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Up-Regulation of Calcitonin Gene-Related Peptide and Receptor Tyrosine Kinase TrkB in Rat Bladder Afferent Neurons following TNBS Colitis

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Abstract

Colonic inflammation has profound effects on the urinary bladder physiology and produces hypersensitivity of bladder afferent neurons and neurogenic bladder overactivity. Calcitonin gene-related peptide (CGRP) expressed in dorsal root ganglia (DRG) plays an important role in mediating sensory perception following visceral inflammation. In the present study, we determined that the expression of CGRP was increased in bladder afferent neurons in lumbosacral DRG following tri-nitrobenzene sulfonic acid (TNBS)-induced colitis in rat. After colitis, the percentage of bladder afferent neurons expressing CGRP was increased in L1 (61.2 ± 2.9 % in colitis vs 37.7 ± 5.1 % in controls; $p < 0.05$) and S1 DRG (26.3 ± 2.3 % in colitis vs 15.5 ± 1.9 % in controls; $p < 0.01$). We also demonstrated that the expression of tyrosine kinase receptor TrkB was increased in L1 (39.7 ± 2.9 % in colitis vs 25.2 ± 4.3 % in controls; $p < 0.05$) and S1 DRG (45.6 ± 3.8 % in colitis vs 38.3 ± 3.6 % in controls; $p < 0.01$) following colitis. CGRP and TrkB were co-stored in a subpopulation of DRG neurons in control and colitic animals and the number of DRG cells co-expressing CGRP and TrkB was significantly increased in L1 (2.7-fold, $p < 0.01$) and S1 DRG (2.4-fold, $p < 0.01$) following colitis. In cultured DRG, exogenous BDNF application significantly increased CGRP expression, which was blocked by TrkB selective inhibitor K252a. These results suggest that up-regulation of CGRP and TrkB in bladder afferent neurons may play a role in colon-to-bladder cross-sensitization following colitis.

Keywords

visceral inflammation; CGRP; neurotrophins; primary afferent neuron; convergence; cross-sensitization

INTRODUCTION

The mechanism underlying a clinical syndrome in which patients with inflammatory bowel disease (IBD) also exhibit urinary tract complications such as some forms of urinary incontinence (Alagiri et al., 1997; Ben-Ami, et al., 2002) is not clear. Studies with animal models of colitis suggested that chronic colonic irritation can lead to neurogenic cystitis as

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Colitis-induced CGRP and TrkB expression in bladder afferent neurons

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manifested by irritative micturition patterns (Pezzzone et al., 2005; Ustinova et al., 2006a). This may be attributable, in part, to a neural cross-talk and/or convergence in the level of primary sensory afferents in dorsal root ganglia (DRG) and spinal cord (Janig and Koltzenburg, 1990; de Groat et al., 1993; Qin et al., 2005; Pezzzone et al., 2005; Ustinova et al., 2006a). In the bladder and bowel interaction, irritation of one visceral organ can activate afferent input to the central nervous system where it can influence efferent output to another. A primary afferent cross interaction has also been observed in visceral-somatic cross-sensitization and postulated to play a role in the cross-activation of visceral and somatic function (Lamb et al., 2006). The notion of neural cross-activation in DRG and spinal cord was supported by studies in animals showing that chemical-induced colitis sensitized bladder afferent neurons in spinal dorsal horn (Qin and Foreman, 2004; Qin et al., 2005) and increased Na⁺ currents in the bladder afferent neurons in DRG (Malykhina et al., 2004). More recent study showed that colitis induced by intracolonic TNBS led to enhanced firing of bladder C-fibers in response to saline bladder distension and increased afferent responses to capsaicin and Substance P (Ustinova et al., 2006a,b).

Neurotrophins and/or neural activity arising in the inflamed colon (Sharkey and Kroese, 2001) may contribute to target organ to neuron interaction and further to neuron-neuron interaction in the primary afferent pathway. Neurotrophins produced in the inflamed organ (Chen et al., 1995; Lowe et al., 1997; Oddiah et al., 1998; Okragly et al., 1999; di Mola et al., 2000) may undergo retrograde transport to the central nervous system (Campenot and MacInnis, 2004) and lead to gene expression and long term changes in neuronal characteristics including: 1) neurotransmitter phenotype; 2) dendrite size and synaptic organization; and 3) innervation density and function in target organs. Recent study showed that local injection of nerve growth factor (NGF) to one peripheral organ caused visceral-somatic cross-sensitization suggesting the role of neurotrophins in the primary afferent cross-activation (Bielefeldt et al., 2006).

Cellular responses mediated by neurotrophins require ligand binding and subsequent recruitment and activation of specific tyrosine kinase receptor Trk (Kaplan and Miller, 1997). NGF preferentially binds to TrkA (Chao, 1992); brain-derived neurotrophic factor (BDNF) binds to TrkB (Berkemeier et al., 1991). Following colitis, the expression levels of NGF and TrkA were greatly increased in the inflamed colon (di Mola et al., 2000). This may change the phenotype of afferent neurons in DRG, alter the production of neuromodulators and neuropeptides in the afferent neurons (Donnerer et al., 1992) and further contribute to visceral hypersensitivity (Delafoy et al., 2003; 2006). Following peripheral inflammation, BDNF was induced in DRG (Cho et al., 1997a, b; Mannion et al., 1999) and may act on DRG neurons through a paracrine mechanism (Lee et al., 1999; Tonra, 1999).

