ARCHIVAL REPORT

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

Monica Tramullas, Beate C. Finger, Rachel D. Moloney, Anna V. Golubeva, Gerard Moloney, Timothy G. Dinan, and John F. Cryan

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 (intraventricular 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 in 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.

From the Laboratory of NeuroGastroenterology (MT, RDM, AVG, GM, TGD, JFC), Alimentary Pharmabiotic Centre; School of Pharmacy (BCF); Department of Psychiatry (TGD); and the Department of Anatomy and Neuroscience (JFC), University College Cork, Cork, Ireland.

Authors MT and BCF contributed equally to this work.

Address correspondence to Professor John F. Cryan, Ph.D., Department of Anatomy and Neuroscience, Western Gateway Building, University College Cork, Room 386, Cork, Ireland; E-mail: j.cryan@ucc.ie.

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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 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 (intraventricular .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 intraventricular 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). All graphs show mean values ± SEM with $*p < .05$; $**p < .01$; $***p < .001$.

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.

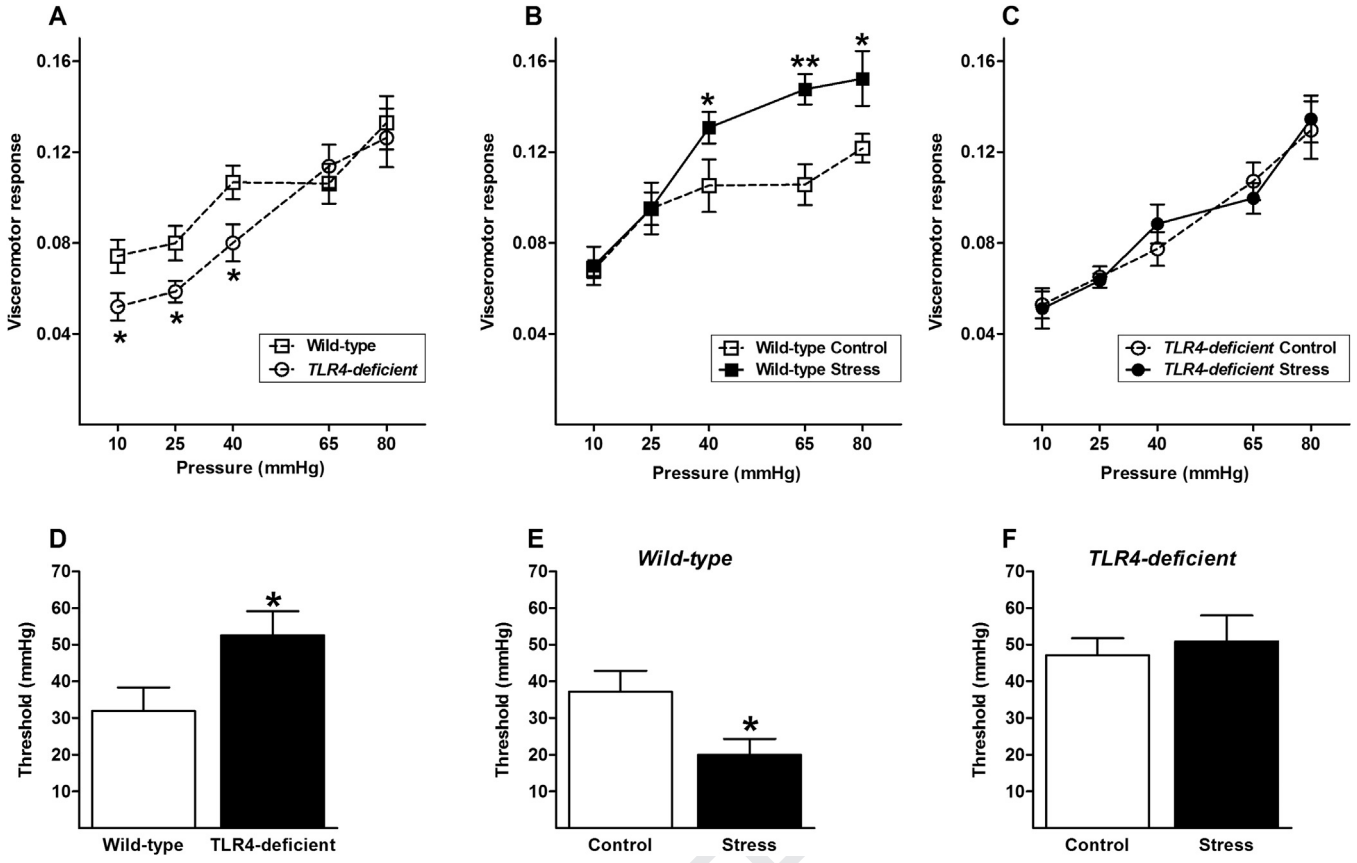

Figure 1. Effects of toll-like receptor 4 (TLR4) deficiency on visceral nociception. Visceral sensitivity to colorectal distension (ascending paradigm from 10 to 80 mm Hg) was assessed as the number of visceromotor responses over pressures. The TLR4-deficient mice presented with decreased visceral pain responses **(A)**, and chronic stress increased visceral pain responses in wild-type mice **(B)**, but TLR4-deficient mice were resistant to stress-induced visceral hypersensitivity **(C)**. Pain thresholds were calculated as the pressure of the distending pulse at which the response evoked exceeded the mean baseline activity plus three times the SD. Mice lacking functional TLR4 demonstrated increased pain threshold **(D)**, whereas chronic stress decreased the pain threshold in wild-type mice **(E)** but not in TLR4-deficient mice **(F)**. $^{*}p < .05$; $^{**}p < .01$; independent sample *t* test or two-way analysis of variance followed by Bonferroni post hoc test. Baseline response to colorectal distension was not different between individual groups/conditions; data represent mean ± SEM.

