ARCHIVAL REPORT

Toll-Like Receptor 4 Regulates Chronic Stress-Induced Visceral Pain in Mice

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Background: Functional gastrointestinal disorders, which have visceral hypersensitivity as a core symptom, are frequently comorbid with stress-related psychiatric disorders. Increasing evidence points to a key role for toll-like receptor 4 (TLR4) in chronic pain states of somatic origin. However, the central contribution of TLR4 in visceral pain sensation remains elusive.

Methods: With pharmacological and genetic approaches, we investigated the involvement of TLR4 in the modulation of visceral pain. The TLR4-deficient and wild-type mice were exposed to chronic stress. Visceral pain was evaluated with colorectal distension. Protein expression levels for TLR4, Cd11b, and glial fibrillary acidic protein (glial cells markers) were quantified in the lumbar region of the spinal cord, prefrontal cortex (PFC), and hippocampus. To evaluate the effect of blocking TLR4 on visceral nociception, TAK-242, a selective TLR4 antagonist, was administered peripherally (intravenous) and centrally (intracerebroventricular and intra-PFC) (n = 10–12/experimental group).

Results: The TLR4 deficiency reduced visceral pain and prevented the development of chronic psychosocial stress-induced visceral hypersensitivity. Increased expression of TLR4 coupled with enhanced glia activation in the PFC and increased levels of proinflammatory cytokines were observed after chronic stress in wild-type mice. Administration of a TLR4 specific antagonist, TAK-242, attenuated visceral pain sensation in animals with functional TLR4 when administrated centrally and peripherally. Moreover, intra-PFC TAK-242 administration also counteracted chronic stress-induced visceral hypersensitivity.

Conclusions: Our results reveal a novel role for TLR4 within the PFC in the modulation of visceral nociception and point to TLR4 as a potential therapeutic target for the development of drugs to treat visceral hypersensitivity.

Key Words: Chronic stress, microglia activation, prefrontal cortex, spinal cord, TLR4, visceral hypersensitivity

Visceral pain is a pronounced and, at times, dominant feature of a variety of gastrointestinal disorders, including irritable bowel syndrome (IBS) (1), many of which are comorbid with stress-related psychiatric disorders. Recurrent, episodic but often unpredictable painful events can exert a disabling impact on daily life and result in impairment of several domains of quality of life (2). Moreover, exposure to life stressors is a well-known key factor affecting the presentation of visceral pain symptomatology (3). To date there are no effective pharmacotherapeutic approaches to selectively treat this visceral hypersensitivity, which in part is because the underlying molecular mechanisms remain largely unknown (4).

Toll-like receptors (TLRs) are a family of pattern-recognition receptors of the innate immune system. The TLRs represent key mediators of innate host defense in the gut, involved in maintaining mucosal as well as commensal homeostasis. Inflammation and altered intestinal homeostasis underlie several diseases affecting the gastrointestinal tract (5). Recent reports have suggested an involvement of peripheral toll-like receptor 4 (TLR4) in patients suffering from IBS (6,7) and in animal models of IBS (8,9). Moreover, growing evidence showing the presence of TLR4 in the enteric nervous system and in the dorsal root ganglia indicate a role for TLR4 in sensory information transmission from the gastrointestinal tract (10,11). However, TLR4 is also expressed within the central nervous system (CNS), predominately in microglia (12). Microglia represent the first line of defense for the CNS, acting as a sensor for pathological events (13).

Recently, data from animal models have suggested that spinal microglia activation is an important component in the facilitation and modulation of the hyper-responsive pain states such as hyperalgesia and allodynia. Therefore, microglia activation is poised to play a key role in the development and maintenance of chronic pain from somatic (14,15) and visceral (16,17) origin. Upon activation by exogenous and endogenous ligands, TLR4 can trigger the activation of microglia (18). This fact coupled with the strong link between microglia activation and pain facilitation have thus suggested a direct role for TLR4 in nociception (19). Indeed, the importance of TLR4 in pain has been emphasized by recent evidence showing a role of spinal TLR4 in the initiation of pathological pain states such as inflammatory (20,21) and neuropathic (21–23) pain in preclinical models. Moreover, blocking TLR4 has prevented (24) and reversed (25) the hyper-responsive phenotypes in animal models of neuropathic pain. However, to our knowledge, the central role of TLR4 on visceral nociception under pathological conditions remains unknown. In addition, whereas preclinical studies have mainly investigated the localization of mechanisms underlying visceral pain within the spinal cord (16,26), little attention has been paid to other pain-related areas within the CNS at a supraspinal level (27).

In the present study, we investigated whether TLR4 exerts a modulatory role in visceral nociception, under physiological and pathological stress-induced conditions. We also evaluated the association of visceral hypersensitivity with TLR4 expression in pain-related areas within the CNS along with microglia activation, a process known to be related to the altered pain sensation.