Previous studies demonstrated that increased expression and phosphorylation of TrkA and TrkB in bladder afferent neurons were associated with cystitis-induced bladder overactivity (Qiao and Vizzard, 2002a). The expression of calcitonin gene-related peptide (CGRP) was also increased in bladder afferent neurons following cystitis (Vizzard, 2001). CGRP is one of the predominant excitatory neurotransmitters in mediating sensory perception. In response to noxious stimuli (Lu and Westlund, 2001; Lu et al., 2005; Delafoy et al., 2006), CGRP was induced in primary afferent neurons in DRG and spinal dorsal horn. In particular, cystitis-induced up-regulation of CGRP in DRG and spinal cord suggests the involvement of CGRP in the sensitization of afferent neuronal pathways of lower urinary tract and a role of CGRP in mediating bladder overactivity (Vizzard, 2001). In previous studies (Qiao et al., 2006), we have demonstrated that BDNF expression was increased in lumbosacral DRG following TNBS-induced colitis in rat. In DRG, CGRP is largely co-stored with BDNF (Luo et al., 2001), whilst it is not clear if these CGRP-containing neurons express TrkB.

In the present study, we identified the colonic and bladder afferent neurons and convergent neurons with dichotomizing projection to both colon and bladder in lumbosacral DRG of rats and determined if colitis-induced bladder overactivity is associated with neurochemical changes in the bladder afferent neurons in DRG. The molecules we examined include CGRP and TrkB. We also characterized the interrelationship between TrkB signaling and CGRP expression in DRG neurons by co-localization study and culture of DRG neurons. Part of the data has been reported as an abstract form in the Digestive Disease Week Annual Meeting.

MATERIALS AND METHODS

Experimental Animals

Adult male Sprague-Dawley rats (150-200 g) (Harlan Sprague Dawley, Inc.) were divided into experimental (colitis induced by intracolonic instillation of TNBS in 50 % EtOH for 7 days, n = 15) and control groups (intracolonic instillation of saline, n = 12; or intracolonic instillation of 50 % EtOH for 7 days, n = 7). Within these groups, some of the animals were injected with two distinct neuronal retrograde tracing dyes (see below for dye injection) to the smooth muscle layer of distal colon and urinary bladder respectively. All experimental protocols involving animal use in this study were approved by the Institutional Animal Care and Use Committee in Virginia Commonwealth University. Animal care was in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress.

Retrograde Labeling

Under anesthesia (2.5 % isoflurane, SurgiVet, Smiths Medical PM, Inc. Waukesha, WI), the rat urinary bladder and distal colon were exposed under a sterile environment with a lower abdominal incision. Neuronal tracing agent Fast Blue (FB, 4 %, weight/volume; Polysciences, Inc. Warrington, PA) was injected into 8 sites (5 μ L per site) in the bladder wall for retrograde labeling of bladder afferent neurons in the DRG. Under the same surgery, dicarbocyanine dye 1,1'dioleyl-3,3,3'-tetramethylindocarbocyanine (DiI, a solution of 25 mg in 50 % methanol; Molecular Probes, Eugene, OR) was injected into 10 sites (4 μ L per site) into the muscle wall of the descending colon (5-7 cm away proximal to the external anal sphincter) to label colonic afferent neurons innervating this area. Sterilized Hamilton syringe (10 μ L size) was used separately for FB or DiI injection. To prevent leakage and labeling of adjacent tissues, the needle was left in place for 30 sec after each injection and a cotton swab was held close to the injection site to wipe off any excess dye that might leak from the needle tip during the needle withdrawal. In this manner, no visible leakage of the dyes was observed after each injection. Injections into the lumen, major blood vessels, or overlying fascial layers were avoided. The incision was closed with 4-0 sutures. The rats were allowed for survival until the harvest of the tissues. We also checked the leakage of the dyes by examining the bladder, colon, and surrounding tissues after euthanasia of the rats. No contamination of FB to colon and other tissues was noticed, and no contamination of DiI to bladder and other tissues was noticed.

Induction of Localized Inflammation

To induce inflammation in the distal colon locally, fasted rats were anesthetized and TNBS was instilled into the lumen of the colon at a dose of 150 mg/kg (60 mg/mL solution in 50 % EtOH) through a syringe-attached polyethylene catheter via the rectum 6 cm proximal to the anus. Animals that received similar volume of 50 % EtOH enema or saline served as control. All colonic instillations were performed under isoflurane (2.5 %) anesthesia. Euthanasia of animals was performed 7 days after intracolonic injection. To ensure exposure of the distal colon to TNBS, rats were held head-down by lifting up the tail for 2 min. In some of the animals, colitis was induced four days after tracing dye injection for a proper transport of these dyes.

According to a previously validated grading scheme for colitis (Morris et al., 1989), we assessed the severity of colitis and only the rats with severe interstitial edema were used for current study. In these rats, TNBS induced extensive ulceration, hyperemia, adhesion, edema, and changes in mucosal architecture. Two to 3-fold increase in the thickness of colonic muscle layer was also noticed in animals with colitis. We also evaluated the urinary bladder in colitic animals compared to control; with colitis, there was no significant sign of bladder inflammation, hypertrophy, and changes in bladder weight (control: 51.25 ± 0.96 mg vs. colitis: 50.33 ± 5.86 mg) that were normally seen in inflamed bladder (Qiao and Vizzard, 2002a). Localized induction of inflammation in the colon with TNBS was also reported in mice and female rats (Lamb et al., 2006; Ustinova et al., 2006b).

