Colitis induces CRF expression in hypothalamic magnocellular neurons and blunts CRF gene response to stress in rats

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Kresse, Adelheid E., Mulugeta Million, Esteban Saperas, and Yvette Taché. Colitis induces CRF expression in hypothalamic magnocellular neurons and blunts CRF gene response to stress in rats. Am J Physiol Gastrointest Liver Physiol 281: G1203–G1213, 2001.—We investigated hypothalamic neuronal corticotropin-releasing factor (CRF) gene expression changes in response to visceral inflammation induced by 2,4,6-trinitrobenzenesulfonic acid (TNB) and acute stress. Seven days after TNB, rats were subjected to water-avoidance stress (WAS) or restraint for 30 min and euthanized. Hypothalamic CRF primary transcripts (heteronuclear RNA, hnRNA) and CRF and arginine vasopressin (AVP) mRNAs were assessed by in situ hybridization. Antisense 35S-labeled cRNA probes against CRF mRNA intronic (AVP) mRNAs were assessed by in situ hybridization. Antisense 35S-labeled cRNA probes against CRF mRNA intronic and exonic sequences and an oligonucleotide probe against the AVP mRNA were used. TNB induced macroscopic lesions and a fivefold elevation in myeloperoxidase activity in the colon. Colitis increased CRF hnRNA and mRNA signals in the magnocellular part of the paraventricular nucleus of the hypothalamus (PVN) and supraoptic neurons, whereas AVP mRNA was not altered. Colitis did not modify CRF hnRNA signal in the parvocellular part of the PVN (pPVN), plasma corticosterone, and serum osmolality levels. However, CRF hnRNA expression in the pPVN and the rise in corticosterone, and defecation induced by WAS or restraint were blunted in colitic rats. These data show that colitis upregulates CRF gene expression in magnocellular hypothalamic neurons but dampens CRF gene transcription in the pPVN and plasma corticosterone responses to environmental acute stressors.

2,4,6-trinitrobenzenesulfonic acid; arginine vasopressin gene expression; supraoptic nucleus; osmolality; heteronuclear corticotropin-releasing factor gene expression; defecation; food intake

corticotropin-releasing factor (CRF) is the primary mediator of the hypothalamic pituitary-adrenal (HPA) endocrine limb of the stress response (50, 53, 54). The dorsal medial parvocellular division of the paraventricular nucleus of the hypothalamus (pPVN) is the main source of hypothalamic CRF-synthesizing cell bodies. These neurons project to the portal capillary plexus in the external zone of the median eminence (50, 57) where CRF release triggers the secretion of adrenocorticotropic hormone (ACTH) from adenohypophysial corticotrophs and, subsequently, glucocorticoid secretion from the adrenal cortex (53, 57). Neurons that synthesize CRF are also found in the magnocellular division of the PVN (mPVN) (56). They are believed to play only a minimal role in regulating ACTH release, but because CRF synthesis is significantly enhanced by increased plasma osmolarity, mPVN CRF may exert autocrine/paracrine effects on vasopressin and oxytocin secretion during conditions of cellular dehydration (47, 56). Therefore, the CRF neuronal population in the PVN (in both pPVN and mPVN) represents an important integration site for convergence of inhibitory and stimulatory inputs and is subject to stimuli-related plasticity (56).

The use of an intron-specific CRF cRNA probe, which hybridizes to heteronuclear CRF RNA (CRF hnRNA) before the excision of the intronic sequences to form mature CRF mRNA, has been validated as an index of the transcriptional activity of the CRF gene in the PVN (18). Several studies in rats showed that CRF hnRNA expression is increased in the pPVN within 30–60 min of glucocorticoid depletion (18), exposure to ether (28, 29), restraint, or intraperitoneal injection of hypertonic saline (34), whereas changes in CRF mRNA expression take longer, reaching a maximum 3–6 h later (35). Repeated exposures to a homotypic stressor (such as restraint) result in the adaptation of the HPA axis, whereby the induction of CRF hnRNA expression and the increase in CRF mRNA in the pPVN as well as the rise in ACTH and corticosterone plasma levels observed after the first period of restraint no longer occur after multiple exposures (34, 35). However, the transcriptional response of the CRF gene to an intraperi-
toney injection of hypertonic saline is maintained in the pPVN after repeated administrations (32). These data suggest that adaptive responses to repeated intermittent homotypic stressors may differ between the exteroceptive and interoceptive nature of the stressor (47). In addition, chronic exposure to the same nonimmune stressor usually facilitates the pituitary-adrenal response to a novel heterotypic stressor (4). In contrast, the HPA axis response to acute stressors is dampened under conditions of chronic immunologic stress of somatic origin, such as arthritis (15).

