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Authors

Taché, Yvette
Million, Mulugeta

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Role of Corticotropin-releasing Factor Signaling in Stress-related Alterations of Colonic Motility and Hyperalgesia

Yvette Taché* and Mulugeta Million

CURE/Digestive Diseases Research Center, and Center for the Neurobiology of Stress, Department of Medicine, Division of Digestive Diseases, University of California Los Angeles, and VA Greater Los Angeles Healthcare System, Los Angeles, California, USA

The corticotropin-releasing factor (CRF) signaling systems encompass CRF and the structurally related peptide urocortin (Ucn) 1, 2, and 3 along with 2 G-protein coupled receptors, CRF₁ and CRF₂. CRF binds with high and moderate affinity to CRF₁ and CRF₂ receptors, respectively while Ucn1 is a high-affinity agonist at both receptors, and Ucn2 and Ucn3 are selective CRF₂ agonists. The CRF systems are expressed in both the brain and the colon at the gene and protein levels. Experimental studies established that the activation of CRF₁ pathway in the brain or the colon recaptures cardinal features of diarrhea predominant irritable bowel syndrome (IBS) (stimulation of colonic motility, activation of mast cells and serotonin, defecation/watery diarrhea, and visceral hyperalgesia). Conversely, selective CRF₁ antagonists or CRF₁/CRF₂ antagonists, abolished or reduced exogenous CRF and stress-induced stimulation of colonic motility, defecation, diarrhea and colonic mast cell activation and visceral hyperalgesia to colorectal distention. By contrast, the CRF₂ signaling in the colon dampened the CRF₁ mediated stimulation of colonic motor function and visceral hyperalgesia. These data provide a conceptual framework that sustained activation of the CRF₁ system at central and/or peripheral sites may be one of the underlying basis of IBS-diarrhea symptoms. While targeting these mechanisms by CRF₁ antagonists provided a relevant novel therapeutic venue, so far these promising preclinical data have not translated into therapeutic use of CRF₁ antagonists. Whether the existing or newly developed CRF₁ antagonists will progress to therapeutic benefits for stress-sensitive diseases including IBS for a subset of patients is still a work in progress.

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Key Words

Colonic motility; Corticotropin-releasing factor; Irritable bowel syndrome; Stress; Visceral pain

Official abbreviations according to UPHARM guideline were used in this review series.

CRF, corticotropin-releasing factor
CRF₁, corticotropin-releasing factor receptor 1
CRF₂, corticotropin-releasing factor receptor 2

Introduction

The influence of emotions on the gastrointestinal tract function have been described many centuries ago.^{1,2} The feelings of

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*Correspondence: Yvette Taché, PhD

Center for Neurovisceral Sciences & Women's Health, VA Greater Los Angeles Healthcare System, CURE Building 115, Room 117, 11301 Wilshire Blvd, Los Angeles, CA 90073, USA

Tel: +1-310-312-9275, Fax: +1-310-268-4963, E-mail: ytache@mednet.ucla.edu

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ORCID: Yvette Taché, <http://orcid.org/0000-0002-1044-5279>.

“butterflies” in the stomach as well as the “gut wrenching” and nauseous feelings and abdominal pain during or following emotional stress are some of the overt manifestations of the intimate brain-gut connections.^{3,4} The past decades have witnessed an increasing recognition of the role played by the bidirectional interaction between the brain and gut in functional bowel diseases.⁵⁻¹³ These interactions occurs through neuronal pathways via the efferent and afferent components of the parasympathetic and sympathetic nervous system,¹⁴⁻¹⁶ hormonal route including the hypothalamic-pituitary-adrenal (HPA) axis¹⁷ and/or components of the immune system and the microbiota.¹⁸ Recent studies also identified a repertoire of neuromediators and receptors in the central nervous system and the gut that are involved in conveying the brain gut interactions in health and diseases.¹⁸ In particular one of the key mediator in the bodily response to various stressors and the brain-gut interactions is the corticotrophin releasing factor (CRF) pathways family.¹⁹⁻²¹

In this review, we will first briefly outlined the components of the mammalian CRF signaling systems that encompasses CRF peptides, receptors and signaling pathways. We will review pre-clinical findings on the actions of CRF in the brain and the colon to mimic stress-related stimulation of colonic secretory-motor function and the development of visceral hyperalgesia as well as the role of CRF receptor subtypes in these colonic responses to stress. Lastly, we will address the translational application of these experimental studies to clinical settings with existing clinical trials using CRF receptor 1 (CRF₁) antagonists for irritable bowel syndrome (IBS).

Corticotropin-releasing Facotor Peptides and Receptors

Corticotropin-releasing Factor Peptides

The mammalian CRF family of peptides consists of 4 distinct paralogs including CRF, and urocortin (Ucn) 1, Ucn2, and Ucn3.²² CRF is a 41-amino acid (a.a.) first isolated from ovine brain by Vale et al²³ The peptide structure is highly conserved among a large variety of mammalian species including human, primate, carnivore and rodents.^{22,24} Similar to CRF, the structure of Ucn1 is highly conserved across mammalian species with rat, mouse and sheep primary sequences sharing 100% identity, and 95% with that of human.²⁵ Subsequently, the simultaneous cloning of 2 novel putative CRF-related peptides by 2 independent groups^{26,27} resulted in divergent nomenclatures that were normal-

ized in the UPHARM guideline.²⁸ The mouse Ucn2, a 38-a.a. peptide, shares 34% homology with human/rat/mouse (h/r/m) CRF and 42% with r/mUcn1.²⁹ Mouse Ucn3 and hUcn3 (also named stresscopin)²⁶ share 90% identity with each other and are more distantly related to h/r/mCRF, h/rUcn1, and mUcn1 (18% and 21% homology respectively).²⁷

