Chronic Stress-Induced Changes in Pro-Inflammatory Cytokines and Spinal Glia Markers in the Rat: A Time Course Study

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Key Words

Chronic stress · Glia · Pro-inflammatory cytokines · Serum · Spinal cord visceral pain

Abstract

Background/Aims: Spinal glia activation has been proposed as one mechanism underlying visceral hyperalgesia in a rodent model of chronic stress. In order to assess the possible role of changes in circulating cytokines and in blood-spinal cord barrier (BSCB) permeability in spinal glia activation, we studied the time course of peripheral and spinal pro-inflammatory cytokines and of spinal and satellite glia markers in response to repeated water avoidance (WA) stress. **Methods:** Spinal cords and dorsal root ganglion cells (DRGs) were collected from control rats, rats exposed to 1-hour WA, or 1-hour WA daily for 5 days or 1-hour WA daily for 10 days. **Results:** We demonstrated a time-dependent change in circulating IL-1 β and spinal IL-1 β , IL-6 and TNF- α in stressed animals compared with controls. We found altered expression of the astrocyte markers GFAP and Connexin 43 in spinal and DRG samples at different time points. Finally, WA was associated with increased BSCB permeability. **Conclusions:** These findings confirm the concept that both peripheral and spinal immune markers are altered after chronic WA and suggest a

possible link between stress-induced increase of peripheral pro-inflammatory cytokines, changes in satellite glial cells, increase in BSCB permeability and increase in spinal pro-inflammatory mediators suggesting glia activation.

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Introduction

Engagement of the central stress system by acute psychological or physical stressors results in activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system, including the sympatho-adrenal axis [1]. The resulting increase in circulating glucocorticoids and catecholamine can influence peripheral immune responses [2]. Depending on the nature and duration of the stressor, stress hormones may either inhibit the production of pro-inflammatory cytokines or boost immune responses via the peripheral production of pro-inflammatory cytokines, including TNF- α , IL-1 β , or IL-6 [3]. Sustained activation of the HPA axis, in association with altered feedback inhibition of the HPA axis response during chronic stress, can lead to disturbance in the balance of pro-inflammatory and anti-inflammatory cytokines.

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In the CNS, glial cells, including microglia and astrocytes, play important roles in immunosurveillance of the microenvironment, monitoring cellular debris, apoptotic cells, alterations in neuronal phenotype and balance of synaptic homeostasis [4]. Based on a series of reports showing that different stressors can induce the release of pro-inflammatory cytokines in the CNS [5, 6], the concept of a profile of 'cytokine signature' in the CNS in response to stress has been suggested [7] and glia has been proposed as the source of those molecules [8]. While the mechanisms by which stress induces glial secretion of cytokines remain unclear, a role for CNS adrenergic processes has been suggested [9]. Other studies have suggested that stress does not tonically increase pro-inflammatory cytokine levels but triggers a shift in the pro-inflammatory phenotype of microglia and responsiveness to further immune challenge [5].

Pro-inflammatory cytokines are important players in both peripheral and central sensitization as demonstrated in a number of experimental studies showing up-regulation of inflammatory cytokines in models of mechanical or thermal hyperalgesia in rats [10–12]. In humans, IL-1 β , IL-6 and TNF- α are elevated after traumatic spinal cord injury [13]. Although the specific triggers of spinal glia reactivity in chronic pain models remain unclear, it is known that glia is reactive in chronic pain conditions and has the capacity to release inflammatory mediators in response to *in vitro* stimulation with cytokines such as IL-1 β [10]. Together, these data suggest a possible interplay between peripheral and CNS inflammatory mediators in the context of chronic pain. Interestingly, recent work has pointed to the possible role of inflammatory cytokines, including IL-1 β , in the modulation of the blood-brain barrier permeability and the clinical implication of its dysfunction in diseases, including neuropathic pain [14].