In gastrointestinal disorders, the symptomatology of visceral pain has been causally associated with exposure to chronic stressful events (26,30,38,39). We have recently established that chronic psychosocial stress induces visceral hypersensitivity in mice (30). Hence, we further investigated whether TLR4 modulates this pathological state of stress-induced visceral pain. As expected, after chronic stress mice with normal TLR4 functionality showed a pronounced increase in visceral pain responses (genotype × stress: $F_{1,28} = 5.100$; $p < .05$) (Figure 1B). However, this sensitization to noxious visceral stimuli was absent in TLR4-deficient mice (Figure 1C), suggesting a key role for TLR4 as a modulator under this pathological state of stress-induced visceral pain. In line with these results, the pain threshold was significantly decreased in wild-type mice after chronic stress but not in TLR4-deficient mice (genotype × stress: $F_{1,28} = 4.7$; $p < .05$) (Figure 1E,F). Interestingly, TLR4-deficient mice responded normally in other aspects to chronic stress (30). Wild-type and TLR4-deficient mice exhibited an equal decrease in the social interaction ratio (stress: $F_{1,30} = 14.63$; $p < .001$), as illustrated by social avoidance in the social interaction test (Figure S1 in Supplement 1), suggesting a stress-induced social avoidance phenotype independent of TLR4 signaling. Moreover, chronic stress caused an overall decrease in adipose mass (stress: $F_{1,43} = 49.41$; $p < .001$) (Figure S2A in Supplement 1), which was more pronounced in mice with TLR4 deficiency (stress × genotype: $F_{1,43} = 10.51$; $p < .01$). Consequently, animals exposed to stress gained a significant amount of lean mass (stress: $F_{1,43} = 12.03$; $p < .01$) (Figure S2B in Supplement 1), with a higher increase in stress mice lacking functional TLR4 (stress × genotype: $F_{1,43} = 12.03$; $p < .01$). Evening corticosterone levels were significantly decreased in all stress animals independent of genotype (stress: $F_{1,28} = 4.04$; $p < .05$) (Figure S2C in Supplement 1) as previously described for this protocol of social defeat/overcrowding stress (30,40).

TAK-242, a Selective TLR4 Antagonist, Reduces Visceral Sensitivity in Wild-Type Mice and Reverses the Visceral Hypersensitive Phenotype in Stressed Mice

We further proved that it is indeed TLR4 that regulates visceral pain perception by administration of a small-molecule and selective TLR4 antagonist, TAK-242. This compound successfully attenuated visceral pain sensation in mice with functional TLR4 when administrated peripherally (treatment: $F_{1,11} = 18.993$; $p < .001$) (Figure 2A) and remained without effects on pain behavior in mice with TLR4 deficiency (Figure 2B). Also blocking TLR4 significantly increased the pain threshold in wild-type mice

