Corticotropin-Releasing Factor Binding Protein – A Ligand Trap?

Olaf Jahn, Jelena Radulovic, Oliver Stiedl, Hossein Tezval, Klaus Eckart and Joachim Spiess*

Department of Molecular Neuroendocrinology, Max Planck Institute for Experimental Medicine, Hermann-Rein-Str. 3, 37075 Goettingen, Germany

Abstract: The actions of the neuropeptide corticotropin-releasing factor (CRF) are modulated by a CRF binding protein (CRFBP). In view of the memory-enhancing effects of CRF, the release of endogenous CRF from CRFBP by CRFBP inhibitors has been suggested as a therapeutical strategy for the treatment of cognitive deficits. This mini-review will summarize recent advances in the field with a focus on the pharmaceutical potential of CRFBP inhibitors.

Keywords: Corticotropin-releasing factor (CRF), CRF binding protein (CRFBP), CRFBP inhibitor, urocortin, cognitive deficits.

INTRODUCTION

Corticotropin-releasing factor (CRF), a C-terminally amidated 41 amino acid neuropeptide originally identified and characterized on the basis of its hypophysiotropic actions [1, 2], is the key mediator of the mammalian response to stressors. Exposure to stressors results in a series of coordinated stress responses organized to enhance the probability of survival and to return the organism to homeostasis. In view of its hormonal function within the neuroendocrine stress response, CRF is an early chemical trigger of the hypothalmus-pituitary-adrenal (HPA) axis whose end point is the release of glucocorticoids, the most important mediators of the metabolic changes associated with the stress response [3]. Outside of the hypothalamus, CRF is produced at many other sites in the mammalian central nervous system (CNS) and serves as neuromodulator in addition to its hypophysiotropic actions [4]. CRF modulates a variety of complex brain functions such as anxiety, learning and memory, locomotor activity, and food intake [4-6]. Moreover, CRF is linked to the pathogenesis of various anxiety, mood, and eating disorders [7, 8]. In the periphery, CRF modulates cardiovascular functions [9] and plays an immunomodulatory role [10]. Taken together, there is a growing body of evidence that CRF integrates the neuroendocrine, autonomic, behavioral, and immunologic responses to stress.

CRF acts through two subtypes, CRF_1 and CRF_2, of a G protein-coupled receptor. The CRF receptor belongs to the secretin-like family of G protein-coupled receptors. The two CRF receptor subtypes differ in their tissue distribution and pharmacological properties. The biology of the CRF receptor has been the subject of numerous recent reviews [11-13]. Additionally, the mammalian CRF system comprises a CRF binding protein (CRFBP) and four different naturally occurring ligands, CRF and the related peptides urocortin I (UcnI) [14], UcnII (also known as stresscopin-related peptide) [15, 16], and UcnIII (also known as stresscopin) [16, 17] (Fig. **1**). Other important nonmammalian members of the CRF peptide family are amphibian sauvagine (Svg) [18] and fish urotensin I [19]. The evolution and physiology of CRF and its related peptides have recently been reviewed [20].

In contrast to the membrane-bound CRF receptors, CRFBP is a secreted 37 kDa protein without significant sequence homology to the CRF receptor or to any other known class of proteins. Although numerous hormone-binding proteins have been identified, including proteins binding peptide hormones such as the insulin-like growth factor (IGF) binding protein (IGFBP) superfamily [21], CRFBP is the only known binding protein with high affinity for a neuropeptide. It was originally identified on the basis of the hypothesis that it may protect the maternal HPA axis from overstimulation by the high levels of CRF produced by the placenta during pregnancy [22, 23]. In humans, CRFBP was found at various peripheral sites such as plasma, placenta, amniotic fluid, and synovial fluid, as well as in brain and pituitary, whereas in rodents and sheep, CRFBP has been detected only in brain and pituitary [24]. In the rat brain, cerebral cortex and hippocampus appear to be the most prominent sites of CRFBP mRNA and protein production as demonstrated by *in situ* hybridization and immunodetection (reviewed in [25]). Although its physiological role – especially in the CNS – still remains elusive, CRFBP has become an interesting target of pharmacological research since it is known that approximately 50 % of the total human brain CRF is bound to CRFBP [26, 27] and, therefore, not available for CRF receptors. Thus, CRFBP represents a pharmacologically significant pool of endogenous ligand. Because of the distinct distribution of CRFBP in the brain, CRF release by CRFBP inhibitors can exhibit CRF receptor subtype-specific actions. In view of the enhancement of learning and memory by activation of hippocampal CRF_1, the release of CRF from CRFBP in the hippocampus potentially is of therapeutical significance to reduce cognitive deficits as observed for example in Alzheimer's disease [26].