Corticotropin-releasing Factor Receptors

CRF and CRF-related peptides exert their biological actions by binding to 2 specific G-protein coupled receptors (GPCR), CRF₁ and CRF₂ (415 a.a. and 411 a.a., respectively).^{22,30} The 2 receptors are encoded by 2 distinct genes³¹ and display differential pharmacological and anatomical profiles indicative of distinct functional roles.^{28,32} CRF₁ and CRF₂ receptors share 70% identity within their species homologues.²⁸ The most variable component is the binding domain that encompasses the N-terminal and the 3 extracellular coils that share only 40% homology between the 2 receptor subtypes. In their N extracellular domains, CRF receptors contain several potential points of N-glycosylation along with several Cys residues that form disulfide bonds closely associated with their functionality.³³

As part of class B1 GPCRs containing exon-intron organization, CRF receptors are subject to extensive alternate splicing. A growing number of CRF₁ receptor splice variants (> 10) with specific amino acid deletions has been identified in the brain and peripheral tissues including the skin, gut and placenta.³⁴⁻³⁶ The main functional CRF₁ receptor is CRF_{1a}, which results from the excision of exon 6. This is a unique feature to humans since in most mammals, the exon 6 is absent and the fully active CRF₁ receptor protein arises from transcription of 13 exons present within the CRF₁ receptor gene sequence.³⁷ In humans, the CRF₂ gene consists of 12 exons and alternate splicing of exon 1 gives rise to three functional membrane isoforms: α (a), β (b) and γ (c) differing in the length of their N-terminal domains while in rodents, only the CRF_{2a} and CRF_{2b} are expressed.²⁸ In addition, recently, five variants of the CRF_{2a} receptors have been identified.³⁸ CRF₁ and CRF₂ receptor variant expression is tissue specific, regulated by specific physiological conditions and affected by environmental stimuli.^{35,38-40} There are growing evidence that these isoforms modulates CRF signaling.^{37,41,42}

Corticotropin-releasing Factor Ligand-receptor Selectivity

Radioligand binding and functional studies, established that the CRF₁ receptor has high affinity to h/r/mCRF (1.9 nM) and

rUcn1 (0.3 nM) but no appreciable binding affinity (> 100 nM) to Ucn2 and Ucn3.^{28,33} In contrast, rUcn, h/mUcn2 and mUcn3 bind CRF₂ receptor with greater affinity (0.3, 1.7/2.1, and 5.0 nM respectively) than h/r/mCRF (30.7 nM), making this receptor subtype highly selective for Ucn signaling.^{25,29,30,33,43} Although CRF is a preferential CRF₁ agonist, no endogenous ligand with selectivity for CRF₁ receptors have been identified. Recently, non-natural peptides, namely cortagine and stressin₁-A have been developed which display selective binding to CRF₁ receptors.^{44,45}

The structural and mechanistic basis of natural peptide agonists interaction with the CRF₁ receptor has been delineated and much advancement has been made as recently reviewed in details.^{33,46} In particular, it is well established that the α -helical conformation of CRF peptides in hydrophobic or amphiphilic milieu plays an important role to convey the biological activity of these peptides. The structure-activity relationship based on CRF₁ mediated ACTH release established that the first 21 a.a. of the N-terminal and 8 residues of the C-terminal regions of CRF are required for the peptide to be biologically active.³³ The current state-of-knowledge posits a “two-step” model of ligand-receptor interaction whereby the C-terminal portion of CRF (about 33-40 a.a.) is essential to bind the extracellular N-terminal domain of the CRF₁ receptor (N-domain). This brings the N-terminus (about 10 a.a.) of the peptide in the optimal orientation to bind to the J-domain which is formed by the seven transmembrane helices and three extracellular loops responsible for the coupling to G_s- and G_i-proteins.^{33,34} Recent studies also indicate that the molecular basis for ligand selectivity involves a single amino acid difference in the N-terminal extracellular domains of the receptors (CRF₁ Glu104/ CRF_{2a} Pro-100) at a site proximate to peptide residue 35 (Arg in CRF/Ucn1, Ala in Ucn2/3).⁴⁷

In contrast to the CRF₁ receptor isoforms,^{34,36} binding characteristics of CRF receptor splice variants, CRF_{2a}, CRF_{2b}, and CRF_{2c} are almost identical with high affinity for Ucn1, Ucn2, and Ucn3, and lower affinity for r/hCRF.^{27,48-50} Unexpectedly, the mouse soluble CRFR_{2a} isoform displays very low affinity for Ucn2 and Ucn3 while binding to Ucn1 (K_i 6.6 nM) and to a lesser extent to CRF (23 nM). Activation of this isoform also inhibits cAMP and extracellular signal-regulated kinases 1 (ERK1/2)-p42,44 responses to Ucn1 and CRF.⁴¹ In contrast, rat CRF_{2a-tr} binds with low affinity to CRF (K_d 12.7 nM) while displaying no affinity to Ucn1.⁵¹

