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CORTICOTROPIN RELEASING FACTOR SIGNALING IN COLON AND ILEUM: REGULATION BY STRESS AND PATHOPHYSIOLOGICAL IMPLICATIONS

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Abstract

It is well established that central corticotropin releasing factor (CRF) signaling mediates the gastrointestinal responses to stress. However, as shown in the brain, both CRF receptors and ligands are also widely expressed in the colon and the ileum of humans and rodents, and stress modulates their expression. Several functional studies documented that peripheral injection of CRF or urocortin stimulates colonic transit, motility, Fos expression in myenteric neurons, and defecation through activation of CRF₁ receptors, whereas it decreases ileal contractility *via* CRF₂ receptors. Additionally, intraperitoneal administration of CRF induces colonic mast cells degranulation *via* both CRF₁ and CRF₂ receptors and increases ion secretion and mucosal permeability to macromolecules, which can in turn promote intestinal inflammation and alter visceral sensitivity. Most peripheral CRF-induced alterations of colonic and ileal functions mimic effects which are observed after stress exposure, and CRF receptor antagonists given peripherally prevent stress-induced GI dysfunction. Furthermore, CRF peptides can reproduce secretomotor and mucosal alterations *in vitro*. Therefore, accumulated clinical and preclinical evidence supports in addition to the brain, a role for peripheral CRF signaling in mediating stress-induced effects on gastrointestinal sensorimotor, mucosal and immune functions, that may be components of underlying mechanisms involved in stress-related impact on inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).

Keywords

corticotropin releasing factor; corticotropin releasing factor receptor; urocortin; stress; colon; ileum; irritable bowel syndrome; inflammatory bowel disease

INTRODUCTION

The biochemical coding of the stress response has been unraveled in the past decades through the identification of a 41-amino acid peptide, corticotropin releasing factor (CRF), and its related peptides, urocortin 1 (Ucn 1), Ucn 2 and Ucn 3. CRF and urocortins exert their biological actions by interacting with CRF₁ and CRF₂ receptors, encoded by two distinct genes (1–3). In particular, CRF plays a crucial role in the stress-related stimulation of the hypothalamo-pituitary-adrenal axis through activation of pituitary CRF₁ receptor and,

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in association with urocortins, acts as a neuromodulator to coordinate the behavioral, autonomic, and visceral efferent limbs of the stress response (1–4).

The gastrointestinal (GI) tract is particularly sensitive to stress. Convergent preclinical evidence has accumulated over the years suggesting that stress-related alterations of GI functions, particularly at the colonic level, are primarily mediated by the activation of brain CRF/CRF₁ signaling (2, 5). However, recent studies point to an equally important contribution of the peripheral CRF signaling locally expressed in the gut to the GI stress response (6–11). In addition, there is increasing experimental and clinical evidence that the induction and progression of inflammatory and functional intestinal disorders are influenced by CRF signaling pathways (12–14). Among the inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD), share many clinical features, but also important differences, such as disease location and histological features (13). CD is a chronic transmural inflammatory disease affecting the terminal ileum and the colon, which is associated with frequent attacks of diarrhea, abdominal pain, nausea and fever, while UC affects primarily the distal colon. Irritable bowel syndrome (IBS) is a highly prevalent functional GI disorder affecting predominantly the colon which is mainly characterized by abdominal pain and discomfort in association with altered bowel habits in the absence of any structural abnormalities (15). Despite differences in their etiologies (16, 17), stress represents a common risk factor in the pathogenesis of IBS and IBD (14, 18, 19). In this review, we will summarize evidence in support of a major role for peripheral CRF signaling in the GI stress response in both humans and rodents, with a special emphasis on the colon and the ileum, and we will discuss the relevance of these preclinical findings in relation with components of stress-related impact on IBS and IBD.

CRF SIGNALING SYSTEM OVERVIEW

Both CRF₁ and CRF₂ receptors are members of the G-protein coupled receptors family. In most cells in the peripheral tissues, the physiological actions of CRF and urocortins involve coupling of both CRF₁ and CRF₂ receptors to G_{α_s} proteins that stimulate cAMP mediated signaling cascades (20). However, couplings to other types of G proteins have also been described (21). The activation of cellular G-protein after CRF receptor stimulation leads to the induction of multiple signaling pathways (MAPK, PK, ERK1/2), which differentially influence neuronal, endothelial, endocrine, smooth muscle, epithelial and immune cell activities (21–24). These differential effects depend on a number of factors including the subtype of CRF receptor activated, the tissue in which the activation occurs and environmental factors. Although CRF₁ and CRF₂ share about 70% amino acid identity, they exhibit differential pharmacologies due to their distinct N-terminal ligand binding domains (20). Hence, CRF binds with the highest affinity to CRF₁ receptors (20) while Ucn 1 shows an equal high affinity to both CRF receptor types and Ucn 2 and Ucn 3 are selective agonists for CRF₂ receptors (20). Several splice variants of CRF receptors have been identified in rodents and humans: CRF₁ (α,β, c-n) of which only CRF_{1α} is functional (21), while both CRF_{2α} and CRF_{2β} are functional (20, 21, 25). In rodents the predominant form expressed in the periphery is CRF_{2β} (26), although several CRF_{2α} isoforms have recently been isolated in the esophagus (25).

It is traditionally accepted that CRF, Ucn 1 and Ucn 2 bioactivities are modulated by binding to the secreted CRF binding protein (CRF-BP) (27, 28). However, additional regulatory pathways have been recently unveiled. The CRF₁ alternative splice variants, for instance, contain truncation and/or deletions that disrupt ligand binding and/or signaling capabilities of functional CRF_{1α} receptors, subsequently affecting CRF and/or urocortins effects in target tissues (25, 29). A soluble CRF_{2α} protein was also identified in mouse brain and rat esophagus and initially thought to behave as a binding protein (30). However,

subsequent studies found that despite its correct translation, this protein is not secreted and may therefore alter the transcription of full-length CRF_{2α} mRNA in cells, instead of acting as a decoy receptor (25, 31). Furthermore, in the mouse heart, a dominant-negative CRF_{2β} splice variant (i.v.-mCRF_{2β}), recently described, was shown to impair mCRF_{2β} function by retaining its cellular location to the endoplasmic reticulum-Golgi complex (32). Together these studies demonstrate that the CRF signaling system is finely tuned by a number of regulatory pathways at the receptors and the ligands levels.

