression show HPA axis disturbances manifested by elevated plasma ACTH and cortisol levels and a disturbed negative feedback regulation (266, 498). Along these lines, enlarged pituitaries and adrenal glands have been found in affected subjects (17, 436). Particular disturbance of the CRF/CRFR system has been consistently reported, including increased levels of CRF in the CSF of many patients and suicide victims suffering from major depression (10, 11, 25, 184, 438). This finding was not confirmed in all studies (320, 417, 478), but a closer examination revealed that subjects with dexamethasone nonsuppression, i.e., impaired feedback regulation, consistently showed higher CRF levels in the CSF compared with those with normal dexamethasone suppression (10). Postmortem studies revealed an upregulation of CRF mRNA expression in the PVN (495, 496) and LC (47, 409), as well as a downregulation of CRFR1 in the prefrontal cortex (408, 437), which is potentially a compensatory regulation due to elevated CSF levels of CRF. This might also lead to desensitization of CRFRs, thus explaining the blunted ACTH response following exogenous CRF challenge (197, 264). Another indication is the observation that antidepressant or electroconvulsive therapy is able to normalize HPA axis activity and CSF levels of CRF. Beyond that, it appears that the maintenance of elevated CSF CRF levels or aberrant HPA axis function, despite successful treatment, could be predictive for relapse into a depressive episode (26, 133, 279, 319).

More recently, human genetic studies also have provided considerable support for the CRF hypothesis of mood and anxiety disorders (44). With the expansion of human genetic studies, the genes for the CRF/CRFR system have been interrogated in greater detail with regards to their potential association with psychiatric disorders. None of the to-date published genome-wide association studies revealed indications of a significant association of CRF/CRFR-related single nucleotide polymorphisms (SNPs) with major depression, bipolar disorder, PTSD, or anxiety disorders. However, testing association of genetic variation in a candidate gene approach revealed that particularly SNPs in the *CRFR1* gene interact with adverse environmental factors to predict risk for the development of stress-related psychiatric disorders. Variations of the *CRFR1* gene appear to moderate the development of depression after childhood trauma (60). Variation in the *CRFR1* gene has been linked to risk for depression in the presence of childhood maltreatment (60, 480). Mainly, the three allele haplotype of *CRFR1* involving the SNPs rs7209436, rs110402, and rs242924 in *intron 1* form a haplotype, where the TAT combination protects severely maltreated individuals against depression. The presence of the rare TAT haplotype shows a significantly decreased risk for adult depressive symptoms in an additive manner in subjects with a history of child abuse (60). This finding was confirmed in another study showing that the TAT haplotype formed by the three SNPs located in *intron 1* of the *CRFR1* gene interact with childhood maltreatment to predict adult depression (480). Interestingly, PTSD subjects with the GG genotype at SNP rs110402 and with a history of moderate to severe childhood abuse showed a significant improvement in symptoms when treated with a CRFR1 antagonist compared with placebo (153). Besides, the TAT haplotype was associated with heightened levels of neuroticism in children who had expe-

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rienced one or two types of maltreatment, but children who had experienced three or four types of maltreatment had similar neuroticism levels as the non-maltreated group (147). Additionally, the SNPs rs110402 and rs242924 were shown to significantly interact with childhood maltreatment to predict the cortisol response in the dexamethasone/CRF test. Maltreated subjects clearly showed an association of the GG genotype of each SNP with elevated cortisol response to the test (611). Moreover, CRFR1 interacts with the promoter region of the 5-HT transporter gene (*5-HTTLPR*) and child abuse to predict current, adult depressive symptoms. Individuals carrying risk alleles in both genes exhibit depressive symptoms at less severe levels of child abuse compared with individuals with no or only one of the risk alleles (508).

