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## Stress and corticotropin releasing factor (CRF) promote necrotizing enterocolitis in a formula-fed neonatal rat model

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<b>Abstract:</b>	<p>The etiology of necrotizing enterocolitis (NEC) is not known. Alterations in gut microbiome, mucosal barrier function, immune cell activation, and blood flow are characterized events in its development, with stress as a contributing factor. The hormone corticotropin-releasing factor (CRF) is a key mediator of stress responses and influences these aforementioned processes. CRF signaling is modulated by NEC's main risk factors of prematurity and formula feeding. Using an established neonatal rat model of NEC, we tested hypotheses that: (i) increased CRF levels—as seen during stress—promote NEC in formula-fed newborn rats, and (ii) antagonism of CRF action ameliorates NEC. Newborn pups were formula-fed to initiate gut inflammation and randomized to: no stress, no stress with subcutaneous CRF administration, stress (acute hypoxia followed by cold exposure—NEC model), or stress after pretreatment with the CRF peptide antagonist Astressin. Dam-fed unstressed and stressed littermates served as controls. NEC incidence and severity in the terminal ileum were determined using a histologic scoring system. Changes in CRF, CRF receptor (CRFRs), and toll-like receptor 4 (TLR4) expression levels were determined by immunofluorescence and immunoblotting, respectively. Stress exposure in FF neonates resulted in 40.0% NEC incidence, whereas exogenous CRF administration resulted in 51.7% NEC incidence compared to 8.7% in FF non-stressed neonates (<math>p &lt; 0.001</math>). Astressin prevented development of NEC in FF-stressed neonates (7.7% vs. 40.0%; <math>p = 0.003</math>). CRF and CRFR immunoreactivity increased in the ileum of neonates with NEC compared to dam-fed controls or FF unstressed pups. Immunoblotting confirmed increased TLR4 protein levels in FF stressed (NEC model) animals vs. controls, and Astressin treatment restored TLR4 to control levels. Peripheral CRF may serve as specific pharmacologic target for the prevention and treatment of NEC.</p>
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3  
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## 39 Abstract

40 The etiology of necrotizing enterocolitis (NEC) is not known. Alterations in gut microbiome,  
41 mucosal barrier function, immune cell activation, and blood flow are characterized events in its  
42 development, with stress as a contributing factor. The hormone corticotropin-releasing factor  
43 (CRF) is a key mediator of stress responses and influences these aforementioned processes. CRF  
44 signaling is modulated by NEC's main risk factors of prematurity and formula feeding. Using an  
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46 seen during stress—promote NEC in formula-fed newborn rats, and (ii) antagonism of CRF action  
47 ameliorates NEC. Newborn pups were formula-fed to initiate gut inflammation and randomized  
48 to: no stress, no stress with subcutaneous CRF administration, stress (acute hypoxia followed by  
49 cold exposure—NEC model), or stress after pretreatment with the CRF peptide antagonist  
50 Astressin. Dam-fed unstressed and stressed littermates served as controls. NEC incidence and  
51 severity in the terminal ileum were determined using a histologic scoring system. Changes in  
52 CRF, CRF receptor (CRFRs), and toll-like receptor 4 (TLR4) expression levels were determined  
53 by immunofluorescence and immunoblotting, respectively. Stress exposure in FF neonates  
54 resulted in 40.0% NEC incidence, whereas exogenous CRF administration resulted in 51.7% NEC  
55 incidence compared to 8.7% in FF non-stressed neonates ( $p < 0.001$ ). Astressin prevented  
56 development of NEC in FF-stressed neonates (7.7% vs. 40.0%;  $p = 0.003$ ). CRF and CRFR  
57 immunoreactivity increased in the ileum of neonates with NEC compared to dam-fed controls or  
58 FF unstressed pups. Immunoblotting confirmed increased TLR4 protein levels in FF stressed  
59 (NEC model) animals vs. controls, and Astressin treatment restored TLR4 to control levels.  
60 Peripheral CRF may serve as specific pharmacologic target for the prevention and treatment of  
61 NEC.

62



## 63 **Introduction**

64 Necrotizing enterocolitis (NEC) is the most common fatal gastrointestinal (GI) disease affecting  
65 premature infants in the developed world [1]. The incidence is 0.3 – 2.4 cases of NEC for every  
66 1,000 live births [2], corresponding to annual costs ranging between \$500 million to \$1 billion in  
67 the United States [3]. No specific therapy is available to treat NEC. Nearly one half of patients  
68 afflicted with NEC develop complications requiring surgical intervention; of these, approximately  
69 50% die [1]. Overall mortality has remained unchanged over the past 30 years [3]. Survivors face  
70 ongoing morbidity due to malnutrition, recurrent small bowel obstructions, liver failure, and  
71 neurocognitive deficits [1, 4].

72  
73 The major processes implicated in NEC's pathogenesis include abnormal bacterial colonization  
74 [5, 6], intestinal barrier dysfunction [4, 7-12], overzealous inflammation [11-16], and ischemia due  
75 to vasoconstriction [17-20]. While these have been well-characterized, their temporal and cause-  
76 effect relationships during NEC's development remain undefined. Widely accepted risk factors  
77 for NEC include prematurity, history of enteral formula feeding [2], and physiologic stress [4, 7].  
78 Protective factors include breast-feeding [4], administration of probiotics [21], and corticosteroid  
79 administration [4, 22-24].

