Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability

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Gareau MG, Jury J, Perdue MH. Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. Am J Physiol Gastrointest Liver Physiol 293: G198–G203, 2007. First published May 17, 2007; doi:10.1152/ajpgi.00392.2006.—Neonatal maternal separation (MS) predisposes adult rats to develop stress-induced mucosal barrier dysfunction/visceral hypersensitivities and rat pups to develop colonic epithelial dysfunction. Our aim was to examine if enhanced epithelial permeability in such pups resulted from abnormal regulation by enteric nerves. Pups were separated from the dam for 3 h/day (days 4–20); nonseparated (NS) pups served as controls. On day 20, colonic tissues were removed and mounted in Ussing chambers. Horseradish peroxidase (HRP) flux was used to measure macromolecular permeability. HRP flux was increased in MS versus NS pups. The enhanced flux was inhibited by the cholinergic muscarinic antagonist atropine and the nicotinic antagonist hexamethonium. The cholinergic component was greater in tissues from MS versus NS pups, suggesting that increased cholinergic activity was responsible for the MS elevated permeability. Western blots and immunohistochemistry of colonic tissues demonstrated increased expression of choline acetyltransferase (Chat) in MS pups, indicating greater synthesis of acetylcholine. Since a previous study indicated that corticotropin-releasing factor (CRF) mediates barrier dysfunction in MS pups, we examined if the two pathways were linked. In MS tissues, nonselective CRF receptor antagonism inhibited the enhanced flux, and the addition of atropine did not produce further inhibition. Using selective receptor antagonists, we identified that CRF receptor 2 was involved in mediating this effect. These findings suggest that CRF, via CRF receptor 2, acts on cholinergic nerves to induce epithelial barrier dysfunction. Our study provides evidence that MS-induced defects persisted past the period of separation and appeared to be maintained by the activation of peripheral CRF receptors (CRF-Rs) (11, 37). Another recent study (10) also implicated cholinergic pathways in MS-induced epithelial abnormalities.

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Adult rats of a stress susceptible strain that are subjected to daily separation from the dam during the neonatal period demonstrate increased colonic permeability (2) and visceral hyperalgesia (28). Adult rats that are not genetically susceptible to stress can also develop similar abnormalities following exposure to a mild acute stressor if they have been subjected to neonatal MS (7, 37). We (11) previously reported that neonatal rats subjected to MS had impaired epithelial barrier function even at the time of separation. Enhanced uptake of antigens during this period might result in sensitization and/or priming for activation of immune cells upon reencounter with these antigens in later life. In our study, the MS-induced defects persisted past the period of separation and appeared to be maintained by the activation of peripheral CRF receptors (CRF-Rs) (11, 37). Another recent study (10) also implicated cholinergic pathways in MS-induced epithelial abnormalities.

The enteric nervous system (ENS) is a separate branch of the autonomic nervous system and is composed of intrinsic nerves containing various neurotransmitters including acetylcholine, monoamines, and neuropeptides. The ENS plays an important role in maintaining normal intestinal function, with neuronal signaling regulating epithelial ion transport and barrier function (8, 24, 25). ENS-stimulated ion secretion and permeability are increased in the jejunum of adult rats following exposure to acute stress (34), with the change being mediated by acetylcholine acting on muscarinic receptors (33). In addition, there is evidence that under normal conditions, baseline colonic ion transport is regulated mainly by acetylcholine in the guinea pig intestine (36). Cholinergic mechanisms have also been identified to maintain baseline epithelial physiology in the mouse jejunum (3).

To our knowledge, the role of submucosal plexus enteric nerves in MS-induced colonic barrier dysfunction has not been examined previously. Therefore, the aim of our present study was to determine if the effect of early life stress on colonic barrier dysfunction, ex vivo, in neonatal distal colonic tissue is mediated by changes in submucosal cholinergic nerves and to determine if the effects of CRF occur via the activation of cholinergic enteric nerves.

METHODS

Animals. Primiparous timed-pregnant Sprague Dawley female rats were obtained from Charles River Laboratories (St. Constant, QC, Canada) on gestational days 13 and 14. Dams were individually housed in cages lined with chip bedding on a 12:12-h light-dark cycle (lights on at 8:00 AM) with free access to food and water. The day of...
birth was considered to be day 1. All procedures were approved by the Animal Care Committee at McMaster University.