Less is known regarding the influence of chronic visceral inflammation on the activity of the HPA axis under basal conditions and in response to acute exteroceptive stress. Previous studies in rats showed that colonic inflammation induced by a single intrarectal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNB) (42) is associated with a rise in corticosterone plasma levels monitored 24 h later (39, 55). This response was no longer detectable at 3 or 6 days after TNB despite the persistence of colitis, suggesting adaptation of the pituitary adrenal axis (39). It is well established that central CRF has a protective action on experimental colitis (8, 14, 39), and steroids are the mainstay in the management of inflammatory bowel disease (58). However, acute repeated stress aggravates colitis (9, 14, 39), indicating possible alterations of the hypothalamic CRF protective mechanisms under these conditions. Changes in CRF gene transcription and synthesis occurring in the PVN during colitis are still unknown. In the present study, we first investigated CRF hnRNA and mRNA signals in the pPVN, mPVN, and supraoptic nucleus (SON) in rats with chronic colitis, using semiquantitative in situ hybridization with probes complementary to intronic and exonic sequences of the rat CRF mRNA (18). Second, we examined whether a state of chronic colitis influences the induction of CRF hnRNA expression in the pPVN (34) and the CRF receptor-mediated increase in corticosterone and distal colonic motor response to an acute exteroceptive stressor (51, 53). Lastly, to gain insight into the underlying mechanisms through which colitis may modulate CRF gene expression in the hypothalamic neurons, we assessed the possible role of osmotic changes by monitoring serum osmolarity and arginine vasopressin (AVP) gene expression in the PVN.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Harlan Laboratories, San Diego, CA) weighing 250–300 g were housed up to five per cage under conditions of controlled temperature (21 ± 2 °C), humidity, and illumination (12:12-h light-dark cycle starting at 6 PM). Animals were given free access to tap water and food (Purina Rat Chow) and allowed to acclimatize starting at 6 PM). Animals were given free access to tap water and food (Purina Rat Chow) and allowed to acclimatize (51, 53). Lastly, to gain insight into the underlying mechanisms through which colitis may modulate CRF gene expression in the hypothalamic neurons, we assessed the possible role of osmotic changes by monitoring serum osmolarity and arginine vasopressin (AVP) gene expression in the PVN.

Treatments

Colitis was induced as previously described (39). TNB (liquid form; Fluka Chemical, Ronkonkoma, NY) diluted in an equal volume of 50% ethanol was instilled intrarectally at a dose of 80 mg/kg in 0.2 ml using a PE-60 cannula, the tip of which was positioned ~8 cm past the anus in fasted and lightly hand-restrained rats. The cannula was left in place for 1 min to ensure that the TNB-ethanol solution was not expelled immediately. Another group of 18-h fasted rats received an equal volume of vehicle (50% ethanol diluted in water, 1:1 vol/vol) under similar conditions. All rats were housed individually after TNB or vehicle treatment. To account for the possible influence of the ethanol content of the vehicle, age-matched rats that received no treatment were kept under similar conditions and served as naive controls (n = 4).

Water-avoidance stress (WAS) was performed as previously described (6). Briefly, rats were individually placed onto a small plastic platform (6 × 6 × 8 cm) located in the middle of a home cage filled with water up to 1 cm below the platform height. Wrap-restraint stress was achieved as previously reported (39) and consisted of wrapping the forelimbs and upper body with gauze secured with paper tape to restrict, but not prevent, movements.

Experimental Protocols

Seven days after TNB or vehicle instillation, groups of rats (n = 8/group) were maintained in their home cage or subjected to WAS or wrap-restraint stress for a 30-min period, weighed, and euthanized by decapitation. Blood was then collected for corticosterone determination. The fecal pellet output over the 30-min stress period was monitored. Brains were quickly removed, snap frozen on dry ice (CO2, −80°C), and stored in a −80°C freezer until being sectioned for in situ hybridization. Colonic tissue (2–8 cm proximal to the anus) was examined for gross morphological changes and kept at −80°C until processed 24 h later for the myeloperoxidase (MPO) activity assay. In a second experiment 7 days after TNB (n = 8) or vehicle (n = 8) administration, CRF (2 μg/kg) or saline was injected intraperitoneally, and 30 min later, rats were euthanized by decapitation and blood was collected for corticosterone measurements. Fecal pellet output over the 30-min period after intraperitoneal CRF was also monitored. In a third experiment, groups of rats (n = 6 rats/group) were administered vehicle or TNB intrarectally or received no treatment. Daily food and water intakes were recorded for 2 days before and for 1, 3, or 7 days after vehicle or TNB. At each time point, rats were euthanized and blood was collected for osmolality assay. To avoid the confounding effects of the circadian pattern (56), rats were euthanized between 10 and 12 AM in all experiments. Because vocalization, smell, handling, and weighing are stressful, rats were euthanized randomly and quickly in a room adjacent to the one where the experiments took place. Although it is difficult to ascertain whether the weighing and handling procedures may have been thoroughly nonstressful, all groups were handled in the same way.

