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EFFECTS OF THE STRESS-RELATED NEUROPEPTIDES UROCORTINS ON
COLONIC EPITHELIAL BARRIER FUNCTION

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biology

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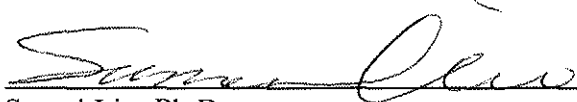
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By Aaron Karo

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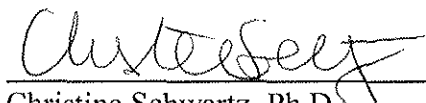
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ABSTRACT

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Urocortins (Ucn1, Ucn2, and Ucn3) belong to the corticotropin-releasing factor (CRF) peptide family which exert their actions on two CRF receptors subtypes (CRF₁ and CRF₂). Ucn1 binds to CRF₁ and CRF₂, whereas Ucn2 and Ucn3 bind to CRF₂. CRF has been implicated in stress-stimulated impairment of intestinal barrier function, but the effects of urocortins on intestinal epithelial barrier function remains unclear. The aims of the study were to investigate the role of urocortins on intestinal epithelial barrier function, and determine which CRF receptor subtype(s) mediates the effects of urocortins. In Ussing flux chambers, transepithelial resistance (TER) and flux of horseradish peroxidase (HRP), were measured as an indicator of paracellular permeability and transcellular permeability, respectively. Application of all three urocortins caused a decrease of TER, and increase in HRP flux, in concentration-dependent manners. Ucn2- and Ucn3-induced TER reduction was only inhibited by the CRF₂ antagonist. Ucn1-, Ucn2-, and Ucn3-induced increase of HRP flux was only inhibited by the CRF₂ antagonist. In conclusion, all three urocortins induce disturbances of colonic epithelial barrier function, which mimic the effects of stress and CRF. The effects of urocortins are mediated by the CRF₂ receptors, which suggest the CRF₂ receptors are regulating colonic barrier function.

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INTRODUCTION

Intestinal Barrier Function

The intestinal barrier is a protective surface in the digestive tract that separates the outside environment (i.e. intestinal lumen) from the body. There are several levels of protection in the intestine. The mucus layer covers the surface intestinal epithelial cells on the luminal side of the intestine (Fig. 1a, b). It contains mucins produced by goblet cells and defensins secreted by Paneth cells (Van Spaendonk et al., 2017). Mucins form a gel-like layer that prevents bacteria from contacting the epithelium, whereas defensins directly kill the bacteria. Next, the intestinal epithelial cells form a physical barrier limiting free exchange of water, ions, and macromolecules between the intestinal lumen and the internal environment of the body. The movement of molecules and ions across the intestinal epithelium occurs by transcellular or paracellular pathway (Odenwald & Turner, 2017). Nutrient absorption, aided by size- and charge-selective channels and membrane transporters, occurs through transcellular transport (Van Spaendonk et al., 2017). The intercellular space between enterocytes allows for less selective paracellular transport and is regulated by controlling the tightness of intercellular junctions (Van Spaendonk et al., 2017). The third layer of protection consists of immune cells, which include microfold cells in the intestinal epithelial layer and antigen presenting cells (e.g. dendritic cells and macrophages) in the lamina propria (Van Spaendonk et al., 2017). The microfold cells are responsible for detecting antigens in the gut lumen and delivering to

dendritic cells and macrophages, which may trigger immune/inflammatory responses (Van Spaendonk et al., 2017). Although the mucus layer and the mucosal immune system all contribute to the protective function, the intestinal epithelium is the primary physical barrier that possesses the property of selective permeability and limits free exchange of water, ions, and macromolecules between the intestinal lumen and the underlying tissues. Perturbation of the intestinal barrier has been linked to many gastrointestinal and systemic disease states, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, diabetes, and multiple sclerosis (Choi, Yeruva, & Turner, 2017; Wells et al., 2017).

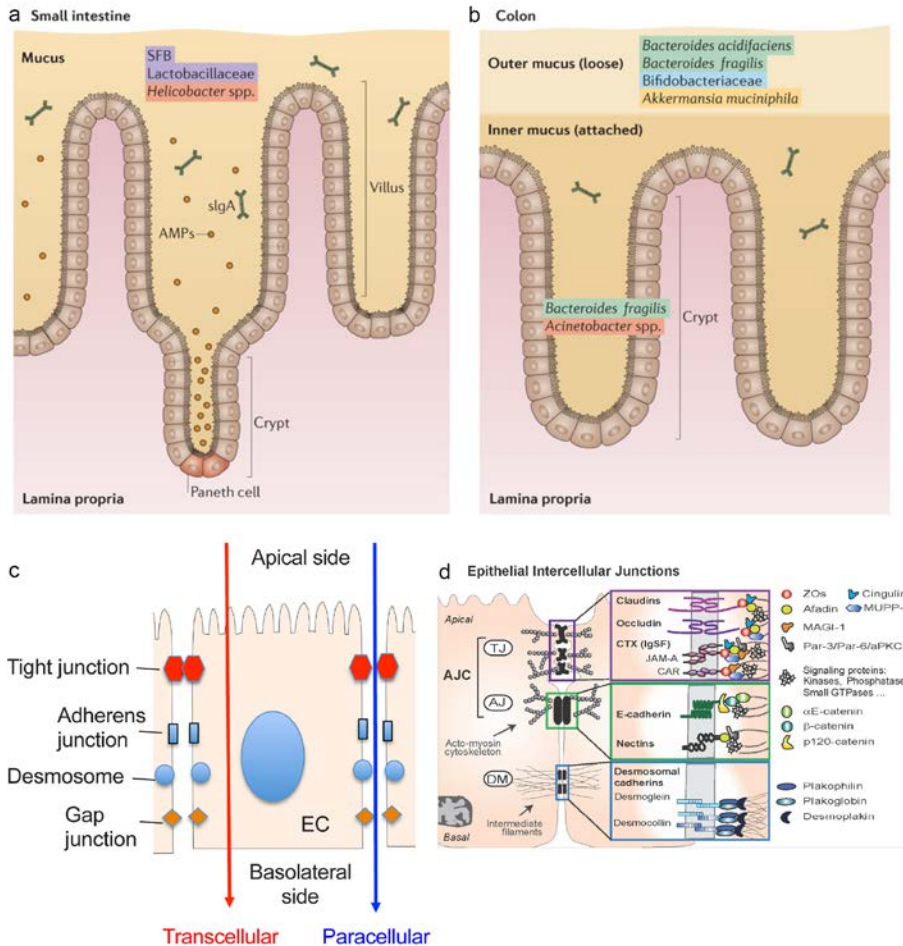


Figure 1. The intestinal epithelial barrier. (a) Small intestinal mucosa. (b) Colonic mucosa. (c) Transcellular and paracellular pathways across the intestinal epithelial cell (EC) layer. (d) Apical junctional complex (AJC). The apical junctional complex includes tight junctions (TJ) and adherens junctions (AJ). Underneath the AJC is the desmosome (DM). NOTE: (a) and (b) modified from Donaldson, Lee, & Mazmanian (2016), (c) modified from Wells et al. (2017), (d) modified from Luissint, Parkos, & Nusrat (2016).

