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Atorvastatin Prevents Neuroinflammation in Chronic Constriction Injury Rats through Nuclear NFκB Downregulation in the Dorsal Root Ganglion and Spinal Cord

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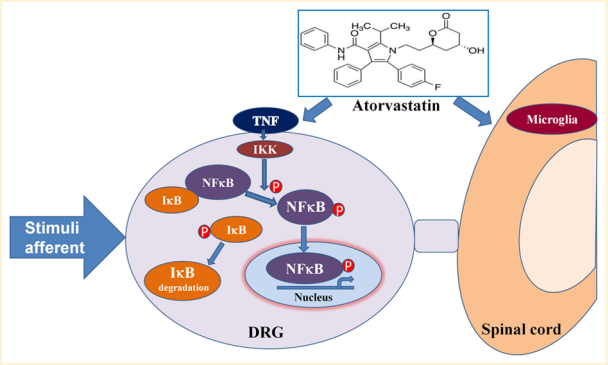
ABSTRACT: Atorvastatin, traditionally used to treat hyperlipidemia, belongs to a class of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors. This study investigated the antineuroinflammatory and antihyperalgesic effects of atorvastatin in dorsal root ganglia (DRG) and spinal cord for chronic constriction injury (CCI) neuropathic pain in rats. Fifty-four Sprague−Dawley rats were divided into three groups including sham, CCI, and CCI+atorvastatin. Rats were orally administered atorvastatin (10 mg/kg/day) once daily for 2 weeks after surgery and sacrificed at days 3, 7, and 14. All animals were assessed for mechanical allodynia and thermal hyperalgesia in both hindpaws. Western blotting, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) were used to detect inflammatory proteins and proinflammatory cytokines at day 7 after surgery. Pain behaviors were significantly reduced in the CCI+atorvastatin group compared to the CCI group. Atorvastatin attenuated CCI-induced inflammatory mediators (pAkt/Akt, COX-2, iNOS, EP1, and EP4) and reduced proinflammatory cytokines TNF-α and IL-1β levels in DRG and spinal cord. Atorvastatin also inhibited nuclear pNFκB activation. Double immunofluorescent staining further demonstrated that pNFκB proteins were decreased by atorvastatin in DRG satellite cells and spinal microglia. Atorvastatin may primarily inhibit the nuclear translocation of pNFκB to prevent CCI-induced peripheral neuropathic pain. Atorvastatin exhibits antineuroinflammatory and antinociceptive properties in the central and peripheral nerve systems.



KEYWORDS: *Atorvastatin, chronic constriction injury, neuropathic pain, pNFκB, neuroinflammation, proinflammatory cytokines*

Peripheral neuropathic pain results from different types of nerve injury including diabetic neuropathy, postherpetic neuralgia, drug-induced neuropathy, and traumatic nerve injury.[1−3] It is generally accepted that peripheral nerve injury results in chronic neuropathic pain associated with hyperresponsiveness of sensory neurons within the dorsal root ganglion and the spinal cord dorsal horn.[4] Schwann cells, macrophages, and activated glia or microglia peripherally and centrally release nitric oxide (NO) and proinflammatory cytokines after peripheral nerve injury.[5] Microglia are representative of the resident mononuclear phagocyte population in the central nerve system (CNS). Microglia share many phenotypical and functional characteristics with peripheral macrophages and monocytes, and may share many immune reactions in the brain.[6,7] Inhibition of hyperactive microglia in the spinal dorsal horn could alleviate neuropathic pain induced by peripheral nerve injury.[8,9] It is likely that an elevated microglia level within the spinal dorsal horn could lead to central sensitization of neurons and enhance spinal nociceptive transmission following peripheral nerve injury.[9,10] Proinflammatory cytokines are often produced in a cascade in which TNF-α is typically made first, causing the induction of IL-1 and IL-6. The effects of proinflammatory cytokines show synergy (especially TNF-α and IL-1) when more than one cytokine is present.[11]

Statins can trigger several neuroprotective signaling pathways. Neuroprotective potency was associated with HMG-CoA reductase blockade[12,13] and neuroprotective effects could be neutralized by either mevalonate or cholesterol administration.[14,15] Treatment with statins after brain damage augmented neurogenesis and synaptogenesis without altering serum cholesterol levels.[16,17] Statins achieved neuroprotection by enhancing the release of neurotrophic factors.[18] Statins also reduce inflammation and microglial activation and restore microvascular parameters in the brain.[19]

Neuropathic pain from nerve injury is a common condition, for which there is currently no definitive effective treatment.[20]

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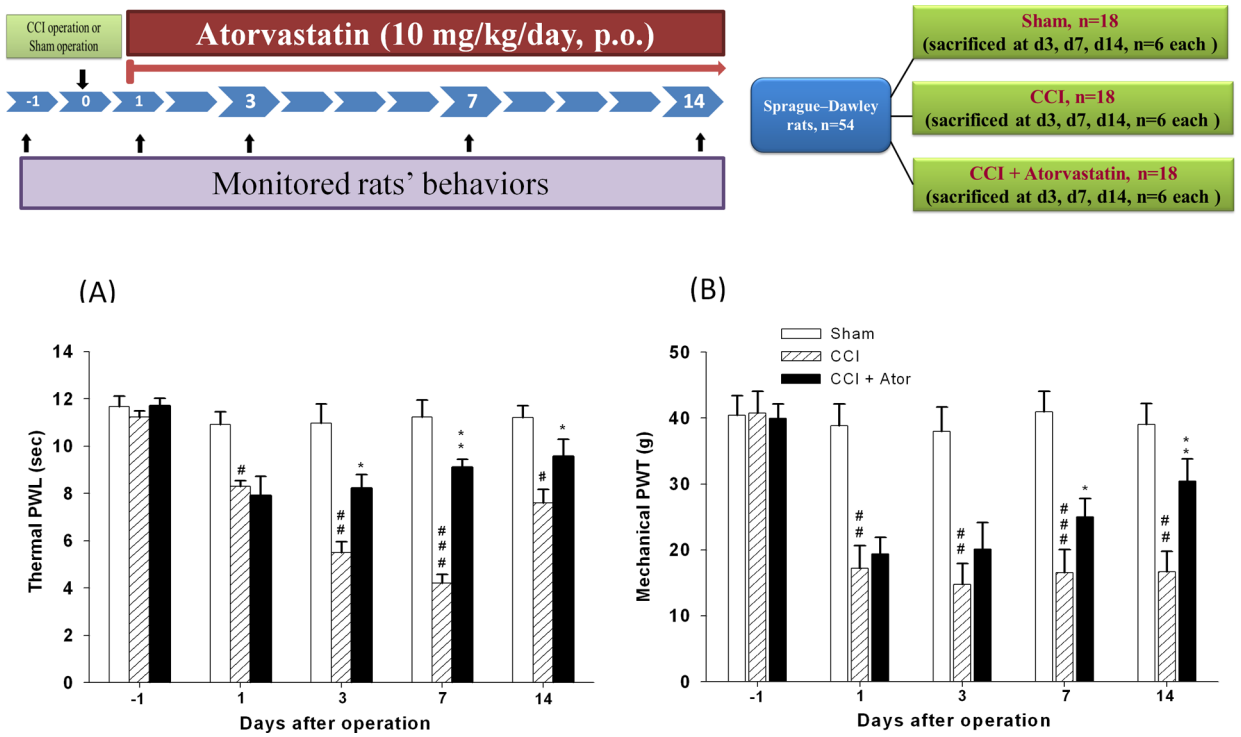

Figure 1. Effects of atorvastatin on thermal paw withdrawal latency (PWL) and mechanical paw withdrawal threshold (PWT) evoked by chronic constriction injury (CCI) in rats. Top panel depicts the experimental design (left) and flow diagram (right) for the CCI model in rats. (A) Thermal PWL and (B) Mechanical PWT were performed 1 day before CCI and at days 1, 3, 7, 14 after surgery. Sham-operated rats were subjected to the same surgical procedure without manipulation of the nerve. Data represent the mean ± SE for 6 rats per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham group at the corresponding time points; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CCI group.