Corticotropin-releasing Factor Receptor Signaling

As members of the class B1 class of GPCR, CRF receptors are primarily coupled to G_{αs}, the hallmark feature known to this GPCR subfamily. Ligand receptor interaction initiates several intracellular cAMP-dependent signaling cascades leading to acute post-translational modification of target proteins by protein kinase A (PKA) in the cytoplasm and gene transcription regulation by cAMP response element-binding proteins activation in the nucleus.⁴⁶ However, CRF ligand-receptor interaction can diverge to a plethora of downstream intracellular effectors, such as guanyl cyclase, ERK1/2, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, ion channels, tyrosine hydroxylase phosphorylation and glycogen synthase kinase 3 beta/Wnt/b-catenin pathway.^{34,46,52} In general, while CRF₁ and CRF₂ receptor signaling is mediated primarily through G_{αs} and thus cAMP as the second messenger,^{33,34} a low affinity binding site for the peptide ligand can also activate G_i- and G_{q/11}-coupled state.^{46,53} The occurrence of various signaling pathways is cell type and ligand specific.⁴⁶ The several described signaling pathways of CRF ligand-receptor interaction have been delineated in several cell lines or in specific organs,⁴⁶ and more recently, in colonic endocrine, neuronal or epithelial cells.^{36,54,55}

Corticotropin-releasing Factor Receptor Antagonists

Key to the assessment of the role of endogenous CRF ligands and CRF receptors in the stress response was the early development of several N-terminally truncated and substituted CRF analogs developed by Jean E Rivier.⁵⁶ Overall, four groups of CRF receptor antagonists have been developed which include the specific, nonselective peptide CRF₁ and CRF₂ receptor antagonists namely α -helical CRF₉₋₄₁, D-Phe¹²CRF₁₂₋₄₁, astressin and the acting astressin-B; the selective peptide CRF₂ receptor antagonists, anti-sauvagine and astressin₂-B;⁵⁶ the recently developed selective peptide CRF₁ antagonist, YAM19 which represents the only peptide CRF₁ receptor antagonist⁵⁷; and the numerous non-peptide CRF₁ antagonists.^{33,58,59} All these antagonists have been widely used as tools to determine the CRF receptor subtypes mediating stress-induced behavioral, endocrine and autonomic responses.^{58,60,61} While upon peripheral administration, the non-peptide CRF₁ receptor antagonists readily cross the blood-brain barrier as recently reviewed,^{59,62} the peptide CRF antagonists have no or very low ability to cross the barrier.⁶³ This al-

allows us to delineate the central and/or peripheral role of CRF receptor upon injection of antagonists into the brain or the periphery respectively. For instance, we showed that intravenous (iv) injection of astressin, at a dose that blocked intravenous CRF-induced delayed gastric emptying, did not influence CRF injected into cisterna magna-induced delayed gastric transit in rats.⁶⁴ Likewise, α -helical CRF₉₋₄₁ infused intravenously did not influence the suppression of pentagastrin stimulated acid secretion evoked by intracerebroventricular (icv) injection of CRF, while, injected icv, the antagonist blocked the icv CRF action in rats.⁶⁵

Role of Brain Corticotropin-releasing Factor Receptor 1 in Stress-related Stimulation of Colonic Motor Function

Brain Distribution of Corticotropin-releasing Factor – Corticotropin-releasing Factor Receptor 1

CRF peptides and receptors are widely distributed in the brain including specific regions linked with anxiogenic and digestive behaviors.^{32,66-69} In particular, major sites of CRF mRNA and CRF immunoreactivity are located in the paraventricular nucleus (PVN) of the hypothalamus, cerebral cortex, amygdalar-hippocampal complex and pontine Barrington's nucleus in rodents and humans.^{67,68} With regards to the brain distribution of CRF receptors, dense CRF₁ receptor expression is found in the forebrain, subcortical limbic structures (more prominently in the septal region) and amygdala, whereas the expression in the hypothalamus is low under basal conditions but markedly up-regulated by stress.^{32,70,71} Moreover, CRF₁ receptors at the gene and protein levels are prominently expressed in the anterior and intermediate lobe of the pituitary.⁶⁰

Activation of Brain Corticotropin-releasing Factor Receptor 1 Is Involved in the Colonic Response to Stress

Consistent with the brain localization of CRF/CRF₁ signaling pathway, convergent preclinical reports point to the role of central CRF₁ receptors in stress-related stimulation of colonic secretory motor function.⁶¹ First, CRF and Ucn1 injected into the cerebrospinal fluid induces the occurrence of colonic spike burst activity, acceleration of colonic transit, decreased in colonic fluid absorption, stimulation of defecation, and induction of diarrhea in freely moving rats, mice and gerbils, mimicking the effects of ex-

posure to acute stressors whereas Ucn2 and Ucn3 under the same conditions have no effect.⁷²⁻⁸⁰ Secondly, pharmacological blockade of CRF receptors using icv injection of peptide CRF₁/CRF₂ receptor antagonists, α -helical CRF₉₋₄₁, D-Phe¹²CRF₁₂₋₄₁, or astressin inhibited the stimulation of defecation, as well as the increase in wet stool and colonic spike-bursts induced by icv CRF, wrap restraint, water avoidance stress (WAS) and/or conditioned fear.^{72,75,76,81-86} Furthermore, the CRF₁ receptor antagonists, CP-154,526, CRA 1000, NBI-27914, NBI-35965, antalarmin, JTC-017, NGD 98-2, and NGD 9002 injected either centrally or peripherally (as they cross the blood brain barrier), dampened icv CRF and various stressors including acute restraint, WAS, elevated plus maze, social intruder or exposure to chronic heterotypic stressors (WAS, forced swim, cold restraint, and restraint-induced stimulation of colonic motor function).^{74,80,84,87-90} By contrast the selective CRF₂ antagonist, astressin₂-B injected centrally at doses that blocked CRF₂ receptor-mediated inhibition of gastric emptying, did not alter restraint stress-related defecation in mice or chronic heterotypic stress.^{74,89} In addition CRF₁ receptor knockout mice showed significantly less defecation in an open field test than did the wild-type littermates.⁹¹ Importantly, brain CRF₁ receptors are not involved in the basal and postprandial regulation of colonic motor function under non-stress conditions.⁹²