80  
81 The peptide hormone corticotropin-releasing factor (CRF), the related urocortin (UCN) peptides,  
82 and their cognate CRF receptors (CRFRs) may play important roles in NEC's development. In  
83 mammals, CRF synthesis and secretion from the hypothalamus into the portal circulation initiates  
84 the response to physiologic and psychologic stress as a part of the hypothalamic-pituitary-adrenal  
85 (HPA) axis [25]. CRFRs (CRF<sub>1</sub> and CRF<sub>2</sub>) are expressed ubiquitously in several cell types and

86 organs [25], and are secreted into the plasma in extracellular vesicles [26]. CRF<sub>1</sub> is predominantly  
87 found in the brain, and its activation by CRF initiates the HPA axis response. CRF<sub>2</sub> is  
88 predominantly present in the periphery, and its activation by UCN1-3 returns stress responses back  
89 to homeostasis by facilitating negative feedback of the HPA axis [27, 28]. Through autocrine,  
90 paracrine, and endocrine mechanisms, CRF and urocortins act via CRFRs to elicit peripheral organ  
91 effects. Spatio-temporal activation of CRFRs and their ligands is nuanced and critical for disease  
92 development and progression [29]. Activation of CRF<sub>1</sub> is associated with pro-inflammatory  
93 events, whereas activation of CRF<sub>2</sub> is associated with anti-inflammatory effects in the GI tract as  
94 well as in mast cells [30].

95

96 Several studies have demonstrated that components of the CRF system modulate GI motility,  
97 barrier function, and inflammation [31-36]; these events also contribute to NEC's pathogenesis.  
98 Luminal bacteria are necessary for NEC to occur [4]. CRF is associated with alterations in luminal  
99 bacterial colonization. Increased levels of endogenous CRF and its exogenous administration are  
100 associated with inhibited small bowel peristalsis [37, 38], altered secretion of luminal mucin [39]  
101 and gastric acid [40], and increased bacterial adherence to epithelial surfaces [39]. These result in  
102 bacterial overgrowth, loss of commensal bacterial species, and selection of pathogenic gram-  
103 negative and gas-forming bacterial organisms in the gut lumen [39, 41-43]. These luminal  
104 defenses have been found deficient in NEC [7, 12, 44], and similar shifts in the luminal  
105 microbiome are characteristic of NEC, in both experimental [5] and clinical [45] settings. In  
106 addition, CRF increases gut barrier permeability via increased expression of toll-like receptor 4  
107 (TLR4) on enterocyte and immunocyte membranes [46, 47]. Downstream effects of TLR4  
108 activation by bacterial endotoxin have been well characterized in NEC [1, 15], and include

109 elaboration of pro-inflammatory cytokines [6], compromise of epithelial tight junctions [2],  
110 enterocyte apoptosis [9], and inhibition of enterocyte migration and restitution [48]. Independent  
111 of its TLR4-related actions, CRF serves as a chemoattractant and activator of mast cells and other  
112 immunocytes. Reciprocal release of nerve growth factor (NGF) from immunocytes promotes  
113 innervation of these cells by the enteric nervous system (ENS), such that subsequent stress-induced  
114 CRF signaling by the ENS sustains a pro-inflammatory state [49-53]. CRF's actions in mast cells  
115 are mediated largely by CRF<sub>2</sub> [30]. Finally, CRF contributes to local vasoconstriction and  
116 enterocyte ischemia by promoting endothelin release, along with decreased endothelial nitric oxide  
117 synthase (eNOS) activity [54, 55]. Ischemic changes seen in NEC were traditionally attributed to  
118 asphyxia and hypoxic events [56]; however, more recent work suggests that NEC's ischemic  
119 insults stem from altered local endothelin-to-nitric oxide ratios [17-19] and endothelin receptor  
120 expression [20] favoring vasoconstriction, with resultant ischemia-reperfusion injury.

121  
122 In addition, clinical factors that affect NEC's incidence are key modulators of CRF signaling. CRF  
123 signaling decreases with administration of probiotics [39, 41, 43], and CRF activity is subject to  
124 negative feedback control by corticosteroids [57]. In contrast, intestinal CRF activity increases in  
125 response to maternal separation and the transition from breast feeding to formula feeding [35, 58,  
126 59]. Endogenous CRF and CRFR levels increase within the GI tract in response to inflammation  
127 [31] and stressful stimuli [52]. Newborn animals appear to be more vulnerable to these changes  
128 [57, 60]. This vulnerability may be accentuated in the setting of prematurity due to compromised  
129 feedback control from an immature HPA axis [57].

130

131 Given the parallels between peripheral CRF pathways and what we know about NEC, it is  
132 attractive to postulate a key role for CRF in NEC's pathogenesis. Peptide-based CRF inhibitors  
133 do not cross the blood-brain barrier and tend to have negligible effects on either central nervous  
134 system or HPA axis function. They do not appear to affect normal GI function. Thus,  
135 pharmacologic inhibition of overstimulated peripheral CRF signaling seems to offer promise for  
136 the prevention and treatment of NEC. In this proof-of-concept study, we sought to test the  
137 hypothesis that over expression of peripheral CRF activation is key for development of  
138 experimental NEC. We utilized a well-described neonatal rat model consisting of formula feeding  
139 and exposure to hypoxia and cold stress.

140

## 141 **Materials and Methods**

### 142 **Materials**

143 All chemicals were purchased from known vendors. CRF peptide and peptide antagonist,  
144 Astressin (AST) were purchased from American Peptide Company, Sunnyvale, CA.

145

### 146 **Animals**

147 Animal experiments were performed using neonatal Sprague Dawley rats (Charles River, Pontage,  
148 MI) and were approved by the Institutional Animal Care and Use Committee of Children's  
149 Hospital Oakland Research Institute. Neonates were delivered spontaneously from timed-  
150 pregnant female rats. Neonates (weight 6-10 g) were randomized into six treatment and two  
151 control groups on postnatal day 3: Group 1: dam-fed unstressed (DF, n = 22); Group 2: dam-fed  
152 **stressed** (DFS, n = 26); Group 3: formula-fed, unstressed (FF, n = 23); Group 4: formula-fed

153 stressed (NEC, n = 25); Group 5: formula-fed unstressed with 30µg/kg CRF administration (CRF,  
154 n = 27); Group 6: formula-fed stressed with 60µg/kg Astressin administration (AST, n = 26).  
155 Neonates in Groups 1 and 2 were housed with a dam and allowed *ad libitum* nursing (Fig.1).