DISTRIBUTION OF CRF LIGANDS AND RECEPTORS IN COLON AND ILEUM: REGULATION BY STRESS

In contrast to the brain, where it has been extensively described, the distribution, expression pattern and regulation of CRF receptors and ligands in the GI tract of mammals is still incomplete. In this section we will focus on what is known in humans and rodents (mouse, rat, guinea-pig) at the colonic and ileal levels.

Expression of CRF receptors in the colon

Both CRF receptors subtypes have been detected at the gene and/or protein level in humans and rodent colons (Table 1). We recently described the expression of CRF₁ mRNA in colonic resections from healthy adults and the presence of CRF₁ immunoreactivity (IR) in lamina propria (LP) cells which were identified by immunohistochemistry as being macrophages and mast cells, as well as in submucosal (SNP) and myenteric neuronal plexus (MNP) (33). This supports earlier data in human colonic resections showing gene expression of CRF₁, CRF_{2α} and to a minor extent CRF_{2β} in isolated lamina propria mononuclear cells (LPMCs), as well as very little CRF_{2α} mRNA in epithelial cells fractions (34). The low gene expression of CRF₂ receptors in healthy human colonic epithelium found in this study is in agreement with recent reports showing little or even no expression in a number of transformed or non-transformed human colonic cell lines (Caco-2, HT-29, NCM460) as well as in human colonic biopsies or xenografts (35, 36). Interestingly, at the protein level, CRF₂ IR has been detected, although weakly, in healthy human colonic epithelial cells (35), and with much higher intensity in LPMCs, SNP, MNP, vascular endothelial cells and vascular smooth muscle cells of blood vessels (37). CRF₁ and CRF₂ receptors were also identified at the gene and protein level in BON cells, a pancreatic carcinoid-derived human endocrine cell line, which share functional similarities with intestinal enterochromaffin cells (36) and in mast cells in sigmoid colon biopsies and in HMC-1 cells, a human mast cell line (38).

In rats, CRF₁ mRNA was mainly found in MNP, SNP, goblet cells and stem cells of the colonic crypts as well as in scattered cells of the surface epithelium and the lamina propria of the proximal colonic mucosa (26, 39, 40). In contrast, CRF₂ expression was essentially detected in the mucosa, localized in the luminal surface of the crypts and in blood vessels of the submucosal layer (40). Immunoreactivity for CRF₁ has been detected in guinea-pigs' colonic MNP and SNP, while CRF₂ IR was only detected in MNP (41, 42).

Expression of CRF ligands in the colon

Most of the studies assessing the distribution of CRF and its related peptides in the colon show that they are expressed in close proximity of the CRF receptors, pointing out the existence of local autocrine/paracrine regulatory loops. For instance, in humans, CRF mRNA was found in the mucosa, mainly localized in enterochromaffin cells (43). In contrast, Ucn 1 IR and mRNA were detected in colonic lamina propria macrophages with a minimal amount in epithelial cells (34). Ucn 3 mRNA and IR were found in the MNP and SNP, in subserosal vascular endothelial and smooth muscle cells, in colonic smooth muscle

layers and in enterochromaffin cells of human colonic tissue, as well as in enteric glial cells (37). As of today, the presence of Ucn 2 in human colon has not been described.

In rats, we recently showed that CRF is expressed in the colon, predominantly in the distal part, with the highest levels of expression in the submucosal plus muscle layers compared to the mucosa (44). CRF IR was detected in individual cells scattered in the mucosa, mainly located in enterochromaffin cells, cells in the crypts and LP, as well as in MNP (33). In earlier studies, the preproCRF IR labeling was detected in rat colonocytes, with weaker detection in colonic bottom crypts and submucosal cells (10). CRF IR was found in MNP and SNP in all segments of the large intestine of guinea-pigs, although CRF IR positive cells were sparse (45). Along with CRF, Ucn 1 mRNA and IR was observed in rat MNP and SNP (46, 47). Of note, in the guinea-pig colon, CRF positive neurons do not bear CRF₁ receptors but the CRF₁ positive neurons are expressed in neuronal neighbors (45). In the rat colon, Ucn 1-positive neurons are co-localized with CRF₁ receptor positive neurons (46). Ucn 2 mRNA was also detected throughout the large intestine of rats in the MNP and the nerve fibers innervating the circular muscle (48). With regards to the peptide, Ucn 2 IR was measurable in all layers of the rat intestinal tract, including the mucosal and submucosal layers, and detected in epithelial cells of the mucosa, as well as in support cells and immune cells of the LP, SNP, MNP (48). Taken together, these data suggest that the colon in humans, rats and guinea-pigs is an important target organ for CRF signaling.

Expression of CRF receptors and ligands in the ileum

Conversely to the colon, the ileal distribution and expression of CRF receptors and ligands have been little investigated in rodents and there are no data in humans, yet. In rats and guinea-pigs, CRF₁ and CRF₂ IR were detected in MNP and SNP (45, 49), as well as in nerve fibers of the longitudinal and circular muscle layers and in mucosal cells of the ileum (49). In mice, CRF₁ mRNA was detected in ileal LP and epithelial cells, while CRF₂ mRNA was only found in a few cells of LP (50).

While the expression of urocortins in the ileum has not been investigated, CRF IR was found to be sparsely distributed in MNP and SNP of the guinea-pig ileum (45). In rats as well, CRF IR was detected in ileal SNP and in LP immune cells and Paneth cells, but not in epithelial cells (51). Lastly, in mice ileum, CRF IR was primarily present in subepithelial cells and a few cells of the epithelial surface (50). Hence, although not extensively studied thus far, the ileum appears to be a potential target for CRF/Ucn signaling.