Clinical and experimental observations suggest that atorvastatin may be useful for treating some pathologic conditions where neurogenic inflammation is predominant,[21,22] but the mechanisms mediating this potential therapeutic effect are poorly understood. In this study, we tested the hypothesis that statins act directly on the sensory neurons to decrease expression of proinflammatory molecules induced by chronic constriction injury (CCI), especially by decreasing the expression of phospho-nuclear factor κB (pNFκB) and cyclooxygenase-2 (COX-2). Dorsal root ganglia (DRG) sensory neurons play a critical role in neuropathic hypersensitivity.[1,23,24] We found that atorvastatin decreased CCI-induced inflammatory mediators and increased neuroprotective factors in the sciatic nerve.[21] We further investigated whether atorvastatin had an impact on neuropathic pain symptoms in the peripheral DRG and central spinal dorsal horn of CCI rats. Atorvastatin was orally administered once daily to CCI rats and its effects on allodynia and hyperalgesia were assessed. In parallel with behavioral experiments, we analyzed biochemical changes induced by atorvastatin in the spinal dorsal horn and DRG neurons following sciatic nerve injury.

RESULTS

Atorvastatin Affects CCI-Induced Thermal Hyperalgesia and Mechanical Allodynia. CCI rats had a marked decrease of thermal paw withdrawal latency (PWL; Figure 1A) and mechanical paw withdrawal threshold (PWT; Figure 1B) from day 1 to day 14, suggesting that CCI caused rapid onset of nociceptive pain (<day 3) and long-lasting neuropathic pain behaviors (hyperalgesia and allodynia). Notably, CCI-induced neuropathic pain was obvious on day 3 and the most severe pain behaviors appeared on day 7. These results were consistent with previous reports.[25,26] There were no significant changes of PWL and PWT in the sham group. The CCI +atorvastatin group (10 mg/kg/day, p.o.) showed markedly increased PWL (day 3 to day 14) and PWT (day 7 to day 14) in comparison with the CCI group (Figure 1). The results suggested that atorvastatin can attenuate neuropathic pain induced by peripheral nerve injury. Since oral administration of atorvastatin started the next day after surgery, this neuropathic rat model can be considered a protection and prevention protocol and thus it is not surprising that atorvastatin is effective from day 3 to day 14 after CCI.

Atorvastatin Decreased Proinflammatory Cytokines TNF-α and IL-1β in DRG and Spinal Cord. Since the rat model of neuropathic pain induced by CCI was apparent on day 3 and most severe on day 7, proinflammatory cytokines were measured at both time points. ELISA kits were used to investigate the effect of atorvastatin on proinflammatory TNF-α and IL-1β in the DRG and spinal cord of CCI rats. In the CCI group, IL-1β (Figure 2) and TNF-α (Figure 3) levels were markedly increased in rat DRG and spinal cord on days 3 and 7 compared to the sham control. The CCI+atorvastatin group showed significantly reduced levels of CCI-elevated cytokines (Figures 2 and 3).

Atorvastatin Decreased Inflammatory Proteins in DRG and Spinal Cord. The time-course alterations of pAkt/Akt, pIκB/IκB, pNFκB, COX-2, EP1, EP4, and iNOS expression after CCI are shown for the DRG (Figure 4) and spinal cord (Figure 5). CCI significantly increased the expression of pAkt/Akt at days 3, 7, and 14. The CCI +atorvastatin group (10 mg/kg/day, p.o.) showed decreased expression of pAkt/Akt at each time point compared to the

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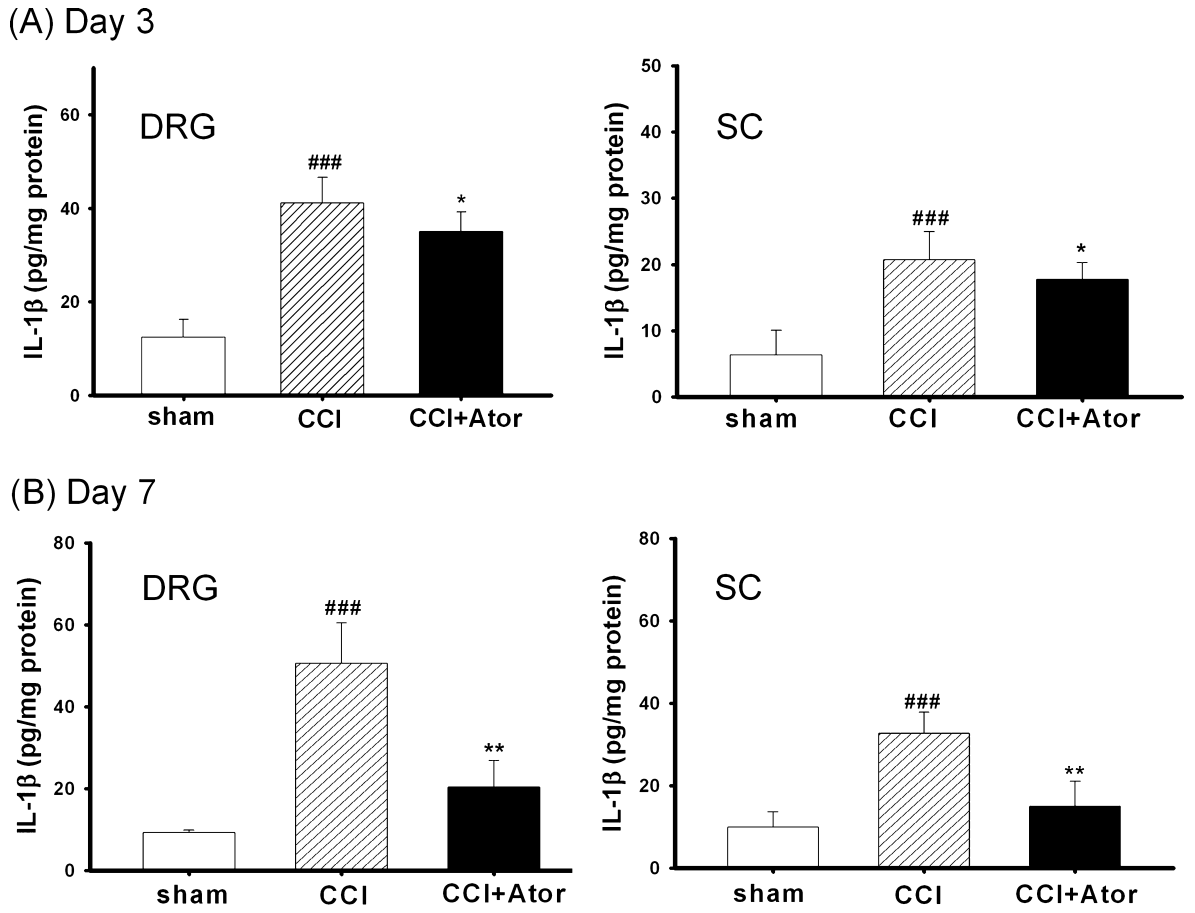

Figure 2. Effects of atorvastatin (Ator, 10 mg/kg/day, p.o.) on IL-1β levels of dorsal root ganglion and spinal cord in CCI rats at (A) day 3 and (B) day 7. IL-1β levels in dorsal root ganglion (DRG) and spinal cord (SC) are depicted. Data represent the mean ± SE for 6 rats per group. $^{###}p < 0.001$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CCI group.

CCI group. IκB activity was determined by the ratio of cytoplasmic pIκB/IκB, a critical step of NFκB activation, which showed a prominent increase after CCI compared with the sham group. Atorvastatin significantly reduced the expression of cytoplasmic pIκB/IκB from day 3 to day 14 after CCI. Nuclear pNFκB also decreased from day 3 to day 14 after atorvastatin treatment. Particularly, COX-2 was markedly increased after CCI compared with the sham group. After atorvastatin treatment, COX-2 expression was dramatically downregulated compared with the CCI group. Even at day 14, the atorvastatin-treated group still exhibited much lower COX-2 expression than the CCI group, indicating its powerful anti-inflammatory potential. The increase in iNOS was considerable after CCI, and this inflammatory protein was reduced by atorvastatin. EP1 and EP4 proteins were also increased after CCI and decreased after atorvastatin administration.

Atorvastatin Decreased pNFκB Immunofluorescent Localization in DRG and Spinal Cord. Double immunofluorescent staining was used to further validate the involvement of pNFκB activation in peripheral nerve injury. We observed that CCI induced increased pNFκB expression in DRG satellite cells (Figure 6) and spinal microglia at day 7 (Figure 7). The CCI+atorvastatin group (10 mg/kg/day, p.o.) showed significantly decreases in the CCI-induced upregulation of pNFκB levels in glial cells (Figures 6 and 7). The relative fluorescence data were quantitated in Figures 6 and 7.