Molecular Composition of the Apical Junctional Complex

Intestinal epithelial cells adhere to each other by intercellular junctions, i.e. tight junctions, adherens junctions, and desmosomes, which make up the apical junction complex (Choi et al., 2017; Wells et al., 2017) (Fig. 1c, d). Tight junctions, located on the apical end of the intercellular spaces, determine paracellular permeability by providing a barrier for larger molecules while allowing for diffusion of water, ions, and smaller molecules through the paracellular pathway (Van Spaendonk et al., 2017; Wells et al.,

2017). The tight junctions are made up of transmembrane proteins, including claudins, occludin, tricellulin, and junctional adhesion molecules (JAM). These proteins all interact with cytoplasmic zona occludens (ZO) that connect the transmembrane tight junction proteins at their cytoplasmic C-terminal strands with F-actin and myosin (Van Spaendonk et al., 2017; Wells et al., 2017) (Fig. 1d). Contraction induced by the interaction of actin myosin microfilaments causes the opening of intercellular space and increases paracellular permeability. Thus, tight junctions are the primary determinant of paracellular permeability. Adherens junctions and desmosomes provide adhesion to maintain cell-cell interactions (Wells et al., 2017). Epithelial cadherin (E-cadherin) is the major component of adherens junctions. E-cadherin is a single spanning transmembrane protein that interacts with the extracellular portion of E-cadherin on adjacent enterocytes. E-cadherin interacts with β -catenin on the cytoplasmic face, which in turn interacts with α -catenin (Van Spaendonk et al., 2017). α -catenin is linked to F-actin cytoskeleton. α -catenin, along with E-cadherin, regulates tight junctions assembly and adds further strength to the apical junctional complex (Odenwald & Turner, 2017). Desmosomes are a type of intercellular junction that provides strong adhesion between adjacent cells. Desmosomes are linked to intracellular intermediate filaments such as keratin to add mechanical strength to intestinal tissue (Van Spaendonk et al., 2017). The role of desmosome in intestinal barrier function is still unclear.

Effects of Stress on Intestinal Barrier Function

It is well known that stress impairs intestinal barrier function. Several investigations across different animal models have demonstrated increased intestinal permeability, ion transport, and mucus secretion with stress (Santos, Benjamin, Yang,

Prior, & Perdue, 2000; Soderholm & Perdue, 2001; Tache & Perdue, 2004). Previously, various physical or psychological stressors have been used in animal studies; but more recently, more psychological models have been chosen to mimic the experience of everyday environmental and life stress of the average person (Soderholm & Perdue, 2001).

Restraint stress, cold restraint stress, and water avoidance stress are the most commonly used protocols to imitate physical and psychological stressors. Rats exposed to restraint stress or cold restraint stress have exhibited intestinal barrier dysfunction by increased transepithelial conductance and permeability to inactive marker molecules (Santos et al., 1999; Saunders, Kosecka, McKay, & Perdue, 1994). In another study, the flux of the macromolecule horseradish peroxidase (HRP) across the intestinal epithelium in rats exposed to restraint stress was assessed (Kiliaan et al., 1998). The study found enhanced HRP flux across intestinal tissues of stressed rats compared with controls. They also found HRP-containing endosomes within enterocytes, goblet cells, and Paneth cells, and HRP located in the paracellular space between adjacent intestinal epithelial cells in stressed rats (Kiliaan et al., 1998), suggesting that HRP is transported via both transcellular and paracellular pathways.

Water avoidance was used as a psychological stressor to determine the effects of psychological stress on epithelial barrier function. Exposure to water avoidance stress (one hour per day for five days) caused an increase in epithelial ion secretion (measured by baseline short-circuit current, I_{sc}), ion permeability (measured by tissue conductance), and macromolecular permeability (measured by HRP flux) in the rat jejunum (Santos et al., 2000). Mast cells are immune cells widely distributed throughout the intestine and are

involved in the regulation of gastrointestinal function. To investigate the role of mast cells in stress-induced increase of intestinal epithelial permeability, mast cell-deficient rats (Ws/Ws) were used (Santos et al., 2000). In Ws/Ws rats, water avoidance stress failed to induce any changes in intestinal baseline ion secretion or intestinal permeability (Santos et al., 2000). This study implicated the important role of intestinal mast cells in stress-induced epithelial pathophysiology and may provide novel target for treatments.

Stress experienced early in life (e.g. maternal deprivation) has been shown to modify immune and gastrointestinal functions. To test effects of maternal deprivation on intestinal epithelial barrier, male Wistar rat pups were separated from the dam during postnatal days 2-14 or left undisturbed with their dam. At 12 weeks of age, colonic paracellular permeability, mast cell production, and expression of mRNA for immunological factors were assessed (Barreau, Ferrier, Fioramonti, & Bueno, 2004). Rats that experienced maternal deprivation had a substantial increase in colonic permeability compared to control rats (Barreau et al., 2004). The increased colonic permeability was associated with bacterial translocation into the visceral organs, increased mast cell density, and increased cytokine mRNA expression (Barreau et al., 2004). The neonatal period (birth to day 14) is a stress hyporesponsive period when stress hormones are low. During this period, applying an intense stressor such as maternal deprivation will outweigh the hyporesponsiveness, leading to long-term colonic epithelial barrier dysfunction (Barreau et al., 2004).

The hypothalamic-pituitary-adrenal (HPA) axis is known to be responsible for the body's overall stress responses. However, the HPA axis is not involved in neonatal maternal deprivation-induced increase in intestinal permeability. Neonatal maternal

deprivation has been shown to increase corticosterone levels in 12-week-old rats, however, a corticoid receptor antagonist had no effect on neonatal maternal deprivation-induced increase of intestinal permeability (Barreau et al., 2007). These results differ from those observed by Meddings & Swain (2000) who examined intestinal permeability after restraint and swimming stress. In this study, adrenalectomy or blockage of glucocorticoid receptors abolished restraint or swimming stress-induced increase of intestinal permeability (Meddings & Swain, 2000).