156

## 157 **Experimental model of necrotizing enterocolitis (NEC)**

158 Experimental NEC was induced as described previously, with a protocol that consisted of maternal  
159 separation, formula feeding, and exposure to hypoxia and cold stress [6, 61, 62]. Briefly, groups  
160 of formula-fed neonates (FF, NEC, CRF, and AST group) were separated from dams on postnatal  
161 day 3 and housed with other members of their treatment group in a temperature-controlled  
162 incubator (34°C). They were gavage fed six times daily with approximately 12.5µL/g body weight  
163 (80-200µL) of a special rodent formula consisting of 15g Similac 60/40 in 75 mL Esbilac canine  
164 milk replacement (Ross Pediatrics, Columbus, OH and Pet-Ag, Hampshire, IL, respectively).  
165 Stress sessions (DFS, NEC, and AST groups) took place twice daily, and consisted of exposure to  
166 a 100% N<sub>2</sub> atmosphere (hypoxia) in a modular incubation chamber (Billups-Rothenberg, Del Mar,  
167 CA) for 60 seconds, followed by exposure to 4°C for 10 minutes (cold stress). Animals were  
168 returned to their incubators immediately after stress sessions (Fig. 1). CRF (30µg/kg) and  
169 Astressin (60µg/kg) in 100µL sterile water were administered twice-daily as subcutaneous  
170 injections. Astressin injections were performed 30 minutes before exposure to hypoxia and cold  
171 stress. Experimental conditions were applied for 48 hours, after which animals were euthanized  
172 and specimens collected. Animals that were injured from aspiration of formula feeds or presumed  
173 gastric or esophageal injury were immediately euthanized and excluded from analysis.

174

175 **Figure 1 Legend. Experimental model of NEC- a timeline.**

176 Neonates were housed with dams for 3 days before being randomized at postnatal day (PND) 3  
177 into 6 groups as shown. Dam-fed controls were randomized to no stress (DF) or hypoxia / cold  
178 stress exposure (DFS), housed with a dam, and allowed *ad libitum* feeding. Formula-fed neonates  
179 were separated from their dams and housed in an incubator. They were randomized to no stress  
180 (FF), hypoxia / cold stress exposure (NEC), no stress with CRF administration (CRF), and hypoxia  
181 / cold stress exposure after pretreatment with Astressin (AST).

182

## 183 **Histology**

184 The GI tract was removed intact from euthanized pups, linearized, and gently flushed with 1mL  
185 of sterile phosphate-buffered saline (10mM PBS, pH 7.4). 40mm of terminal ileum was removed.  
186 The distal 20mm was flushed with fresh fixative (4% paraformaldehyde in PBS with 5% sucrose)  
187 and immersed in room-temperature fixative for 6 hours. The proximal 20mm was snap-frozen in  
188 liquid nitrogen and stored at -80°C for subsequent analysis (see below). Fixed specimens were  
189 rinsed and dehydrated in serial dilutions of ethanol in PBS (5, 10, 20, 50, and 70% ethanol),  
190 processed, embedded in paraffin, and sectioned at 5µm for microscopic analysis by the Mouse  
191 Pathology Core of the Helen Diller Cancer Center at the University of California at San Francisco.  
192 Sections were stained with hematoxylin and eosin (H&E) for light microscopy analysis. Paraffin-  
193 embedded slides were stored at room temperature for further analysis.

194

## 195 **Analysis of mucosal injury**

196 Mucosal injury and presence of NEC were assessed using 5µm H&E-stained sections of intestine  
197 ~~by researchers (GW and RB)~~ blinded to the treatment groups. Pathologic changes in intestinal  
198 architecture were evaluated via a NEC scoring system developed for use in neonatal rats [61, 62].

199 Histologic changes in ileum were scored on a scale of 0-3; 0 = normal, 1 = mild inflammation,  
200 separation of the villous core without other abnormalities, 2 = moderate inflammation, villous core  
201 separation, submucosal edema, and epithelial sloughing, and 3 = severe, denudation of epithelium  
202 with loss of villi, full-thickness necrosis, or perforation. Animals with histologic scores  $\geq 2$  were  
203 defined as having developed NEC (Fig. 2).

204

205 **Figure 2 Legend. Mucosal injury and NEC scoring system.** Representative H&E-stained  
206 terminal ileum sections to show mucosal injury consistent with NEC. (a) Normal intestinal  
207 mucosa: score 0. (b) Mild inflammation: score 1. (c) Moderate inflammation consistent with  
208 NEC: score 2. (d) Severe inflammation consistent with NEC: score 3.

209

## 210 **Antibodies**

211 Primary antibodies: The primary and secondary antibodies, dilutions used, and sources were as  
212 follows: Antibodies from Santa Cruz Biotechnology, Santa Cruz, CA: CRFR<sub>1/2</sub> (sc-1757; goat;  
213 1:500) [63],  $\beta$ -actin (A2228; mouse; 1:5,000) [29], TLR4 (M16) (sc-12511; goat; 1:1000), and  
214 CRF (rabbit; 1:5,000; Courtesy of Prof. W. Vale:) were used. Secondary antibodies: For  
215 immunofluorescence staining goat anti-rabbit conjugated to Rhodamine Red-X or FITC (Jackson  
216 ImmunoResearch) at 1:500 dilution was used. For Western blot analyses donkey anti-goat/rabbit  
217 conjugated to Alexa Fluor 680 (Thermo Fisher Scientific) and donkey anti-mouse conjugated to  
218 IRDye 800 (Rockland Immunochemicals, Pottstown, PA) at 1:20,000 dilution were used.

219

## 220 **Western Blot Analysis**

221 Ileum tissue samples were homogenized in RIPA buffer supplemented with protease inhibitor  
222 cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktails (Sigma-Aldrich). Total  
223 protein concentration was determined using the Bradford assay with bicinchoninic acid (BCA)  
224 reagent (Millipore Sigma, St. Louis, MO). Total protein (40 µg) was resolved by 10% SDS-PAGE,  
225 transferred to polyvinylidene difluoride membranes (PVDF, Immobilon-FL; Millipore, Billerica,  
226 MA) and blocked for 1 h at room temperature in Odyssey Blocking Buffer (Li-COR Biosciences,  
227 Lincoln, NE). Membranes were incubated with primary antibodies overnight at 4°C. Membranes  
228 were washed for 30 min (1 × PBS, 0.1% Tween20) and incubated with secondary antibodies for 1  
229 h at room temperature. Blots were analyzed and quantified with the Odyssey Infrared Imaging  
230 System.