Measurements

Fecal output. The colonic motor response was assessed by monitoring the cumulative number of fecal pellets excreted over the 30-min period.
Food and water intake. Daily Purina chow and water intake were measured once per day between 8 and 9 AM to the nearest 0.1 g and 0.1 ml, respectively.

Plasma corticosterone and serum osmolarity. Trunk blood was collected into chilled tubes containing EDTA (1.5 mg/ml; for corticosterone assay) or without EDTA (for serum osmolarity assay). Samples were centrifuged at 2,000 rpm at 4°C for 10 min and stored at −20°C for further determination of plasma corticosterone (Peptides Biology Laboratory, Salk Institute, La Jolla, CA) and serum osmolarity using freezing point depression (Advanced Instrument, Norwood, MS; Veterans Affairs Greater Los Angeles Healthcare System, West Los Angeles Health Care Center, Pathology and Laboratory Medicine).

Colitis. In each rat, the entire colon was excised and opened longitudinally. Colitis was assessed by macroscopic damage scoring and quantification of granulocyte infiltration by measuring the MPO activity as previously described (39). For macroscopic scoring, the colon was rinsed thoroughly in ice-cold normal saline and visually examined according to criteria previously described in detail (37,39), through which ulcerations (0–10) and diarrhea (0–2) were rated and added to the wall thickness (in mm) of the colon. An area corresponding to 2–8 cm proximal to the anus was used for MPO assay as a measure of tissue infiltration by granulocytes (30, 39, 42). Samples were immediately rinsed in ice-cold saline (0.9%), blot dried, and kept on dry ice (−80°C). Measurements were performed within 24–48 h after sampling, which has been described previously (12) to be essential for reliable quantification. The tissue (200–400 mg) was homogenized in 10% (wt/vol) 20 mM phosphate buffer (pH 7.4) and centrifuged at 8,000 g for 20 min at 4°C. The supernatant, which contained <5% of the total MPO activity, was discarded. The pellet was rehomogenized in 50 mM acetic acid (pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide. MPO activity was assessed by measuring the H2O2-dependent oxidation of 3,3′,5′-tetramethylbenzidine and expressed as units per milligram of tissue. One unit of enzyme activity was defined as the amount of MPO present that would produce a change in absorption of 1.0 at a wavelength of 655 nm for 1 min at 37°C.

In Situ Hybridization

Labeling of AVP oligonucleotide probe. A 48-mer, complementary to the nucleotides that code for the last 16 amino acids of the glycoprotein precursor that are not shared with oxytocin, was synthesized at the Institute for Molecular Biology at the University of California (Los Angeles, CA). The probe was 3′-end labeled using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and α-[32P]dATP (REN DuPont, Boston, MA) to a specific activity of 0.8–1.5 × 106 cpm/μg and purified using BioSpin-6 (Bio-Rad) purification columns. The probe used in this study has been shown previously (43) to specifically recognize AVP. Control experiments using preincubation of tissue sections with RNase to predigest any CRF mRNA or an excess of unlabeled probe showed the absence of specific hybridization signals (data not shown).

cRNA probe synthesis. The pGEM-4 plasmid containing the exonic sequence (CRF mRNA) of the rat CRF cDNA (1.2 kb, gift from Dr. Kelly Mayo, Northwestern University, Evanston, IL) was linearized with Hind III to generate a template for the transcription of a specific antisense probe. A pGEM-3 plasmid containing the sequence of the single intron of the rat CRF gene (530 bp; kindly provided by Dr. Stanley Watson, University of Michigan, Ann Arbor, MI) was linearized with Hind III to transcribe a probe specifically detecting the primary transcript of the CRF gene (CRF hnRNA). Radioactive cRNA probes were synthesized by incubation of 1 μg linearized plasmid with the appropriate polymerase enzymes in standard labeling buffer (Promega, Madison, WI), including 8 mM dithiothreitol, 40 U RNase inhibitor (RNasin, Promega), 25 mM ATP/CTP/GTP, 5 mM unlabeled UTP, and 200 μCi 35S-labeled UTP (REN DuPont). Nonincorporated nucleotides were removed using BioSpin-6 chromatography columns (Bio-Rad). The specific activities of probes used for analysis were usually 7–9 × 106 cpm/μg. We also generated sense probes that were hybridized with some of the sample sections to control for specificity and never yielded specific hybridization signals.