In addition, CRFR1 SNPs were found to be associated with panic disorder (305, 658) and with the response to antidepressant treatment (368, 375). The authors tested the association of the haplotype-tag SNPs rs1876828, rs242939, and rs242941 with antidepressant treatment in Mexican-Americans. It appeared that only patients in the high-anxiety group showed a significant association of the GAG haplotype with treatment response (368). Similar results were obtained in Han Chinese patients with major depression. Again, only in high-anxiety patients was the GAG haplotype predictive for a successful response to fluoxetine treatment (375). Another study revealed that individuals carrying the G-allele of rs242939 or the haplotype GGT may be highly susceptible to recurrent major depression when exposed to negative life events (374). Studies in rhesus macaques revealed evidence for an association of SNPs in the *CRFR1* gene (rs242939) and metabolic activity in the intraparietal sulcus and precuneus. These findings suggest that genetic variation in *CRFR1* affects the risk for affective disorders by influencing the function of the neural circuit underlying anxious temperament. In addition, differences in gene expression or in the protein sequence involving exon 6 may play an important role and suggests that variation in *CRFR1* may influence brain function even before any childhood adversity (374).

For *CRFR2*, only a limited number of positive, but also negative, associations with depression and anxiety-related phenotypes have been reported so far (44). A recent study identified a rare variant of *CRFR2* in a family with bipolar disorder, which tracked with the affected status. This nonsense mutation causes a premature stop, leading to a COOH-terminally truncated *CRFR2*. The lack of the COOH-terminal 28 amino acids has functional consequences, including reduced cell surface expression and altered intracellular signaling (123). In addition, testing 16 candidate HPA axis SNPs identified rs28365143 in the *CRF-BP* gene as predictive with respect to antidepressant treatment. Patients homozygous for the G allele had better remission and response rates, as well as reduced symptoms. Interestingly, the homozygous G allele carriers had better treatment outcomes with selective serotonin reuptake inhibitors but not for the combined 5-HT and norepinephrine reuptake inhibitors (447). This finding confirms an earlier *CRF-BP* gene variant association with response to citalopram in African American and Hispanic depressed patients. However, the association was found for a different SNP, rs10473984 (45).

Preclinical and clinical findings conclusively supported the CRF/CRFR system as a promising target for the treatment of psychiatric disorders and stimulated the development of CRFR1 antagonists and realization of a significant number of clinical trials (211, 539). The first clinical study published was a Phase IIa open-label trial designed to assess the safety but also efficacy of NBI-30775/R121919. The compound improved depression symptoms in the Hamilton Depression Rating Scale similar to a serotonergic antidepressant (680) and additionally improved sleep parameters (243). Another upside, NBI-30775/R121919 did not have major impact on HPA axis activity when tested in the CRF challenge test (341). Despite the encouraging clinical results, the further development of NBI-30775/R121919 was discontinued because of liver enzyme elevation observed in some healthy probands. The CRFR1 antagonist NBI-34041 tested in a Phase I study demonstrated safety and tolerability without affecting basal HPA axis regulation. NBI-34041 showed an improved resistance against psychosocial stress (280). A subsequent Phase IIb, double-blind, placebo-controlled trial used the CRFR1-specific antagonist CP-316,311 in patients with recurrent major depression. However, the treatment did not improve Hamilton Depression Rating Scale scores compared with a placebo-treated control group. The study was even stopped prematurely due to the lack of efficacy of CP-316,311 (46). Similarly, a subsequent large, randomized, and placebo-controlled clinical trial of patients with generalized anxiety disorder was unable to demonstrate any therapeutic effect of the CRFR1 antagonist pexacerfont (BMS-562086) (120). An earlier study using the same antagonist for treatment of major depression was completed in 2007 without reporting any results (578). Similarly, the development of several compounds, e.g., ONO-2333Ms, SSR125543, and others, was discontinued due to lack of efficacy in controlled trials for major depression (211, 311, 539, 578). Most recently, the first evaluation of a CRFR1 antagonist for the treatment of PTSD also failed. The antagonist verucerfont (GSK561679) did not show any superiority over placebo (153, 154).