Brain Sites of Action of Corticotropin-releasing Factor-induced Stimulation of Colonic Motor Function

Brain nuclei responsive to CRF leading to the stimulation of colonic motor function (increased in tonic and phasic colonic motility, decreased colonic transit time and induction of watery fecal output) have been localized in the hypothalamus (PVN, arcuate nucleus) and pontine areas, namely the locus coeruleus (LC)/Barrington nucleus complex,⁹³⁻⁹⁶ which are also brain nuclei involved in central CRF-induced anxiety and depression.^{97,98} Anatomical support for the role of pontine and PVN CRF signaling pathways in the regulation of pelvic organ functions came from tracing studies showing that a proportion of CRF immunoreactive neurons in the Barrington's nucleus and PVN are linked transynaptically to the colon.^{99,100} Of interest is the growing pharmacological evidence that a number of brain peptides influencing food intake, such as neuropeptide Y, glucagon-like peptide, cocaine- and amphetamine-regulated transcript (CART), and ghrelin, act in the brain to stimulate colonic motor function through CRF receptor activation-dependent mechanisms in the

hypothalamus.¹⁰¹⁻¹⁰⁶

Brain Corticotropin-releasing Factor and Colonic Response: Peripheral Mechanisms

Peripheral mechanisms involved in colonic secretory motor stimulation in response to central CRF or stress (restraint or WAS) is independent from the activation of the HPA axis and brought about through the activation of sacral parasympathetic outflow and related input to the enteric nervous system (ENS) activity.^{73,82,85} The ganglionic blocker, chlorisondamine prevented and subdiaphragmatic vagotomy attenuated the stimulation of colonic transit, phasic and tonic contractions, and defecation induced by CRF injected icv or into the PVN while bretylium (noradrenergic blocker) had no effect.^{73,95,96,103} Effector mechanisms within the colon involved parasympathetic cholinergic mediated activation of colonic serotonin (5-HT) interacting with 5-HT₃ and 5-HT₄ receptors. This is supported by the increase in 5-HT content in the feces of the rat proximal colon in response to icv CRF¹⁰⁷ and pharmacological approach using 5-HT₃ antagonists (granistron, ramosteron, ondansetron and azasetron), the 5-HT₄ antagonist, SB-204070 and the muscarinic antagonist, atropine that all blocked icv CRF-induced colonic motor stimulation and decreases in colonic fluid loss.^{76,78,88,107}

Role of Colonic Corticotropin-releasing Factor Receptors in Stress-related Stimulation of Colonic Motor Function —

Activation of Peripheral Corticotropin-releasing Factor Receptor 1 Contributes to the Colonic Response to Stress

Functional studies showed that CRF and Ucn1 injected peripherally are as potent as injected centrally to increase propagative clustered spike-burst activity in the proximal colon and to stimulate distal colonic transit, defecation and to induce prominent diarrhea in rodents.^{54,72,76,108-115} The stimulation of colonic secretory-motor function after peripheral administration of CRF or Ucn1 involves CRF₁ receptors in rats and mice. This is supported by the fact that elective CRF₁ agonist, cortagine or stressin1 injected intraperitoneally stimulates colonic motor function and induces diarrhea while the CRF₂ agonists, Ucn2 has not effect under the same conditions.^{54,108,110,111,116,117}

The colonic CRF-CRF₁ signaling may have relevance as peripheral effector of the acute stress response. There is evidence

that stressors that stimulate colonic motor function such as endotoxin or early maternal separation upregulates CRF-CRF₁ signaling in the colon.^{118,119} In addition peripherally injected peptide antagonists α -helical CRF₉₋₄₁, astressin or astressin-B blocked or blunted the stimulation of distal colonic transit and high amplitude high frequency contraction and fecal pellet output induced by acute restraint or WAS in rats or mice.^{72,76,110,113,115}

Corticotropin-releasing Factor Receptor 1 in the Colon: Mechanisms to Stimulate Propulsive Motor Function

Recent studies establishing the expression of both CRF₁ receptor and ligands in the colon at the gene and protein levels in various cells including neuronal (ENS), endocrine (enterochromaffin cells) and immune cells (mast cells, eosinophiles, T-helper lymphocytes in the lamina propria) in rodents.^{54,116,118,120-122} This provides a neuroanatomical substrata pointing toward a local role CRF signaling pathways as recently reviewed.¹²³

Consistent with a local action, intraperitoneal injection of CRF-induced colonic stimulation is not affected by ganglion blockade, in contrast with the evidence on the role of the autonomic nervous system in mediating central CRF-induced stimulation of colonic propulsive motor function.^{73,96} Further support for direct action within the colon came from *in vitro* studies in isolated colonic rat preparations or isolated rat colonic muscle strips where CRF increased basal myoelectrical peristaltic activity and phasic contractions and electric field stimulation off-contractions.^{110,122,124}