Perfusion and Tissue Harvesting

Intracardiac perfusion was performed for euthanasia of animals. Under anesthesia (3–4 % isoflurane), animals were euthanized via perfusion first with oxygenated Krebs buffer (pH 7.4) (95 % O₂, 5 % CO₂) followed by 4 % paraformaldehyde. After perfusion, the spinal cord and DRG were quickly removed and postfixed for 6 h. Tissue was then rinsed in phosphate buffered saline (0.1 M PBS, pH 7.4) and placed in ascending concentrations of sucrose (20 %) for cryoprotection. DRG and spinal cord from level L1–S1 were identified and DRG was sectioned parasagittally at a thickness of 20 μ m. Tissues from control and experimental animals were handled in an identical manner.

Culture of DRG Neurons

L1 and S1 DRG from untreated rats were identified and freshly dissected out and disrupted by enzymatic digestion for 40 min at 31 °C in Dulbecco's modified Eagle's medium (DMEM) containing enzyme mixture (collagenase, 4 mg/mL, Sigma; protease, 4 mg/mL, Sigma). The isolated cells were plated at 3 ganglia per well in a 4-well chamber glass slides (Lab-Trk, Nunc Inc., Naperville, IL) in DMEM supplemented with 10 % fetal bovine serum (DMEM-10), 200 units/mL penicillin, 200 μ g/mL streptomycin, and 100 μ g/mL gentamycin. All cultures were maintained in a 10 % CO₂ environment at 37 °C. Ara C (10 μ M) was added after 48 h of culture and maintained until experiment. Attached DRG cells were then fasted with DMEM prior to the incubation with BDNF (100 ng/mL, Promega) for a variety of time courses. Trk selective inhibitor K252a (Calbiochem) was used at 100 nM concentration. At the designated time points, cells were washed with PBS and fixed for 2 h with 4 % paraformaldehyde. The fixed cells were washed with 0.1 M PBS and ready for immunocytochemistry.

Immunostaining

DRG sections from control and experimental animals were processed for BDNF, TrkB and/or CGRP immunoreactivity using an on-slide immunostaining technique. Tissues from all groups of animals (control and experimental) treated at the same time block were processed simultaneously. Cultured DRG neurons were immunostained with CGRP and PGP9.5 antibodies. Briefly, samples were incubated for 1 h at room temperature with blocking solution containing 3 % normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBST (0.3 % Triton X-100 in 0.1 M PBS, pH 7.4). Samples were then incubated in primary antibodies diluted in PBST containing 5 % normal donkey serum overnight at 4 °C. The following antibodies were used: rabbit anti-CGRP (1:1000, Chemicon International Inc., CA), rabbit anti-TrkB (1:500, Santa Cruz, CA), rabbit anti-BDNF (1:500, Santa Cruz, CA), mouse anti-CGRP (1:2000, Abcam Inc. MA), goat anti-TrkB (1:100, Santa Cruz, CA) and rabbit anti-PGP9.5 (1:1000, Chemicon). After rinse (3 $\times$ 10 min with 0.1 M PBS), tissues were incubated with fluorescence-conjugated species-specific secondary antibody Alexa 594 or 488 (1:500, Molecular Probes, Eugene, OR) for 2 h at room temperature. Following wash, the slides were coverslipped with Citifluor (Citifluor Ltd., London). For sections containing DiI, Triton X-100

in all buffers used was substituted by 0.3 % Tween-20 for the prevention of DiI fading. The specificity of the antibodies and background staining level were evaluated with staining in the absence of primary or secondary antibody. The specificity of the antibodies was also determined by CGRP pre-absorption assay (Fig. 1a and b) and TrkB western blot (Fig. 1c). All the control experiments showed that CGRP and TrkB antibodies used in the current study exhibited high specificity in DRG.

Assessment of Positively Stained DRG Cells

For the DRG cells labeled with neuronal tracing dye, a non-labeled DRG from animals processed on the same day were used as background level. DRG cells exhibiting specific fluorescence (DiI, red; FB, blue) that was greater than background level were counted as positive. Only cells with a visible nucleus were counted. CGRP, BDNF and TrkB immunoreactive cells were counted at a similar manner. CGRP, BDNF and TrkB staining observed in experimental tissue was compared to that observed from experiment-matched negative controls. DRG cells exhibiting cytoplasmic CGRP, BDNF or TrkB staining that was greater than the background level observed in experiment-matched negative controls were considered positively stained. Positively counted cells were not further divided into categories of different staining intensities.

Data Analysis

Positively stained cells were counted independently by two investigators under a Nikon fluorescence photomicroscope with a multiband filter set for simultaneous visualization of DiI and Alexa 594 (red, ex 590 ± 22.5 nm/em 660 ± 16 nm), FB (UV, excitation wavelength of 340-380 nm) and Alexa 488 green fluorophores. DRG from control and experimental rats were grouped and counted according to the date of experiment. For double or triple staining analysis, cells expressing multiple dyes were counted simultaneously. We have chosen every third section for one specific antibody to avoid double counting. The number of DRG cells expressing FB or DiI or the percent of bladder afferent neurons (FB labeled) expressing CGRP or TrkB was presented as mean $\pm$ SEM. Comparisons were made using ANOVA, followed by Dunnett's test.