231

## 232 **Immunofluorescence and microscopy**

233 Terminal ileum sections (5µM thick) from experimental and control groups were deparaffinized  
234 in xylene and rehydrated in ethanol series. Sections were incubated in blocking buffer containing  
235 1x PBS, 0.3% Triton X-100, 10% normal goat serum for 1 h at room temperature followed by  
236 incubated with primary antibodies (anti-CRF and anti-CRF<sub>1/2</sub>) overnight at 4°C. Sections were  
237 washed and incubated with fluorescent secondary antibodies (conjugated to Rhodamine Red-X  
238 and FITC) for 1 h at room temperature. Images were acquired using an epi-fluorescence  
239 microscope (20x and 40x objectives) and images were captured using AxioVision Imaging  
240 software.

241

## 242 **Statistical analysis**



243 The incidence of NEC was determined for each treatment group and expressed as percentage  $\pm$   
244 standard error. Groups were compared using directional Chi square analysis and Fischer's exact  
245 test using Stata SE software (StatCorp; College Station, TX). *P* values  $< 0.05$  were considered  
246 statistically significant. All data are representative of at least three independent experiments  
247 (biological replicates), involving 12 litters of neonatal rats.

248

## 249 **Results**

### 250 **Formula feeding by itself causes mild inflammation in neonates**

251 Stress in dam-fed neonates or formula feeding in absence of other factors may not be sufficient to  
252 cause overt NEC-like disease in rodents or humans. To test this notion, neonates were left in their  
253 home cages with dams without any handling or stress (dam-fed; DF) or exposed to acute hypoxia  
254 and cold stress (DFS). As expected, terminal ileum histology was normal in DF pups and after 72  
255 hours of exposure to stressors, none (0/26) of the pups in DFS group developed histologic findings  
256 consistent with NEC (Fig. 3a-b, g). Formula feeding without stress exposure (FF) resulted in mild  
257 inflammatory changes in the terminal ileum with 17 of 23 pups demonstrating mild inflammation  
258 with vacuolization of villi and two of 23 ( $8.7 \pm 5.9\%$ ) developed NEC (Fig. 3c, g).

259

### 260 **Formula feeding combined with hypoxia and cold stress exposure** 261 **causes NEC-like changes in the gut morphology**

262 We confirmed that formula feeding combined with acute exposure to hypoxia and cold stress  
263 (NEC) over 48 hours caused overt changes in gut histopathology. Moderate to severe  
264 inflammatory changes occurred in 10 of 25 pups ( $40.0 \pm 9.8\%$ ) animals in the NEC group, with

265 nearly all specimens demonstrating some degree of inflammatory change (23 of 25 pups; Fig. 3d,  
266 g). Histopathological changes were also accompanied by gross changes in the small bowel that  
267 showed erythema, edema, full thickness necrosis, and perforation in pups with NEC compared  
268 with DF unstressed controls (Fig. 4a). Gross changes observed in rat gut were similar to those  
269 seen in preterm newborn human infants with complicated NEC (Fig. 4b).

270

271 **Figure 3 Legend. Formula feeding and exposure to acute stressors causes frank NEC-like**  
272 **histopathological damage in rats.** Representative H&E-stained micrographs showing villi  
273 damage in NEC. (a-c) Terminal ileum sections of DF, DFS, and FF unstressed pups showed  
274 normal gut histology with well-preserved villi structure. (d) Inflammatory changes were present  
275 in terminal ileum of FF neonates exposed to stressors (NEC) or (e) FF unstressed pups with CRF  
276 administration. (f) Pretreatment with Astressin in FF pups prevented stress-induced changes in  
277 ileum histopathology and prevented development of NEC. DF = dam-fed, unstressed; DFS = dam  
278 fed, stressed; FF = formula-fed unstressed; NEC = formula-fed, stressed; CRF = formula-fed,  
279 unstressed with 30µg/kg of sc CRF administration; AST = formula-fed, stressed with 60µg/kg of  
280 sc CRF antagonist, Astressin administration. Scale Bar = 100µM. (g) Stack bar graph  
281 summarizing numbers of neonates with 0-3 scores within control or treatment groups.

282

283 **Figure 4 Legend. Experimental model of necrotizing enterocolitis.** (a) Formula feeding  
284 combined with hypoxia and cold stress exposure induced gross changes in the small bowel  
285 including erythema, edema, and full thickness necrosis. (b) Gross intra-operative findings  
286 representative of complicated NEC in a premature human neonate.

287

288 **CRF and CRF receptor expression is increased after induction of**  
289 **NEC**

290 We next asked if NEC is associated with increased expression of CRF and its receptors in the  
291 terminal ileum. Immunofluorescence staining revealed diffuse and low levels of CRF and CRFR  
292 immunoreactivity (CRF-IR and CRF<sub>1/2</sub>-IR, respectively) in the dam-fed unstressed group (Fig. 5a;  
293 DF). Exposure to hypoxia and cold stress in dam-fed animals increased CRF-IR in the villi (Fig.  
294 5b; DFS). CRF and CRFRs co-localized in the ileum (Fig. 5, Merge). In the formula-fed  
295 unstressed group, again CRF-IR co-localized with its receptors along the basolateral aspects of  
296 villous enterocytes, along with some staining within villi and in the submucosal and myenteric  
297 plexuses (Fig. 5c; FF). Induction of NEC resulted in clear, discrete and multiple points of co-  
298 localization of CRF-IR with its receptors within submucosal and myenteric plexuses, and in the  
299 villi (Fig. 5d; NEC), and omission of primary antibody (negative control) did not show any signal  
300 (Fig. 5e). Analysis of the sections at higher magnification revealed CRF-IR and CRF<sub>1/2</sub>-IR co-  
301 localization in the villus tip with little to no staining in the neurons of the submucosal or myenteric  
302 plexuses in DF control neonates. In NEC neonates, CRF-IR and CRF<sub>1/2</sub>-IR expression increased  
303 in the villi and around the center corresponding to the location of enteric neurons coursing  
304 alongside blood vessels, and was diffused and disorganized. Expression was also clearly evident  
305 in the neurons of the plexuses (Fig. 5f). This finding suggests an association between the  
306 development of NEC and increased CRF and CRFR expression within the enteric nervous system  
307 as well as the enterocytes.

