ABSTRACT

GIBSON, AMELIA JIN KYUNG. Mast Cell Corticotropin-Releasing Factor Receptor Subtypes, CRF_1 and CRF_2, Differentially Modulate Stress-induced Intestinal Permeability. (Under the direction of Dr. Adam Moeser).

Psychological stress is gaining recognition as an important predisposing or inciting factor in the development of gastrointestinal disease. The shift from a protective response intended to maintain homeostasis to one that results in profound consequences and the development of disease is unclear. The stress response is mediated by corticotropin-releasing factor (CRF) that may be released both centrally and peripherally. The bi-directional communication between the brain and gut serves as the link between stress perception and gastrointestinal function. Mast cells have been identified as important mediators of the brain-gut axis, and previous research has demonstrated that intestinal mast cells play a critical role in mediating stress-induced gastrointestinal dysfunction although the mechanism remains unclear. Mast cells have been shown to express both CRF receptor subtypes, CRF_1 and CRF_2, and can be activated through binding of either receptor. However, the influence of CRF_1 and CRF_2 on mast cell function during the stress response is yet to be elucidated. Here we demonstrate a dichotomous role of CRF receptor subtypes in mast cell activation in response to psychological stress. Furthermore, we have identified a novel protective role for mast cell CRF_2 in modulating stress-induced intestinal permeability. Mast cell-deficient mice repleted with CRF_1 -/- bone marrow-derived mast cells (BMMCs) were completely protected from stress-induced increases in intestinal permeability; whereas, mice repleted with CRF_2 -/- BMMCs developed a severe loss of intestinal barrier function. To further characterize the mechanism by which CRF receptor subtypes control mast cell activation, we used an *in vitro*

system of mast cell degranulation by IgE and antigen cross-linking in the mucosal mast cell line, rat basophil leukemia cells (RBL-2H3). Prior to stimulation, RBL-2H3 cells were pretreated with CRF receptor agonists and antagonists then assessed for degranulation by beta-hexosaminidase release. Absence of CRF_1 signaling prevented degranulation; whereas, the loss of CRF_2 signaling enhanced degranulation, indicating that CRF receptor subtypes differentially regulate mast cell degranulation pathways. These data indicate that targeting individual mast cell CRF receptor subtypes may lead to novel therapies in the treatment of stress-induced gastrointestinal disease.

Mast Cell Corticotropin-Releasing Factor Receptor Subtypes, CRF_1 and CRF_2, Differentially Modulate Stress-induced Intestinal Permeability

by
Amelia Gibson

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2013

APPROVED BY:

_____	_____
Adam Moeser DVM, PhD Committee Chair	Sam Jones DVM, PhD
_____	_____
Anthony Blikslager DVM, PhD	Jody Gookin DVM, PhD

DEDICATION

To my family and friends for supporting me during this process.

BIOGRAPHY

Amelia Gibson received a Bachelor of Science in Microbiology from the University of Tennessee in 2004. After working in London, England, Amelia moved to Raleigh, NC to pursue a dual master's degree in Microbial Biotechnology and Business Administration at North Carolina State University. Following a year of teaching, Amelia returned to NCSU to pursue a PhD in Comparative Biomedical Sciences and perform the research that is presented here.

ACKNOWLEDGMENTS

I would first like to acknowledge my mentor, Dr. Adam Moeser, for giving me a great project and devoting his time, energy, and resources on my intellectual and professional growth. Secondly, I would like to sincerely thank Dr. Sam Jones who went above and beyond to find funding for me during my first year of study. Without him, I would not be here, and I appreciate his belief in me and support throughout the entirety of my time in the program. I would like to thank Dr. Jody Gookin and Dr. Anthony Blikslager for sitting on my committee and continuously providing support and constructive criticism throughout this project. Finally, I would like to thank the members of the Moeser Lab, specifically Dr. Beth Overman, Dr. Liz Lennon, and Laura Edwards for providing their friendship, expertise, and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

CHAPTER 1: MAST CELLS AS MEDIATORS OF STRESS-INDUCED GASTROINTESTINAL DYSFUNCTION

Abstract

Mast cells were first characterized in 1878 by Paul Ehrlich and much research has focused on their role in modulating the allergic response. However, as research interest in mast cell function and activation broadens, this unique cell type is being recognized as an important mediator of normal physiological processes and as critical effector cells in the development of disease. Within the gastrointestinal tract, mast cells reside just below the intestinal epithelium, poised to respond to pathogens and mount a protective inflammatory response. However, mast cells are also associated with the development of notable gastrointestinal disorders, including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) through unclear mechanisms. Their role in the development of these disorders and the exacerbation of symptoms are believed to be through stress signaling pathways, leading to the release of preformed or *de novo* synthesized mediators that may alter gastrointestinal motility, secretion, barrier function, and visceral nociception. Animal models utilizing mast cell stabilizers and the subsequent amelioration of stress-induced changes in GI function indicate that mast cells may be a therapeutic target in the treatment of IBS and IBD. Here we discuss the effects of stress on gastrointestinal function and the role of the mast cell in this response.

Introduction

Mast cells are derived from hematopoietic progenitor cells that circulate in the blood and migrate to tissues where they mature into two subtypes, connective tissue or mucosal mast cells, that differ in morphology and mediator content (1,2,3). Mast cells are dispersed throughout most tissues but are critically located at the host-environment interface, such as along mucosal surfaces and blood vessels and adjacent to nerve cells and lymphatic vessels, allowing them to quickly respond to a variety of external stimuli (4). Activation of mast cells leads to the release of preformed mediators as well as stimulates the production of *de novo* synthesized mediators. These mediators fall into four major groups, proteoglycans, proteases, amines, and arachidonic acid metabolites (3). Of the most well studied mediators, primarily known for their role in allergy and inflammation, preformed mediators include histamine, serine proteases, tryptase, chymase, and various cytokines (5). *De novo* synthesized mediators include prostaglandins, leukotrienes, chemokines, and select cytokines. As a consequence of the broad range of mast cell mediators, the ability of these cells to respond quickly, and their location within the tissue, mast cell activation can have profound effects on both distant and neighboring tissues.

Within the gastrointestinal tract, mast cells modulate gastrointestinal function and contribute to the pathophysiology of GI disorders associated with stress, namely irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (6). More recently, it has been demonstrated that mast cells express receptors for neuropeptides such as corticotropin-releasing factor (CRF), Substance P, and the urocortins, indicating that mast cells may act as a critical link between the brain and peripheral tissues (7,8,9). These neuropeptides have

been demonstrated to stimulate mast cells to release mediators; yet, the physiological relevance of mast cell-neuropeptide signaling is yet to be elucidated. In this review, the relationship between stress neuropeptide signaling on mast cell activation and the contribution to intestinal disorders will be discussed.

Stress and the Role of CRF Family Peptides in Modulating Gastrointestinal Function

The stress response is an important adaptive mechanism needed to protect an organism from disturbances in the environment (10). This response is characterized by the release of chemical mediators whose primary function is to return an organism to homeostatic conditions. A successful adaptive response requires both the ability to respond to a stressor as well as the ability to appropriately control the stress response. Both human and animal models have demonstrated that stress can have a profound effect on gastrointestinal function characterized by alterations in secretory properties, motility, and barrier function (11). Research in animal models of stress have demonstrated elevated levels of the stress neuropeptide, corticotropin-releasing factor (CRF), in peripheral tissues following stress (12,13,14,15). Consequently, studies on stress-induced changes in GI function have focused on the role of CRF and its related peptides in modulating these effects.

CRF Family of Neuropeptides

The central effectors of the stress response are CRF and norepinephrine with peripheral limbs of the stress response regulated by the hypothalamic-pituitary-adrenal (HPA) axis and the

sympathetic and adrenomedullary systems (16). Perceived or real threats to homeostasis trigger the release of CRF from the hypothalamus. CRF may then act on the pituitary gland to stimulate the release of anti-inflammatory glucocorticoids from the adrenal cortex. Within the GI tract, both central and peripheral CRF signaling pathways have proven to be important in regulating homeostatic and disease processes.

CRF is a 41-amino acid peptide that can be released both centrally and peripherally (17,18,19,20). CRF and its receptors subtypes, CRF_1 and CRF_2, have been identified in several regions of the gastrointestinal tract, including the small intestine, large intestine and gastric myenteric plexus (21,14,22-24). The CRF receptor subtypes are class B, 7-transmembrane G protein-coupled receptors produced from distinct genes with each receptor subtype having multiple splice variants (25). Although these receptors share 70% amino acid homology, CRF binds with a tenfold greater affinity to CRF_1 than CRF_2 (25,26). Signaling through CRF receptor subtypes is primarily linked to the activation of adenylate cyclase through the Gs subunit but there is evidence to suggest that there may be tissue-specific coupling to additional G subunits (27,28).

In addition to binding to CRF, CRF receptor subtypes can bind to additional members of the CRF family of neuropeptides, urocortin (Ucn), urocortin 2 (Ucn2), and urocortin 3 (Ucn3). These peptides are expressed centrally and peripherally but differ in tissue distribution and receptor binding affinity (29,30,31). Ucn is a 40-amino acid peptide that binds to CRF_1 and CRF_2 (32). Ucn2 and Ucn3 are both 38-amino acid peptides that selectively bind to CRF_2 (29,31). In addition to structural similarities to CRF, the urocortins have also been shown to alter GI function (33-35).

The Effect of Stress on Gastrointestinal Motility

Under normal conditions, digesta is moved through the GI tract in a manner that promotes nutrient and water absorption. Gastrointestinal motility is known to be altered by exposure to psychological stress in human cases as well as in animal models (9,36,37). Typically, consequences of stress are manifested by an inhibition of gastric emptying and stimulation of colonic motor functions. Animal studies in rats and dogs have demonstrated that solid meal gastric emptying is delayed by acute stress (38,39,40). A study conducted by Fukado *et al* demonstrated that psychological stress increased colonic motility in healthy volunteers as well as in patients with Irritable Bowel Syndrome (IBS) (41). Additionally, studies in rodents utilizing restraint stress and water avoidance stress further support the influence of psychological stress in altering colonic transit accompanied by increased fecal output (42,43).

Evidence suggests that CRF plays a pivotal role in regulating gastrointestinal motility in response to stress. Restraint stress is known to increase CRF mRNA in the amygdale and paraventricular nucleus, leading to altered gastrointestinal motility (44). Exogenous application of CRF to the central nervous system attenuated gastric emptying and acid secretion (45,40). Intravenous injection of CRF into human subjects increased colonic motility in healthy volunteers as well as in IBS patients (46). Studies also demonstrate that CRF receptor subtypes differentially influence gastrointestinal motility. Activation of CRF_1 by CRF or Ucn via intracerebroventricular injection increased colonic motility, decreased colonic transit time, and induced defecation in rodents, a response that was completely blocked by treatment with the non-selective CRF antagonists, α-helical CRF 9-41 or astressin

(47,48,37,42). Conversely, blockade of CRF_2 by the selective antagonist, astressin2b, had no effect on CRF-induced increase in colonic motility (49). Within the upper GI tract, CRF_2 is the primary mediator of delayed gastric emptying associated with stress. Intravenous and intraperitoneal injection of Ucn, which binds to both receptor subtypes, induced delayed gastric emptying which was blocked by the CRF_2 antagonist, antisauvagine-30 (50,34,30,51).

The Effect of Stress on Secretion

Secretion of ions and water is an important physiologic process allowing the dispersal of digestive enzymes and proper digestion. Additionally, the ability to induce secretion of water and ions when exposed to intestinal pathogens represents an innate defense mechanism against intestinal infection (52). Stress is known to impact normal secretory processes in the GI tract. In human studies utilizing intestinal perfusion techniques, psychological and physical stress reduced water absorption and stimulated chloride secretion in the jejunum (53,54,55). Rodent studies using restraint stress and cold, restraint stress led to increased chloride secretion over unstressed controls and enhanced fluid secretion (56,57). A porcine model of early life stress, early weaning stress, demonstrated that early weaned pigs had elevated short circuit current (*Isc*) values compared to later weaned controls, indicative of increased secretory activity (58).

In order to examine the role of CRF in colonic epithelial physiology, Santos *et al* subjected rodents to restraint stress and pre-treated with a CRF antagonist, α-helical CRF 9-41 (59). Restraint stress induced chloride secretion in the colon, a response that was blocked by treatment with α-helical CRF 9-41. The effects of restraint stress on ion secretion were

mimicked by intraperitoneal injection of CRF. Taken together, these data indicate that CRF is the primary mediator regulating stress-induced changes in secretory activity. Furthermore, CRF_1 has been identified as the receptor subtype responsible for enhanced secretory activity and the associated clinical symptom of diarrhea. Saunders *et al* found that intravenous injection of CRF resulted in a dose-dependent increase in secretory diarrhea and fecal fluid content in rats (60). This effect was abolished by pretreatment with the CRF_1 antagonist, CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]et hylamine). Treatment with the selective CRF_1 agonist, Stressin 1, led to enhanced secretory activity, providing additional evidence that CRF_1 mediates secretion in response to stress (61).

The Effect of Stress on Intestinal Permeability

The intestinal barrier acts as a physical, enzymatic, and immunologic barrier against the onslaught of antigenic compounds found in the gut lumen (6). The constant onslaught of antigenic compounds found in ingested food, an individual's microbiota, and potentially pathogenic microorganisms warrants the need for a regulatory mechanism between the internal and external environment (62). The intestinal barrier accomplishes this through its distinct structure that allows the selective passage of beneficial molecules, such as water and nutrients, while excluding potentially harmful substances found in the lumen.

The intestinal barrier is comprised of a single layer of columnar epithelial cells that are connected by junctional complexes, the adherens junctions and tight junctions (63). The adherens junctions maintain cell-cell contact and allow communication between cells while

the tight junctions regulate intestinal permeability to molecules between the paracellular space. Loss of barrier function leads to increased intestinal permeability whereby molecules that are normally restricted to the intestinal lumen enter the blood stream. Psychological stress has been shown to increase intestinal permeability, contributing to the pathophysiology of gastrointestinal disorders such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and bacterial gastroenteritis (6,64). Evidence of stress-induced changes in intestinal permeability has been gathered using both small and large animal models. Rodents subjected to restraint stress and cold, restraint stress exhibited increased jejunal and colonic permeability to mannitol, ^{51}Cr-EDTA, horseradish peroxidase (HRP), and the bacterial peptide, fMLP (56,59,65,66). In a porcine model of early weaning stress, intestinal permeability to 3H-mannitol was increased in piglets subjected to early weaning stress (15,67).

Not surprisingly, CRF has been implicated as the primary mediator of stress-induced intestinal permeability. Restraint stress-induced intestinal permeability was ameliorated by pretreatment with a CRF receptor antagonist, α-helical CRF (9-41) (59). Furthermore, increased intestinal permeability, similar to that induced by restraint stress, was seen *in vivo* in rats intraperitoneally injected with CRF and *ex vivo* when intestinal tissue was treated with CRF on the Ussing Chamber (59,68). These findings in rodent studies were supported in a large animal, porcine, model where *ex vivo* treatment of porcine jejunal tissue with CRF resulted in increased intestinal permeability to 4kD FITC-dextran and piglets pretreated with α-helical CRF (9-41) were protected from stress-induced increases in intestinal permeability (69,58). Studies using the porcine model of early weaning stress indicate that CRF_1 may be

the primary receptor subtype mediating increased intestinal permeability in response to stress; piglets subjected to early weaning stress had increased CRF_1 expression in intestinal tissues compared to later weaned controls (67).

Intestinal Mast Cells: Effector Cells in Stress-induced GI Dysfunction and Disease

Research has shown a link between acute and chronic psychological stress to a variety of diseases including obesity, heart disease, cancer, asthma, and gastrointestinal disorders (70,71,72,73,17). The gastrointestinal tract is highly sensitive to stress with alterations in motility, permeability, secretion, and visceral hypersensitive documented in animal and human studies (6). Not surprisingly, stress has been shown to play an important role in exacerbating the symptoms or inciting the onset of both functional and inflammatory gastrointestinal disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (74,75). Unfortunately, the precise mechanism contributing to the pathobiology of these diseases is yet to be elucidated.

Mast cells have been identified as an important cell type in regulating the physiologic response to stress in the GI tract and represent a critical link between the brain and gut (6). Mast cells are located adjacent to peripheral nerve endings and express receptors for neuropeptides, including substance P, neurotensin, and CRF (76,77,78,79,80,81). Animal models of stress have demonstrated that mast cell mediators are elevated in response to stress and the physiologic changes associated with stress are ameliorated by mast cell stabilization (81). Consequently, mast cell activation and subsequent release of selective mediators may represent the critical link between stress and gastrointestinal disease.

Mast Cell Role in the Brain-Gut Axis

The brain-gut axis involves the bi-directional communication between the central nervous system and the peripheral, enteric and autonomic, nervous systems whereby stress perception and experience are relayed to the GI tract (82). Mast cells act as the one of the primary effector cells of the brain-gut axis and are capable of releasing a wide array of mediators following activation by stress (82,6). As previously discussed, mast cells express receptors for the neuropeptide, CRF, which acts as the primary mediator of the stress response. Although mast cells are known to be activated by stress and express CRF receptors, little is known about mast cell-CRF signaling in the stress response. *In vitro* studies by Cao et al demonstrated that mast cells can be directly activated by CRF to release VEGF (7). Secretion of VEGF was CRF_1 dependent and mediated by increased cAMP production by adenylate cyclase.

Mast cells can be activated by other neurotransmitters that are released during the stress response including substance P, neurotensin, nerve growth factor, pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) (83). Substance P is an important neurotransmitter involved in the perception of pain through activation of the neurokinin-1 receptor (NK_1) (84). Electron micrograph images of rat jejunum reveal a close association between mast cells and substance P-positive nerves (85). Mast cell activation by substance P may occur through NK_1 signaling pathways at low concentrations (86) or independent of the receptor via direct activation of G proteins (87). The response to substance P is variable and may (88) or may not (89) induce degranulation.

In instances where degranulation occurs, histamine (90,91), serotonin (92), and prostanoids (93,94) are released.

Neurotensin is a neuromodulator and neurotransmitter with actions in the central nervous system and in peripheral tissues (95). Within the GI tract, neurotensin is primarily stored in the endocrine-like N cells of the intestinal mucosa and regulate physiologic and pathophysiologic processes such as ion secretion, motility, and inflammation (96,97). Neurotensin has tissue-specific effects and is known to inhibit motility and secretion in the stomach and small intestine (98,99) while enhancing motility and secretion in the large intestine (100,101,102). In a rodent model of immobilization stress, plasma levels of neurotensin were increased leading to colonic mucin release, prostaglandin E2 (103) release, and mast cell activation (104,105). Mast cells express the neurotensin receptor, NT-R, and activation by neurotensin leads to the release of histamine by a receptor dependent mechanism (106,107).

Nerve growth factor (NGF) is a neurotrophin involved in the maintenance of neuronal populations throughout the body (108,109). Central and peripheral levels of NGF are drastically altered during times of stress and vary by tissue (110,111). In a rodent model of early life stress and subsequent adult stress, nerve growth factor levels in the brain were reduced in maternally deprived rats subjected to forced swim and open field tests in adulthood (112). Dichotomously, in peripheral tissues of the colon, levels of nerve growth factor were increased in rats subjected to chronic stress, leading to increased visceral hypersensitivity (113). Stress-induced visceral hypersensitivity was ameliorated when rats were pre-treated with an anti-NGF antibody or an NGF receptor antagonist prior to stress.

Mast cells express the NGF receptor, tyrosine receptor (TrkA), and can be activated to induce neurite growth, a process that is NGF-dependent (114). NGF has been demonstrated to influence mast cell differentiation (115), size, number (116,117), and survival (118). Additionally, mast cells can synthesize NGF and participate in autocrine signaling that may contribute to their pathophysiologic role in stress-related diseases (119).