Study design. Neonatal rat pups were exposed to the MS protocol from day 4 until day 19 and killed the following day. Male and female pups were killed at the same time each day to limit the effects of circadian rhythm. Conscious rat pups were killed by decapitation, and colonic tissue was excised. Segments of the distal colon were mounted in Ussing chambers for permeability experiments. Additional tissue was collected and processed for Western blot analysis and immunohistochemistry.

Maternal separation. Dams and their litters (culled to 12 pups) were randomly assigned to the MS protocol or to the control non-separated (NS) protocol. MS pups were individually separated from the dam for 3 h/day from days 4 to 19 of life (as described below), whereas control NS pup remained in their home cage with the dam and left undisturbed. MS pups were removed from the home cage and taken into a separate room, where they were placed into individual compartments in plastic cages lined with chip bedding. Cages were maintained at 37 ± 0.5°C by placing them on a temperature-controlled heating pad. The separation procedure was performed at the same time each day to minimize the effects of circadian rhythm. All procedures were approved by the Animal Care Committee at McMaster University.

Ussing chamber experiments. Segments of the distal colon were opened along the mesenteric border, cut into flat sheets, and mounted in Ussing chambers (WP Instruments, Narco Scientific, Mississauga, ON, Canada), with a 0.6-cm² serosal tissue area exposed to 8 ml of circulating oxygenated Krebs buffer maintained at 37°C. The buffer consisted of (in mM) 115 NaCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 KH₂PO₄, and 25 NAHCO₃ at pH 7.35 ± 0.02. Additionally, glucose (10 mM) was added to the serosal buffer as a source of energy, which was balanced by mannitol (10 mM) in the mucosal buffer.

Macromolecular permeability. Horseradish peroxidase (HRP, 44 kDa) was used as a model protein probe to examine macromolecular permeability. Type VI HRP (Sigma Chemical, St. Louis, MO) was added (10⁻⁶ M) to the luminal buffer once equilibrium was reached, 15 min after tissues had been mounted. Serosal samples (500 µl) were taken at 30-min intervals for 2 h and replaced with fresh buffer to maintain constant volume. The enzymatic activity of HRP was measured using a modified Worthington method as previously described (15) using a kinetic assay, which consisted of 20-µl samples aliquoted in duplicate in 96-well plates. Phosphate buffer (containing 0.003% hydrogen peroxide and 80 mg/ml o-dianisidine) was added (200 µl), and the enzymatic activity was determined based on the rate of increase in optical density at 460 nm for 15 min. The flux of HRP from the mucosal to the serosal side of the chamber was calculated as the average value of two consecutive stable flux periods (60–90 and 90–120 min). HRP flux was expressed as pico moles per centimeter squared per hour.

Role of cholinergic nerves. The cholinergic antagonists atropine (muscarinic antagonist, 10⁻⁵ M, Sigma) and hexamethonium (nicotinic antagonist, 10⁻³ M, Sigma) or the general neurotoxin tetrodotoxin (10⁻⁶ M) were administered to the serosal side of the chamber. Following a 15-min pretreatment period, HRP was added, and the flux determined as above. To determine if abnormalities were due to changes in epithelial receptors or signaling, the muscarinic agonist betahanechol (10⁻⁶ M, Sigma) was added in the presence of tetrodotoxin 15 min prior to the addition of HRP for flux determination. To determine the role of CRF and if CRF acts via activating cholinergic neurons, tissues were treated with a nonselective CRF-R antagonist, α-helical CRF₉₋₄₁ (10⁻⁶ M, Sigma), in the presence or absence of atropine or tetrodotoxin (added at least 15 min prior). To specifically address the subtype of CRF-R involved, selective receptor antagonists were used, including antalarmin [selective for CRF-R1, 10⁻⁶ M (40), Sigma] and antisaugagine-30 [selective for CRF-R2, 10⁻⁶ M (40), Sigma]. In contrast to antisaugagine-30, antalarmin does not dissolve in water or saline and therefore was dissolved in DMSO. (Additional studies validated that a similar quantity of DMSO alone had no detrimental effects on gut function in vitro.) The concentrations of the antagonists were those found to be maximally effective based on previous studies (9, 33, 40) or preliminary experiments.