308  
309 **Figure 5 Legend. CRF and CRF receptor immunoreactivity (IR) is increased in the terminal**  
310 **ileum of neonates with NEC.** (a-e) Representative immunostained section from DF, DFS, FF

311 and NEC ileum. CRF-IR (red) and CRFR (CRF<sub>1/2</sub>-IR, green) was evident in the villi, blood  
312 vessels, and neurons within the myenteric plexuses of FF and NEC groups, but only low, diffuse  
313 staining was seen in DF and DFS control groups. (f) Higher magnification (63x) confocal images  
314 revealed differences in staining pattern in CRF-IR and CRF<sub>1/2</sub>-IR in DF controls versus NEC  
315 groups.

316

### 317 **Exogenous CRF promotes NEC-like changes in the gut morphology**

318 Having confirmed our hypothesis that hypoxia and cold stress exposure (NEC group) results in  
319 increased levels of CRF, we asked if CRF alone is sufficient to initiate NEC-like disease. We  
320 administered CRF in formula-fed unstressed mice instead of hypoxia and cold stress exposure  
321 (CRF group). As predicted,  $51.9 \pm 9.6\%$  (14 of 27) of neonates developed NEC (Fig. 3a-b). This  
322 increase in incidence reached statistical significance compared to DF, DFS, and FF groups ( $p <$   
323  $0.001$ , Fig. 6).

324

325 **Figure 6 Legend. Incidence of necrotizing enterocolitis among treatment groups.** The  
326 incidence of NEC was determined for each treatment group and expressed as percentage  $\pm$  standard  
327 error. Groups were compared using directional Chi square analysis and Fischer's exact test. \*: FF  
328 versus NEC groups,  $p = 0.006$ ; \*\*: FF versus CRF groups,  $p < 0.001$ ; #: NEC versus AST groups,  
329  $p = 0.033$ . DF = dam-fed, unstressed; DFS = dam fed, stressed; FF = formula-fed unstressed; NEC  
330 = formula-fed, stressed; CRF = formula-fed, unstressed with  $30\mu\text{g}/\text{kg}$  of sc CRF administration;  
331 AST = formula-fed, stressed with  $60\mu\text{g}/\text{kg}$  of sc CRF antagonist, Astressin administration.

332

333 **CRF antagonism with Astressin prevents development of NEC-like**  
334 **changes in the gut morphology**

335 We reasoned if exogenous CRF was sufficient to promote NEC-like inflammation and gross gut  
336 edema, antagonism of CRF even in formula-fed neonates exposed to hypoxia and cold stress  
337 should ameliorate these changes. As predicted, **AST** administration abrogated development of  
338 NEC in 92% of the pups with 24 of 26 pups showing no or low-grade inflammation (score 0-1,  
339 Fig. 3). AST treatment significantly reduced NEC incidence to  $7.7 \pm 5.2\%$  (2 of 26) versus  $51.9$   
340  $\pm 9.6\%$  (14 of 27) in the CRF group and  $40.0 \pm 9.8\%$  (10 of 25) in the NEC group ( $p = 0.0033$ ;  
341 Fig. 6).

342

343 **Astressin treatment decreases toll-like receptor 4 (TLR4) levels**

344 CRF is known to increase expression of TLR4, which in turn causes changes in gut permeability.  
345 Next, we confirmed using western blotting that TLR4 levels were increased in ileum of neonates  
346 with NEC compared with DF unstressed controls. Since treatment with Astressin prevented  
347 development of NEC, we ascertained TLR4 levels in ileum of neonates in AST group and found  
348 expression levels to be similar to those seen in DF unstressed control group (Fig. 7). This data  
349 suggests that antagonism of CRF with Astressin was sufficient to downregulate TLR4 levels.

350

351 **Discussion**

352 Necrotizing enterocolitis is a major cause of morbidity and mortality in premature neonates.  
353 Despite being first described over 100 years ago, no specific treatments have been developed. The  
354 role of stress in the development of NEC has been established. However, little is known about the

355 role of key mediators of the stress axis—such as CRF and CRFRs—in NEC. In this study, we  
356 demonstrated that (i) stress in combination with formula-feeding, but neither alone, cause NEC-  
357 like histologic changes; (ii) over- and mis-expression of CRF is associated with the development  
358 of NEC; and (iii) CRF antagonism is sufficient to markedly decrease NEC incidence in an  
359 experimental animal model. While most studies that employed a similar NEC model,  
360 demonstrated NEC rates over 50% [6, 61, 62]; ours did not reach that level. This is likely explained  
361 by the fact that we limited our experimental conditions to 48 hours after randomization, while  
362 others applied their models over a 72-hour period.

363

364 Formula feeding is known to increase expression of the components of the CRF system, whereas  
365 administration of probiotics, breast milk, and corticosteroids decrease their expression [39, 41, 43,  
366 57]. Spatio-temporal activation of CRFRs and their ligands is nuanced and critical for  
367 development of several GI disorders that include inflammatory bowel disease and functional GI  
368 diseases [29]. Stress-induced alterations in GI motility and diarrhea are well described, and gut-  
369 specific elimination of CRF ameliorates these symptoms. Activation of CRF<sub>1</sub> is associated with  
370 pro-inflammatory events, whereas activation of CRF<sub>2</sub> is associated with anti-inflammatory effects  
371 in the GI as well as in mast cells [30]. Newborn animals appear to be more vulnerable to these  
372 changes [60]. In this study, we found increased expression of CRF in enteric neurons in the  
373 terminal ileum of rats with NEC. Co-localization of CRF and CRFR expression within submucosal  
374 and myenteric plexuses and also within the core of villi was more robust, albeit disorganized in  
375 rats with NEC, whereas organized basolateral localization was evident in the ileum of control rats.  
376 This is the first report to demonstrate upregulation of CRF immunoreactivity in the gut of rats with  
377 experimental NEC. Although previous literature has suggested that CRF activity might play a role

378 in neonatal intestinal injury and repair [64], here, we demonstrate unequivocally that stress-  
379 induced increases in CRF are sufficient to increase NEC incidence and severity.