DISCUSSION

This study demonstrated that oral administration of atorvastatin prevented or reduced CCI-related neuropathic pain features in the sciatic nerve.[21] In this study, we also investigated the possible mechanisms of atorvastatin pain relief in DRG neurons and the spinal dorsal horn in CCI-induced neuropathic rats. Our major findings are (1) atorvastatin significantly reduced the severity of thermal hyperalgesia and mechanical allodynia in the rat CCI model, and (2) atorvastatin decreased proinflammatory cytokines TNF-α and IL-1β and down-regulated inflammatory signaling proteins (pAkt, pIκB, pNFκB, COX-2, EP1/EP4 and iNOS) in the DRG and spinal cord. These findings provide biochemical evidence that atorvastatin could have an important role preventing nerve injury-associated neuropathic pain. Atorvastatin also had anti-inflammatory actions in different models of neuronal damage.[27,28]

In this CCI model, rats were administered with 10 mg/kg atorvastatin by oral gavage once daily. It appears that the dose used in the rats is several-fold higher than the recommended human daily dose (10−80 mg/day). The translation of drug doses from animal studies to human studies should not be based on body weight alone, which can result in inappropriate comparisons between studies. Notably, the animal doses should not be extrapolated to a human equivalent dose by a simple conversion based on body weight. It is generally accepted that body surface area-based dose calculation is the most suitable

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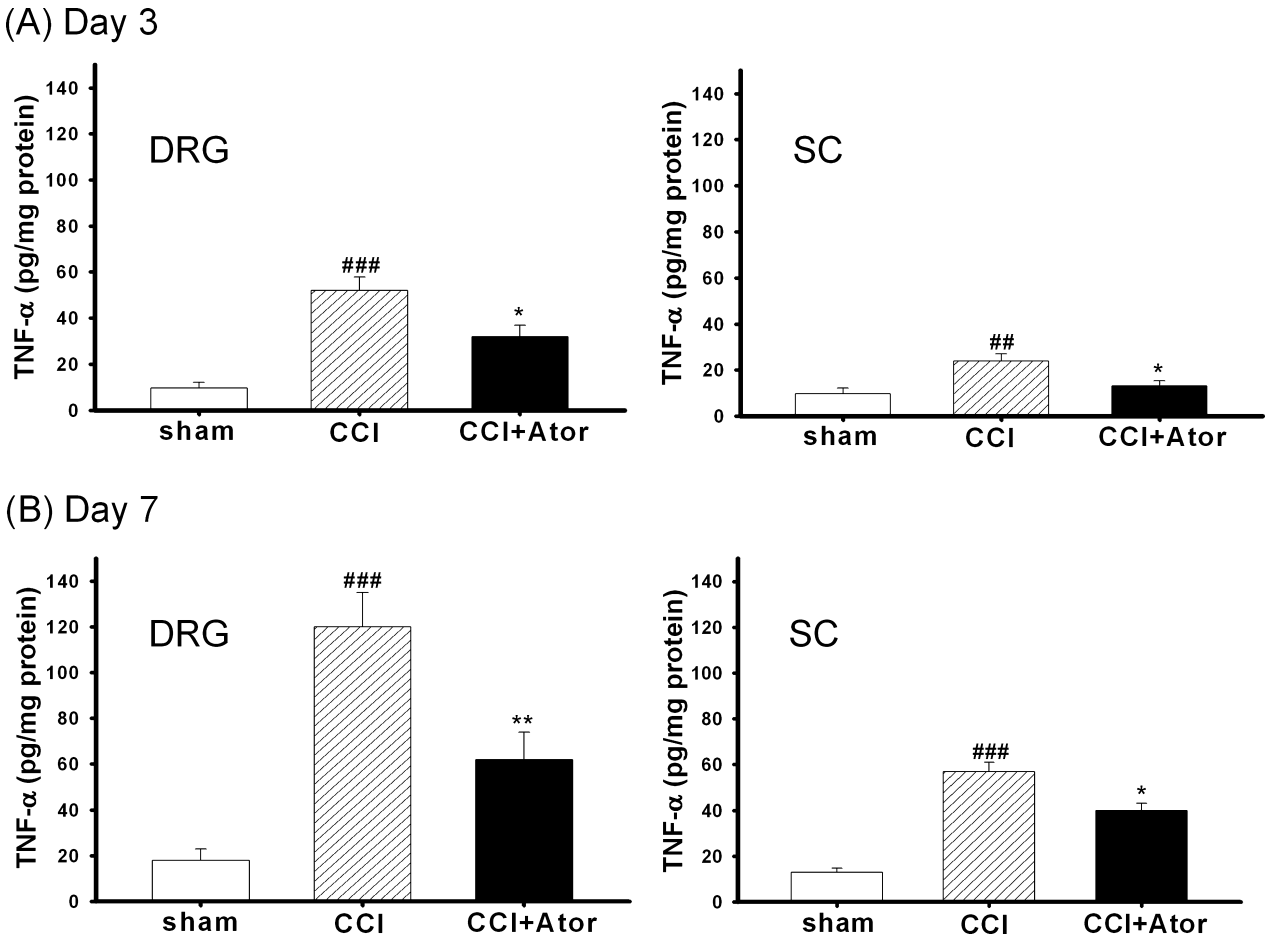

Figure 3. Effects of atorvastatin (Ator, 10 mg/kg/day, p.o.) on TNF-α levels of dorsal root ganglion and spinal cord in CCI rats at (A) day 3 and (B) day 7. TNF-α levels in dorsal root ganglion (DRG) and spinal cord (SC) are depicted. Data represent the mean ± SE for 6 rats per group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CCI group.

method for conversion of drug doses between species.[29] Body surface area correlates well across many mammalian species with several physiological parameters such as blood volume, basal metabolism, caloric expenditure, oxygen utilization, circulating plasma proteins, and so forth. Using the formula for dose translation based on body surface area, the 10 mg/kg atorvastatin used in rats in this study corresponds to the calculated human equivalent dose for atorvastatin of 1.62 mg/kg, which equates to a 97.2 mg dose of atorvastatin for an adult (60 kg).[29] This dosage of atorvastatin is close to the maximum recommended human daily dose (80 mg/day).

Neuropathic pain is characterized by spontaneous pain, allodynia, and hyperalgesia.[30] Thermal hyperalgesia and mechanical allodynia in CCI rats were lower than those in the sham group rats, demonstrating that CCI rats exhibited behavioral signs of hyperalgesia resulting from noxious thermal and mechanical stimuli.[9] It appears that Aδ and C sensory neurons may participate in thermal stimuli-evoked peripheral nerve injury-induced neuropathic pain.[31] Mechanical allodynia is due to the sensitization of the central nervous system, and it is characterized by hyperalgesia to mechanical stimuli. Aβ sensory neurons may contribute to mechanical stimuli-evoked peripheral neuropathy in animal models.[32] In our study, oral administration of atorvastatin reversed CCI-induced mechanical allodynia and thermal hyperalgesia, suggesting that it could influence the neuronal activity of primary afferent sensory fibers. We observed that the most severe CCI-induced pain behaviors occurred on day 7, and thus, we performed the biochemical experiments on the same day to explore the possible mechanisms of atorvastatin relief of nerve injury-evoked neuropathic pain. In this work, atorvastatin reduced CCI-induced thermal hyperalgesia on day 3 and mechanical allodynia on day 7, suggesting that it could be more sensitive to the small-diameter sensory Aδ and C fibers.