Mast Cell Activation during the Stress Response

It is evident that mast cell function can be altered by neurotransmitters released in response to stress; however, the exact mechanism remains unknown. Animal models indicate that mast cells are profoundly influenced by psychological stress in a manner that contributes to the pathophysiology of notable GI diseases (6). Specifically, it has been demonstrated that psychological stress leads to mast cell activation and degranulation in the intestine (81,120). Santos et al found that rats subjected to water avoidance stress had increased number of mast cells and an increase in the proportion of activated mast cells (121). Furthermore, they identified mast cells as the critical regulator of stress-induced increases in colonic epithelial permeability. Wild type, but not mast cell-deficient rats, experienced significant increases in the flux of horseradish peroxidase. These findings were supported in a large animal, porcine model of early life stress in which piglets subjected to early weaning exhibited marked elevation in the mast cell protease, tryptase, as well as increased evidence of mast cell degranulation (67). Piglets treated 30 minutes prior to weaning with a mast cell stabilizer, cromolyn, were protected from stress-induced barrier dysfunction.

Mast Cells and Irritable Bowel Syndrome

Stress is believed to play an important role in the development of functional gastrointestinal disorders such as irritable bowel syndrome (IBS). IBS is a disease of unknown etiology with patients typically experiencing abdominal pain and disturbances in bowel habit (122). Susceptibility to this disease is believed to be dependent on genetic factors (123) although environmental factors and stress may be involved in altered gut function associated with IBS (124).

Altered motor function leading to diarrhea or constipation is reported in individuals with IBS (125). Psychological stress is known to alter gastrointestinal motility in human cases as well as in animal models, typically manifested by impaired gastric emptying and increased colonic motility (9,36,37). Alterations in intestinal motility are associated with mast cell activation and increased release of mast cell mediators, including proteases, prostaglandins, leukotrienes, and serotonin (5-HT) (126). Rats treated with mast cell degranulators exhibited increased motility that was antagonized by treatment with a serotonin antagonist as well as cyclooxygenase and lipoxygenase inhibitors (127). Additionally, PAR-2 activation has been shown to regulate GI motility (128). PAR-2 is a G-protein coupled receptor that becomes activated upon protease cleavage at an enzymatic site on the extracellular amino terminus (129). Mast cell tryptase can activate PAR-2 and studies on rate intestinal tissue indicates that mast cell tryptase can activate intestinal myocytes through PAR-2 signaling, a pathway that may contribute to motility disturbances (130).

Visceral hypersensitivity is reported in 21-65% of patients with IBS (131,132) and growing evidence indicates that mast cells play an important role in modulating this response

(133). Mast cell numbers are increased in the large intestine in animal models of visceral hypersensitivity and human patients display increased mast cell numbers in the small intestine (134), large intestine (135,136), and rectum (137). Mast cell activation and release of tryptase and histamine are elevated in jejunal fluid (134) and supernatants from colonic biopsies of patients with IBS (136,138). Barbara *et al* demonstrated that the number of mucosal mast cells and proximity to sensory nerves are positively correlated with abdominal pain (136) and mast cell mediators, including, tryptase and histamine, are known to activate enteric nerves, leading to neuronal hyperexitability (139). Additionally, supernatants from patients with IBS excited human submucosal neurons, an effect that was abrogated by a 5-HT_3 receptor antagonist, histamine receptor blockade, and protease inhibition (140). Treatment with the mast cell stabilizers, keitotifen and doxantrazole, alleviated visceral hypersensitivity and intestinal symptoms in patients with IBS (141) as well as stress-induced visceral hypersensitivity in rats (142).

Mast Cells and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), comprised of Crohn's disease and ulcerative colitis, is a debilitating intestinal disorder of unknown etiology. The susceptibility to the development of IBD and the recurrence of symptoms are believed to involve a combination of genetic predisposition, environmental factors, and an exaggerated immune response (143). Environmental factors, such as psychological stress, are thought to play an important role in the development of IBD as the penetrance in monozygotic twins is less than 50% in Crohn's disease and less than 20% in ulcerative colitis (144). Much research has focused on the

inflammatory response in models of IBD and in human subjects. Consequently, the intestinal epithelium and permeability to luminal contents in IBD has been heavily scrutinized and identified as a key factor in the pathophysiology of the disease.

The intestinal barrier functions to limit the exposure of potentially antigenic substances to the underlying tissue. Defects in intestinal permeability have been identified in uninflamed tissue from patients with Crohn's disease (145). As previously discussed, psychological stress can increase intestinal permeability, but the mechanism remains unknown. Mast cells are gaining recognition as a key mediator of stress-induced permeability and consequently, a critical effector cell in the development of the symptoms of IBD (6). Patients with IBD show an increase in mast cell activity and degranulation, leading to an exaggerated mast cell response that can intensify inflammation (146). Animal models of colitis demonstrate similar increases in mast cell number, mast cell activation and subsequent exaggerated inflammatory response (147,148). These studies demonstrate that mast cells contribute to chronic inflammation in the gut.

Animal models of stress-induced changes in intestinal permeability confirm the role of mast cells in regulating intestinal permeability. Research conducted in piglets connected alterations in intestinal permeability to stress-induced mast cell activation (67). Piglets subjected to early life stress displayed decreased small intestinal barrier function demonstrated by increased paracellular flux to mannitol when samples were mounted on Ussing chambers. This loss of barrier function was restored when piglets were treated with a mast cell stabilizer prior to weaning, indicating a role of mast cells in regulating stress-induced intestinal permeability. The effects of mast cell activation in a rat model of chronic

stress showed similar results to the piglet model of early life stress (121). Wild type and mast cell-deficient rats were subjected to water avoidance stress for five consecutive days. Tissue samples mounted on Ussing chambers from stressed rats showed an increase in colonic permeability to horseradish peroxidase compared to mast cell-deficient rats. Furthermore, chronic stress led to an increase in mucosal mast cells numbers and in the proportion of activated mast cells in colonic tissue compared to unstressed controls.

The mechanisms of mast cell-mediated increases in intestinal permeability are incompletely understood. Although mast cells release a variety products that can influence epithelial permeability under various physiologic and pathophysiologic circumstances, the exact mast cell mediators released upon stress have not been fully characterized. However, select mast cell mediators have been identified in contributing to changes in intestinal permeability. DeMaude et al demonstrated in a mouse model of acute stress that mast cell products, specifically IFNγ, impair colonocyte differentiation and reduces tight junction mRNA (149). Nerve growth factor released by mast cells was also shown to alter intestinal permeability in rats (150). Rats subjected to early life stress by neonatal maternal deprivation experienced increased gut permeability following exogenous treatment with CRF in adulthood. This response was ameliorated by pretreatment with the mast cell stabilizer, doxantrazole, as well as by treatment with anti-NGF.

Mast cell tryptase has been identified as a mediator that controls intestinal permeability with elevations in mucosal tryptase demonstrated in piglets that experienced intestinal barrier function in response to early weaning stress (67). Jacob et al utilized *in vitro* colonocyte studies to determine the effects of mast cell tryptase on colonocyte function

and PAR-2 activation (151). Colonocytes that were treated with mast cell supernatants exhibited increased paracellular permeability to macromolecules and redistribution in tight junction proteins. When mast cells were co-cultured with colonoytes, mast cell degranulation increased paracellular permeability, an effect that was abolished by tryptase inhibition.

Conclusion

Physical and psychological stressors have a dramatic impact on gastrointestinal physiology as evidenced by human and animal models where secretion, intestinal barrier function, and motility are altered (6). These changes in normal, homeostatic GI function can contribute to the onset and exacerbation of notable GI disease. It is evident that neuroendocrine signaling within the gut, mediated by CRF, plays a critical role in modulating GI function in both health and disease but the mechanism is yet to be elucidated. Mast cells are emerging as an extremely versatile cell type capable of influencing both normal and pathological processes. Mast cells can be activated by neurotransmitters released during the stress response and mast cell mediators can alter GI function (152). The dysregulation of mast cell activation during the stress response represents a critical link between stress-induced changes in GI function. Animal models using mast cell stabilizers and subsequent amelioration of stress-induced changes in GI function indicate that mast cells are a promising therapeutic target. As more research into activation and regulation of mast cells during the stress response is performed, it is hoped that a better understanding of this unique cell type will lead to new treatments for a variety of gastrointestinal diseases.

References

1 Irani, A. A., Schechter, N. M., Craig, S. S., DeBlois, G. & Schwartz, L. B. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A* 83, 4464-4468, (1986)

.

2 Shanahan, F., Denburg, J. A., Bienenstock, J. & Befus, A. D. Mast cell heterogeneity. *Can J Physiol Pharmacol* 62, 734-737, (1984).

3 Kitamura, Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu Rev Immunol* 7, 59-76, (1989).

4 Abraham, S. N. & St John, A. L. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol* 10, 440-452.

5 Bischoff, S. C. Physiological and pathophysiological functions of intestinal mast cells. *Semin Immunopathol* 31, 185-205, (2009).

6 Soderholm, J. D. & Perdue, M. H. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 280, G7-G13, (2001).

7 Cao, J. *et al.* Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. *J Immunol* 174, 7665-7675, (2005).

8 van der Kleij, H. P. *et al.* Functional expression of neurokinin 1 receptors on mast cells induced by IL-4 and stem cell factor. *J Immunol* 171, 2074-2079, (2003).

9 Larauche, M., Kiank, C. & Tache, Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 60 Suppl 7, 33-46, (2009).

10 Chrousos, G. P. Stress, chronic inflammation, and emotional and physical well-being: concurrent effects and chronic sequelae. *J Allergy Clin Immunol* 106, S275-291, (2000).

11 Konturek, P. C., Brzozowski, T. & Konturek, S. J. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol* 62, 591-599.

12 Lytinas, M. *et al.* Acute stress results in skin corticotropin-releasing hormone secretion, mast cell activation and vascular permeability, an effect mimicked by intradermal corticotropin-releasing hormone and inhibited by histamine-1 receptor antagonists. *Int Arch Allergy Immunol* 130, 224-231, (2003).

13 Cao, J., Boucher, W., Kempuraj, D., Donelan, J. M. & Theoharides, T. C. Acute stress and intravesical corticotropin-releasing hormone induces mast cell dependent vascular endothelial growth factor release from mouse bladder explants. *J Urol* 176, 1208-1213, (2006).

14 Kawahito, Y. *et al.* Local secretion of corticotropin-releasing hormone by enterochromaffin cells in human colon. *Gastroenterology* 106, 859-865, (1994).

15 Smith, F. *et al.* Early weaning stress impairs development of mucosal barrier function in the porcine intestine. *Am J Physiol Gastrointest Liver Physiol* 298, G352-363.

16 O'Connor, T. M., O'Halloran, D. J. & Shanahan, F. The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia. *QJM* 93, 323-333, (2000).

17 Bhatia, V. & Tandon, R. K. Stress and the gastrointestinal tract. *J Gastroenterol Hepatol* 20, 332-339, (2005).

18 Vale, W., Spiess, J., Rivier, C. & Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213, 1394-1397, (1981).

19 Asakura, H., Zwain, I. H. & Yen, S. S. Expression of genes encoding corticotropin-releasing factor (CRF), type 1 CRF receptor, and CRF-binding protein and localization of the gene products in the human ovary. *J Clin Endocrinol Metab* 82, 2720-2725, (1997).

20 Ekman, R. *et al.* Biosynthesis of corticotropin-releasing hormone in human T-lymphocytes. *J Neuroimmunol* 44, 7-13, (1993).

21 Liu, S. *et al.* Distribution and chemical coding of corticotropin-releasing factor-immunoreactive neurons in the guinea pig enteric nervous system. *J Comp Neurol* 494, 63-74, (2006).

22 Yuan, P. Q., Wu, S. V., Wang, L. & Tache, Y. Corticotropin releasing factor in the rat colon: expression, localization and upregulation by endotoxin. *Peptides* 31, 322-331.

23 Chatzaki, E. *et al.* CRF receptor type 1 and 2 expression and anatomical distribution in the rat colon. *J Neurochem* 90, 309-316, (2004).

24 Porcher, C., Juhem, A., Peinnequin, A., Sinniger, V. & Bonaz, B. Expression and effects of metabotropic CRF1 and CRF2 receptors in rat small intestine. *Am J Physiol Gastrointest Liver Physiol* 288, G1091-1103, (2005).

25 Bale, T. L. & Vale, W. W. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* 44, 525-557, (2004).

26 Perrin, M. *et al.* Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proc Natl Acad Sci U S A* 92, 2969-2973, (1995).

27 Grammatopoulos, D. K., Randeva, H. S., Levine, M. A., Kanellopoulou, K. A. & Hillhouse, E. W. Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. *J Neurochem* 76, 509-519, (2001).

28 Blank, T. *et al.* Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J Neurosci* 23, 700-707, (2003).

29 Reyes, T. M. *et al.* Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A* 98, 2843-2848, (2001).

30 Hsu, S. Y. & Hsueh, A. J. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat Med* 7, 605-611, (2001).

31 Lewis, K. *et al.* Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A* 98, 7570-7575, (2001).

32 Vaughan, J. *et al.* Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378, 287-292, (1995).

33 Nozu, T., Martinez, V., Rivier, J. & Tache, Y. Peripheral urocortin delays gastric emptying: role of CRF receptor 2. *Am J Physiol* 276, G867-874, (1999).

34 Million, M. *et al.* Human urocortin II, a new CRF-related peptide, displays selective CRF(2)-mediated action on gastric transit in rats. *Am J Physiol Gastrointest Liver Physiol* 282, G34-40, (2002).

35 Martinez, V., Wang, L., Million, M., Rivier, J. & Tache, Y. Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides* 25, 1733-1744, (2004).

36 Rao, S. S., Hatfield, R. A., Suls, J. M. & Chamberlain, M. J. Psychological and physical stress induce differential effects on human colonic motility. *Am J Gastroenterol* 93, 985-990, (1998).

37 Stengel, A. & Tache, Y. Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. *Annu Rev Physiol* 71, 219-239, (2009).

38 Gue, M., Peeters, T., Depoortere, I., Vantrappen, G. & Bueno, L. Stress-induced changes in gastric emptying, postprandial motility, and plasma gut hormone levels in dogs. *Gastroenterology* 97, 1101-1107, (1989).

39 Nakade, Y. *et al.* Restraint stress delays solid gastric emptying via a central CRF and peripheral sympathetic neuron in rats. *Am J Physiol Regul Integr Comp Physiol* 288, R427-432, (2005).

40 Tache, Y., Maeda-Hagiwara, M. & Turkelson, C. M. Central nervous system action of corticotropin-releasing factor to inhibit gastric emptying in rats. *Am J Physiol* 253, G241-245, (1987).

41 Fukudo, S. & Suzuki, J. Colonic motility, autonomic function, and gastrointestinal hormones under psychological stress on irritable bowel syndrome. *Tohoku J Exp Med* 151, 373-385, (1987).

42 Maillot, C., Million, M., Wei, J. Y., Gauthier, A. & Tache, Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* 119, 1569-1579, (2000).

43 Martinez, V., Rivier, J. & Tache, Y. Peripheral injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks peripheral CRF- and abdominal surgery-induced delayed gastric emptying in rats. *J Pharmacol Exp Ther* 290, 629-634, (1999).

44 Gomez, F., Manalo, S. & Dallman, M. F. Androgen-sensitive changes in regulation of restraint-induced adrenocorticotropin secretion between early and late puberty in male rats. *Endocrinology* 145, 59-70, (2004).

45 Lenz, H. J., Raedler, A., Greten, H., Vale, W. W. & Rivier, J. E. Stress-induced gastrointestinal secretory and motor responses in rats are mediated by endogenous corticotropin-releasing factor. *Gastroenterology* 95, 1510-1517, (1988).

46 Fukudo, S., Nomura, T. & Hongo, M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. *Gut* 42, 845-849, (1998).

47 Gue, M., Junien, J. L. & Bueno, L. Conditioned emotional response in rats enhances colonic motility through the central release of corticotropin-releasing factor. *Gastroenterology* 100, 964-970, (1991).

48 Martinez, V. & Tache, Y. Role of CRF receptor 1 in central CRF-induced stimulation of colonic propulsion in rats. *Brain Res* 893, 29-35, (2001).

49 Martinez, V., Wang, L., Rivier, J., Grigoriadis, D. & Tache, Y. Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J Physiol* 556, 221-234, (2004).

50 Martinez, V., Wang, L., Rivier, J. E., Vale, W. & Tache, Y. Differential actions of peripheral corticotropin-releasing factor (CRF), urocortin II, and urocortin III on gastric emptying and colonic transit in mice: role of CRF receptor subtypes 1 and 2. *J Pharmacol Exp Ther* 301, 611-617, (2002).

51 Wang, L., Martinez, V., Rivier, J. E. & Tache, Y. Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. *Am J Physiol Regul Integr Comp Physiol* 281, R1401-1410, (2001).

52 Barrett, K. E. & Keely, S. J. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* 62, 535-572, (2000).

53 Barclay, G. R. & Turnberg, L. A. Effect of psychological stress on salt and water transport in the human jejunum. *Gastroenterology* 93, 91-97, (1987).

54 Barclay, G. R. & Turnberg, L. A. Effect of cold-induced pain on salt and water transport in the human jejunum. *Gastroenterology* 94, 994-998, (1988).

55 Barclay, G. R. & Turnberg, L. A. Effect of moderate exercise on salt and water transport in the human jejunum. *Gut* 29, 816-820, (1988).

56 Saunders, P. R., Kosecka, U., McKay, D. M. & Perdue, M. H. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am J Physiol* 267, G794-799, (1994).

57 Empey, L. R. & Fedorak, R. N. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins Leukot Essent Fatty Acids* 38, 43-48, (1989).

58 Moeser, A. J. *et al.* Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *Am J Physiol Gastrointest Liver Physiol* 292, G173-181, (2007).

59 Santos, J. *et al.* Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. *Am J Physiol* 277, G391-399, (1999).

60 Saunders, P. R., Maillot, C., Million, M. & Tache, Y. Peripheral corticotropin-releasing factor induces diarrhea in rats: role of CRF1 receptor in fecal watery excretion. *Eur J Pharmacol* 435, 231-235, (2002).

61 Teitelbaum, A. A., Gareau, M. G., Jury, J., Yang, P. C. & Perdue, M. H. Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. *Am J Physiol Gastrointest Liver Physiol* 295, G452-459, (2008).

62 Keita, A. V. & Soderholm, J. D. The intestinal barrier and its regulation by neuroimmune factors. *Neurogastroenterol Motil* 22, 718-733.

63 Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 9, 799-809, (2009).

64 Cameron, H. L. & Perdue, M. H. Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. *J Pharmacol Exp Ther* 314, 214-220, (2005).

65 Kiliaan, A. J. *et al.* Stress stimulates transepithelial macromolecular uptake in rat jejunum. *Am J Physiol* 275, G1037-1044, (1998).

66 Zhao, J. *et al.* A protease inhibitor against acute stress-induced visceral hypersensitivity and paracellular permeability in rats. *Eur J Pharmacol* 654, 289-294.

67 Moeser, A. J., Ryan, K. A., Nighot, P. K. & Blikslager, A. T. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *Am J Physiol Gastrointest Liver Physiol* 293, G413-421, (2007).

68 Santos, J. *et al.* Stress neuropeptides evoke epithelial responses via mast cell activation in the rat colon. *Psychoneuroendocrinology* 33, 1248-1256, (2008).

69 Overman, E. L., Rivier, J. E. & Moeser, A. J. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. *PLoS One* 7, e39935.

70 Koch, F. S., Sepa, A. & Ludvigsson, J. Psychological stress and obesity. *J Pediatr* 153, 839-844, (2008).

71 Rozanski, A., Blumenthal, J. A. & Kaplan, J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy. *Circulation* 99, 2192-2217, (1999).