380

381 We further demonstrated that exogenous CRF administration promotes the development of NEC  
382 even in absence of external stressors, primarily via mucosa epithelial inflammation leading to villi  
383 loss, submucosal edema, necrosis, and perforation. In support of this pro-inflammatory role of  
384 CRF, pharmacological antagonism of CRF action was protective; we found NEC incidence was  
385 decreased by ~81% in rats after CRF antagonism even in the face of formula-feeding and  
386 exogenous stressors. CRF antagonism was accompanied by less severe mucosal injury compared  
387 to rats with NEC. Previous studies have described the role of CRF activation in various  
388 inflammatory gut disorders and in NEC's key pathologic processes [37-43, 46, 47, 49-51, 53-55];  
389 here, we show that antagonizing the actions of CRF can prevent development of experimental  
390 NEC.

391

392 Alteration in intestinal barrier permeability is a hallmark of human NEC. TLR4 activation,  
393 bacterial overgrowth, and vasoconstriction are thought to promote gut ischemia and intestinal  
394 barrier permeability in NEC. Increased TLR4 expression and activation are key steps in NEC's  
395 development, and have been shown to precede overt histologic signs of inflammation in  
396 experimental NEC [6, 9, 61]. Similar to others, we found TLR4 expression was increased in ileum  
397 of rats with NEC compared with controls. CRF antagonism markedly decreased TLR4 expression.  
398 Other studies have shown contribution of CRF in modulating mast cells and gut function including  
399 motility and permeability [33, 34, 65, 66]. While this study did not ascertain the contribution of  
400 immune versus non-immune TLR4 in promoting NEC, CRFRs are present in both immune and

401 non-immune cells of the gut. Both endocrine and paracrine actions of CRF have been described  
402 in these cell types.

403

404 Stress in neonates has been shown to be associated with mucosal injury in a variety of gut  
405 disorders. CRF and CRFR activation have been shown to be key modulators in the brain-gut axis.  
406 This study demonstrates that CRF activation plays a role in the development of experimental NEC  
407 via increased receptor localization and disorganization leading to mucosal injury. These findings  
408 suggest that at least in the setting of experimental NEC, specific antagonism of CRF in the  
409 peripheral tissues ameliorates NEC's incidence and severity, and holds promise for pharmacologic  
410 prevention of this disease.

411

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413

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- 656

Figure 1.

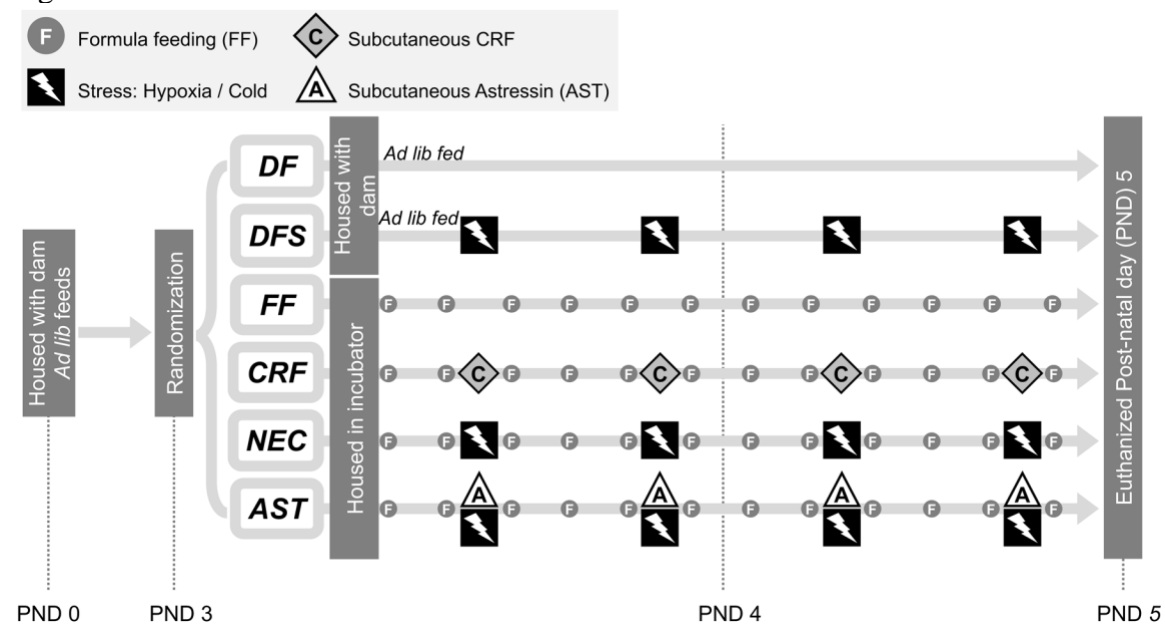


Figure 2.