Digital images were obtained using a digital camera attached to a Nikon fluorescence photomicroscope. Exposure times were held constant when acquiring images from control and experimental rats processed and analyzed on the same day. Images were imported into Adobe Photoshop where groups of images were assembled.

RESULTS

Identification of colonic and bladder primary afferent neurons in lumbosacral DRG before and after colitis

Under the fluorescence microscope, colonic afferent neurons (labeled with DiI, Fig. 2a, red cells) and bladder afferent neurons (labeled with FB, Fig. 2b, blue cells) were visualized and counted from lumbosacral L1-L6 and S1 DRG in control and TNBS animals (Fig. 2d and e). The lumbosacral segments of DRG were chosen based on previous reports that colonic and bladder afferent neurons were mainly located in L1, L2, L6 and S1 DRG (Applebaum et al., 1980; Keast and De Groat, 1992). The L3-L5 DRG was used as internal controls. We found that L1, L2, L6 and S1 DRG contained both FB and DiI cells although the density of the dyes was diverse. In S1 DRG, both FB and DiI cells were intensely labeled (Fig. 2a and b). In L6 DRG, all FB cells were intensely labeled while DiI cells exhibited mixture of intensity. In L1 and L2 DRG, both FB and DiI cells exhibited mixture of intensity. In L3-L5 DRG, few or no cells (0-2 cells per section) were labeled with either FB or DiI. In the present study, we counted all the cells labeled regardless of the intensity as shown in Figure 2. There were no significant

changes in the number of cells labeled with DiI or FB after colitis compared to segment-matched control (Fig. 2d and e). These numbers were averaged from all the DRG sections obtained. The data was not corrected with the area of the different DRG sections even though the L5 is the largest and S1 is the smallest. Results showed that both DiI and FB cells were present in L1, L2, L6 and S1 DRG with 10-30 neurons labeled per section; this was significantly higher than the number of cells labeled in L3-L5 DRG. In L1, L6 and S1 DRG, there were 3 to 5 neurons co-labeled with DiI and FB (Fig. 2c, purple cells, and 2f), which comprised 5-27 % of FB or DiI-labeled neurons (Table 1), suggesting the convergence of colon and bladder afferents. No or few double-labeled cells were present in L2-L5 DRG.

CGRP immunoreactivity in bladder afferent neurons before and after colitis

To examine the distribution of CGRP in bladder afferent neurons before and after colitis, we first assessed the effects of neuronal tracing dye FB injection on CGRP-IR cell profiles in DRG. In L1 DRG from control animals, 33.2 ± 2.6 cells per section expressed CGRP before dye injection and 34.4 ± 3.1 cells per section expressed CGRP after dye injection. Intracolonic instillation of 50 % EtOH (vehicle control) had no effects on CGRP expression in DRG compared to saline when L1 (EtOH: 33.3 ± 4.0 vs saline: 33.4 ± 2.6 cells per section) DRG were examined. Therefore, we considered the changes in CGRP-IR cell profiles in bladder afferent neurons were due to the effects of intracolonic instillation of TNBS and colonic inflammation. After colitis, the number of cells expressing CGRP was increased in L1 DRG (72.8 ± 8.3 cells per section, $p < 0.05$) compared to control.

In all DRG examined, a subpopulation of bladder afferent neurons (Fig. 3a, blue cells labeled with FB) expressed CGRP immunoreactivity (Fig. 3b and c, cells indicated with green arrows). Not all bladder afferent neurons expressed CGRP (Fig. 3a-c, cells indicated with orange arrow) or all CGRP-immunoreactive neurons were bladder afferent neurons (Fig. 3a-c, cells indicated with yellow arrow). In control animals (saline and 50 % EtOH-treated combined), 15-38 % of FB cells expressed CGRP (specifically in L1 DRG: 37.7 ± 5.1 %, L6 DRG: 21.1 ± 2.8 % and S1 DRG: 15.5 ± 1.9 %). After TNBS-induced colitis, significant increases in the percentage of FB cells expressing CGRP were observed in L1 DRG (61.2 ± 2.9 %, $p < 0.05$) and S1 DRG (26.3 ± 2.3 %, $p < 0.01$) compared to the matched segments from control animals (Fig. 3d). Colitis did not cause significant changes in the percentage of FB cells expressing CGRP in L6 (22.9 ± 3.3 %, $p = 0.69$) DRG compared to control (Fig. 3d).

Some of the DRG neurons with dichotomizing projection to both bladder and colon (convergent neurons, co-labeled with DiI and FB in Fig. 4a and b) also expressed CGRP (Fig. 4a-d, white arrow). Following colitis, the percentage of the convergent neurons expressing CGRP was significantly increased in L1 DRG (5.2-fold, $p < 0.05$) and S1 DRG (1.5-fold, $p < 0.05$) but not in L6 DRG (Fig. 4e).