In the present study, we propose to assess changes in pro-inflammatory cytokines in a model of stress-induced exacerbated visceral nociception. Using a validated chronic psychological stress model, e.g. repeated water avoidance (WA) stress, we aimed to test the overall hypothesis that (a) chronic psychological stress leads to increased circulating levels of pro-inflammatory cytokines and changes in dorsal root ganglion (DRG)-satellite glia cell phenotype and (b) the changes in circulating cytokine profile are associated with increased blood-spinal cord barrier (BSCB) permeability and increases in spinal cord pro-inflammatory cytokines are suggestive of glia activation.

Material and Methods

Adult male Wistar rats (250–275 g; Harlan, Inc.) were maintained on a normal light-dark cycle, and provided with food and water *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at the VA Greater Los Angeles Healthcare System. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978).

WA Stress

The model of chronic WA stress consists in placing each animal on a small platform (8 × 8 × 10 cm) affixed to the center of a Plexiglas cage (25 × 25 × 45 cm) filled with tepid water (25°C) to within 1 cm of the top of the platform for 1 h per session. Animals are not directly exposed to the water and when placed on the platform, avoid the aversive stimulus (water) by remaining on the platform. The rats were placed on the platform and in case they fell, they were immediately removed from the water, dried, and placed again on the platform. The rats learned quickly to avoid the water and never fell more than once or twice and if so, only during the first exposure to the stressor. The model of chronic WA stress (10 days) is a validated model of chronic psychological stress-induced visceral hyperalgesia and has been described in detail previously [15, 16].

Five groups of rats were studied: one control group in which rats were handled daily. In another group, rats were exposed to a single 1-hour WA session and sacrificed 1 h later. A third group was exposed to a single WA for 1 h but sacrificed 24 h later. A fourth group was exposed to WA for 1 h on 5 consecutive days and sacrificed 24 h later. Finally, a fifth group was exposed to WA for 1 h on 10 consecutive days and sacrificed 24 h later. A summary of the experimental groups is provided in figure 1. At the time of euthanasia, the spinal cords were hydroextruded with iced saline, lumbar L6/S1 spinal segments and DRGs were dissected and processed for Western blotting and enzyme-linked immunosorbent assay (ELISA). Blood samples were collected from the trunk and serums were processed for cytokine measurements using standard ELISA. Different sets of experiments with the same group paradigm (control, 1 h after 1 WA, 24 h after 1 WA, 24 h after 5 WA and 24 h after 10 WA) were performed for the Evans Blue and FITC-dextran experiments.

Western Blotting

Spinal cord and DRG samples were transferred to extraction buffer (50 mM Tris buffer, pH 8.0, containing 3% SDS, 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA), Protease inhibitor cocktail (1:100; Sigma), and Phosphatase inhibitor cocktail I and II (1:100; Sigma), and immediately homogenized by sonication. 25 μ g protein was electrophoresed on 3–8% NuPage Tris-Acetate gels, transferred to polyvinylidene fluoride membranes.

Blots from spinal samples were probed with primary antibodies anti-GFAP (1:65,000; Millipore) [17, 18], anti-Iba1 (1:500; WAKO) [19], and anti-Connexin 43 (1:1,000; Cell Signaling) [20]. Blots from DRG samples were probed with primary antibodies anti-GFAP (1:65,000; Millipore) [17, 18], and anti-Connexin 43 (1:1,000; Cell Signaling) [20]. Membranes were stripped and re-blotted with anti-actin (1:1,000; Cell Signaling). The intensity of

Fig. 1. Schematic diagram illustrating the different groups of animals used in the study. WA: Water avoidance stress.

immunoreactive bands was quantified using Image Quant software (Molecular Dynamics, Sunnyvale, Calif., USA) and expressed relative to actin.

Enzyme-Linked Immunosorbent Assay

Commercially available kits were used to perform ELISA for IL-1 β , IL-6, and TNF- α (Invitrogen, Camarillo, Calif., USA) [21–23]. The sensitivity for each assay was 3 pg/ml, 5 pg/ml, 4 pg/ml for IL-1 β , IL-6, and TNF- α , respectively. Serum samples were collected from blood (500 μ l) and centrifuged at 10,000 rpm for 15 min at 4°C. Spinal cord and DRG samples were collected and homogenized in buffer (0.5% Triton X, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% SDS). Protein concentration for tissue samples was measured using the standard BCA method (Thermo Scientific, Rockford, Ill., USA). All samples were run in duplicates and absorbance was measured at 450 nm in a microplate reader (Bio Rad, Model 680).