Convergent evidence substantiates that the peripheral stimulatory action of CRF or Ucn1 involved the increase ENS neuronal activity in cholinergic, nitrenergic and serotonergic neurotransmission promoting peristaltic through the activation of CRF₁ receptors on ENS in rats or guinea pigs.^{54,107,116,122,125} First, the use of laser captured combined with reverse transcriptase polymerase chain reaction or immunohistochemical detection in myenteric whole mount preparation of the colon revealed that CRF₁ receptors are primarily expressed at the gene and protein levels on myenteric neurons compared with other layers of the rat or guinea colon under basal conditions.^{54,116,122,125} Second, the neuronal blocker, tetrodotoxin abolished Ucn1-evoked phasic contractions in rat colonic smooth muscle strips,¹²² indicative of an ENS mediated component. Third, when injected intraperitoneally, CRF in rats or partial restraint in mice induces Fos expression, a marker of neuronal activation,¹²⁶ in CRF₁ receptor expressing cholinergic and nitrenergic myenteric neurons in

the proximal colon while Ucn2 under the same conditions did not induce Fos.^{54,116,117,127,128} It is to note that atropine, a muscarinic blocker, does not affect intraperitoneal CRF-induced neuronal activation in colonic myenteric ganglia, indicating that the Fos response is not secondary to the activation of muscarinic receptors either on the myenteric ganglia (which possess both nicotinic and muscarinic receptors) or on colonic smooth muscle cells but rather to a direct effect on enteric neurons.¹²⁷ Additional support came from electrophysiological recording showing that direct administration of CRF or Ucn1 onto colonic myenteric and submucosal plexus preparations of guinea pig excites both myenteric and submucosal neurons through CRF₁ receptor.^{125,129,130} Other studies in rat submucosal neuron preparation showed that CRF induces a tetrodotoxin sensitive and CRF₁ receptor mediated neuronal activation monitored by the increase in intracellular calcium.¹³¹

Other peripheral molecular pathways of the potent stimulation of colonic secretory motor function by peripheral injection of CRF may include the crosstalk between the enteric neuroendocrine and immune systems. CRF added *in vitro* to rat colonic section up-regulates IL-6 in the colonic tissues and potentiates IL-6 induced submucosal neuron activity of the colon through CRF₁ receptor.¹³¹

Corticotropin-releasing Factor Receptor 2 in the Colon: Modulation of Stress-related Stimulation of Propulsive Motor Function

The CRF₂ ligands, Ucn1 and CRF₂ receptors are also expressed in rodent colon.^{54,55,123,132-135} The CRF₂ receptors are localized at the gene and protein levels in cells of the rodent colonic ENS⁵⁴ although less prominently than that CRF₁ receptor^{130,131} and human colonic lamina propria mononuclear cells,¹³⁵ subepithelial mast cells¹³⁶ and epithelial cells⁵⁵ supporting a local action to influence neuronal and immune responses.

Recent studies indicate that intraperitoneal pretreatment with Ucn2 acting through CRF₂ receptors inhibits peripheral CRF-or Ucn1 induced stimulation of colonic motor function while pre-treatment with astressin₂-B or genetic deletion of CRF₂ in mice exacerbated the restraint stress or intraperitoneal CRF and Ucn1 induced stimulatory colonic responses.^{54,137} These data are indicative that acute stress-related colonic stimulation engages not only the colonic CRF₁-mediated enteric stimulatory pathway but also CRF₂ to dampen the colonic response to stress. This interaction is occurring in myenteric neurons as evidenced by the localization of CRF₂ receptors on CRF₁ expressing myenteric

neurons and the reduction of CRF-induced ERK1/2 signaling in the rat colon myenteric primary neurons by CRF₂ receptors activation.⁵⁴ The differential effects of CRF₁ and CRF₂ receptors are also reported to exist in stress-induced intestinal mucosal barrier function alterations. In pigs exposed to early-weaning stress, intestinal barrier dysfunction and hypersecretion is mediated through CRF₁ activation whereas activation of peripheral CRF₂ plays a protective role in the alterations of intestinal barrier function in response to early life stress.¹³⁸

Role of Brain Corticotropin-releasing Factor Receptors in Stress-related Visceral Hyperalgesia

Corticotropin-releasing Factor Receptor 1 Signaling Is Involved in the Stress-related Hyperalgesia

Gué et al¹³⁹ provided the first evidence that CRF injected *icv* induced visceral hyperalgesia to colorectal distention (CRD) in rats, mimicking the response induced by partial restraint stress. Furthermore, the *icv* injection of the CRF₁/CRF₂ antagonists, α -helical CRF₉₋₄₁ prevented the effects of restraint pointing to the involvement of central CRF signaling in acute stress-related development of visceral hyperalgesia.¹³⁹ Subsequent studies using selective CRF₁ antagonists,⁵⁹ namely NBI-35965, NBI-27914, NBI-30775, CP-154,526, CP-376395, antalarmin, JTC-017, DMP-696, NGD 98-2, or NGD 9002 supported the involvement of CRF₁ subtype in the hyperalgesic response to CRD induced by *icv* CRF injection^{80,140} or in a variety of experimental models inducing visceral hyperalgesia including acute or repeated exposure to WAS alone or combined with neonatal maternal separation, consecutive sets of nociceptive CRD, repeated daily CRD 6 weeks after the development of colitis, intracolonic infusion of 0.5% acetic acid, or performing the CRD in a high-anxiety rat strain, the Wistar Kyoto (WKO).^{80,90,140-152} Likewise in CRF₁ receptor knockout mice, the visceral motor response to phasic CRD is reduced.¹⁴⁹ These consistent pre-clinical reports indicate that CRF₁ signaling plays a critical role in the visceral hyperalgesia occurring in different contexts: early life adverse events, repeated psychological stress in adulthood, chronic high anxiety as well as peripherally initiated mechanisms associated with previous colonic inflammation or repeated nociceptive CRD.

