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Wnt/β-catenin signaling regulates brain-derived neurotrophic factor release from spinal microglia to mediate HIV_1 gp120-induced neuropathic pain

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Abstract

HIV-associated neuropathic pain (HNP) is a common complication for AIDS patients. The pathological mechanism governing HNP has not been elucidated, and HNP has no effective analgesic treatment. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family related to the plasticity of the central nervous system. BDNF dysregulation is involved in many neurological diseases, including neuropathic pain. However, to the best of our knowledge, the role and mechanism of BDNF in HNP have not been elucidated. In this study, we explored this condition in an HNP mouse model induced by intrathecal injection of gp120. We found that Wnt3a and β-catenin expression levels increased in the spinal cord of HNP mice, consequently regulating the expression of BDNF and affecting hypersensitivity. In addition, the blockade of Wing-Int/β-catenin signaling, BDNF/TrkB or the BDNF/p75NTR pathway alleviated mechanical allodynia. BDNF immunoreactivity was colocalized with spinal microglial cells, which were activated in HNP mice. Inhibition of spinal microglial cell activation by minocycline relieved mechanical allodynia in HNP mice. This study helped to elucidate the role of the Wing-Int/β-catenin/BDNF signaling axis in HNP and may establish a foundation for further research investigating the Wing-Int/β-catenin/BDNF signaling axis as a target for HNP treatment.

Keywords

Wing-Int, spinal cord, brain-derived neurotrophic factor, microglia, HIV-associated neuropathic pain

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Introduction

In 2018, in an estimated 37.9 million HIV/AIDS cases reported by the World Health Organization (https://www.who.int/gho/hiv/en/), up to 90% of the patients experienced pain without effective analgesic treatment.[1] HIV can infect the nervous system of patients, causing neurological dysfunction.[2,3] HIV-associated neuropathic pain (HNP) is a major complication for HIV-1/AIDS patients,[4–6] subjecting patients to suffering and a reduction in quality of life.[7] HNP has been attributed to the combined effect of viral products (e.g. gp120, Tat) and host cell-derived factors (e.g. proinflammatory cytokines).[8] Accumulating evidence suggests that an alteration in the expression of some neurotrophic factors contributes to HNP disease.[9,10] However, the molecular mechanisms by which these neurotrophic factors lead to these features have not been elucidated.

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic factor family and plays a crucial function in the survival and differentiation of

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nerve cells.[11,12] BDNF is divided into protype (proBDNF) and mature (mBDNF) to exert different effects on different receptors. In addition, dysregulation of BDNF is associated with a variety of neurological disorders, including neuropathic pain (NP).[13–15] Coull et al.[16] reported that the activation of spinal microglia and the release of BDNF caused pain in peripheral nerve injury. Despite this nerve injury, spinally released BDNF is essential to neuroplasticity and NP. BDNF in the central nervous system also plays a key role in mediating the transition from acute pain to chronic pain.[17] The possibility that BDNF may be an important neurotrophic factor involved in HNP has not been confirmed to date. Autopsy results showed increased expression of BDNF in brain tissues of patients with neuroAIDS, which is caused by HIV_1 gp120,[18] suggesting that BDNF is correlated with HNP. In addition, the spinal cord is the primary center for the transmission and integration of pain information. However, studies investigating changes in the expression of BDNF in the spinal cords of HNP patients or animals and the effects of these changes have not been reported. Therefore, an investigation into the spinal BDNF profile and its role in HNP is strongly warranted.

HIV_1 gp120 is the common toxic protein used to study HNP.[6,19] Intrathecal injection (i.t.) of gp120 in mice is a classic animal model for studying HNP.[20–26] Our previous in vitro studies showed that HIV_1 gp120 activates mouse microglia (BV2) and upregulates BDNF expression via the Wing-Int (Wnt)/β-catenin signaling pathway.[10] The microglia are directly related to pain.[10,16] Therefore, these results indicate that the Wnt/β-catenin/BDNF signal axis plays an important role in the development of HNP lesions.

This article seeks to determine the role and mechanism of the spinal Wnt/β-catenin/BDNF signaling axis in the gp120-induced HNP model. We found that the Wnt/β-catenin signaling pathway upregulates the production of BDNF in spinal microglia and that spinal BDNF subsequently interacts with TrkB and p75NTR receptors to affect mechanical allodynia in HNP mice. These findings may help to elucidate the mechanism of HNP and establish a foundation for further research identifying drug targets for the effective treatment of HNP.

Materials and Methods

Animals

All animals were adult ICR mice (7 weeks of age and weighing 22 ± 2 g) purchased from Shanghai Slake Laboratory Animals Co., Ltd. All procedures used in this study were approved by the Zhejiang Sci-Tech University Animal Experimental Ethics Committee. Animals were housed in cages (3–5 mice per cage) and given free access to rodent chow and water ad libitum in a room maintained at 24°C and a 12 h/12 h light–dark cycle.

Drug administration

HIV_1 gp120 (Bal) (HIV_1/Clade B) (IT-001-002p) was purchased from Immune Technology Corp (USA). gp120 purified protein was reconstituted at 1 mg/mL in sterile filtered PBS (0.22-μm pore filters) only and aliquoted on ice (5 μL/tube) and stored at −80°C. Before usage, HIV_1 gp120 was immediately diluted to a final concentration of 20 ng/μL in PBS (100 ng/animal).[24,26]

Recombinant human BDNF (Bachem: 4038290, 5 ng/animal), recombinant mouse Wnt3a (R&D: 1324-WN-010/CF, 100 ng/animal), recombinant mouse DKK1 (R&D: 5897-DK-010, 1 μg/animal), recombinant human TrkB-Fc (R&D: 688-TK-100, 250 ng/animal), or TAT-Pep5 (Sigma: 506181, 40 μg/animal) was first dissolved as a concentrated stock solution in 0.01 M PBS. IWR-1 (Sigma: I0161, 50 μM/animal) or minocycline (Sigma: M9511, 50 μg/animal) were dissolved in DMSO or in 0.9% sterile physiological saline.[27,28] All the drugs were aliquoted in small volumes and stored at −80°C. Drugs were immediately diluted with PBS or 0.9% saline to reach final concentrations before administration.

Antibodies

The antibodies used for immunofluorescence staining were mouse anti-BDNF (1:300, Santa Cruz Biotech# sc-65514), rabbit anti-GFAP (1:300, Abcam#ab7260), rabbit anti-Iba1 (1:300, Abcam#ab178846), rabbit anti-NeuN (1:400, Abcam#ab177487), goat anti-rabbit IgG H&L(Cy3) (1:200, Abcam#ab6939), and goat anti-mouse IgG H&L (FITC) (1:100, Abcam#ab6785). The antibodies used for Western blotting (WB) were rabbit anti-BDNF (1:1000, Santa Cruz Biotech#sc-546), mouse anti-Wnt3a (1:1000, R&D Systems#MAB1324-050), mouse anti-β-catenin (1:5000, BD#610153), mouse anti-tubulin (1:10,000, proteintech#HRP-66031), rabbit anti-GFAP (1:1000, Abcam#ab7260), rabbit anti-Iba1 (1:1000, Abcam#ab178846), rabbit anti-NeuN (1:5000, Abcam#ab177487), rabbit anti-TrkB (1:1000,CST# 80E3), rabbit anti-p75 NGF (1:5000, Abcam#ab52987), goat anti-sortilin (1:1500,R&D Systems#AF2934), goat anti-rabbit IgG H&L (HRP) (1:30,000, Abcam# ab97051), goat anti-mouse IgG H&L (HRP) (1:30,000, Abcam#ab97023), and donkey anti-goat IgG H&L (HRP) (1:10,000,Abcam#ab6885).