Measurement of BSCB Permeability

Groups of stressed rats and controls were assigned to FITC-dextran or Evans Blue experiments to assess changes in BSCB permeability.

FITC-Dextran

Stressed rats from different groups and controls were fasted for 6 h and gavaged with 4 kDa FITC-labeled dextran diluted in saline (Sigma-Aldrich, St. Louis, Mo., USA; 500 mg/kg, 125 mg/ml). After 1 h, the rats were sacrificed and the spinal cords were hydroextruded with iced saline, and lumbar L6/S1 spinal segments were isolated and homogenized in PBS. FITC-dextran concentration in homogenates was determined by spectrophotometry (excitation wavelength: 485 nm; emission wavelength: 535 nm; SpectraMax M2; Molecular Devices, Sunnyvale, Calif., USA). The size of FITC-dextran is 4 kDa and its passage into the spinal cord (after permeation into the blood) is indicative of permeability to small molecules of similar size.

Evans Blue Assay

BSCB permeability was determined by Evans Blue extravasation into the spinal cord in separate groups of stressed and control rats. Evans Blue dye (2%, 4 ml/kg) was infused through the jugu-

lar vein of anaesthetized rats. After 45 min, the animals were perfused via a cardiac perfusion with PBS. Spinal cord tissues were immediately hydroextruded with iced saline and the lumbar spinal cord (L6/S1) was dissected. Tissues were incubated in 600 μ l formamide (Sigma-Aldrich) at 60°C for 72 h. Evans Blue concentration was then determined by spectrophotometry at 620 nm. Evans Blue is known to bind to albumin and its passage into the spinal cord is representative of the passage of molecules of sizes close to 68 kDa and lower.

Statistical Analysis

Data from control rats were normalized to 100% and data from stressed animals were expressed as percentages of the control response. Data were tested for normal distribution using the Kolmogorov-Smirnov normality test and were analyzed using a one-way ANOVA followed by Dunnett's post-test comparing all groups to the control. Correlation studies between different markers were not possible because each time point corresponded to a different group of rats.

Results

Stress Induced Changes in Plasma Cytokines

A one-way ANOVA was used to test the effect of time on plasma cytokine levels. Serum levels of IL-1 β were significantly different across time, $F(4,42) = 6.095$ ($p = 0.006$). Dunnett's post-hoc comparisons indicated that the serum levels of IL-1 β at 1 h (126.9 ± 9.4 , $p < 0.05$), 24 h after 1 WA (127.9 ± 6 , $p < 0.05$) and 24 h after 5 WA (121.3 ± 6.5 , $p < 0.05$) were significantly increased compared with non-stressed controls (100 ± 3.9 ; $n = 6$ –12/group; fig. 2a). However, the levels of circulating IL-6 and TNF- α were not significantly different in any of the stressed groups compared with controls (for IL-6: $n = 6$ –12/group and for TNF- α : $n = 6$ –12/group; fig. 2b, c). The absolute plasma cytokine levels were within the

Fig. 2. Plasma cytokine levels measured by ELISA. **a** Circulating level of IL-1 β , $n = 6-12$ in each group, * $p < 0.05$, significantly different from controls, ANOVA followed by Dunnett's test. **b** Circulating level of IL-6, $n = 6-12$ in each group. **c** Circulating level of TNF- α , $n = 6-11$. Data are means $\pm$ SEM normalized to controls.

Fig. 3. Spinal cord cytokine levels measured by ELISA. **a** Spinal cord level of IL-1 β , $n = 6-12$ in each group. **b** Spinal cord level of IL-6, $n = 6-12$ in each group. **c** Spinal cord level of TNF- α , $n = 6-12$. Data are means $\pm$ SEM normalized to controls. * $p < 0.05$, significantly different from controls, ANOVA followed by Dunnett's test.

range described in previous papers [24, 25] with lower values of 43.7 ± 3 pg/ml for IL-1 β , 42.6 ± 5 pg/ml for IL-6 and 24.8 ± 3 pg/ml for TNF- α .