By contrast, the central effects of CRF₂ receptor activation on

visceral pain are scant. In a recent study Lee et al¹⁵² indicates that perfusion of selective CRF₂ receptor antagonist, astressin₂-B into the anterolateral BNST reduced visceral motor response to low CRD pressure in rats. Further studies are needed to assess the contribution of brain CRF₂ receptors in the modulation of visceral pain.

Mechanisms Involved in Corticotropin-releasing Factor Receptor 1 Mediated Prevention of Visceral Hyperalgesia

Brain sites responsive to CRF and CRF₁ antagonist to influence stress-related visceral hyperalgesia encompass the hippocampus, anterior lateral bed nucleus of the stria terminalis (BNST) and the central amygdala (CeA). One study showed that the CRF₁/CRF₂ antagonists, α -helical CRF₉₋₄₁ microinjected into the hippocampus or peripheral injection of the CRF₁ antagonist, JTC-017 decreased abdominal contraction frequency evoked by nociceptive tonic CRD along with the anxiety response to CRD in rats.⁹⁰ There is also recent evidence that the CRF₁ antagonist, CP-376395 microinjected into the anterolateral BNST after repeated WAS reduced the visceral motor response to CRD.¹⁵² Converging studies also point CeA as a responsive site to the CRF₁ antagonist, CP-376395 to reduced visceral hyperalgesia to CRD in the WKO rat strain.¹⁵³ Conversely CRF microinjected into the CeA increased sensitivity to CRD performed at 20 to 80 mmHg in Wistar rats through CRF₁ receptor as shown by the blockade of CRF action by the intra-CeA injection of CP-154,526.¹⁵⁴ Neuroanatomical evidence that CRF is densely expressed in cell bodies and axon terminals of cortical amygdala projecting to widespread regions of the basal forebrain and pontine/brainstem including the locus coeruleus (LC) support the notion that endogenous CRF acts as a ligand to act on CRF₁ receptor in the CeA.^{155,156} Moreover CRF-like immunoreactivity and gene expression in CeA are increased in response to CRD¹⁵⁷ and various conditions inducing visceral pain or hyperalgesia.¹⁵⁸⁻¹⁶¹

The underlying mechanisms through which blockade of brain CRF₁ receptor activation influences the development of stress or mechanosensitization-related visceral hyperalgesia are still not fully understood. One component may involve the dampening of pontine noradrenergic output from the visceral to the forebrain. Recent electrophysiologic reports in anesthetized rats showed that the peptide antagonist D-Phe¹²CRF₁₂₋₄₁, or astressin injected into the cerebrospinal fluid or directly into the LC and the selective CRF₁ antagonist, NBI-35965, injected iv prevented

the activation of the LC noradrenergic neurons responsive to both central injection of CRF and CRD.¹⁶²⁻¹⁶⁴ Consistent with electrophysiological demonstration of LC noradrenergic silencing by CRF receptor antagonists, the CRF₁ antagonist, JTC-017 reduces noradrenaline release in the hippocampus and CeA induced by CRD.^{90,154} The release of noradrenaline in the cortical and limbic rostral efferent projections from the LC or CeA¹⁶⁵ is known to induce arousal and anxiogenic responses along with hypervigilance to visceral input which is a commonly found feature in IBS patients.^{166,167}

Role of Colonic Corticotropin-releasing Factor Receptors in Stress-related Hyperalgesia

Corticotropin-releasing Factor Receptor 1

Recent reports suggest that enhanced CRF/CRF₁ receptor signaling in the colon, in addition to central activation, bears relevance as part of the peripheral efferent components responsible for the induction of visceral hyperalgesia. There is evidence that peripheral injection of CRF induces visceral hypersensitivity to CRD, an effect reproduced by the intraperitoneal administration of the selective CRF₁ agonist, cortagine in rats and mice.¹¹⁷ Conversely in rats repeatedly exposed to WAS for 10 days, peripheral injection of peptide CRF antagonist astressin before each stress session prevented the development of visceral hyperalgesia supporting the participation of a peripheral component to the development of visceral hyperalgesia.¹⁴⁴ This is further supported by the demonstration that the visceral hyperalgesia induced by cortagine injected peripherally is abolished by peripheral, but not central, injection of astressin at an equivalent dose.¹¹⁷

Several mechanisms are involved in the visceral hyperalgesia linked with the activation of CRF₁ receptors in the colon. Peripheral CRF induces colonic mast cells degranulation.¹⁶⁸⁻¹⁷⁰ which can in turn lead to the release of several preformed or newly synthesized mediators eg, histamine, tryptase, prostaglandin E₂, and nerve growth factor known to activate or sensitize sensory afferents.^{171,172} The involvement of colonic mast cell is further supported by the use of mast cell stabilizer, doxantrazole, that prevented acute stress-induced colonic hypersensitivity to a second set of CRD.^{139,169} Additionally, peripheral CRF signaling activation disrupts the intestinal epithelial barrier and CRF₁ receptor antagonists block stress-induced increases in intestinal permeability.^{123,168,173} Such CRF receptor mediated alteration of

intestinal permeability increases the penetration of soluble factors (antigens) into the lamina propria, which can result in nociceptors sensitization.¹⁷⁴ Increased intestinal permeability is indeed a phenomenon that appears as a prerequisite for the development of visceral hyperalgesia in rodents.¹⁷⁵