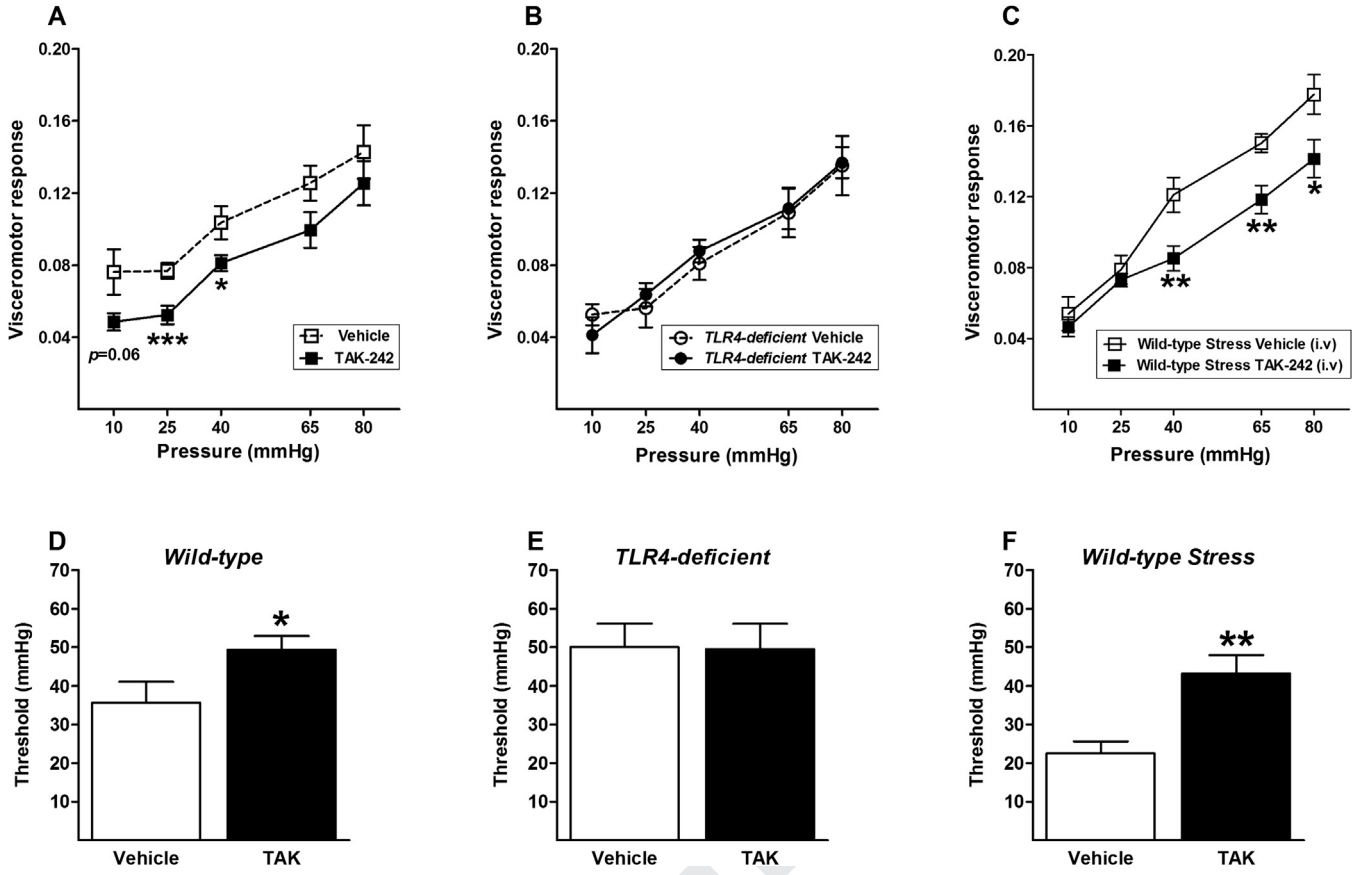

Figure 2. TAK-242 decreases visceral pain and reverses the visceral hypersensitive phenotype in stressed mice. Administration of the toll-like receptor 4 (TLR4) antagonist TAK-242 decreased visceral pain responses in mice with functional TLR4 when administered peripherally (intravenous 10 mg/kg) **(A)** but not in TLR4-deficient mice **(B)**. Similarly, administration of TAK-242 elevated pain thresholds in wild-type mice **(D)** but was ineffective in TLR4-deficient mice **(E)**. Moreover, administration of TAK-242 peripherally (intravenous 10 mg/kg) after exposure to chronic stress decreased the pain response to control levels **(C)**. Also, the antagonist normalized the stress-induced decrease in the pain threshold in wild-type mice **(F)**. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; independent sample *t* test or one-way analysis of variance followed by Bonferroni post hoc test; baseline response to colorectal distension was not different between individual groups/conditions; data represent mean ± SEM.

($t = 2.107$; $p < .05$) (Figure 2D) but was ineffective in TLR4-deficient mice ($t = .0556$; $p =$ ns) (Figure 2E). Similarly, administration of TAK-242, robustly counteracted chronic stress-induced visceral hypersensitivity (IV: treatment: $F_{1,38} = 209$; $p < .001$) (Figure 2C). Along with the decreased visceral pain responses, TAK-242 administration significantly increased the pain threshold after chronic stress ($t = 3.67$; $p < .01$) (Figure 2F).

Chronic Psychosocial Stress Increases LPS-Induced Stimulation of Spleen Cytokines and Peripheral TAK-242 Administration Counteracts Inflammatory Phenotype in Stressed Mice

After spleen stimulation with LPS, a TLR4 agonist, wild-type mice exposed to chronic psychosocial stress exhibited an increased spleen level of IL6 ($F_{2,37} = 14.33$; $p < .001$) (Figure 3) and TNFα ($F_{2,35} = 3.92$; $p < .05$) compared with the control group. Moreover, peripheral administration of TAK-242 inhibited LPS-stimulated release of spleen IL6 and TNFα after chronic stress.