BDNF immunoreactivity in DRG before and after colitis

We previously reported that the levels of BDNF mRNA and protein were increased in L1 and S1 DRG after colitis (Qiao et al., 2006). Here we showed that BDNF immunoreactivity was constitutively expressed in lumbosacral DRG in control animals (Fig. 5a) with a significant increase (Fig. 5b) after colitis. In control animals, there were 19.8 ± 4.5 cells per section expressed BDNF in L1 DRG and the number of cells expressing BDNF was increased to 42.1 ± 12.2 per section ($p < 0.01$) after colitis. In S1 DRG, colitis vs control was 41.6 ± 14.3 vs 18.7 ± 2.1 cells per section ($p < 0.01$). There was no change in the number of cells expressing BDNF in L6 DRG after colitis (35.2 ± 16.5 cells per section) compared to control (28.6 ± 8.3 cells per section).

TrkB immunoreactivity in whole DRG and bladder afferent neurons before and after colitis

We further examined the changes in BDNF high affinity receptor TrkB in L1 and S1 DRG before and after colitis (Fig. 6a and d). In control (saline) animals, TrkB-immunoreactive cell profiles were observed in L1 (22.3 ± 3.4 cells per section) and S1 (15.4 ± 3.7 cells per section) DRG. In vehicle (50 % EtOH)-treated animals, there were no significant changes in the number of DRG cells expressing TrkB immunoreactivity in L1 (19.6 ± 7.6 cells per section) and S1 (20.2 ± 2.4 cells per section) DRG compared to saline control. In TNBS-treated animals, the number of DRG cells expressing TrkB immunoreactivity was significantly increased in L1 (55.4 ± 7.8 cells per section, $p < 0.01$) and S1 (32.8 ± 5.0 cells per section, $p < 0.01$) DRG compared to saline and vehicle controls.

To determine if TrkB immunoreactivity, like CGRP, was also expressed in bladder afferent neurons, we examined the percentage of FB-labeled bladder afferent neurons expressing TrkB in L1 and S1 DRG before and after colitis. The immunostaining showed that a subpopulation of FB-labeled neurons (Fig. 6b, blue cells) were TrkB-immunoreactive (arrows-indicated purple cells in Fig. 6c, merged from 6a and b). In control animals, 25.2 ± 4.3 % of FB cells from L1 DRG expressed TrkB; and 38.3 ± 3.6 % of FB cells from S1 DRG expressed TrkB. After colitis, significant increases in the percentage of FB cells expressing TrkB were observed in L1 (39.7 ± 2.9 %, $p < 0.05$) and S1 (45.6 ± 3.8 %, $p < 0.01$) DRG (Fig. 6e).

Co-localization of CGRP and TrkB in DRG

The co-localization of CGRP and TrkB in L1 and S1 DRG was examined with double immunostaining before and after colitis. In all DRG examined, a subpopulation of TrkB-immunoreactive cells (Fig. 7a and c, red and yellow cells) also expressed CGRP (Fig. 7c, yellow cells merged from 7a and 7b). In L1 DRG from control animals, there were 6.1 ± 1.7 cells per section co-expressing TrkB and CGRP; in S1 DRG from control animals, there were 5.7 ± 1.1 cells per section co-expressing TrkB and CGRP. After colitis, the number of DRG cells per section co-expressing TrkB and CGRP was significantly increased in L1 (16.7 ± 2.1 , $p < 0.01$) and S1 (13.8 ± 0.5 , $p < 0.01$) DRG (Fig. 7d). There were no changes in the percentage of TrkB cells expressing CGRP (Fig. 7e).

Effects of BDNF on CGRP expression in cultured DRG

To examine if TrkB-mediated signaling is involved in CGRP expression in DRG, we incubated cultured L1 and S1 DRG neurons with BDNF (Fig. 8a and b) for a variety of time course. The total number of DRG neurons was visualized with immunostaining of PGP9.5, a specific neuronal marker (Fig. 8a and b, red and yellow cells). CGRP expression level was presented as the percentage of PGP9.5-immunoreactive DRG neurons expressing CGRP (Fig. 8a and b, yellow cells) before (Fig. 8a) and after (Fig. 8b) BDNF application. Acute BDNF treatment did not cause significant changes in the percentage of DRG neurons expressing CGRP; however, overnight incubation with BDNF significantly augmented CGRP expression (1.5-fold, $p < 0.05$) in cultured DRG neurons (Fig. 8c). Pre-incubation with K252a, a selective antagonist of Trk, effectively blocked BDNF-induced CGRP expression in the DRG neurons (Fig. 8d).

DISCUSSION

The present studies demonstrate that colitis-induced colon-to-bladder sensory cross-sensitization involves the up-regulation of CGRP and TrkB in bladder afferent neurons. In these studies, we showed that colitis induced significant increases in the percentage of bladder afferent neurons expressing CGRP in L1 and S1 DRG but not L6 DRG. This may be due to increased BDNF expression in L1 and S1 but not L6 DRG (Qiao et al., 2006 and the present study). To determine the role of BDNF in inducing CGRP expression in bladder afferent

neurons following colitis, we examined the expression of BDNF high affinity receptor TrkB in lumbosacral DRG and showed that 1) TrkB immunoreactivity was significantly increased in the L1 and S1 DRG following colitis; 2) the expression of TrkB in bladder afferent neurons was increased following colitis; and 3) the number of cells co-expressing CGRP and TrkB was significantly increased in L1 and S1 DRG following colitis compared to control. In cultured DRG neurons, BDNF significantly induced CGRP expression, which was attenuated by Trk selective inhibitor K252a.