Regulation of colonic and ileal CRF receptors and ligands expression by stress

While convergent functional evidence accumulates in favor of a major role of peripheral CRF signaling system in the GI stress response, details on how and where the peripheral CRF signaling is recruited by stress still remains unclear. Several recent reports suggest that stress of either interoceptive (infection, inflammation) or exteroceptive (psychological or physical stress) origin can, as it has been shown in the brain, affect the expression of CRF signaling in the GI tract. In human, exposure to *Clostridium difficile* toxin A was reported to increase the expression of CRF₂ mRNA and IR in HT-29 colonocytes and colonic xenografts (35). Similar upregulations in response to *Clostridium difficile* toxin A perfusion in an ileal loop were observed in mice for CRF₁ and CRF₂ mRNA (50, 52) and CRF mRNA in both rats and mice (50, 51). Colonic inflammation induced by trinitrobenzene sulfonic acid in rats also increased Ucn 2 expression in a large population of infiltrating immune cells (macrophages) (48). In our own studies, we found that rat colonic CRF mRNA was upregulated in response to an intraperitoneal injection of lipopolysaccharide (LPS) (44).

Differential expression of peripheral CRF signaling could also be the cause of higher or lower susceptibility to stress in individuals. In support of this hypothesis, Wistar Kyoto and Sprague-Dawley, two strains of rats with diverse anxiety sensitivities, were recently reported to exhibit differential profiles of CRF₁ and CRF₂ receptor expression in their colon under basal conditions and following an acute stress such as colorectal distension (CRD) or exposure to an open field (53). These results open new venues of investigation, particularly in light of the recent description of alternative splice variants for both receptors (21, 25).

ROLE OF CRF SIGNALING PATHWAYS IN THE COLONIC AND ILEAL RESPONSES TO STRESS

Propulsive motor function

When injected peripherally, CRF strongly alters colonic motility and transit in several mammalian species including rodents and humans (2). Clinical studies show that systemic injection of CRF induces a colonic motility response that includes the occurrence of clustered contractions in the descending and sigmoid colon, which is more prominent in IBS patients than in healthy controls (54). In rat colon, peripheral injection of CRF and Ucn 1 increases clustered spike-burst propagative activity (55, 56) and stimulates distal colonic transit and defecation (55, 57, 58). Similarly to peripheral CRF injections, acute physical or psychological stress in humans increases colonic propulsive motor function (59–62) although in other studies, increase or no change have been reported (63–65). In rodents, acute stress (restraint, water avoidance stress (WAS)) has been clearly established to stimulate colonic transit and defecation (for review see (2)). In contrast to the colon, very little is known about the effect of peripheral CRF or stress on the ileum in humans. In rodents, only a few studies have specifically focused on the ileum and all show an inhibitory effect of stress on ileal contractility (66–68). Convergent studies to characterize the CRF receptors involved in these processes have established that the stimulation of colonic motility after peripheral administration of CRF and Ucn 1 involves CRF₁ receptors in rats and mice (2), while in contrast the inhibition of ileal phasic contractions involves activation of CRF₂ receptors in rats (49, 51).

At the colonic level, the stimulation of motility and transit induced by peripheral CRF injection in conscious rodents is not affected by ganglion blockade, suggesting that the effects are peripherally mediated (69). Similarly, the functionality of the peripheral CRF signaling system in the colon during stress is supported by reports that peripherally injected peptide antagonists which do not cross the blood-brain-barrier, namely α -helical CRF₉₋₄₁ or astressin, block or blunt the stimulation of distal colonic transit and fecal pellet output induced by acute wrap restraint or WAS in rats (55, 58, 70, 71). Further support for a peripherally-restricted action of CRF peptide when injected peripherally is that it can be reproduced *in vitro* in colonic and ileal preparations. In an isolated colonic rat preparation, CRF increased basal myoelectrical peristaltic activity (55, 72) and increased phasic contractions and electric field stimulation off-contraction in isolated colonic muscle strips (46), whereas CRF and Ucn 1 inhibited the phasic contractions in ileal circular muscle strips (49). In guinea-pig ileum however, CRF was found to either stimulate the circular muscle activity in strips (73) and increase contractions in longitudinal muscle myenteric plexus preparation (74), or to have no influence on ileal smooth muscle strips contraction (75), highlighting possible species differences between rats and guinea-pigs ileum response.

Convergent evidence suggest a major role of enteric neurons in the mediation of peripheral CRF effects on colonic and ileal motility as also found for the colonic response to acute stress (76). First, the neuronal blocker, tetrodotoxin abolishes Ucn 1-evoked phasic contractions in colonic smooth muscle strips (46), indicative of an enteric nervous system

(ENS)-mediated event. Second, when injected intraperitoneally in rats, CRF induces Fos expression, a marker of neuronal activation, in cholinergic and nitrenergic myenteric neurons in the colon (77). Atropine, a muscarinic blocker, does not affect CRF-induced neuronal activation, indicating that it is not secondary to the activation of muscarinic receptors either on the myenteric ganglia (which possess both nicotinic and muscarinic receptors) or on colonic muscles (77) but rather to a direct effect on enteric neurons. In agreement with this, atropine prevents the CRF-induced ileal muscle contractions of guinea-pig preparations confirming that CRF does not act directly on the smooth muscle but exerts an excitatory action on the myenteric plexus *via* acetylcholine release (74). Third, direct administration of CRF or urocortins in ileal and colonic myenteric and submucosal plexus preparation of guinea pig excites both myenteric and submucosal neurons as monitored by electrophysiological recordings (41, 42, 78). In conscious rats, Fos expression in colonic myenteric neurons in response to intraperitoneal CRF is prevented by administration of the non-selective antagonist astressin and the selective CRF₁ antagonist CP-154,526 and reproduced by intraperitoneal injection of the selective CRF₁ agonists, stressin₁-A and cortagine suggesting a major participation of CRF₁ receptors in these effects (7, 39, 77). In the guinea-pig colon as well, the predominant expression of functional CRF₁ receptors, relative to CRF₂ receptors, along with the expression of CRF₁ receptors ligands in colonic enteric neurons (45–47) suggest that CRF₁ signaling is the main modulator in the ENS (41). Interestingly, in the rat ileum, Fos activation in response to activation of CRF₁ receptors is restricted to the submucosal enteric neurons, showing differential modulation of ileal *vs.* colonic enteric neurons by CRF ligands (39). In the colon, CRF and Ucn 1 action on the ENS to increase the contractility is related to the enhancement of cholinergic, nitrenergic and serotonergic neurotransmission (39, 46). To date, the effector mechanisms involved in the inhibitory effect of CRF and Ucn 1 on rat ileum remain unknown.