Cytokines in the Spinal Cord

The levels of IL-1 β , TNF- α and IL-6 were assessed in spinal cord extracts from the 5 groups. A one-way ANOVA was used to test the effect of time on spinal cytokine expression. The spinal level of IL-1 β varied depending on time, $F(4,47) = 4.033$ ($p = 0.0068$). Dunnett's post-hoc comparisons indicated that IL-1 β was significantly increased at 1 h after 1 WA (222.8 ± 29.1) compared with

controls (100 ± 22.4 , $p < 0.05$; $n = 6-12$ /group; fig. 3a). Similarly, IL-6 varied depending on time, $F(4,40) = 3.448$ ($p = 0.0164$). Dunnett's post-hoc comparisons indicated that IL-6 was significantly higher 1 h after 1 WA (145.5 ± 10.6) compared with controls (100 ± 9.9 , $p < 0.05$; $n = 6-12$ /group; fig. 3b). There was no significant difference in the expression of IL-1 β and IL-6 at any other time points compared with controls. The level of spinal TNF- α varied depending on time, $F(4,42) = 4.457$ ($p = 0.0043$). Dunnett's post-hoc comparisons indicated that spinal TNF- α was increased at 24 h after 1 WA (128.2 ± 11.1) and at 24 h after 10 WA (141.6 ± 14.6) compared with

Fig. 4. Protein expression in spinal cord extracts measured by Western blotting. **a** Spinal cord level of GFAP, $n = 6-12$. **b** Spinal cord level of Connexin 43 (Cn43), $n = 6-12$. **c** Spinal cord level of Iba1, $n = 6-12$. Data are means $\pm$ SEM normalized to controls. * $p < 0.05$, significantly different from controls, ANOVA followed by Dunnett's test. **d** Representative blots.

controls (100 ± 8.3 , $p < 0.05$; $n = 6-12$ /group; fig. 3c). The absolute spinal cytokine levels were within the range described in previous papers [23, 26] with lower values of 5.3 ± 0.7 pg/ml for IL-1 β , 7.5 ± 1 pg/ml for IL-6 and 5.0 ± 1 pg/ml for TNF- α .

GFAP, Iba1 and Connexin 43 in the Spinal Cord and DRGs

In the spinal cord, a one-way ANOVA showed changes in GFAP across time, $F(4,44) = 9.733$ ($p = 0.0001$). Dunnett's post-hoc comparisons showed an increased level of GFAP observed 24 h after 5 WA (133.6 ± 3.7 , $p < 0.05$), while a significant decrease was found 24 h after 10 WA (50 ± 4.2 , $p < 0.05$) compared with controls (100 ± 11.1 ; $n = 6-12$ /group; fig. 4a). Connexin 43 significantly changed across time, $F(4,31) = 5.240$ ($p = 0.0024$), and a Dunnett's post-hoc comparisons showed the level at 24 h after 10 WA was decreased to 66.3 ± 1.7 compared with controls (100 ± 9.9 , $p < 0.05$; $n = 6-12$ /group; fig. 4b). Iba1 showed no changes in the stressed groups compared with controls ($n = 6-12$ /group; fig. 4c). Representative blots for each time point are presented in figure 4d.

In DRGs, a one-way ANOVA showed changes in GFAP across time, $F(4,31) = 5.504$ ($p = 0.0018$). Dunnett's post-hoc comparisons revealed that GFAP was dramatically increased 24 h after 10 WA (315.3 ± 67.5) compared with controls (100 ± 21.35 , $p < 0.01$; $n = 6-10$ /group; fig. 5a), while Connexin 43 was significantly increased 24 h after 5 WA (168 ± 18.35) compared with controls (100 ± 13.4 , $p < 0.05$; $F(4,31) = 2.809$, $p = 0.0424$; $n = 6-10$ /group; fig. 5b). Representative blots for each time point are presented in figure 5c.

