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Up-regulation of brain-derived neurotrophic factor is regulated by extracellular signal-regulated protein kinase 5 and by nerve growth factor retrograde signaling in colonic afferent neurons in colitis

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Abstract

Brain-derived neurotrophic factor (BDNF) plays an essential role in sensory neuronal activation in response to visceral inflammation. Here we report that BDNF up-regulation in the primary afferent neurons in the dorsal root ganglia (DRG) in a rat model of colitis is mediated by the activation of endogenous extracellular signal-regulated protein kinases (ERK) 5 and by nerve growth factor (NGF) retrograde signaling. At 7 days of colitis, the expression level of BDNF is increased in conventional neuronal tracing dye Fast Blue labeled primary afferent neurons project to the distal colon. In these neurons, the phosphorylation (activation) level of ERK5 is also increased. In contrast, the level of phospho-ERK1/2 is not changed in the DRG during colitis. Prevention of the ERK5 activation in vivo with an intrathecal application of the MEK inhibitor PD98059 significantly attenuates the colitis-induced increases in BDNF expression in the DRG. Further studies show that BDNF up-regulation in the DRG is triggered by NGF retrograde signaling which also involves activation of the MEK/ERK pathways. Application of exogenous NGF exclusively to the compartment containing DRG nerve terminals in an ex vivo ganglia-nerve preparation has markedly increased the BDNF expression level in the DRG neuronal cell body that is placed in a different compartment; this BDNF elevation is attenuated by U0126, PD98059 and a specific ERK5 inhibitor BIX02188. These results demonstrate the mechanisms and pathways by which BDNF expression is elevated in primary sensory neurons following visceral inflammation that is mediated by increased activity of ERK5 and is likely to be triggered by the elevated NGF level in the inflamed viscera.

Keywords

brain-derived neurotrophic factor; extracellular signal-regulated kinase; nerve growth factor; retrograde; primary afferent neuron

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INTRODUCTION

Visceral inflammation induces considerable neuronal plasticity in the primary afferent pathways that may underlie the inflammation-induced sensory hypersensitivity. In animal models with colitis induced by chemicals such as tri-nitrobenzene sulfonic acid (TNBS), zymosan, acetic acid, mustard oil, or dextran sulphate sodium, there are increased levels of neuroactive compounds in the dorsal root ganglia (DRG) accompanied with increased sensory hypersensitivity (Burton and Gebhart, 1995; Coutinho et al., 1996; Larsson et al., 2006; Qiao et al., 2008; Qiao and Grider, 2010; Xia et al., 2012). This may underlie the mechanism by which patients with visceral inflammation such as active ulcerative colitis often experience pain, urgency, and incontinence (Bernstein et al., 1996; Drewes et al., 2006). The brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor (NGF) family, is enriched in the sensory neuronal cell body in the DRG and is implicated to participate in sensory neuronal activation in a variety of animal models (Mannion et al., 1999; Obata and Noguchi, 2006; Lin et al., 2011; Cao et al., 2012; Xia et al., 2012). In peripheral inflammation-induced hyperalgesia, spinal intrathecal injection of BDNF/TrkB antisense or antiserum significantly blocks inflammation-induced somatic pain (Groth and Aanonsen, 2002; Matayoshi et al., 2005). Studies by us and others also show that neutralization of the endogenous BDNF attenuates colitis-induced visceral hypersensitivity (Delafooy et al., 2006; Xia et al., 2012). Direct measurement of BDNF expression levels in these animal models demonstrates marked up-regulation of BDNF messenger as well as BDNF protein in the sensory neuronal bodies in the DRG (Qiao et al., 2008) and in capsaicin-sensitive nociceptive neurons (Xia et al., 2012). BDNF generated by the DRG neurons can undergo anterograde transport to the central nerve terminals in the spinal dorsal horn and its release can increase synapse efficacy thereby contributing to central sensitization (Qiao et al., 2008; Wang et al., 2012).

