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Activation of corticotropin-releasing factor neurons and microglia in paraventricular nucleus precipitates visceral hypersensitivity induced by colorectal distension in rats

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ABSTRACT

Visceral hypersensitivity is a major contributor to irritable bowel syndrome and other disorders with visceral pain. Substantial evidence has established that glial activation and neuro-glial interaction play a key role in the establishment and maintenance of visceral hypersensitivity. We recently demonstrated that activation of spinal microglial toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor κB (NF-κB) signaling facilitated the development of visceral hypersensitivity in a rat model developed by neonatal and adult colorectal distensions (*CRD*s). Hypothalamic paraventricular nucleus (PVN) plays a pivotal role in the pathogenesis of chronic pain. In this study, we examined the mechanism by which microglia and neurons in PVN establish and maintain visceral hypersensitivity and the involvement of TLR4 signaling. Visceral hypersensitivity was precipitated by adult colorectal distension (*CRD*) only in rats that experienced neonatal *CRD*s. Visceral hypersensitivity was associated with an increase in the expression of c-fos, corticotropin-releasing factor (CRF) protein and mRNA in PVN, which could be prevented by intra-PVN infusion of lidocaine or small interfering RNA targeting the CRF gene. These results suggest PVN CRF neurons modulate visceral hypersensitivity. Adult *CRD* induced an increase in the expression of Iba-1 (a microglial marker), TLR4 protein, and its downstream effectors MyD88, NF-κB, as well as proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) only in rats that experienced neonatal *CRD*s. Intra-PVN infusion of minocycline, a nonselective microglial inhibitor, attenuated the hyperactivity of TLR4 signaling cascade, microglial activation, and visceral hypersensitivity. Taken together, these data suggest that neonatal *CRD*s induce a glial activation in PVN. Adult *CRD* potentiates the glial and CRF neuronal activity, and precipitates visceral hypersensitivity and pain. TLR4 signaling and proinflammatory cytokines TNF-α and IL-1β may participate in neuro-glial interaction during the pathogenesis of visceral hypersensitivity.

1. Introduction

Visceral hypersensitivity is a major contributor to irritable bowel syndrome (IBS), a prevalent functional gastrointestinal disorder characterized by abnormal bowel habits and chronic abdominal pain (Chey et al., 2015). The pathogenesis of visceral hypersensitivity remains speculative due to undetectable structural abnormalities in the peripheral organs. Accumulating evidence has established that glial activation and neuro-glial interaction play a key role in the establishment and maintenance of persistent pain in animal models (Zhang et al., 2015) and chronic pain patients (Loggia et al., 2015). Microglial cells, the resident

Abbreviations: AWR, abdominal withdrawal reflect; ACTH, adrenocorticotrophin hormone; CORT, corticosterone; CRF, corticotropin-releasing factor; *CRD*, colorectal distension; EDTA, ethylenediaminetetraacetic acid; EMG, electromyography; HPA, hypothalamic–pituitary–adrenal axis; IBS, irritable bowel syndrome; IκB, inhibitor of kappa B; IL-1β, interleukin-1β; MyD88, myeloid differentiation factor 88; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PVN, paraventricular nucleus; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

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macrophages in the central nervous system (Tang, 2014), can detect pathological events in CNS, and release a large number of proinflammatory cytokines that act detrimentally or beneficially on the surrounding neurons (Eyo and Wu, 2013; Ji et al., 2014; Kettenmann et al., 2011) to induce visceral hypersensitivity and neuropathic pain (Shi et al., 2012; Tsuda et al., 2005; Watkins et al., 2001).

It is now well-established that chronic pain, such as inflammatory pain, neuropathic pain, and cancer pain, is an expression of neural plasticity and sensitization throughout the peripheral and central nervous systems (Ji et al., 2014). The paraventricular nucleus (PVN) integrates multiple sources of afferent inputs to generate integrated autonomic outputs for pain and analgesia regulation. Corticotrophin-releasing factor (CRF) originating from the hypothalamic PVN elevates adrenocorticotropin hormone (ACTH) and corticosteroid levels via the hypothalamic–pituitary–adrenal (HPA) axis (Bravo et al., 2011; de Kloet et al., 2005). Early life stress can lead to permanent dysregulation of the PVN neuroplasticity and HPA axis (Amath et al., 2012; Green et al., 2011; Van den Bergh et al., 2008). We have previously reported that neonatal colorectal distension (*CRD*), an early life stress, induce visceral hypersensitivity and elevations in the plasma cortisol level and CRF expression in PVN (Chen et al., 2015; Yu et al., 2014). CRF and the HPA neuraxis modulate neuropathic pain (Fu and Neugebauer, 2008) and analgesia (Lariviere and Melzack, 2000), and the PVN may be a pivotal region modulating visceral hypersensitivity. However, the direct evidence of microglia and neurons in PVN on the pathogenesis of visceral hypersensitivity and pain is not fully understood.