Intracerebroventricular TAK-242 Reduces Visceral Sensitivity in Wild-Type Mice

Although TAK-242 is a very small molecule and highly lipophilic, it is not known whether TAK-242 could cross the brain blood barrier. Due to this fact we aimed to investigate whether TLR4 is modulating visceral pain at the central level. The central administration of TAK-242 into the lateral ventricle (intraventricular) significantly reduced visceral pain sensation in mice with functional TLR4 (treatment: $F_{1,17} = 29.836$; $p < .001$) (Figure 4A) and increased the pain threshold ($t = 2.687$; $p < .05$) (Figure 4C). These results imply a central modulatory role for TLR4 in visceral nociception.

Visceral Hypersensitivity Is Associated with Increased TLR4 Expression in the PFC and Hippocampus But Not in Lumbar Region of Spinal Cord

Even though other pathological pain states as inflammatory and neuropathic pain have been associated with alterations of TLR4 at the spinal cord level (20–23), spinal TLR4 expression has not been studied in models of visceral hypersensitivity. We assessed the levels of TLR4 in the spinal cord in the model of visceral hypersensitivity in CRD-naïve mice, given that our data also identify TLR4 as an important target in the central modulation of visceral nociception on a behavioral level. In the lumbar region of the spinal cord, protein levels of TLR4 were unchanged in stressed mice ($t = .484$; $p =$ ns) (Figure S3) when compared with the control group. Whether TLR4 within the CNS is involved in stress-induced visceral hypersensitivity at central level, TLR4 expression could be modified in other brain areas implicated in pain perception.

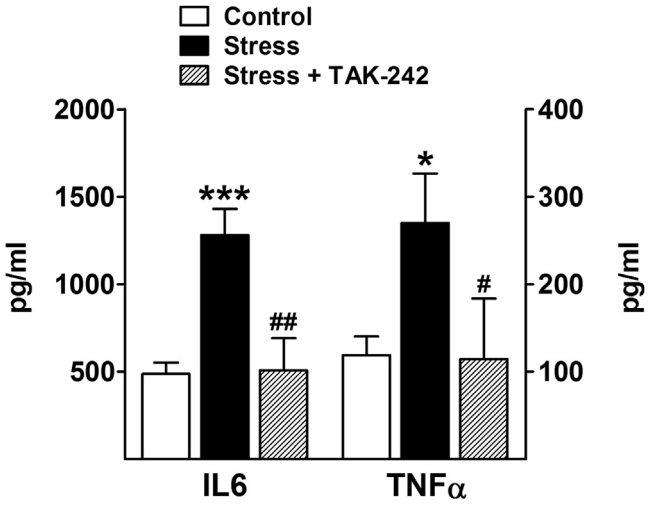

Figure 3. Effects of chronic stress on cytokine levels after lipopolysaccharide (LPS) spleen stimulation. Cytokine levels of interleukin (IL)6 and tumor necrosis factor (TNF)α in spleen cell culture supernatant after stimulation with LPS (1 μg/mL, 37°C, 24 hours) were measured in wild-type mice or control subjects exposed to chronic psychosocial stress (24 hours after the last stress session) or 1 hour after TAK-242 intravenous administration after the exposure to chronic psychosocial stress. Exposure to chronic psychosocial stress significantly increased spleen levels of IL6 and TNFα after the stimulation with LPS, compared with control mice. Moreover, peripheral administration of TAK-242 blocked the LPS-induced stimulation of spleen IL6 and TNFα. $^{*}p < .05$; $^{***}p < .001$ versus control; $^{\#}p < .05$; $^{\#\#}p < .01$ versus stress; one-way analysis of variance followed by Bonferroni post hoc test; data represent mean ± SEM.

Thus, to further pinpoint whether TLR4 was altered in other pain-related regions within the CNS, we evaluated the levels of expression of TLR4 in the PFC and the hippocampus, two relevant brain areas involved in pain modulation and vulnerable to exhibit changes in response to stress (41). In addition, recent evidence has shown the importance of PFC and hippocampus activation in response to colorectal stimulation (27,42,43). As shown in Figure 5 and Figure S4 in Supplement 1, the levels of TLR4 were significantly increased in the PFC ($t = 4.91$; $p < .01$) (Figure 5A) and hippocampus ($t = 3.41$; $p < .01$) (Figure S4A) after chronic stress, when compared with control mice. Furthermore, as shown in Figure 5D, immunofluorescence staining confirmed the expression of TLR4 in the PFC.