The rat BDNF promoter III has a CRE element (5'-TCACGTCA-3') that is activated by the transcription factor cAMP response element-binding (CREB) (Tao et al., 1998). CREB activation is associated with its phosphorylation on Ser¹³³ by ERK1/2 in cultured hippocampal neurons and brain slices (Impey et al., 1998; Obrietan et al., 1999). ERK is a subfamily of the mitogen-activated protein kinases (MAPK) and includes isoforms ERK1, ERK2 and the big MAPK ERK5. The activated ERK5 initiates a phosphorylation cascade through activation of p90 ribosomal S6 kinase which ultimately results in activation of CREB (Watson et al., 2001; Wang and Tournier, 2006). Several lines of evidence have established that activation of ERK in the primary afferent pathway regulates sensory hypersensitivity following peripheral inflammation or nerve injury. For example, in response to noxious stimulation of the peripheral tissue or electrical stimulation to the peripheral nerve, ERK1/2 is activated in the spinal cord dorsal horn neurons (Ji et al., 2002; Qiao et al., 2008), and both ERK1/2 and ERK5 are demonstrated to be phosphorylated in the DRG (Obata et al., 2003; 2004; Qiao and Gulick, 2007). Intrathecal injection of MEK inhibitor U0126 or PD98059, which specifically attenuates ERK1/2 and ERK5 activities (Kamakura et al., 1999), has significantly alleviated pain behavior induced by inflammation to hind paw (Ji et al., 2002; Obata et al., 2003), viscera (Galan et al., 2003; Cruz et al., 2007; Lai et al., 2011) or joint (Schrader et al., 2006). The present study demonstrates that downstream target of PD98059 could be to suppress BDNF production in the DRG thereby inhibiting sensory activity.

The MEK/ERK pathway is a primary target of NGF signaling. A unique characteristic of NGF is the retrograde transport of NGF/TrkA or its signaling by which NGF derived from the target tissue can influence cellular physiology in the remote neuronal cell body (Howe and Mobley, 2005). It is extensively reported that peripheral inflammation increases NGF expression in the inflamed distal organ in humans as well as in animal models (Woolf et al.,

1994; Lowe et al., 1997; di Mola et al., 2000; Vizzard, 2000; Stanzel et al., 2008; Qiao and Grider, 2010). The expression of NGF and TrkA are increased in both neural and non-neural structures of bowel in patients with Crohn's disease or ulcerative colitis (di Mola et al., 2000), and NGF mRNA is also elevated in the distal colon post colitis induction in rat (Stanzel et al., 2008; Qiao and Grider, 2010). We recently demonstrate that TrkA expression level is increased in specifically labeled colonic afferent neurons during colitis which is due to retrograde transport of TrkA from the peripheral afferent nerve terminals located in the distal colon to the colonic afferent neurons located in the DRG (Qiao and Grider, 2010). Up-regulation of TrkA in DRG during colitis may enhance the responsiveness of these neurons to NGF and increase NGF-initiated signal transduction such as activation of ERK and its signaling targets in these neurons.

A study with systemic drug intervention by injecting antiserum of either NGF or BDNF intraperitoneally to rats in TNBS-induced colitis suggests a possible interaction of the NGF and BDNF pathways in colitis-induced visceral hypersensitivity (Delafoy et al., 2006). The present study utilizes the same animal model as well as an ex vivo two-compartmented culture approaches in showing that colitis-induced BDNF up-regulation in the lumbosacral DRG is mediated by an increased activity of ERK5 in colonic afferent neurons. Retrograde NGF has a role in elevating the ERK5-BDNF axis in sensory neurons.

MATERIALS AND METHODS

Experimental animals and reagents

Adult male Sprague-Dawley rats (150–200 g) from Harlan Sprague Dawley, Inc. (Indianapolis, IN) were used. 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 as well as to reduce the number of animals used. General chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Induction of colonic inflammation

To induce inflammation in the distal colon, 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 on day 7 after TNBS treatment. To ensure exposure of the distal colon to TNBS, rats were held head-down by lifting up the tail for 2 min.