Several studies have demonstrated that TNF-α,[33] IL-1β,[34] and pNFκB[35] contribute to the transmission of nociceptive signals in inflammatory and neuropathic states. Either direct release or indirect induction of the release of proinflammatory mediators such as TNF-α and IL-1β are involved in many aspects of inflammation, including increasing vascular permeability, cell migration, edema development, fever, and hyperalgesia.[36] TNF-α and IL-1β can be released from astrocytes, microglial cells, and neurons in the spinal cord after nerve injury. Thus, inhibition of spinal and brain TNF-α and IL-1β signaling might reduce inflammatory, neuropathic or cancer pain. Abundant evidence has shown that activation of both astrocytes and microglial cells can synthesize various neuroexcitatory substances such as proinflammatory cytokines TNF-α and IL-1β, which increase the expression of COX-2 and iNOS, further leading to prostaglandins (PGs) and NO syntheses that potentiate pain transmission and modulation.[37,38] In this study, atorvastatin significantly decreased proinflammatory cytokines in the DRG and spinal cord,

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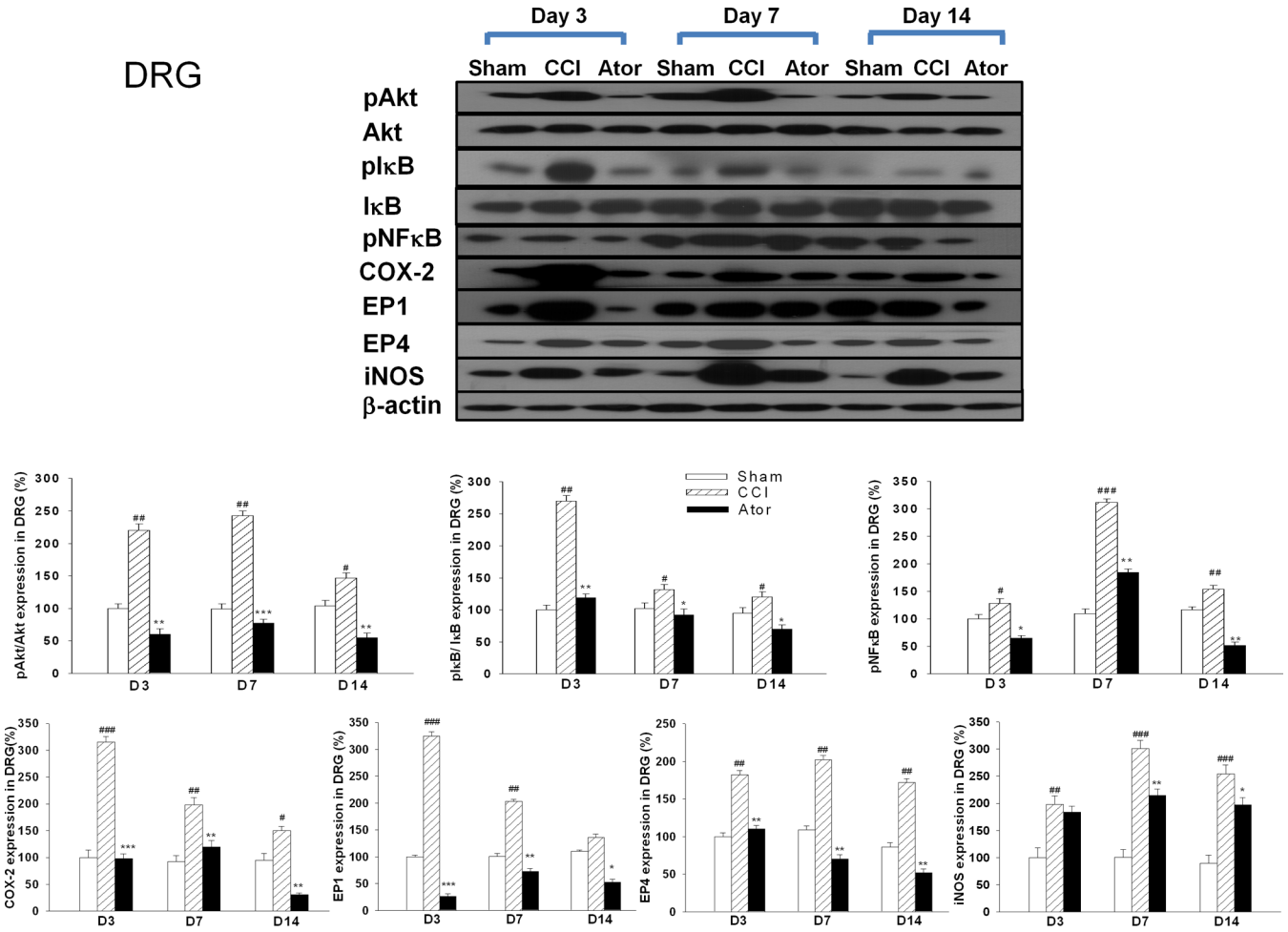

Figure 4. Effects of atorvastatin (Ator, 10 mg/kg/day, p.o.) on the expression of pAkt/Akt, pIκB/IκB, pNFκB, COX-2, iNOS, EP1, and EP4 proteins in dorsal root ganglion (DRG) induced by CCI. pNFκB protein expression was determined from the DRG nuclear portion. Upper panel: Western blots for pAkt/Akt, pIκB, IκB, pNFκB, COX-2, iNOS, EP1, and EP4 proteins from DRG. Bar charts show the relative density of pAkt/Akt, pIκB/IκB, pNFκB, COX-2, EP1, EP4, and iNOS proteins in sham, CCI, and Ator-treated rats at days 3, 7, and 14. Band intensity was quantified by densitometry and indicates the percentage change relative to the sham group (100%). Data represent the mean ± SE for 6 rats per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CCI group.

suggesting that it could inhibit inflammatory or neuropathic pain from peripheral nerve injury.

Akt, a serine/threonine-specific protein kinase, is also known as protein kinase B (PKB) and plays a key role in DRG neuron cell survival.[39] Small- to medium-sized DRG neurons express pAkt, with upregulation after intraplantar injection of capsaicin or electrical stimulation.[40] Phosphorylation of Akt is induced by tumor necrosis factor α (TNF-α) receptor-dependent and independent mechanisms.[41] In addition, the involvement of the PI3K/Akt pathway in the expression of inflammatory mediators in microglia through the activation of NF-κB has been reported.[42] PGE2 exerts its effect by acting via 4 subtypes of the PGE receptor, EP1 to EP4.[43] EP receptor antagonists were shown to reduce hyperalgesia and allodynia in rodents.[44] One report revealed that EP1 mRNA is expressed in rat DRG neurons.[45] DRG neuronal cells, Schwann cells and satellite cells are activated in response to inflammation.[46] Inflammatory mediators such as PGs, NO and substance P have been shown to enhance the sensitivity of primary afferents and spinal cord neurons.[47] Upregulation of spinal microglia induced by peripheral nerve injury results in central sensitization in lamina II neurons and induces thermal hyperalgesia behaviors.[10] In CCI rats, atorvastatin decreased the expression of inflammatory proteins (cytoplasmic pIκB/IκB, nuclear pNFκB, COX-2, iNOS, EP1, and EP4) in the DRG neurons and spinal cord. Double immunofluorescent staining further confirmed that pNFκB proteins were largely eliminated by atorvastatin treatment at day 7 after CCI. Results from Western blot and immunofluorescence support the animal behavioral findings, suggesting that atorvastatin had protective and/or therapeutic effects on CCI-induced hyperalgesia and allodynia.

It is generally accepted that spinal glial cell activation is both necessary and sufficient for inducing neuropathic pain.[48] Additionally, activated microglia and astrocytes are implicated in the initiation and maintenance of spinal nociceptive sensitization in neuropathic pain states.[49] A previous report demonstrated that cross-talk between spinal neurons and glial cells is the most important mechanism underlying the development of neuropathic pain.[50] Spinal glial activation has been confirmed in various animal neuropathic and inflammatory pain models.[51] Thus, inhibition of spinal glial activation is now another option for treating neuropathic pain. Microglia are a source of multiple cytokines including IL-1β, IL-6 and TNF-α, which contribute to different features of pathological pain.[52] Microglia also express COX-2 and iNOS, leading to the synthesis of PGs and NO and enhanced pain sensitivity.[53]