In situ hybridization histochemistry. Sections of 14-μm thickness were cut in a cryostat and thaw mounted onto Probe-On slides (Fisher Scientific), air dried for 10 min, and stored in sealed boxes in a −20°C freezer. Hybridization histochemistry was carried out in every fourth serial section throughout the extent of the PVN with 4 rats for each group. AVP oligonucleotide probe. This was carried out as previously described in detail (43). Briefly, the radiolabeled probe (106 cpm/100 μl−slide−1) was diluted into the hybridization buffer consisting of 1× Denhardt’s solution, 0.25 mg/ml yeast transfer RNA (Sigma), 0.5 mg/ml salmon testes DNA (Sigma), 10% dextran sulfate, 200 mM dithiothreitol, and 50% formamide, applied to the slides, and incubated at 42°C. After the hybridization, the slides were rinsed in four 15-min washes of 1× SSC (1× SSC = 0.15 M NaCl and 0.015 mM sodium citrate, pH 7.0) at 55°C, dehydrated in ethanol, and air dried. Subsequently, dehydrated and air-dried slides were dipped in nuclear track emulsion (NTB-2, Kodak, Rochester, NY; diluted 1:1 with distilled water), exposed at 4°C for 10 days, developed in D-19 developer (Kodak), lightly counterstained with thionine, and coverslipped with Permount.

In situ hybridization with riboprobes. In brief, frozen tissue sections were brought to room temperature, air dried for 30 min, and subsequently fixed in phosphate-buffered fixative containing 4% paraformaldehyde for 10 min. Rinses in PBS (10 mM) and 1 M thriethanolamine (TEA, pH 8.0, 2 min) were followed by acetylation in 0.25% acetic anhydride (in 0.1 M TEA for 10 min), rinses in 2× SSC, and dehybridge/delipidation through graded concentrations of ethanol (50%, 70%, 95%, and 100%) and chloroform (5 min). Probes were diluted in hybridization solution (as described above) to yield 4 × 106 cpm/100 μl. After addition of 200 mM dithiothreitol, 0.5 mg/ml sheared salmon testes DNA, and 0.5 mg/ml polyadenylic acid, 100 μl of the mixture were spotted onto air-dried slides, coverslipped, and incubated at 60°C overnight (16–20 h) in sealed boxes. The next day, coverslips were removed in 4× SSC at room temperature, and the slides were rinsed in decreasing concentrations of SSC, followed by digestion of nonhybridized probe with RNase A (20 μg/ml, in Tris-EDTA buffer with 0.5 M NaCl at 37°C for 30 min) and one high-stringency rinse in 0.1× SSC at 55°C. Subsequently, slides were processed for dipping in nuclear track emulsion as described above.

Analysis. Semiquantitative densitometric analysis of relative levels of CRF hnRNA and CRF mRNA expression was carried out on emulsion-coated slides under dark- and bright-field illumination over the confines of counterstained cell profiles. Optical density (OD) readings (e.g., integrated mean gray values of areas corresponding to the area of a single cellular profile) were taken at regularly spaced intervals (70 μm) using personal computer-driven image analysis software (OPTIMAS, version 5.1). Determinations were made on six to eight sections through the midportion of the PVN and
through the SON in each animal (1.700–1.900 mm posterior to bregma). The values were corrected for nonspecific background labeling by determination of specific labeling ratios (OD values over labeled cells divided by density values of equivalent background areas) whereby labeling exceeding three times the background values was considered specific and average values were determined. Because densitometric analysis revealed no significant differences of CRF hnRNA or CRF mRNA expression in the hypothalamic nuclei between naive and vehicle-pretreated rats, both groups were pooled as one control group for statistical analysis.

### Statistical Analysis

Results are expressed as means ± SE. Comparisons between groups were performed by ANOVA, followed by a Bonferroni-Dunn post hoc test to assess statistical significance between multiple pair-wise comparisons. \( P < 0.05 \) was considered significant.

### RESULTS

#### Colonic Inflammatory Response

In vehicle-treated rats, MPO activity was low and there were no macroscopic damage lesions in the colon (Table 1). Seven days after TNB treatment, the colon was inflamed, as characterized by the thickening of the colonic wall, the presence of mucosal lesions, and a fivefold increase in MPO activity compared with vehicle-treated rats (Table 1). After 7 days of colitis, exposure to WAS for 30 min did not influence the damage score and the MPO activity. Stress alone had no effect on these parameters in vehicle-treated rats (Table 1).