Drug and particular alcohol dependence are additional indications for CRFR1 antagonists (681). Similar to depression, ethanol withdrawal, but also withdrawal of psychostimulants, opiates and benzodiazepines, is associated with enhanced CRF neurotransmission. Activation of the CRF system may contribute to the withdrawal/negative effect

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stage of the addiction cycle. CRF-related circuits may represent a common pathway underlying the observed comorbidity of alcohol dependence and major depression. In the so-called "self-medication" hypothesis, Markou and colleagues (396) suggested that drug use might be motivated by negative reinforcement mechanisms to relieve depression-like symptoms by the anxiolytic and dysphoria relieving effects. However, CRFR1 antagonists verucerfont and pexacerfont have also been disproven in their efficacy as treatments for stress-induced craving for alcohol (347, 549, 560).

Taken together, one has to admit that the enormous expectations were unpredictably disappointed by the collective failure of CRFR1 antagonists, leaving behind a perplexity from which the field has not yet fully recovered. CRFR1 antagonists have joined the queue where most drug developments for psychiatric disorders that started with great expectations end up (311).

Nevertheless, in light of the large body of evidence supporting the hypothesis that the CRF/CRFR system plays an important role in the etiology and pathophysiology of stress-related disorders, the development of refined intervention strategies is the future challenge (267, 454). The currently accepted view is that CRFR1 antagonists have the best prospect as effective antidepressants or anxiolytics in those patients suffering from a dysregulated CRF/CRFR system. Accordingly, a respective stratification of patients would represent a major breakthrough. Currently, the stratification is an unsolved difficulty, as for most psychiatric disorders, reliable biomarkers are basically missing. Assays to interrogate CRF/CRFR system disturbances have been proposed, including *1*) the measurement of CRF levels in the CSF, *2*) the assessment of HPA axis function using the dexamethasone/CRF stimulation (279), or *3*) the characterization of changes in sleep profile using EEG measures (243). However, it remains unclear to what extent these assays have the required specificity to act as a reliable proxy for the central CRF/CRFR system that would qualify them as a biomarker. None of these assays was considered in previously conducted clinical trials, not to mention that these biomarker concepts are far from being implemented as diagnostic tests in routine clinical practice. Alternatively, a pharmacogenomic strategy could be a possibility to overcome the current situation using, for instance, SNPs in the *CRFR1* gene to stratify patients according to their probability to respond to pharmacotherapy (45). The observation that the GAG haplotype related to SNPs rs1876828, rs242939, and rs242941 in high-anxiety patients is positively associated with the antidepressant response points in this direction (368).

Considering the wealth of promising preclinical data, other stress-related pathologies, such as neurodegenerative disorders, should be considered valid targets for CRFR1 antagonist treatment. Particularly, the earlier discussed implication of CRFR1 in Alzheimer's disease could profit from already approved CRFR1 antagonists (673).

In addition, the optimization of small molecules antagonizing CRFR1 activity possesses further potential for improvement of therapeutic effectiveness. Existing CRFR1 antagonists are based on low-affinity lead compounds identified by a limited number of high-throughput screens of chemical libraries (13, 257, 548). Hence, it is not surprising that most high-affinity, small-molecule antagonists share a similar chemical scaffold that serves to position the functional groups at the right distance and in the right orientation within the binding pocket. Consequently, CRFR1 antagonists seemingly bind to the same binding site (H199 in TM3 and M276 in TM5; **FIGURE 10**) and act as allosteric modulators blocking the action of peptide ligands. Binding within the TMD stabilizes the inactive state, leaving the receptor unable to transduce signals; thus these compounds act as inverse agonists (213, 253, 454). Interestingly, the structurally different small-molecule NBI 77173 acts as a partial agonist stabilizing a weak active state, which is blocked from transition into a fully active state (253). An important aspect concerns the binding kinetics of small-molecule antagonists, i.e., kinetics of association with, and dissociation from, the receptor. Careful analyses of receptor association and dissociation revealed remarkable differences between lead compounds, which were consistent with in vivo pharmacodynamics, as assessed by the suppression of ACTH release in antagonist-treated rats (179). These previously unrecognized differences might have contributed to the variable outcome of clinical trials and suggest that the pharmacology of CRFR1 antagonists could be advanced by maximizing receptor residence time, e.g., by slowing antagonist dissociation (179). Similarly, the exact assessment of the antagonist dosage would be crucial to determine optimal CRFR1 occupancy in the brain. Ligands for positron emission tomography would allow exact monitoring, but seemingly these are still under development and not yet established for clinical use (376, 684). The recently unveiled detailed three-dimensional structure of CRFR1 (261) opens previously not existing opportunities for rational drug design. Novel compounds might be engineered to act on the CRFR1 at different binding sites and thereby might be able to influence downstream signaling in a more differential manner (146).