Soderholm et al. (2002) reported that neonatal maternal separation also exacerbates intestinal epithelial barrier dysfunction when encountering a mild stress later in life. Neonatal Sprague-Dawley rats were separated for three hours per day at 4-21 days of age (Soderholm et al., 2002). Between days 80 and 90, separated and control rats were exposed to a mild acute stress (30-min water avoidance stress) or sham stress (Soderholm et al., 2002). Ussing chamber experiments demonstrated increased ion secretion, transepithelial conductance, and transepithelial transport of macromolecules in the colon of separated rats with minimal effects in control rats (Soderholm et al., 2002). The non-selective corticotropin-releasing factor (CRF) receptor antagonist (α -helical CRF (9-41)) injected 20 minutes before the acute stress prevented stress-induced changes previously observed (Soderholm et al., 2002). This implies that early life trauma predisposes the adults to increased sensitivity to mild stress regarding intestinal epithelial barrier function and that CRF plays an important role in this process.

Another early life stressor that has been observed is early weaning in piglets. Moeser et al. (2007) took weaned 19-day-old pigs and assessed jejunal and colonic tissues for epithelial barrier function and ion transport in the Ussing chambers. The study

found an increase in short-circuit current (i.e. increased ion secretion) that was sensitive to tetrodotoxin, a neuronal blocker, and indomethacin, a non-steroidal anti-inflammatory drug that blocks prostaglandin production (Moeser et al., 2007). This suggests that the enteric nervous system and prostaglandin synthesis pathways are activated due to the stress of weaning. In addition, decreased transepithelial resistance and increased permeability in jejunum and colon were observed in weaned piglets compared to unweaned control pigs (Moeser et al., 2007). Weaning caused an increase in CRF₁ receptor expression in jejunum and colon of the weaned pigs, and the non-selective CRF receptor antagonist α -helical CRF (9-41) abolished weaning-induced changes in baseline ion secretion and intestinal permeability (Moeser et al., 2007).

The impairment of intestinal barrier function by stress has also been observed in humans. One study observed small intestine permeability using healthy volunteers subjected to different types of psychological stress. The study found that acute psychological stress in the form of public speech increased small intestine permeability. Small intestinal permeability was measured by a two-hour lactulose-mannitol urinary excretion test. Stress-induced increase of small intestinal permeability was inhibited by the mast cell stabilizer, disodium cromoglycate, suggesting the involvement of mast cells (Vanuytsel et al., 2014).

CRF and Urocortins

The primary pathway that initiates the stress response is the hypothalamic-pituitary-adrenal axis. CRF is a 41-amino acid neuropeptide that is released in the hypothalamus during stress. Once released, CRF acts on the anterior pituitary to cause the release of adrenocorticotrophic hormone (ACTH). ACTH acts on the adrenal cortex to

promote the release of glucocorticoids, which help maintain or reinstate homeostasis during stress. Besides the hypothalamus, CRF has been found in many other brain regions (Henckens, Deussing, & Chen, 2016) and in peripheral organs and tissues including the gut (Barreau et al., 2007; Kawahito et al., 1994; Liu et al., 2006). CRF has been implicated in stress-related functional changes in the gut, such as increased colonic motility, hypersecretion, and impaired intestinal barrier function (Tache & Perdue, 2004).

Urocortins (Ucns) are members of the CRF family of neuropeptides. Three urocortins (Ucn1, 2, and 3) have been identified and they all present in the gastrointestinal tract (Chang et al., 2011; Chang et al., 2007; Mahajan, Liao, Barkan, Takahashi, & Bhargava, 2014). It has been known that CRF mediates the stress responses in the gut, whereas Ucn2 mediates the stress-coping response by inhibiting stress-induced hypermotility in the colon (Gourcerol et al., 2011). Urocortins have also been found to play a role in gut inflammation (Mahajan et al., 2014).

CRF and CRF-related peptides exert their biological actions by binding to two G-protein coupled receptors, CRF₁ and CRF₂. CRF bind with higher affinity to CRF₁ and with lower affinity to CRF₂ (Hauger et al., 2003). Ucn1 shows an equally high affinity to both CRF₁ and CRF₂ and demonstrates higher affinity to both CRF receptors than CRF (Hauger et al., 2003). Ucn2 and Ucn3 exclusively bind to the CRF₂ receptor and are considered as selective agonists for the CRF₂ receptor (Hauger et al., 2003; Stengel & Tache, 2010). CRF₁ and CRF₂ receptors may co-exist as dimers to coordinate cellular responses (Mahajan et al., 2014).

Role of CRF in Stress-induced Disruption of Intestinal Barrier Function

CRF has been implicated in stress-induced disruption of intestinal barrier function. Peripheral administration of CRF in rats via intraperitoneal injection (i.p.) increased colonic ion secretion, colonic transepithelial conductance, and HRP flux, which mimicked the effects of restraint stress (Santos et al., 1999). The effects of CRF on colonic permeability and HRP flux were dose-dependent (Santos et al., 1999). Treatment with α -helical CRF (9-41) (i.p.), a non-selective peptide CRF receptor antagonist that does not penetrate the blood-brain barrier, inhibited both the restraint stress and CRF-induced increase of colonic ion secretion, tissue conductance, and HRP flux (Santos et al., 1999). This observation suggests the role of peripheral CRF receptors in stress or CRF-induced disruption of intestinal barrier function. The direct peripheral action of CRF was confirmed by a follow-up study, which showed that exogenously adding CRF to the serosal side of colonic tissue mounted in the Ussing chamber, a device used for *in vitro* measurement of intestinal permeability, mimicked stress-induced intestinal barrier defects (Saunders et al., 2002).