### Hypothalamic CRF hnRNA and mRNA Responses to Acute WAS in Vehicle- and TNB-Treated Rats

In vehicle-treated rats, positive CRF hnRNA hybridization signal was low under resting conditions in the pPVN and virtually nonexistent in the mPVN (Fig. 1A) and SON (not shown). CRF hnRNA expression was selectively induced in the pPVN after 30 min of WAS (Fig. 1C). Semiquantitative analysis of autoradiographic data indicated that acute WAS resulted in a significant 4.3-fold elevation of CRF hnRNA signal in the pPVN but not in the mPVN (Fig. 1C) and SON (Fig. 2). Colitis reduced the rise in CRF primary transcript induced by WAS (Figs. 1D and 2) but did not alter the CRF hnRNA signal in the pPVN of rats not exposed to the acute stress (Fig. 1B). Interestingly, in TNB-treated rats, CRF hnRNA signal was present in the mPVN (Fig. 1, B and D) and SON (not shown) when monitored 7 days after administration. CRF hnRNA signal in the mPVN and SON was increased 2.5- and 3.3-fold, respectively, in TNB- vs. vehicle-treated groups. These changes were not altered by acute exposure to WAS on day 7 after TNB (Fig. 2).

In the pPVN, CRF mRNA signal was clearly visible under resting conditions (Fig. 3A) and no obvious changes were observed in TNB-treated rats or in vehicle- or TNB-treated rats exposed to WAS (Fig. 4). In contrast, colitis increased by 4.7- and 5.3-fold the CRF mRNA signal in magnocellular neurons of both the

### Table 1. Acute WAS on day 7 after TNB-induced colitis does not affect severity of colonic inflammation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Macroscopic Lesion Score</th>
<th>MPO, U/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.9 ± 0.5</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>TNB</td>
<td>14.3 ± 1.8*</td>
<td>31.4 ± 5.2*</td>
</tr>
<tr>
<td>Vehicle + WAS</td>
<td>1.8 ± 0.4</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td>TNB + WAS</td>
<td>14.0 ± 2.2*</td>
<td>33.6 ± 6.7*</td>
</tr>
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</table>

Values represent means ± SE of 6–8 rats/group. Rats fasted for 18 h were treated with intracolonic vehicle (25% ethanol, −0.2 ml) or 2,4,6-trinitrobenzenesulfonic acid (TNB; 80 mg/kg, −0.2 ml) and 7 days later were exposed to 30-min water-avoidance stress (WAS). MPO, myeloperoxidase. *\( P < 0.05 \) compared with vehicle or vehicle + WAS (by ANOVA).
mPVN (Figs. 3B and 4) and SON, respectively (Figs. 3D and 4), where little or no CRF mRNA could be detected in vehicle-treated animals (Figs. 3C and 4).

In vehicle-treated rats (n = 4), the AVP mRNA hybridization signal, described as specific labeling ratios, was high in both the mPVN (11.3 ± 0.1) and SON (11.4 ± 0.2) and was not modified 7 days after TNB treatment (n = 4) in the mPVN (11.3 ± 0.2) and SON (11.4 ± 0.2) (Fig. 5). No AVP mRNA signal was detected in the pPVN in vehicle- or TNB-treated groups (Fig. 5).

Corticosterone and Colonic Motor Responses

In vehicle- and TNB-treated rats, the basal plasma corticosterone levels were similar (100 ± 37 and 92 ± 23 ng/ml, respectively). These relatively high basal plasma corticosterone levels might reflect handling during weighing just before euthanasia and the decapitation procedures. Regardless, in vehicle-treated rats, exposure to 30 min of WAS or wrap-restraint stress induced a similar 3.5-fold significant increase in plasma corticosterone levels (361 ± 54 and 344 ± 17 ng/ml, respectively; Fig. 6A). However, in TNB-treated rats, the corticosterone response to WAS and wrap-restraint stress was attenuated, and values were no longer significantly different from the controls (Fig. 6A). Corticosterone values 30 min after the intraperitoneal injection of CRF (2 μg/kg) were not significantly different between vehicle- (401.3 ± 38.9 ng/ml, n = 4) and TNB-treated groups (357.7 ± 61.6 ng/ml, n = 4) but were significantly elevated compared with intraperitoneal injection of saline in both vehicle- (110.8 ± 33.7 ng/ml, n = 4) and TNB-treated groups (50.3 ± 23.4 ng/ml n = 4). Values of corticosterone were not significantly different between vehicle and TNB groups after intraperitoneal injection of saline, although corticosterone levels tended to be lower in the TNB than in the vehicle group.