Intrathecal injection (i.t.)

ICR mice were anesthetized with 2.5% isoflurane for induction and 1% for maintenance. After trimming the

fur on the lower back of the mouse, the skin was sterilized with 75% alcohol. A 25-μL microsyringe (Shanghai Gaoge Industry and Trade Co., Ltd.) was inserted into the L5–L6 intervertebral space. A sudden tail twist or leg kick was used as an indicator of successful penetration of the needle tip into the vertebral canal at the cauda equina level. The drug was slowly delivered for 15 s, and the needle was left in situ for an additional 15 s before withdrawal.[6,24]

Von Frey filament test

Mice were first habituated in a $13.2 \times 5 \times 4$ cm Plexiglas box for three continuous days prior to behavioral testing. On the testing day, mice were acclimatized for 30–40 min in individual Plexiglas compartments with a wire mesh bottom. A 4.08 filament was used in the initial measurement. A test was performed by touching the center area of the hind paws perpendicular to the filament with an appropriate force until the filament was slightly bent and remained bent for 2 s. Based on the response to the initial filament (4.08/1.0 g), the next larger filament was used in the next test in response to the withdrawal of its paws. The next smaller filament was used in the next test in case the animal withdrew its paw in response to the initial filament. This process was continued until the animal's pain threshold was determined. Signs of a noxious withdrawal response included abrupt withdrawal, lateral moving, shaking, lifting, licking, or biting of the touched area of the hind paw.[6]

Western blotting

Mice were anesthetized and transcardially perfused with cold PBS. The L4–L6 lumbar spinal cord segments were collected for WB. The total amount of protein was extracted from the tissue samples by cell lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl pH 7.4, 1% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA pH 8.0) containing the protease inhibitor PMSF (Sigma) on ice. After centrifugation (12,000 r/min, 10 min), the supernatant was collected, and the protein concentration was detected using the BCA Protein Assay Kit (Beyotime). Equal amounts of protein were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis at 80 V for 30 min followed by 120 V for 90 min. Then, the proteins were transferred onto a PVDF membrane at 4°C by 100 V for 90 min. The membranes were blocked with 5% skim milk for 120 min and then incubated overnight with the primary antibodies at 4°C. The membranes were then washed using Tris buffered saline supplemented with Tween-20 (TBST) 3×10 min followed by incubation with the secondary antibodies (conjugated with horseradish peroxidase) for 1 h at room temperature. Then, the membrane was washed with TBST three times for 10 min each time and developed with enhanced chemiluminescence, and WB images were measured using ImageJ software.

Immunohistochemistry

The L4–L6 lumbar spinal cord segments were collected for immunohistochemistry analysis and postfixed for 2 h at 4°C in 4% paraformaldehyde. The paraffin-embedded sections were dewaxed, and antigenic heat was repaired. The sections were first blocked with 0.3% Triton X-100 in 10% goat serum for 1 h at 20–25°C. The sections were then incubated overnight at 4°C with the primary antibodies. After incubation, the sections were washed three times for 15 min in TBST and incubated for 2 h at approximately 20–25°C with the corresponding secondary antibodies. Next, the sections were again washed three times for 15 min in TBST. The immunofluorescence images were captured with a confocal scanning laser microscope (Model number: FV1200, Manufacturer: Nikon).

Statistical analysis

All statistical analyses were carried out using Prism7 (GraphPad) software and are presented as the mean ± SEM. We used Student's t test, one-way analysis of variance (ANOVA), or two-way ANOVA to evaluate the significant differences in the group data in all the experiments; $p < 0.05$ was considered to be significant.

Results

I.t. of gp120 activates spinal Wnt/β-catenin signaling and upregulates spinal BDNF expression

Previous work has established that i.t. of HIV_1 gp120 protein induces hyperalgesia and mechanical allodynia in animals.[29,30] Indeed, in this study, following gp120 administration, mice showed a progressive decrease in mechanical withdrawal threshold tested by von Frey filaments (Figure 1(a)). Mechanical allodynia was observed at 1 h (***$p < 0.001$; gp120 group vs. vehicle group; two-way ANOVA; $n = 6$) after gp120 injection, peaked at 2 h, and lasted for more than 6 h. Therefore, the HNP animal model was successfully established by i.t. gp120.

In this study, the expression profiles of Wnt3a, β-catenin and BDNF were analyzed in the mouse spinal cord at different time points (0 h, 1 h, 2 h, 4 h, 6 h) after i.t. of HIV_1 gp120 protein. The expression of Wnt3a (*$p < 0.05$; gp120 group vs. vehicle group; one-way ANOVA; $n = 3$), β-catenin (**$p < 0.01$, ***$p < 0.001$; gp120 group vs. vehicle group; one-way ANOVA; $n = 3$), and BDNF (*$p < 0.05$, **$p < 0.01$; gp120 group vs. vehicle group; one-way ANOVA; $n = 3$) in the

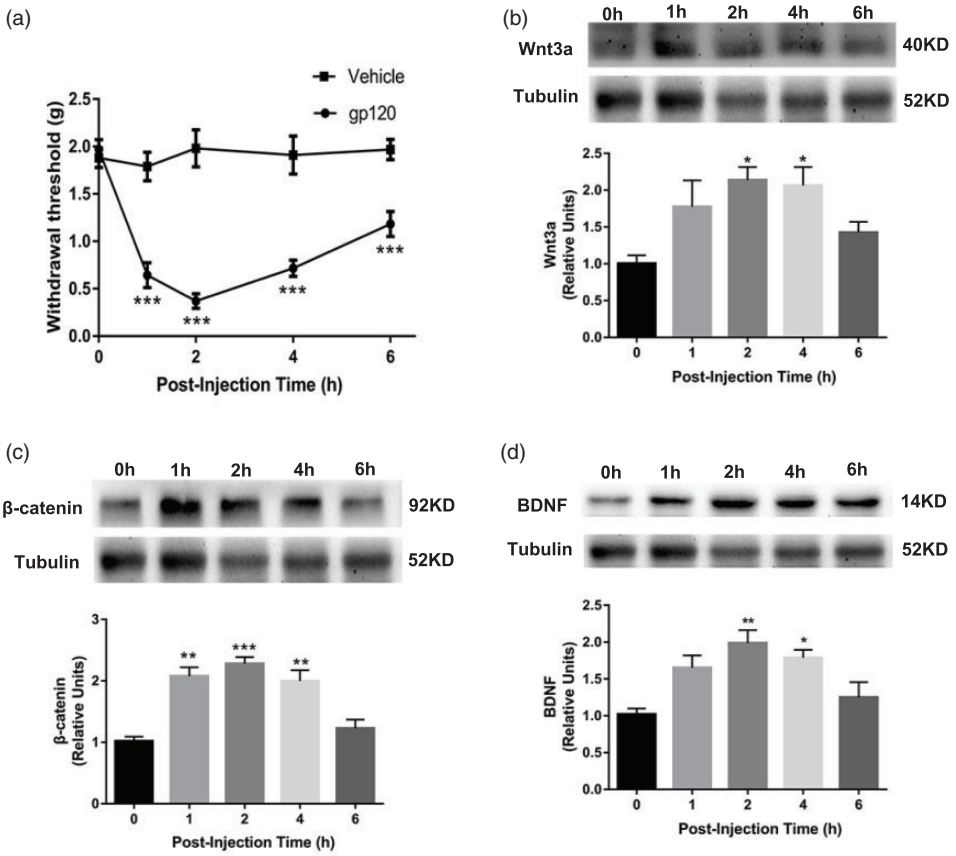

Figure 1. I.t. administration of gp120-induced mechanical allodynia in mice, activated spinal Wnt/β-catenin signaling and upregulated spinal BDNF expression. (a) I.t. administration of gp120-induced mechanical allodynia in mice, demonstrated as reduced tactile paw withdrawal thresholds. Mechanical allodynia persisted for at least 6 h. I.t. vehicle (PBS, 5 μL) had no effect on tactile allodynia in sham animals during a 6-h time course (***$p < 0.001$; vs. vehicle group; two-way ANOVA; $n = 6$ mice in each group). Time course of Wnt3a (b) and β-catenin (c) protein levels after i.t. administration of gp120 showed that Wnt3a and β-catenin protein began to rise after 1 h of gp120 injection and peaked at 2 h. (d) Time course of BDNF protein levels after i.t. gp120. After 1 h of gp120 injection, spinal BDNF began to rise and peaked at 2 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; vs. time point 0 group; one-way ANOVA; $n = 3$). Data are expressed as the mean ± SEM.
BDNF: Brain-derived neurotrophic factor.