Retrograde labeling

Under anesthesia (2.5 % isoflurane), the rat distal colon was exposed with a lower abdominal incision. The neuronal tracing agent, Fast Blue (FB, 4 %, weight/volume; Polysciences, Inc. Warrington, PA), was injected into 10 sites (4 μ L per site) in 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. A sterilized Hamilton syringe (10 μ L size) was used for injection. Three days after FB was injected, TNBS was instilled to the distal colon to induce inflammation. Thus, euthanasia of animals was performed on day 10 after FB injection.

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 µm. Tissues from control and experimental animals were handled in an identical manner.

Immunostaining

DRG sections from control and experimental animals were processed with primary antibodies rabbit anti-BDNF (1:500, Santa Cruz, CA), rabbit anti-phospho-ERK1/2 (1:400, Cell Signaling Technology Inc. Danvers, MA), or rabbit anti-phospho-ERK5 (1:300, Cell Signaling Technology Inc.) overnight at 4 °C followed with fluorescence-conjugated species-specific secondary antibody Alexa 594 or Alexa 488 for 2 hours at room temperature. Following washing, the slides were coverslipped with Citifluor (Citifluor Ltd., London). Immunostaining in the absence of primary or secondary antibody was processed for background evaluation. The specificity of the antibodies was also evaluated with western blot (Qiao and Gulick, 2007; Qiao et al., 2008). Tissues from all groups of animals (control and experimental) treated at the same time block were processed simultaneously.

DRG cells were counted under a Zeiss fluorescent photomicroscope with a multiband filter set for simultaneous visualization of Alexa 594 (red, ex 590 ± 22.5 nm/em 660 ± 16 nm) and FB (UV, excitation wavelength of 340–380 nm). DRG cells with a visible nucleus were identified, and the cells that exhibited greater fluorescence than background level were counted as positive. Within the specific segment of DRG (such as L1 DRG), the similar size of sections were chosen with the microscope built-in grids and all the positive cells were counted in the sections and expressed as number of cells per section. We have chosen every third section for one specific antibody to avoid double counting. For each DRG, 4–6 sections were counted and the number of cells per section was averaged as one point.

Protein extraction

Freshly dissected DRGs were homogenized in solubilization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 100 mM NaF supplemented with protease inhibitor cocktail (P8340, 1:100, Sigma-Aldrich) and phosphatase inhibitor cocktail 1 (P2850, 1:100, Sigma-Aldrich). The homogenate was centrifuged at 20,200 g for 10 min at 4 °C, and the supernatant was removed to a fresh tube for further analysis. The protein concentration was determined using Bio-Rad DC protein assay kit.

Western blot

Proteins were separated on a 7.5–15 % SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5 % milk in Tris-buffered saline for 1 hour and then incubated with phospho-ERK1/2 (1:1000) or phospho-ERK5 (1:1000) antibody followed by horseradish peroxidase-conjugated secondary antibody. The bands were identified by ECL. For internal loading control, the same membrane was stripped and re-probed with antibody against the non-phosphorylation form of ERK1/2 (1:1000) or ERK5 (1:1000). The ECL-exposed films were digitized, and densitometric quantification of immunoreactive bands was performed using the software FluorChem 8800 (Alpha Innotech, San Leandro, CA). The expression level of the target protein in control animal from each independent experiment was considered as 1, and the relative expression level of the target

protein in experimental animals was adjusted as a ratio to control animals. The level of the phospho-bands was normalized with the level of non-phospho-bands obtained using the same membrane. For phospho-ERK1/2, the p42 and p44 bands were analyzed together.

Enzyme-linked immunosorbent assay (ELISA)

BDNF ELISA kit (Promega Corporation) was used for this examination. Briefly, polystyrene microtiter plates (96-well) were pre-coated according to manufacture's instruction. The protein extracts were sequentially treated with 1 N HCl and then neutralized with 1 N NaOH. The treated samples and BDNF standard were incubated sequentially with chicken anti-human BDNF pAb (1:500), anti-IgY HRP conjugate (1:200) and TMB One Solution (3,3',5,5'-tetramethylbenzidine). The absorbance at 450 nm was measured within 30 min of stopping the reaction and the BDNF levels were expressed as pg BDNF per mg total protein calculated against the standard curve.