72 Thaker, P. H. *et al.* Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 12, 939-944, (2006).

73 Sandberg, S. *et al.* Positive experiences and the relationship between stress and asthma in children. *Acta Paediatr* 91, 152-158, (2002).

74 Suarez, K., Mayer, C., Ehlert, U. & Nater, U. M. Psychological stress and self-reported functional gastrointestinal disorders. *J Nerv Ment Dis* 198, 226-229.

75 Mawdsley, J. E. & Rampton, D. S. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* 54, 1481-1491, (2005).

76 Spanos, C. *et al.* Stress-induced bladder mast cell activation: implications for interstitial cystitis. *J Urol* 157, 669-672, (1997).

77 Theoharides, T. C. *et al.* Corticotropin-releasing hormone induces skin mast cell degranulation and increased vascular permeability, a possible explanation for its proinflammatory effects. *Endocrinology* 139, 403-413, (1998).

78 Skofitsch, G., Savitt, J. M. & Jacobowitz, D. M. Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and mesentery. *Histochemistry* 82, 5-8, (1985).

79 Fewtrell, C. M. *et al.* The effects of substance P on histamine and 5-hydroxytryptamine release in the rat. *J Physiol* 330, 393-411, (1982).

80 Alexacos, N. *et al.* Neurotensin mediates rat bladder mast cell degranulation triggered by acute psychological stress. *Urology* 53, 1035-1040, (1999).

81 Castagliuolo, I. *et al.* Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am J Physiol* 271, G884-892, (1996).

82 Farhadi, A., Fields, J. Z. & Keshavarzian, A. Mucosal mast cells are pivotal elements in inflammatory bowel disease that connect the dots: stress, intestinal hyperpermeability and inflammation. *World J Gastroenterol* 13, 3027-3030, (2007).

83 Theoharides, T. C. & Cochrane, D. E. Critical role of mast cells in inflammatory diseases and the effect of acute stress. *J Neuroimmunol* 146, 1-12, (2004).

84 De Felipe, C. *et al.* Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* 392, 394-397, (1998).

85 Stead, R. H. *et al.* Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. *Proc Natl Acad Sci U S A* 84, 2975-2979, (1987).

86 Krumins, S. A. & Broomfield, C. A. Evidence of NK1 and NK2 tachykinin receptors and their involvement in histamine release in a murine mast cell line. *Neuropeptides* 21, 65-72, (1992).

87 Columbo, M., Horowitz, E. M., Kagey-Sobotka, A. & Lichtenstein, L. M. Substance P activates the release of histamine from human skin mast cells through a pertussis toxin-sensitive and protein kinase C-dependent mechanism. *Clin Immunol Immunopathol* 81, 68-73, (1996).

88 Li, W. W. *et al.* Substance P signaling controls mast cell activation, degranulation, and nociceptive sensitization in a rat fracture model of complex regional pain syndrome. *Anesthesiology* 116, 882-895.

89 Janiszewski, J., Bienenstock, J. & Blennerhassett, M. G. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. *Am J Physiol* 267, C138-145, (1994).

90 Hagermark, O., Hokfelt, T. & Pernow, B. Flare and itch induced by substance P in human skin. *J Invest Dermatol* 71, 233-235, (1978).

91 Maggi, C. A. The effects of tachykinins on inflammatory and immune cells. *Regul Pept* 70, 75-90, (1997).

92 Lam, F. Y. & Ferrell, W. R. Mediators of substance P-induced inflammation in the rat knee joint. *Agents Actions* 31, 298-307, (1990).

93 Jacques, L., Couture, R., Drapeau, G. & Regoli, D. Capillary permeability induced by intravenous neurokinins. Receptor characterization and mechanism of action. *Naunyn Schmiedebergs Arch Pharmacol* 340, 170-179, (1989).

94 Abelli, L. *et al.* Microvascular leakage induced by substance P in rat urinary bladder: involvement of cyclo-oxygenase metabolites of arachidonic acid. *J Auton Pharmacol* 12, 269-276, (1992).

95 Vincent, J. P., Mazella, J. & Kitabgi, P. Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* 20, 302-309, (1999).

96 Kitabgi, P., Kwan, C. Y., Fox, J. E. & Vincent, J. P. Characterization of neurotensin binding to rat gastric smooth muscle receptor sites. *Peptides* 5, 917-923, (1984).

97 Zhao, D., Pothoulakis, C. & Abba, J. K. in *Handbook of Biologically Active Peptides* 1085-1090 (Academic Press, 2006).

98 Thor, K. & Rokaeus, A. Antroduodenal motor response induced by (Gln4)-neurotensin in man. *Acta Physiol Scand* 118, 369-372, (1983).

99 Read, N. W. *et al.* Effect of infusion of nutrient solutions into the ileum on gastrointestinal transit and plasma levels of neurotensin and enteroglucagon. *Gastroenterology* 86, 274-280, (1984).

100 Bueno, L., Fioramonti, J., Fargeas, M. J. & Primi, M. P. Neurotensin: a central neuromodulator of gastrointestinal motility in the dog. *Am J Physiol* 248, G15-19, (1985).

101 Calam, J., Unwin, R. & Peart, W. S. Neurotensin stimulates defaecation. *Lancet* 1, 737-738, (1983).

102 Brown, D. R. & Treder, B. G. Neurohormonal regulation of ion transport in the porcine distal jejunum. Actions of neurotensin and its natural homologs. *J Pharmacol Exp Ther* 249, 348-357, (1989).

103 Pothoulakis, C., Castagliuolo, I. & Leeman, S. E. Neuroimmune mechanisms of intestinal responses to stress. Role of corticotropin-releasing factor and neurotensin. *Ann N Y Acad Sci* 840, 635-648, (1998).

104 Carraway, R. *et al.* Neurotensin stimulates exocytotic histamine secretion from rat mast cells and elevates plasma histamine levels. *J Physiol* 323, 403-414, (1982).

105 Castagliuolo, I. *et al.* Colonic mucin release in response to immobilization stress is mast cell dependent. *Am J Physiol* 274, G1094-1100, (1998).

106 Barrocas, A. M., Cochrane, D. E., Carraway, R. E. & Feldberg, R. S. Neurotensin stimulation of mast cell secretion is receptor-mediated, pertussis-toxin sensitive and requires activation of phospholipase C. *Immunopharmacology* 41, 131-137, (1999).

107 Cochrane, D. E. Peptide regulation of mast-cell function. *Prog Med Chem* 27, 143-188, (1990).

108 Levi-Montalcini, R. The nerve growth factor 35 years later. *Science* 237, 1154-1162, (1987).

109 Lindsay, R. M. & Harmar, A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* 337, 362-364, (1989).

110 Alleva, E., Aloe, L. & Bigi, S. An updated role for nerve growth factor in neurobehavioural regulation of adult vertebrates. *Rev Neurosci* 4, 41-62, (1993).

111 Aloe, L., Alleva, E. & Fiore, M. Stress and nerve growth factor: findings in animal models and humans. *Pharmacol Biochem Behav* 73, 159-166, (2002).

112 Reus, G. Z. *et al.* Maternal deprivation induces depressive-like behaviour and alters neurotrophin levels in the rat brain. *Neurochem Res* 36, 460-466.

113 Winston, J. H., Xu, G. Y. & Sarna, S. K. Adrenergic stimulation mediates visceral hypersensitivity to colorectal distension following heterotypic chronic stress. *Gastroenterology* 138, 294-304 e293.

114 Nilsson, G. *et al.* Human mast cells express functional TrkA and are a source of nerve growth factor. *Eur J Immunol* 27, 2295-2301, (1997).

115 Matsuda, H. *et al.* Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *J Exp Med* 174, 7-14, (1991).

116 Aloe, L. The effect of nerve growth factor and its antibody on mast cells in vivo. *J Neuroimmunol* 18, 1-12, (1988).

117 Barreau, F., Salvador-Cartier, C., Houdeau, E., Bueno, L. & Fioramonti, J. Long-term alterations of colonic nerve-mast cell interactions induced by neonatal maternal deprivation in rats. *Gut* 57, 582-590, (2008).

118 Kawamoto, K. *et al.* Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the trk proto-oncogene receptor. *Blood* 86, 4638-4644, (1995).

119 Leon, A. *et al.* Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci U S A* 91, 3739-3743, (1994).

120 Soderholm, J. D. *et al.* Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 123, 1099-1108, (2002).

121 Santos, J., Yang, P. C., Soderholm, J. D., Benjamin, M. & Perdue, M. H. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* 48, 630-636, (2001).

122 Longstreth, G. F. *et al.* Functional bowel disorders. *Gastroenterology* 130, 1480-1491, (2006).

123 Morris-Yates, A., Talley, N. J., Boyce, P. M., Nandurkar, S. & Andrews, G. Evidence of a genetic contribution to functional bowel disorder. *Am J Gastroenterol* 93, 1311-1317, (1998).

124 Matricon, J. *et al.* Review article: Associations between immune activation, intestinal permeability and the irritable bowel syndrome. *Aliment Pharmacol Ther* 36, 1009-1031.

125 Camilleri, M. *et al.* Prospective study of motor, sensory, psychologic, and autonomic functions in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 6, 772-781, (2008).

126 Santos, J., Guilarte, M., Alonso, C. & Malagelada, J. R. Pathogenesis of irritable bowel syndrome: the mast cell connection. *Scand J Gastroenterol* 40, 129-140, (2005).

127 Fargeas, M. J., Theodourou, V., Fioramonti, J. & Bueno, L. Relationship between mast cell degranulation and jejunal myoelectric alterations in intestinal anaphylaxis in rats. *Gastroenterology* 102, 157-162, (1992).

128 Zhao, A. & Shea-Donohue, T. PAR-2 agonists induce contraction of murine small intestine through neurokinin receptors. *Am J Physiol Gastrointest Liver Physiol* 285, G696-703, (2003).

129 Cottrell, G. S., Amadesi, S., Schmidlin, F. & Bunnett, N. Protease-activated receptor 2: activation, signalling and function. *Biochem Soc Trans* 31, 1191-1197, (2003).

130 Corvera, C. U. *et al.* Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest* 100, 1383-1393, (1997).

131 Mayer, E. A. *et al.* Functional GI disorders: from animal models to drug development. *Gut* 57, 384-404, (2008).

132 Camilleri, M. *et al.* Candidate genes and sensory functions in health and irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 295, G219-225, (2008).

133 Santos, J., Alonso, C., Guilarte, M., Vicario, M. & Malagelada, J. R. Targeting mast cells in the treatment of functional gastrointestinal disorders. *Curr Opin Pharmacol* 6, 541-546, (2006).

134 Guilarte, M. *et al.* Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut* 56, 203-209, (2007).

135 Piche, T. *et al.* Mast cells and cellularity of the colonic mucosa correlated with fatigue and depression in irritable bowel syndrome. *Gut* 57, 468-473, (2008).

136 Barbara, G. *et al.* Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 126, 693-702, (2004).

137 Park, J. H. *et al.* Mucosal mast cell counts correlate with visceral hypersensitivity in patients with diarrhea predominant irritable bowel syndrome. *J Gastroenterol Hepatol* 21, 71-78, (2006).

138 Cenac, N. *et al.* Role for protease activity in visceral pain in irritable bowel syndrome. *J Clin Invest* 117, 636-647, (2007).

139 Traver, E., Torres, R., de Mora, F. & Vergara, P. Mucosal mast cells mediate motor response induced by chronic oral exposure to ovalbumin in the rat gastrointestinal tract. *Neurogastroenterol Motil* 22, e34-43.

140 Buhner, S. *et al.* Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. *Gastroenterology* 137, 1425-1434, (2009).

141 Klooker, T. K. *et al.* The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome. *Gut* 59, 1213-1221.

142 Gue, M. *et al.* Stress-induced visceral hypersensitivity to rectal distension in rats: role of CRF and mast cells. *Neurogastroenterol Motil* 9, 271-279, (1997).

143 Xavier, R. J. & Podolsky, D. K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448, 427-434, (2007).

144 McGuckin, M. A., Eri, R., Simms, L. A., Florin, T. H. & Radford-Smith, G. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis* 15, 100-113, (2009).

145 Soderholm, J. D. *et al.* Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? *Gastroenterology* 117, 65-72, (1999).

146 Farhadi, A. *et al.* Reduced immunostaining for c-kit receptors in mucosal mast cells in inflammatory bowel disease. *J Gastroenterol Hepatol* 22, 2338-2343, (2007).

147 Iba, Y., Sugimoto, Y. & Kamei, C. Participation of mast cells in colitis inflammation induced by dextran sulfate sodium. *Methods Find Exp Clin Pharmacol* 24, 15-18, (2002).

148 Kleinschmidt, S., Harder, J., Nolte, I., Marsilio, S. & Hewicker-Trautwein, M. Chronic inflammatory and non-inflammatory diseases of the gastrointestinal tract in

cats: diagnostic advantages of full-thickness intestinal and extraintestinal biopsies. *J Feline Med Surg* 12, 97-103.

149 Demaude, J., Salvador-Cartier, C., Fioramonti, J., Ferrier, L. & Bueno, L. Phenotypic changes in colonocytes following acute stress or activation of mast cells in mice: implications for delayed epithelial barrier dysfunction. *Gut* 55, 655-661, (2006).

150 Barreau, F. *et al.* Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. *J Physiol* 580, 347-356, (2007).

151 Jacob, C. *et al.* Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 280, 31936-31948, (2005).

152 Caso, J. R., Leza, J. C. & Menchen, L. The effects of physical and psychological stress on the gastro-intestinal tract: lessons from animal models. *Curr Mol Med* 8, 299-312, (2008).

CHAPTER 2: MAST CELL CRF RECEPTOR 1 MEDIATES STRESS-INDUCED INTESTINAL PERMEABILITY AND ENHANCES MAST CELL DEGRANULATION

Abstract

Life stress and the onset of gastrointestinal diseases have a clear link; however, the precise mechanisms are poorly understood. Previous studies demonstrate that stress triggers intestinal barrier dysfunction via corticotropin-releasing factor (CRF) and intestinal mast cells. Mast cells express both CRF receptor subtypes (CRF_1 and CRF_2); yet, the functional role of mast cell-specific CRF receptors during the stress response remains unknown. Here we investigate the role of mast cell CRF_1 in stress-induced intestinal permeability and mast cell activation. Mast cell deficient mice (Wsh) repleted with WT or CRF_1 -/- bone marrow-derived mast cells (BMMCs) were subjected to 3 hours of restraint stress. Paracellular flux of FITC-dextran (FD4) was measured in Ussing Chambers as an index of intestinal permeability. Restraint stress increased FD4 flux ($p<0.05$) in Wsh mice repleted with WT BMMCs; however, Wsh mice repleted with CRF_1 -/- BMMCs did not exhibit any changes in FD4 flux in response to stress. To determine the differences in WT and CRF_1 -/- BMMCs that may influence intestinal permeability, mast cell degranulation pathways were studied in a mucosal mast cell line, rat basophil leukemia cells (RBL-2H3). RBL-2H3 cells were pretreated with a CRF_1 agonist or antagonist prior to stimulation, and beta-hexosaminidase release was measured as an indicator of degranulation. Blockade of CRF_1 prevented beta-hexosaminidase release; whereas, pretreatment with the CRF_1 agonist increased mast cell degranulation. These data demonstrate that CRF_1 promotes mast cell degranulation and stress-induced intestinal permeability, providing the basis for a novel preventative and therapeutic target for stress-related GI disorders.

Introduction

The physiological response to stressors, either physical or psychological, serves to maintain homeostasis and ultimately improve survival of the individual; however, an extended or dysregulated stress response may lead to profound consequences (1). Psychological stress is known to exacerbate or initiate symptoms of a variety of diseases including obesity, heart disease, cancer, asthma, and gastrointestinal disorders (2-7). The gastrointestinal tract has proven to be extremely susceptible to stress and significant changes in gut motility, secretion, absorption, visceral nociception, and epithelial permeability have been well documented in animal models of stress (8-10). Not surprisingly, human patients suffering from stress-related GI disorders, including Irritable Bowel Syndrome (IBS) and the Inflammatory Bowel Diseases (IBD), experience similar changes in gut function (11,12). Although there is a clear link between psychological stress, altered GI function, and GI disease, the exact mechanism mediating the pathophysiology of these diseases remains unknown.

The stress response is regulated by activation of the hypothalamic-pituitary-adrenal (HPA) axis; whereby, corticotropin-releasing factor (CRF) is released from the hypothalamus in response to a stressor (13). CRF may then stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and the subsequent downstream release of glucocorticoids from the adrenal cortex. CRF is a 41 amino acid peptide found centrally and peripherally that acts through two receptor subtypes, CRF_1 and CRF_2 (14-17). Signaling through CRF receptor subtypes leads to activation of adenylate cyclase through the Gs

subunit, although there is evidence to suggest that there may be tissue-specific coupling to additional G subunits (18,19).

CRF signaling during the stress response is believed to be the primary mediator of stress-related changes in GI function, leading to increased colonic motility, ion secretion, and intestinal permeability (20). Many of these changes have been attributed to CRF_1 signaling; however, the cell type mediating this response is yet to be elucidated (21,22-24). Mast cells have been identified as a critical effector cell in the intestinal stress response with many stress-induced changes in GI function being prevented by mast cell stabilization (25-28). Mast cells express functional CRF receptors, but a paucity of information is available on the role of these receptors in influencing mast cell function (29). Given that mast cells modulate GI function and express CRF_1, we hypothesized that mast cell CRF_1 regulates stress-induced intestinal permeability. Consequently, the objective of this study was to evaluate the specific role of mast cell CRF_1 in controlling mast cell activation and regulating intestinal permeability during the stress response.

Materials and Methods

Murine Animal Study Protocol. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. For murine studies, C57BL/6 (WT) mice, CRF_1 -/- mice, and mast cell deficient mice, $Kit^{Wsh\text{-}Wsh}$ (Wsh) were used. WT mice and Wsh mice were supplied by Jackson Laboratories. CRF_1 -/- mice were a gift from the Rivier lab (J.E.F. Rivier, The Salk Institute, La Jolla CA).

Restraint Stress Protocol. Mice subjected to restraint stress were placed in a transparent 50 mL plastic conical tube modified with air holes for the indicated time of 1, 3, or 6 hours. Control mice were left in their cages for equal time as stressed mice without food and water. Stressed and control mice were immediately sacrificed following restraint stress by CO_2 inhalation and colonic tissue was collected.

Bone Marrow Mast Cell Preparation. Bone marrow from femurs of 8-12 week old congenic WT mice and CRF_1 -/- mice were flushed out and grown in RPMI medium containing 10% FBS (Invitrogen, Calsbad, CA), penicillin and streptomycin (Invitrogen, Carlsbad, CA), 100X HEPES (Invitrogen, Carlsbad, CA), 100X nonessential amino acids (Invitrogen, Carlsbad, CA), 100 mM sodium pyruvate (Invitrogen), recombinant IL-3 (5 ng/ml; R&D Systems, Minneapolis, MN) and stem cell factor (5 ng/ml; R&D Systems, Minneapolis, MN) for a period of 4 weeks, at which time the resulting cell cultures are expected to be >98% MCs determined by toluidine blue staining and flow cytometry.

Mast Cell Repletion Experiments. For mast cell repletion of Wsh mice, $1x10^7$ WT or CRF_1 -/- bone marrow-derived mast cells (BMMCs) in sterile PBS were injected intraperitoneally into 4-6 week old mice. Repletion of the intestine was allowed to occur for 12-14 weeks at which time repleted mice were subjected to restraint stress or used as controls. Mast cell repletion was verified by postmortem histological analysis of intestinal tissue.