Enhanced TLR4 Expression and Visceral Hypersensitivity Is Associated with Glia Activation in PFC But Not in Hippocampus

So far, studies have linked the etiology of visceral pain with glial cell activation, including microglia and astrocytes in the CNS (16,44). Because TLR4 is predominantly expressed on microglia within the CNS (12), we hypothesize a direct link between the activation of microglia and the enhanced expression of TLR4 in the PFC and the hippocampus and, hence, the modulation of visceral pain. Indeed, the levels of activated microglia within the PFC showed the same profile of increase as described for TLR4, after chronic stress (stress: $F_{1,18} = 24.90$; $p < .001$) (Figure 5B). However, exposure to chronic stress in TLR4-deficient mice also triggered an increase in microglia (Figure 5B) without facilitating an increase in visceral pain (Figure 1C,F). By contrast, in the hippocampus, the levels of expression of the microglial marker Cd11b were unchanged after chronic stress (stress: $F_{1,19} = .07$; p = ns) (Figure S4B in Supplement 1). Because TLR4 is also expressed on astrocytes, GFAP expression (an astrocyte marker) was analyzed in the PFC. After chronic stress, the expression of GFAP was significantly increased in the PFC in wild-type mice ($t = 4.74$; $p < .01$) (Figure 5C). These results suggest that the upregulation of TLR4 along with glia activation in the PFC might be involved in processing painful stimuli at a central level.

Intra-PFC TAK-242 Counteracts Visceral Hypersensitivity Phenotype in Stressed Mice

To further confirm the regulatory role of TLR4 in the PFC in visceral nociception, TAK-242 was administered bilaterally into the PFC after exposure to chronic stress. As shown in Figure 4, TAK-242 robustly counteracted chronic stress-induced visceral hypersensitivity (treatment: $F_{1,27} = 34.56$, $p < .001$) (Figure 4B) and normalized the stress-induced decrease in the pain threshold in wild-type mice ($t = 4.56$; $p < .001$) (Figure 4D). These results indicate that TLR4 is functionally involved in visceral pain modulation in the PFC in a model of stress-induced visceral hypersensitivity.

Chronic Psychosocial Stress Increases Proinflammatory Cytokines Levels in PFC in Wild-Type But Not in TLR4-Deficient Mice

Pathological pain is now understood in the context of inflammation (central and peripheral) associated with tissue damage (45). As a consequence of TLR4 activation, numerous proinflammatory cytokines are released, triggering the initiation and maintenance of chronic pain states (19,20,22). Because TLR4 is enhanced in the PFC, we assessed the mRNA expression levels of TNFα, IL1-β, and IL6 in the PFC in wild-type and TLR4-deficient mice after chronic stress (Table 1). The mRNA levels of TNFα ($F_{3,31} = 3.35$, $p < .05$) and IL1-β ($F_{3,31} = 4.75$, $p < .01$) were significantly increased in wild-type mice exposed to chronic stress but not in TLR4-deficient mice. The mRNA levels of IL6 were unchanged in all the conditions studied.

Discussion

There is a growing awareness that visceral pain is not only a common symptom in gastrointestinal disorders but also a key determinant of disease severity and clinical impact (46). As a consequence, the search for therapeutic targets to treat visceral pain has become a major goal in preclinical and clinical research alike (47). However, this has proven to be challenging, because the underlying mechanisms leading to the expression of this form of pain remain relatively undefined (4).

In the present study, we provide novel evidence that TLR4 is a key modulator of visceral pain and thereby a potential therapeutic target for the treatment of visceral hypersensitivity. We demonstrate that TLR4 deficiency reduced visceral sensitivity and prevented the development of stress-induced visceral hypersensitivity. Moreover, mice with normal TLR4 functionality exposed to chronic psychosocial stress exhibited visceral hypersensitivity. Our findings are in agreement with data showing a key role for TLR4 in the induction of hypersensitive phenotypes in mouse models of inflammatory and neuropathic pain (19–23). With a pharmacological approach, we further confirmed that it is indeed TLR4 that regulates visceral pain perception either peripherally and centrally by administration of a small-molecule and selective TLR4 antagonist, TAK-242. This compound successfully attenuated visceral pain sensation in wild-type mice and also reversed the hyperalgesic phenotype presented in mice exposed