The role of specific CRF receptor subtypes in stress and CRF related peptides-induced disruption of intestinal barrier function has been investigated using selective CRF₁ or CRF₂ receptor agonists and antagonists. In rats, both acute (restraint, cold restraint) and chronic (water avoidance stress 1 h/day for 5-10 days, neonatal maternal separation) stress increased intestinal permeability (Barreau et al., 2007; Kiliaan et al., 1998; Santos et al., 2000; Santos et al., 1999; Saunders et al., 1994; Saunders et al., 2002; Soderholm et al., 2002). Stress-induced increase of intestinal permeability was suppressed by pretreatment of rats with peripheral administration of the non-selective

CRF receptor antagonist, α -helical CRF (9-41) (Santos et al., 1999; Saunders et al., 2002; Soderholm et al., 2002), or the selective CRF₁ antagonist, SSR-125543 (Barreau et al., 2007), suggesting the role of CRF₁ receptors in stress-induced increase of intestinal permeability. Peripheral administration of selective CRF₁ receptor agonist, cortagine, in rats mimicked the effects of stress on intestinal permeability (Larauche et al., 2009). In human colonic biopsies mounted in Ussing chambers, CRF increased HRP flux across the colonic epithelium but did not affect transepithelial resistance or ⁵¹Cr-EDTA flux, indicating an increase in transcellular permeability (Wallon et al., 2008). The effect of CRF on HRP flux in human colonic biopsies was abolished by α -helical CRF (9-41) and partially inhibited by the selective CRF₁ receptor antagonist, antalarmin (Wallon et al., 2008), further supporting the role of CRF₁ receptor in modulation of intestinal permeability.

There have been several studies documenting that CRF₂ receptors, on the contrary, may mediate stress-induced impairment in intestinal barrier function. Gareau et al. (2007) reported that neonatal maternal separation-induced increase of HRP flux in the rat colon was blocked by the non-selective CRF receptor antagonist α -helical CRF (9-41) and the selective CRF₂ receptor antagonist antisauvagine-30, but was unaffected by the selective CRF₁ receptor antagonist antalarmin. Antisauvagine-30 also blocked the effects of chronic peripheral administration of CRF on colonic transepithelial resistance and HRP flux in rats (Teitelbaum, Gareau, Jury, Yang, & Perdue, 2008), further supporting a role of CRF₂ receptor in modulating both paracellular and transcellular permeability. Interestingly, selective blockade of CRF₂ receptors with astressin 2B exacerbated early weaning stress-induced intestinal barrier dysfunction in pigs (Smith et al., 2010),

implicating a protective role of CRF₂ receptor in maintaining the intestinal barrier. Taken together, these data suggest that activation of peripheral CRF receptors by stress has an impact on intestinal permeability. However, previous studies using CRF₁ or CRF₂ receptor antagonists have yielded contradictory results on the role of each CRF receptor subtypes in modulation of intestinal barrier function. CRF₁ or CRF₂ knockout mice should be used in the future to delineate the specific role of each of the receptor subtypes.

Significance

Gastrointestinal diseases, such as IBD and IBS, affect 70 million Americans annually ("Digestive Diseases Statistics for the United States", 2017). IBS has many negative impacts on quality of life including disruptions in relationships, diet, travel, and sleep. A variety of factors influence a person's chances of developing IBS, but long-term stressful events are strongly suspected to cause IBS. The details of the mechanisms by which stress leads to IBS are unknown, but evidence suggests that CRF and CRF-related peptides, Ucn1, 2, and 3, mediate many forms of the stress-related gastrointestinal dysfunction (Bhargava, 2011; Chang et al., 2011; Lewis et al., 2001). The role of CRF in stress-induced disruption of intestinal epithelial barrier function has been clarified. However, it is still unclear what role Ucn1, 2, and 3 plays in this process. The present research focused on the effects of Ucn1, 2, and 3 on colonic epithelial barrier function by activating CRF₁ and/or CRF₂ receptors, which may cause "leaky" gut. Results of the research will bridge the gap in knowledge of the stress effects on gastrointestinal function. Understanding of these pathways will aid in advancement of treatments for stress-related gastrointestinal disorders by targeting the signaling pathways used by the CRF and CRF-related peptides.

SPECIFIC AIMS

This study had two specific aims. Specific Aim 1 was to determine the effects of Ucn1, 2, and 3 on colonic epithelial permeability. If urocortins were involved in stress-induced disruption of colonic epithelial barrier function, then exogenously added urocortins would mimic the effects of stress and cause an increase in colonic epithelial permeability. C57BL/6 mice were used in this study. Effects of urocortins on colonic epithelial permeability were assessed by the Ussing Chamber technique. Specific Aim 2 was to determine which CRF receptor subtype(s) mediate(s) the effects of Ucn1, 2, and 3 on colonic epithelial permeability using selective CRF₁ and CRF₂ receptor antagonists.

MATERIALS AND METHODS

Animals

Forty-five adult male C57BL/6 mice were used in the study. The mice were purchased from Envigo RMS, Inc. (Madison, WI) and maintained on a 12-hour light/dark cycle with controlled temperature in the animal facility at the Health Science Center, University of Wisconsin-La Crosse (UWL). Animals had *ad libitum* access to chow and water until the days of experiments. On the day of the experiment, the mouse was euthanized by CO₂ inhalation followed by cervical dislocation. The proximal colon was removed and used for measuring intestinal permeability using the Ussing chamber technique. All procedures in this project were approved by the Institutional Animal Care and Use Committee at UWL (protocol 6-17).

Mucosa/submucosa Tissue Dissection

The proximal colon was removed from euthanized mice and placed in Krebs solution. The Krebs solution (pH 7.4) consists of 120.9 mM NaCl, 5.9 mM KCl, 14.4 mM NaHCO₃, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 11.5 mM glucose, and 25 mM CaCl₂. The colon was opened along the mesenteric boarder. The flat sheet of the colonic tissue was dissected to remove the serosa and muscularis externa layers. The remaining mucosa/submucosa preparation was used for measuring colonic epithelial ion secretion and permeability using the Ussing chamber technique.

Ussing Chamber Recording

The Ussing chambers were used to measure intestinal permeability. The chambers were set up based on the standard protocol (Gareau et al., 2007). After dissecting the mucosa/submucosa preparation, the preparation was divided into 1-cm long segments, mounted onto slider inserts (cross-sectional area of 0.3 cm²), and placed into the Ussing chamber vertically with the luminal side facing the left chamber and the basolateral side facing the right chamber (Fig. 2A). Tissues were bathed in 37°C oxygenated Krebs solution (3 ml/chamber) and allowed to equilibrate for 30 min. Each Ussing chamber was equipped with a pair of Ag/AgCl electrodes connected via agar-KCl bridges for measurement of transmural potential difference (PD). A second pair of electrodes was connected to an automated voltage clamp amplifier (VCC MC6, Physiological Instruments, San Diego, CA) to inject the required short-circuit current (I_{sc}) to maintain a zero-potential difference. I_{sc} (μA/cm²) was recorded continuously by a computer connected to the voltage clamp system and used to measure intestinal ion secretion. Transepithelial resistance (TER, Ωcm²) was calculated according to Ohm's law. TER predominately reflects paracellular ion permeability of the intestinal epithelium. A lower TER indicates a higher paracellular ion permeability, and vice versa. Sample recordings of I_{sc} and TER were shown in Fig. 2B and 2C.