The roles of peripheral CRF and CRFBP in the mechanism of human parturition have been extensively reviewed [28-30] and will not be covered here. The present mini-review will provide an update on biochemical, pharmacological, and structural aspects of CRFBP, and will

*Address correspondence to this author at the Department of Molecular Neuroendocrinology, Max Planck Institute for Experimental Medicine, Hermann-Rein-Str. 3, 37075 Goettingen, Germany; Tel: +49-551-3899-258; Fax: +49-551-3899-359; E-mail: spiess.office@mail.em.mpg.de

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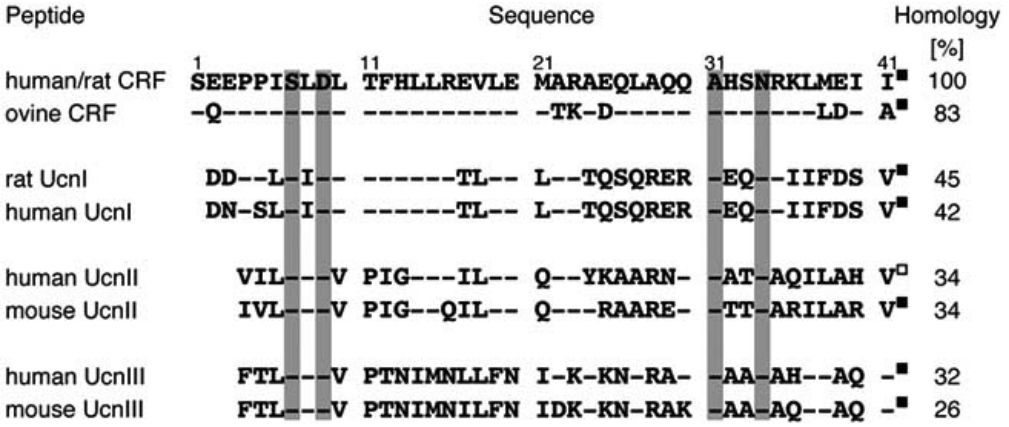

Fig. (1). ***Amino acid sequence alignment of selected mammalian members of the CRF peptide family.*** The sequences are shown in comparison to human/rat CRF. A dash marks an identical amino acid. Residues that are homologous between the CRF peptides are underlaid in grey. A C-terminal amide group is indicated by a filled square. Since the corresponding precursor protein of human UcnII lacks a known amidation site [16], C-terminal amidation is putative for this peptide and therefore indicated by an open sqare.

focus on the potential functions of this protein in the CNS, as well as on the therapeutical potential of CRFBP inhibitors.

BIOCHEMISTRY OF CRFBP

CRFBP was originally purified from human plasma [31] and subsequently cloned from human liver and rat brain cDNA libraries [32]. Human and rat CRFBP cDNAs display a high degree of sequence homology and encode precursor proteins consisting of 322 amino acids with one putative N-linked glycosylation site and eleven conserved cysteine residues (Fig. **2**) [32, 33]. The first cysteine residue is contained within the putative signal sequence, while the other ten cysteine residues form five sequential disulfide bridges which are essential for ligand binding activity [34, 35]. Highly homologous cDNAs encoding mouse [36] and sheep [37] CRFBP have also been cloned (Fig. **2**). In all four mammalian species serving as sources of mRNA coding for CRFBP and used for cDNA cloning, the corresponding proteins share an average amino acid homology of 86 %. The positions of the cysteine residues and the single glycosylation site are strictly conserved (Fig. **2**).

Mass spectrometric peptide mapping and N-terminal sequencing of recombinant rat CRFBP [35] provided