Disorders of the peripheral organs as well as injuries or diseases of the nervous system (i.e. chronic spinal cord injury, cystitis, colitis) can produce neurogenic bladder overactivity coupled with increases in neurochemicals and the excitability of C-fibers and neurons (Kruse and de Groat, 1993; Yoshimura and de Groat, 1999; Malykhina et al., 2004; Qiao and Vizzard, 2002a,b;2004; Ustinova et al., 2006). The underlying mechanism of the neurogenic bladder overactivity resulting from a wide variety of factors such as nerve injury and diseases is not clear. The symptoms that patients with IBD also present with abnormal bladder function may be due to the similar spinal innervation of colon and bladder and sensory cross interaction in the level of spinal cord and DRG. In the present study, we first examined the segmental distribution pattern of colonic and bladder primary afferents in lumbosacral DRG with double dye retrograde tracing techniques and demonstrated that the colonic and bladder afferent neurons were located in L1, L2, L6 and S1 DRG. Recent studies on the characterization of the primary afferent innervation of the mouse colon with FB retrograde labeling demonstrated that colonic afferents in mouse were located in L1, L2, L6 and S1 DRG. The present study and previous reports showed that bladder afferent neurons were mainly located in DRG at the spinal levels L1-L2 and L6-S1 in rat (Applebaum et al., 1980; Keast and de Groat, 1992; Bennett et al., 1996; Qiao and Vizzard, 2002a,b), and these DRG also contain primary afferents that innervate the distal colon in rat (Keast and de Groat, 1992; Traub et al., 1999). The two peaks of labeling are likely to be due to innervation from the hypogastric/splanchnic and pelvic afferents (Berthoud et al., 2004; Bielefeldt et al., 2005) respectively. The present study used two distinct dyes FB and DiI labeling for bladder and colonic afferents respectively and the number of cells labeled per section was comparable with previous studies (Bennett et al., 1996; Vizzard, 2001) that reported six to 25 labeled cells in DRG sections after FB retrograde labeling of the rat bladder but a little higher than the one reported by Keast and de Groat (1992). This may be due to the amount of dye injected (total volume: 5-10 μ L in reported study vs. 40 μ L in present study) and/or the number of injection sites in the bladder wall. In the present study, we injected same amount of dye with the same number of injection sites to each animal studied and showed that with identical manners in the dye injection, there were no significant changes in the number of cells labeled in DRG section before and after colitis. With double labeling techniques, we also demonstrated that 3-5 neurons per DRG section were labeled with both dyes, along with earlier reports (Keast and de Groat, 1992; Christianson et al., 2004), suggesting a dichotomizing projection to both colon and bladder. The existence of convergent neurons was not due to cross contamination of the dye to adjunct tissues based on the postmortem examination. As well, there were no significant changes in the number of dichotomizing neurons in DRG section before and after colitis. Based on these studies, we characterized the changes in CGRP and TrkB in bladder afferent neurons in L1, L6 and S1 DRG before and after colitis in order to determine the correlation between neurotrophins and CGRP with colitis-induced neurogenic bladder overactivity.

CGRP is an important nociceptive marker and plays a major role in mediating hypersensitivity in many systems (Smith et al., 1992; Nahin and Byers, 1994; Zhang et al., 2001; Winston et al., 2003). Following peripheral inflammation, more neurons had detectable CGRP expression in the innervating DRG, and mice lacking α -CGRP failed to develop secondary hyperalgesia after inflammation (Smith et al., 1992; Zhang et al., 2001). Administration of a CGRP receptor antagonist greatly attenuated the visceromotor reflex in rats with colonic inflammation (Plourde

et al., 1997) and reduced TNBS-induced colonic hypersensitivity (Delafoy et al., 2006). The association of CGRP expression in lower urinary tract following chemical cystitis-induced bladder overactivity has been reported (Vizzard, 2001), however, the involvement of CGRP in colitis-induced cross-activation of bladder afferent neurons is not clear. In the present study, we examined the level of CGRP immunoreactivity in bladder afferent neurons following TNBS colitis. Significant increases in the percentage of bladder afferent neurons expressing CGRP were observed in L1 and S1 DRG following 7 days of colitis. The changes in CGRP level in response to peripheral inflammation or injury have been controversial but are reported to have a time-dependent fashion: short-term (up to 72 h) induction of colitis decreased CGRP content in colonic afferent neurons (Traub et al., 1999) but chronic inflammation of bladder induced CGRP up-regulation in bladder afferent neurons (Vizzard, 2001); acute somatic inflammation (6 h) decreased CGRP content in dorsal spinal cord (Galeazza et al., 1995) while chronic somatic injury (up to several months) increased the level of CGRP in DRG and spinal cord (Smith et al., 1992; Hanesch et al., 1993; Galeazza et al., 1995). We recently characterized CGRP mRNA level in lumbosacral DRG before and after colitis and found that CGRP mRNA was increased in L1 and S1 DRG at both 3 and 7 days after colitis (unpublished data). Therefore, the time-dependent differential changes in CGRP expression in the DRG may be due to initial release of CGRP centrally and then balanced with increased CGRP production after chronic inflammation or injury. Recent studies reported that addition of CGRP to DRG neurons enhances TTX-resistant (TTX-R) Na⁺ currents (Natura, 2005). Over-expression of CGRP in bladder afferent neurons with colitis may also be associated with bladder afferent sensitization such as colitis-induced increase in TTX-R Na⁺ currents in these neurons (Malykhina et al., 2004).