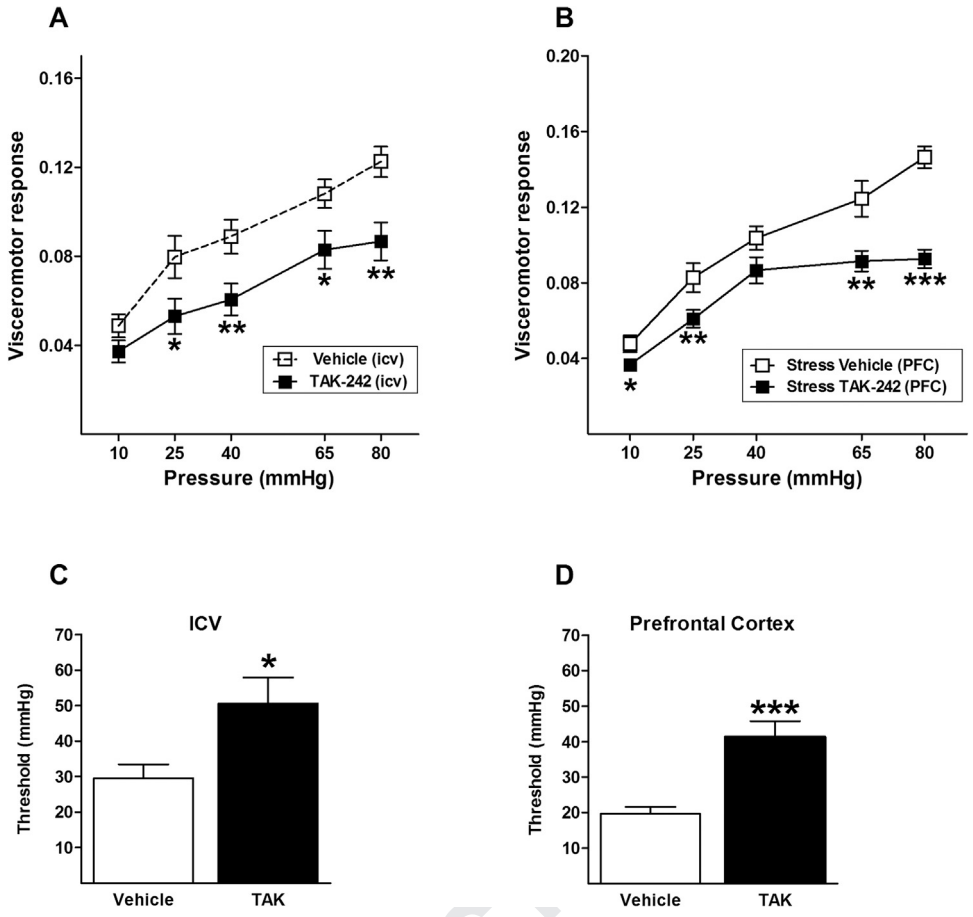

Figure 4. Central administration of TAK-242 decreases visceral pain in wild-type mice and into the prefrontal cortex (PFC) counteracts the visceral hypersensitivity phenotype in stressed mice. Administration of the toll-like receptor 4 antagonist TAK-242 decreased visceral pain responses in mice with functional toll-like receptor 4 when administered centrally (intraventricular [ICV] .04 mg/2 μL) **(A)**. Also, central administration of TAK-242 elevated pain thresholds in wild-type mice **(C)**. Administration of TAK-242 into the PFC after exposure to chronic stress decreased the pain response to control levels **(B)**. Moreover, the antagonist normalized the stress-induced decrease in the pain threshold in wild-type mice **(D)**. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; independent sample *t* test or one-way analysis of variance followed by Bonferroni post hoc test; baseline response to colorectal distension was not different between individual groups/conditions; data represent mean ± SEM.

to chronic stress. That TAK-242 counteracts visceral hypersensitivity and also reduces visceral sensitivity under basal conditions is relevant, because the efficacy of TAK-242 has been largely evaluated in laboratory mice (48) and human clinical trials (49) in the context of treating sepsis. However, beyond this, the analgesic effect of TAK-242 has not to our knowledge been described yet. TLR4 is expressed in the enteric nervous system and in the dorsal root ganglia (10,11) and thus, peripheral blocking of TLR4 could impede the sensory information transmission from the gastrointestinal tract to the CNS. Additionally, peripheral administration of TAK-242 blunted LPS-induced release of spleen IL6 and TNFα after chronic stress. Interestingly, proinflammatory cytokines like IL6 and TNFα are important players in both peripheral and central sensitization process, and they are well-established to contribute to initiating and maintaining chronic pain states (19,45). TAK-242 also decreased visceral pain sensation when administered into the lateral ventricle, indicating that TLR4 has a critical role in visceral pain processing at central level. Furthermore, our data showed that the observed visceral hypersensitivity after exposure to chronic stress is associated with increased TLR4 protein levels within the PFC and the hippocampus in CRD-naïve mice, two brain areas involved in pain modulation (50). Increasing evidence from human (42) and nonhuman (43) functional imaging studies have demonstrated a distinct pattern of PFC and hippocampus activation in response to visceral pain (27). It is noteworthy that the PFC is a highly vulnerable region in response to stress, undergoing neurochemical and structural alterations that determine the deficits in PFC-mediated behaviors (41). Thus, TLR4 could exhibit an important regulatory function in processing painful stimuli in the PFC. Our data are in agreement with a recently published study in which TLR4 was upregulated in the PFC in mice after repeated restraint/acoustic stress exposure (48). Nonetheless, the superficial layers of the spinal dorsal horn have also been associated with the modulation of abdominal pain (16,26). In our study, TLR4 levels in the lumbar region of the spinal cord were unchanged under chronic stress conditions in the model of visceral hypersensitivity. However, we cannot rule out the presence of alterations in TLR4 expression in the spinal cord after stress exposure, because dorsal (containing mainly nociceptive neurons) and ventral parts of the spinal cord were taken for western blot analysis. Nevertheless, a recently published report has shown a specific differential profile for the involvement of the spinal TLR4 in inflammatory and neuropathic pain (21).