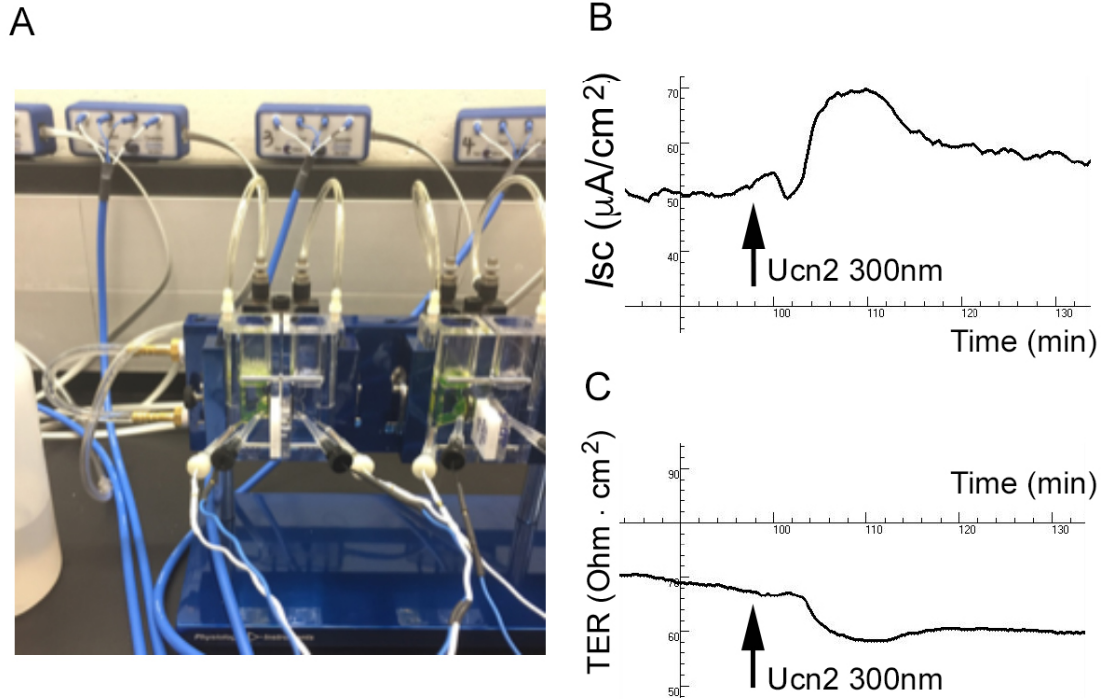


Figure 2. Ussing chamber recording. (A) Ussing chamber setup. (B) Sample recording of short-circuit current (I_{sc}). (C) Sample recording of transepithelial resistance (TER).

Colonic transcellular permeability to macromolecules was determined by measuring mucosal-to-serosal flux of HRP (44 kDa). HRP was added to luminal chambers to reach a concentration of 10 μM . Samples (300 μl) were taken from the serosal chambers every 30 min for 1 h to obtain the baseline HRP flux. Equal volume of Krebs solution, maintained at 37°C, was added back to the chamber to keep volume constant. The amounts of HRP in the samples were quantified using the 1-StepTM Ultra TMB-ELISA substrate solution and the optical density of the reaction product was measured at 465 nm. Mucosal-to-serosal flux of HRP is expressed as $\text{ng/ml/cm}^2/\text{h}$.

Experimental Protocol for Specific Aim 1

The effects of Ucn1, Ucn2, and Ucn3 on colonic I_{sc} , TER, and HRP flux were investigated in Specific Aim 1. Different concentrations of Ucn1, Ucn2, or Ucn3 were added to basolateral chambers one hour after HRP addition. The working concentrations of urocortins were 30 nM, 100 nM, 300 nM, and 1 μ M. I_{sc} and TER were recorded continuously for another hour. Samples (300 μ l) were taken from the serosal chambers every 30 min for another hour and replaced with equal volume Krebs solution. I_{sc} , TER, and HRP flux after addition of urocortins were compared with the baseline values. At the end of the experiment, carbachol, a non-selective acetylcholine muscarinic receptor agonist, was added to the serosal chamber in a working concentration of 10 μ M to test tissue viability. An increase in I_{sc} indicated that the tissue was still in good condition.

Experimental Protocol for Specific Aim 2

The CRF receptor subtype that mediates the effects of Ucn1, Ucn2, or Ucn3 on colonic epithelial permeability was determined in Specific Aim 2. The selective CRF₁ receptor antagonist NBI27914 (working concentration 10 μ M), or the selective CRF₂ receptor antagonist antisauvagine-30 (working concentration 1 μ M), was added to basolateral chambers 15 min prior to Ucn1, 2, or 3 treatment. I_{sc} , TER, and HRP flux after addition of Ucn and CRF receptor antagonist were compared with the values obtained when treated with Ucn alone.

RESULTS

Actions of Ucn1, Ucn2, or Ucn3 on Colonic Epithelial Permeability

The transepithelial tissue resistance was recorded to study actions of Ucns on colonic paracellular permeability. Application of Ucn1 (30 nm – 1 μ M), Ucn2 (30 nm – 1 μ M), or Ucn3 (30 nm – 1 μ M) to the basolateral side of the colonic mucosa/submucosa preparation caused a concentration-dependent decrease of TER (Fig. 3A-C), indicating an increase in colonic paracellular permeability. At a concentration of 1 μ M, Ucn3 caused the greatest decrease in TER, followed by Ucn2 and Ucn1 (Fig. 3D). The effect of Ucn3 (1 μ M) on TER was significantly greater than that caused by Ucn1 at the same concentration (Fig 3D).

The HRP flux rate was used to measure colonic transcellular permeability. All three Ucns caused an increase in HRP flux from the luminal to basolateral side of the colonic mucosa in a concentration-dependent manner; with the greatest flux rate for all Ucns occurring at a concentration of 1 μ M (Fig. 4A-C). At the concentration of 1 μ M, Ucn1, Ucn2, and Ucn3 caused similar magnitudes of increase in HRP flux (Fig. 4D).