Together these data support the possibility that stress-induced alterations of colonic and ileal motility are linked to the activation of the peripheral CRF signaling in enteric neurons, with excitatory effects being mediated through CRF₁ which in turn recruit the cholinergic, nitrenergic and serotonergic transmission and inhibitory effects through CRF₂ receptors.

Epithelial barrier function in relation with enteroendocrine, epithelial and immune cells

The gut wall is protected by 1) a barrier composed of a single layer of polarized intestinal epithelial cells tightly sealed by tight junctions that regulate the passage of fluid, antigens and macromolecules as well as secrete mucus, antimicrobial peptides and immunoglobulins to limit bacterial colonization, and 2) a well developed immune system. A breach in the gut wall due to alterations in secretion and/or epithelial barrier physical disruption allows pathogens to get access to the lamina propria, which can in turn affect the local immune activity, participating in the onset and maintenance of intestinal diseases (79, 80).

The GI tract contains a variety of cells which participate in the innate defense mechanisms of the epithelium as well as in the control of intestinal secretory, motor and immune functions. Despite their low numbers in the GI tract, enterochromaffin cells, a subtype of enteroendocrine cells, store more than 80% of the organism's serotonin (5-HT) (81). Enterochromaffin cells act as sensor cells that signal every luminal change (acidity, osmolarity, nutrients, pathogens, bacterially-derived toxins) by releasing 5-HT in the gut (81, 82). The release of 5-HT that can occur constitutively and following stimulation activates nerves fibers, which besides affecting gut motility and the sensory response, can also cause the secretion of mucus from goblet cells and an increase in passive water flux to wash away any pathogens or noxious agents (83). There is considerable evidence that peripheral release of 5-HT is involved in stress-and central CRF-mediated alterations in rodent GI functions (71, 84–90). However, the role of peripheral CRF in these effects has been little addressed. Nevertheless, two pieces of evidence point towards enterochromaffin

cells being a potential direct target of the CRF signaling system peripherally. First, enterochromaffin cells in the human colon express CRF and Ucn 3 (37, 43). Second, *in vitro* studies using BON cells, an enterochromaffin cell-like cell line, showed they respond to rat/human CRF and the selective CRF₂ ligand, human Ucn 3 with cAMP formation and release of serotonin (36).

Goblet cells, that are present in the ileum and the colon, secrete and release mucus in the lumen, which protects the mucosa of close bacterial interaction/penetration by forming a coating layer over the epithelium (91). The direct influence of peripheral CRF signaling activation on mucus release has not been directly assessed, however, the presence of CRF₁ receptors on goblet cells and the fact that stress and peripheral injection of CRF induces mucus depletion and reduces the number of goblet cells in rat distal colon (70, 92, 93) suggest the likelihood of a direct action of CRF or urocortins on mucin secretion (40).

The intestinal epithelium also contributes to host defense by producing antimicrobial peptides (AMPs) (94, 95). A number of cells in the gastrointestinal tract can secrete AMPs, including epithelial cells, mast cells or Paneth cells, specialized cells which are located deep in the small intestine crypts (94–96). It is currently unknown whether the peripheral CRF signaling plays any role in the release of AMPs under conditions of stress. However, few studies in human and rodents suggest that this innate defense mechanism can be recruited under conditions of stress and that its defect could participate to the pathogenesis of IBD and IBS (97–101). Thus, in women, acute cold stress was shown to induce a significant release of α -defensin in the jejunum (102). In mice and rats, Paneth cells' secretory activity in the small intestine is affected by infective and nutritional stress (103). Whether this is also the case for psychological or physical stress is not known. Recent data showing a decreased release of antimicrobial peptides in the skin after a psychological stress support the possibility that stress might affect this protective pathway in the gut as well (104).

In addition to mucosal and enteroendocrine cells, the colon and the ileum in particular are home to a variety of resident immune cells such as mast cells, lymphocytes, and macrophages which are known to occupy a key role in the control of intestinal immunity, and which have been identified as targets of CRF signaling in other organs such as the skin, lungs and brain (12). Their modulation by peripheral CRF in the gut has been recently investigated. In a number of tissues including the gut, mast cells have been found to express the CRF receptors (38, 105–107), and their activation by either urocortins or CRF (105) leads to the selective release of cytokines and other pro-inflammatory mediators (108) well established to affect the epithelial barrier function (10, 109–111). Several studies documented that peripheral CRF affects colonic epithelial function in human (38) and rats (112–114) *via* recruitment (115) and activation of mast cells and both CRF₁ and CRF₂ receptors (38, 112–114). The influence of CRF signaling on ileal mast cells has been less investigated. In our recent studies, we found that in mice treated with the selective CRF₁ agonist, cortagine, the colon responds with increased TGF- β expression known to be a potent modulator of human intestinal mast cell effector functions (116). In addition, the ileum exhibits a dose-related interferon γ (IFN γ) response indicating T cell and/or natural killer (NK) cell activation, which is followed by tight junction deregulation and dose-dependent apoptotic loss of different cell populations (Kiank *et al*, unpublished data). Thus, in the ileum, cells other than mast cells may be responsible for CRF signaling mediated immune stimulation. Earlier studies indicated that CRF stimulates the proliferation of human lymphocytes by increasing interleukin (IL)-2 receptor expression and enhancing the production of IL-1 and IL-2 (117). Thus, the intestinal lymphocyte activation may be triggered by local CRF signaling.