spinal cord of mice gradually increased from 0 h until it peaked at 2 h (Figure 1(b)–(d)) with 2.1-, 2.3-, and 2.0-fold increases for Wnt3a, β-catenin, and BDNF, respectively. Interestingly, although the magnitudes of the peak increase differed among Wnt3a, β-catenin, and BDNF in the spinal cord of HNP mice, these proteins displayed similar temporal profiles of upregulation (Figure 1(b)–(d)). Therefore, the Wnt/β-catenin signaling pathway is critical for HNP-induced BDNF expression in the mouse spinal cord.

Wnt/β-catenin signaling pathway regulates the expression of spinal BDNF and affects mechanical allodynia in HNP mice

Our previous study showed that gp120 stimulates BDNF expression in BV2 cells via the Wnt/β-catenin signaling pathway.[10] In this study, the results in Figure 1(b)–(d) show that i.t. HIV_1 gp120 upregulated the expression of Wnt3a, β-catenin, and BDNF in the spinal cord of treated mice. Therefore, it was hypothesized that in HNP animals, the Wnt/β-catenin signaling pathway may regulate the expression of BDNF and thus affect mechanical allodynia. Therefore, the following line of experiments was performed.

We first activated the Wnt/β-catenin signaling pathway by i.t. Wnt3a. WB results showed that β-catenin (*$p < 0.05$; Wnt3a group vs. vehicle group; one-way ANOVA; $n = 3$) and BDNF (*$p < 0.05$; Wnt3a group vs. vehicle group; one-way ANOVA; $n = 3$) protein levels in the spinal cord were significantly increased 2 h after i.t. Wnt3a relative to the control group, at 1.8 times and 2.2 times, respectively (Figure 2(a)–(b)). Interestingly, the upregulation levels of β-catenin and BDNF proteins in the spinal cord of i.t. gp120 mice were similar to those of i.t. Wnt3a mice. Meanwhile, i.t. of not only Wnt3a but

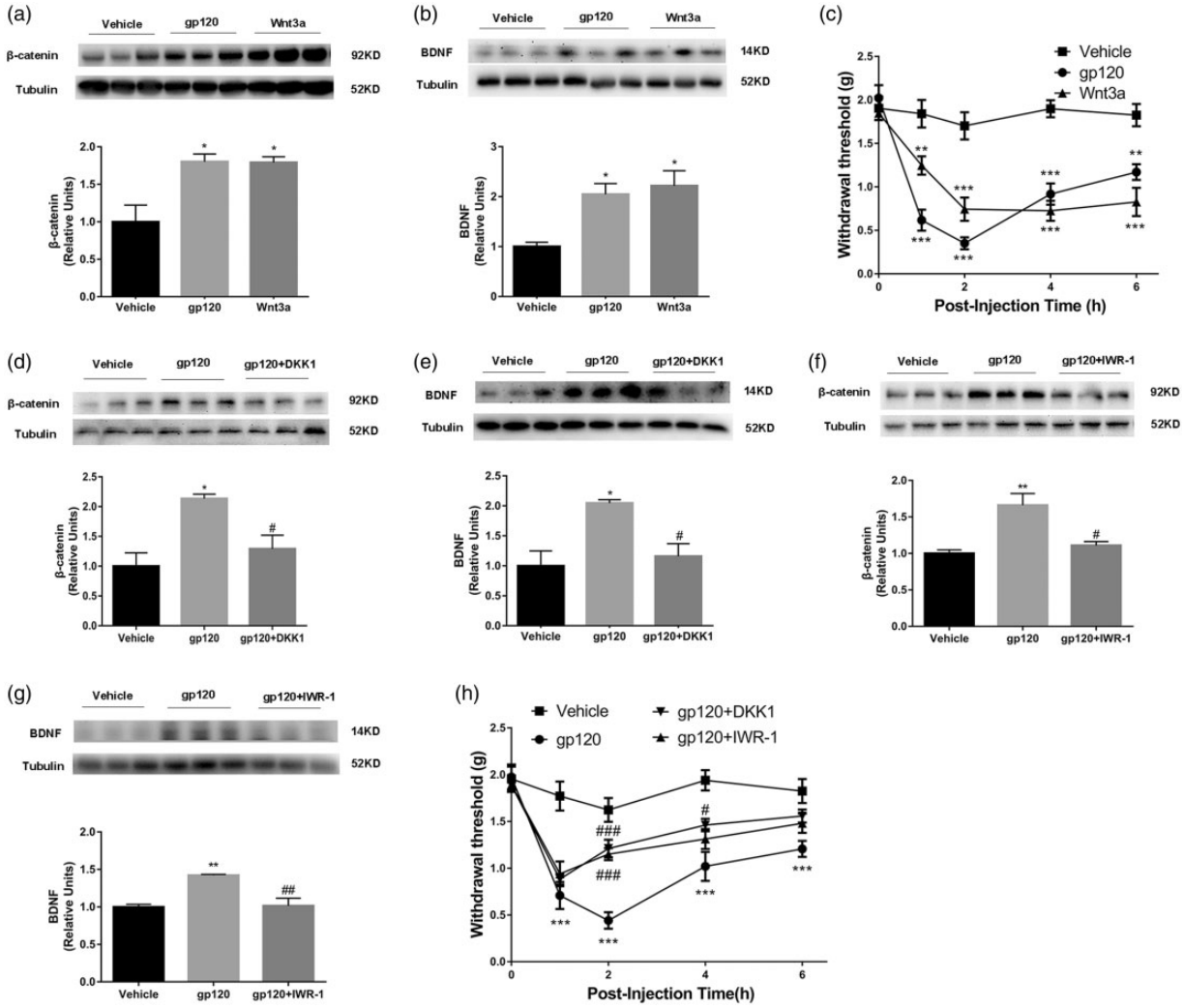

Figure 2. Effects of activation or blocking of Wnt/β-catenin on mechanical allodynia and BDNF expression in mice. WB showed that intrathecal injection of Wnt3a led to upregulation of β-catenin (a) and BDNF (b) in the mouse spinal cord ($^{*}p < 0.05$; vs. vehicle group; one-way ANOVA; $n = 3$). (c) I.t. administration of gp120 or Wnt3a induced mechanical allodynia in mice. After i.t. administration of gp120 or Wnt3a, mice displayed significantly reduced paw withdrawal thresholds, indicating the presence of tactile allodynia. The mechanical allodynia in the groups treated with gp120 and Wnt3a persisted for at least 6 h ($^{**}p < 0.01$, $^{***}p < 0.001$; vs. vehicle group; two-way ANOVA; $n = 6$). WB analysis showed that DKK1 impaired gp120-induced upregulation of β-catenin (d) and BDNF protein (e). In addition, WB analysis showed that IWR-1 impaired gp120-induced upregulation of β-catenin (f) as well as BDNF protein (g) ($^{*}p < 0.05$, $^{**}p < 0.01$; vs. vehicle group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; vs. gp120 group; one-way ANOVA; $n = 3$). (h) DKK1 or IWR-1 reversed gp120-induced mechanical allodynia in mice ($^{***}p < 0.001$; vs. vehicle group, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$; vs. gp120 group; two-way ANOVA; $n = 6$). Data are expressed as the mean ± SEM.
BDNF: Brain-derived neurotrophic factor; DKK1: Dickkopf-1; IWR-1: endo-IWR 1.