In vehicle- or TNB-treated rats not exposed to acute stress, no or few fecal pellets were noted during the 30-min observation period. WAS and wrap-restraint stress for 30 min significantly increased the number of pellets to 7.0 ± 0.6 and 4.0 ± 0.2 pellets/30 min, respectively, in vehicle-treated rats (Fig. 6B). The colonic motor response to WAS and wrap-restraint stress was significantly reduced by 57% and 50%, respec-
tively, in TNB-treated rats (Fig. 6B). It is to be noted that on day 7, colitic rats had formed and countable pellets, although of softer consistency than the vehicle-treated rats.

**Food and Water Intake**

The daily food and water intakes were 21.7 ± 0.6 g/day and 32.8 ± 1.2 ml/day, respectively, for the 2 days before treatment (n = 18; pooled data of 3 groups); these values remained similar for the 7 following days in the nontreated group (Fig. 7). Intrarectal instillation of the vehicle (25% ethanol, n = 6) significantly reduced food and water intake by 12.3% and 25%, respectively, on day 1 after treatment, whereas thereafter values were no longer significantly different from those of the naive control group (Fig. 7, A and B). In contrast, TNB resulted in a peak inhibition of food intake at days 1 (66.5%) and 2 (63.0%) after enema; thereafter food intake remained lower in TNB- vs. vehicle-pre-treated group (−42.5% at day 3) but returned to a control level by day 6 after treatment (Fig. 7A). TNB also induced a maximal inhibition of water intake on day 1 (73.5%) after treatment, which decreased on day 2 (33.6%) and reached control values on days 6–7 (Fig. 7B). The water-to-food intake ratio in basal states was 1.5 ± 0.1 and remained at this level in both the naive and vehicle-treated groups throughout the 7-day experimental period (Fig. 7C). After TNB treatment, on the other hand, the water-to-food intake ratio increased significantly on day 2 (2.9 ± 0.6, P < 0.05) and was no longer different from that of the vehicle-treated group on day 3 (1.9 ± 0.2) after treatment and thereafter (Fig. 7C).

**Serum Osmolarity**

There was no significant difference in serum osmolarity between vehicle- and TNB-treated groups or compared with basal values (293 ± 2 mosmol/kgH2O). Serum osmolarity levels on days 1, 3, and 7 post treatment were 295.3 ± 2.6, 292.8 ± 3.7, and 293.5 ± 1.2 mosmol/kgH2O, respectively, in vehicle-pre-treated rats and 297.5 ± 1.3, 295.3 ± 2.1, and 294 ± 1.6 mosmol/kgH2O, respectively, in TNB-pre-treated rats (P > 0.05 between and within groups of 6 rats/group). The slight
increase in serum osmolarity on day 1 after TNB did not reach statistical significance.

DISCUSSION

This study was designed to characterize changes in hypothalamic CRF gene expression associated with experimental colitis and the HPA axis response to acute psychological stress under conditions of colitis for 7 days in rats. We found low basal CRF hnRNA expression in the pPVN and mPVN, and there was a substantial pool of CRF mRNA located in the dorsal medial pPVN, where it is constitutively expressed as previously reported (21, 34). Acute exposure to WAS provoked a rapid and selective induction of CRF hnRNA expression in the pPVN associated with activation of the adrenal secretion. This was indicated by a 4.3-fold increase in CRF hnRNA signal intensity in the pPVN and concordantly elevated plasma corticosterone levels at 30 min after the onset of WAS. However, no CRF hnRNA expression was detected in the mPVN and SON after the acute stress. The induction of CRF hnRNA signal in the pPVN occurred with a rapid onset and was not associated with a corresponding change in the pool of CRF mRNA. Existing evidence indicates that several hours of lag time exist between an acute stress stimulus and the increase in CRF mRNA expression in the pPVN, whereas the onset of CRF gene expression using intron-directed in situ hybridization is detectable within 15 min after stress (22, 32, 33, 35, 44). A rapid increase of CRF hnRNA in the pPVN along with a rise in plasma corticosterone levels have been reported in response to exposure to ether, restraint, and osmotic (intraperitoneal injection of hypertonic saline) or immune challenges (bacterial endotoxin) (22,
Intracolonic instillation of TNB induced, at adapted to chronic inflammation of visceral origin. These findings indicate that various exteroceptive (restraint or water avoidance) or interoceptive (immune or osmotic) stressors result in a rapid increase in CRF gene transcription selectively in pPVN neurons that is correlated with pituitary-adrenal activation (18, 22). Although the brain pathways involved in the stimulation of the HPA axis by WAS have not yet been fully established, the activation of brain stem noradrenergic cell groups may play a role. WAS induces Fos expression in regions of the pontine A6 and medullary A1 and A2 noradrenergic catecholaminergic groups (6, 40). Direct noradrenergic projections from the A2 region to the pPVN mainly around the CRF-containing neurons (10, 31) as well as the stimulation of CRF hnRNA and mRNA expression by norepinephrine delivered into the PVN (23, 24) have been well established.