Drug delivery to animals

Resiniferatoxin (RTX), an ultrapotent analog of capsaicin, was dissolved in 10 % Tween-80, 10 % ethanol, and normal saline and injected systemically as we have reported previously (Qiao and Grider, 2010) at a dose of 300 µg/kg body weight one week prior to TNBS induction to produce long-lasting desensitization of unmyelinated nociceptive C-fiber afferents (Szallasi et al., 1989; Dray et al., 1990; Craft et al., 1995). RTX-induced desensitization was examined by the eye movements in response to RTX solution dropped to the surface of the eye (Szallasi et al., 1989). PD98059 in 20 % DMSO stock was diluted to 100 µg/mL with saline and filled into an osmotic-pump (Alzet 2001) connected to an intrathecal (i.t.) catheter (No. 0007740, Alzet) for long-term drug infusion. Control animals received the same concentration of DMSO in saline. The tip of the catheter was positioned at the L4-L5 spinal level and was confirmed after euthanasia of the animals. This delivery site was chosen because of its position located in the middle of L1 and S1 segments where we found that BDNF was up-regulated during colitis. This procedure was done under anesthesia (2.5 % isoflurane).

DRG-spinal nerve two-compartmented culture

We used a Campenot chamber and a modification of the method of Delcroix et al (2003) to study the DRG-spinal nerve complex. The Campenot chamber has two large compartments on both sides and a narrow one in the middle. After the chamber was sealed onto the bottom of a cell culture plate, the middle compartment was filled with 1 % agarose serving as a divider and the two large compartments containing Dulbecco's Modified Eagle Medium (DMEM) were used for separation of the ganglion and nerve terminals of the isolated S1 DRG-spinal nerve complex. 2–3 layers of small filter papers soaked with DMEM were lightly placed on top of the nerve segment that crossed over the center compartment ("bridge"). The culture plate was then placed into cell culture incubator at 37°C. After 3 h of quiescent time, the nerve terminals were treated with NGF.

Statistical analysis

The results from each study were presented as mean ± SE. Comparison between control and experimental groups was made by using a one-way ANOVA followed by the Dunnett's test. Comparison between multiple groups was made by using a one-way ANOVA followed by the Newman-Keuls's test. When two groups were compared, a student's *t* test was used. Differences between means at a level of *p* 0.05 were considered to be significant.

RESULTS

Up-regulation of BDNF in colonic afferent neurons during colitis

Inflammatory irritation of the distal colon causes considerable changes in the primary afferent pathways projecting to the viscera. This includes the up-regulation of BDNF in specifically labeled colonic afferent neurons in the DRG (Fig. 1). We have previously shown that thoracolumbar and lumbosacral DRGs contain colonic primary afferent neurons (Qiao and Grider, 2007), and the expression level of BDNF was demonstrated to be increased in the L1 and S1 DRG at 7 days post colitis induction (Qiao et al., 2008). The present study shows that at 7 days of colitis the percentage of colonic afferent neurons (Fig. 1A, FB-labeled blue cells from L1 DRG) in the L1 and S1 DRGs expressing BDNF (Fig. 1B, red cells) was also increased (Fig. 1D). To examine whether colitis-induced neural up-regulation in the distal colon has a role in BDNF up-regulation in the colonic afferent neurons, we pre-treated the animals with RTX to block the activity of capsaicin-sensitive fibers (presumably C-fibers). Results showed that prolonged pre-administration of RTX reversed colitis-induced BDNF up-regulation in the colonic afferent neurons (Fig. 1E).