Murine Ussing Chamber Studies. Segments of proximal colon were harvested in oxygenated (95% O_2-5% CO_2) Ringer solution (in mmol: 109.8 NaCl, 5.3 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 2.4 Na_2HPO_4 and 0.4 NaH_2PO_4). Tissues were mounted in 0.3 cm^2 aperture Ussing chambers and bathed on the mucosal and serosal sides with 5 mL of Ringer solution. 10 mM glucose was added to the serosal side and osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solution was oxygenated (95% O_2-5% CO_2) and maintained at a temperature of 37 °C. Barrier function was determined by measuring transepithelial electrical resistance (TER, $\Omega.cm^2$) and mucosal permeability by mucosal-serosal FITC-Dextran (FD4) flux. TER data was collected for 90 minutes using 3% KCl agar electrodes (Physiologic Instruments. Acquire & Analyze). After a 15 minute equilibration period on the chambers, 5mg of FD4 was added to the mucosal side. Following an additional 15 minute equilibration period, samples from the serosal side were collected at 15 minute intervals in triplicate for a total of 60 minutes. The presence of FD4 fluorescence intensity of each sample was measured by fMax Fluorescence Microplate Reader (Molecular Devices) and concentrations were determined from standard curves generated by serial dilution of

FD4. Data for FD4 flux were presented as the average rate of FD4 flux over the one hour time period in ug/min.

Flow Cytometry. C-kit and FcƐRI expression of BMMCs were analyzed by flow cytometry. Cells were blocked with anti-Fcγ/RIIb/CD-16-2 (2.4G2; Santa Cruz Biotechnology, Santa Cruz, CA) then stained with PE-conjugated anti-CD117 (BD Pharmingen, San Jose, CA) and APC-conjugated anti- FcƐRI (eBioscience, San Diego, CA). Cells were analyzed using fluorescence-activated cell sorting (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) with data acquisition by Cellquest Pro.

Activation of Bone Marrow-Derived Mast Cells by A23187 and Tryptase Release. $1x10^5$ CRF_1 -/- BMMCs were plated in Tyrode's Buffer. Cells were treated with 500 ng of A21387 (Sigma Aldrich, St. Louis, MO) for 30 mins. Tryptase release was measured using the Thiobenzyl Ester Substrate Assay as previously described (30).

*Corticosterone Assay***.** Serum collected from mice subjected to 3 hours of restraint stress was analyzed for corticosterone levels using a rat and mouse radioimmunoassay (MP Biomedicals, Inc. Orangeburg, NY).

*CRF Intraperitoneal Injections***.** Wsh mice were repleted with WT or CRF_1 -/- BMMCs (as previously described). Mice were intraperitoneally injected with saline or 150 ug/kg of CRF at 12-14 weeks post-repletion. Sections of colon were collected at 4 hours post-injection and

mounted on the Ussing Chamber. Mucosal-to-serosal FD4 flux was monitored as a measure of intestinal permeability.

RBL-2H3 CRF Receptor Agonist and Antagonist Beta-hexosaminidase Assay. Rat basophil leukemia cells (RBL-2H3) were provided by the Abraham lab (S.N. Abraham, Duke University, Durham, NC). Cells were seeded at $5.0x10^4$ cells per well in a 96 well plate and incubated with 1.5 ug/ml anti-DNP IgE (Sigma Aldrich, St. Louis, MO) overnight. Cells were then washed, resuspended in Tyrode's buffer and pre-treated with CRF 1/2 receptor agonist (CRF) at the indicated concentrations (J.E.F. Rivier, The Salk Institute, La Jolla CA) or a CRF_1 antagonist (Antalarmin, Sigma Aldrich, St. Louis, MO) at the indicated concentrations for 10 mins. DNP-HSA was added at 30 ng/ml to induce degranulation. Control wells received an equal volume of Tyrode's buffer. After one hour, the β-hexosaminidase released into the supernatants and in cell lysates was quantified by hydrolysis of p-nitrophenyl *N*-acetyl-β-D-glucosamide (Sigma-Aldrich, St Louis, MO) in 0·1 M sodium citrate buffer (pH 4·5) for 60 min at 37°. The percentage of β-hexosaminidase release was calculated as a percentage of the total content.

Histology. Sections of murine colon were collected immediately after euthanasia and opened along the anti-mesenteric border. Tissue was frozen in OCT, cut in 5 uM sections, fixed in Carnoy's solution, and stained with toluidine blue.

Electron Microscopy. 1 x 10^6 BMMCs were fixed in McDowell's and Trump's 4F:1G fixative (31). After 2 rinses in 0.1 M sodium phosphate buffer (pH 7.2), samples were placed in 1% osmium tetroxide in the same buffer for 1 hr at room temperature. Samples were rinsed 2 times in distilled water and dehydrated in an ethanolic series culminating in two changes of 100% acetone. Tissues were then placed in a mixture of Spurr (32) resin and acetone (1:1) for 30 min, followed by 2 hr in 100% resin with 2 changes. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70ºC for 8 hrs to 3 days. Semi-thin (0.25-0.5 um) sections were cut with glass knives and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (70-90 nm) sections were cut with a diamond knife, stained with methanolic uranyl acetate followed by lead citrate and examined with a transmission electron microscope.

Immunofluorescent Staining and Confocal Analysis. RBL-2H3 cells and CRF_1 -/- cells were labeled with a goat anti-CRF_1 primary antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were subsequently stained using a secondary antibody, donkey anti-goat/FITC (1:1000; Jackson Immunoresearch, West Grove, PA). Nuclei were stained using TO-PRO3 iodide 642/661 (1:1000: Invitrogen, Carlsbad, CA). Confocal images were obtained with a 3-Laser Nikon Confocal Laser Scanning Instrument (Nikon Instruments). Images were obtained using EZ-C1 Nikon software (Silver Version 2.01).

Statistical Analyses. Data are reported as means ± SEM based on the experimental number (n). Data were analyzed by using a t-test or standard one-way ANOVA (Sigmastat, Jandel

Scientific, San Rafael, CA). A post hoc Fisher LSD was used to determine differences between treatments following one way ANOVA. Results were considered significant at $P < 0.05$.

Results

Restraint Stress Increases Intestinal Permeability and Serum Corticosterone Levels

Restraint stress is a commonly used murine model of psychological stress known to alter gastrointestinal function (33). To determine the optimal time required to induce changes in intestinal permeability, wild type (WT) mice were subjected to 1h, 3h, or 6h of restraint stress. Intestinal permeability was assessed by mucosal-serosal flux of FITC-dextran (FD4) through colonic tissue mounted on Ussing chambers. Mice subjected to 3h and 6h of restraint stress exhibited significant increases in intestinal permeability compared to unstressed, control mice (Fig. 1A). 3h of restraint stress induced the greatest change in colonic permeability, and histological analysis revealed that the intestinal epithelium was not altered in response to stress (Fig.1B). Consequently, subsequent experiments utilized 3h restraint stress.

Corticosterone is a hormone released by the adrenal cortex during the stress response, downstream of CRF signaling (34). To ensure that restraint stress activates the central stress response, serum corticosterone levels of control (unstressed) mice and mice subjected to restraint stress were analyzed. Restraint stress significantly increased serum corticosterone levels in WT mice (Fig. 2).

Intestinal Mast Cells Regulate Stress-induced Intestinal Permeability

Psychological stress is known to alter the intestinal barrier, but the mechanism remains unknown (35). Mast cells have been identified as an important mediator of gut function. To determine the role of mast cells in regulating intestinal permeability during

stress, mast cell-deficient mice (Wsh) and mast cell repletion experiments were performed. First, we demonstrated that mast cell deficiency did not affect the stress response. Wsh mice subjected to 3h of restraint stress displayed similar elevations in serum corticosterone compared to stressed, WT mice (Fig. 2). Wsh mice were repleted with WT bone marrow-derived mast cells (BMMCs) by intraperitoneal injection. To ensure that mast cell repletion was successful, intestinal tissue was collected after a 12 week period. Sections of ileum stained with toluidine blue demonstrated the presence of mast cells in intestinal tissue of repleted mice (Fig. 3).

To determine the role of mast cells in modulating stress-induced intestinal permeability, WT BMMC-repleted mice were subjected to 3h of restraint stress. Colonic tissue was collected and assessed for changes in barrier function by transepithelial electrical resistance (TER) and intestinal permeability to FD4. Restraint stress resulted in a significant decrease in TER in WT, Wsh, and Wsh repleted mice (Fig. 4A). Intestinal permeability to FD4 was unaffected in stressed Wsh mice; however, FD4 flux was increased in WT mice subjected to restraint stress (Fig. 4B). Increased FD4 flux was also exhibited in Wsh mice repleted with WT BMMCs.

Mast Cell CRF_1 Mediates Stress-induced Increases in Intestinal Permeability

We have previously demonstrated that intestinal CRF signaling mediates intestinal permeability in response to stress and that CRF_1 is upregulated during this response (36). Given these findings in combination with the results from Fig 4, we hypothesized that mast cell CRF_1 may be playing a critical role in regulating stress-induced increases in intestinal

permeability. To assess the role of mast cell CRF_1 in the stress response, we repleted Wsh mice with CRF_1 -/- BMMCs. We first demonstrated that rodent mast cells express CRF_1. A mucosal mast cell line, rat basophil leukemia cells (RBL-2H3), and WT BMMCs were stained with anti-CRF_1 (Fig. 5). Both RBL-2H3 cells and WT BMMCs displayed diffuse expression of CRF_1.

We then sought to characterize CRF_1 -/- BMMCs to ensure that the absence of CRF_1 would not affect their function. Toluidine blue staining (Fig, 6A) and electron micrographs (Fig. 6B) confirmed similar morphology between WT and CRF_1 -/- BMMCs. Both types of BMMCs displayed defined nuclei and prominent granules characteristic of mast cells. Expression of two common mast cell receptors, FcεRI and c-kit, were assessed by flow cytometry, demonstrating similar expression between WT and CRF_1 -/- BMMCs (Fig. 6B). Mast cell function was evaluated by calcium ionophore (A23187)- induced degranulation, as measured by tryptase release. WT and CRF_1 -/- BMMCs released similar levels of tryptase following stimulation by A23187 (Fig. 6D).

Given that WT and CRF_1 -/- BMMCs were phenotypically and functionally similar, we wanted to determine how the absence of mast cell CRF_1 signaling would influence intestinal permeability. Wsh mice repleted with WT BMMCs or with CRF_1 -/- BMMCs were subjected to 3h restraint stress and assessed for changes in intestinal permeability. Restraint stress resulted in a significant decrease in TER in mice repleted with WT or CRF_1 -/- BMMCs (Fig. 7A). Interestingly, intestinal permeability to FD4 was not increased in Wsh mice repleted with CRF_1 -/- BMMCs as it was in the case of WT BMMC-repleted mice (Fig. 7B). To further confirm that CRF signaling through the mast cell was responsible for

the changes in intestinal permeability, Wsh mice repleted with WT BMMCs or CRF_1 -/- BMMCs were intraperitoneally injected with CRF. WT BMMC-repleted mice treated with CRF displayed a similar increase in intestinal permeability to mice subjected to restraint stress (Fig. 8 and Fig. 7B). Additionally, CRF treatment of Wsh mice repleted with CRF_1 -/- BMMCs mimicked the results from restraint stress where absence of mast cell CRF_1 -/- prevented stress-induced increases in intestinal permeability (Fig. 8).

Mast cell CRF_1 enhances IgE-induced degranulation

To determine the effect of CRF_1 signaling on mast cell function, we evaluated mast cell degranulation using an *in vitro* model of mast cell activation in a rat mucosal mast cell line, RBL-2H3 cells. Mast cells were stimulated to degranulate by cross-linking of the FcεRI receptor via IgE binding. RBL-2H3 cells were treated with CRF (CRF1/2 agonist) or antalarmin (CRF_1 antagonist) prior to IgE crosslinking and assessed for degranulation, as measured by beta-hexosaminidase release. 10 nM CRF pretreatment of RBL-2H3 cells significantly increased beta-hex release compared to IgE treated cells (Fig. 9A). Conversely, blockade of CRF_1 by antalarmin (1 uM and 100 nM) decreased IgE-induced degranulation.

Discussion

The link between stress-related gastrointestinal disorders, such as Irritable Bowel Syndrome (IBS) and inflammatory bowel disease (IBD), and mast cell activation is becoming more apparent, yet the mechanism remains unknown (11,37-42). Activation of mast cells leads to the release of preformed and *de novo* synthesized mediators, a response that is dependent on the type of stimuli (43,44). During psychological stress and in response to stress peptides, it has been demonstrated that mast cells are activated to release mediators that increase intestinal permeability, an effect that is completely prevented by mast cell stabilization (25-28). Although it is known that mast cells express both CRF receptor subtypes, the role of the individual mast cell CRF receptors in regulating intestinal barrier function during the stress response is yet to be elucidated (29,45,46). By using a mouse model of restraint stress and mast cell repletion with CRF_1 -/- BMMCs, we were able to determine the role of mast cell CRF_1 in mediating stress-induced intestinal permeability. Further, we identified the influence of CRF_1 signaling on a mast cell degranulation pathway.

Restraint stress is a commonly used psychological stressor in rodent models (33). We confirmed that 3h of restraint stress was sufficient to induce a central stress response, measured by serum corticosterone levels, and elicit a peripheral pathophysiologic response in the intestine, as assessed by intestinal permeability to FD4. Mast cells have previously been demonstrated to increase intestinal permeability both by stress and by peripheral dosing with CRF, but much of that research has focused on the use of pharmacologic mast cell stabilization by doxantrazole and cromolyn that may have nonspecific effects (28,47,48,49). Additional studies comparing stress-induced intestinal permeability in wild type and mast

cell-deficient rodents confirm findings from mast cell stabilization, but may be confounded by compensatory mechanisms arising from mast cell deficiency (26,27). Here, we provide definitive evidence that mast cells are required for stress-induced increases in intestinal permeability in a mouse model that limits confounding or compensatory mechanisms. Mast cell-deficient (Wsh) mice repleted with WT BMMCs were compared to both WT and Wsh mice. Restraint stress induced a significant increase in intestinal permeability to FD4 in WT mice but not in Wsh mice. Stress-induced intestinal permeability was restored, to similar levels as WT mice, in Wsh mice repleted with WT BMMCs, indicating that mast cells are the primary cell type mediating intestinal permeability during the stress response. TER decreased in all mice subjected to restraint stress, suggesting that mast cell activation during stress has a specific effect on paracellular permeability to macromolecules but not on the permeability of ions.

Signaling through CRF_1 has been demonstrated to influence intestinal dysfunction through unclear mechanisms (21,22-24). To determine the role of mast cell CRF_1 in this response, we first confirmed the expression of CRF_1 on murine BMMCs. BMMCs from CRF_1 -/- mice were harvested and compared to WT BMMCs to ensure that mast cell morphology, phenotype, and function were not compromised as a result of CRF_1 deficiency, ensuring that all stress-induced intestinal changes were due to the absence of mast cell CRF_1 signaling and not an inherent difference between cell types. We utilized the mast cell repletion model with WT BMMCs and CRF_1 -/- BMMC and found that loss of mast cell CRF_1 signaling protected against stress-induced intestinal permeability. These data indicate that CRF_1 on the mast cell, specifically, mediates changes in intestinal permeability in

response to stress. TER was decreased in all stressed mice, indicating that permeability to macromolecules is regulated by mast cell CRF_1; however, permeability to ions is regulated by other mechanisms. Chronic peripheral administration of CRF elicits colonic permeability similar to that resulting from psychological stress, suggesting that CRF is the primary neuropeptide involved in intestinal permeability changes from stress (26). To confirm these findings in our model, WT BMMC- repleted and CRF_1 -/- BMMC-repleted Wsh mice were dosed with CRF by intraperitoneal injection. Intestinal permeability to FD4 was assessed and results mimicked those induced by restraint stress, providing evidence that CRF signaling through mast cell CRF_1 regulates stress-induced intestinal permeability.

It has been previously demonstrated that psychological stress activates mast cells to degranulate in the intestine, but is unclear whether this is via direct signaling through mast cell CRF receptors (25,50,48). To determine the role of mast cell CRF_1 in regulating mast cell degranulation, we utilized a well-studied mast cell degranulation pathway induced by IgE cross-linking of FcεRI in a mucosal mast cell line, RBL-2H3 cells. CRF binds to both receptors but with different binding affinities. Pretreatment with the lowest concentration of CRF (10 nM) led to a significant increase in mast cell degranulation. Furthermore, blockade of CRF_1 by the selective antagonist, antalarmin, impaired mast cell degranulation. These data indicate that mast cell CRF_1 enhances mast cell degranulation.

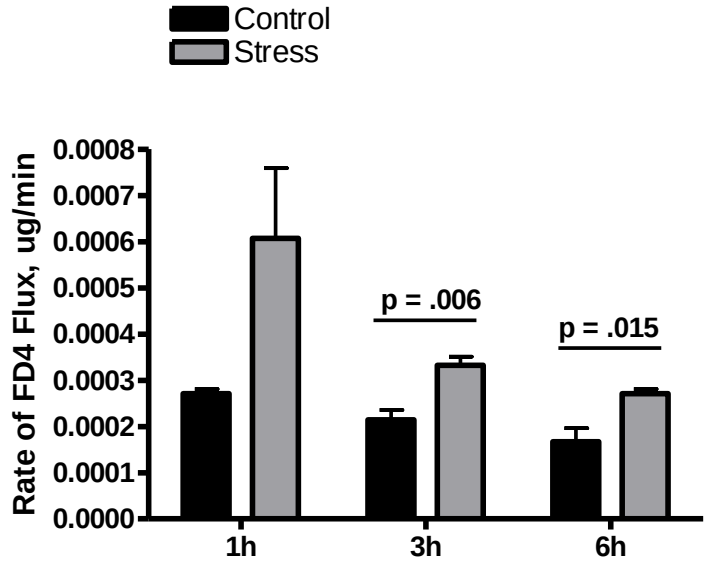
Overall these findings demonstrate that mast cell CRF_1 mediates stress-induced intestinal permeability and mast cell CRF_1 signaling promotes degranulation. CRF_1 regulation of mast cell degranulation may provide the link between stress, altered GI dysfunction, and the development of GI disease. Additional studies will need to be

performed to determine the mediators released through mast cell CRF_1 signaling that influence intestinal permeability, although previous research has demonstrated that mast cell tryptase and mast cell TNFα alter the intestinal barrier (51,49). Based on our findings, selective inhibition of mast cell CRF_1 may represent a novel preventative therapeutic target in the treatment of stress-related GI disease.

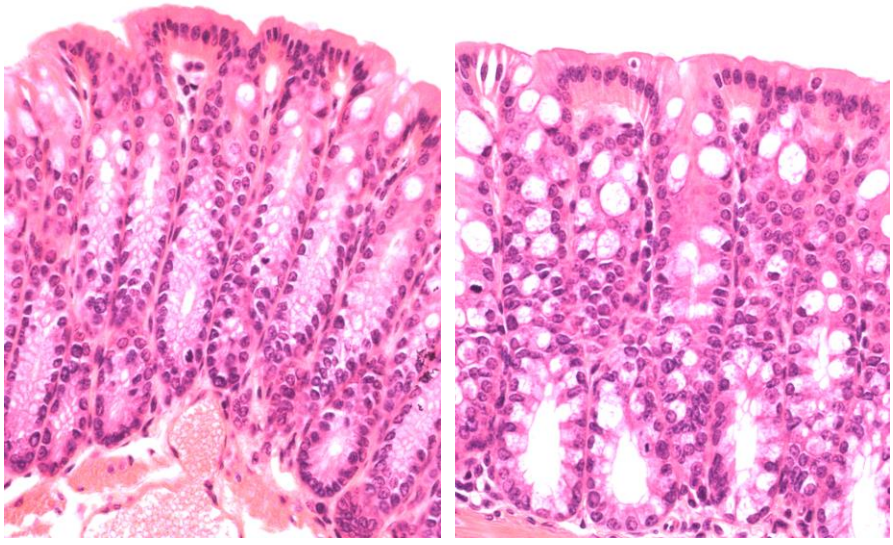
Figure 1. Restraint stress induces increased intestinal permeability to FD4.