also gp120 induced mechanical allodynia in the mice ($^{**}p < 0.01$, $^{***}p < 0.001$; gp120 group or Wnt3a group vs. vehicle group; two-way ANOVA; $n = 6$; Figure 2(c)). Moreover, the behavioral change trend exhibited a striking similarity between i.t. Wnt3a mice and i.t. gp120 mice. These preliminary results suggest that HIV_1 gp120 may activate the Wnt/β-catenin signaling pathway to regulate spinal BDNF expression thereby affecting mechanical allodynia.

To further investigate whether the Wnt/β-catenin signaling pathway is involved in the expression of spinal BDNF and mechanical allodynia, two drugs that inhibited Wnt/β-catenin signaling pathways were used: (1) IWR-1, a small molecule that promotes β-catenin degradation by abrogating Axin2 turnover and thus disrupting Wnt pathway responses, and (2) DKK-1, an antagonistic inhibitor of the Wnt signaling pathway that acts by isolating the LRP coreceptor to prevent it

from facilitating the activation of the Wnt signaling pathway. To specifically target the Wnt/β-catenin signaling pathway in the spinal cord, the drugs were delivered into the spinal fluid by intrathecal administration.

DKK1 (200 ng/μL, 5 μL) or IWR-1 (20 ng/μL, 5 μL) was i.t. 30 min prior to gp120, and the spinal cord was collected 2 h after i.t. gp120. DKK1 abolished the gp120-induced upregulation of β-catenin ($^{\#}p < 0.05$; gp120 + DKK1 group vs. gp120 group; one-way ANOVA; $n = 3$) and BDNF ($^{\#}p < 0.05$; gp120+DKK1 group vs. gp120 group; one-way ANOVA; $n = 3$) (Figure 2(d)–(e)). Similarly, IWR-1 attenuated the gp120-induced upregulation of β-catenin ($^{\#}p < 0.05$; gp120+IWR-1 group vs. gp120 group; one-way ANOVA; $n = 3$) and BDNF ($^{\#\#}p < 0.01$; gp120+IWR-1 group vs. gp120 group; one-way ANOVA; $n = 3$) (Figure 2(f)–(g)). These data suggest that the Wnt/β-catenin signaling pathway is critical for spinal cord BDNF expression in the HNP model.

In the behavioral studies, separate groups of mice received i.t.s of one of the following drug combinations with a 30-min interval between the first and second drug injection: PBS (5 μL)/PBS (5 μL); PBS (5 μL)/gp120 (20 ng/μL, 5 μL); IWR-1 (20 ng/μL, 5 μL)/gp120 (20 ng/μL, 5 μL); DKK1 (200 ng/μL, 5 μL)/gp120 (20 ng/μL, 5 μL). In the control group, two consecutive injections of PBS did not result in mechanical allodynia (Figure 2(h)). Injection of PBS/gp120 caused mechanical allodynia that lasted for more than 6 h postinjection. However, IWR-1 and DKK1 blocked gp120-induced mechanical allodynia (Figure 2(h)). For instance, gp120-induced mechanical hyperalgesia in mice was significantly reversed 2 h ($^{\#\#\#}p < 0.001$; gp120+DKK1 group or gp120+IWR-1 group vs. gp120 group; two-way ANOVA; $n = 6$) after i.t. of IWR-1 and DKK1. Therefore, these data demonstrate that the Wnt/β-catenin signaling pathway affects mechanical allodynia.

In this section, activation of the Wnt/β-catenin signaling pathway upregulates BDNF expression in the spinal cord and induces mechanical allodynia in mice. Blocking the Wnt/β-catenin signaling pathway reverses the upregulation of BDNF and alleviates mechanical allodynia induced by HIV_1 gp120. In summary, the Wnt/β-catenin signaling pathway affects mechanical allodynia caused by gp120 via the regulation of BDNF expression in the spinal cord.

Spinal BDNF upregulation is critical for the expression of nociceptive behavior in HNP mice

To further investigate the role of spinal BDNF in HNP, a series of experiments were performed. According to the mouse nociceptive behavior (Figure 1(a)) and the temporal profile of BDNF expression (Figure 1(d)) in HNP mice, we selected the time-point of 2 h after i.t. gp120 to detect BDNF expression compared with the control group. WB results showed that i.t. of gp120 led to mBDNF ($^{*}p < 0.05$; gp120 group vs. vehicle group; t test; $n = 3$) and proBDNF ($^{*}p < 0.05$; gp120 group vs. vehicle group; t test; $n = 3$) upregulation in the mouse spinal cord within 2 h (Figure 3(a)). Moreover, immunofluorescence results from the control spinal cord showed that BDNF was expressed at low immunofluorescence intensity but increased considerably in the experimental spinal cord examined at 2 h ($^{*}p < 0.05$; gp120 group vs. vehicle group; t test; $n = 3$) after gp120 injection (Figure 3(b)). These results suggest that BDNF functionally contributes to gp120-induced nociceptive behaviors. To further confirm the roles of BDNF in mouse behavioral changes, recombinant BDNF (1 ng/μL, 5 μL) was injected intrathecally into wild-type mice. We found that recombinant BDNF directly caused mechanical allodynia that lasted for more than 6 h ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; BDNF group vs. vehicle group; two-way ANOVA; $n = 6$; Figure 3(c)). These results demonstrate that HNP is correlated with the upregulation of spinal BDNF induced by gp120. Spinal BDNF upregulation is critical for the expression of nociceptive behavior in HNP mice.

Spinal microglia activate and colocalize with BDNF in HNP mice

The above results reveal that upregulation of spinal BDNF plays an important role in HNP. However, it has not been determined where the increased spinal BDNF from HNP mice is. To answer this question, the following tests were carried out. First, the protein levels of GFAP (astrocyte marker), Iba1 (microglia marker), and NeuN (neuron marker) in the spinal cord were determined. The results of WB analysis showed that Iba1 was upregulated in the spinal cord of HNP mice with a 3.4-fold increase in Iba1 ($^{**}p < 0.01$; gp120 group vs. vehicle group; t test; $n = 3$), whereas GFAP and NeuN upregulation was not observed in the spinal cord of HNP mice (Figure 4(a)–(c)). To further confirm the activation of microglia in HNP mice, an immunofluorescence analysis was performed. In addition, immunofluorescence of the spinal cord for GFAP, Iba1, and NeuN was also performed. Iba1-labeled microglia displayed hypertrophic morphologies, whereas astrocytes and neurons were not observed in the spinal cord of HNP mice (Figure 4(d)). These results proved that microglia are reactive in the spinal cords of mice following i.t. administration of gp120.