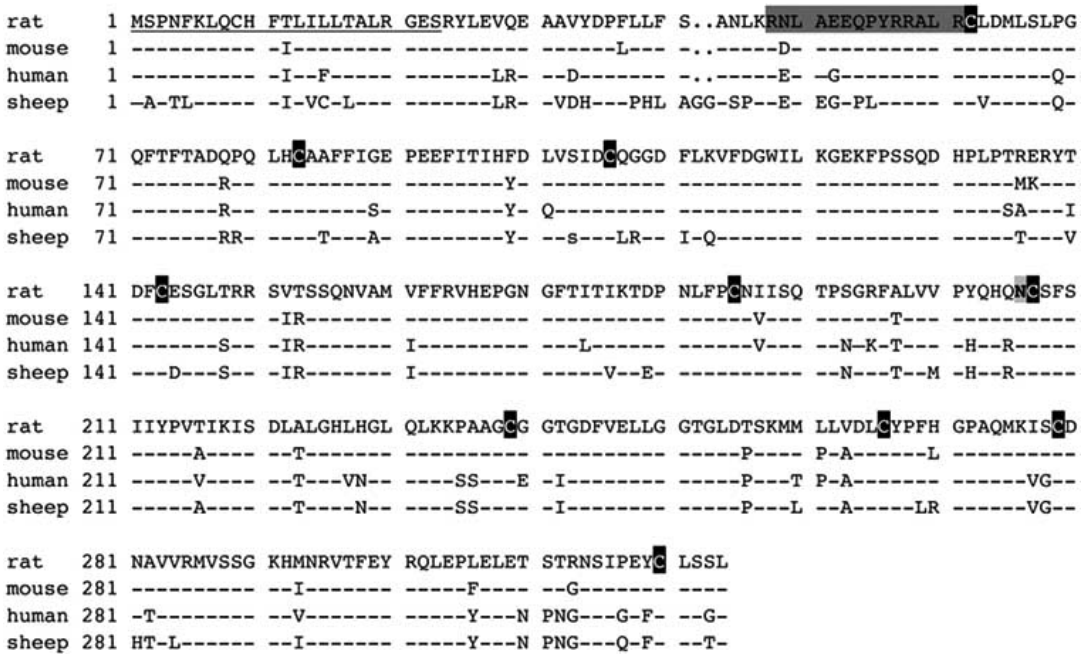

Fig. (2). ***Amino acid sequence alignment of mammalian CRFBP.*** The sequences are shown in comparison to rat CRFBP. A dash marks an identical amino acid. Dots indicate deletions or insertions. The signal sequence identified with recombinant rat CRFBP is underlined. The ten conserved Cys residues involved in the formation of disulfide bridges are underlaid in black. The single, conserved N-linked glycosylation site is shown in bold font and is underlaid in light grey. On the basis of a photoaffinity labeling approach, the ligand-binding site has been mapped to the stretch of amino acids of rat CRFBP which is underlaid in dark grey (see text for details).

evidence that the signal peptide consists of 23 amino acids (Fig. **2**). The finding of Arg^{24} as the N-terminal amino acid of the purified protein was in agreement with the results obtained by a prediction algorithm for the identification of signal peptides and their cleavage sites [38]. It is therefore likely that the conserved residue Arg^{24} is the N-terminal amino acid of all mammalian versions of CRFBP, although Tyr^{25} has been previously proposed as N-terminal residue of recombinant human and rat CRFBP [32].

On the basis of different protein species observed by gel electrophoresis and the presence of the conserved motif –Asn–Cys–Ser–, a consensus site for N-linked glycosylation, it was proposed that human and rat CRFBP are glycosylated at Asn^{206} (Fig. **2**) [35, 39]. By using mass spectrometric and other analytical techniques, it was shown that recombinant human CRFBP carries one N-linked carbohydrate of the complex type [40]. The glycosylation was found not to be important for CRFBP activity as indicated by indistinguishable binding characteristics of CRFBP and its nonglycosylated analogs [40]. Analysis of purified recombinant rat CRFBP by electrospray mass spectrometry revealed a heterogeneous protein mixture consisting of five protein species that differed by an average mass increment of 162 Da (Fig. **3**). This characteristic pattern of microheterogeneity is compatible with the assumption that rat CRFBP carries one N-linked carbohydrate of the high-mannose type [41]. The high-mannose structure of the oligosaccharide was confirmed on the basis of the mass spectrometric characterization of the isolated proteolytic glycopeptide using selective fragmentation of the carbohydrates by in-source collision-induced dissociation (O. Jahn, K. Eckart, J. Spiess; unpublished data). For both human and rat CRFBP, no evidence for O-glycosylation was found [35, 40]. The different types of N-glycosylation identified for human and rat CRFBP, respectively, imply species differences that may reflect the different distribution patterns for this protein (see above). However, the oligosaccharide structures of recombinant glycoproteins can be influenced by the host cell lines used [42] and the cell culture conditions applied [43]. Thus, the biological significance of these findings remains unclear until data on the types of N-glycosylation of endogenous CRFBP from various species are available.