Retrograde NGF signaling increased BDNF expression in the DRG neurons via MEK/ERK pathway

In the inflamed distal colon, the expression level of NGF was increased which also facilitated TrkA retrograde transport and increased TrkA expression in colonic afferent neurons in the DRG (Qiao and Grider, 2010). To examine if the retrograde NGF signaling had a role in BDNF expression in the DRG, we utilized an ex vivo ganglia-nerve preparation in a two-compartmented culture. At 12 h after NGF (50 ng/mL) was added to the compartment containing S1 DRG nerve terminals, the expression level of BDNF in the DRG neuronal cell bodies located in another compartment was examined. Our results show that retrograde NGF increased BDNF expression in the DRG neurons (compare Fig. 2B to A; Fig. 2F). Blockade of the MEK/ERK pathway with either U0126 (2 μ M) (compare Fig. 2C to B) or PD98059 (5 μ M) (compare Fig. 2D to B) significantly reduced BDNF level in the DRG soma resulting from NGF retrograde signaling (Fig. 2F). Since U0126 and PD98059 can block both ERK1/2 and ERK5 activities (Kamakura et al., 1999), we used a newly developed ERK5 inhibitor BIX02188 (Tatake et al., 2008; Obara et al., 2009) and found that BIX02188 (30 μ M, Selleck Chemicals LLC, Houston, TX) was also able to attenuate NGF-induced BDNF up-regulation in the DRG soma (compare Fig. 2E to B, Fig. 2F).

Activation of ERK5 but not ERK1/2 in colonic afferent neurons in colitis

Western blot results showed that the level of phospho-ERK5 was significantly increased in the DRG (L1+S1) (Fig. 3A) with a 3-fold increase at 7 days post colitis induction when compared to control (Fig. 3B). The level of phospho-ERK1/2 was not changed in these DRGs at 7 days post colitis induction (Fig. 3C, D). To examine whether ERK1/2 was activated at an earlier time point, we examined 3 days of colitis and found that colitis did not change the phosphorylation level of ERK1/2 in the DRG (Fig. 3E, F). This observation was confirmed with immunohistochemistry (Fig. 4A to D) showing that the number of DRG neurons expressing phospho-ERK5 was significantly increased in the L1 (Fig. 4E) and S1 DRGs (Fig. 4F), while there was no change in the number of L1 and S1 DRG neurons expressing phospho-ERK1/2 (Fig. 4E, F). Further examination showed that the phospho-ERK5 immunoreactivity (Fig. 5A) was expressed in colonic afferent neurons labeled with neuronal tracing dye FB (Fig. 5B) in small diameter ($\sim 20 \mu$ m) neurons with a 3-fold increase in the percentage of specifically labeled colonic afferent neurons expressing phospho-ERK5 (Fig. 5D).

Activation of ERK5 regulated BDNF expression in vivo

To examine if the activation of ERK5 in the DRG during colitis contributes to BDNF expression in the DRG, MEK/ERK inhibitor PD98059 was infused intrathecally one day before the colitis induction and continuously infused for 7 days with an Alzet mini-pump. The BDNF immunoreactive neurons were counted in the L1 and S1 DRG from animals treated with vehicle (DMSO) + 50 % EtOH (Figure 6A), vehicle + TNBS (Figure 6B), PD98059 + EtOH (Figure 6C), or PD98059 + TNBS (Figure 6D). Results showed that colitis increased BDNF expression in both L1 and S1 DRG from animals treated with vehicle (Fig. 6E); however, colitis failed to increase BDNF expression in the L1 and S1 DRG from animals receiving continuous PD98059 intervention (Fig. 6E). The results that PD98059 treatment attenuated BDNF expression in the DRG was confirmed with ELISA measurement of BDNF content in these DRGs (Fig. 7).