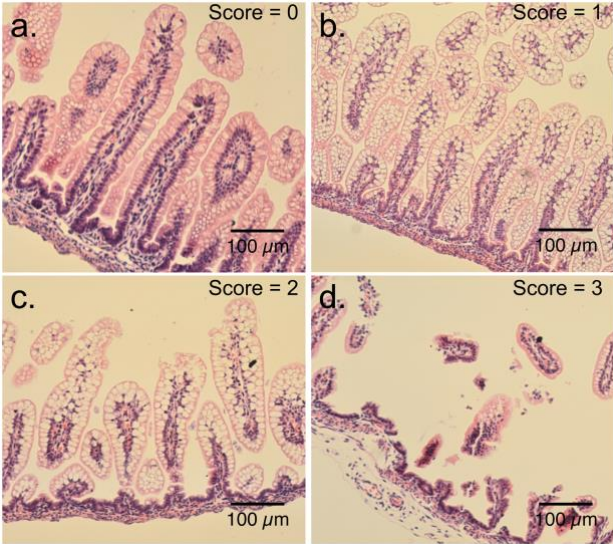




Figure 3

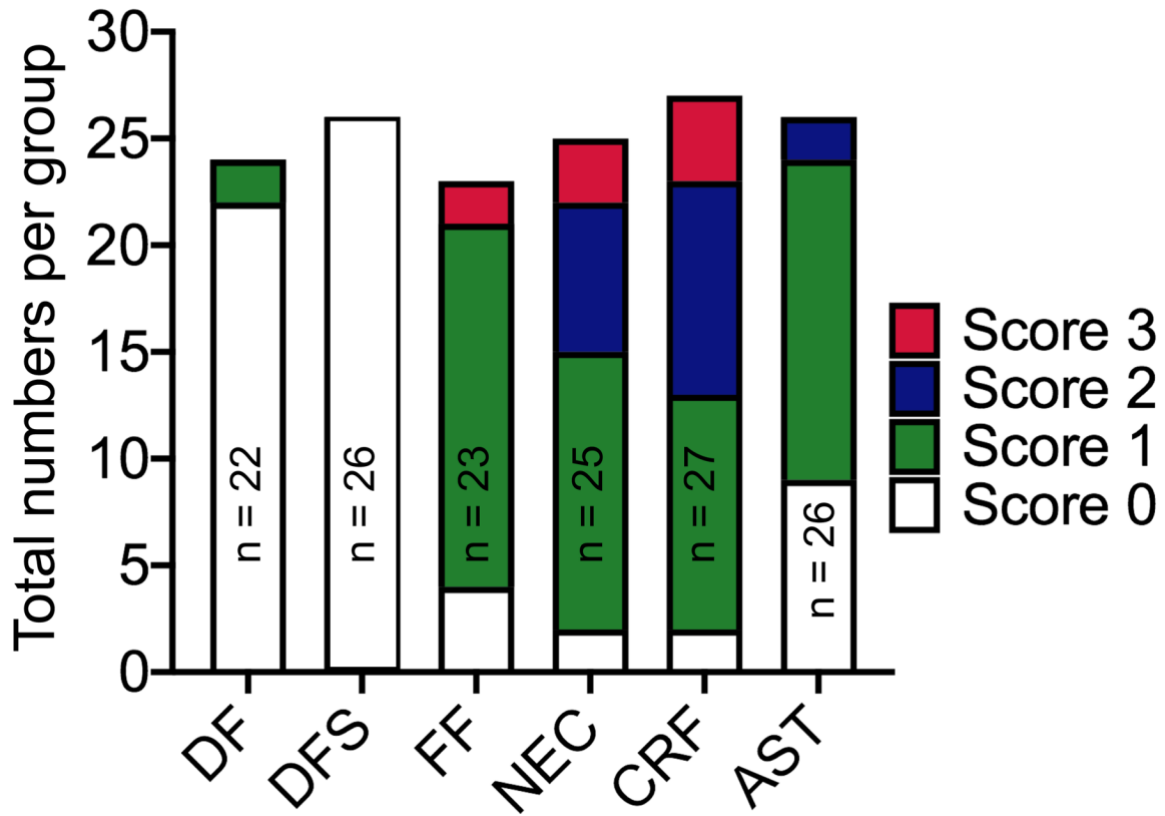
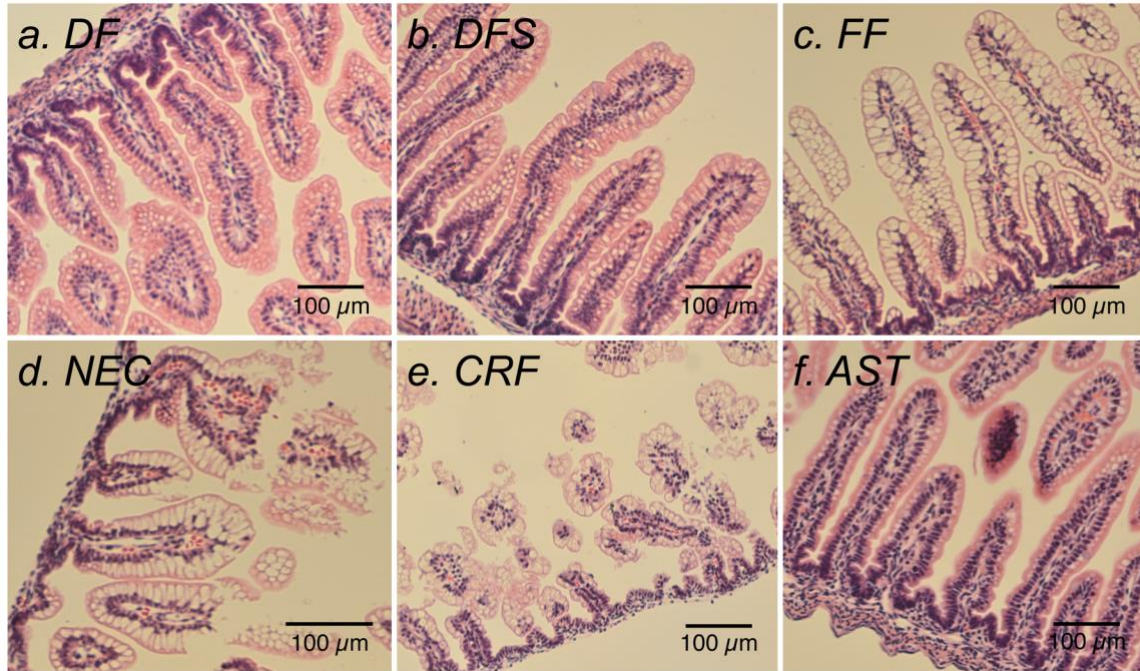
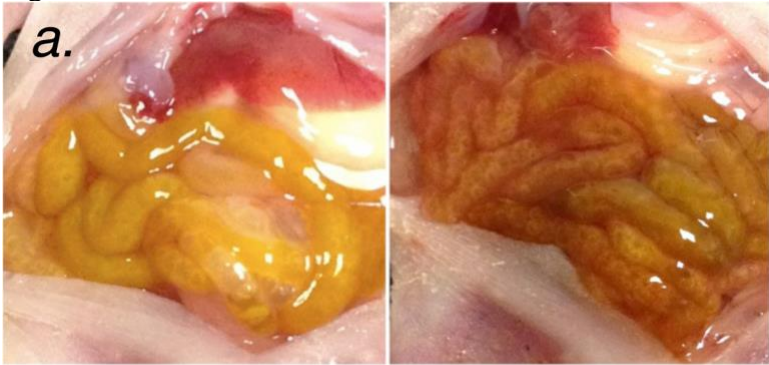