Macrophages are also a target for peripheral CRF signaling *via* CRF₂ receptor and may influence the mucosal immune homeostasis *via* this pathway (118). For instance, in the presence of CRF, Ucn 1 and Ucn 2, murine macrophages were shown to transiently inhibit a LPS-induced TNF- response, which was followed by a second phase of heightened TNF- α production (119). It was also demonstrated that low doses of Ucn 1 and Ucn 2 (10^{-10} - 10^{-8} M) enhance macrophages apoptosis which may trigger an anti-inflammatory response whereas high doses did not (120). Additional evidence for the anti-inflammatory effects of peripheral CRF was demonstrated in human monocyte-derived dendritic cells, which express both CRF₁ and CRF₂ receptors, and responded to CRF treatment with an attenuated IL-18 production which is anti-inflammatory by promoting a Th1 shift of the T cell response (121). Taken together, these data hint for time- and dose-dependency of CRF receptor ligands to modulate inflammatory processes (for review see (12)).

A number of reports also suggest that stress, like CRF, can recruit and activate mast cells (93, 122–125), neutrophils (93), eosinophils (126) and mononuclear cells (93, 127) in the jejunal, ileal and colonic mucosa. In human jejunum, acute pain induced by cold exposure induces mast cell degranulation (102, 128). Compared to healthy controls, IBS patients, who present high levels of psychological stress, exhibit higher numbers of mucosal mast cells in their jejunum (129) and colon (130, 131), as well as CD8+ T lymphocytes in the colon (132). In mice exposed to acute restraint combined with noise stress, the colonic mast cell degranulation involves the over-production of IFN γ (133) and chronic restraint stress increases eosinophils expressing CRF in the jejunum, which participate to the recruitment of mast cells and epithelial barrier dysfunction (126). Repeated exposure of rats to WAS also induces hyperplasia and activation of mast cells, causes an infiltration of neutrophils and mononuclear cells, and increased myeloperoxidase activity in both the ileal and colonic mucosa which is associated with bacterial adhesion and penetration into enterocytes (93, 127, 134). When acute restraint stress occurred under conditions of preexistent colonic inflammation induced by dinitrobenzene sulfonic acid, it evoked an inflammatory relapse with significant macroscopic colonic damage, increased myeloperoxidase activity and significant infiltration of mucosal and submucosal T cells (135). Likewise, in mice this heightened stress-induced reactivation of experimental colitis was shown to depend on T helper cell activation (136), suggesting recruitment of T cells in stress conditions that may participate to intestinal inflammation.

Thus, CRF signaling can transfer stress signals to enteroendocrine, mucosal and immune cells which are resident or infiltrate the GI tract during inflammation. Mast cells, macrophages and mononuclear cells appear as likely target cells of CRF signaling (for review see (12)) which can have both, pro-and anti-inflammatory effects and may thus have damaging or protective effects on intestinal homeostasis. In addition, there seems to be a site specificity of CRF signaling effects which might be at the origin of a different stress response in the colon and ileum. Therefore, the final outcome of activation of CRF/CRF₁ or Ucn 2/CRF₂ paracrine circuit on intestinal secretory and motor function or on the inflammation appears to be highly organ/tissue-specific and context-dependent.

Ion and water secretion

The maintenance of an appropriate fluid balance, assured by ion and water secretion, is essential to the normal function of the small and large intestines. As indicated earlier, this capacity for high ion and water secretion in the intestine is a defense mechanism serving to flush out pathogens, thereby preventing mucosal adhesion, which is commonly observed as the phenomenon of diarrhea (137). Intraperitoneal CRF administration induces a watery diarrhea with rapid onset in rats (138). This diarrhea is antagonized by selective CRF₁ antagonists (138) and mimicked by a peripheral injection of the selective CRF₁ agonist,

cortagine, in both rats and mice (7, 39). Similarly, restraint stress induces watery diarrhea in rats (89, 139).

Water moves passively at the epithelial level thanks to an active ion transport assured by villus epithelial cells that are absorptive (*via* sodium) and crypt epithelial cells which are secretory (*via* chloride). Changes in this active ion transport across the tissue, or secretory response, can be measured *in vivo* by the double- or triple-lumen closed-segment perfusion technique (102, 140, 141) or *in vitro* using Ussing chambers by measuring the short circuit current (Isc). In humans, studies on intestinal secretory function have been limited to the influence of psychological or physical stress on the jejunum, where dichotomous listening was found to cause a reduction in net water absorption coupled with net sodium/chloride secretion (140). Other studies showed that acute cold stress induces a chloride-related decrease in peak secretory response in women with moderate background stress (102). In rats, both acute intraperitoneal administration of CRF and chronic subcutaneous administration of CRF increase the basal colonic Isc (113, 115). This increase in Isc was reproduced by chronic peripheral administration of the selective CRF₁ agonist stressin-1 and the selective CRF₂ ligand, Ucn 3, suggesting a role of both CRF receptor subtypes in this alteration. However, intriguingly, pretreatment with antisauvagine, a selective CRF₂ antagonist, did not have any effect on chronic CRF-induced Isc increase (115). Whether the dose of antagonist used was inefficient to block CRF effect *in vivo* remains uncertain as no dose-response studies were performed. Similar to the effects of peripheral injections of CRF ligands, exposure of rodents to either acute or chronic stress increases basal and stimulated Isc in the colon (113, 127, 142–144) as well as in the ileum (93, 127, 145) and this alteration in Isc is abolished by pretreating rats with the peripheral non-selective CRF antagonists astressin or α -helical CRF₉₋₄₁ (112, 113, 142). Together, these data suggest the participation of both CRF₁ and CRF₂ receptors in the alterations of epithelial secretory response induced by stress and activation of peripheral CRF signaling.