PHARMACOLOGY OF CRFBP

The structure activity relationship between peptides of the CRF family and their receptors has been investigated in numerous studies (reviewed in [13]). Whereas the stretch of amino acids 3 to 41 is required for full biological potency, N-terminal truncation of CRF by 8 to 11 amino acid residues leads to CRF antagonists. The C-terminal part of the CRF molecule including the C-terminal amide group is required for high affinity binding to CRF receptors. The ligand requirements of CRFBP are different from those for the CRF receptor subtypes. By N- and C-terminal truncation of human/rat CRF (h/rCRF) or rat UcnI (rUcnI), it has been demonstrated that the central part of the peptides, residues 6 to 33 of h/rCRF or 5 to 32 of rUcnI, is sufficient for high affinity binding to CRFBP [35, 44]. These differences in the

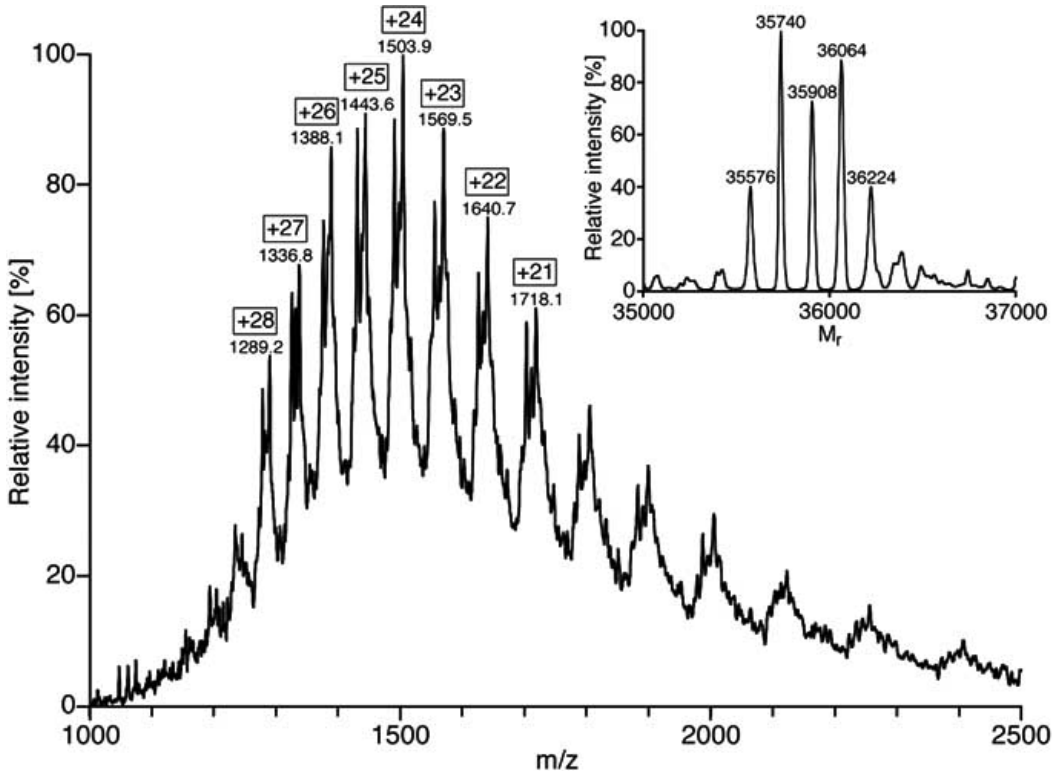

Fig. (3). ***Electrospray mass spectrum of purified recombinant rat CRFBP.*** The protein was sprayed from an aqueous mixture of acetonitrile (50 %) and acetic acid (1 %). The mass spectrum was recorded on a Waters Micromass Autospec-T four-sector tandem mass spectrometer equipped with a NanoES interface upgrade. The deconvoluted mass spectrum is shown in the inset. The deconvolution was performed on the basis of the charge states $[M+21H]^{21+}$ to $[M+28H]^{28+}$.