Mice were subjected to 1h, 3h, or 6h of restraint stress. Colonic tissue was mounted an Ussing chambers. Intestinal permeability was assessed by mucosal-serosal flux of FD4. Values represent means ± SE for n = 4-6 mice/treatment. A) 3h and 6h or restraint stress resulted in a significant increase in FD4 flux compared to unstressed controls with the greatest change occurring after 3h of restraint stress. Data were analyzed using a *t*-test to compare differences between restraint stress time points and their respective controls. B) Colonic sections from 3h control and 3h restraint stressed mice were fixed in 10% formalin and stained with H&E. 3h restraint stress did not alter intestinal epithelium compared to 3h control.

A



B



3h Control 3h Stress

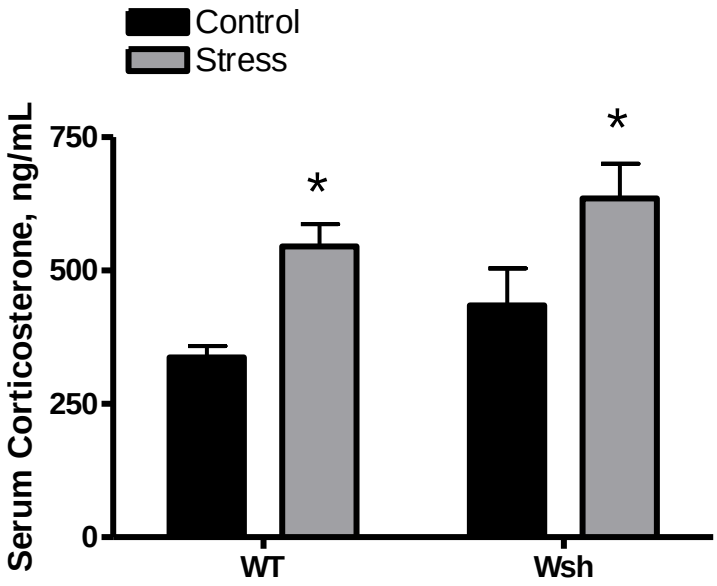


Figure 2. Serum corticosterone levels in wild type (WT) and mast cell-deficient mice (Wsh) subjected to 3 hours of restraint stress.

Values represent means ± SE for n=4-5 mice/treatment. Restraint stress induced elevations in serum corticosterone levels in both WT and Wsh mice. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbols (*) differ by $p<0.05$.

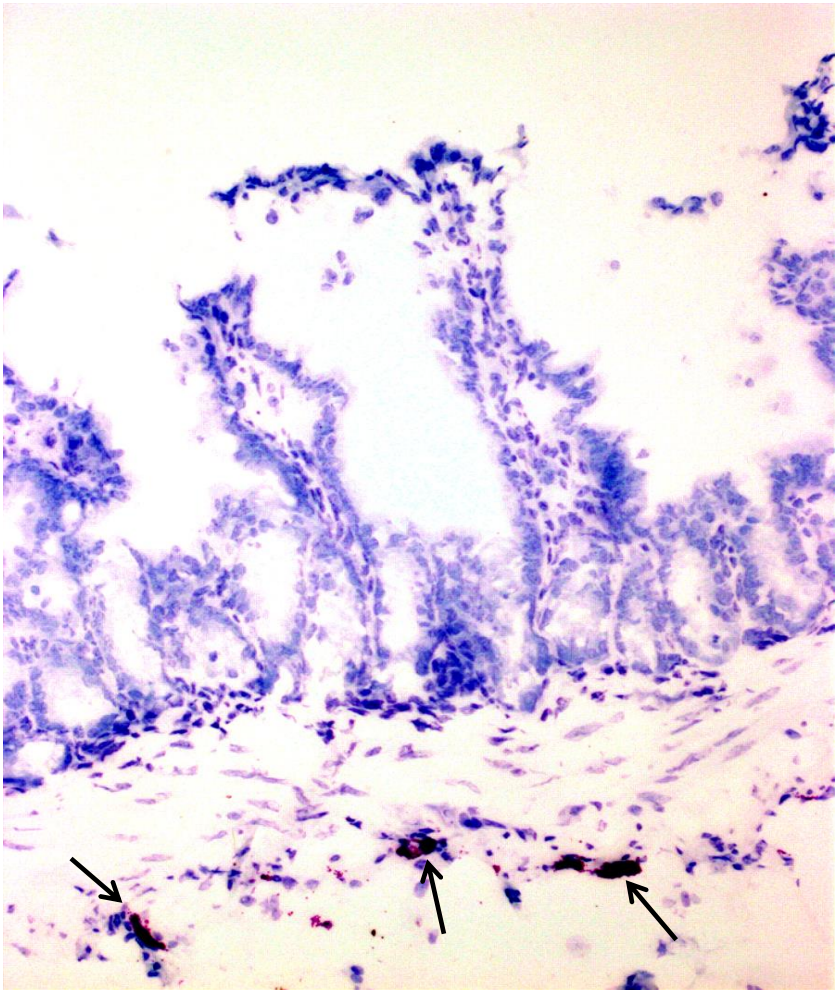
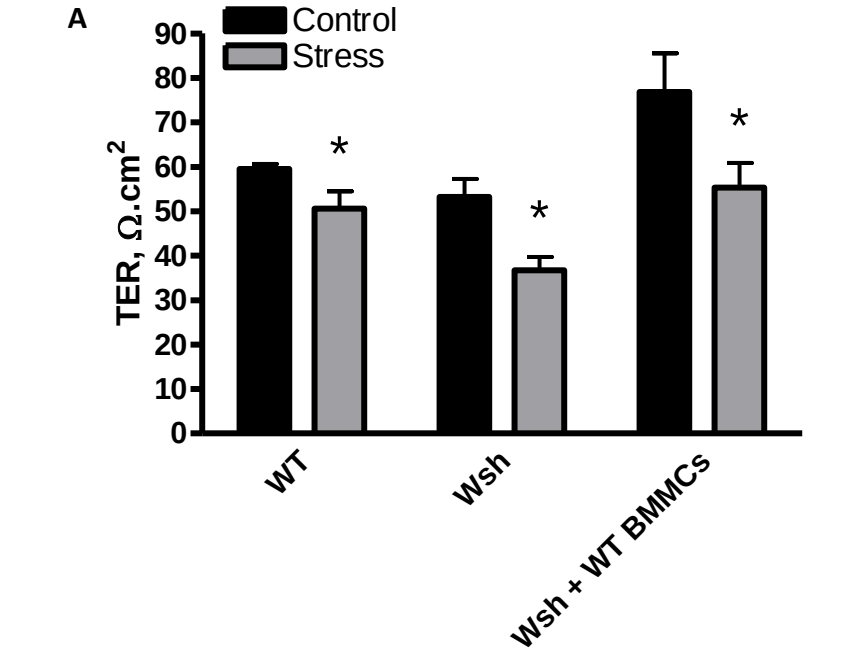
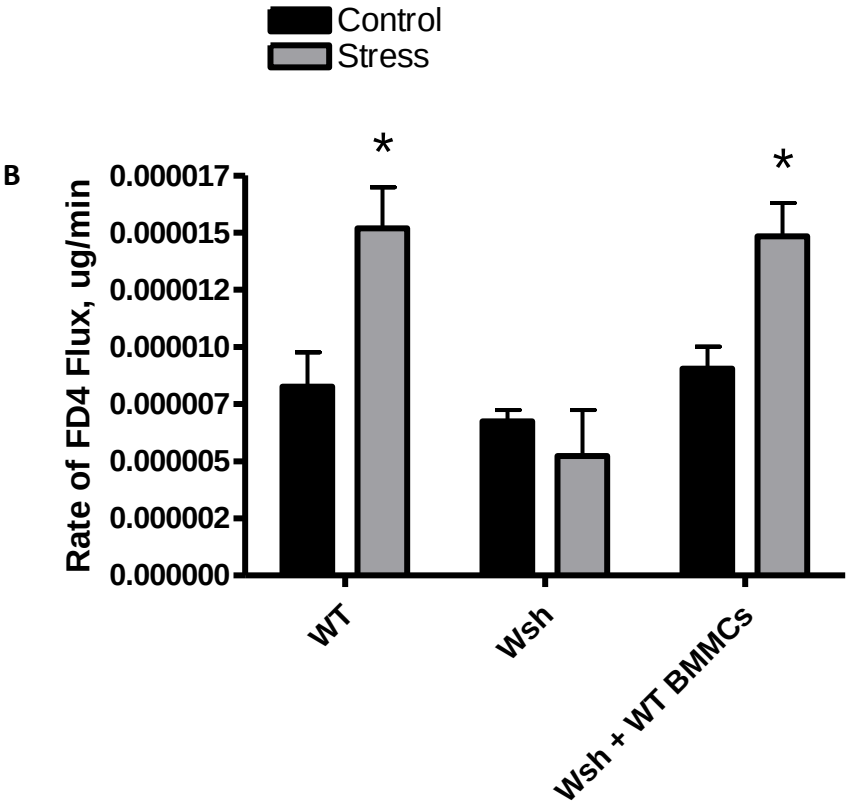


Figure 3. Histological analysis of ileal tissue from Wsh mice repleted with WT BMMCs. Toluidine blue staining of ileal tissue demonstrates successful repletion of Wsh mice with WT BMMCs, indicated by black arrows.

Figure 4. Intestinal mast cells mediate stress-induced increases in intestinal permeability to FD4.

WT, Wsh, and Wsh mice repleted with WT bone marrow-derived mast cells (BMMCs) were subjected to 3 h of restraint stress. Colonic tissue was mounted on Ussing chambers and assessed for changes in barrier function by transepithelial electrical resistance (TER) and intestinal permeability to FD4. Values represent means ± SE for n=4-5 mice/treatment. A) Restraint stress led to a decrease in TER in WT, Wsh, and Wsh + WT BMMCs. B) Restraint stress induced increases in intestinal permeability to FD4 in WT and Wsh mice repleted with WT BMMCs mice. Wsh mice were protected from stress-induced increases in FD4 flux. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbols (*) differ by $p<0.05$.


A
Control
Stress
TER, Ω.cm2
90
80
70
60
50
40
30
20
10
0
*
*
*
WT
Wsh
Wsh + WT BMMCs


Control
Stress
B
Rate of FD4 Flux, ug/min
0.000017
0.000015
0.000012
0.000010
0.000007
0.000005
0.000002
0.000000
*
*
WT
Wsh
Wsh + WT BMMCs

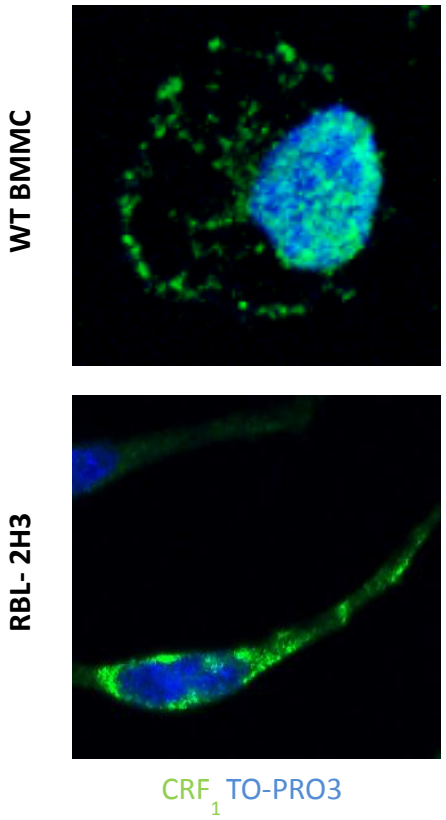
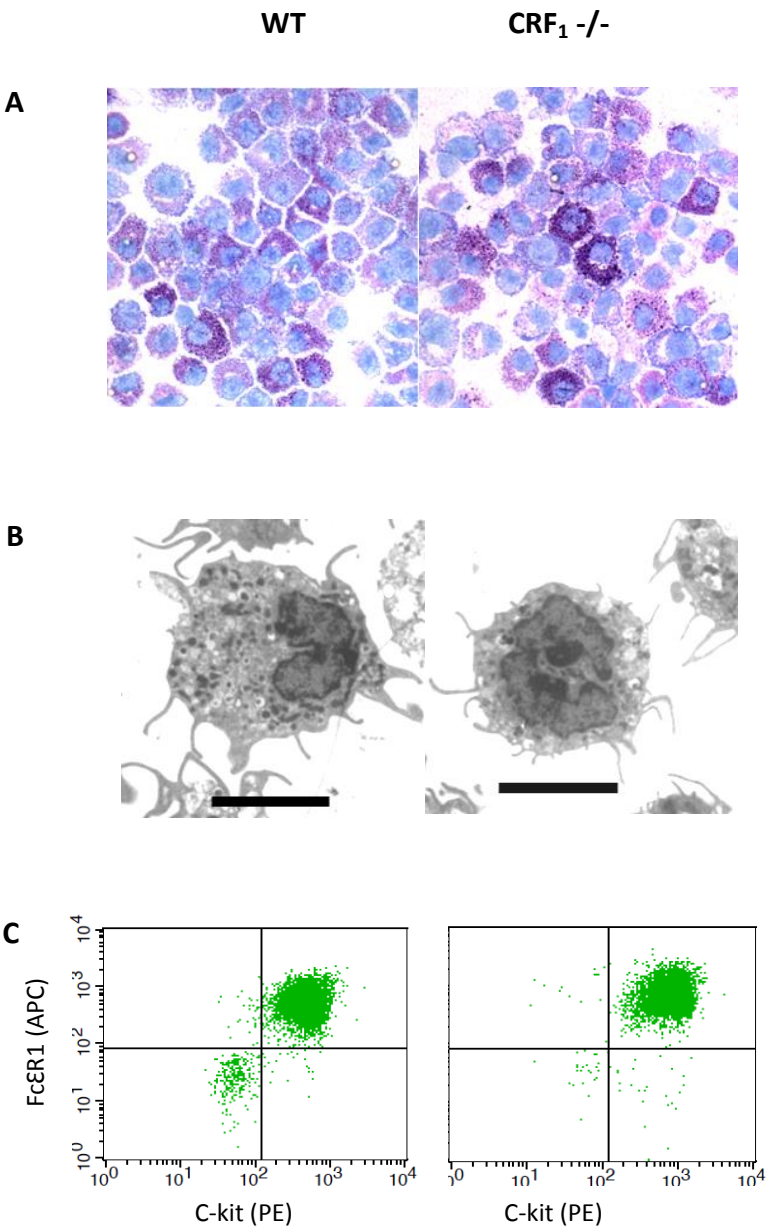


Figure 5. BMMCs and RBL-2H3 cells express CRF_1.

WT BMMCs and RBL-2H3 cells were stained for CRF_1 expression (green) and with a nuclear stain, TO-PRO3 (blue).

Figure 6. Histological and functional analysis of WT and CRF_1 -/- bone marrow-derived mast cells.

Wild type and CRF_1 -/- mast cells display similar morphologies as evidenced by A) Toluidine blue staining and B) electron microscopy. C) WT and CRF_1 -/- BMMCs express mast cell surface receptors FcεRI and c-kit. D) WT and CRF_1 -/- BMMCs release similar levels of tryptase following A23187 treatment. Values represent means ± SE for n = 3 wells/treatment. Tryptase release was assessed in 3 separate experiments. Data were analyzed using a *t*-test to compare differences between A23187 stimulated BMMCs and their respective controls. Symbols (*) differ by $p<0.05$.


WT
CRF1 -/-
A
B
C
FcεR1 (APC)
C-kit (PE)
C-kit (PE)

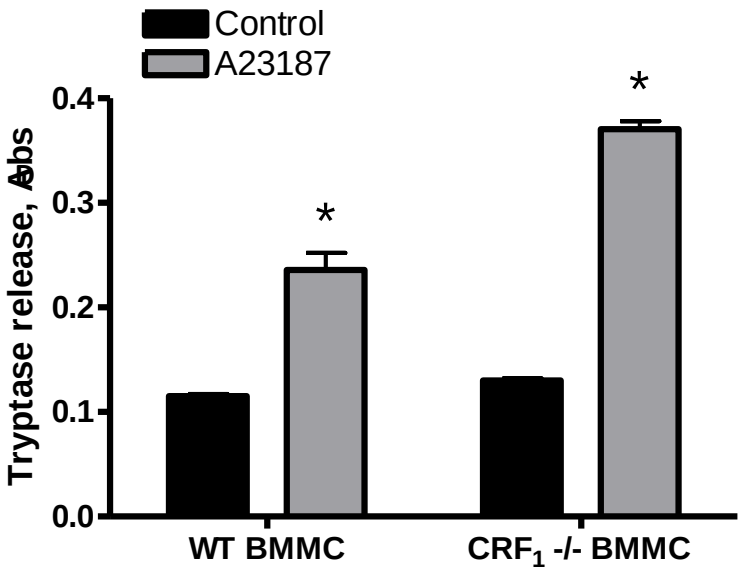
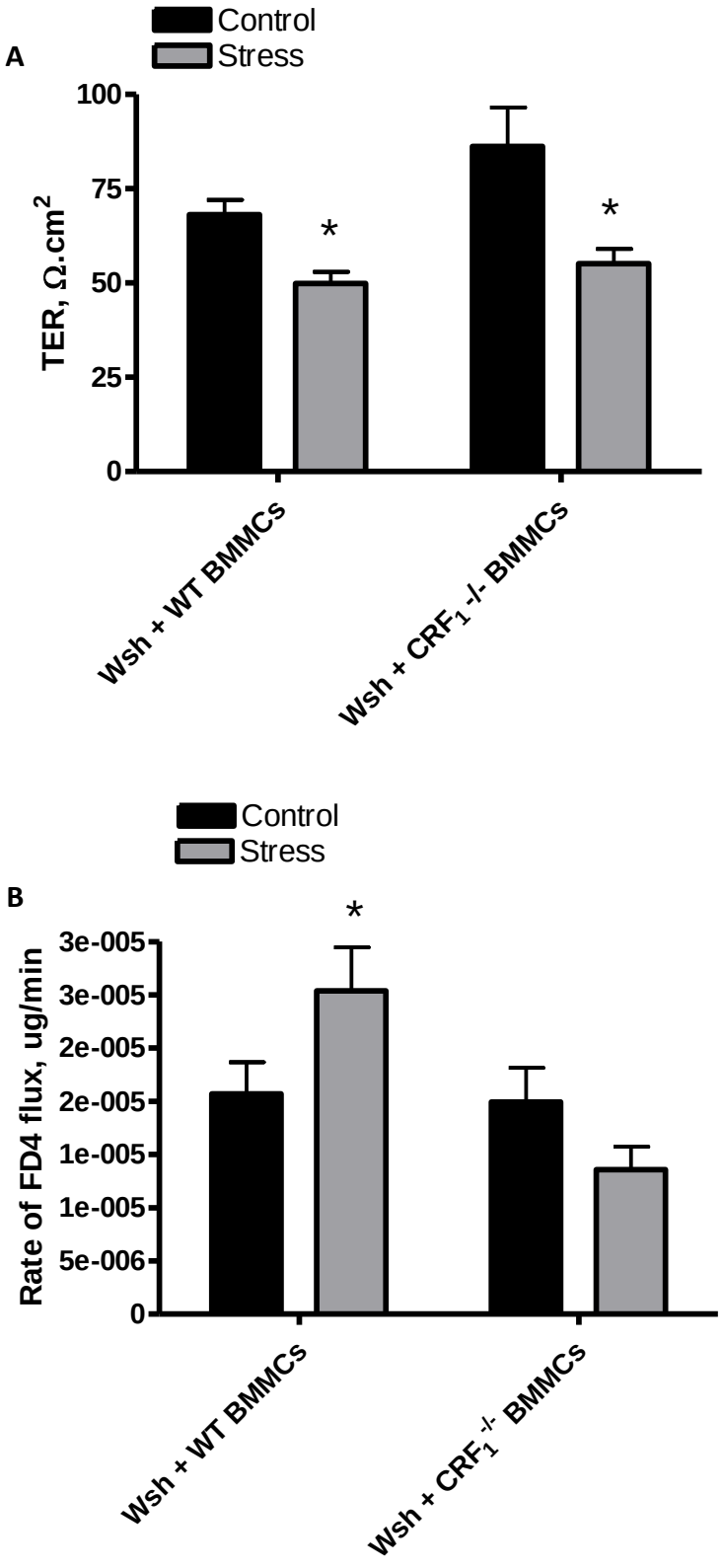

Control
A23187
Tryptase release, Abs
0.4
0.3
0.2
0.1
0.0
*
*
WT BMMC
CRF1 -/- BMMC

Figure 7. Mast cell CRF_1 modulates stress-induced intestinal permeability.