In recent years, we have seen a plethora of studies demonstrating a high regional or cell-type specificity of effects, which are difficult to translate into an adequate treatment (244). In this context, it is also important to emphasize the need for a more critical debate and evaluation of preclinical data. It is imperative to take into account the complete picture: the selective perception of some results and ignorance of others will not be productive. One example is the somewhat ignored contrary outcomes of behaviors in the

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forced swim test: some CRF overexpressing mouse lines show increased floating interpreted as behavioral despair (321), but several others show no effect (215) or even decreased floating (384, 628), which is in agreement with the effects observed following central application of the CRFR1-specific agonist cortagine (602). The latter would surprisingly suggest that a CRFR1 agonist has relevant antidepressant potential. Similarly, the variable outcomes of local virus-based CRF overexpression reveals a complexity, which reinforce the impression that the novel tools implemented in preclinical research provide an increasingly more detailed picture with which the development of pharmacotherapy is currently not able to keep up pace.

VIII. CONCLUSIONS AND FUTURE PERSPECTIVES

The significance of CRF-related peptides and their receptors as a central hub of the physiological stress response system has attracted major research efforts over the past few decades. Since the discovery of CRF in 1981, our understanding of the system has significantly advanced, often driven by technological leaps that provided novel experimental possibilities to address previously unsolved mysteries. Many current uncertainties will be illuminated in the near future by currently implemented technologies. For example, most refined expression maps of the brain and periphery or developmental stages in rodents but also humans can be expected in the course of the current single-cell sequencing boom (355, 452). Latest improvements in cryo-electron microscopy have overcome the obstacles of X-ray crystallography to solve structures of TM molecules, such as GPCRs (338). This development has led to a burst of publications reporting structures of GPCRs, including the detailed structure of CRFR1. This will shed light on structural problems, such as ligand receptor interactions and mechanisms of signal transduction. In addition, these results will supply the blueprint for intelligent design of novel agonists or antagonists (57). This could ultimately help to overcome the limited efficacy of the CRFR1 antagonists, which found their way into clinical trials, as they are all based on uniform core chemical structure. The development of chemogenetic and optogenetic effectors, in conjunction with viral vectors (e.g., lentivirus, AAV, rabies virus) and Cre driver lines, has opened up new possibilities to address the CRF system with previously unmet precision on the circuit level (195, 273, 290). Current publications are most likely only the prelude to a surge of studies addressing how CRF-related peptides and receptors or their hosting neurons shape neuroendocrine, autonomic, or behavioral stress responses. However, it is foreseeable that additional tools are required, e.g., providing access to UCN1, UCN2, and CRF-BP cells, as well as the possibility to simultaneously address two or more constituents of the CRF system by using different site-specific recombinases (e.g., FLPo, Dre). Mouse mutants have enormously contributed in the last 2 decades to dissect the CRF system. Novel conditional mouse lines, such as floxed CRF and CRFR2 mice, which are vulnerable to conditional inactivation, have just been recently established and are about to be fully explored. Moreover, an appropriate model of CRF overexpression is overdue. Surprisingly, all attempts to model CRF hyperactivity do not meet expectations, as the many currently available mouse models or viral approaches are compromised by significant ectopic expression. A broad spectrum of mouse genetic and viral vector-based tools is required to unravel, e.g., the connectivities of ligand and receptor-expressing cells, and to elucidate how these circuits impinge on the stress response and, vice versa, how they are themselves shaped by it.