DISCUSSION

Previous physiological studies by us and others have revealed critical roles of BDNF in mediating sensory hypersensitivity during colitis by blocking endogenous BDNF action with a BDNF neutralizing antibody (Delafoy et al., 2006; Xia et al., 2012). The present study shows that BDNF is enriched in the colonic afferent neurons in the DRG and its expression level is elevated in these neurons attributed by increased activity of capsaicin-sensitive primary afferents in colitis. Up-regulation of sensory BDNF by colitis is demonstrated to be attenuated by inhibition of ERK activity, specifically the big MAPK ERK5 activity with intrathecal application of MAPK kinase inhibitor PD98059. During colitis, the ERK5 is activated in colonic afferent neurons. We show that pre-treatment with PD98059, U0126 or a ERK5 specific inhibitor BIX02188 also blocks the retrograde NGF-induced BDNF expression in the sensory neuronal soma in an ex vivo ganglia-nerve two-compartmented preparation. This is in agreement with those done in disassociated DRG neuronal culture showing that retrograde NGF-induced neuronal survival response is mediated by activation of ERK5 in the neuronal cell body (Watson et al., 2001).

There is increasing evidence in demonstrating roles of the neurotrophin systems in mediating sensory sensitivity and inflammatory pain (Woolf et al., 1994; Groth and Aanonsen, 2002; Matayoshi et al., 2005; Delafoy et al., 2006; Qiao and Grider, 2010; Xia et al., 2012). This is also true with inflammatory bowel disease, which demonstrates an increased expression level of NGF and its receptor in the inflamed colon of patients (di Mola et al., 2000), as well as in animals with experimental colitis (Stanzel et al., 2008; Qiao and Grider, 2010). The elevated neurotrophin system especially the NGF/TrkA in the inflamed colon affects gut physiology through at least two pathways: 1) influences of gut motility by regulating enteric nervous system; and 2) modulation of gut sensitivity by regulating plasticity of the primary afferent pathways. Primary afferent neurons that innervate the distal colon are mainly located in DRGs at the thoracolumbar (T13-L2) and lumbosacral (L6-S1 in rats) spinal levels (Keast and de Groat, 1992; Traub et al., 1999; Berthoud et al., 2004; Qiao and Grider, 2007). Spinal afferents arise from the target organ with cell bodies in the DRG projecting into the dorsal horn of the spinal cord (Grundy, 2002), where they are thought to play a major role in nociception. In response to peripheral irritation, neuropeptides and neurotransmitters are generated in the DRG by sensory neurons and their release can result in neurogenic hypersensitivity and pain (Allen et al., 1997; Bauer et al., 2009; Qiao and Grider, 2009; Seal et al., 2009). Among these neuroactive compounds, our previous studies demonstrate that BDNF is up-regulated in L1 and S1 DRGs during colitis (Qiao et al., 2008). The present study continues this line of research and is undertaken to examine the signaling pathways that facilitate BDNF expression in the DRG.

One of the convincing results in supporting a role of neural activity arising from the inflamed colon (Sharkey and Kroese, 2001) in BDNF up-regulation in the DRG lies in the inhibitory effect of prolonged RTX treatment on colitis-induced BDNF up-regulation in the specifically labeled colonic afferent neurons. Systemic RTX produces long-lasting desensitization of capsaicin sensitive primary afferents (presumptive C-fibers) and has been widely used to study the action of nociceptive C-fiber afferents (Szallasi et al., 1989; Dray et al., 1990; Craft et al., 1995; Xia et al., 2012). In humans, RTX has also been suggested for therapeutic intervention of visceral disorders (Cruz et al., 1997). Our results with systemic administration of RTX suggest that increase in BDNF expression in the colonic afferent neurons requires the activation of unmyelinated C-fiber colonic afferents that are typically stay “silent” at physiologic state. Up-regulation of the neural signals in the inflamed distal colon can affect BDNF expression in the DRG via retrograde transport; this may involve a unique signal mediated by NGF. To support this notion, the present study utilizes a two-compartmented culture preparation demonstrating that retrograde NGF indeed triggers BDNF expression in the DRG neuronal soma. In DRG mass culture, NGF treatment also increases BDNF expression in the TrkA/CGRP peptidergic DRG neurons and almost 90 % of TrkA DRG neurons express BDNF (Michael et al., 1997). We have shown that during colitis the TrkA expression undergoes retrograde transport and is increased in colonic afferent neurons (Qiao and Grider, 2010). Thus these neurons are able to respond to NGF to induce BDNF expression during colitis.