Wsh mice repleted with WT BMMCs or CRF_1 -/- BMMCs were subjected to 3h of restraint stress. Colonic tissue was mounted on Ussing chambers and assessed for changes in barrier function, TER, and intestinal permeability to FD4. Values represent means ± SE for n = 6-7 mice/treatment. A) Restraint stress induced decreases in TER in Wsh mice repleted with WT BMMCs and Wsh mice repleted with CRF_1 -/- BMMCs. B) Intestinal permeability to FD4 was increased in Wsh mice repleted with WT BMMCs; whereas, Wsh mice repleted with CRF_1 -/- BMMCs were protected from stress-induced increases in intestinal permeability. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbols (*) differ by $p<0.05$.


A
Control
Stress
TER, Ω.cm2
100
75
50
25
0
*
*
Wsh + WT BMMCs
Wsh + CRF1 -/- BMMCs
B
Control
Stress
Rate of FD4 flux, ug/min
3e-005
3e-005
2e-005
2e-005
1e-005
1e-005
5e-006
0
*
Wsh + WT BMMCs
Wsh + CRF1 -/- BMMCs

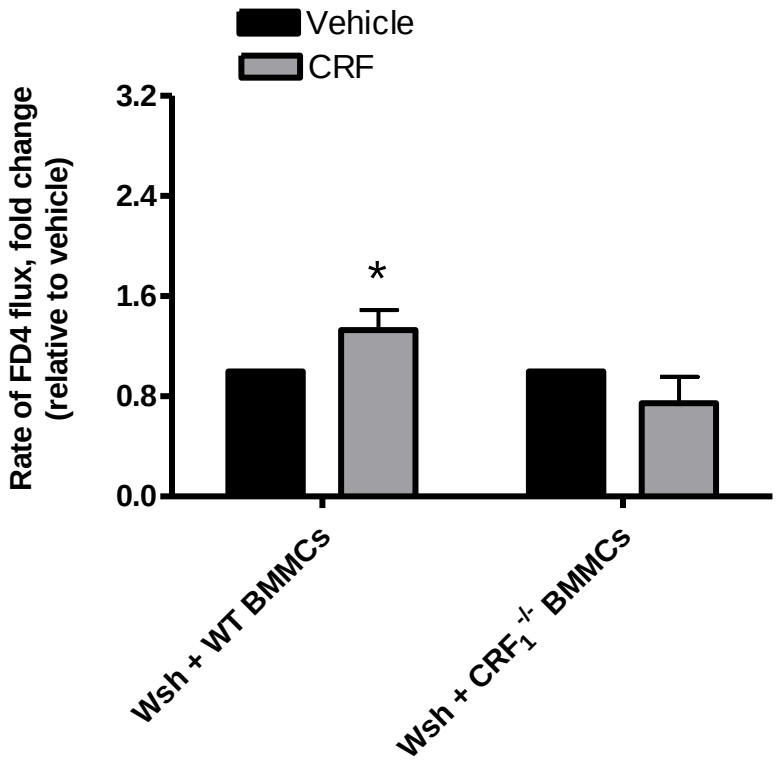
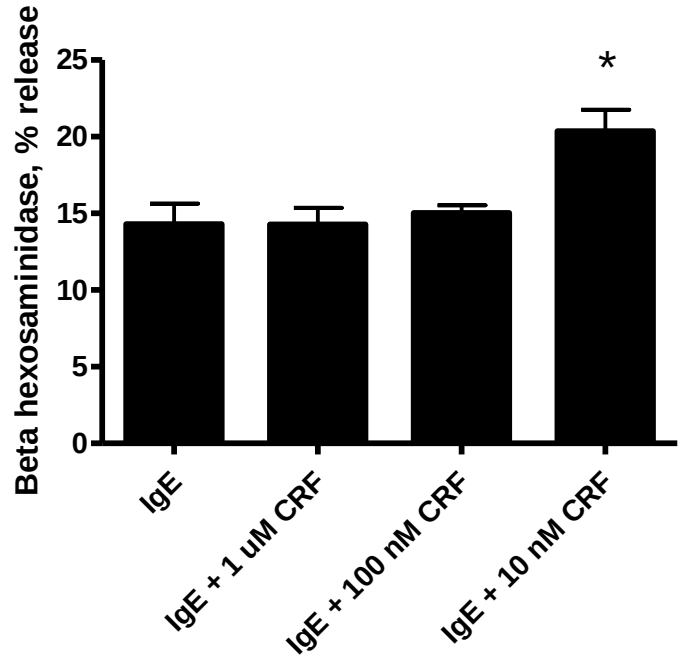


Figure 8. Mast cell CRF_1 regulates intestinal permeability induced by exogenous CRF. Wsh mice repleted with WT BMMCs or CRF_1 -/- BMMCs were intraperitoneally injected with 150 ug/kg of CRF. Colonic tissue was collected 4 hours post-injection and mounted on Ussing chambers. Intestinal permeability was assessed by mucosal-serosal flux of FD4. Values represent means ± SE for n = 4 mice/treatment. CRF treatment induced increased intestinal permeability to FD4 in Wsh mice repleted with WT BMMCs but not in Wsh mice repleted with CRF_1 -/- BMMCs. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbols (*) differ by $p<0.05$.

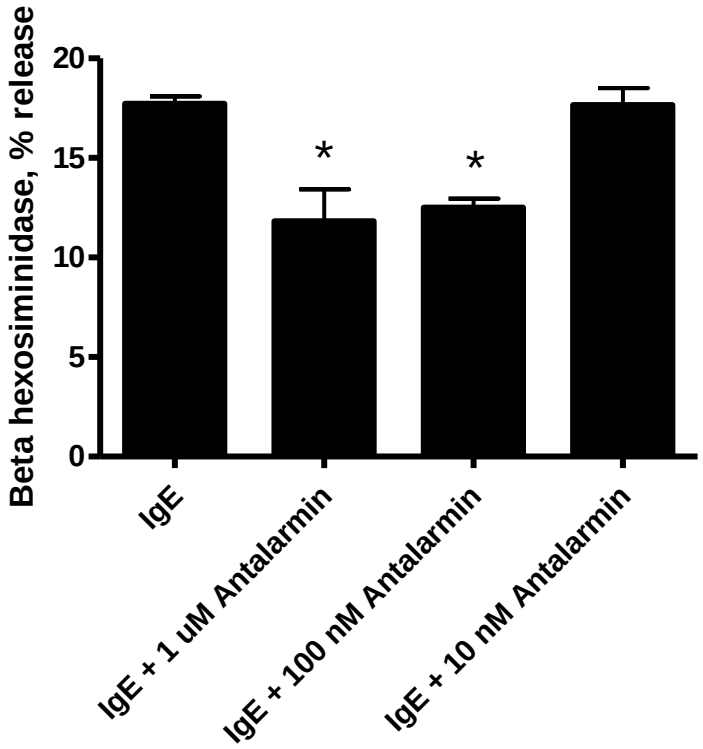
Figure 9. Mast cell CRF_1 controls IgE-induced degranulation.

RBL-2H3 cells were pretreated with CRF (CRF1/2 agonist) or antalarmin (CRF_1 antagonist) prior to stimulation with IgE. Degranulation was measured by beta-hexosaminidase release. Values represent means ± SE for n = 3 wells/treatment. Beta-hexosaminidase release was assessed in 3 separate experiments. A) Pretreatment with 10 nM CRF enhanced IgE-induced beta-hexosaminidase release. B) Pretreatment with antalarmin at 1 uM and 100 nM impaired IgE-induced degranulation of beta-hexosaminidase. Data were analyzed using a 1-way ANOVA and differences between treatment groups were compared using a Fisher LSD post-hoc test. Symbols (*) differ by $p<0.05$.

A


Beta hexosaminidase, % release
25
20
15
10
5
0
*
IgE
IgE + 1 uM CRF
IgE + 100 nM CRF
IgE + 10 nM CRF

B


Beta hexosiminidase, % release
20
15
10
5
0
*
*
IgE
IgE + 1 uM Antalarmin
IgE + 100 nM Antalarmin
IgE + 10 nM Antalarmin

References

1 McEwen, B. S. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res* 886, 172-189, (2000).

2 Bhatia, V. & Tandon, R. K. Stress and the gastrointestinal tract. *J Gastroenterol Hepatol* 20, 332-339, (2005).

3 Thaker, P. H. *et al.* Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 12, 939-944, (2006).

4 Rozanski, A., Blumenthal, J. A. & Kaplan, J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy. *Circulation* 99, 2192-2217, (1999).

5 Koch, F. S., Sepa, A. & Ludvigsson, J. Psychological stress and obesity. *J Pediatr* 153, 839-844, (2008).

6 Sandberg, S. *et al.* Positive experiences and the relationship between stress and asthma in children. *Acta Paediatr* 91, 152-158, (2002).

7 Steptoe, A. & Kivimaki, M. Stress and cardiovascular disease. *Nat Rev Cardiol*.

8 Saunders, P. R., Hanssen, N. P. & Perdue, M. H. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar-Kyoto rats. *Am J Physiol* 273, G486-490, (1997).

9 Saunders, P. R., Kosecka, U., McKay, D. M. & Perdue, M. H. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am J Physiol* 267, G794-799, (1994).

10 Konturek, P. C., Brzozowski, T. & Konturek, S. J. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol* 62, 591-599.

11 Mawdsley, J. E. & Rampton, D. S. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* 54, 1481-1491, (2005).

12 Suarez, K., Mayer, C., Ehlert, U. & Nater, U. M. Psychological stress and self-reported functional gastrointestinal disorders. *J Nerv Ment Dis* 198, 226-229.

13 Bonfiglio, J. J. *et al.* The corticotropin-releasing hormone network and the hypothalamic-pituitary-adrenal axis: molecular and cellular mechanisms involved. *Neuroendocrinology* 94, 12-20.

14 Chalmers, D. T., Lovenberg, T. W. & De Souza, E. B. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci* 15, 6340-6350, (1995).

15 Spiess, J., Rivier, J., Rivier, C. & Vale, W. Primary structure of corticotropin-releasing factor from ovine hypothalamus. *Proc Natl Acad Sci U S A* 78, 6517-6521, (1981).

16 Vale, W., Spiess, J., Rivier, C. & Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213, 1394-1397, (1981).

17 Chen, R., Lewis, K. A., Perrin, M. H. & Vale, W. W. Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A* 90, 8967-8971, (1993).

18 Grammatopoulos, D. K., Randeva, H. S., Levine, M. A., Kanellopoulou, K. A. & Hillhouse, E. W. Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. *J Neurochem* 76, 509-519, (2001).

19 Blank, T. *et al.* Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J Neurosci* 23, 700-707, (2003).

20 Larauche, M., Kiank, C. & Tache, Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 60 Suppl 7, 33-46, (2009).

21 Stengel, A. & Tache, Y. Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. *Annu Rev Physiol* 71, 219-239, (2009).

22 Larauche, M. *et al.* Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *Am J Physiol Gastrointest Liver Physiol* 297, G215-227, (2009).

23 Maillot, C., Million, M., Wei, J. Y., Gauthier, A. & Tache, Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* 119, 1569-1579, (2000).

24 Barreau, F. *et al.* Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. *J Physiol* 580, 347-356, (2007).

25 Castagliuolo, I. *et al.* Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am J Physiol* 271, G884-892, (1996).

26 Teitelbaum, A. A., Gareau, M. G., Jury, J., Yang, P. C. & Perdue, M. H. Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. *Am J Physiol Gastrointest Liver Physiol* 295, G452-459, (2008).

27 Santos, J., Yang, P. C., Soderholm, J. D., Benjamin, M. & Perdue, M. H. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* 48, 630-636, (2001).

28 Santos, J. *et al.* Stress neuropeptides evoke epithelial responses via mast cell activation in the rat colon. *Psychoneuroendocrinology* 33, 1248-1256, (2008).

29 Cao, J. *et al.* Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. *J Immunol* 174, 7665-7675, (2005).

30 Krishnaswamy, G. & Chi, D. S. *Mast cells : methods and protocols*. (Humana Press, 2006).

31 McDowell, E. M. & Trump, B. F. Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch Pathol Lab Med* 100, 405-414, (1976).

32 Spurr, A. R. A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26, 31-43, (1969).

33 Glavin, G. B., Pare, W. P., Sandbak, T., Bakke, H. K. & Murison, R. Restraint stress in biomedical research: an update. *Neurosci Biobehav Rev* 18, 223-249, (1994).

34 McEwen, B. S. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol* 583, 174-185, (2008).

35 Soderholm, J. D. & Perdue, M. H. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 280, G7-G13, (2001).

36 Moeser, A. J. *et al.* Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *Am J Physiol Gastrointest Liver Physiol* 292, G173-181, (2007).

37 Fox, C. C. *et al.* Enhancement of human intestinal mast cell mediator release in active ulcerative colitis. *Gastroenterology* 99, 119-124, (1990).

38 Barbara, G. *et al.* Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 126, 693-702, (2004).

39 Guilarte, M. *et al.* Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut* 56, 203-209, (2007).

40 O'Sullivan, M. *et al.* Increased mast cells in the irritable bowel syndrome. *Neurogastroenterol Motil* 12, 449-457, (2000).

41 King, T., Biddle, W., Bhatia, P., Moore, J. & Miner, P. B., Jr. Colonic mucosal mast cell distribution at line of demarcation of active ulcerative colitis. *Dig Dis Sci* 37, 490-495, (1992).

42 Dvorak, A. M., Monahan, R. A., Osage, J. E. & Dickersin, G. R. Mast-cell degranulation in Crohn's disease. *Lancet* 1, 498, (1978).

43 Bischoff, S. C. Physiological and pathophysiological functions of intestinal mast cells. *Semin Immunopathol* 31, 185-205, (2009).

44 Gilfillan, A. M. & Tkaczyk, C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* 6, 218-230, (2006).

45 Papadopoulou, N. G. *et al.* Regulation of corticotropin-releasing hormone receptor-2 expression in human cord blood-derived cultured mast cells. *J Mol Endocrinol* 35, R1-8, (2005).

46 Cao, J., Cetrulo, C. L. & Theoharides, T. C. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. *Mol Pharmacol* 69, 998-1006, (2006).

47 Santos, J. *et al.* Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. *Am J Physiol* 277, G391-399, (1999).

48 Moeser, A. J., Ryan, K. A., Nighot, P. K. & Blikslager, A. T. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *Am J Physiol Gastrointest Liver Physiol* 293, G413-421, (2007).

49 Overman, E. L., Rivier, J. E. & Moeser, A. J. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. *PLoS One* 7, e39935.

50 Soderholm, J. D. *et al.* Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 123, 1099-1108, (2002).

51 Jacob, C. *et al.* Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 280, 31936-31948, (2005).

CHAPTER 3: MAST CELL CRF RECEPTOR 2 PLAYS A CRITICAL PROTECTIVE ROLE IN MODULATING STRESS-INDUCED INTESTINAL PERMEABILITY BY SUPPRESSION OF MAST CELL DEGRANULATION PATHWAYS

Abstract

Psychological stress is gaining recognition as a predisposing factor in the development of gastrointestinal diseases. The initiation of disease and the development of symptoms have been linked to stressful life events, yet the mechanism remains unclear. Previous studies have demonstrated that CRF_1 regulates GI function in response to stress and CRF signaling. Our group has further demonstrated that mast cell CRF_1 modules changes in intestinal permeability in response to stress and exogenous CRF. Mast cells express both CRF_1 and CRF_2, yet little is known about mast cell CRF_2 signaling in the stress response. Here we investigate the role of mast cell CRF_2 in regulating stress-induced intestinal permeability and mast cell activation. Mast cell deficient mice (Wsh) repleted with WT or CRF_2 -/- bone marrow-derived mast cells (BMMCs) were subjected to 3 hours of restraint stress. Paracellular flux of FITC-dextran (FD4) was measured in Ussing Chambers as an index of intestinal permeability. Restraint stress increased FD4 flux ($p<0.05$) in Wsh mice repleted with WT BMMCs. Interestingly, Wsh mice repleted with CRF_2 -/- BMMCs exhibited a marked increase in FD4 flux in response to stress. To determine the differences in WT and CRF_2 -/- BMMCs that may influence intestinal permeability, mast cell degranulation pathways were studied in a mucosal mast cell line, rat basophil leukemia cells (RBL-2H3). RBL-2H3 cells were pretreated with a CRF_2 agonist or antagonist prior to stimulation, and beta-hexosaminidase release was measured as an indicator of degranulation. Blockade of CRF_2 significantly increased beta-hexosaminidase release; whereas, pretreatment with the CRF_2 agonist impaired mast cell degranulation. These data demonstrate that CRF_2 prevents mast cell degranulation and stress-induced intestinal

permeability, indicating a novel protective role of mast cell CRF_2. These findings provide the basis for mast cell CRF_2 as a therapeutic target for stress-induced GI diseases.

Introduction

The physiological response to stressors is intended to be protective and return an individual to homeostatic conditions (1). A dysregulated or hyper responsive stress response can lead to severe consequences within the gastrointestinal tract, resulting in changes in basic gut function, including increased gut motility, secretion, absorption, visceral nociception, and epithelial permeability (2-4). The onset and exacerbation of GI disorders, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), are associated with alterations in gut function (5,6). Much emphasis has been placed on the consequences of a hyperactive stress response, yet little is known about resolution or homeostatic control of this response.

The stress response is regulated by activation of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing factor (CRF) is released from the hypothalamus in response to a stressor, leading to the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and glucocorticoids from the adrenal cortex (7). CRF is a 41 amino acid peptide found centrally and peripherally that acts through two receptor subtypes, CRF_1 and CRF_2 (8-11). CRF preferentially binds to CRF_1 (12), and signaling through CRF_1 is believed to be the primary pathway involved in the development of stress-related GI dysfunction, resulting in increased colonic motility, visceral hypersensitivity, ion secretion, and intestinal permeability (13,14,15-17).

The role of CRF_2 signaling during the stress response is yet to be elucidated, but research in CRF_2 -/- mice demonstrate a hypersensitive stress phenotype (18,19); whereas, CRF1 -/- mice display a blunted response to stress (20,21). Within the GI tract, CRF_2 is known to dampen visceral pain and inhibit motility (22,23). Taken together, these studies

indicate that CRF_2 may function to dampen CRF_1 in the stress response, but the primary cell type involved remains unclear.