Furthermore, BDNF expression in spinal cord neurons was examined. Double staining showed that BDNF was colocalized with microglia, occasionally in small amounts with astrocytes, but it was not colocalized with neurons (Figure 4(e)). These results suggested that

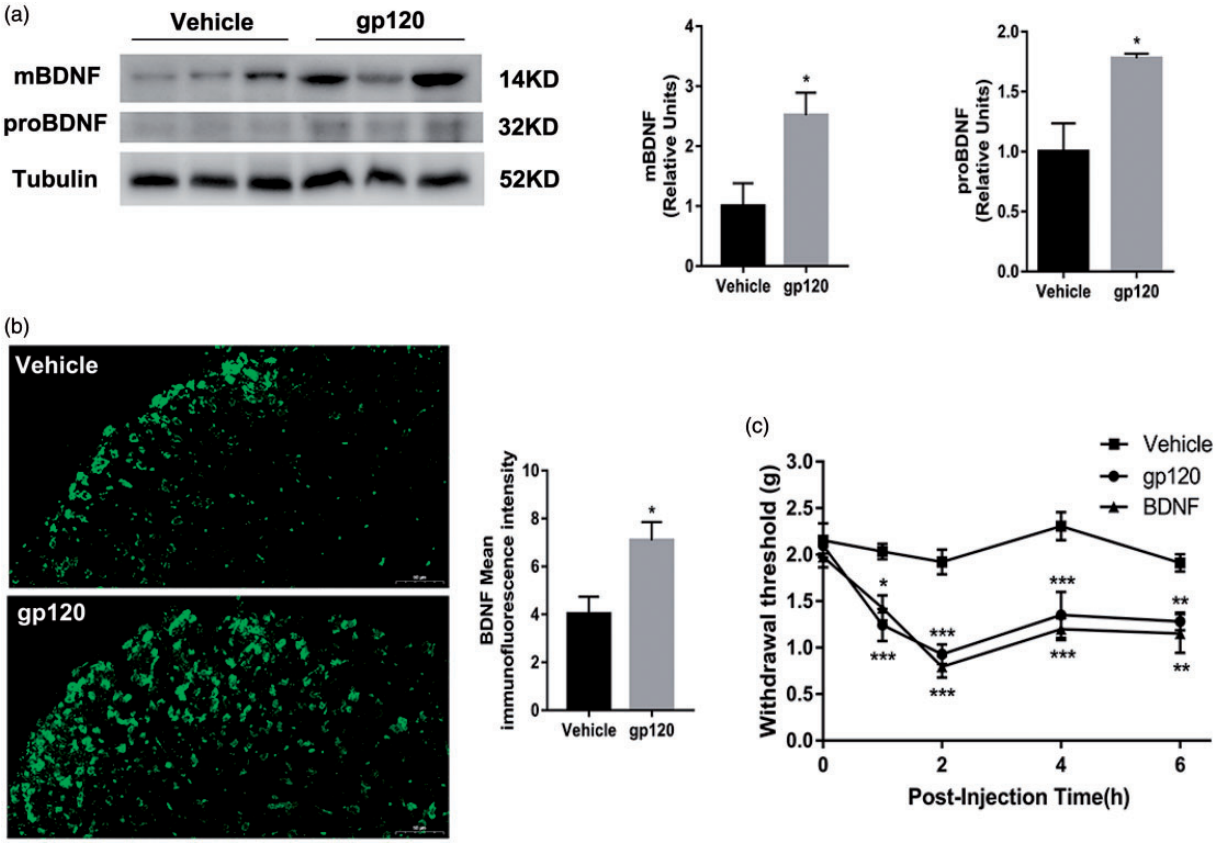

Figure 3. Spinal BDNF upregulation is critical for the expression of nociceptive behavior in HNP mice. (a) The expression of mBDNF and proBDNF in the spinal cord was upregulated 2 h after i.t. gp120 (*$p < 0.05$; vs. vehicle group; *t* test; $n = 3$). (b) Immunofluorescence showed the expression of BDNF protein (green) in the spinal cord. The fluorescence intensity increased greatly in the experimental spinal cord examined at 2 h after gp120 injection (*$P < 0.05$; vs. vehicle group; *t* test; $n = 3$). (c) Intrathecal administration of exogenous BDNF (1 ng/μL, 5 μL) caused mechanical allodynia, which persisted for at least 6 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; vs. vehicle group; two-way ANOVA; $n = 6$). Scale bar: 50 μm. Data are expressed as the mean ± SEM.
BDNF: Brain-derived neurotrophic factor.

the upregulation of BDNF might mainly be caused by microglia, which leads to NP.

Spinal microglial activation is critical for mechanical allodynia and BDNF upregulation in HNP mice

Next, we attempted to determine the role of microglia in gp120-induced HNP. The gp120-induced increases in Iba1 (#$p < 0.05$; gp120 + Mino group vs. gp120 group; one-way ANOVA; $n = 3$) and BDNF (#$p < 0.05$; gp120 + Mino group vs. gp120 group; one-way ANOVA; $n = 3$) proteins diminished when we cotreated mice with minocycline (Figure 5(a)–(b)) to inhibit microglial activation. In the behavioral studies, separate groups of mice received i.t.s of one of the following drug combinations with a 30-min interval between the first and second drug injection: PBS (5 μL)/PBS (5 μL); PBS (5 μL)/gp120 (20 ng/μL, 5 μL); and minocycline (10 μg/μL, 5 μL)/gp120 (20 ng/μL, 5 μL). In the control group, two consecutive injections of PBS did not result in mechanical allodynia (Figure 5(c)). Injection of PBS/gp120 caused mechanical allodynia. However, minocycline blocked gp120-induced mechanical allodynia (###$p < 0.001$; gp120 + Mino group vs. gp120 group; two-way ANOVA; $n = 6$; Figure 5(c). For instance, gp120-induced mechanical hyperalgesia in mice was significantly reversed 2 h after i.t. of minocycline. Therefore, these data demonstrate that minocycline reversed mechanical allodynia and BDNF upregulation in HNP mice.

BDNF receptor TrkB and p75NTR upregulate and affect mechanical allodynia in HNP mice

To examine the expression of BDNF receptors, including TrkB, p75NTR, and sortilin, tissue lysates from the spinal cord were used for WB analysis 2 h after i.t. administration of gp120. Increased levels of TrkB (**$p < 0.01$; gp120 group vs. vehicle group; *t* test; $n = 3$) and p75NTR (*$p < 0.05$; gp120 group vs. vehicle group; *t* test; $n = 3$) were detected in the spinal cord 2 h