Expression of choline acetyltransferase by Western blot analysis. The expression of choline acetyltransferase (ChAT) protein was determined using Western blot analysis. Distal colonic tissue samples were collected, weighed, and frozen at −80°C until further use. Samples were then homogenized in glass tubes containing 1 ml of mammalian protein extraction reagent (Pierce Chemical) containing protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Indianapolis, IN) for 20 s on ice. Homogenates were centrifuged, and supernatants were collected and stored at −80°C. Protein quantification was determined by a Bio-Rad Bradford microplate assay (Bio-Rad, Mississauga, ON, Canada) immediately prior to use. Samples (20 µg protein) in reducing/loading buffer were boiled and electrophoresed using 10% SDS-PAGE mini-gels (Bio-Rad). Separated proteins were then transferred to a nitrocellulose film (Bio-Rad) by electroblotting and blocked for 1 h in 5% low-fat Carnation powdered milk with Tris-buffered saline-0.1% Tween 20 (TBST). Blots were then incubated for 1 h with mouse monoclonal ChAT antibody (Chemicon, Temecula, CA; 1:1,000) or rabbit polyclonal anti-protein gene product (Pgp9.5 (Chemicon; 1:2,000) antibody. After a thorough wash with TBST, blots were incubated in secondary antibodies conjugated with HRP (anti-mouse IgG and anti-rabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Following a wash, immunoreactive proteins were then visualized by enhanced chemiluminescence (ECL; Perkin-Elmer, Woodbridge, ON, Canada) for 1 min and exposed to Kodak XBL film (Kodak, Rochester, NY). Membranes were probed for ChAT first, stripped with Tris-SDS-β-mercaptoethanol (pH 6.8), and then re-probed for Pgp9.5 as a protein loading control. Films were scanned, and densitometry was performed using Image J software (National Institutes of Health, Bethesda, MD) and expressed as the mean density of ChAT normalized to the mean density of Pgp9.5.

Expression of ChAT by immunohistochemistry. Pieces of distal colonic tissue were collected and immediately fixed in formalin. Following 48 h of fixation, tissues were embedded in paraffin and sliced into 5-µm sections. Slides were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 10% hydrogen peroxide in methanol for 20 min. Sections were then incubated for 10 min with universal blocking solution (Dako, Mississauga, ON, Canada), followed by an incubation with 50 µg/ml normal rat serum in antibody diluting buffer for 30 min to block any nonspecific antibody binding. Subsequently, slides were incubated overnight with 15 µg/ml of mouse anti-ChAT antibody (Chemicon). Sections were then treated with a biotinylated rabbit anti-mouse IgG for 90 min, followed by an incubation with alkaline phosphatase (AP)-conjugated streptavidin (1:100) for 30 min and stained with fast red for 90 s. Counterstain was performed with hematoxylin for 4 min, and slides were examined under a light microscope. A nonspecific mouse IgG was used as the negative control. Positive cells were identified and counted as cells per crypt. Five crypts were counted per piece of tissue, and 3 tissues were counted per animal (×200 magnification).

Statistics. Results are expressed as means ± SE. Groups were compared using ANOVA. Post hoc analysis was performed using the Mann-Whitney test. Differences of P < 0.05 were considered to be significant.

RESULTS

Cholinergic antagonists blocked the increased macromolecular permeability in MS pups. MS caused an increase in HRP flux across colonic tissues that was dramatically (~5-fold) and significantly (P < 0.005) increased compared with that in NS pups (Fig. 1). Atropine, alone or in combination with hexamethonium, significantly reduced the increased flux to control

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values or below (Fig. 1). In MS tissues, the effect of combined administration of atropine and hexamethonium on HRP flux was significantly greater than that observed with atropine alone, suggesting that the two pathways (muscarinic and nicotinic) were not completely in series. Both antagonists also reduced the flux in tissues from NS pups, although no cumulative effect was observed. The cholinergic component of the response was greater in tissues in MS versus NS pups (24.5 vs. 3.8 pmol·cm⁻²·h⁻¹). These findings indicate that cholinergic mechanisms were responsible for the enhanced macromolecular permeability in separated pups.