Previous (Qiao et al., 2006) and the current studies showed that BDNF expression level was significantly increased in L1 and S1 but not L6 DRG following colitis. The CGRP promoter contains a complex proximal element that is cAMP- and ras-responsive (Lanigan and Russo, 1997), suggesting a possibility of neurotrophins in inducing CGRP expression in DRG. To examine if BDNF expression contributes to increased CGRP expression in bladder afferent neurons, we examined, in the present study, the distribution of BDNF high affinity receptor TrkB in bladder afferent neurons before and after colitis. Results showed that TrkB immunoreactivity was significantly increased in the whole DRG (1.6-1.8 fold, $p < 0.01$) and in FB-labeled L1 and S1 bladder afferent neurons (1.6-fold in L1 DRG, $p < 0.01$) after colitis. We also demonstrated that the number of cells co-expressing TrkB and CGRP was greatly increased in DRG following colitis. Interestingly, the current study showed that there were no changes in the percentage of TrkB cells expressing CGRP before and after colitis, suggesting that CGRP expression was proportionally increased along the up-regulation of TrkB. Increased TrkB expression in DRG neurons may enhance the responsiveness of these neurons to BDNF therefore induces CGRP expression. The current studies also suggest that the increased TrkB and CGRP was not only located in the bladder afferent neurons but also in the non-labeled neurons. For example, TrkB-immunoreactive cell profile was increased in the bladder afferent neurons by a smaller number (colitis: 12 vs control: 7 cells per section); whereas TrkB-immunoreactive cell profile was increased in the whole DRG by a larger number (colitis: 55 vs control: 20 cells per section). Similar results were shown with CGRP expression in the DRG. These data suggest that increased BDNF after colitis may act on DRG neurons including bladder afferent neurons and induce CGRP expression in TrkB-containing cells.

Following colitis, NGF and TrkA were greatly increased in the inflamed colon (di Mola et al., 2000), which may change the phenotype of afferent neurons in DRG and increase the production of neuromodulators (i.e. BDNF) in sensory neurons through retrograde transport. Following peripheral or visceral inflammation, significant increases in BDNF and receptor TrkB mRNA in DRG and spinal cord have also been reported (Cho et al., 1997a,b; Lee et al., 1999; Mannion et al., 1999; Qiao and Vizzard, 2002a). Previous studies with cystitis rat model

(Vizzard, 2001; Qiao and Vizzard, 2002a) suggest that increased expression of TrkB and CGRP in bladder afferent neurons may contribute to cystitis-induced bladder overactivity. As well, the present study suggests that increased TrkB and CGRP expression in bladder afferent neurons may play a role in colon-to-bladder cross-sensitization following colitis.

The role of NGF in regulating CGRP in sensory neurons has been extensively reported. Injection of NGF (alphaNGF) antiserum decreased the levels of CGRP protein expressed in DRG (Shadiack et al., 2001). NGF immunoneutralization greatly blocked the activity of CGRP nerve fibers (Sevcik et al., 2005) and attenuated the up-regulation of CGRP in spinal cord induced by spinal cord injury (Christensen and Hulsebosch, 1997; Krenz et al., 1999; Sevcik et al., 2005). However, the contribution of BDNF, a central neuronal modulator, in the transcriptional regulation of CGRP in DRG is unknown. Unlike NGF, BDNF expression in sensory neurons may fit into a local autocrine and paracrine mechanism rather than the classic target derived model following peripheral inflammation (Lee et al., 1999; Michael et al., 1999; Tonra, 1999; Kim et al., 2001). Therefore, we hypothesize that increased BDNF in DRG following colitis may act on bladder afferent neurons and bladder/colonic convergent neurons, induce CGRP expression; and this may further contribute to bladder hypersensitivity. In DRG, 55 % of BDNF-immunoreactive neurons were co-localized with CGRP (Luo, et al., 2001); and in rat trigeminal ganglion, 54 % of BDNF-immunoreactive neurons contained CGRP (Ichikawa et al., 2006), suggesting a possible role of BDNF in regulating CGRP in these neurons. In the present study, incubation of BDNF with cultured DRG augmented CGRP expression in these neurons. Increased expression of TrkB in bladder afferent neurons may lead to increased responsiveness of these neurons to BDNF, which may play a role in the regulation of CGRP expression in these neurons.

In summary, the present study demonstrated that colitis-induced bladder cross-sensitization involved neural cross interaction in the level of primary afferents as was expressed as neurochemical and neurotransmitter alterations. Up-regulation of CGRP in bladder afferent neurons with colitis may result from increased expression of BDNF in lumbosacral DRG. Increased expression of TrkB in bladder afferent neurons after colitis increases the response of these neurons to BDNF and initiates signal transduction; this may lead to CGRP over-expression and further contribute to bladder hypersensitivity such as increased membrane excitability. Taken together, the present studies demonstrated that neurotrophins play an important role in target organ-to-neuron and neuron-to-neuron interaction, which may contribute to the neurogenic cystitis resulting from colitis.

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Figure 1.