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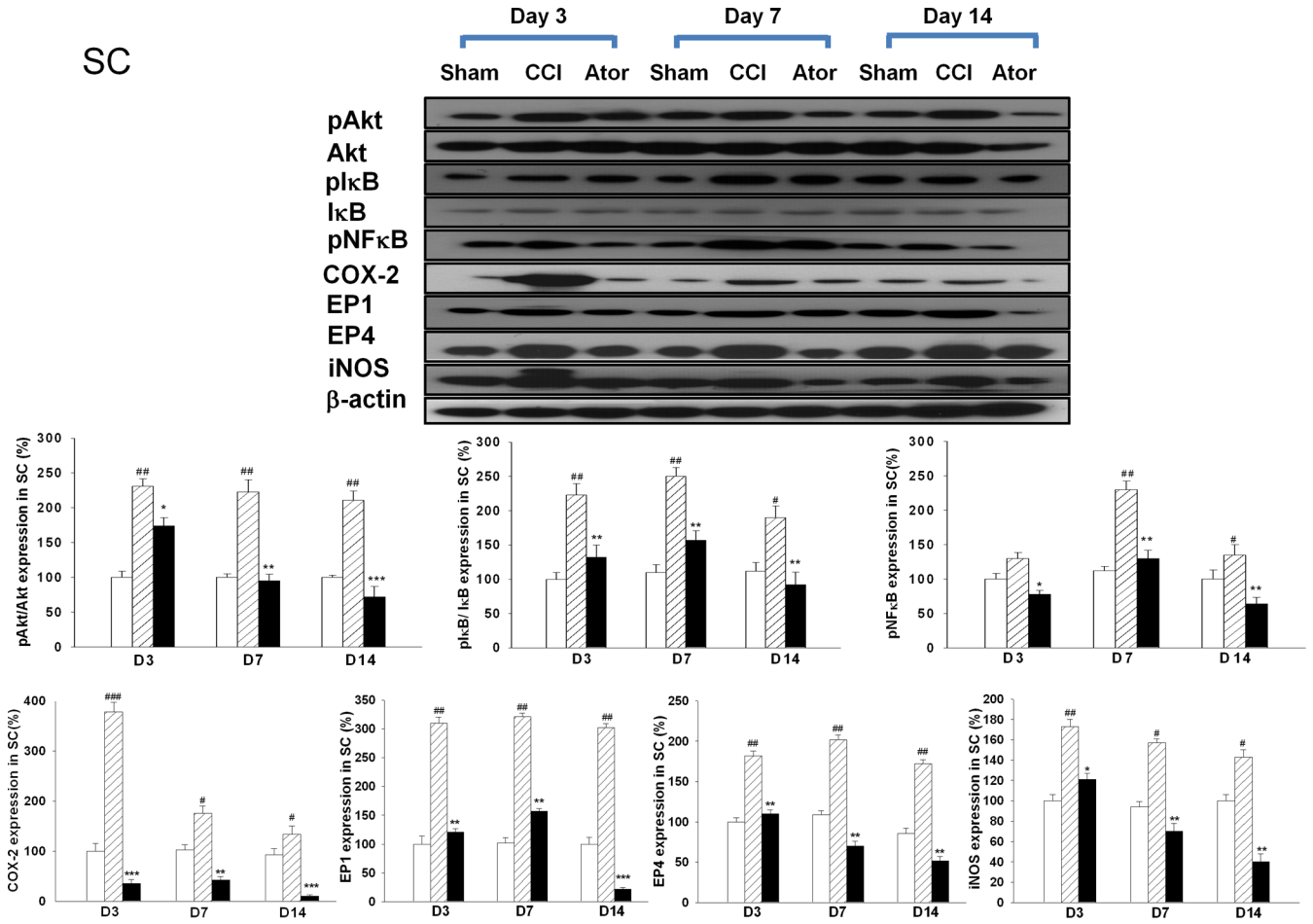

Figure 5. Effects of atorvastatin (Ator, 10 mg/kg/day, p.o.) on the expression of pAkt/Akt, pIκB/IκB, pNFκB, COX-2, iNOS, EP1, and EP4 proteins in spinal cord (SC) induced by CCI. pNFκB protein expression was determined from the SC nuclear portion. Upper panel: Western blots for pAkt/Akt, pIκB, IκB, pNFκB, COX-2, iNOS, EP1, and EP4 proteins from SC. Bar charts show the relative density of pAkt/Akt, pIκB/IκB, pNFκB, COX-2, EP1, EP4, and iNOS proteins in sham, CCI, and Ator-treated rats at days 3, 7, and 14. Band intensity was quantified by densitometry and indicates the percentage change relative to the sham group (100%). Data represent the mean ± SE for 6 rats per group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with CCI group.

Furthermore, activation of pNFκB by cytokines in microglia and astrocytes further facilitates the release of inflammatory cytokines.[54,55] In this study, we observed that atorvastatin inhibits spinal and DRG glial cell activation, and accordingly the NFκB activity is strikingly reduced. Our findings suggest that atorvastatin could be invaluable for preventing CCI-induced inflammatory and neuropathic pain.

CONCLUSIONS

Atorvastatin reduces proinflammatory cytokine release and inflammatory protein expression and blocks pNFκB activation in spinal microglia and DRG satellite cells, the predominant responses that contribute to preventing and/or treating neuroinflammation. As such, this study provides evidence that atorvastatin could be a good candidate for the control of peripheral nerve injury-induced neuropathic pain.

METHODS

Animals. Male Sprague–Dawley rats (n = 54, 250–300 g), provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan), were used in this study. Rats were adapted to the animal facility for a week. The humidity, temperature and light cycle were controlled, and food and water were provided ad libitum. This study was approved by the Animal Care and Use Committee of Kaohsiung Medical University.

Chronic Constriction Injury Surgery. Bilateral chronic constriction injury surgery was carried out as described previously.[9,21,56] Briefly, 54 rats were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (40 mg/kg) and the common sciatic nerve was exposed at the level of the middle thigh by blunt dissection through the biceps femoris muscle. Proximal to the sciatic trifurcation, approximately 7 mm of nerve was dissected and freed, and then three ligatures of 4–0 chromic catgut were placed around the sciatic nerve. This produced blanching of the nerve and a considerable reduction of blood flow in the sciatic nerve secondary to occlusion of the epineural vasculature. In sham-operated rats, the same surgical procedure was followed but no ligatures were placed.

Experimental Design. All rats were acclimated to the laboratory environment for at least 1 h before behavioral testing. Rats were fed with atorvastatin (10 mg/kg/day, Pfizer Inc., Freiburg, Germany) suspended in phosphate buffered saline (PBS) or PBS alone via oral gavage once daily[57] beginning on the next day following surgery for 14 days. Animals were sacrificed at day 3, day 7, and day 14 after surgery. The DRG and spinal dorsal horn neurons from each group were taken for further experiments. A dosage of 10 mg/kg/day atorvastatin was used based on our previous study[21] and that of Pathak and coworkers.[13,55] Pathak et al. demonstrated that a ceiling effect was found at a dose of 10 mg/kg atorvastatin with the 3 and 30 mg/kg doses being less effective in a rat CCI model.[13]

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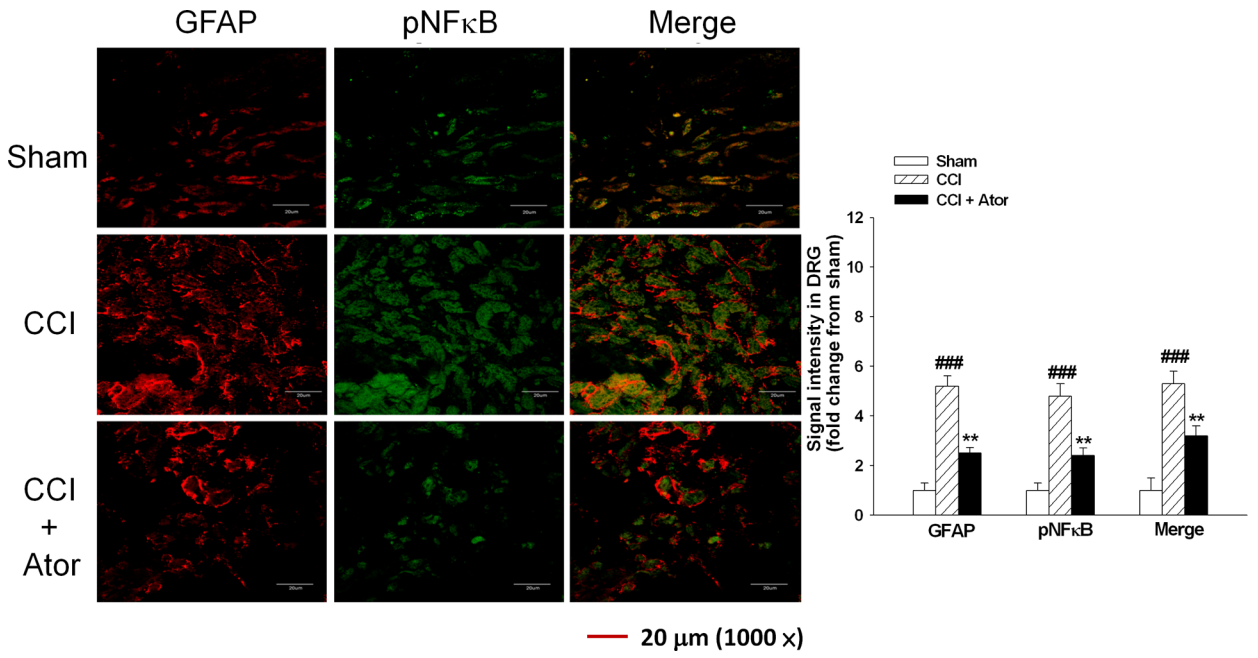