Stress Induced Changes in BSCB Permeability

A one-way ANOVA revealed that Evans Blue concentration varied in the different groups, $F(4,32) = 4.374$ ($p = 0.0062$). Dunnett's post-hoc comparisons showed an increased concentration at 24 h after 5 WA sessions (9.5 ± 1.5) compared with controls (4.84 ± 0.3 , $p < 0.05$), suggesting an increased permeability to molecules of a size comparable to albumin (fig. 6a). There was no significant difference between the other stress groups and controls ($n = 5-9$ /group). In a different series of experiments, the concentration of FITC-dextran was signifi-

Fig. 5. Protein expression in DRG extracts measured by Western blotting. **a** DRG level of GFAP, $n = 6-10$. **b** DRG level of Connexin 43 (Cn43), $n = 6-10$. Data are means $\pm$ SEM normalized to controls. * $p < 0.05$, ** $p < 0.01$, significantly different from controls, ANOVA followed by Dunnett's test. **c** Representative blots.

Fig. 6. Measures of blood-spinal cord permeability **a** Evans Blue extravasation in the spinal cord, $n = 5-9$. **b** FITC-dextran extravasation in the spinal cord, $n = 6-11$. Data are means $\pm$ SEM normalized to controls. * $p < 0.05$, significantly different from controls, ANOVA followed by Dunnett's test.

cantly higher in spinal cord samples collected 24 h after 1 WA (0.2 ± 0.002 , $p < 0.05$) and from samples collected 24 h after 5 WA (0.24 ± 0.013 , $p < 0.001$) compared with controls (0.17 ± 0.005), $F(4,39) = 7.580$ ($p = 0.0001$), suggesting an increased permeability to small-size molecules at these time points ($n = 6-11$ /group; fig. 6b).

Discussion

In the present study, we were able to demonstrate a time-dependent profile in the levels of circulating IL-1 β and spinal IL-1 β , IL-6 and TNF- α in stressed animals compared with non-stressed controls. In addition, we found temporal changes in the expression of the astrocytic marker GFAP in spinal and DRG samples. We also

identified that WA stress is associated with a transient increase in BSCB permeability.

We reported an increased plasma concentration of IL-1 β at 1 h after 1 WA, 24 h after 1 WA and 24 h after 5 WA. We found no changes in other circulating pro-inflammatory cytokines (IL-6 and TNF- α) in response to stress at any time points. These results point towards a rapid increase in IL-1 β in the circulation in response to a single stress session (within 1–24 h after 1 WA stress session), which seems to be maintained after 5 WA sessions. The changes in circulating IL-1 β were significant ($p < 0.05$) though it should be noted that the amplitude of the changes does not correspond to an ‘inflammatory profile’ per se as can be seen in models of peripheral inflammation for example [27]. These findings are consistent with other reports showing that, while the effect of stress on the immune system and the pattern of stress-induced cytokine increase in the circulation and in the CNS is depending on the nature of the stressor, IL-1 β appears to be one key cytokine that is consistently increased after stress exposure in both blood and brain in several stress models [28, 29]. It is unlikely that circulating IL-1 β originates from a peripheral site of inflammation or injury since we previously demonstrated that the model of chronic WA stress is not associated with significant gastrointestinal structural alterations or inflammation [30]. However, it is possible that our results reflect a cell-mediated immune response to stress as described in other studies [31]. The effect of stress on cell-mediated immune response may include trafficking or redistribution of peripheral lymphocytes between different immune compartments, including the spleen, in which changes in the expression of cell adhesion molecules plays a significant role [32]. Although we did not measure circulating lymphocytes or cytokines from extracted circulating lymphocytes, it is possible that they contribute to the increased circulating IL-1 β in our stress model [33]. Cytokines in the blood may also originate from chromaffin cells of the adrenal medulla [34], or may derive from high CNS concentrations [35].