Because TLR4 is predominately expressed in microglia within the CNS (12) and microglia activation has been linked to the

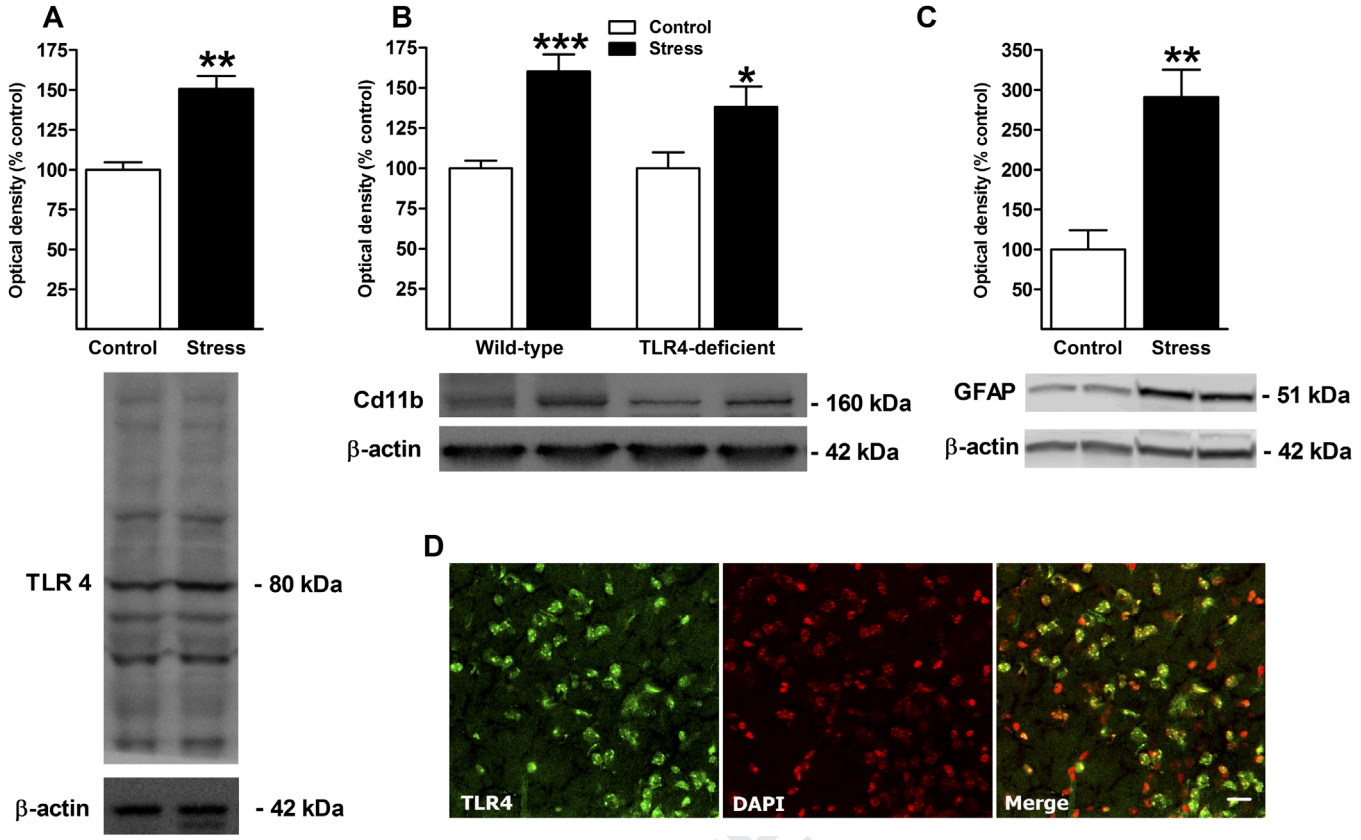

Figure 5. Effects of chronic stress on toll-like receptor 4 (TLR4) expression and glia activation in the prefrontal cortex (PFC). Western blot analysis for TLR4, Cd11b (microglia marker), and glial fibrillary acidic protein (GFAP) (astrocyte marker) in the PFC protein expression of TLR4 was significantly increased in the frontal cortex **(A)** after chronic stress when compared with control mice. Microglia expression (Cd11b) in the PFC was significantly increased in wild-type but also was moderately increased in TLR4-deficient mice after chronic stress **(B)**. Also, GFAP expression was significantly increased after exposure to chronic stress **(C)**. Representative image of TLR4 immunoreactivity in the PFC of wild-type mice **(D)**. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar: 50 μm. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; independent sample *t* test or two-way analysis of variance followed by Bonferroni post hoc test; data represent mean ± SEM expressed as percentage of the control group.