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Methods and Materials

Animals

Male wild-type C3H/HeN and TLR4-deficient mice (C3H/HeJ) were used in this study (5–6 weeks old upon arrival). The C3H/HeJ do not express functional TLR4, because of naturally occurring mutations in the *Tlr4* gene (28). Mice were split into separate cohorts for behavioral testing (colorectal distension [CRD]) and for harvesting of samples (naïve or post-stress) for mRNA and protein level analysis. Mice were group-housed (five/cage) except for social defeat studies where mice were singly housed. Male CD1 mice ($n = 40$, 9–10 weeks old) were used as aggressors in the social defeat procedure. Water and food were available ad libitum to all mice throughout the whole study. The holding room was temperature (21 ± 1°C) and humidity (55 ± 10%) controlled and under a 12-hour light/dark cycle (lights on 7:00 AM). All animals were supplied by Harlan (Derby, UK).

All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Chronic Social Defeat/Overcrowding Procedure

This chronic stress procedure was carried out as described previously (29,30). Briefly, mice were exposed to an unpredictable mixed model of social defeat and overcrowding sessions for 19 days. Subsequently, all mice underwent social interaction testing (Supplement 1).

Social Interaction Test

Twenty-four hours after the last stress, social avoidance behavior was assessed as described previously (29–31) in the social interaction test (Supplement 1).

Colorectal Distension

Colorectal distension is a procedure frequently used in mice (32) and humans (33) to assess visceral pain. The CRD was carried out as described previously (30,34) (Supplement 1).

Corticosterone Assay

Plasma corticosterone concentrations were measured with commercially available enzyme immunoassay kits (Assay Designs, Ann Arbor, Michigan) according to the instructions of the manufacturer, as described in Supplement 1.

Surgery and Drug Administration

Mice were anaesthetized with isoflurane (1.5–2%) and placed in a stereotaxic frame. The skull was exposed, and permanent guide cannulas (22G) were implanted unilaterally above the lateral ventricle (from bregma: anterior-posterior −.45 mm, medial/lateral 1.0 mm, dorsal/ventral [DV] −2.0 mm) and bilaterally above the prelimbic cortex region (from bregma: anterior-posterior +1.9 mm, medial/lateral +/−.5 mm, DV −2.0 mm) according to the atlas of Franklin and Paxinos (35) and fixed on the skull with dental cement. A 28G dummy cannula was inserted in the guide cannula to prevent clogging. Mice were allowed to recover 5 days after the surgery; the weight changes were monitored daily. Microinjections were performed with a 28G injection cannula extended .5 mm beyond the tip of the guiding cannula (the final DV coordinate −2.5 mm) attached to flexible plastic tubing and a Gastight Hamilton syringe.

TAK-242 (Discovery Fine Chemicals, Wimborne, United Kingdom), a small-molecule and selective TLR4 antagonist (36), was dissolved in a fat emulsion, 50% soybean oil (Sigma-Aldrich, Dublin, Ireland) in saline, and prepared fresh daily. Administrations were performed centrally into the lateral ventricle (intracerebroventricular .02 mg/μL, 2 μL) and in the prefrontal cortex (PFC) (.02 mg/μL, .5 μL in each hemisphere) 20 min before CRD or peripherally into the tail vein (10 mg/kg, 100 μL) 1 hour before CRD. After the intracerebroventricular and PFC experiments, cannula placement was verified by injection of ink followed by brain dissection to determine ventricular flow of the ink or placement verification in the PFC. Data from animals with incorrectly placed cannulas were discarded from the experiments.

Spleen Cytokine Assays

Measurements of proinflammatory cytokines interleukin (IL)6 and tumor necrosis factor (TNF)α on spleen cells cultured with lipopolysaccharide (LPS) (Sigma-Aldrich) was carried out with custom mouse Multi-Spot 96-Well Plates (Meso Scale Discovery, Rockville, Maryland) according to instructions of the manufacturer as described in Supplement 1.

Western Blot

Western blot was performed as previously described (37) to determinate the protein levels of TLR4, Cd11b, and glial fibrillary acidic protein (GFAP) (Supplement 1).

Immunofluorescence Staining

The CRD-naïve mice were sacrificed without anesthesia, and the brains were snap-frozen and stored at −80°C. Slices were incubated with anti-TLR4 overnight followed by the incubation with the secondary antibody conjugated with Alexa488 (Supplement 1).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from PFC samples with the Qiagen RNeasy Lipid Mini Kit (Qiagen, Valencia, California). Complementary DNA was synthesized with 1 mg total RNA with random primers. Quantitative changes in messenger RNA (mRNA) levels were estimated by real time-polymerase chain reaction (Supplement 1).

Statistical Analysis

Statistical differences between groups were analyzed by repeated measures one- or two-way analysis of variance followed by Bonferroni post hoc test. Independent-sample *t* tests were used to compare two independent groups. All tests were performed at a significance level of $p < .05$. All analysis was carried out with SPSS 18.0 for windows (SPSS, Chicago, Illinois).

Results

TLR4 Deficiency Reduces Visceral Nociceptive Responses and Prevents Development of Stress-Induced Visceral Hypersensitivity

Mice deficient in TLR4 underwent CRD to assess visceral pain, in an exploration of whether TLR4 plays a modulatory role in visceral nociception. Absence of functional TLR4 triggered a decrease in visceral sensitivity (genotype: $F_{1,14} = 7.611$; $p < .05$) (Figure 1A) along with an increased pain threshold ($t = 2.228$; $p < .05$) (Figure 1D). These results are consistent with a hypoalgesic phenotype when compared with wild-type mice, suggesting a modulatory role of TLR4 in visceral nociception in response to colonic stimulation.

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