In the spinal cord, we observed increased concentrations of IL-1 β and IL-6 at 1 h after 1 WA only, while TNF- α was increased 24 h after 1 WA and 24 h after 10 WA only. Our results point towards a rapid and transient increase in pro-inflammatory mediators in the spinal cord in response to a single stress session (within 1–24 h after 1 WA stress session), while the only cytokine showing changes after 10 days of stress was TNF- α . This cytokine profile in the spinal cord does not reflect the changes of IL-1 β observed in the serum from day 1 to 24 h after day

5, suggesting the lack of direct peripheral origin of spinal cytokines. In the CNS, cytokines may be of neuronal or glial origins (including microglia and astrocytes) and play an important role in the sensitization of nociceptive signals [36]. In the present study, our data showed no changes in the protein level of Iba1 at any time point (commonly used as one marker of microglia). However, we have previously demonstrated that chronic WA stress leads to p38 activation in spinal microglia 24 h after 10 WA and that spinal microglia play a functional role in the expression of visceral hyperalgesia [18]. In addition, previous studies have reported the potential of glia to release mediators without change in Iba1 phenotype [37, 38]. It is also known that stress alone can promote glia activation in the CNS, or prime glia to further stimulation [8]. Our present results also showed a biphasic change in GFAP expression with an increased GFAP protein level 24 h after 5 WA followed by a decreased level compared with controls 24 h after 10 WA. These results are consistent with our previous findings showing a decreased GFAP protein level and GFAP immunohistochemistry intensity 24 h after 10 WA [36]. Together, these results suggest a possible implication of spinal glia activation and the release of glia mediators in the CNS immune response to chronic stress. We also found decreased spinal glutamate transporter expressions after stress and supporting evidence for a role of the glutamatergic system in our model [18].

IL-1 β and TNF- α have been implicated in the pathophysiological changes occurring in various disease states, including rheumatoid arthritis, neuropathic pain or inflammatory bowel disorders [35, 39]. In the spinal cord, the central role of IL-1 β to the development of chronic pain is well established [40–42]. Elevated spinal IL-1 β was reported in both human [13] and rat [43] following spinal cord injury. It has been proposed that the pro-nociceptive effect of IL-1 β is mediated through the up-regulation of other pro-nociceptive mediators (NGF, CGRP) or the modulation of neuronal excitability by affecting receptors such as TRPV1, sodium channels, GABA or NMDA receptors. In a recent paper, spinal IL-1 β released from astrocytes was found to enhance NR1 phosphorylation to facilitate inflammatory pain [44]. Similarly, increasing evidence suggest a critical role of TNF- α in the pathogenesis of pain, including neuropathic pain and inflammatory pain; and while the peripheral effect of TNF- α on nociceptor sensitization is well established [45], the role of TNF- α in central sensitization is increasingly supported by a growing literature. TNF- α expression is induced in spinal cord glial cells in several models of chron-

ic pain [46], and intrathecal injections of TNF- α or a TNF- α inhibitor have been found to trigger and inhibit hyperalgesic response, respectively [12, 39]. Recent work identified the critical role of TNF- α in spinal synaptic activity and in the control of NMDAR activity [12].

In the chronic WA stress model, TNF- α is increased in the spinal cord 24 h after 1 WA and 24 h after 10 WA. Interestingly, these 2 time points correspond to the time points at which we previously reported visceral hyperalgesia [30, 47] suggesting a possible link between spinal TNF- α and stress-induced sensitization of visceral nociception. Although our study is limited by (1) the lack of statistical correlation between the level of TNF- α and visceral hyperalgesia (observations in different rat groups from different experiments precluding correlation analysis) and (2) the lack of pharmacological studies evaluating the role of cytokine inhibitors on peripheral and spinal markers and visceral sensitivity, one may speculate about the role of IL-1 β and TNF- α in the modulation of the visceral nociceptive response. Further mechanistic studies using transgenic animals or testing the effects of cytokine receptor antagonists or blocking antibodies are warranted to confirm this hypothesis.