In contrast to acute stress, repeated intermittent stressors can result in preserved or desensitized HPA axis responses, depending on the stress paradigm (32, 34, 35). The present study shows that the HPA axis adapted to chronic inflammation of visceral origin. Intracolonic instillation of TNB induced, at day 7, macroscopic lesions as well as a fivefold increase in MPO activity in the distal colon as previously reported (39). However, CRF hnRNA and mRNA signals in the pPVN and plasma corticosterone levels were not significantly different in TNB- vs. vehicle-treated groups. A similar regimen of TNB administration elevates plasma corticosterone at day 1, whereas the increase was no longer observed 3 or 6 days later (39, 55). These data, along with the present demonstration of unchanged expression of CRF primary transcript and CRF mRNA in the pPVN and circulating corticosterone, indicate that the HPA axis is adapted to chronic colitis. However, we showed that the neuroendocrine response to acute exteroceptive stressors is blunted in colitic rats. Specifically, the induction of CRF hnRNA expression in the pPVN 30 min after the onset of WAS in TNB-treated rats was only one-half of that observed in the vehicle-treated group. This was accompanied by a 48% reduction in the rise of corticosterone. Likewise, 30-min wrap-restraint stress resulted in a lower corticosterone response in TNB- than in vehicle-treated rats. In models of chronic adjuvant-induced inflammatory arthritis, blunted CRF mRNA expression in the PVN and glucocorticoid response to acute restraint or noise was also reported (1, 15, 16). This contrasts with repeated nonimmune stressors, which, regardless of their ability to induce adaptation, invariably facilitate the mounting of HPA axis response to a heterotypic acute stressor (4, 35). Collectively, these data suggest that the rapid CRF gene transcription induced by an acute stressor in the pPVN is dampened in the presence of preexisting chronic inflammation of visceral or somatic origin.

The impaired HPA axis response to WAS in colitic rats is likely to reside primarily at the hypothalamic rather than pituitary level. This is supported by the reduced induction of CRF hnRNA expression in the pPVN after WAS for 30 min. In addition, the pituitary responsiveness to exogenous CRF was unchanged in rats with colitis as shown by the similar increases in plasma corticosterone levels induced by an intraperitoneal injection in both vehicle- and TNB-treated rats. It remains possible, however, that the corticosterone responses may not have reflected differences in ACTH release. Consequently, our data cannot completely rule out a possible participation of the pituitary. Regardless, our findings indicate that colitis for 7 days reduces ACTH secretagogue release from PVN neurons in response to an acute exteroceptive stimuli. This phenomenon may be caused by an alteration of the sensitivity of PVN neurons to stress-related afferent inputs and/or a reduction in the magnitude of the afferent input itself, such as decreased noradrenergic stimulation, leading to a decreased HPA axis response (52).

Interestingly, the autonomic response was also dampened by colitis, as shown by the reduced fecal output response to acute restraint stress and WAS in TNB-treated rats. Consistent evidence indicates that the stimulation of colonic motor function induced by WAS involves activation of specific brain CRF receptors and the sacral parasympathetic nervous system (36, 40, 41, 51). The reduced colonic motor response to water avoidance may therefore reflect decreased activation of CRF pathways in the PVN of TNB-treated rats. We (39, 40) previously observed that young female Lewis rats known to exhibit impaired hypothalamic CRF response to acute stressors (7) display lower activation of the sacral parasympathetic nucleus and defecation after WAS compared with the Fischer strain. However, 5–7 days after TNB colitis, a decreased contractility of longitudinal and circular smooth muscle activity (13, 19), loss of neurons within the myenteric ganglia (45), and reduced myenteric nerve function (25) are reported in the isolated distal colon of rats. Thus in TNB-treated rats, in addition to the blunted hypothalamic CRF response to stress, the above peripheral alterations may also contribute to the decreased colonic motor response to acute stress.