Mast cell activation is believed to be critical in regulating stress-induced changes in GI function (24-27). Mast cells express functional CRF receptors, and previous research by our group has identified mast cell CRF_1 as mediating stress-induced increases in intestinal permeability, but the role of mast cell CRF_2 in this response is unknown (28). Consequently, the objective of this study was to evaluate the specific role of mast cell CRF_2 in controlling mast cell activation and regulation of stress-induced intestinal permeability.

Materials and Methods

Murine Animal Study Protocol. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. For murine studies, C57BL/6 (WT) mice, CRF_2 -/- mice, and mast cell deficient mice, $Kit^{Wsh\text{-}Wsh}$ (Wsh) were used. WT mice and Wsh mice were supplied by Jackson Laboratories. CRF_2 -/- mice were a gift from the Rivier lab (J.E.F. Rivier, The Salk Institute, La Jolla CA).

Restraint Stress Protocol. Mice subjected to restraint stress were placed in a transparent 50 mL plastic conical tube modified with air holes for 3 hours. Control mice were left in their cages for equal time as stressed mice without food and water. Stressed and control mice were immediately sacrificed following restraint stress by CO_2 inhalation and colonic tissue was collected.

Bone Marrow Mast Cell Preparation. Bone marrow from femurs of 8-12 week old congenic WT mice and CRF_2 -/- mice were flushed out and grown in RPMI medium containing 10% FBS (Invitrogen, Calsbad, CA), penicillin and streptomycin (Invitrogen, Carlsbad, CA), 100X HEPES (Invitrogen, Carlsbad, CA), 100X nonessential amino acids (Invitrogen, Carlsbad, CA), 100 mM sodium pyruvate (Invitrogen), recombinant IL-3 (5 ng/ml; R&D Systems, Minneapolis, MN) and stem cell factor (5 ng/ml; R&D Systems, Minneapolis, MN) for a period of 4 weeks, at which time the resulting cell cultures are expected to be >98% MCs determined by toluidine blue staining and flow cytometry.

Mast Cell Repletion Experiments. For mast cell repletion of Wsh mice, $1x10^7$ WT or CRF_2 -/- bone marrow-derived mast cells (BMMCs) in sterile PBS were injected intraperitoneally into 4-6 week old mice. Repletion of the intestine was allowed to occur for 12-14 weeks at which time repleted mice were subjected to restraint stress or used as controls. Mast cell repletion was verified by postmortem histological analysis of intestinal tissue.

Murine Ussing Chamber Studies. Segments of proximal colon were harvested in oxygenated (95% O_2-5% CO_2) Ringer solution (in mmol: 109.8 NaCl, 5.3 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 2.4 Na_2HPO_4 and 0.4 NaH_2PO_4). Tissues were mounted in 0.3 cm^2 aperture Ussing chambers and bathed on the mucosal and serosal sides with 5 mL of Ringer solution. 10 mM glucose was added to the serosal side and osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solution was oxygenated (95% O_2-5% CO_2) and maintained at a temperature of 37 °C. Barrier function was determined by measuring transepithelial electrical resistance (TER, $\Omega.cm^2$) and mucosal permeability by mucosal-serosal FITC-Dextran (FD4) flux. TER data was collected for 90 minutes using 3% KCl agar electrodes (Physiologic Instruments. Acquire & Analyze). After a 15 minute equilibration period on the chambers, 5mg of FD4 was added to the mucosal side. Following an additional 15 minute equilibration period, samples from the serosal side were collected at 15 minute intervals in triplicate for a total of 60 minutes. The presence of FD4 fluorescence intensity of each sample was measured by fMax Fluorescence Microplate Reader (Molecular Devices) and concentrations were determined from standard curves generated by serial dilution of

FD4. Data for FD4 flux were presented as the average rate of FD4 flux over the one hour time period in ug/min.

Flow Cytometry. C-kit and FcƐRI expression of BMMCs were analyzed by flow cytometry. Cells were blocked with anti-Fcγ/RIIb/CD-16-2 (2.4G2; Santa Cruz Biotechnology, Santa Cruz, CA) then stained with PE-conjugated anti-CD117 (BD Pharmingen, San Jose, CA) and APC-conjugated anti- FcƐRI (eBioscience, San Diego, CA). Cells were analyzed using fluorescence-activated cell sorting (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) with data acquisition by Cellquest Pro.

Activation of Bone Marrow-Derived Mast Cells by A23187 and Tryptase Release. $1x10^5$ WT or CRF_2 -/- BMMCs were plated in Tyrode's Buffer. Cells were treated with 500 ng of A21387 (Sigma Aldrich, St. Louis, MO) for 30 mins. Tryptase release was measured using the Thiobenzyl Ester Substrate Assay as previously described (29).

*Corticosterone Assay***.** Serum collected from mice subjected to 3 hours of restraint stress was analyzed for corticosterone levels using a rat and mouse radioimmunoassay (MP Biomedicals, Inc. Orangeburg, NY).

*CRF Intraperitoneal Injections***.** Wsh mice were repleted with WT or CRF_2 -/- BMMCs (as previously described). Mice were intraperitoneally injected with saline or 150 ug/kg of CRF at 12-14 weeks post-repletion. Sections of colon were collected at 4 hours post-injection and

mounted on the Ussing Chamber. Mucosal-to-serosal FD4 flux was monitored as a measure of intestinal permeability.

RBL-2H3 CRF Receptor Agonist and Antagonist Beta-hexosaminidase Assay. Rat basophil leukemia cells (RBL-2H3) were provided by the Abraham lab (S.N. Abraham, Duke University, Durham, NC). Cells were seeded at $5.0x10^4$ cells per well in a 96 well plate and incubated with 1.5 ug/ml anti-DNP IgE (Sigma Aldrich, St. Louis, MO) overnight. Cells were then washed, resuspended in Tyrode's buffer and pre-treated with a CRF_2 agonist (Ucn2) at the indicated concentrations (J.E.F. Rivier, The Salk Institute, La Jolla CA) or a CRF_2 antagonist (astressin 2b, J.E.F. Rivier, The Salk Institute, La Jolla CA) at the indicated concentrations for 10 mins. DNP-HSA was added at 30 ng/ml to induce degranulation. Control wells received an equal volume of Tyrode's buffer. After one hour, the β-hexosaminidase released into the supernatants and in cell lysates was quantified by hydrolysis of p-nitrophenyl *N*-acetyl-β-D-glucosamide (Sigma-Aldrich, St Louis, MO) in 0·1 M sodium citrate buffer (pH 4·5) for 60 min at 37°. The percentage of β-hexosaminidase release was calculated as a percentage of the total content.

Electron Microscopy. 1 x 10^6 BMMCs were fixed in McDowell's and Trump's 4F:1G fixative (30). After 2 rinses in 0.1 M sodium phosphate buffer (pH 7.2), samples were placed in 1% osmium tetroxide in the same buffer for 1 hr at room temperature. Samples were rinsed 2 times in distilled water and dehydrated in an ethanolic series culminating in two changes of 100% acetone. Tissues were then placed in a mixture of Spurr (31) resin and

acetone (1:1) for 30 min, followed by 2 hr in 100% resin with 2 changes. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70ºC for 8 hrs to 3 days. Semi-thin (0.25-0.5 um) sections were cut with glass knives and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (70-90 nm) sections were cut with a diamond knife, stained with methanolic uranyl acetate followed by lead citrate and examined with a transmission electron microscope.

Immunofluorescent Staining and Confocal Analysis. RBL-2H3 cells and CRF_2 -/- cells were labeled with a goat anti-CRF_2 primary antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were subsequently stained using a secondary antibody, donkey anti-goat/FITC (1:1000; Jackson Immunoresearch, West Grove, PA). Nuclei were stained using TO-PRO3 iodide 642/661 (1:1000: Invitrogen, Carlsbad, CA). Confocal images were obtained with a 3-Laser Nikon Confocal Laser Scanning Instrument (Nikon Instruments). Images were obtained using EZ-C1 Nikon software (Silver Version 2.01).

Statistical Analyses. Data are reported as means ± SEM based on the experimental number (n). Data were analyzed by using a t-test or standard one-way ANOVA (Sigmastat, Jandel Scientific, San Rafael, CA). A post hoc Fisher LSD was used to determine differences between treatments following one way ANOVA. Results were considered significant at $P < 0.05$.

Results

Mast Cell CRF_2 Protects Against Stress-induced Intestinal Permeability

CRF signaling has previously been demonstrated to be important in regulating mucosal barrier function during stress, yet little is known about the role of CRF_2 in this response (32). To assess the role of mast cell CRF_2 in the stress response, we repleted Wsh mice with CRF_2 -/- BMMCs. We first demonstrated that rodent mast cells express CRF_2. A mucosal mast cell line, rat basophil leukemia cells (RBL-2H3), and WT BMMCs were stained with anti-CRF_2 (Fig. 1). Both RBL-2H3 cells and WT BMMCs displayed diffuse expression of CRF_2.

We then sought to characterize CRF_2 -/- BMMCs to ensure that the absence of CRF_2 would not affect their function. Toluidine blue staining (Fig, 2A) and electron micrographs (Fig. 2B) confirmed similar morphology between WT and CRF_2 -/- BMMCs. Both types of BMMCs displayed defined nuclei and prominent granules characteristic of mast cells. Expression of two common mast cell receptors, FcεRI and c-kit, were assessed by flow cytometry, demonstrating similar expression between WT and CRF_2 -/- BMMCs (Fig. 2C). Mast cell function was evaluated by calcium ionophore (A23187)- induced degranulation, as measured by tryptase release. WT and CRF_2 -/- BMMCs released similar levels of tryptase following stimulation by A23187 (Fig. 2D).

Given that WT and CRF_2 -/- BMMCs were phenotypically and functionally similar, we wanted to determine how the absence of mast cell CRF_2 signaling would influence intestinal permeability. Wsh mice repleted with WT BMMCs or with CRF_2 -/- BMMCs were subjected to 3h restraint stress and assessed for changes in intestinal permeability.

Restraint stress resulted in a significant decrease in TER in mice repleted with WT or CRF_2 -/- BMMCs (Fig. 3A). Wsh mice repleted with WT BMMCs displayed an increase in intestinal permeability to FD4 following restraint stress (3B). Interestingly, intestinal permeability to FD4 was markedly increased by nearly 2-fold in Wsh mice repleted with CRF_2 -/- BMMCs compared to their unstressed controls. To further confirm that CRF signaling through the mast cell was responsible for the changes in intestinal permeability, Wsh mice repleted with WT BMMCs or CRF_2 -/- BMMCs were intraperitoneally injected with CRF. WT BMMC-repleted mice treated with CRF displayed a similar increase in intestinal permeability to mice subjected to restraint stress (Fig. 4 and Fig. 3B). Additionally, CRF treatment of Wsh mice repleted with CRF_2 -/- BMMCs mimicked the results from restraint stress where absence of mast cell CRF_2 -/- resulted in an exacerbated response to stress and profound increase in intestinal permeability (Fig. 4).

Mast Cell CRF_2 Dampens IgE-induced Degranulation

To determine the effect of CRF_2 signaling on mast cell function, we evaluated mast cell degranulation using an *in vitro* model of mast cell activation in a rat mucosal mast cell line, RBL-2H3 cells. Mast cells were stimulated to degranulate by cross-linking of the FcεRI receptor via IgE binding. RBL-2H3 cells were treated with Ucn2 (CRF_2 agonist) or astressin2b (CRF_2 antagonist) prior to IgE crosslinking and assessed for degranulation, as measured by beta-hexosaminidase release. 10 uM and 5 uM Ucn2 pretreatment of RBL-2H3 cells significantly reduced beta-hex release compared to IgE treated cells (Fig. 5A). Conversely, blockade of CRF_2 by astressin2b (1 nM) enhanced IgE-induced degranulation.

Discussion

Prominent GI diseases including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) have been linked to psychological stress and mast cell activation, yet the precise mechanism remains unknown (5,33-38). Psychological stress and stress peptides are known to activate mast cells to release mediators that increase intestinal permeability (24-27). Although it is known that mast cells express both CRF receptor subtypes, the role of the individual mast cell CRF receptors in regulating intestinal barrier function during the stress response is yet to be elucidated (28,39,40). We sought to characterize the role of mast cell CRF_2 in regulating stress-induced intestinal permeability using a mouse model of restraint stress and mast cell repletion with CRF_2 -/- BMMCs. In order to determine the effect of mast cell CRF_2 on mast cell function, we studied the impact of mast cell CRF_2 signaling on mast cell degranulation.

It has previously been demonstrated that human mast cells derived from cord blood and a human mast cell line, HMC-1, express functional CRF_2 (28). We confirmed that rodent mast cells, BMMCs and a rat mucosal mast cell line (RBL-2H3), also express CRF_2. Signaling through CRF_1 has been demonstrated to influence intestinal dysfunction (14,15-17), and we previously reported that mast cell CRF_1 modulates these changes. To determine the role of mast cell CRF_2 in regulating stress-induced intestinal permeability, we harvested BMMCs from CRF_2 -/- mice and compared them to WT BMMCs. Mast cell morphology, phenotype, and function were assessed and no significant differences were identified. Consequently, we believe CRF_2 deficiency does not alter mast cell function and any physiologic changes are due to the absence of CRF_2 signaling, not mast cell dysfunction.

The role of mast cell CRF_2 in stress-induced intestinal permeability was assessed by comparing mast cell-deficient (Wsh) mice repleted with WT BMMCs versus those repleted with CRF_2 -/- BMMC. Our findings indicate that the absence of mast cell CRF_2 signaling exacerbate stress-induced intestinal permeability. These data provide evidence that CRF_2 on the mast cell, specifically, protects against increased intestinal permeability during the stress response. TER was decreased in all stressed mice, indicating that permeability to macromolecules is regulated by mast cell CRF_2; however, permeability to ions is regulated by other mechanisms. Chronic peripheral administration of CRF elicits colonic permeability similar to that resulting from psychological stress, suggesting that CRF is the primary neuropeptide involved in intestinal permeability changes from stress (25). To determine the effects of CRF signaling through mast cell CRF_2, WT BMMC- repleted and CRF_2 -/- BMMC-repleted Wsh mice were dosed with CRF by intraperitoneal injection. Intestinal permeability to FD4 was assessed and results mimicked those induced by restraint stress, providing evidence that CRF signaling through mast cell CRF_2 protects against stress-induced intestinal permeability.

It has been previously demonstrated that psychological stress activates mast cells to degranulate in the intestine, but is unclear whether this is via direct signaling through mast cell CRF receptors (24,41,42). Our previous research indicates that mast cell CRF_1 promotes mast cell degranulation. To determine the role of mast cell CRF_2 in regulating mast cell degranulation, we utilized a well-studied mast cell degranulation pathway induced by IgE cross-linking of FcεRI in a mucosal mast cell line, RBL-2H3 cells. Ucn2 binds to CRF_2 with greater affinity and acts as a selective CRF_2 agonist (12). Pretreatment with the lowest

concentration of Ucn2 at 10 uM and 5 uM resulted in a significant decrease in beta-hexosaminidase release and impaired mast cell degranulation. Blockade of CRF_2 by the selective antagonist, astressin2b, enhanced mast cell degranulation. These data indicate that mast cell CRF_2 limits mast cell degranulation.

Overall these findings demonstrate that mast cell CRF_2 protects against stress-induced intestinal permeability and mast cell CRF_2 signaling inhibits degranulation pathways. CRF_2 regulation of mast cell degranulation may indicate a homeostatic regulation in the dampening of CRF_1 responses that are known to mediate gastrointestinal dysfunction. A limited number of studies in rodent models provide evidence that these receptors do work in opposition. Research using CRF receptor knockout mice indicate differences in systemic stress responsiveness, where CRF_1 -/- mice have a blunted stress response and CRF_2 -/- mice are hyperresponsive (20,21,23). Within the GI tract, intravenous injection of a CRF_2 agonist prevents visceral pain associated with colorectal distension, a response that is associated with CRF_1 signaling (22). Here we provide evidence, for the first time, that CRF_2 signaling on the mast cell prevents GI dysfunction induced by stress. Further studies will need to be conducted to determine the interaction between mast cell CRF_1 and CRF_2 signaling pathways during the stress response. Based on our findings, selective agonism of mast cell CRF_2 may represent a novel therapeutic target in prevention and treatment of stress-related gastrointestinal disease.

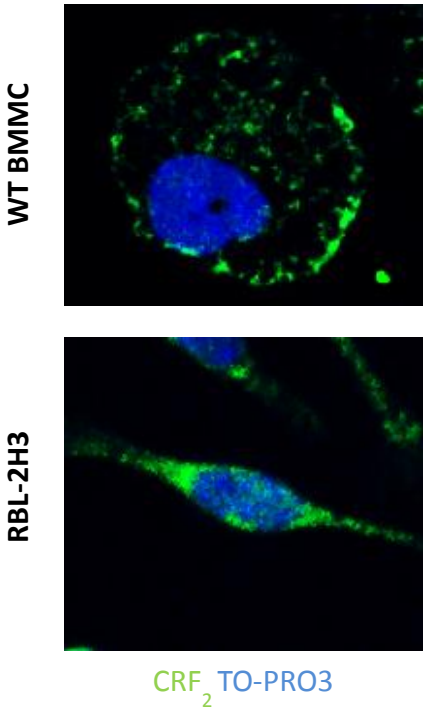
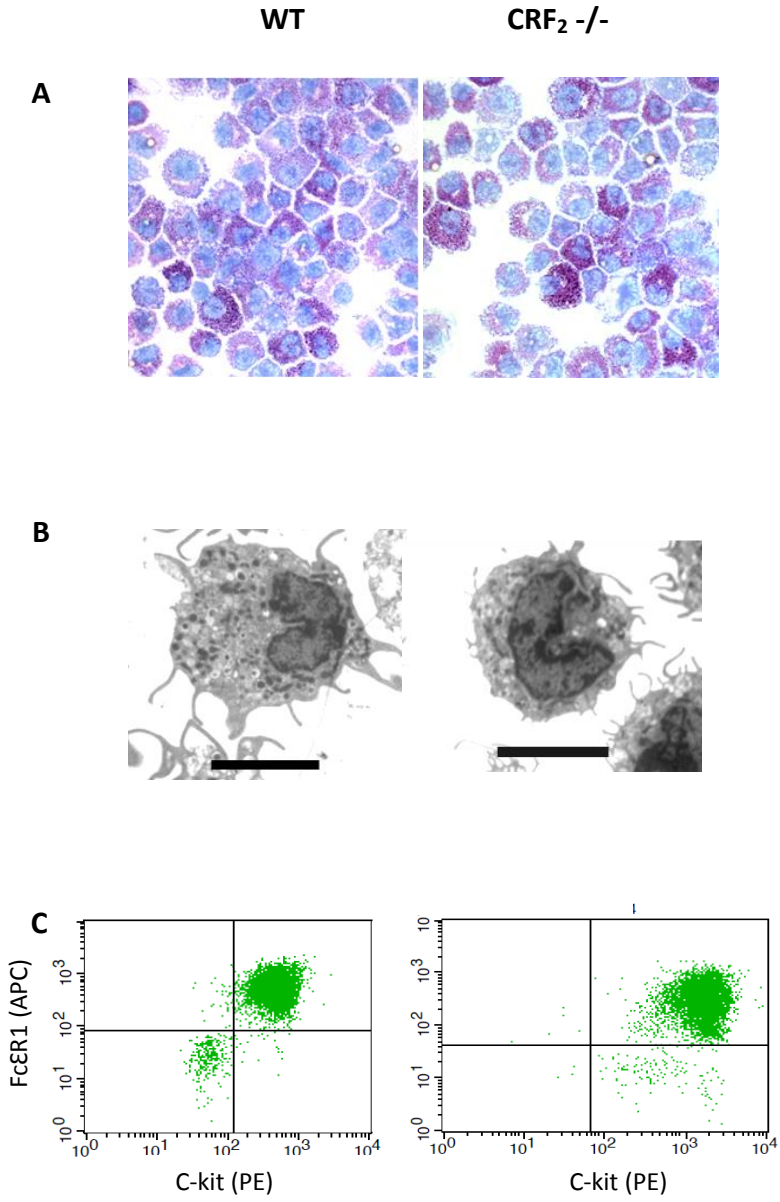


Figure 1. BMMCs and RBL-2H3 cells express CRF_2.