While glia is accepted as a potential source of cytokines in the CNS, it remains unclear what signals are causing glia activation and release of pro-inflammatory cytokines, in particular in models of stress-induced CNS glia activation. Evidence exists supporting the ability of peripheral cytokines to transduce their signals across the blood-brain barrier (BBB) [48], and thereby, triggering a series of signaling pathways in the CNS. This has been described for IL-1 β which is thought to induce the elevation of CNS IL-1 β through several indirect mechanisms such as stimulation of vagal or other nerve afferents [49, 50], immune cell trafficking from blood to brain with subsequent secretion [51], induction of mediators release from BBB cells [52], interaction with circumventricular organs [53] or directly crossing the BBB. In a recent paper [54], it was demonstrated that regional neural activation defined a gateway for autoreactive T cells to cross the BBB via an effect of IL-6 in endothelial cells in the spinal cord supporting a role for neural mechanisms in neuroimmune activation in the CNS.

In our study, we found a simultaneous increase in IL-1 β in the circulation and in the spinal cord in the early stress exposure, with increased circulating IL-1 β outlasting changes in spinal IL-1 β . In view of these results, it is difficult to conclude whether peripheral IL-1 β may be a triggering factor in the increase of spinal IL-1 β . However, it is possible that an increase in spinal TNF- α (which oc-

curred only at 24 h after 1 WA and 24 h after 10 WA) may be consecutive to the transduction of peripheral IL-1 β signals from the periphery to the CNS. This is supported by our observations that the BSCB permeability is increased in our model of chronic WA stress at 24 h after 1 WA. We showed increased permeability to small molecules (4 kDa) 24 h after 1 WA and 24 h after 5 WA, while permeability to Evans Blue (which binds to albumin (68 kDa)) was significantly increased 24 h after 5 WA (which may be connected to the prior passage of smaller molecules enabled 24 h after 1 WA). Surprisingly, the significant increase of BSCB permeability to large molecules matched the increased expression of spinal GFAP at 24 h after 5 WA. It is possible that changes in astrocyte phenotypes, although associated with increased GFAP, may influence BSCB permeability at day 5. The role of Connexin 43 (a gap junction protein involved in astrocyte networking) in the control of BBB permeability has been previously discussed [55]. However, while Connexin 43 is predominantly associated with astrocytic gap junctions, Connexin 43 in endothelial cells may have cell autonomous effects on their responses to permeability-enhancing signals, independent of any effects on intercellular communication (colocalization with the tight junction molecules occludin, claudin, and ZO-1) [56], which may explain the different time course observed for stress-induced changes in BSCB permeability and changes in expression of spinal Connexin 43 and GFAP in our model after 10 WA.

Our findings, showing a trend for sustained increased levels of Connexin 43 in DRGs throughout the 10 WA period and significantly higher levels of GFAP at 24 h after 10 WA in DRGs, suggest that satellite glia cells (SGCs, glial cells present in sensory neurons in DRGs) may respond to input from either spinal or peripheral origin. It has been previously observed that increased gap junction-mediated coupling between SGCs (as indicated by increased Connexin 43 expression) contributes to increased sensory neuron coupling and augmented neuronal excitability. Interestingly, SGCs were also found capable of releasing cytokines such as IL-1 β and TNF- α [57, 58]. Although the sensitization of DRG neurons is well documented in rat models of visceral pain induced by inflammation [59], the role of SGCs in visceral pain has received little attention. A recent study showed that gap junction blockers injected directly into DRGs abolish colonic inflammation-induced changes in SGCs and sensory neurons and significantly reverse visceral pain behavior, suggesting a role of SGCs in the modulation of visceral nociception after inflammation [60]. Our data indicate SGCs activation and increased coupling, sug-

gested by increased Connexin 43 in response to chronic WA stress in DRGs. The role of these changes in spinal glia activation and increase in visceral nociception in the chronic WA stress model needs to be further investigated.

In conclusion, the present study establishes a profile of circulating and spinal cytokines during the course of 10 days WA stress associated with changes in spinal astrocytes, BSCB permeability, and DRG SGC activation. While the data provide a mostly descriptive analysis of those changes, they also provide the necessary framework to further evaluate the functional role of peripheral and spinal cytokines in stress-induced spinal glia activation and the development of visceral hyperalgesia.

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Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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