Figure 6. Double immunofluorescent staining for GFAP (DRG satellite cell marker) and pNFκB in the dorsal root ganglion (DRG) at day 7 after chronic constriction injury (CCI). Expression of pNFκB proteins was significantly increased in the CCI group compared with the sham and CCI +Ator group. Administration of atorvastatin (Ator, 10 mg/kg/day, p.o.) attenuated CCI-enhanced pNFκB proteins. Quantitative fluorescence data are depicted. Data represent the mean ± SE for 6 rats per group. $^{###}p < 0.001$ compared with sham group; $^{**}p < 0.01$ compared with CCI group. Scale bar, 20 μm; magnification, 1000×.

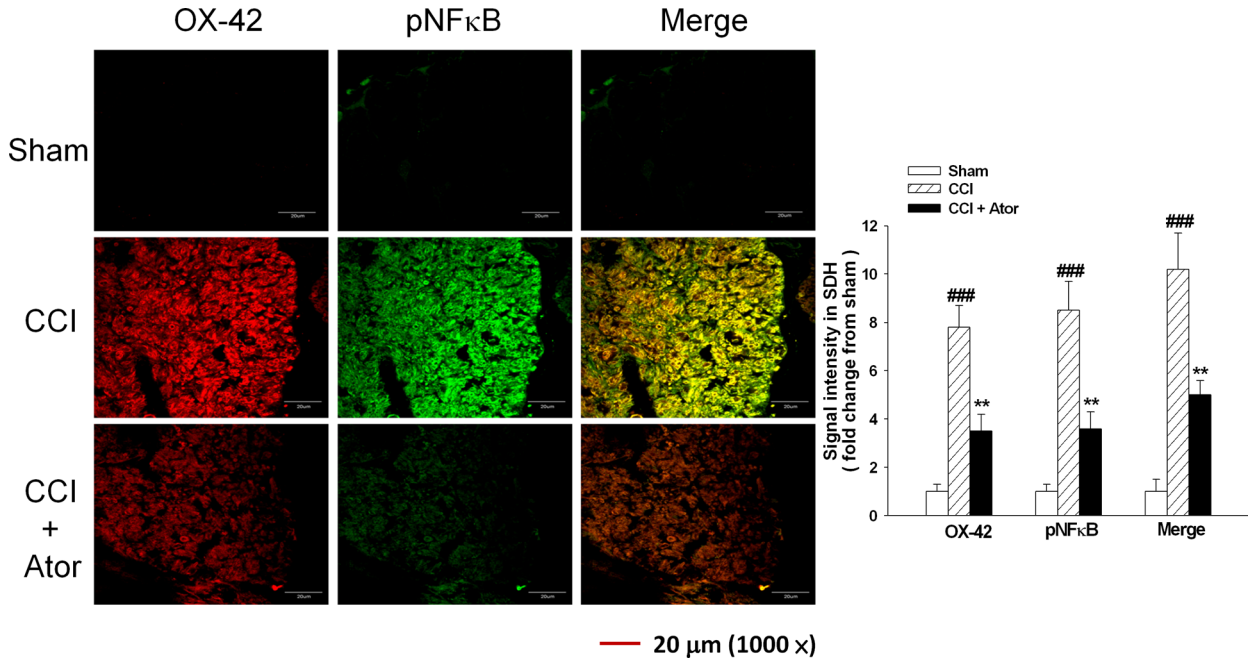

Figure 7. Double immunofluorescent staining for OX-42 (spinal microglia marker) and pNFκB in the central spinal dorsal horn (SDH) at day 7 after chronic constriction injury (CCI). Expression of pNFκB proteins was significantly increased in the CCI group compared with the sham and CCI +Ator group. Administration of atorvastatin (Ator, 10 mg/kg/day, p.o.) attenuated CCI-enhanced pNFκB proteins. Quantitative fluorescence data are depicted. Data represent the mean ± SE for 6 rats per group. $^{###}p < 0.001$ compared with sham group; $^{**}p < 0.01$ compared with CCI group. Scale bar, 20 μm; magnification, 1000×.

Assessment of Thermal Hyperalgesia and Mechanical Allodynia. Allodynia (exaggerated response to normally non-noxious stimuli) and hyperalgesia (decreased threshold to noxious stimuli) were evaluated in the sham, CCI, and CCI+atorvastatin groups. Paw withdrawal latency (PWL) was used to evaluate thermal hyperalgesia.[58] Thermal hyperalgesia was assessed by placing the hind paw on a radiant heat source and measuring the PWL under low-intensity heat set to a cutoff time of 30 s using an IITC analgesiometer (IITC Inc., Woodland Hills, CA). In addition, a series of calibrated von Frey filaments were used to measure the paw withdrawal threshold (PWT). Rats were housed in a wire mesh cage (18 × 25 × 18 cm). The mechanical allodynia threshold was defined as the minimal force (*g*) eliciting a withdrawal response. The average force evoking reliable withdrawals was recorded as PWT.[59]

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ELISA Analysis of TNF-α and IL-1β. Commercially available Quantikine ELISA kits were employed to quantify the TNF-α and IL-1β concentrations spectrophotometrically following the manufacturer's instructions (R&D Systems, Minneapolis, MN). At different time points (days 3 and 7) the spinal cord and DRG were removed, homogenized in lysis buffer with protease inhibitors, and centrifuged to separate the insoluble pellet from the supernatant. The Bradford protein assay was used to determine total protein concentration in the supernatant employing the sandwich enzyme immunoassay technique. The intensity of color measurement was proportional to the amount of TNF-α and IL-1β. Consequently, the sample values could be read off the standard curve in the sandwich ELISA formats.

Western Blot Analysis and Protein Extraction. All specimens extracted at different time points were frozen in liquid nitrogen and stored at −80 °C. Each sample was lysed in ice-cold (4 °C) lysis buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100, 0.2 mM EDTA, 10 mM NaF, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 0.4 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride, and 1 mM sodium orthovanadate) and then centrifuged at 20,000 g for 30 min at 4 °C. For Western blot analysis an equal volume of sample buffer (2% sodium dodecylsulfate (SDS), 10% glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol and 50 mM Tris-HCL, pH 7.2) was added to the sample, which was then loaded onto 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Temecula, CA). The membrane was blocked with 5% milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h and then incubated with Akt (1:1000 dilution; Cell Signaling Technology, Danvers, MA), phosphate Akt (pAkt) (Ser473, 1:500 dilution; Cell Signaling Technology, Danvers, MA), pNFκB (1:500 dilution; Millipore, Temecula, CA), IκB, iNOS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-IκB (pIκB), COX-2 (1:1000 dilution; Abcam, London, UK), EP1, EP4 (1:500 dilution; Cayman, Ann Arbor, MI), and β-actin (1:2000 dilution; Sigma-Aldrich, Saint Louis, MO). After the secondary antibody was conjugated with HRP (1:5000 dilutions in 5% milk) for 1 h, the signals on the membrane were identified using ECL-plus luminal solution and exposed to X-ray film for autoradiography.

Immunofluorescent Staining. Animals were perfused with 4% paraformaldehyde in 0.1 M PBS. Spinal cord and DRG were removed and postfixed overnight. Tissues were cryoprotected in 0.1 M PBS containing 30% sucrose and cryostat cut into 15 μm thick sections. The sections were blocked for 1 h at 22 °C in 0.1 M PBS containing BSA and 0.1% Triton-X before overnight incubation at 4 °C with the primary antibodies anti-GFAP (DRG satellite cells marker, Millipore, Temecula, CA), anti-OX-42 (spinal microglia marker, Millipore, Temecula, CA), and anti-pNFκB (Millipore, Temecula, CA), followed by secondary species-specific fluorescent antibodies (Jackson ImmunoResearch Lab, Inc., West Grove, PA) for 1 h at 22 °C. Images were viewed on a Zeiss Axiovert 200 M inverted microscope and captured on a Zeiss LSM 510 confocal microscope (Jena, Germany). Quantitative analyses of fluorescent images were performed using Zeiss AxioVision 4.8 software.