structural requirements for ligand binding allow the design of CRFBP-specific peptide fragments which bind with high affinity to CRFBP, but not to the CRF receptor. This structure activity relationship was the basis for the development of peptidic CRFBP inhibitors which are capable to displace specifically endogenous CRF from CRFBP [26]. This displacement results in an increased availability of "free" CRF to act as agonist at the CRF receptor (see below). The synthetic CRF fragment h/rCRF^{633} has been accepted as the peptidic lead compound of CRFBP inhibitors [26, 44]. Instead of N- and C-terminal truncation of CRF-like peptides, the C-terminal desamidation of h/rCRF or rUcnI (Fig. **1**) to generate h/rCRF(OH) and rUcnI(OH) is an alternative approach for the design of CRFBP inhibitors. Since the presence of the C-terminal amide group is required for high-affinity binding to the CRF receptor, but not to CRFBP, this modification generates CRFBP-specific ligands. As can be concluded on the basis of their pharmacological profiles (Table **1**), h/rCRF(OH) and h/rCRF$^{6-33}$ are useful inhibitors due to their high specificity for CRFBP, whereas rUcnI(OH) exhibits some residual affinity to the CRF receptor.

Table 1. Binding of h/rCRF, rUcnI, and their Related CRFBP Inhibitors to CRFBP and CRF Receptors

	IC_{50} [nM]		
Peptide	**rat CRFBP**	**rat CRF_1**	**mouse $CRF_{2\beta}$**
h/rCRF	0.54	1.6	42
h/rCRF(OH)	0.93	1600	> 3000
h/rCRF$^{6-33}$	1.9	> 3000	> 3000
rUcnI	0.98	0.17	0.86
rUcnI(OH)	1.1	110	260

Affinity constants were generated by competition binding assays using scintillation proximity assay techniques as described in [45].

Although h/rCRF and its sheep ortholog ovine CRF (oCRF) differ by only seven amino acids (Fig. **1**), the affinity of h/rCRF to CRFBP is higher than that of oCRF by approximately three orders of magnitude. The stretch of residues 22 to 25, –Ala–Arg–Ala–Glu–, of h/rCRF was found to be responsible for the high affinity of h/rCRF [44], in contrast to the low affinities of oCRF and Svg containing the sequences –Thr–Lys–Ala–Asp– and –Glu–Lys–Gln–Glu– instead [35, 44]. On the basis of these findings, it was recently established that the first amino acid of the four amino acid motif –Ala–Arg–Ala–Glu– serves as a switch enhancing or preventing high affinity binding to CRFBP [45]. This switch role was recognized on the basis of the observation that [Glu^{22}]h/rCRF is not bound with high affinity to CRFBP in contrast to h/rCRF naturally containing Ala^{22} or [Ala^{21}]Svg, which both exhibit high affinity [45]. The crucial contribution of Ala^{22} of h/rCRF to high affinity binding may result from its localization within the hydrophobic patch of the amphiphilic α-helix proposed to be formed by CRF-related peptides [46-49]. Assuming that the hydrophobic patch is important for binding to CRFBP, the interaction may be disturbed due to the introduction of the charged bulky Glu residue in [Glu^{22}]h/rCRF. Since the affinity of both h/rCRF and [Glu^{22}]h/rCRF to either CRF receptor subtype is not decreased by this single amino acid exchange, and their subtype selectivity is not changed either, this switch can be applied to discriminate between CRFBP and CRF receptors. This strategy permits the synthesis of CRF analogs to selectively target CRFBP or CRF receptors without cross-reaction and was already successfully applied to design [Glu^{11,16}]astressin [45], "acidic astressin", a CRF antagonist of higher solubility than the original astressin.

STRUCTURAL INSIGHT INTO LIGAND BINDING OF CRFBP

So far, no three-dimensional structure information by nuclear magnetic resonance (NMR) or crystallography data is available for CRFBP. Moreover, molecular modeling approaches are rendered difficult, in part due to the fact that there is no suitable template to be used for molecular modeling since CRFBP does not display significant sequence homology to any other known class of proteins. However, detailed knowledge on the ligand-binding site of CRFBP is of great importance to facilitate the design of new peptidic and non-peptidic CRFBP inhibitors. Recently, the ligand-binding site of rat CRFBP was identified using a photoaffinity labeling approach [50]. In this study, new photoreactive analogs of the CRFBP inhibitor h/rCRF$^{6-33}$ were employed in combination with different mass spectrometric techniques [51, 52] to directly determine contact sites between residues of CRF and its binding protein on the level of the single amino acids involved. Thereby, the binding site of h/rCRF$^{6-33}$ on CRFBP was mapped to a 14 amino acid stretch N-terminally of Cys^{62}, participating in the first disulfide bridge (Fig. **2**). On the basis of the results of photoaffinity labeling experiments using a bifunctional photoprobe, an anti-parallel alignment of the α-helical peptide h/rCRF$^{6-33}$ and the N-terminal domain of CRFBP during binding has been proposed [50]. In view of the characterization of the conformation of the ligand-binding site and the binding mechanism, these findings will greatly facilitate computer-simulated ligand-docking techniques as soon as the three-dimensional structure of CRFBP has been elucidated.