Corticotropin-releasing Factor Receptor 2

In contrast, activation of CRF₂ by intraperitoneal injection of the selective CRF₂ peptide agonist, Ucn2, as well as sauvagine, a non selective CRF₁/CRF₂ agonist with higher affinity to CRF₂, blunt repeated CRD- induced visceral sensitization.^{146,176} This indicates opposite effects of CRF₁ and CRF₂ signaling in the mechanosensitization of the colon. Similarly, CRF₂ activation by in-

traperitoneally injected Ucn2 suppressed CRD- induced spinal phosphorylation of ERK 1/2 and visceral pain, although the exact site of CRF₂ activation to inhibit spinal ERK 1/2 activity is not known.¹⁷⁶

Corticotropin-releasing Factor Signaling in Healthy and Irritable Bowel Syndrome Subjects

Colonic Responses to Peripheral Corticotropin-releasing Factor Signaling

The actions of peripheral CRF on the colon reported in experimental studies have been largely reproduced in healthy subjects or IBS patients along with preliminary evidence of the beneficial effect of peripheral injections of peptide CRF antagonist (Tables 1 and 2). With regards to the intestinal motility, one clinical report showed that systemic administration of CRF increases the motility index in the descending colon in both healthy and IBS groups with a significant higher response in the IBS groups.¹⁷⁷

Reports assessing changes in visceral pain established that systemic injection of the preferential CRF₁ agonist, ovine CRF lowered pain thresholds to repetitive rectal distensions and enhanced the intensity of discomfort sensation to CRD in healthy humans.¹⁷⁷⁻¹⁷⁹ Beneficial effects of systemic injection of α -helical CRF₉₋₄₁ was reported in a setting of electrical rectal stimulation which evoked abdominal pain and anxiety in IBS patients compared with healthy subjects.¹⁸⁰ There is also clinical evidence that systemic administration of the peptide antagonist normalizes the altered EEG activities in IBS patients under basal and in response to CRD.¹⁸¹

Table 1. Clinical Studies Showing Mimicry Between Activation of the Corticotropin-releasing Factor Signaling System and Irritable Bowel Syndrome-related Symptoms in Humans

CRF agonists stimulate/activate/increase in healthy humans or IBS patients	References
HPA axis	21
Colonic motility	21
Permeability/bacterial translocation	184
Viscerosensitivity	178,179
Mast cells	184
Anxiety/depression	208
Inflammation	183
CRF antagonists reverse IBS-associated increase of	
Anxiety +/-	194
Motility +/-	21
Permeability	184
Viscerosensitivity	21

CRF, corticotrophin-releasing factor; IBS, irritable bowel syndrome; HPA, hypothalamic-pituitary-adrenal.

Table 2. Preclinical Studies Highlighting the Relevance of Corticotropin-releasing Factor Receptor 1 Blockade to Reduce Irritable Bowel Syndrome-like Symptoms (see reviews^{21,123,209,210})

Clinical features of IBS-diarrhea predominant patients	CRF ₁ antagonists in experimental animals block stress-related
Co-morbidity with anxiety and depression	Anxiety/depression
Hypervigilance	CRD-induced locus coeruleus activation-induced hypervigilance
Changes in autonomic functions (↓ vagal, ↑ sympathetic)	Autonomic responses (↓ vagal, ↑ sympathetic)
Increased bowel movements/diarrhea	Increased colonic motility/defecation/diarrhea
Ion transport dysfunction	Colonic mucosal barrier dysfunction
Change in mast cells (number, and activation)	Activation of mast cells
Increase barrier permeability	Increase barrier permeability/antigen translocation
Lower pain threshold to colorectal distention	Hypersensitivity to colorectal distention

IBS, irritable bowel syndrome; CRF₁, corticotrophin-releasing factor receptor 1; CRD, colorectal distention.

Similar to rodent data, CRF receptors have been identified in human colonic biopsies and enteric nervous system.^{136,182,183} CRF administered intravenously activates subepithelial mast cells, and stimulates transcellular uptake of protein antigens in colonic biopsies of healthy subjects.¹⁸⁴ Since increased uptake of antigen-sized macromolecules is associated with inflammation, and there is increasing evidence that IBS patients display a low graded colonic inflammation, intraepithelial lymphocytes, mast cell degranulation and increased permeability, these changes may be consistent with enhanced colonic CRF signaling.^{185,186} Additionally, recent evidence showed the expression of CRF₁ receptors and variants on human enterochromaffin-like cells, the BON subclone 1 cells. In these cells, CRF induces a CRF₁ receptor mediated stimulation of 5-HT release and up regulated the expression of 5-HT synthesizing enzyme, tryptophan hydroxylase.³⁶ Considering the important role of 5-HT in the pathophysiology of IBS,¹⁸⁷ this may represent an additional site of action of CRF to increase colonic motility and visceral pain in human subjects.

Does Targeting Corticotropin-releasing Factor Receptor Have Therapeutic Values?