Three major signaling pathways are activated by NGF in neurons: the ERK pathway, the PI 3-kinase/Akt pathway, and the PLC γ pathway (Segal, 2003). Activation of ERK (i.e. ERK1/2, ERK5) or PI 3-kinase pathway enhances gene expression through the activation of transcription factor CREB (Watson et al., 2001; Perkinson et al., 2002; Segal, 2003). Activation of the PLC γ pathway leads to Ca²⁺ and Na⁺ influx through the activation of ion channels, Ca²⁺ release from stores, and further leads to CREB activation (Blum and Konnerth, 2005). Another feature of neurotrophin signaling is the retrograde transport of target-derived neurotrophins (Barker et al., 2002). A key pathway activated by Trk in the cell body after retrograde transport involves ERK5 (Watson et al., 2001). In the present study, we show that the level of phospho-ERK5 is increased in the DRG examined by western blot and also in colonic afferent neurons examined by immunohistochemistry, suggesting an activation of the NGF retrograde signaling within the colonic sensory reflex pathway. It is noteworthy that our study in comparison has shown no change in the phosphorylation level of ERK1/2 in the L1 and S1 DRGs as examined. This is consistent with our earlier studies in an animal model of bladder inflammation (Qiao and Gulick, 2007) but is in discrepancy with results obtained in hind paw inflammation or nerve injury in showing an increase in phospho-ERK1/2 in the DRG (Averill et al., 2001; Doya et al., 2005; Obata et al., 2004). In these animal models, phospho-ERK1/2 is not only expressed in DRG neurons but also in the surrounding satellite cells and is rapidly increased in response to intense noxious peripheral stimuli or electrical stimuli on C-fibers within a few minutes (Ji et al., 1999; Dai et al., 2002). In the present study, the phosphorylation levels of ERK1/2 are examined with western blot at 3 days and 7 days post colitis induction, but do not show changes. It is not clear if we have missed the window of detecting ERK1/2 activation in the DRG at a different time point. Prolonged continuous intrathecal infusion of PD98059 can block both ERK1/2 and ERK5 activities (Kamakura et al., 1999) during colitis. Suppression of BDNF by PD98059 in the present study suggests that the elevated ERK5 activity, and possibly ERK1/2 if activated at earlier time points in the DRG post colitis induction, contributes to sensory BDNF up-regulation during colitis.

Previous studies have suggested a modulatory role of BDNF in regulating inflammation associated hyperalgesia. For example, peripheral or visceral inflammation significantly increases the expression levels of BDNF and receptor TrkB in the DRG and spinal cord

(Cho et al., 1997a,b; Lee et al., 1999; Mannion et al., 1999; Qiao and Vizzard, 2002; Qiao and Grider, 2010). A recent study shows that BDNF(+/-) mice exhibit a weaker visceral response to colorectal distension and a lower sensitivity in the colon with colitis than BDNF(+/+) mice (Yang et al., 2010). The present study identifies a signaling pathway involving ERK5 activation in primary afferent neurons leading to BDNF up-regulation in the DRG. These studies along with our previous findings (Qiao and Grider, 2010) provide a coherent model of an in vivo signal transduction by which neural components arising in the inflamed distal organ activate primary afferent neurons via retrograde transport. Particularly in colitis, this model includes up-regulation of the NGF/TrkA system in the inflamed distal colon and their retrograde transport to the DRG (Qiao and Grider, 2010) where they activate ERK5 and lead to BDNF expression in colonic afferent neurons.