MS increased permeability was not mediated by epithelial cell abnormalities. To determine if the abnormal cholinergic regulation of permeability in MS tissue was due to changes in enteric nerves or the epithelium, we administered the cholinergic muscarinic agonist bethanechol to the serosal side of the Ussing chamber. Bethanechol was added in the presence of tetrodotoxin to eliminate a neural contribution. [Tetrodotoxin prevented any change in short-circuit current in response to electrical field stimulation (data not shown).] In the presence of bethanechol and tetrodotoxin, HRP flux was similar in tissues from NS and MS rat pups (7.3 ± 2.7 vs. 7.1 ± 1.7 pmol·cm⁻²·h⁻¹, mean ± SE, n = 9–10), suggesting that the enhanced permeability was not due to altered muscarinic receptor expression or signaling in epithelial cells. Similarly, the administration of tetrodotoxin alone did not cause significant differences between NS and MS tissue (4.6 ± 1.1 vs. 1.7 ± 0.7 pmol·cm⁻²·h⁻¹, n = 5–7).

ChAT protein expression was enhanced by MS. To quantify the synthesis of acetylcholine, ChAT protein was examined in the neonatal rat colon using Western blot analysis and immunohistochemistry. Western blots revealed an increase in ChAT protein when normalized to the neuronal marker PGP9.5 (Fig. 2A), suggesting increased expression within enteric nerves. Immunostaining with ChAT antibody using full-thickness tissue showed greater immunoreactivity in MS tissue compared with NS controls (NS: 3.6 ± 0.6 cells/crypt and MS: 8.6 ± 0.4 cells/crypt, 15 crypts/animal, n = 4). There was also increased expression clearly evident in the epithelium (Fig. 2B).

![Fig. 1. Effect of cholinergic antagonists on maternal separation (MS)-induced increases in horseradish peroxidase (HRP) flux. Atropine (ATR; 10⁻⁵ M) was added alone or in combination with hexamethonium (HEX; 10⁻⁵ M) directly to Ussing chambers. After a 15-min incubation period, HRP was added to the mucosal side, and samples were obtained from the serosal side every 30 min for 2 h. Values indicate means ± SE; n = 6–12. *P < 0.05 compared with nonseparation (NS) vehicle (VEH) control; #P < 0.05 compared with MS VEH control; $P < 0.05 compared with MS with ATR.](http://ajpgi.physiology.org/)

![Fig. 2. Expression of choline acetyltransferase (ChAT). A: Western blot analysis for ChAT was performed on homogenized full-thickness distal colonic tissue. Blots were stripped and reprobed for the neuronal marker protein gene product (PGP)9.5. A significant increase in ChAT levels was observed as measured by densitometry and normalized to PGP9.5. B: immunostaining for ChAT was performed on paraffin-embedded circular sections of MS and NS distal colons. Increased staining was observed in the epithelium of MS tissue compared with NS controls as visualized by light microscopy (magnification: ×200). Values indicate means ± SE; n = 7–8. *P < 0.05 compared with NS.](http://ajpgi.physiology.org/)
fore, MS may result in the increased production of acetylcholine due to greater ChAT enzyme activity.

**MS-enhanced permeability inhibited by CRF antagonism was not further reduced by neural/cholinergic antagonism.** Treatment of MS tissue with a nonselective CRF-R antagonist significantly reduced the enhanced flux of HRP compared with vehicle treatment (Fig. 3A), indicating that MS-induced release of CRF was involved in maintaining the barrier defect. Treatment with the CRF-R antagonist had no effect on NS tissue (6.7 ± 1.1 vs. 5.8 ± 2.0 pmol·cm⁻²·h⁻¹, mean ± SE, n = 5–10). Treatment with either atropine or tetrodotoxin prior to the addition of the CRF-R antagonist produced no significant additional inhibitory effect on permeability. These findings suggest that both CRF and acetylcholine increase permeability via the same pathway.

The administration of selective CRF-R antagonists in vitro revealed that the CRF-R2 antagonist but not the CRF-R1 antagonist produced significant inhibition of the flux of HRP (Fig. 3B), suggesting that the CRF-R2 subtype mediates the MS-induced enhanced macromolecular permeability in neonatal rat pups.