Strong evidence for a direct peripheral action of CRF peptides on epithelial secretory response comes from *in vitro* studies, performed in Ussing chambers. While the only study performed in humans did not show any effect of the direct administration of CRF on colonic biopsies from healthy controls on Isc (38), in rat colonic tissue, CRF has been found to consistently increase the baseline Isc (112, 113). *In vitro*, treatment with α -helical CRF₉₋₄₁ or astressin prevented the CRF-induced increase in Isc (112, 114), further supporting a role for peripheral CRF receptors located at the submucosal/mucosal level. Of note, maximal epithelial Isc responses in rat colonic tissue have been obtained with sauvagine, a CRF agonist with high affinity to both CRF receptors administered *in vitro* at doses 200–1000 fold lower than CRF, suggesting a predominant CRF₂ effect in the modulation of secretory epithelial functions in the rat colon (114).

It is well established that enteric neuronal reflex pathways within the enteric nervous system which control chloride secretion involve acetylcholine and serotonin (146). In support of such pathways in the effect of peripheral CRF on secretory function, hexamethonium and atropine (nicotinic and muscarinic blockers, respectively) but not bretylium (adrenergic blocker) prevented CRF-induced Isc increase in rat colonic tissue (112). Mast cells also play a key role in the CRF-induced secretory epithelial alterations as colonic tissue pretreated with doxantrazole or issued from mast cells deficient rats do not exhibit Isc changes following CRF exposure in Ussing chambers (112, 115). A number of studies also show that the watery diarrhea induced by stress or exogenous central administration of CRF in rats depends on serotonin release and the contribution of 5-HT₃ receptors (88, 90), but whether this pathway is also recruited in response to peripheral CRF signaling activation is still undetermined.

Taken together, these data established the involvement of peripheral CRF signaling in the modulation of secretory function under stress *via* activation of both CRF₁ and CRF₂ receptors, activation of cholinergic enteric neurons, mast cells and possibly serotonergic pathways.

Permeability

Trafficking of molecules through the intestinal epithelial barrier occurs *via* two routes: paracellular (between cells) which controls ion selectivity, nutrients and solute permeability and/or transcellular (through cells) which allows transport of large molecules (antigens, immunoglobulins) through epithelial cells (147). The integrity of the epithelial barrier is regulated by a complex protein system that constitutes tight junctions (for review see (148)). Gross changes in epithelial permeability are measured *in vivo* by the mannitol/lactulose ratio or lumen-to-blood ratio of macromolecules such as albumin. Gut paracellular permeability is measured *in vivo* using radioactive or fluorescent probes of more than 10 Å in size (⁵¹Cr-EDTA, FITC-dextran, FI-sulfonic acid) (149, 150) and *ex vivo* using Ussing chambers to assess the flux of FITC-dextran, the transepithelial resistance (TER) or the conductance ($G=1/TER$), which reflects paracellular ion exchange and tissue viability. Gut transcellular permeability is assessed *ex vivo* *via* measurements of horseradish peroxidase (HRP, 44 kDa, 50–60 Å) flux or endocytosis.

So far, the effects of peripheral CRF administration on epithelial barrier function in humans have not been assessed. In rats, however, acute or chronic peripheral CRF administration stimulates colonic HRP flux *ex vivo* or ⁵¹Cr-EDTA flux *in vivo* (10, 113, 115). This increase in colonic HRP flux *ex vivo* appears to be CRF₂-mediated (115). In contrast, in our own *in vivo* experiments, we found that selective peripheral activation of CRF₁ receptors in rats increased the colonic permeability as monitored by Evans blue permeation from lumen to blood (7). Thus far, there is no report on the influence of the peripheral CRF signaling on ileal epithelial permeability. However, our recent experiments indicate that the activation of either peripheral CRF receptors induces the loss of the epithelial barrier in the ileum by reducing the expression of integral tight junctions proteins and altering the expression of IFN- γ and IL-10 (Kiank *et al.*, unpublished data). These effects seem to be related to the recruitment of CRF₂ pathways by CRF₁ activation and appear to be dose-dependent, as shown by the protective effect of peripheral CRF₁ signaling activation at low doses (Kiank *et al.*, unpublished data).

In humans and rodents, stress also induces alterations of epithelial permeability. Acute cold stress exposure enhances jejunal permeability to antigenic macromolecules (blood-to-lumen albumin permeability) and enhances the blood-to-lumen mannitol and xylose permeability in healthy controls (102, 141). Alterations of permeability have also been shown *in vitro* in colonic biopsies of both post-infectious and non post-infectious diarrhea-predominant IBS patients (151, 152) and *in vivo* in the small intestine (153). Nevertheless, a direct causal relationship between increased permeability and stress has not been established in these studies. In rodents, both acute (restraint, WAS, cold) and chronic stress (WAS 5–10 days, maternal separation) increase the paracellular and transcellular permeability in the colon (10, 112, 113, 127, 133, 142–144, 154, 155) and in the ileum (93, 127, 145). At the colonic level, this alteration is abolished by pretreatment of rats with the peripheral administration of the non selective CRF antagonists astressin or α -helical CRF₉₋₄₁ (10, 112, 113, 142) or the selective CRF₁ antagonist, SSR-125543 (10), supporting the participation of CRF₁ receptors in the modulation of colonic permeability.