In vitro studies have their worthiness, and we would not understand the complexity of signaling modalities of CRF-related neuropeptides and CRFRs without the groundwork achieved by approaches involving heterologous expression. This type of approach will remain important, but it can be envisioned that the implementation of human-induced pluripotent stem cells and neuronal cultures or organoids derived thereof might provide additional facets. In vitro studies have demonstrated that CRFR-mediated signal transduction largely relies on the cellular context, i.e., availability of signaling molecules or interaction partners, a fact that must be addressed in vivo. This includes the important question: where in a given neuron are CRFRs located on the subcellular level, and whether this has consequences on CRFR signaling with respect to potential signaling microdomains? From earlier studies in the hippocampus and PVN, we have obtained a fundamental understanding of how CRF release is promoted, but for most brain structures this is still far from being even approximately understood. Where and under which circumstances CRF neurons release their cargo and how released CRF-related peptides activate their receptors remain a fundamental question.

In general, the translation of the increasingly more detailed comprehension of brain structure and function on the molecular, cellular, and circuit scale back to the organismic or behavioral level is a major future challenge. In the case of the CRF system, one future goal is to understand how changes on the micro-, meso- and marco-scale convey variations on the organismic level to promote adaptation. To master the meaningful integration and interpretation of different data levels, it will be mandatory to involve computational and systems neuroscience (30, 137). It can be expected that the current technological advances will be further promoted by the worldwide efforts of national and international initiatives to understand brain function and disorders (214).

It is obvious that the understanding of a stress response system on the organismic level relies on in vivo models.

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However, it has become evident that many of our current behavioral models are limited in their significance and by their anthropomorphic interpretation. The Porsolt forced swim test (483) is a prime example where immobility or floating behavior is misinterpreted as a measure for depression or despair (439). The test shows high predictive validity and reproducibility in screening for drugs acting on biogenic monoamines. However, other prerequisites of a valid animal model, i.e., construct and face validity, are obviously lacking in this acute test: depression is a disease developing over longer periods, and there is no disease symptom that would reflect the readout immobility. Moreover, the test is used to acutely test the effects of drugs, whereas antidepressants require several weeks to be effective in patients. The suggestion that the switch from swimming to floating/immobility is an adaptive response involving learning and memory processes, which guides the shift from active to passive coping strategies, seems more reasonable (418).

We need more refined behavioral tests with better ethological validity that provide more objective and quantitative measures (190, 563, 663). In addition, the ANS could provide another, currently largely neglected, entry point to quantitatively evaluate stress responses. Particularly the modulatory effect of CRF on the ANS has surprisingly not been further pursued after its initial description soon after the discovery of CRF. To what extent the stress response affects CRF-related peptides and receptors on the epigenetic level, and thereby regulates their expression levels, is another urgent question.

Finally, we believe that the therapeutic potential of the CRF/CRFR system has to be reconsidered. The negative outcome of previous clinical trials of CRFR1 antagonists has unfortunately stalled additional efforts, despite the fact that preclinical work has, in the meantime, established a more developed view on the complexity of the system. Careful evaluation of failed clinical trials, e.g., assessing genetic profiles of subjects included in the trial, might help to identify more successful strategies. A personalized approach based on alterations in the CRF system might be necessary for preselection of subjects matching a CRFR1 antagonist treatment. Moreover, the cross-talk between CRFR1-specific signal transduction and other GPCRs, e.g., 5-HT_{2A} receptors, suggests that adjunctive treatments of CRFR1 antagonists, together with small molecules affecting other neurotransmitter/modulator systems, might provide additional means to revive a clinical perspective.

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