**DISCUSSION**

This study shows for the first time that exposing rat pups to neonatal MS resulted in the development of increased macromolecular permeability that was mediated largely by cholinergic pathways. Compared with NS controls, MS pups showed an elevated HRP flux in the colon, which was inhibited by muscarinic and nicotinic receptor antagonists. These effects did not appear to be mediated by increased muscarinic receptors or epithelial cells. We identified enhanced expression of ChAT, implying that increased synthesis of acetylcholine may be induced in rat pups exposed to MS. Blockade of CRF-Rs also reduced HRP flux, with no further inhibition by tetrodotoxin or atropine. These results suggest that both CRF and acetylcholine are acting along the same pathway and that excess CRF resulting from MS may enhance permeability by acting on cholinergic neurons.

We (10) previously reported that electrical field stimulation of enteric nerves resulted in a significantly greater short-circuit current response in tissues from MS rat pups compared with NS control pups, suggesting an MS-induced alteration in the ENS regulation of ion transport. In that study, the short-circuit current response to stimulation of muscarinic receptors while blocking nerves was similar in tissues from both rat groups, suggesting an increased cholinergic activity mediating secretory state. Here, we showed that similar cholinergic mechanisms appear to be involved in the increased epithelial permeability resulting from exposure of rat pups to MS.

Acetylcholine is a classical neurotransmitter used by the parasympathetic system to communicate with the ENS as well as within intrinsic enteric nerves. It acts both neurally and nonneurally in mediating its excitatory effects. Nonneuronal acetylcholine has also been identified in epithelial, endothelial, and immune cells in the gut, where its release is mediated by an organic cation transporter rather than vesicular release, as in neurons (43). To determine if stimulation of cholinergic receptors was mediating the increased permeability, blockade of muscarinic and nicotinic receptors with atropine and hexamethonium was performed. Pretreatment with atropine alone or in combination with hexamethonium significantly reduced the elevated permeability in MS tissue, reducing the value to control levels. Hexamethonium, in the presence of atropine, produced a greater inhibition than with atropine alone, indicating that some cholinergic nerves (likely interneurons) act on effector neurons to release transmitters other than acetylcholine, possibly containing substance P or VIP. Furthermore, the cholinergic component, as determined by the difference in permeability values without and with cholinergic antagonists, was much greater in the colon of MS pups than the component observed in the colon of NS rat pups. These findings indicate that MS results in elevated cholinergic transmission in the ENS. This suggests that the muscarinic and nicotinic pathways are in parallel rather than in series, perhaps indicating a role for extrinsic postganglionic cholinergic drive in mediating barrier function.

To determine if acetylcholine production was increased and if it was of neuronal or nonneuronal origin, we employed Western blot analysis and immunohistochemistry to examine protein expression and localization. ChAT was used to indicate the presence of acetylcholine, since it is essential in the synthesis of the neurotransmitter and is a specific marker of cholinergic nerves and acetylcholine-containing cells. ChAT has been identified by immunohistochemistry in the cell bodies and nerve terminals of both submucosal and myenteric plexus neurons (27); in addition, the cell membrane, endosomes,
cytoskeleton, nucleus, and mitochondria of epithelial cells have also been shown to be immunoreactive (16). In our study, densitometry analysis of Western blots revealed a significant increase in the expression of ChAT relative to the expression of PGP9.5, a neuronal marker, suggesting that increased enzyme levels were present in the enteric nerves of MS pups. In addition, immunohistochemistry revealed an increase in ChAT immunoreactivity localized within the epithelium in MS tissue compared with NS tissue. This increase in both neuronal and nonneuronal acetylcholine production suggests that an increase in available acetylcholine was mediating the barrier dysfunction in MS rat pups. As a result of the experimental design employed, we cannot clearly determine whether an increase in neuronal sprouting is observed in our model. Nerve growth factor (NGF) is increased in neonatal rat pups exposed to MS (1) and can induce neurite sprouting (13) as well as ChAT activity (22). Since NGF was not examined in this study, we cannot determine whether increased neuronal sprouting was involved in maintaining the observed increase in ChAT. Recent publications have found a splice variant of ChAT called pChAT, which has been shown to be exclusively localized to neuronal cells and fibers in intramural ganglia (23) and submucosal ganglia (6, 39). Few publications exist characterizing this protein, and a commercially available antibody is not yet available. However, use of this variant in the future would be useful in determining the neuronal to nonneuronal contribution of ChAT to the MS-induced dysfunction.