etiology of somatic (15,49) and visceral (16,17) pain, we showed that stress-induced visceral hypersensitivity and enhanced TLR4 expression in the PFC were associated with microglia activation, as assessed through the increased levels of Cd11b. Interestingly, although TLR4-deficient mice displayed no stress-induced visceral hypersensitivity, we also observed an increase in microglia activation in these mice after exposure to chronic stress, indicating a dissociation between the functional nociceptive changes and microglia activation. It is worth noting that previous reports showed decreased microglia activation in TLR4-deficient mice after nerve injury (22). By contrast, no changes in microglia levels were found in the hippocampus after chronic stress, either in wild-type or TLR4-deficient mice. Along with microglia activation in the PFC, the expression of GFAP, an astrocyte marker, was also increased in wild-type mice exposed to chronic stress.

Table 1. Cytokine mRNA Expression in PFC of Wild-Type and TLR4

	Wild-Type		TLR4-Deficient	
	Control	Stress	Control	Stress
TNFα	1.0 ± .23	2.15 ± .46[a]	.80 ± .35	.86 ± .22
IL1β	1.0 ± .12	1.40 ± .08[b]	.87 ± .07	.95 ± .14
IL6	1.0 ± .17	1.25 ± .11	1.18 ± .24	1.24 ± .15

Cytokine messenger RNA (mRNA) expression (expressed as fold change vs. the wild-type control group) in prefrontal cortex (PFC) of wild-type and toll-like receptor 4 (TLR4) exposed to either chronic psychosocial stress or nonstressed (control) procedure. IL, interleukin; TNF, tumor necrosis factor.

[a] $p < .05$ versus control wild-type; one-way analysis of variance followed by Bonferroni post hoc test; data represent mean ± SEM.

[b] $p < .01$ versus control wild-type; one-way analysis of variance followed by Bonferroni post hoc test; data represent mean ± SEM.

To further confirm the concept that it is TLR4 within the PFC that is responsible for mediating chronic stress-induced visceral hypersensitivity, we administered TAK-242 directly into the PFC in animals that underwent our psychosocial stress paradigm and successfully blunted the visceral hypersensitivity. It is noteworthy that after stress, when visceral hypersensitivity is present in mice with functional TLR4, mRNA levels of TNFα and IL1β were increased in the PFC. Thus, intra-PFC blockade of TLR4 could prevent the increased mRNA expression of TNFα and IL1β after chronic stress. Our data indicated that, from a part of the peripheral role of TLR4 in visceral pain modulation, TLR4 shows an important regulatory function in processing painful stimuli in the PFC. Additionally, our data are consistent with the fact that the blockade of TLR4 with specific molecules reverses the hyper-responsive states in models of neuropathic pain (25).

It is of further interest to consider possible ligands of TLR4 within the CNS that might modulate visceral sensitivity. Reports

have shown that the exposure to chronic stress triggers central release of a variety of mediators from damage cells and oxidative stress, including heat shock protein 70 mRNA (51,52) and saturated fatty acids (53). These particular molecules can act as endogenous mediators binding to TLR4 (53,54) and might therefore be associated with visceral hypersensitivity through the release of proinflammatory cytokines as one plausible mechanism. Several pain states are associated with excessive inflammation in both the periphery and the CNS, which contribute to the initiation and maintenance of persistent pain (45,55). In our study, the release of proinflammatory cytokines in the periphery and the enhanced mRNA expression of cytokines in the PFC could be involved in the mechanisms underlying stress-induced visceral hypersensitivity. In addition, chronic stress also disrupts the intestinal barrier (56–58), making it leaky and increasing the circulating levels of immunomodulatory bacterial cell wall components such as LPS, the main activator of TLR4. However, the nature of the mediators involved in TLR4-mediated changes in nociception (22) as well as the underlying mechanisms remain unclear and warrant future research efforts (59).

Hence, we demonstrated that TLR4 is required to modulate visceral pain under physiological conditions and also for the initiation of pathological visceral pain states. In addition, the selective pharmacological blockade of TLR4 in the PFC with TAK-242 was able to counteract the hyper-responsive phenotype in an animal model of stress-induced visceral hypersensitivity, indicative of a novel role of TLR4 especially within the PFC in visceral pain modulation. Thus, blocking TLR4 might be a potential strategy to treat visceral hypersensitivity. Given the clinical availability of TAK-242, human trials are warranted to test the efficacy of TLR4 antagonists in functional gastrointestinal disorders associated with visceral hypersensitivity, such as IBS.

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