Additional support to a peripherally restricted action of CRF signaling on epithelial permeability changes has also been obtained *in vitro*. In human colonic biopsies mounted in Ussing chambers, CRF administered on the serosal side induced an increased uptake of HRP

by endocytosis, sign of an increased transcellular permeability, but did not affect paracellular permeability as assessed by permeation of ^{51}Cr -EDTA and TER (38). This result contrasts with the increase in both paracellular and transcellular permeability observed in rat colonic tissue after exposure to CRF, sauvagine or Ucn 3 *in vitro* (112–114). This could be related to species differences in the effector mechanisms recruited in the periphery, but those are still not well known in humans. In rats however, it is shown that enteric nerves and mast cells are involved in the alterations of colonic permeability induced by peripheral CRF administration (38, 112, 115). Interestingly, alterations in paracellular permeability are mediated by adrenergic and nicotinic nerves, whereas cholinergic, adrenergic and nicotinic nerves all participate to the alterations of transcellular permeability (112). The CRF receptors and effector mechanisms involved in the alterations of epithelial permeability in the ileum remain unknown.

Thus, stress-induced activation of peripheral CRF receptors has an important impact on permeability at both the ileal and colonic levels. Together, these data suggest the participation of both CRF₁ and CRF₂ receptors in the alterations of epithelial permeability barrier in response to activation of peripheral CRF signaling or stress, with recruitment of mast cells, nicotinic, cholinergic and adrenergic enteric nerves.

EPITHELIAL DYSFUNCTION-INDUCED IMMUNE ACTIVATION

Altered integrity of the intestinal epithelial cellular layer and tight junctions deregulation may have systemic consequences and seem to be a key factor leading to the dysfunction of several organs such as lung, liver, gut, or kidney associated during septic complication that are caused by decontrolled inflammatory processes (156). Intriguingly, exposure of Fisher rats to stress before induction of a stroke by middle cerebral artery occlusion is associated with a worse stroke outcome which is linked with colonic inflammation and bacterial translocation into different organs such as mesenteric lymph nodes, spleen, liver, and lung (157). Together these data emphasize the potential clinical relevance of bacterial translocation in severely sick patients.

It is widely accepted that stress can trigger local inflammatory processes in the gut and influences the clinical course of gastrointestinal disorders such as peptic ulcer, IBS or IBD. The increased passage of antigens, commensal microorganisms or even pathogens in the lamina propria subsequent to an epithelial barrier breach, contributes to the development of inflammatory processes (80, 112, 114, 133, 145, 158, 159). An enhanced bacterial translocation was demonstrated during chronic psychological stress and during colitis, which seems to exaggerate the course of colonic inflammation (160–162). *E. coli* bacteria are also present in the mucosa during ileal inflammation and Toll-like receptor 4 (TLR4) signaling exacerbates ileitis *via* enhancing the local release of IFN γ and nitric oxide from immune cells, thus further damaging the local tissue (163). Finally, it was shown that intestinal inflammation results in a prolonged impairment of colonic epithelial secretion, which increases bacterial translocation (164) and may further boost inflammatory processes triggering chronic diseases such as IBD. The underlying role of stress-related colonic activation of CRF signaling and related effects on local immune cells under these conditions is still to be investigated.

VISCERAL PAIN

A role for peripheral CRF signaling in the development and expression of visceral pain is well documented by several reports in both humans and rodents (7, 18, 165–169). A systemic injection of the preferential CRF₁ agonist, ovine CRF (170) lowers pain thresholds to repetitive rectal distensions in healthy humans (165, 166). In rats, peripheral injection of CRF induces visceral hypersensitivity to CRD (169), an effect reproduced by the

intraperitoneal administration of the selective CRF₁ agonist, cortagine in rats and mice (7). Likewise acute stress (cold, noise) and chronic stressful events have also been shown to increase the visceral sensitivity to rectosigmoid distension in humans, in particular IBS patients (171, 172). In rodents as well, several studies support a strong association between stress (acute or chronic) and increased visceral sensitivity to CRD (123, 124, 150, 173–176).

Converging evidence support the involvement of peripheral CRF₁ receptors in these effects. First, intravenous administration of the non-selective and peripherally-restricted CRF receptors antagonists, α -helical CRF₉₋₄₁ or astressin reduced visceral hyperalgesia in diarrhea-predominant IBS patients subjected to colonic electrical stimulation (167, 168). In earlier experiments with rats repeatedly exposed to WAS for 10 days, we found that peripheral administration of astressin before each stress session could prevent the development of visceral hyperalgesia supporting the participation of a peripheral component to the development of visceral hypersensitivity (177). Lastly, the visceral hyperalgesia induced by peripheral injection of cortagine in rats is abolished by peripheral, but not central, administration of the non selective CRF receptor antagonist astressin at equivalent dose (7). To date, there are no reports about peripheral CRF signaling activation effects on visceral sensitivity in the ileum. Pain associated fibers (myenteric AH neurons) which are known to respond to CRF *via* CRF₁ receptors activation (42) have however been described in the ileum (42, 178) and open the possibility that peripheral CRF signaling may also contribute to the pain and discomfort in the ileal segment.

Peripheral CRF induces mast cells degranulation (10) which can in turn lead to the development of visceral hypersensitivity *via* the release of several preformed or newly generated mediators (*e.g.* histamine (151, 179), tryptase (179), prostaglandin E₂ (180), nerve growth factor (NGF) (123)) that can activate or sensitize sensory afferents (181, 182). Additionally, the disruption of the intestinal epithelial barrier by peripheral CRF signaling activation as detailed earlier may increase the penetration of soluble factors (antigens) into the lamina propria, which can lead to nociceptors sensitization as shown for stress (151, 152) and may be independent from mast cell activation, as suggested for persistent stress (182, 183). Increased intestinal permeability is indeed a phenomenon that appears as a prerequisite for the development of visceral hypersensitivity in both humans and rodents (150, 152, 184).

Taken together, these reports suggest that enhanced peripheral activation of CRF/CRF₁ signaling in addition to central activation bears relevance as part of the peripheral efferent components responsible for altering the colonic visceral responses to stress *via* mast cells recruitment and release of inflammatory mediators which alter the epithelial barrier function.