As to the subunit structure of CRFBP, it has been proposed on the basis of gel filtration data that human CRFBP dimerizes after association with its ligand [53]. However, when photoaffinity labeling in combination with chemical cross-linking was used to investigate the composition of the CRFBP-binding complex, it was found that one molecule of h/rCRF was bound to the rat CRFBP monomer [50]. Whether this observation also holds for human CRFBP has to be established.

CRFBP – A LIGAND TRAP?

On the basis of numerous *in vitro* and *in vivo* studies that suggest an inhibitory role for CRFBP, this protein may represent a passive ligand trap which terminates the actions of CRF and its analogs. However, in view of the significant effects of CRFBP inhibitors, CRFBP may also serve as a ligand reservoir for pharmacological intervention. Whether the release of CRF from CRFBP is also of physiologic significance, remains to be established. In view of some effects of CRFBP inhibitors that are inconsistent with a

simple elevation of CRF levels, it is suggested that CRFBP may have additional active functions in the brain. Monitoring of the immediately early gene product FOS in the brain following intracerebroventricular (i.c.v.) infusion of h/rCRF$^{6-33}$ suggests a limited capacity of the CRFBP inhibitor to activate neurons bearing CRF receptors [54]. On the basis of the distinct FOS production in CRFBP-positive neurons, it was proposed that CRFBP could play a role in signaling by CRF-related peptides independently from CRF receptor activation [54]. In a very recent study on synaptic transmission of midbrain dopamine neurons, CRFBP was found to be required for the CRF-mediated potentiation of the N-methyl-D-aspartate (NMDA) receptor signaling [55]. This study provides the first evidence that CRFBP seems to serve an active role in a CRF-mediated process. Further studies will be needed to discover additional CRFBP activities that possibly do not involve CRF or CRF receptors as reported for the IGFBP system (reviewed in [56]).

ROLE OF CRFBP IN METABOLIC AND CARDIOVASCULAR REGULATION

Besides its essential roles in HPA axis function as well as learning and anxiety, the CRF system has been implicated in affecting metabolic (reviewed in [57, 58]) and cardiovascular regulation (reviewed in [9]). CRFBP is widely expressed in different brain areas including some hypothalamic substructures involved in the regulation of these functions [59-61]. Therefore, CRFBP with its capacity to reduce the available pool of endogenous CRF has become a target to study effects on food intake, body weight, and body temperature regulation, as well as heart rate and blood pressure regulation.

The potential role of CRFBP in metabolic regulation has been recently studied in food-deprived obese and lean Zucker rats [61]. In this study, enhanced expression of the gene encoding CRFBP was induced in the medial preoptic area and the basolateral amygdala by food deprivation and, to a lesser extent, obesity. The enhanced gene expression is matched with an increased production of CRFBP. Thus, it was hypothesized that induction of the CRFBP gene may occur as a feedback mechanism to reduce energy expenditure and stimulate food intake [61]. The concept of a feedback system between CRF and CRFBP production is demonstrated in transgenic mice overexpressing the CRFBP gene. They constitutively produce elevated levels of CRFBP in the anterior pituitary. In these CRFBP-overexpressing mice created by Burrows and colleagues [62], levels of mRNA coding for CRF and vasopressin are substantially elevated. However, although these mice display an attenuated circadian fluctuation of food consumption and body weight, their 24 h weight change is similar to their wild type littermate controls [62]. Long-term weight gain profiles were not obtained in this study. In a similar study, the CRFBP-overexpressing mice created by Lovejoy and colleagues [63] were found to exhibit an increased weight gain with a sexually dimorphic time of onset. Food intake studies would be needed to clarify whether increased food intake or decreased energy expenditure contribute to the altered weight gain observed in these transgenic mice. Investigations of CRFBP-deficient mice revealed a decreased body weight in male, but not female mice [64]. This weight reduction was attributed to reduced food intake.