Statistical Analyses. All data were expressed as the mean ± SE for six animals per group. The behavior data and the Western blot results were analyzed by one-way analysis of variance (ANOVA). When suitable, a Tukey-Kramer pairwise comparison was used for post hoc analysis. $P < 0.05$ was considered statistically significant.

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Notes

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ABBREVIATIONS

pAkt, phospho-Akt; CCI, chronic constriction injury; COX-2, cyclooxygenase-2; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; pIκB, phospho-IκB; IL-1β, interleukin-1β; pNFκB, phospho-nuclear factor κB; NO, nitric oxide; iNOS, inducible nitric oxide synthase; PGE2, prostaglandin E2; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α

REFERENCES

(1) Woolf, C. J., and Mannion, R. J. (1999) Neuropathic pain: Aetiology, symptoms, mechanisms, and management. *Lancet 353*, 1959−1964.

(2) Jensen, T. S., Gottrup, H., Sindrup, S. H., and Bach, F. W. (2001) The clinical picture of neuropathic pain. *Eur. J. Pharmacol. 429*, 1−11.

(3) Woolf, C. J., and Salter, M. W. (2000) Neuronal plasticity: Increasing the gain in pain. *Science 288*, 1765−1769.

(4) Hains, B. C., Saab, C. Y., Klein, J. P., Craner, M. J., and Waxman, S. G. (2004) Altered sodium channel expression in second-order spinal sensory neurons contributes to pain after peripheral nerve injury. *J. Neurosci. 24*, 4832−4839.

(5) Ledeboer, A., Sloane, E. M., Milligan, E. D., Frank, M. G., Mahony, J. H., Maier, S. F., and Watkins, L. R. (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain 115*, 71−83.

(6) Hanisch, U. K., and Kettenmann, H. (2007) Microglia: Active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci 10*, 1387−1394.

(7) Mildner, A., Schmidt, H., Nitsche, M., Merkler, D., Hanisch, U. K., Mack, M., Heikenwalder, M., Bruck, W., Priller, J., and Prinz, M. (2007) Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat. Neurosci 10*, 1544−1553.

(8) Li, S. S., Zhang, W. S., Ji, D., Zhou, Y. L., Li, H., Yang, J. L., Xiong, Y. C., Zhang, Y. Q., and Xu, H. (2014) Involvement of spinal microglia and interleukin-18 in the anti-nociceptive effect of dexmedetomidine in rats subjected to CCI. *Neurosci. Lett. 560*, 21−25.

(9) Dai, Z. K., Lin, T. C., Liou, J. C., Cheng, K. I., Chen, J. Y., Chu, L. W., Chen, I. J., and Wu, B. N. (2014) Xanthine derivative KMUP-1 reduces inflammation and hyperalgesia in a bilateral chronic constriction injury model by suppressing MAPK and NFkappaB activation. *Mol. Pharmaceutics 11*, 1621−1631.

DOI: 10.1021/acschemneuro.5b00032
ACS Chem. Neurosci. 2015, 6, 889−898

(10) Huang, C. Y., Chen, Y. L., Li, A. H., Lu, J. C., and Wang, H. L. (2014) Minocycline, a microglial inhibitor, blocks spinal CCL2-induced heat hyperalgesia and augmentation of glutamatergic transmission in substantia gelatinosa neurons. *J. Neuroinflammation 11*, 7.
(11) Watkins, L. R., and Maier, S. F. (2003) Glia: A novel drug discovery target for clinical pain. *Nat. Rev. Drug Discovery 2*, 973−985.
(12) McTaggart, F., Buckett, L., Davidson, R., Holdgate, G., McCormick, A., Schneck, D., Smith, G., and Warwick, M. (2001) Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. *Am. J. Cardiol 87*, 28B−32B.
(13) Pathak, N. N., Balaganur, V., Lingaraju, M. C., Kant, V., Latief, N., More, A. S., Kumar, D., Kumar, D., and Tandan, S. K. (2014) Atorvastatin attenuates neuropathic pain in rat neuropathy model by down-regulating oxidative damage at peripheral, spinal and supraspinal levels. *Neurochem. Int. 68*, 1−9.
(14) Zacco, A., Togo, J., Spence, K., Ellis, A., Lloyd, D., Furlong, S., and Piser, T. (2003) 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors protect cortical neurons from excitotoxicity. *J. Neurosci. 23*, 11104−11111.
(15) Michikawa, M., and Yanagisawa, K. (1999) Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. *J. Neurochem. 72*, 2278−2285.
(16) Chen, J., Zhang, Z. G., Li, Y., Wang, Y., Wang, L., Jiang, H., Zhang, C., Lu, M., Katakowski, M., Feldkamp, C. S., and Chopp, M. (2003) Statins induce angiogenesis, neurogenesis, and synaptogenesis after stroke. *Ann. Neurol. 53*, 743−751.
(17) Chen, J., Zhang, C., Jiang, H., Li, Y., Zhang, L., Robin, A., Katakowski, M., Lu, M., and Chopp, M. (2005) Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J. Cereb. Blood Flow Metab. 25*, 281−290.
(18) Lu, D., Goussev, A., Chen, J., Pannu, P., Li, Y., Mahmood, A., and Chopp, M. (2004) Atorvastatin reduces neurological deficit and increases synaptogenesis, angiogenesis, and neuronal survival in rats subjected to traumatic brain injury. *J. Neurotrauma 21*, 21−32.
(19) Reis, P. A., Estato, V., da Silva, T. I., d'Avila, J. C., Siqueira, L. D., Assis, E. F., Bozza, P. T., Bozza, F. A., Tibirica, E. V., Zimmerman, G. A., and Castro-Faria-Neto, H. C. (2012) Statins decrease neuro-inflammation and prevent cognitive impairment after cerebral malaria. *PLoS Pathog 8*, e1003099.
(20) Saarto, T., and Wiffen, P. J. (2005) Antidepressants for neuropathic pain. *Cochrane Database Syst. Rev.*, CD005454.
(21) Chu, L. W., Chen, J. Y., Yu, K. L., Cheng, K. I., Wu, P. C., and Wu, B. N. (2012) Neuroprotective and anti-inflammatory activities of atorvastatin in a rat chronic constriction injury model. *Int. J. Immunopathol. Pharmacol. 25*, 219−230.
(22) Gillon, J. T., Smith, S. E., and Lowden, M. R. (2013) Atorvastatin as novel treatment for neuropathic pain: A case report. *Clin. J. Pain 29*, e46−48.
(23) Suzuki, R., and Dickenson, A. H. (2000) Neuropathic pain: Nerves bursting with excitement. *NeuroReport 11*, R17−21.
(24) Zimmermann, M. (2001) Pathobiology of neuropathic pain. *Eur. J. Pharmacol. 429*, 23−37.
(25) Zhou, C., Shi, X., Huang, H., Zhu, Y., and Wu, Y. (2014) Montelukast attenuates neuropathic pain through inhibiting p38 mitogen-activated protein kinase and nuclear factor-kappa B in a rat model of chronic constriction injury. *Anesth. Analg. 118*, 1090−1096.
(26) Khan, J., Ramadan, K., Korczeniewska, O., Anwer, M. M., Benoliel, R., and Eliav, E. (2015) Interleukin-10 levels in rat models of nerve damage and neuropathic pain. *Neurosci. Lett. 592*, 99−106.
(27) Piermartiri, T. C., Figueiredo, C. P., Rial, D., Duarte, F. S., Bezerra, S. C., Mancini, G., de Bem, A. F., Prediger, R. D., and Tasca, C. I. (2010) Atorvastatin prevents hippocampal cell death, neuro-inflammation and oxidative stress following amyloid-beta(1−40) administration in mice: evidence for dissociation between cognitive deficits and neuronal damage. *Exp. Neurol. 226*, 274−284.
(28) Castro, A. A., Wiemes, B. P., Matheus, F. C., Lapa, F. R., Viola, G. G., Santos, A. R., Tasca, C. I., and Prediger, R. D. (2013) Atorvastatin improves cognitive, emotional and motor impairments induced by intranasal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration in rats, an experimental model of Parkinson's disease. *Brain Res. 1513*, 103−116.
(29) Reagan-Shaw, S., Nihal, M., and Ahmad, N. (2008) Dose translation from animal to human studies revisited. *FASEB J. 22*, 659−661.
(30) Chen, N. F., Huang, S. Y., Lu, C. H., Chen, C. L., Feng, C. W., Chen, C. H., Hung, H. C., Lin, Y. Y., Sung, P. J., Sung, C. S., Yang, S. N., Wang, H. M., Chang, Y. C., Sheu, J. H., Chen, W. F., and Wen, Z. H. (2014) Flexibilide obtained from cultured soft coral has anti-neuroinflammatory and analgesic effects through the upregulation of spinal transforming growth factor-beta1 in neuropathic rats. *Mar. Drugs 12*, 3792−3817.
(31) Bian, D., Ossipov, M. H., Zhong, C., Malan, T. P., Jr., and Porreca, F. (1998) Tactile allodynia, but not thermal hyperalgesia, of the hindlimbs is blocked by spinal transection in rats with nerve injury. *Neurosci. Lett. 241*, 79−82.
(32) Campbell, J. N., and Meyer, R. A. (2006) Mechanisms of neuropathic pain. *Neuron 52*, 77−92.
(33) He, Z. W., Wei, W., Li, S. P., Ling, Q., Liao, K. J., and Wang, X. (2014) Anti-allodynic effects of obtusifolin and gluco-obtusifolin against inflammatory and neuropathic pain: Possible mechanism for neuroinflammation. *Biol. Pharm. Bull. 37*, 1606−1616.
(34) Mei, X. P., Sakuma, Y., Xie, C., Wu, D., Ho, I., Kotani, J., and Xu, L. X. (2013) Depressing Interleukin-1beta Contributed to the Synergistic Effects of Tramadol and Minocycline on Spinal Nerve Ligation-Induced Neuropathic Pain. *Neurosignals 22*, 30−42.
(35) Zhou, X., Cheng, H., Xu, D., Yin, Q., Cheng, L., Wang, L., Song, S., and Zhang, M. (2014) Attenuation of Neuropathic Pain by Saikosaponin a in a Rat Model of Chronic Constriction Injury. *Neurochem. Res. 39*, 2136−2142.
(36) Nathan, C. (2002) Points of control in inflammation. *Nature 420*, 846−852.
(37) Moalem, G., and Tracey, D. J. (2006) Immune and inflammatory mechanisms in neuropathic pain. *Brain Res. Rev. 51*, 240−264.
(38) Pan, H. C., Yang, D. Y., Ou, Y. C., Ho, S. P., Cheng, F. C., and Chen, C. J. (2010) Neuroprotective effect of atorvastatin in an experimental model of nerve crush injury. *Neurosurgery 67*, 376−388 discussion 388−379..
(39) Lawlor, M. A., and Alessi, D. R. (2001) PKB/Akt: A key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci. 114*, 2903−2910.
(40) Pezet, S., Spyropoulos, A., Williams, R. J., and McMahon, S. B. (2005) Activity-dependent phosphorylation of Akt/PKB in adult DRG neurons. *Eur. J. Neurosci 21*, 1785−1797.
(41) Toth, C., Martinez, J. A., Liu, W. Q., Diggle, J., Guo, G. F., Ramji, N., Mi, R., Hoke, A., and Zochodne, D. W. (2008) Local erythropoietin signaling enhances regeneration in peripheral axons. *Neuroscience 154*, 767−783.
(42) Lee, J. Y., Jhun, B. S., Oh, Y. T., Lee, J. H., Choe, W., Baik, H. H., Ha, J., Yoon, K. S., Kim, S. S., and Kang, I. (2006) Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of PI 3-kinase/Akt and NF-kappaB activation in murine BV2 microglial cells. *Neurosci. Lett. 396*, 1−6.
(43) Narumiya, S., and FitzGerald, G. A. (2001) Genetic and pharmacological analysis of prostanoid receptor function. *J. Clin. Invest. 108*, 25−30.
(44) Kawahara, H., Sakamoto, A., Takeda, S., Onodera, H., Imaki, J., and Ogawa, R. (2001) A prostaglandin E2 receptor subtype EP1 receptor antagonist (ONO-8711) reduces hyperalgesia, allodynia, and c-fos gene expression in rats with chronic nerve constriction. *Anesth. Analg. 93*, 1012−1017.
(45) Ng, K. Y., Wong, Y. H., and Wise, H. (2011) Glial cells isolated from dorsal root ganglia express prostaglandin E(2) (EP(4)) and prostacyclin (IP) receptors. *Eur. J. Pharmacol. 661*, 42−48.