Toll-like receptor 4 (TLR4) is expressed in microglial cells (Lee et al., 2013). Stimulation of TLR4 activates adapter protein myeloid differentiation factor 88 (MyD88) and nuclear factor (NF)-κB, and increases the production and release of proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (O'Neill and Bowie, 2007; Qin et al., 2013). Activation of the spinal microglial TLR4/MyD88/NF-κB signaling pathway facilitates the development of visceral hypersensitivity induced by neonatal and adult *CRD*s (Chen et al., 2015). It is generally accepted that TLR4 signaling participates in microglial activation and pathological pain occurrence (Lee et al., 2013; Tanga et al., 2005), and PVN may be one target region where TLR4 signaling modulates neuro-glial interaction and visceral hypersensitivity.

Glial activation and neuro-glial interaction are essential for the establishment and maintenance of chronic pain (Ji et al., 2013, 2014; Sorge et al., 2015). Considering the involvement of microglia and the PVN in the pathogenesis of visceral hypersensitivity (Chen et al., 2015), we hypothesized that the microglia and CRF neurons in PVN participate in the pathogenesis of visceral hypersensitivity, and TLR4/MyD88/NF-κB signaling pathway may be involved in this pathogenic process. The result would provide novel insights into the neuronal and molecular bases for the development and precipitation of visceral hypersensitivity, and may pave a new avenue for developing therapeutic approaches against IBS.

2. Materials and methods

2.1. Subjects

Preweanling male Sprague–Dawley neonatal rats (younger than 8 days) were obtained from the Experimental Animal Center of Xuzhou Medical College (Xuzhou, China) and housed in groups with adult female rats until the pups were 25 days old. After separation from the females, the male rats were housed in groups of four per standard Plexiglas cage and had *ad libitum* access to food and water. Rats were examined daily and weighed once per week for 2 months or until their weight reached 200–250 g and subsequently assigned to different groups. During the testing session, rats were housed in standard Plexiglas cages and maintained on a standard 12 h light–dark cycle (lights on at 07:00 AM), with constant temperature and humidity (22 °C and 50%, respectively) and *ad libitum* access to food and water. All procedures were conducted in accordance with the guidelines as described in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 8023, revised 1978) and the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at Xuzhou Medical College.

2.2. Reagents

The CRF-siRNA oligonucleotide sequence (underline shows target sequence) is 5′-TCAGGAAACTGATGGAGATTATCTCGAGATAATCTCCATCAGTTTCCTGTTTTTTC-3′. The non-silencing scrambled siRNA sequence is 5′-TTCTCCGAACGTGTCACGT-3′. The Lentiviral (LV)-siRNA package was constructed by Shanghai GeneChem Co., Ltd (Shanghai, China). Minocycline, fluorocitrate, lidocaine and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO). Rabbit polyclonal anti-MyD88 (sc-11356), mouse monoclonal anti-NF-κB p65 (sc-8008), and rabbit polyclonal anti-inhibitor of kappa B (IκB)-α (sc-371) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-CRF (ab8901), goat polyclonal anti-Iba1 (ab5076), and rabbit polyclonal anti-TLR4 (ab13556) were purchased from Abcam (Cambridge, UK). Glial fibrillary acidic protein (GFAP) mouse monoclonal antibody (#3670) was purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-beta actin monoclonal antibody (TA-09), alkaline phosphatase goat anti-rabbit IgG (ZB-2308), alkaline phosphatase horse anti-mouse IgG (ZB-2310), and alkaline phosphatase rabbit anti-goat IgG (ZB-2311) were purchased from ZSGB-BIO (Beijing, China). PageRuler Plus Prestained Protein Ladder (SM1811) was purchased from Fermentas (Hanover, MD). Primary antibody dilution buffer (P0023A), secondary antibody dilution buffer (P0023D), phenylmethanesulfonyl fluoride (ST506), BCA protein assay kit (P0012), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (P0015), and BCIP/NBT alkaline phosphatase color development kit (C3206) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.3. Development of visceral hypersensitivity rats with neonatal and adult CRDs

The visceral hypersensitivity, as observed in IBS patients, was induced by adult *CRD* in rats that experienced neonatal *CRD*s as described previously (Zhang et al., 2015). In brief, *CRD* rats received neonatal *CRD*s on postnatal days 8, 10, and 12 using an angioplasty balloon (length, 20.0 mm; diameter, 3.0 mm) inserted into the descending colon and upper rectum. The balloon was distended with 0.3 ml water at a pressure of 60 mmHg for 1 min before deflation and withdrawal. The distention was repeated twice separated by 30 min. Rats in the sham group were handled in a manner similar to those in the *CRD* group with balloon insertion except that distension pressure was not applied. Rats in the adult *CRD* group (control) received no neonatal distension. An adult *CRD* was applied 8 weeks later to all rats in which an 80 mmHg (1 min) distention was applied twice with a 5-min interval, or as described in text in *CRD*, sham and control rats.

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