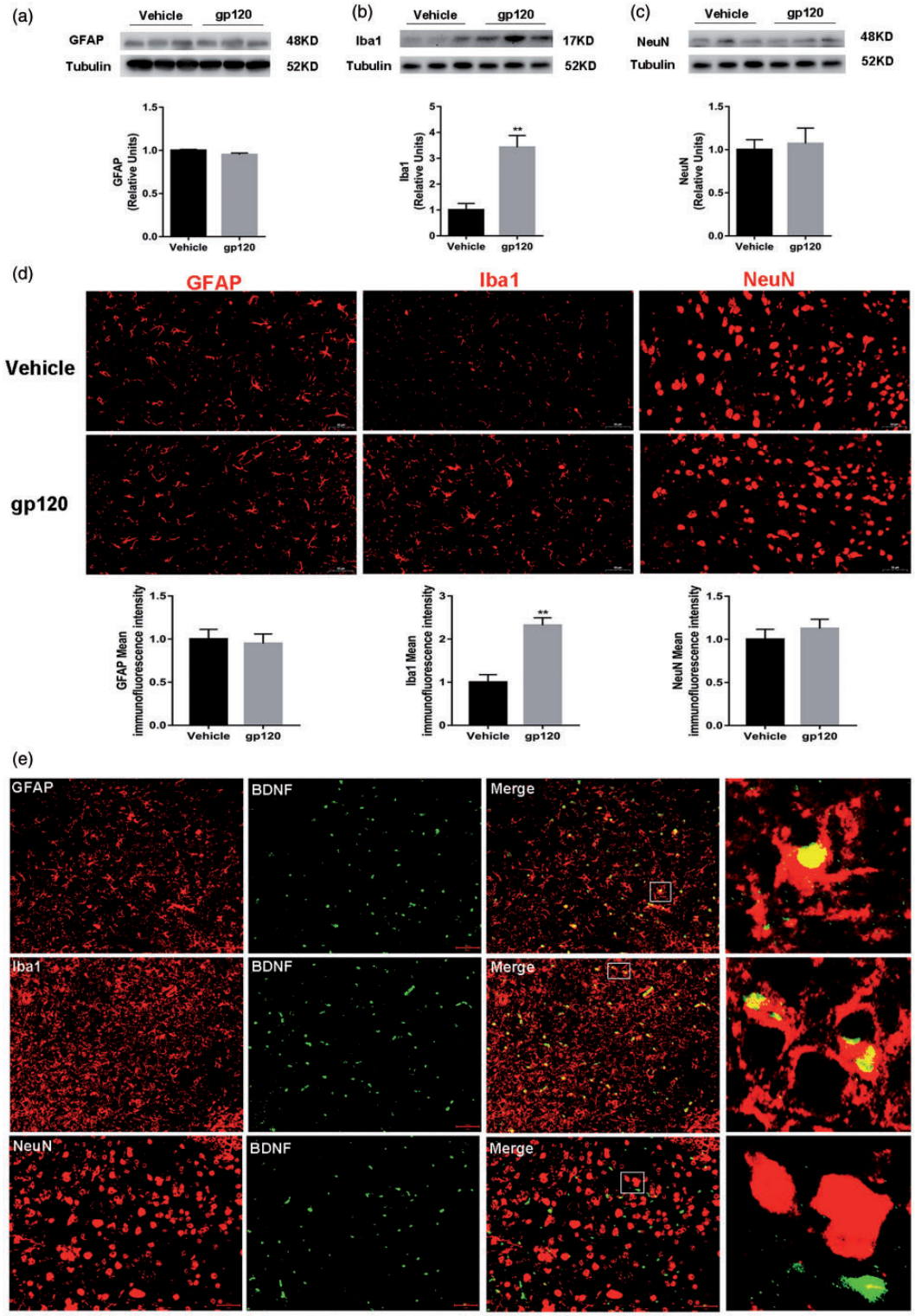

Figure 4. Spinal BDNF was expressed in activated microglia. (a–c) The expression levels of GFAP, Iba1, and NeuN in the spinal cord 2 h after i.t. administration of gp120. WB analysis showed that Iba1 was upregulated in the spinal cord of HNP mice, whereas GFAP and NeuN upregulation was not observed in the spinal cord of HNP mice (**$p < 0.01$; vs. vehicle group; *t* test; $n = 3$). (d) Immunofluorescence showed an increased number of microglia 2 h after intrathecal injection of gp120, whereas GFAP and NeuN were not observed in the spinal cord of HNP mice (**$p < 0.01$; vs. vehicle group; *t* test; $n = 3$). Scale bar: 50 μm. (e) BDNF (green) colocalized with microglia (Iba1, red) and, occasionally, a small amount of astrocytes (GFAP, red) but not neurons (NeuN, red). Spinal cord tissues were collected 2 h after intrathecal injection of gp120. Scale bar: 100 μm. Data are expressed as the mean ± SEM.
GFAP: glial fibrillary acidic protein; BDNF: brain-derived neurotrophic factor.

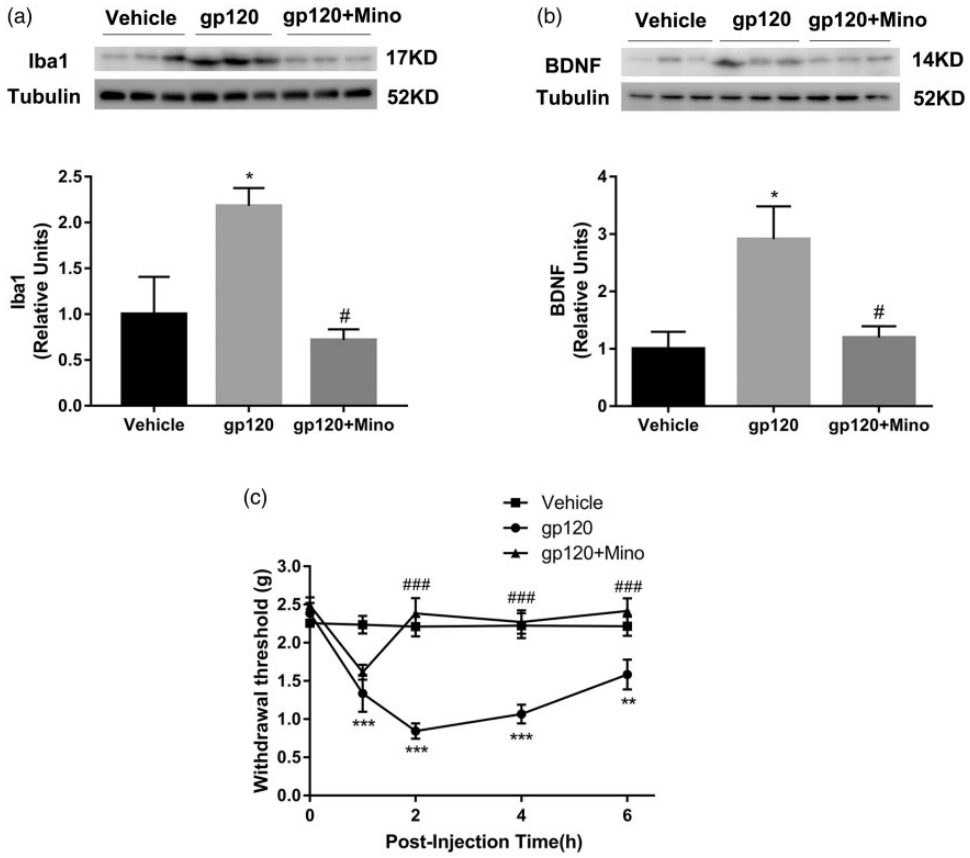

Figure 5. Microglial activation is crucial for gp120-induced pain. WB analysis showed that minocycline prevented gp120-induced upregulation of Iba1 (a) and BDNF protein (b), ($^{*}p < 0.05$; vs. vehicle group, $^{\#}p < 0.05$; vs. gp120 group; one-way ANOVA; $n = 3$). (c) Intrathecal injection of minocycline 30 min before gp120 reversed gp120-induced mechanical allodynia in mice ($^{**}p < 0.01$, $^{***}p < 0.001$; vs. vehicle group, $^{\#\#\#}p < 0.001$; vs. gp120 group; two-way ANOVA; $n = 6$). Data are expressed as the mean ± SEM.
BDNF: Brain-derived neurotrophic factor; Mino: minocycline.

after i.t. gp120 (Figure 6(a)–(b)) with a 1.4- and 1.5-fold increase, respectively. However, the receptor sortilin was not upregulated in the spinal cord (Figure 6(c)). It has therefore been suggested that the occurrence and maintenance of pain could be reduced by giving BDNF high-affinity receptor TrkB antagonist.[31] However, whether blocking the TrkB receptor could reverse mechanical allodynia in HNP mice has not been determined. To test this hypothesis, TrkB-Fc, which scavenges endogenous BDNF, was injected intrathecally 0.5 h before i.t. gp120.[27] As shown in Figure 6(d), TrkB-Fc blocked gp120-induced mechanical allodynia ($^{\#\#\#}p < 0.001$; gp120 + TrkB-Fc group vs. gp120 group; two-way ANOVA; $n = 6$). Similarly, the p75NTR inhibitor TAT-Pep5 reversed gp120-induced mechanical allodynia in mice ($^{\#\#\#}p < 0.001$; gp120 + TAT-Pep5 group vs. gp120 group; two-way ANOVA; $n = 6$; Figure 6(e)). These results indicated that the expression of TrkB and p75NTR in the spinal cord was upregulated, TrkB-Fc or TAT-Pep5 attenuated mechanical allodynia produced by i.t. administration of gp120.