DOI: 10.1021/acschemneuro.5b00032
ACS Chem. Neurosci. 2015, 6, 889−898

(46) Siemionow, K., Klimczak, A., Brzezicki, G., Siemionow, M., and McLain, R. F. (2009) The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion. *Spine (Philadelphia) 34*, 1631−1637.
(47) Durrenberger, P. F., Facer, P., Casula, M. A., Yiangou, Y., Gray, R. A., Chessell, I. P., Day, N. C., Collins, S. D., Bingham, S., Wilson, A. W., Elliot, D., Birch, R., and Anand, P. (2006) Prostanoid receptor EP1 and Cox-2 in injured human nerves and a rat model of nerve injury: A time-course study. *BMC Neurol. 6*, 1.
(48) Scholz, J., and Woolf, C. J. (2007) The neuropathic pain triad: Neurons, immune cells and glia. *Nat. Neurosci 10*, 1361−1368.
(49) Raghavendra, V., Tanga, F., and DeLeo, J. A. (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J. Pharmacol. Exp. Ther. 306*, 624−630.
(50) Austin, P. J., and Moalem-Taylor, G. (2010) The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. *J. Neuroimmunol. 229*, 26−50.
(51) Old, E. A., and Malcangio, M. (2012) Chemokine mediated neuron-glia communication and aberrant signalling in neuropathic pain states. *Curr. Opin. Pharmacol. 12*, 67−73.
(52) Perry, V. H., Nicoll, J. A., and Holmes, C. (2010) Microglia in neurodegenerative disease. *Nat. Rev. Neurol. 6*, 193−201.
(53) Han, H. E., Kim, T. K., Son, H. J., Park, W. J., and Han, P. L. (2013) Activation of Autophagy Pathway Suppresses the Expression of iNOS, IL6 and Cell Death of LPS-Stimulated Microglia Cells. *Biomol. Ther. (Seoul) 21*, 21−28.
(54) D'Agostino, G., La Rana, G., Russo, R., Sasso, O., Iacono, A., Esposito, E., Mattace Raso, G., Cuzzocrea, S., Loverme, J., Piomelli, D., Meli, R., and Calignano, A. (2009) Central administration of palmitoylethanolamide reduces hyperalgesia in mice via inhibition of NF-kappaB nuclear signalling in dorsal root ganglia. *Eur. J. Pharmacol. 613*, 54−59.
(55) Pathak, N. N., Balaganur, V., Lingaraju, M. C., More, A. S., Kant, V., Kumar, D., Kumar, D., and Tandan, S. K. (2013) Antihyperalgesic and anti-inflammatory effects of atorvastatin in chronic constriction injury-induced neuropathic pain in rats. *Inflammation 36*, 1468−1478.
(56) Bennett, G. J., and Xie, Y. K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain 33*, 87−107.
(57) Ye, Y., Martinez, J. D., Perez-Polo, R. J., Lin, Y., Uretsky, B. F., and Birnbaum, Y. (2008) The role of eNOS, iNOS, and NF-kappaB in upregulation and activation of cyclooxygenase-2 and infarct size reduction by atorvastatin. *Am. J. Physiol.: Heart Circ. Physiol. 295*, H343−351.
(58) Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain 32*, 77−88.
(59) Hatashita, S., Sekiguchi, M., Kobayashi, H., Konno, S., and Kikuchi, S. (2008) Contralateral neuropathic pain and neuropathology in dorsal root ganglion and spinal cord following hemilateral nerve injury in rats. *Spine (Philadelphia) 33*, 1344−1351.

DOI: 10.1021/acschemneuro.5b00032
ACS Chem. Neurosci. 2015, 6, 889−898