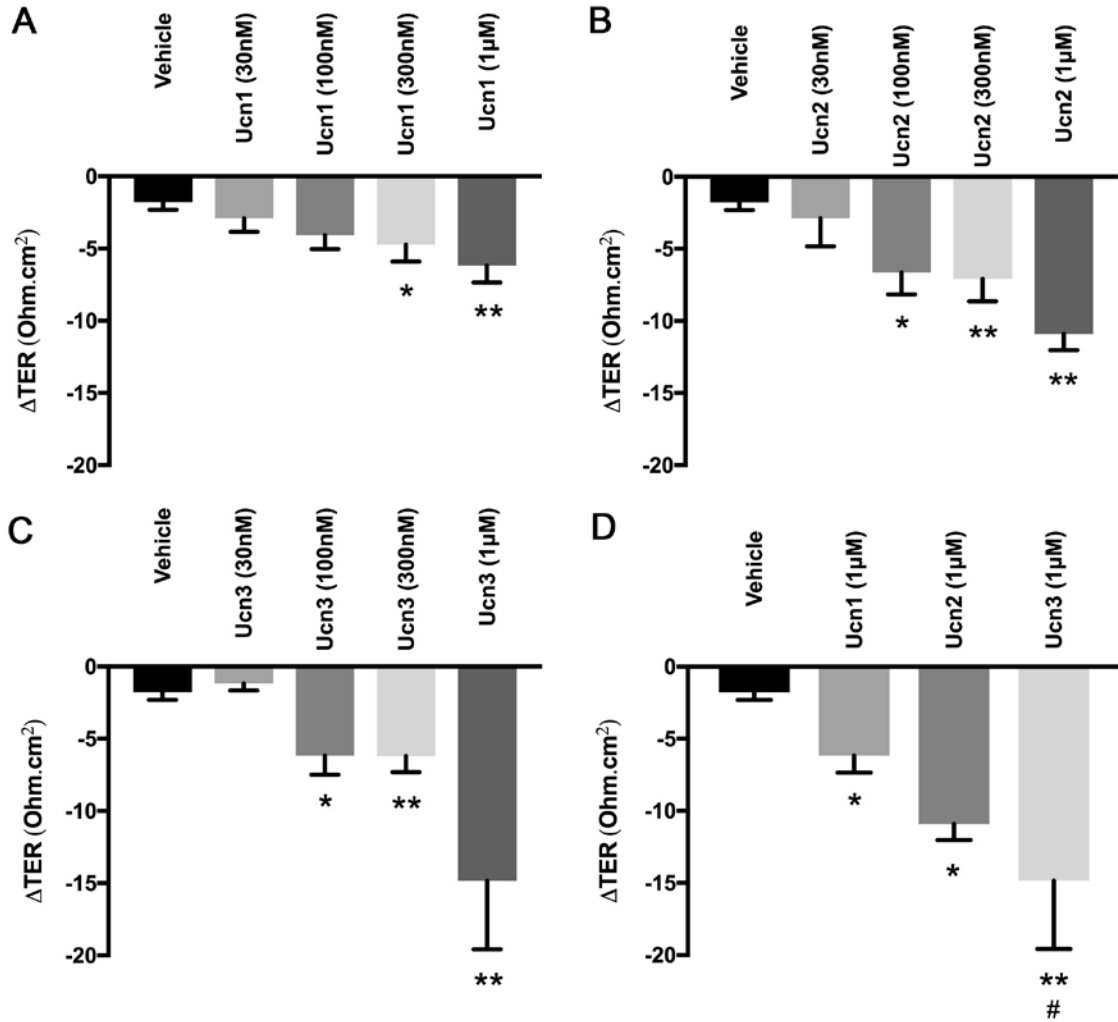


Figure 3. Effects of urocortin 1 (Ucn1), Ucn2, and Ucn3 on the transepithelial tissue resistance (TER) in the mouse proximal colon. Ucn1 (A), Ucn2 (B), and Ucn3 (C) all caused a concentration-dependent decrease of TER in the mouse proximal colon. (D) At the concentration of 1 μ M, Ucn3 caused greater decrease of TER than Ucn1. Bars represent means \pm SE; n = 5-9 tissues/group. * $P < 0.05$ vs. vehicle; ** $P < 0.01$ vs. vehicle; # $P < 0.05$ vs. Ucn1.