The potential role of CRFBP in metabolic regulation has been also addressed by pharmacological experiments using the CRFBP inhibitor h/rCRF$^{6-33}$. I.c.v. injections of h/rCRF$^{6-33}$ were found to blunt excessive weight gain in two animal models of obesity [65]. Suppressed food, but not water intake was confirmed by chronic i.c.v. injection of h/rCRF$^{6-33}$ in obese, but not lean Zucker rats [66].

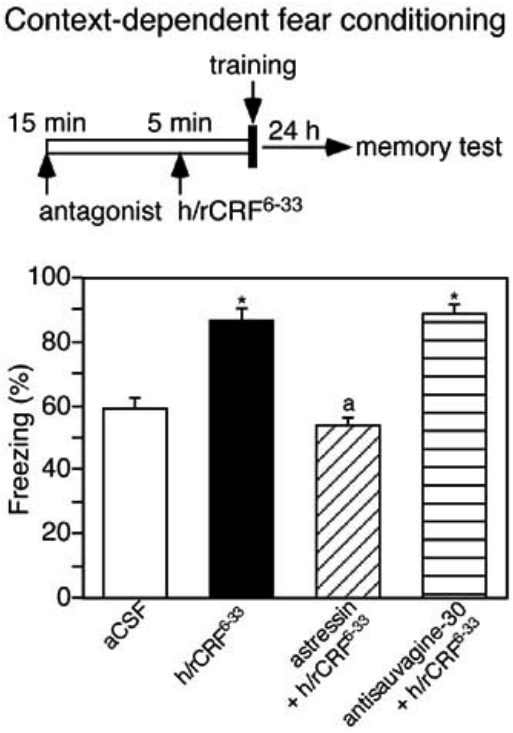

Fig. (4). ***Enhancement of conditioned fear by release of endogenous CRF from hippocampal CRFBP.*** Mice were trained in the fear conditioning paradigm by a single 3-min exposure to a context followed by a 2-s footshock. Freezing, defined as the absence of any movements exept for those related to respiration or heartbeat, indicated the acquisition of conditioned fear. Freezing was measured 24 h later by re-exposing the mice to the conditioning context. Mice were injected with the CRFBP inhibitor h/rCRF$^{6-33}$ 5 min before training. Vehicle or antagonists were injected 15 min before training. Astressin but not antisauvagine-30 prevented the enhancing effect of h/rCRF^{633} on fear conditioning, as indicated by significant attenuation of freezing behavior (J. Radulovic and J. Spiess, unpublished data). Statistically significant differences: $^{*}p < 0.01$ vs vehicle; $^{a}p < 0.01$ vs h/rCRF$^{6-33}$.

It was observed that i.c.v administration of CRF elicits a profound tachycardia in rats [67] that was interpreted to reflect the autonomic activation in response to stress in this species (reviewed in [9]). However, i.c.v. injection of h/rCRF$^{6-33}$ in rats does not mediate the tachycardia and hypertension that is elicited by centrally administered CRF itself [65, 68]. In agreement with these observations, the lack of heart rate effects mediated by i.c.v. injected h/rCRF$^{6-33}$ was confirmed in mice (O. Stiedl, J. Spiess; unpublished data). This lack of action of CRFBP inhibitors may be explained by the differential anatomic distribution of CRF

receptors and CRFBP in the brain. Thus far, no role of central CRFBP in cardiovascular regulation under physiological conditions has been established.

ROLE OF CRFBP IN LEARNING AND ANXIETY FORMATION

An important role of CRFBP in cognitive functions has been first recognized by Behan and coworkers [26] who demonstrated that displacement of CRF from CRFBP significantly improved learning in rats. I.c.v. application of the CRFBP inhibitor h/rCRF$^{6-33}$ resulted in an enhancement of spatial learning [26], visual discrimination [69], passive avoidance [69], and fear conditioning [70, 71]. Intrahippocampal microinfusions of both h/rCRF and the CRFBP inhibitor h/rCRF$^{6-33}$ resulted in an enhanced acquisition of conditioned fear through CRF_1 [70], as revealed by the ability of the nonselective CRF antagonist astressin [45, 72], but not the CRF_2-selective antagonist antisauvagine-30 [73] to block their effects (Fig. **4**). These results indicate that a substantial amount of endogenous CRF is bound to the CRFBP in the mouse brain.

On the basis of the lowered CRF levels in Alzheimer's disease [74, 75], it has been speculated that the memory deficit of Alzheimer patients could be explained – at least in part – by a reduced CRF stimulation [26]. Since CRFBP levels are unchanged in Alzheimer's disease [26], CRFBP inhibitors passing through the blood brain barrier should be considered as potential drugs in this disease condition.