Most noticeable was the observation that chronic colitis induced a state of continuous CRF gene expression selectively in magnocellular hypothalamic neurons. Seven days after TNB, the mPVN and SON displayed substantial upregulation of CRF hnRNA and mRNA signals. The primary phenotype of magnocellular neurons in the PVN and SON consists of AVP- and oxytocin-positive cells (20). In the mPVN, AVP-expressing neurons are encircled by a ring of oxytocin-immunoreactive cells (5, 20), whereas in the SON, AVP neurons are located in the caudal ventral part and oxytocin cells in the rostral and dorsal regions (20). The distribution of CRF hnRNA and mRNA signals induced by colitis appears to be widespread within the mPVN and SON, suggesting that the phenotype of cells expressing the CRF gene may overlap with that of both AVP and oxytocin cell populations. One condition that is known to increase CRF mRNA in magnocellular hypothalamic neurons is extended dehydration (26, 56). During the first days after TNB administration, food intake was reduced as previously reported (3, 38).
Similarly, water consumption was reduced. While it is possible that these changes may have triggered fluid balance disturbances, we failed to observe detectable alteration in serum osmolarity at days 1, 3, or 7 after TNB administration. In addition, despite the decreased water and food intake on the first few days after TNB instillation, rats maintained an adequate, followed by a higher, water-to-food intake ratio during the early part of colitis. By days 6–7 after TNB, the food and water intake values and ratios of TNB-treated rats were identical to those of controls. Lastly, the patterns of hypothalamic CRF and AVP mRNA induced by dehydration and colitis are not similar. Under conditions of osmotic stress resulting from salt loading for 7 days, CRF mRNA increases mainly in the pool of oxytocin neurons of the mPVN and SON but decreases in the pPVN (56). There is also a characteristic sustained elevation of AVP mRNA expression in the pPVN persisting after rehydration (2, 56). In contrast, we found that colitis induced CRF hnRNA and mRNA upregulation throughout the mPVN and SON, whereas CRF hnRNA expression in the pPVN and AVP mRNA in the mPVN were not modified. Together, these results indicate that the induction of CRF synthesis in hypothalamic magnocellular neurons by colitis is likely to result from other additional factors, which are specific to chronic visceral inflammation. Recent reports indicate that experimental colitis induced by TNB increases blood-brain barrier permeability (17) and endothelial adhesion molecule expression in the brain (46) as well as the Th1 pattern of cytokine expression in the colon (49). Whether any of these changes could be part of the mechanisms influencing the upregulation of CRF mRNA synthesis in the mPVN warrants further investigation. Studies (48) have shed light on the possible role of brain cytokine-induced release of prostaglandins influencing CRF gene expression in neuropeptide neurons of the PVN after acute peripheral immune challenges. However, little is known regarding the mechanisms at play influencing CRF gene expression in the PVN in response to chronic inflammation.

In summary, we established that 30 min of WAS resulted in the expression of CRF primary transcript selectively in the pPVN, increased plasma corticosterone levels, and activation of colonic motor function. Chronic colitis for 7 days induced phenolplasticity selectively in the magnocellular neurons of the mPVN and SON where upregulation of CRF gene biosynthesis was observed, as evidenced by the increase in CRF hnRNA and mRNA signals. In contrast, colitis did not influence the low basal levels of CRF primary transcript in the pPVN under resting conditions and basal corticosterone levels. However, colitis blunted the increase in CRF hnRNA expression in the PVN as well as the increase in circulating corticosterone and stimulation of defecation in response to WAS for 30 min.

Perspectives

The impaired HPA axis response to acute environmental stressors may have implications for the understanding of stress-related adverse effects on the outcome of colitis. It is well established that stress exacerbates the course of inflammatory bowel disease in humans and experimental animals (9, 11, 14, 39), whereas glucocorticoids exhibit a beneficial effect on colitis management (58). Colitis-induced suppression of CRF gene activation in the pPVN and plasma corticosterone level dampens the counterregulatory anti-inflammatory mechanisms during exteroceptive stress. This blunting of the anti-inflammatory limb may thus contribute to the stress-related worsening of colitis by leaving unchecked the proinflammatory factors triggered by acute stress. TNB after 7 days also increased CRF hnRNA and mRNA selectively in magnocellular neurons of the mPVN and SON, whereas no changes were observed in the pPVN. These results indicate that alterations in hypothalamic CRF gene expression induced by colitis are regulated by different mechanisms in parvocellular and magnocellular neurons. There is neuroanatomic evidence that neural catecholaminergic input to the magnocellular neurons of the mPVN and SON derives primarily from A1 medullary regions, whereas projections from the A2 regions reach prominence to the population of neurons in the pPVN (10). Whether differential activation of these medullary noradrenergic cell groups during chronic colitis could account for the differential pattern of CRF gene expression in the pPVN and mPVN is worth investigating.

These observations open new venues to delineate the generating mechanisms and implications of hypothalamic magnocellular CRF expression under conditions of chronic colitis. To the best of our knowledge, this represents the first demonstration that a nonosmotic chronic challenge results in the expression of CRF gene expression in hypothalamic magnocellular cell groups.

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