Figure 4.



*Dam-fed, unstressed (DF)*

*Formula-fed, stressed (NEC)*



Figure 5

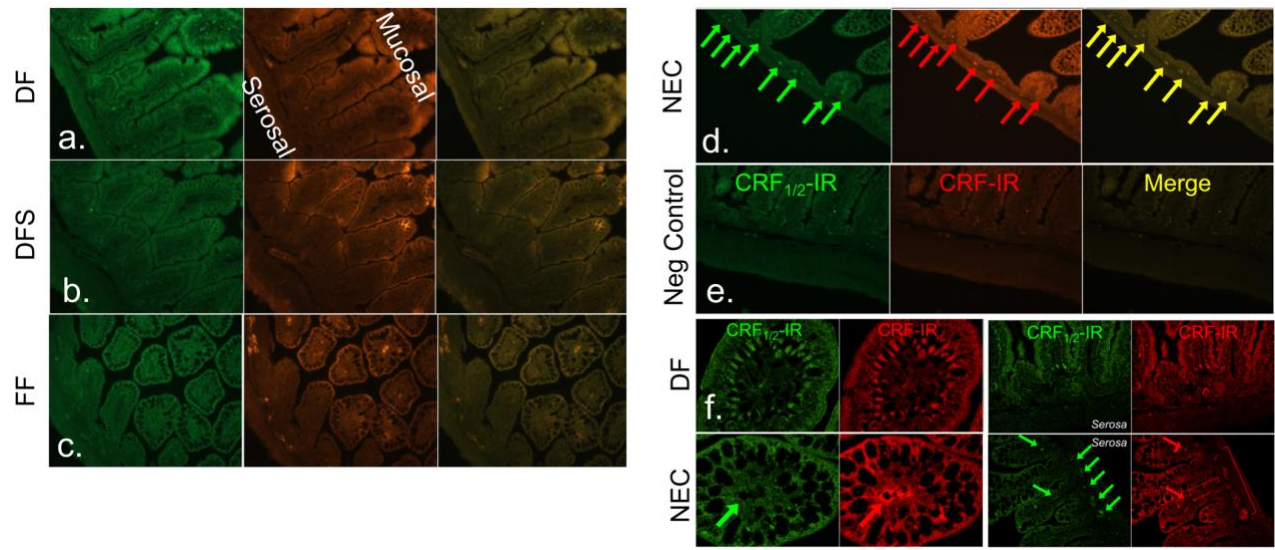


Figure 6.

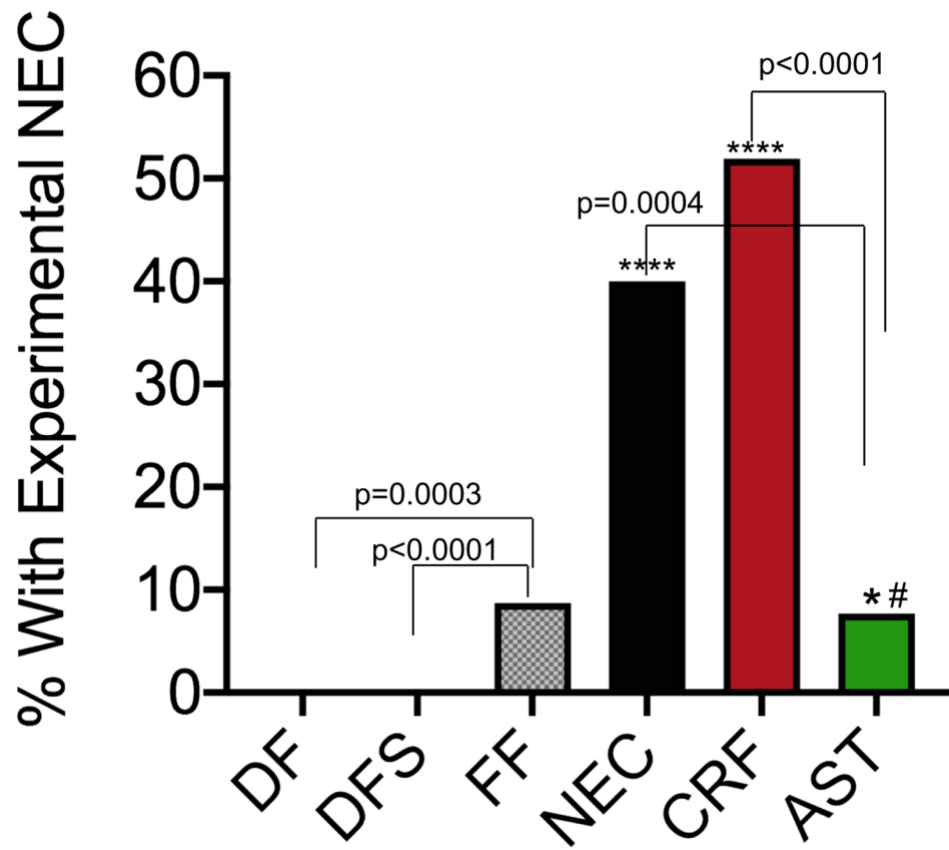


Figure 7