Taking into account that CRF significantly enhances neuronal excitability [76] possibly resulting in epileptiform activity [77], it is suggested that binding of CRF to CRFBP may prevent neuronal hyperexcitability. In line with this view are recent findings demonstrating up-regulation of CRFBP in the entorhinal cortex following seizures [78].

Even at high doses (25 µg (5.3 nmol) per rat) h/rCRF^{633} did not elicit anxiogenic-like effects typically produced by lower doses of the native receptor ligand h/rCRF [26, 69, 79, 80]. It is probable that these differences are caused by the differential distribution of CRFBP and CRF receptors. CRFBP is relatively enriched in brain areas involved in learning and memory such as cerebral cortex and hippocampus compared to some limbic and brain stem areas involved in emotional responses (reviewed in [25]). Therefore, administration of CRFBP inhibitors leads to a functional dissociation of cognition-enhancing effects from stress-like and anxiogenic effects of exogenously applied CRF agonists. In conclusion, individual brain functions such as memory and anxiety modulated by CRF can be pharmacologically targeted by CRFBP inhibitors.

Apart from the pharmacological approaches described above, the role of CRFBP *in vivo* was also investigated by transgenic mouse models of CRFBP gene overexpression [62, 63] and CRFBP deficiency [64]. The phenotypes of these mutant mice have recently been reviewed in detail [25]. Interestingly, CRFBP-deficient mice display increased anxiety-like behavior. This finding was surprising in view of pharmacological experiments reporting that CRFBP inhibitors injected into the brain ventricles do not enhance anxiogenic actions. This discrepancy may be due to the missing "buffer" function of CRFBP in the CRFBP-deficient mouse or limited access of the CRFBP inhibitors to brain areas involved in the regulation of anxiety-like behavior.

Studies with rats demonstrated a significant dissociation between cognitive and anxiogenic roles of CRFBP during aging. I.c.v. injection of h/rCRF$^{6-33}$ significantly improves active avoidance learning of aged rats [69]. It can be speculated that the reduction of CRF available for receptor binding is responsible for impaired learning of aged animals. In contrast, anxiety-like behavior is reduced in old rats, and the observed lowered anxiety-like behavior is accompanied by significant reductions of CRF and CRFBP in the amygdala [81]. On the basis of these data, it appears that the CRF system undergoes regional alterations (with aging) which can significantly alter individual functions of the CRF systems in behavior.

THERAPEUTICAL POTENTIAL OF BINDING PROTEIN INHIBITORS

By definition, binding protein inhibitors specifically bind to the binding protein(s), but not to the cell surface receptors of a particular ligand. Consequently, these compounds are able to displace and thereby release endogenously bound ligand from binding proteins functioning as ligand pools. If binding proteins and receptors are co-localized, this process results in elevated levels of "free" ligand that can activate the receptors. The released ligand acts in a spatially limited fashion and the elicited effects may be therefore substantially differ from those observed after administration of receptor agonists. In general, the use of binding protein inhibitors to locally elevate agonist levels has the advantage that side effectswhich may occur after global receptor activation by exogenously applied agonist are limited. A similar strategy targeted at a binding protein has also been applied to elevate the levels of "free" endogenous IGF known to elicit neuroprotective and regenerative effects [82]. In that study, i.c.v. administration of IGFBP inhibitors exhibited a potent neuroprotective action in a clinically relevant model of stroke [82].

So far, the most significant limiting factor in the use of peptidic CRFBP inhibitors is their inability to cross the blood brain barrier, which makes them unsuitable for systemic administration. Strategies which need to be applied to overcome this limitation may include intranasal peptide administration and development of non-peptidic CRFBP inhibitors which readily enter the brain. Although there is evidence that intranasal administration of IGF has been successful in a rodent model of stroke [83, 84], the general applicability of this route of delivery of peptides into the brain is questionable (reviewed in [85]). In view of the development of non-peptidic CRFBP inhibitors, the identification of the ligand-binding site of CRFBP (see above) may represent the first step towards a structure-based design and refinement of potential lead compounds against CRFBP. On the basis of these results, a minimal CRFBP fragment that contains the ligand-binding site and retains biological activity may have to be designed to resolve the conformation of the ligand-binding site as recently presented for IGFBP [86]. The knowledge on the localization of the CRF-binding site will greatly facilitate computer-simulated ligand-docking techniques as soon as three-dimensional structure information on CRFBP becomes available.

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