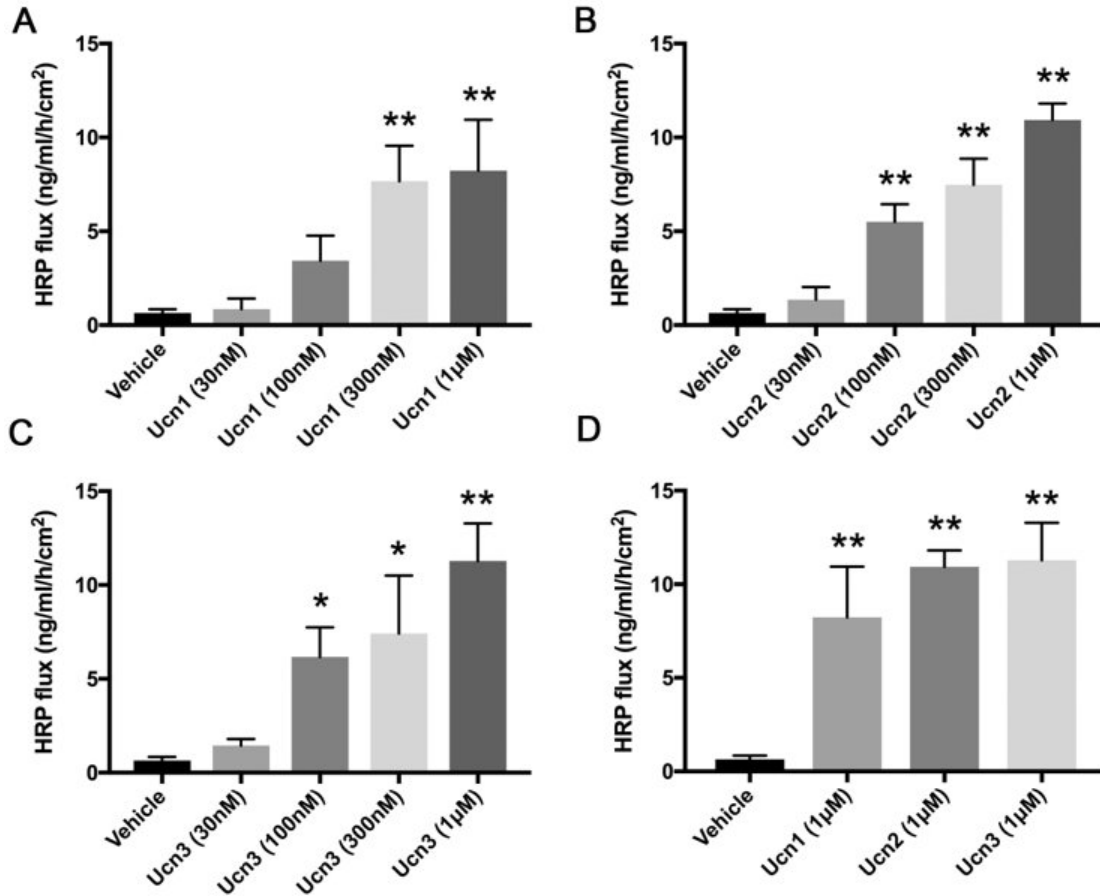


Figure 4. Effects of urocortin 1 (Ucn1), Ucn2, and Ucn3 on horseradish peroxidase (HRP) flux across the mouse proximal colonic mucosa. Ucn1 (A), Ucn2 (B), and Ucn3 (C) all caused a concentration-dependent increase of HRP flux. (D) At the concentration of 1 μ M, Ucn1, Ucn2, and Ucn3 caused similar magnitudes of increase in HRP flux. Bars represent means \pm SE; n = 5-13 tissue/group. * $P < 0.05$; ** $P < 0.01$ vs. vehicle.

Involve ment of the CRF₁ and CRF₂ Receptor Subtypes

Since Ucn1 binds to both CRF₁ and CRF₂ receptors (Hauger et al., 2003), and Ucn2 and Ucn3 selectively bind to the CRF₂ receptor subtype (Hauger et al., 2003; Stengel & Tache, 2010), involve ment of the CRF receptor subtypes in Ucn1-, Ucn2-, and Ucn3-evoked increase of colonic paracellular and transcellular permeability was investigated with the selective CRF₁ receptor antagonist NBI 27914 and the selective

CRF₂ receptor antagonist antisauvagine-30. Ucn1-induced TER reduction was inhibited by NBI 27914 and antisauvagine-30, however, the inhibition didn't reach a statistically significant level (Fig. 5). Ucn2- and Ucn3-induced TER reduction was not affected by NBI 27914, but was significantly inhibited by antisauvagine-30 (Fig.5). In addition, Ucn1-, Ucn2-, and Ucn3-induced increase of HRP flux was not affected by the CRF₁ antagonist, NBI 27914, but was significantly inhibited by the CRF₂ antagonist, antisauvagine-30. (Fig. 6).

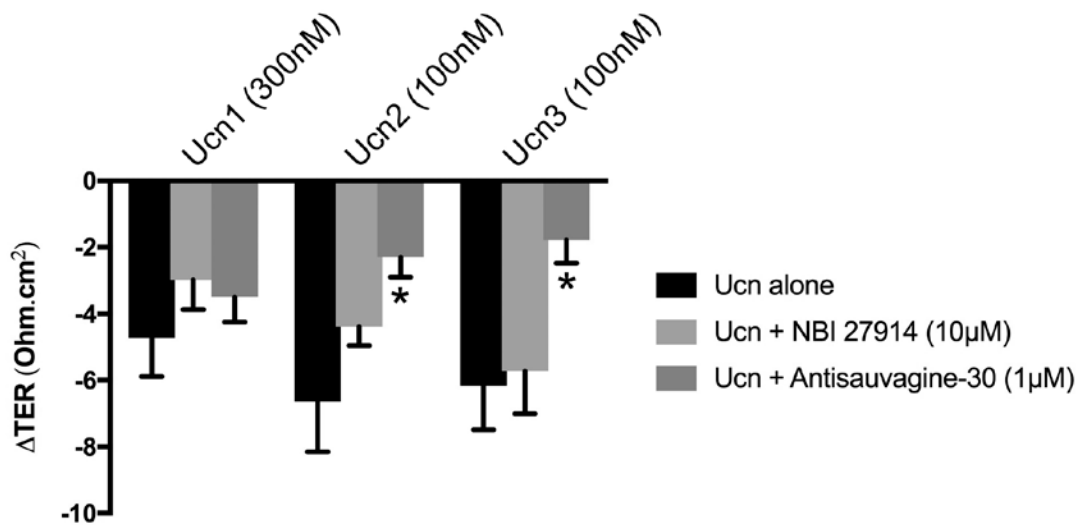


Figure 5. Effects of the corticotropin-releasing factor receptor 1 (CRF₁) and the corticotropin-releasing factor receptor 2 (CRF₂) antagonists on urocortin 1 (Ucn1), Ucn2, or Ucn3-induced suppression of transepithelial tissue resistance (TER) in the mouse proximal colon. Ucn1-induced suppression of TER was not affected by the CRF₁ antagonist, NBI 27914, or the CRF₂ antagonist, antisauvagine-30. Ucn2- and Ucn3-induced suppression of TER was not affected by NBI 27914, but was significantly inhibited by antisauvagine-30. Bars represent means \pm SE; n = 5-8 tissue/group * P < 0.05 vs. Ucn alone.