The vast amount of preclinical studies showing efficacy of CRF₁ antagonists in various experimental IBS models raised high expectation regarding potential translational applications of CRF₁ antagonists for therapeutic benefit in stress-sensitive human disorders. However, while a large number of small molecules CRF₁ antagonists have been disclosed and used in a preclinical setting,⁵⁹ only a few compounds have progressed to clinical trials.¹⁸⁸ Indeed, many of these small molecule CRF₁ antagonists encountered several setbacks related to unattractive pharmacokinetics, prominent tissue accumulation due to lipophilicity, long elimination half-life, high protein binding, or reactive metabolite formation^{59,189} that precluded their clinical testing. Efforts are ongoing to overcome these issues as shown by recently disclosure of new compounds that are less lipophilic with improved pharmacokinetic properties due to the substitution of the propyl groups at the C7 position of the pyrazolol[1,5 α]pyrimidine core with heterocycles.^{33,189-192} The CRF₁ antagonists which are or have reached clinical trials include CP-316311 (Pfizer), pexacerfont (Bristol-Myers Squibb), GSK-876008 and GS-561679 (GlaxoSmithKline), R317573 (Johnson & Johnson), and few others, see review.^{58,59} CRF₁ antagonists such as NBI-30545 and NBI-35965 have the distinct advantage to be orally active and water soluble.^{58,59,80,192}

Several clinical studies provided proof-of-concept that oral administration of CRF₁ antagonists dampened stress-related biological responses in healthy human subjects. NBI-34041/SB-723620 given at 100 mg/day for 14 days reduced the Trier Social Stress Test and mental arithmetic test-induced rise in plasma levels of ACTH and cortisol in healthy male volunteers while not influencing basal circadian release of ACTH.¹⁹³ Further clinical testing, however, was not continued due to preclinical toxicology studies. In a clinical model of generalized anxiety induced by 20-min inhalation of 7.5% CO₂, preliminary studies in 12 healthy subjects showed that R317573 (40 mg once daily) given for 7 days significantly reduced panic symptom score and generalized anxiety compared to placebo.¹⁹⁴ Positron emission tomography (PET) studies indicated that the acute administration of R317573 at 30 and 200 mg results in dose-related changes in regional glucose metabolism in regions relevant to mood and anxiety disorders.¹⁹⁵ A recent clinical study using functional magnetic resonance imaging showed that an acute oral administration of CRF₁ antagonist, GW876008 (single dose of 20 or 200 mg orally) dampened the amygdala activation produced by the anticipation of visceral pain compared to placebo drug in IBS female patients.¹⁹⁶ Of note, CRF-CRF₁ signaling in the CeA is also well established to be a key component of the neuronal circuitry contributing to anxiety-like behavior¹⁹⁷ and therefore well positioned to drive the reciprocal relationship between visceral pain and affective mood.¹⁹⁸ This may provide the neuroanatomical and biochemical substrata to speculate that overactivity of the CRF-CRF₁ in the CeA may underlie the comorbidity of subset of IBS patients who display hypersensitivity to CRD¹⁹⁹ and mood disorders.²⁰⁰

However, whether the existing or newly developed CRF₁ antagonists⁵⁹ will progress to show therapeutic benefits in subsets of IBS is still to be established. An early clinical trial in IBS-diarrhea predominant patients did not show beneficial effect of oral administration of the CRF₁ antagonist paxacerfont (BMS-562086) on IBS symptoms although a dose-related trend to reduce visceral pain was observed at 25 mg and 100 mg for 2 weeks.²⁰¹ Several aspects may have been suboptimal including the dosing regimen as the CRF₁ mediated ACTH levels were not monitored in this clinical trials.²⁰¹ The lack of information on the central and peripheral occupancy of CRF₁ antagonists in humans in the absence of PET ligand as well as the potential role of the more than 10 splice variants of the CRF₁ receptor (some of which are functional)^{36,40} are additional factors. Efforts are undergoing to test radioligand for the CRF₁ receptor to allow rational dose

selection relying on large occupancy in the brain and colon. Importantly CRF₁ antagonists may exert beneficial effects only under conditions of elevated brain/colonic CRF/CRF₁ signaling pathways that cannot be identified in the heterogeneous populations tested so far.

Conclusion

Both experimental and clinical studies in healthy subjects support that the activation of CRF₁ pathways recaptures cardinal features of IBS-diarrhea symptoms (anxiogenic/hypervigilance behavior, colonic mast cell activation and serotonin release, stimulation of colonic propulsive motor function and watery diarrhea, and visceral hyperalgesia) (Table 1). Another key preclinical evidence arises from pharmacological interventions whereby blockade of brain or peripheral CRF₁ receptors prevent/blunt the development of the above CRF or stress-related functional or cellular alterations.^{123,202} Selective CRF₁ antagonists or CRF₁/CRF₂ antagonists abolished or reduced exogenous CRF and stress-induced anxiogenic/depressive behavior, stimulation of colonic motility, mucus secretion, mast cell activation, defecation, diarrhea, and pain related to colonic hyperalgesia (Table 2). Therefore, the conceptual framework supports that sustained activation of the CRF₁ system at central and/or peripheral sites may be one component underlying IBS symptoms. Targeting these mechanisms by CRF₁ antagonists provided a relevant novel therapeutic venue. However so far these promising preclinical data have not translated yet into therapeutic use of CRF₁ antagonists. Therefore, whether the existing or newly developed CRF₁ antagonists will progress to therapeutic benefits for stress-sensitive diseases including IBS for a subset of patients is still a work in progress. Of note is also the inhibitory effects of CRF₂ receptor activation on stress-related gut motility and sensitization, as a gut stress coping system and the possible CRF₁-CRF₂ cross talk in the colon^{54,176,203} and the brain²⁰⁴ that need to be taken into consideration in targeting CRF signaling for gut centric intestinal secretomotor alterations and visceral pain therapeutic purposes. CRF being pivotal in the body's response to stressful stimuli and the extensive preclinical data indicating that dysregulation of CRF-CRF₁ system is implicated in the etiology and maintenance of several stress-sensitive disorders as recently reviewed,^{20,205-207} a better understanding of CRF signaling is likely to provide novel insights in the otherwise challenging pathophysiology of functional gut diseases such as IBS.

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