WT BMMCs and RBL-2H3 cells were stained for CRF_2 expression (green) and with a nuclear stain, TO-PRO3 (blue).

Figure 2. Histological and functional analysis of bone marrow-derived mast cells.

Wild type and CRF_2 -/- mast cells display similar morphology as evidenced by A) Toluidine blue staining and B) electron microscopy. C) WT and CRF_2 -/- BMMCs express mast cell surface receptors FcεRI and c-kit. D) WT and CRF_2 -/- BMMCs display similar tryptase release in response to A23187.


WT
CRF2 -/-
A
B
C
FcεR1 (APC)
C-kit (PE)
C-kit (PE)

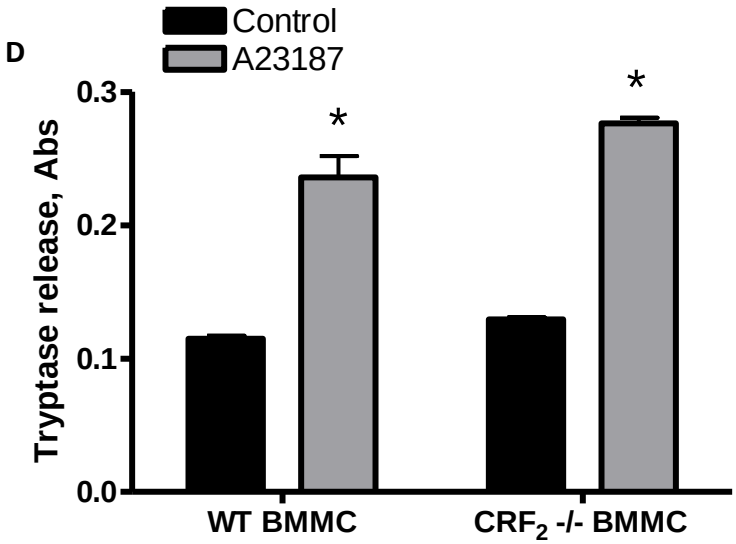
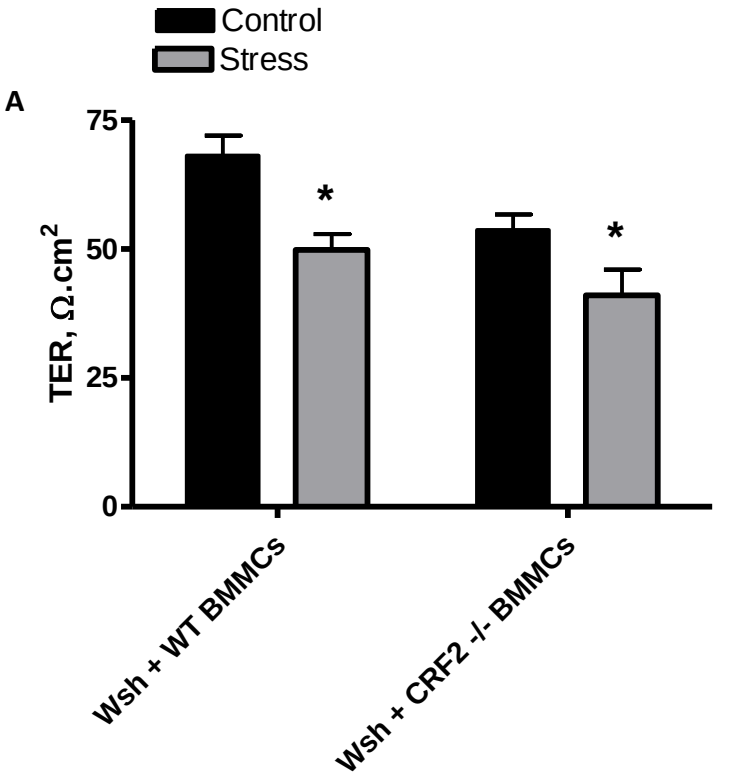
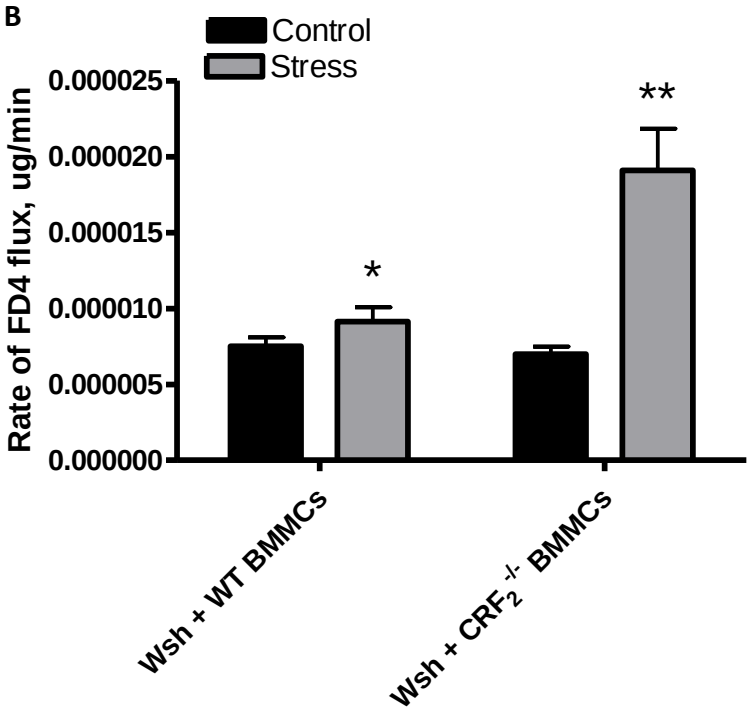

D
Control
A23187
Tryptase release, Abs
0.3
0.2
0.1
0.0
*
*
WT BMMC
CRF2 -/- BMMC

Figure 3. Mast cell CRF_2 protects against stress-induced intestinal permeability.

Wsh mice repleted with WT BMMCs or CRF_2 -/- BMMCs were subjected to 3h of restraint stress. Colonic tissue was mounted on Ussing chambers and assessed for changes in barrier function, TER and intestinal permeability to FD4. Values represent means ± SE for n = 6-7 mice/treatment. A) Restraint stress induced decreases in TER in Wsh mice repleted with WT BMMCs and Wsh mice repleted with CRF_2 -/- BMMCs. B) Intestinal permeability to FD4 was increased in Wsh mice repleted with WT BMMCs. Wsh mice repleted with CRF_2 -/- BMMCs displayed an exacerbated increase in intestinal permeability. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbols (*) differ by $p<0.05$ and symbols (**) differ by $p<0.01$.


Control
Stress
A
75
50
25
0
TER, Ω.cm2
*
*
Wsh + WT BMMCs
Wsh + CRF2 -/- BMMCs


B
Control
Stress
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
Rate of FD4 flux, ug/min
*
**
Wsh + WT BMMCs
Wsh + CRF2 -/- BMMCs

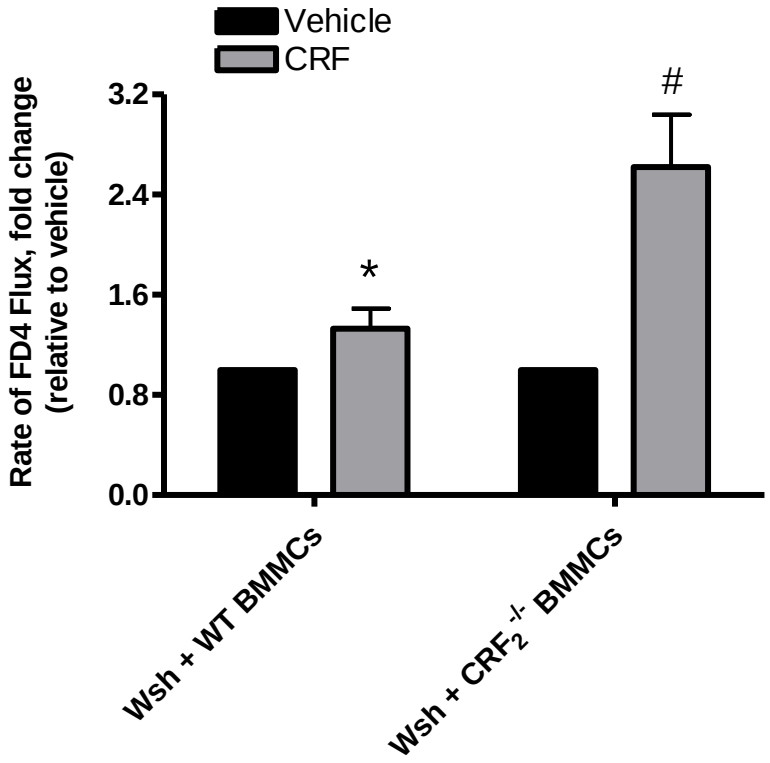
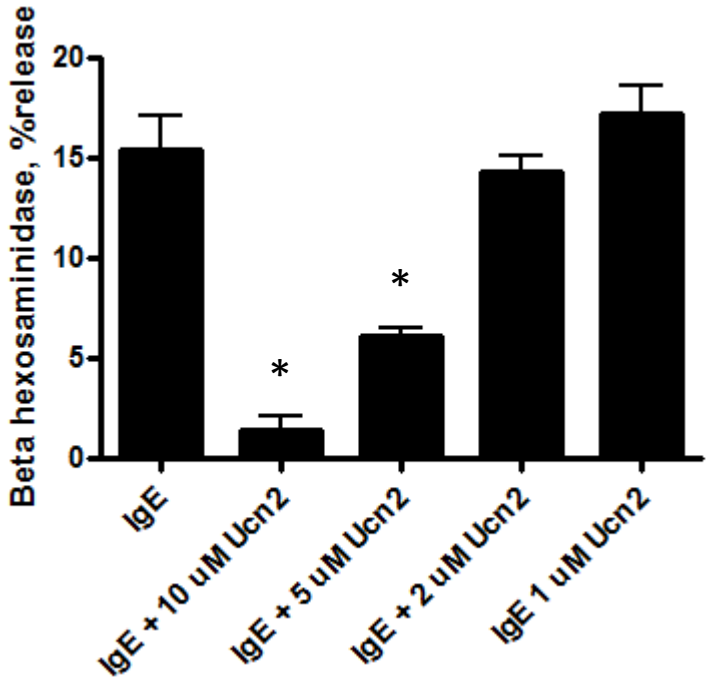


Figure 4. Mast cell CRF_2 protects against intestinal permeability induced by exogenous CRF. Wsh mice repleted with WT BMMCs or CRF_2 -/- BMMCs were intraperitoneally injected with 150 ug/kg of CRF. Colonic tissue was collected 4 hours post-injection and mounted on Ussing chambers. Intestinal permeability was assessed by mucosal-serosal flux of FD4. Values represent means ± SE for n = 4-5 mice/treatment. CRF treatment induced increased intestinal permeability to FD4 in Wsh mice repleted with WT BMMCs. CRF-induced permeability was exacerbated in mice repleted with CRF_2 -/- BMMCs. Data were analyzed using a *t*-test to compare differences between stressed mice and their respective controls. Symbol (*) differ by $p<0.05$ and symbol (#) differs by $p<0.01$.

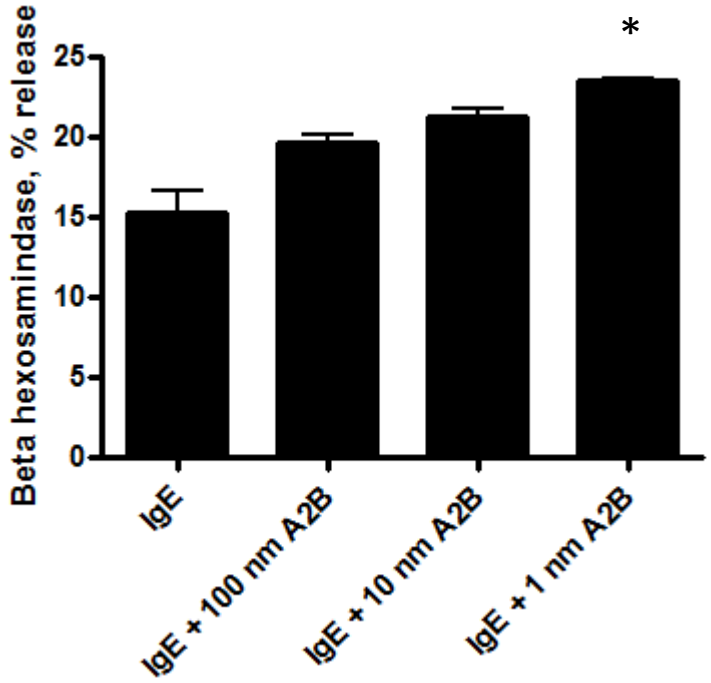
Figure 5. Mast cell CRF_2 dampens IgE-induced degranulation.

RBL-2H3 cells were pretreated with Ucn2 (CRF2 agonist) or Astressin2b (A2B, CRF_2 antagonist) prior to stimulation with IgE. Degranulation was measured by beta-hexosaminidase release. Values represent means ± SE for n = 3 wells/treatment. Beta-hexoaminidase release was assessed in 3 separate experiments. A) Pretreatment with Ucn2 at 10 uM and 5 uM impaired IgE-induced degranulation. B) Pretreatment with A2B at 1 nM enhanced degranulation in IgE stimulated RBL-2H3 cells. Data were analyzed using a 1-way ANOVA and differences between treatment groups were compared using a Fisher LSD post-hoc test. Symbols (*) differ by $p<0.05$.

A


Beta hexosaminidase, %release
20
15
10
5
0
*
*
IgE
IgE + 10 uM Ucn2
IgE + 5 uM Ucn2
IgE + 2 uM Ucn2
IgE 1 uM Ucn2

B


Beta hexosamindase, % release
25
20
15
10
5
0
*
IgE
IgE + 100 nm A2B
IgE + 10 nm A2B
IgE + 1 nm A2B

References

1 McEwen, B. S. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res* 886, 172-189, (2000).

2 Saunders, P. R., Hanssen, N. P. & Perdue, M. H. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar-Kyoto rats. *Am J Physiol* 273, G486-490, (1997).

3 Saunders, P. R., Kosecka, U., McKay, D. M. & Perdue, M. H. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am J Physiol* 267, G794-799, (1994).

4 Konturek, P. C., Brzozowski, T. & Konturek, S. J. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol* 62, 591-599.

5 Mawdsley, J. E. & Rampton, D. S. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* 54, 1481-1491, (2005).

6 Suarez, K., Mayer, C., Ehlert, U. & Nater, U. M. Psychological stress and self-reported functional gastrointestinal disorders. *J Nerv Ment Dis* 198, 226-229.

7 Bonfiglio, J. J. *et al.* The corticotropin-releasing hormone network and the hypothalamic-pituitary-adrenal axis: molecular and cellular mechanisms involved. *Neuroendocrinology* 94, 12-20.

8 Chalmers, D. T., Lovenberg, T. W. & De Souza, E. B. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci* 15, 6340-6350, (1995).

9 Spiess, J., Rivier, J., Rivier, C. & Vale, W. Primary structure of corticotropin-releasing factor from ovine hypothalamus. *Proc Natl Acad Sci U S A* 78, 6517-6521, (1981).

10 Vale, W., Spiess, J., Rivier, C. & Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213, 1394-1397, (1981).

11 Chen, R., Lewis, K. A., Perrin, M. H. & Vale, W. W. Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A* 90, 8967-8971, (1993).

12 Bale, T. L. & Vale, W. W. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* 44, 525-557, (2004).

13 Larauche, M., Kiank, C. & Tache, Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 60 Suppl 7, 33-46, (2009).

14 Stengel, A. & Tache, Y. Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. *Annu Rev Physiol* 71, 219-239, (2009).

15 Larauche, M. *et al.* Cortagine, a CRF1 agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. *Am J Physiol Gastrointest Liver Physiol* 297, G215-227, (2009).

16 Maillot, C., Million, M., Wei, J. Y., Gauthier, A. & Tache, Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* 119, 1569-1579, (2000).

17 Barreau, F. *et al.* Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. *J Physiol* 580, 347-356, (2007).

18 Bale, T. L. *et al.* Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* 24, 410-414, (2000).

19 Coste, S. C. *et al.* Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet* 24, 403-409, (2000).

20 Smith, G. W. *et al.* Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* 20, 1093-1102, (1998).

21 Timpl, P. *et al.* Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet* 19, 162-166, (1998).

22 Million, M. *et al.* CRF2 receptor activation prevents colorectal distension induced visceral pain and spinal ERK1/2 phosphorylation in rats. *Gut* 55, 172-181, (2006).

23 Tache, Y. & Perdue, M. H. Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. *Neurogastroenterol Motil* 16 Suppl 1, 137-142, (2004).

24 Castagliuolo, I. *et al.* Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am J Physiol* 271, G884-892, (1996).

25 Teitelbaum, A. A., Gareau, M. G., Jury, J., Yang, P. C. & Perdue, M. H. Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. *Am J Physiol Gastrointest Liver Physiol* 295, G452-459, (2008).

26 Santos, J., Yang, P. C., Soderholm, J. D., Benjamin, M. & Perdue, M. H. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* 48, 630-636, (2001).

27 Santos, J. *et al.* Stress neuropeptides evoke epithelial responses via mast cell activation in the rat colon. *Psychoneuroendocrinology* 33, 1248-1256, (2008).

28 Cao, J. *et al.* Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. *J Immunol* 174, 7665-7675, (2005).

29 Krishnaswamy, G. & Chi, D. S. *Mast cells : methods and protocols*. (Humana Press, 2006).

30 McDowell, E. M. & Trump, B. F. Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch Pathol Lab Med* 100, 405-414, (1976).

31 Spurr, A. R. A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26, 31-43, (1969).

32 Moeser, A. J. *et al.* Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *Am J Physiol Gastrointest Liver Physiol* 292, G173-181, (2007).

33 Fox, C. C. *et al.* Enhancement of human intestinal mast cell mediator release in active ulcerative colitis. *Gastroenterology* 99, 119-124, (1990).

34 Barbara, G. *et al.* Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 126, 693-702, (2004).

35 Guilarte, M. *et al.* Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut* 56, 203-209, (2007).

36 O'Sullivan, M. *et al.* Increased mast cells in the irritable bowel syndrome. *Neurogastroenterol Motil* 12, 449-457, (2000).

37 King, T., Biddle, W., Bhatia, P., Moore, J. & Miner, P. B., Jr. Colonic mucosal mast cell distribution at line of demarcation of active ulcerative colitis. *Dig Dis Sci* 37, 490-495, (1992).

38 Dvorak, A. M., Monahan, R. A., Osage, J. E. & Dickersin, G. R. Mast-cell degranulation in Crohn's disease. *Lancet* 1, 498, (1978).

39 Papadopoulou, N. G. *et al.* Regulation of corticotropin-releasing hormone receptor-2 expression in human cord blood-derived cultured mast cells. *J Mol Endocrinol* 35, R1-8, (2005).

40 Cao, J., Cetrulo, C. L. & Theoharides, T. C. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. *Mol Pharmacol* 69, 998-1006, (2006).

41 Soderholm, J. D. *et al.* Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 123, 1099-1108, (2002).

42 Moeser, A. J., Ryan, K. A., Nighot, P. K. & Blikslager, A. T. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *Am J Physiol Gastrointest Liver Physiol* 293, G413-421, (2007).