PATHOPHYSIOLOGICAL RELEVANCE OF PERIPHERAL CRF SIGNALING IN IBS AND IBD

As outlined, strong preclinical evidence suggests that activation of the peripheral CRF signaling, by mimicking stress effects, exerts a key role in the alterations of colonic and ileal propulsive motor function, visceral hypersensitivity and the epithelium including secretion, barrier and immune functions *via* activation of CRF₁ receptors in the colon or CRF₂ receptors, or both, in the ileum. Multiple potential pathways recruited by stress in the periphery have been reported. Although early results in humans suggest that the peripheral CRF signaling might participate to the immune components in IBD (13, 14, 185) and it is also very likely involved in the pathogenesis of IBS (11, 186–191), additional clinical data are required to validate this concept.

To date only two double-blind, placebo-controlled clinical trials using CRF₁ antagonists in IBS patients have been performed and the results are inconclusive (192, 193). Additional studies are required to determine whether the first phase IIa clinical trial negative results reflect differential efficacy of CRF₁ antagonists, or a lack of translational application of stress-related mechanisms to the pathophysiology of IBS. Of importance, in human tissues, there is evidence of alternative splicing of CRF₁ receptors leading to eleven isoforms and dimerization of the receptors along with differential regulation under pathophysiologic conditions. This creates additional regulatory elements in the CRF₁ signaling pathways which have been shown to have biological relevance (194). The expression and regulation of alternative splicing of CRF₁ receptors in the colon, their biological actions and interaction with CRF₁ antagonists are unknown and may be additional components to take into account in light of these clinical trials. A better understanding of the molecular aspect of the peripheral CRF signaling system could pave the road to the development of novel therapeutic options to relieve stress- and inflammatory-sensitive bowel disorders.

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Abbreviations

5-HT	serotonin
AMPs	antimicrobial peptides
CD	Crohn's disease
CRD	colorectal distension
CRF	corticotropin releasing factor
CRF₁	corticotropin releasing factor receptor 1
CRF₂	corticotropin releasing factor receptor 2
CRF-BP	CRF binding protein
ENS	enteric nervous system
G	conductance
GI	gastrointestinal
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFNγ	interferon γ
IL	interleukin
IR	immunoreactivity
Isc	short circuit current
LP	lamina propria
LPMCs	lamina propria mononuclear cells
LPS	lipopolysaccharide

MAPK	mitogen activated protein kinase
PK	protein kinase
ERK1/2	extracellular signal-regulated kinase 1/2
MNP	myenteric neuronal plexus
NK	natural killer
PAR-2	protease activated receptor 2
SNP	submucosal neuronal plexus
TER	transepithelial resistance
TLR4	Toll-like receptor 4
UC	ulcerative colitis
Ucn	urocortin
WAS	water avoidance stress

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Table 1

Distribution of CRF receptors and ligands (mRNA and immunoreactivity) in human and rodent colon and ileum.

Receptors	COLON		ILEUM	
	Human	Rodent	Human	Rodent
CRF ₁	SNP (33)	Goblet cells	(26,39,40) ND	Epithelial cells (50,52)
	MNP	Crypts (stem cells)		LP
	LP (mast cell, macrophages)	SNP (33,34,38)	(41)	SNP (45,49)
		MNP		MNP
CRF ₂		LP (few cells)	(40)	
		Epithelium (few cells)		
	Epithelial cells (weak)	(34–36)	(40) ND	LP cells (weak) (50,52)
	SNP (37)	Crypts (luminal surface)		MNP (40,45)
	MNP	Submucosal layer (blood vessels)		SNP
	SML			
VEC				
VSMC				
Ligands	LP (und. inflammatory cells) (34)			
	LP (mast cells) (38)			
Ligands	COLON		ILEUM	
	Human	Rodent	Human	Rodent
CRF	Mucosa (43)	Epithelial cells	(10,44) ND	Epithelial cells (weak) (50)
	Enterochromaffin cells	Mucosa		(50,51)
		Submucosa/muscle layer		Paneth cells
				MNP
Ucn 1		LP		SNP (45)
		MNP	(44–47)	LP
		SNP		
	Epithelial cells (weak) (34)	MNP	(46,47) ND	ND
LP macrophages	SNP			

Ligands	COLON		ILEUM	
	Human	Rodent	Human	Rodent
Ucn 2	ND	Epithelial cells MNP SNP Muscle layer	(48) ND	Epithelial cells MNP SNP Muscle layer
Ucn3	Enterochromaffin cells	(37) ND	ND	ND
	MNP			
	SNP			
	VEC			
	VSMC			
	SML			
	Enteric glial cells			

SNP: submucosal neuronal plexus, MNP: myenteric neuronal plexus, VEC: vascular endothelial cells, VSMC: vascular smooth muscle cells, SML: smooth muscle layer, LP: lamina propria, ND: non determined.

Table 2

Effects of peripheral CRF₁ or CRF₂ receptor signaling on motility, secretion, permeability, inflammation and pain perception in colon and ileum.

	COLON		ILEUM	
	CRF ₁	CRF ₂	CRF ₁	CRF ₂
Motility	↑ (7,46,55–58,72,77,195)	?	↑ (73,74)	↓ (49,51)
Enteroendocrine and mucosal cells secretion	?	↑ (36)	?	?
Ion and water secretion	↑ (7,112–115)	↑ (112,114,115,196)	?	?
Permeability	↑ (7,10,38)	↑ (38,113–115)	↑ (Kiank unpublished) ↓ (Kiank unpublished)	↑ (Kiank unpublished)
Inflammation	↑ (197)	↑ (35,48,52)	↑ (51,117,119) (Kiank unpublished) ↓ (Kiank unpublished)	↑ (35,118,119) ↓ (119,120)
Pain	↑ (7,165–169)	?	?	?

↑ Peripheral CRF signaling activation increases measured activation state;

↓ Peripheral CRF signaling decreases measured activation state; ? No direct reports about peripheral CRF signaling effects on mentioned functional system in the specific gut segment