Pre-absorption of CGRP antibody shows that the CGRP antibody used in this study specifically recognized α -CGRP in DRG. Compare b: after absorption to a: before absorption. Calibration bar = 80 μ m. c, western blot of TrkB shows the specificity of TrkB antibody used in the present study.

Figure 2.
Distribution of colonic afferent neurons and bladder afferent neurons in lumbosacral dorsal root ganglia. Colonic afferent neurons (a, red cells) and bladder afferent neurons (b, blue cells) were retrogradely labeled with DiI and FB respectively. In the merged image (c), purple cell (green arrow) indicates convergent neurons with projection to both colon and bladder. Colonic afferent neurons (d and f) and bladder afferent neurons (e and f) are mainly distributed in L1, L2, L6 and S1 DRG but not L3-L5 DRG. TNBS colitis has no effects on the number of neurons labeled for colonic afferents (n = 6 animals) or bladder afferents (n = 9 animals) compared to control (n = 5-7 animals). Sample photomicrographs show S1 DRG from control animal. Calibration bar = 80 μ m.

Figure 3.
Distribution of CGRP immunoreactivity in bladder afferent neurons before and after colitis. The distribution of CGRP (b, green cells) in bladder afferent neurons (a, FB-labeled blue cells) was characterized by examining the percentage of FB-labeled neurons expressing CGRP. In the merged image (c), bladder afferent neurons that contain CGRP are indicated with green arrows. Histogram (d) shows that the percentage of bladder afferent neurons expressing CGRP was significantly increased in L1 and S1 DRG following colitis ($n = 9$) compared to control ($n = 7$). There was no change in the percentage of bladder afferent neurons expressing CGRP in L6 DRG. Panel e indicates the result of pre-absorption of CGRP antibody. Sample photomicrographs show L1 DRG from control animal. Calibration bar = 80 μm . NS, not significant.

Figure 4.
Distribution of CGRP immunoreactivity in convergent neurons with projection to both colon and bladder. Colonic (a, red cells) and bladder afferent neurons (b, blue cells) were labeled with DiI or FB respectively. The distribution of CGRP (c, green cells) in the neurons with dichotomizing projection to both colon and bladder was characterized by examining the percentage of DiI and FB double-labeled neurons expressing CGRP. In the merged image (d), the convergent afferent neuron that expressed CGRP is indicated with white arrow. Histogram (e) shows that the percentage of convergent afferent neurons expressing CGRP was significantly increased in L1 and S1 DRG following colitis (n = 6) compared to control (n = 5). There was no change in the percentage of the convergent afferent neurons expressing CGRP

in L6 DRG. Sample photomicrographs show S1 DRG from TNBS animal. Calibration bar = 80 μ m. NS, not significant.

Figure 5.

Expression of brain-derived neurotrophic factor in lumbosacral DRG before and after colitis. Immunostaining of BDNF in L1, L6 and S1 DRG from control (a) and colitic (b) rats showed that BDNF was constitutively expressed in all the DRG examined (arrows in a and b). Histogram (c) showed that the BDNF cell profiles was significantly increased in L1 and S1 DRG but not in L6 DRG after colitis compared to control. Calibration bar = 100 μ m. NS, not significant.

Figure 6.
Distribution of TrkB immunoreactivity in the whole DRG and bladder afferent neurons before and after colitis. The distribution of TrkB (a, red cells) in bladder afferent neurons (b, FB-labeled blue cells) was characterized by examining the percentage of FB-labeled neurons expressing TrkB. In the merged image (c), bladder afferent neurons that contain TrkB are indicated with white arrows (purple cells). Histogram d shows TrkB cell profiles in the whole DRG before and after colitis. Histogram e shows that the percentage of bladder afferent neurons expressing TrkB was significantly increased in L1 and S1 DRG following colitis ($n = 7$) compared to control ($n = 6$). Sample photomicrographs show L1 DRG from control animal. Calibration bar = 80 μm .

Figure 7.
Co-localization of TrkB and CGRP in lumbosacral DRG before and after colitis. DRG sections were double stained with TrkB (a, red cells) and CGRP (b, green cells) antibodies simultaneously. The cells co-expressing TrkB and CGRP (c, yellow cells, white arrows) were counted from L1 and S1 DRG before ($n = 4$) and after colitis ($n = 5$). Histogram (d) shows significant increases in the number of L1 and S1 DRG cells co-expressing TrkB and CGRP after colitis compared to control. Sample photomicrographs show L1 DRG from TNBS animal. Calibration bar = 100 μ m.

Figure 8.

Effects of BDNF on CGRP expression in cultured DRG neurons. The L1 and S1 DRG from control animals were dissociated with enzyme and incubated with BDNF (100 ng/mL) for a variety of time course. CGRP expression was normalized with the total number of neurons (red and yellow cells) containing PGP9.5, a neuron specific marker. In the merged images (a and b), neurons containing CGRP are pseudocolored yellow (arrows) and neurons without CGRP are pseudocolored red. Histograms (c and d) show that exogenous BDNF significantly induced CGRP expression after 12 h of incubation, and BDNF-induced CGRP was attenuated with Trk selective inhibitor K252a (100 nM). Calibration bar = 80 μ m.

Table 1

Percentage of FB or DiI neurons with dichotomizing projection to both colon and bladder.

	DiI+FB/FB (%)	DiI+FB/DiI (%)
L1	7.3	20.8
L2	5	4.6
L6	11.9	27.6
S1	25	21.7