CRF is an important hormone in the stress response. It has been shown to modulate gastrointestinal activity by acting at both central (19) and peripheral sites (20, 37, 42). A single intraperitoneal injection of CRF in adult rats resulted in similar gut pathophysiology, including increased ion secretion and permeability, to that observed in rats exposed to acute restraint stress (32). In that study, CRF-induced changes were abolished by pretreatment with atropine and hexamethonium, suggesting the involvement of cholinergic pathways. We previously identified that CRF plays a major role in regulating colonic epithelial function in both adult (37) and immature rats (11) following neonatal exposure to MS. A nonselective CRF-R antagonist, administered intraperitoneally to adult rats (subjected to MS as neonates), inhibited epithelial pathophysiology induced by mild stress (37), and the same antagonist injected subcutaneously to immature rats daily prior to the separation period inhibited the elevated baseline ion secretion and permeability (11). A recent study by Barreau et al. (2) provided evidence that CRF-R1 mediates MS-induced changes in paracellular gut permeability in adult rats. In preliminary work using selective CRF-R antagonists, we identified CRF-R2 as the dominant receptor subtype involved in maintaining the dysfunction in transcellular macromolecular permeability in rat pups exposed to early life stress. It appears that each CRF-R subtype regulates different aspects of gastrointestinal physiology (38). Activation of CRF-R1 is responsible for stress and central/peripheral CRF-induced increased colonic transit (19, 20, 30), MS-induced visceral hyperalgesia (35), and central CRF-induced colonic hypersensitivity (12). Alternatively, the activation of CRF-R2 is involved in central CRF-induced delayed gastric emptying (19) and prevention of visceral pain (21). To our knowledge, this is the first report linking CRF-R2 specifically to changes in transcellular permeability. More extensive studies focusing on CRF-R subtypes and transcellular permeability are currently underway.

In addition to the release of CRF as part of the HPA-axis response to stress, extrinsic CRF-positive neurons originating from Barrington’s nucleus and signaling to the distal colon have been identified (29, 41). The presence of these neurons suggests that a centrally mediated mechanism may regulate colonic CRF. In the periphery, CRF has been identified by immunohistochemistry in the colon within enterochromaffin cells (14) and enteric nerves (18). Colocalization of CRF-positive immunoreactive cell bodies within the submucosal ganglia with VIP suggests that these are secretomotor neurons (18). A combination of local and extrinsic signals may be involved in mediating the effect of CRF on colonic epithelial function. CRF-Rs were also localized in the periphery, with expression on enteric nerves (5, 18), crypt epithelial cells (5), and mast cells (4). The effects of CRF to modify colonic epithelial physiology may occur either directly on the colonic epithelium or indirectly by activating cholinergic nerves to release acetylcholine. In our study, the administration of the nonselective CRF-R antagonist α-helical CRF(9-41) to MS colonic tissues in Ussing chambers caused a significant reduction in HRP flux. Pretreatment with atropine or tetrodotoxin resulted in no additional inhibition. Taken together, these results suggest that the mechanism involved in the MS-induced barrier dysfunction was dependent on CRF activation (via CRF-R2) of enteric nerves to release acetylcholine, which, in turn, activated epithelial cells to increase the permeability of the epithelium. Mast cell hyperplasia is observed in neonatal rats following exposure to MS (1), suggesting that mast cells may also be involved in altered CRF responses, although this point was not examined in the present study and is the focus of future experiments.

In conclusion, findings from this study suggest that exposure of neonatal rat pups to MS results in enhanced synthesis of acetylcholine and a hypercholinergic state in the colon. Macromolecular permeability is increased by acetylcholine released in response to extrinsic signals from the central nervous system and/or intrinsic signals from the ENS. In either case, the responsible neurotransmitter is likely to be CRF, acting via CRF-R2. These findings help clarify the link between the gut and the brain. Preventing cholinergic activation may be a strategy for treating patients with certain brain-gut disorders.

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