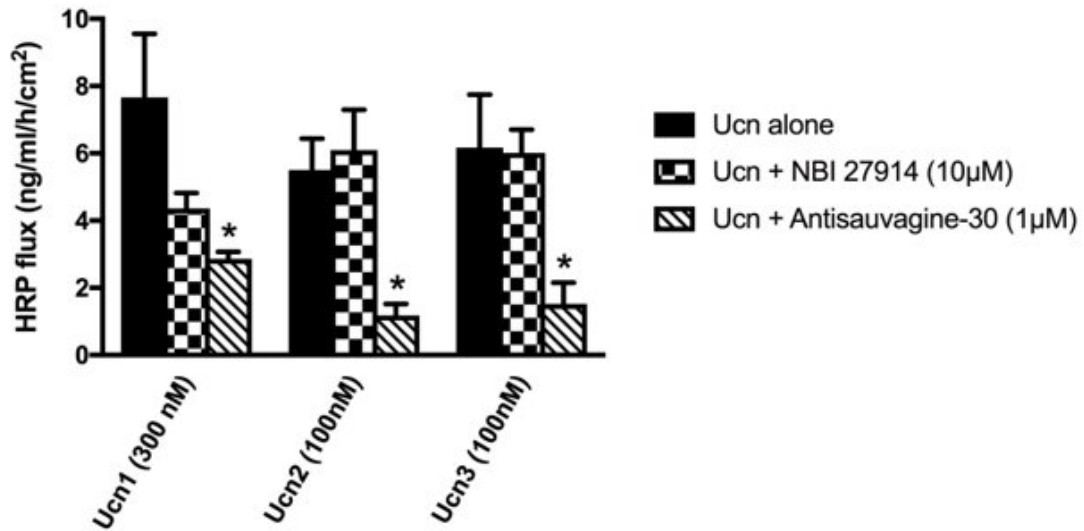


Figure 6. Effects of the corticotropin-releasing factor receptor 1 (CRF₁) and the corticotropin-releasing factor receptor 2 (CRF₂) antagonists on urocortin 1 (Ucn1), Ucn2, or Ucn3-induced increase of HRP flux across the mouse proximal colonic mucosa. Ucn1-, Ucn2-, and Ucn3-induced increase of HRP flux was not affected by the CRF₁ antagonist, NBI 27914, but was significantly inhibited by the CRF₂ antagonist, antisauvagine-30. Bars represent means \pm SE; n = 5-8 tissue/group * P < 0.05 vs. Ucn alone.

DISCUSSION

Stress causes dysfunction of the intestinal barrier. The role of peripheral CRF in stress-induced disruption of intestinal epithelial barrier function has been extensively studied (Barreau et al., 2007; Santos et al., 1999; Saunders et al., 2002; Teitelbaum et al., 2008; Vanuytsel et al., 2014; Wallon et al., 2008). Nevertheless, prior to our study, the role of CRF-related peptides, including Ucn1, Ucn2, and Ucn3, in the regulation of intestinal barrier function has not been reported. In addition, previous studies failed to reach consensus on which specific CRF receptor subtype (CRF₁ or CRF₂) is responsible for mediating stress- or CRF-induced disruption of intestinal epithelial barrier function (Barreau et al., 2007; Gareau et al., 2007; Larauche et al., 2009; Smith et al., 2010; Teitelbaum et al., 2008; Wallon et al., 2008). Here we show that Ucn1, Ucn2, and Ucn3 all induce disturbances in colonic epithelial barrier function, which mimic the effects of stress and CRF. This study also revealed that CRF₂ receptors in the colon mediate Ucn1, Ucn2, and Ucn3-induced disturbances of colonic epithelial barrier function.

Our results indicate that exogenously added Ucn1, Ucn2, and Ucn3 all caused colonic barrier dysfunction similar to that observed following exposure to stress, which includes a decrease in TER and an increase in HRP flux. TER predominantly reflects paracellular ion permeability (Clarke, 2009), which is limited by the tight junctional complex between adjacent epithelial cells. The concentration-dependent decrease in TER caused by urocortins suggests that as the level of stress increases, colonic paracellular permeability increases too, which would exacerbate IBD/IBS symptoms if occurring *in*

in vivo. Although Ucn1, Ucn2, and Ucn3 all resulted in a decrease in TER, Ucn3 caused the greatest decrease in TER, followed by Ucn2 and Ucn1 when tested at the same concentration (1 μ M), suggesting a predominant role of the CRF₂ receptors in regulating colonic paracellular permeability.

HRP is a protein (40 kD) that is commonly used as a tracer to assess transcellular transport across the intestinal epithelium in Ussing chambers (Kiliaan et al., 1998). Ucn1, Ucn2, and Ucn3 all resulted in a concentration-dependent increase in HRP flux across the colonic epithelium, and the effects of the three urocortins on HRP flux were comparable with each other. Increasing HRP flux indicates an increase in transcellular permeability which allows for macromolecules and pathogens to cross the colonic epithelial barrier into the body, which would cause IBD/IBS symptoms *in vivo*.

Ucn1 is a non-selective CRF receptor agonist and can activate both CRF₁ and CRF₂ receptor subtypes. Ucn2 and Ucn3 are selective agonists for CRF₂ receptors. The potent effects of Ucn2 and Ucn3 on TER and HRP flux across the colonic epithelium suggest a role of peripheral CRF₂ receptors in regulating both paracellular and transcellular permeability. Our experiments using the selective CRF₂ receptor antagonist, validated the role of CRF₂ receptors in colonic epithelial permeability. Ucn2- and Ucn3-induced decrease in TER and increases in HRP flux were suppressed by the addition of CRF₂ antagonist, antisauvagine-30, but were unaffected by the addition of CRF₁ antagonist, NBI 27914. Although Ucn1-induced decrease of TER was not statistically affected by NBI 27914 or antisauvagine-30, Ucn1-induced increase in HRP flux was suppressed by antisauvagine-30. Thus, it can be postulated that Ucn1, Ucn2, and Ucn3

exert their effects on colonic epithelial barrier function by activating the peripheral CRF₂ receptors in the gut.

Our studies were carried out *in vitro* on isolated mouse colonic tissues from unstressed animals. The effects of urocortins on colonic epithelial permeability were observed by adding exogenous Ucn1, Ucn2, or Ucn3 to the Ussing chambers. Thus, one of the limitations of our study is that these findings may not directly translate to the situations of stress. However, a recent study by Li et al. (2017) found that chronic stress upregulated gene expression of CRF₂ receptor and the specific CRF₂ receptor ligand Ucn2 in the pig colon along with impairment of intestinal barrier, suggesting that the effects of stress on gut permeability may be predominately mediated through the CRF₂ receptor. Future studies using CRF₂ receptor knockout mice would need to be done to validate the role of CRF₂ in stress-induced impairment of intestinal barrier function.

Many gastrointestinal disorders show sex difference. For example, IBS occurs more often in women than men. However, most preclinical animal research, including our study, has been performed on male animals exclusively. Studies have shown sex difference in stress-induced increase of intestinal permeability. Human females tend to have increased intestinal permeability to macromolecules when exposed to acute cold pain stress (Alonso et al., 2012). In addition, female mice demonstrated greater intestinal permeability in response to acute restraint stress (Mackey et al., 2016). Early weaning stress caused a greater increase in intestinal permeability in female pigs compared with male pigs (Pohl et al., 2017). All these studies suggest that biological sex is an important factor that determines the susceptibility to stress-induced disruption of intestinal barrier function. Further research to elucidate differences between male and female mice in

baseline intestinal permeability and its responses to stress or stress-related peptides would provide valuable information in determining sex predisposition to IBD/IBS.

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