Discussion

Elucidating the mechanisms of NP, especially the early stage of pain, is important for the development of effective drugs for early treatment. Our study reveals that Wnt/β-catenin signaling regulates BDNF release from spinal microglia to mediate HNP. Briefly, i.t. gp120 activates the Wnt/β-catenin signaling pathway to regulate the expression and release of microglial BDNF, which may lead to NP after binding to TrkB or p75NTR receptors. The principle findings were four fold: (1) i.t. administration of gp120 activated the Wnt/β-catenin signaling pathway, which upregulated spinal BDNF expression to induce NP. (2) Activation of the Wnt/β-catenin signaling pathway by Wnt3a upregulated spinal BDNF expression and mechanical allodynia in mice. Blockade of the Wnt/β-catenin signaling pathway in the spinal cord attenuated the gp120-induced upregulation of BDNF and reversed mechanical allodynia. (3) In the induction of neuropathic pain by i.t. administration of gp120, microglial cells were activated and colocalized with BDNF,

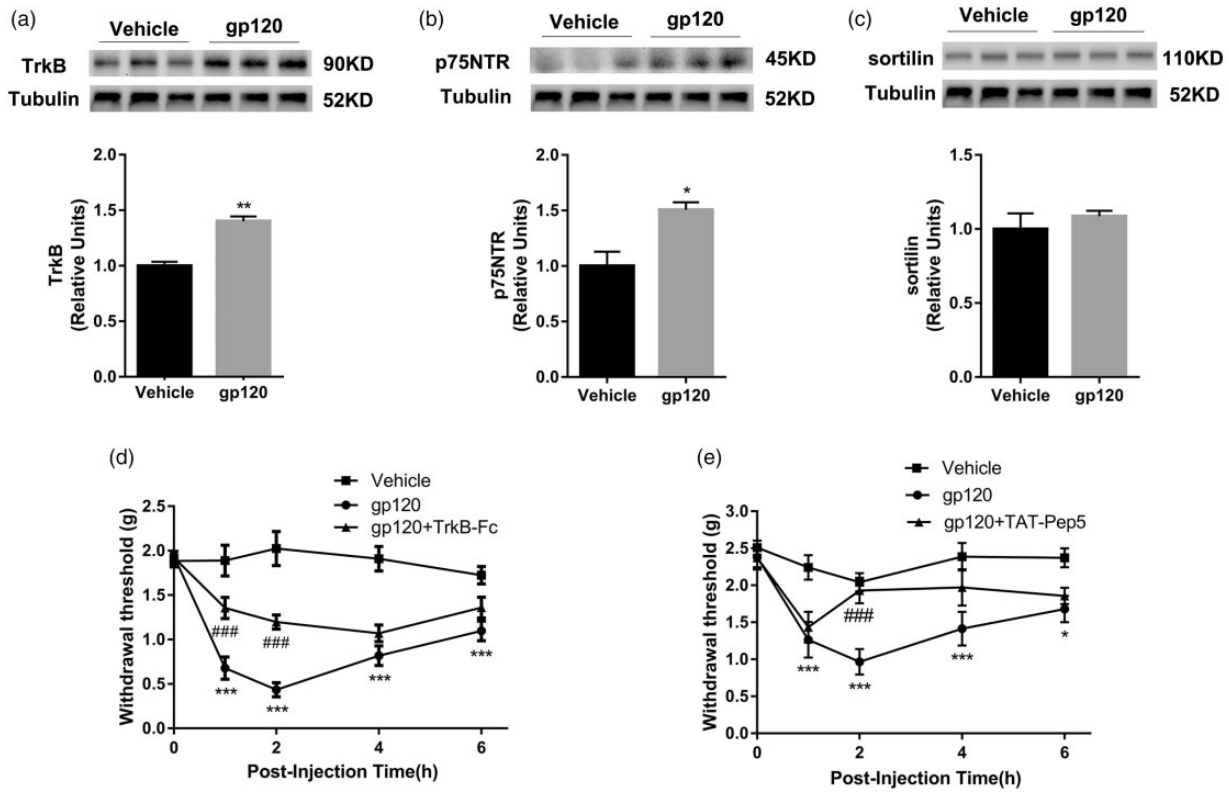

Figure 6. TrkB and p75NTR were upregulated in the spinal cord of HNP mice, and blockade of the BDNF receptor reversed mechanical allodynia. (a–c) The expression levels of TrkB, p75NTR, and sortilin receptor in the spinal cord 2 h after i.t. administration of gp120. WB analysis showed that TrkB and p75NTR were upregulated in the spinal cord of HNP mice, whereas sortilin was not observed in the spinal cord of HNP mice ($^{*}p < 0.05$, $^{**}p < 0.01$; vs. vehicle group; *t* test; $n = 3$). (d) Intrathecal injection of TrkB-Fc 30 min before gp120 reversed gp120-induced mechanical allodynia in mice. (e) TAT-Pep5 reversed gp120-induced mechanical allodynia in mice ($^{*}p < 0.05$, $^{***}p < 0.001$; vs. vehicle group, $^{\#\#\#}p < 0.001$; vs. gp120 group; two-way ANOVA; $n = 6$). Data are expressed as the mean ± SEM.
BDNF: Brain-derived neurotrophic factor.

and inhibition of microglial cell activation blocked the expression of BDNF and reversed mechanical allodynia. (4) Usage of the BDNF scavenger TrkB-Fc or TAT-Pep5 reversed the mechanical allodynia induced by i.t. gp120 in mice. Studies have proven that spinal BDNF plays a crucial role in central sensitization and the induction and persistence of neuropathic pain.[17,32] These findings may reveal the pathogenesis of NP induced by gp120.