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Highlights

- The level of BDNF is increased in the colonic afferent neurons in colitis
- ERK5 but not ERK1/2 is activated in the colonic afferent neurons in colitis
- Retrograde NGF signal increases BDNF expression in sensory neurons
- ERK5 mediates colitis- and NGF-induced BDNF up-regulation in sensory neurons

Figure 1. Increases in BDNF expression in colonic afferent neurons during colitis were attenuated by prolonged pre-treatment with RTX

TNBS treatment resulted in BDNF up-regulation in conventional neuronal tracing dye FB-labeled colonic afferent neurons (A, blue cells) in the DRG. The percentage of FB cells expressing BDNF immunoreactivity (B, red cells; C, merged purple cells, white arrows) was not changed at 3 days post colitis induction and was increased at 7 days post colitis induction (5 animals for each group; *, $p < 0.05$). Colitis-induced BDNF up-regulation in colonic afferent neurons was blunted by RTX pre-treatment (E; *, $p < 0.05$ vs all groups. Three animals were used for each group of vehicle treatment; five animals were used for each group of RTX treatment). Colonic afferent neurons were combined from the L1 and S1 DRGs. Yellow arrows indicated colonic afferent neurons that did not express BDNF. Bar=30 μ m.

Figure 2. Retrograde NGF increased BDNF expression in sensory neurons, which was mediated by the MEK/ERK pathway

In two-compartmented DRG-nerve culture, NGF (50 ng/mL) was added to the chamber containing the sensory axonal terminals. The expression of BDNF was examined in the DRG neuronal soma located in another chamber. After 12 h of NGF treatment, the level of BDNF was increased in the sensory neuronal cell bodies by 3 folds (compare B to A) when compared to control (E). The role of the MEK/ERK pathway in retrograde NGF-triggered BDNF up-regulation was determined by pre-treatment of the ganglia with inhibitors U0126 (C), PD98059 (D) or BIX02188 (E). All inhibitors significantly reduced NGF-evoked BDNF up-regulation in the ganglia when compared to vehicle treatment (F). Results were from 4 independent experiments for each treatment. Bar= 80 μ m. *, $p < 0.05$ vs all groups.

Figure 3. Western blot of ERK in the L1 and S1 DRGs

Western blot results showed that TNBS treatment increased the phosphorylation (activation) level of ERK5 (p-ERK5) in the DRG by 3 fold (*, $p < 0.05$) (A, B); while there was no change in the level of p-ERK1/2 in the DRG at 7 days of colitis (C, D). The level of phospho-ERK1/2 in the DRG was not changed either at 3 days of colitis (E, F). $n=4$ for each experimental group. L1 and S1 DRGs from each animal were combined for analysis.

Figure 4. Changes in ERK immunoreactivity in the L1 and S1 DRGs during colitis
Immunostaining of p-ERK5 (A, B) and p-ERK1/2 (C, D) revealed that both kinases were expressed in small diameter DRG neurons or nucleus (arrows). The number of DRG cells expressing p-ERK5 was increased in L1 (E) and S1 (F) DRGs. There were no changes in the number of cells expressing p-ERK1/2 in the DRG (E, F). Photomicrographs were from S1 DRG. *, $p < 0.05$; $n = 4$ for each experimental group. Bar = 100 μm .

Figure 5. Immunoreactivity of ERK5 was increased in colonic afferent neurons during colitis
 A subpopulation of p-ERK5 (A, red cells) was expressed in colonic afferent neurons (B, blue cells) in the L1 and S1 DRGs. At 7 days of colitis, the percentage of colonic afferent neurons expressing p-ERK5 was increased when compared to vehicle-treated control (D; *, $p < 0.05$; $n = 4$). Bar = 50 μ m. Colonic afferent neurons were combined from the L1 and S1 DRGs from each animal.

Figure 6. Effects of PD98059 on BDNF immunoreactivity in the DRG during colitis

At 7 days of colitis, the number of BDNF cells was increased in the L1 and S1 DRGs (A, B, E). Spinal intrathecal PD98059 reversed the colitis-induced increases in BDNF expression in DRG (D, E). $n=3$ to 5 animals for each treatment. *, $p<0.05$ vs all groups.

Figure 7. BDNF content in the L1 and S1 DRGs

The BDNF level was examined by ELISA showing that spinal intrathecal PD98059 reversed the colitis-induced increases in BDNF expression in DRG. n=3 to 5 animals for each treatment. *, $p < 0.05$ vs all groups.