Neuropathology of HNP

HIV-associated chronic pain is acquired during the progression of AIDS. HNP is chronic and long-term, and it is a common complication of HIV patients. Most HIV-1 patients remain neurologically unimpaired during early pre-AIDS stages. Generally, becoming seropositive after HIV infection requires three to six weeks, and this period is known as seroconversion. After the seroconversion period, HIV infection enters a latency phase called the asymptomatic period, which usually lasts for 8–10 years.[33] Neurological pathologies are noted during this stage, especially in the white matter, although the pathological changes are not consistent. Microglial activation, astrocytosis, and myelin pallor are observed in the white matter during this stage.[34,35] In the AIDS stage, autopsies found that 80%–100% of AIDS patients had neuropathological changes in the CNS.[36,37] The HIV virus infects the microglia and astrocytes of the nervous system,[2] and the infected host cells synthesize the HIV viral proteins gp120 and Tau. These proteins are toxic and cause cells to secrete excitatory amino acids, ROS, inflammatory factors, and BSNFs, which are the main causes of neuroAIDS.[10] The toxicity of the HIV virus protein VPR is also one of the causes of HNP, which can induce peripheral nerve damage.[19] In particular, gp120 has direct neurotoxicity, which can cause neuronal damage and apoptosis.[38] Furthermore, gp120 promotes the release of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) from the spinal cord to induce neuroinflammation, activates DRG neurons and induces allodynia through chemokine receptors.[6,39] Also, gp120 activates microglia and astrocytes in the dorsal horn of the spinal cord to induce pain.[6,40]

Relationship between Wnt/β-catenin signals and HNP

Studies have also shown that Wnt is a highly conserved secretory signaling family protein in evolution that plays an important role in nervous system development.[26,41–43] It has also been demonstrated that dysregulation of Wnt proteins or other components of the Wnt signaling pathway results in many diseases.[44,45] For example, the upregulation of Wnt signaling in cancer patients promotes the invasion, metastasis, and metabolism of cancer cells[46] as well as inflammation, as Wnt signaling is downregulated in the brains of patients with Alzheimer's disease.[47] The Wnt signaling pathway is also involved in HNP lesions.[22,24] HIV_1 gp120 activates neurons, leading to rapid synthesis and secretion of Wnt proteins.[48] Consistent with this notion, Tang et al.[49] found that Wnt3a, Wnt5a, and catenin were mainly expressed in neurons in the SCDH of HNP patients and HNP mice,[26] indicating that Wnt signaling is activated in HNP neural circuits. Further studies have demonstrated that the Wnt5a/CaMKII and Wnt5a/JNK signaling pathways are critical for gp120 to induce proinflammatory cytokine expression in mouse SCDH. The Wnt5a/CaMKII pathway is required for the upregulation of IL-1β and the Wnt5a/JNK pathway and for further upregulation of TNF-α. However, IL-6 is regulated by both pathways.[22,24] Discoveries made from the Wnt signaling pathway were used to study HNP mechanisms, with existing studies demonstrating the role and mechanism of Wnt5a-mediated noncanonical signaling pathways in HNP. Wnt3a and β-catenin expression were upregulated in HNP upregulation, but the role of the classical Wnt/β-catenin signaling pathway in HNP was unclear. Interestingly, our study showed that the Wnt/β-catenin signaling pathway is activated in NP induced by gp120, and activation of the Wnt/β-catenin signaling pathway by Wnt3a also induced mechanical allodynia in mice. In keeping with this possibility, Wnt3a is upregulated in SDH in patients with chronic pain of HIV.[49] In animal models, Wnt3a is expressed in the superficial layers of the mouse SCDH.[26] In addition, blockade of the Wnt signaling pathway by DKK1 and IWR-1 reversed gp120-induced mechanical allodynia. In effect, these studies show that the Wnt/β-catenin signaling pathway plays an important role in gp120-induced mechanical allodynia.

Wnt/β-catenin/BDNF signaling axis is involved in HNP pain

BDNF is an important molecule in the development of chronic pain and modulates synaptic plasticity. Previous studies have shown that Ca^{2+} influx activates cAMP response element-binding protein (CREB) transcription factors to regulate the transcriptional expression of BDNF.[50] However, in addition to being regulated by Ca^{2+}/CREB, BDNF may be regulated by other pathways, such as Wnt/β-catenin.[27,51] In HNP model mice, we found that the upregulation of Wnt3a and β-catenin protein in the spinal cord was similar to the upregulation trend of BDNF. Interestingly, our previous in vitro studies showed that gp120 could induce the upregulation of Wnt3a, β-catenin, and BDNF expression in BV2 cells, while the blockade of the Wnt/β-catenin signaling pathway attenuated the upregulation of BDNF.[10] Considering these phenomena, it can be speculated that HIV_1 gp120 may participate in HNP by activating the Wnt/β-catenin signaling pathway to regulate BDNF in vivo. Our recent research has proven that in the capsaicin-induced pain model, the Wnt/β-catenin signal transduction pathway regulates BDNF expression and is involved in the pain neural circuit.[27] In this study, we found that in gp120-induced NP, the expression of BDNF in the spinal cord is upregulated. Moreover, HNP mouse mechanical pain was reversed using the BDNF scavengers TrkB-Fc and TAT-Pep5. Activation of the Wnt/β-catenin signaling pathway upregulated BDNF expression and blockade of the Wnt/β-catenin signaling pathway downregulated BDNF expression and reversed mechanical allodynia. Therefore, our results confirm that the Wnt/β-catenin signaling pathway is involved in gp120-induced NP by regulating BDNF.

Microglia release BDNF to participate in HNP pain

The involvement of glial cells in the occurrence and maintenance of NP is one of the major advances made in pain research, especially microglial activation in NP.[52,53] A number of studies suggest that activated microglia secrete multiple proinflammatory cytokines and BDNF that lead to neuroinflammation and central sensitization at the spinal level and NP through microglia–neuron interactions.[54–56] Tang et al.[40] found activation of astrocytes instead of microglia in chronic pain HIV patients together with the observation of significant increases in the proinflammatory cytokines TNF-α and IL-1β. However, microglia and astrocytes were found to be activated in animal models with i.t. gp120 after three weeks.[6] It was identified from this study that microglia were activated 2 h after i.t. gp120, while astrocytes were not. By contrast, astrocyte activation was not detected (as judged by the GFAP level). In considering this discrepancy, it is worth noting that in i.t. gp120 model, microglia were restricted immediately after administration. Previous studies have shown that gp120-stimulated microglial activation may return to a resting state later.[25] In addition, microglia might be activated instantaneously in the early stages of HIV-associated pain development. HIV_1-infected microglia might have returned to a resting state after human death. Our study also

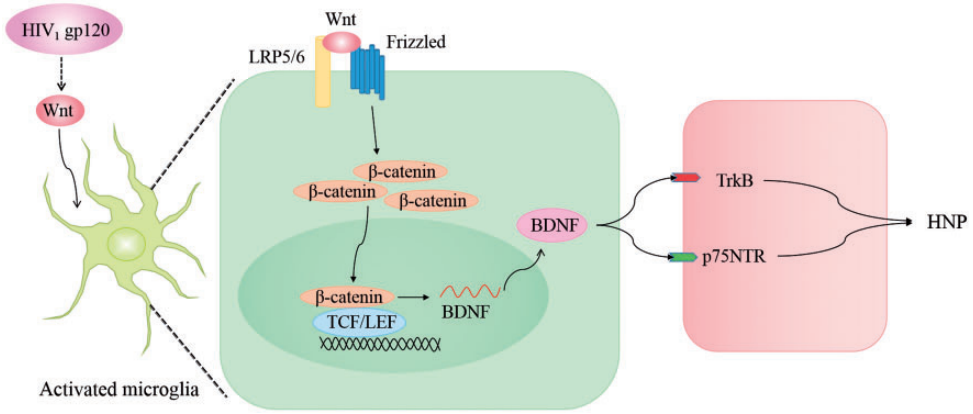

Figure 7. Wnt/β-catenin signaling regulates BDNF release from spinal microglia to participate in HIV-associated neuropathic pain. HIV_1 gp120 activates neurons, leading to rapid synthesis and secretion of Wnt, and then Wnt protein binds to frizzled receptor to activate microglia. Activation of Wnt/β-catenin signaling leads to BDNF transcription and expression in microglia. BDNF from microglia binds to the TrkB or p75NTR receptor to induce HNP.

found that BDNF colocalized with spinal microglial cells in the gp120-induced NP model. This finding indicates that activated microglia secrete BDNF. Minocycline, a microglial inhibitor, inhibited the activation of microglia, reversed mechanical allodynia in HNP mice and inhibited the upregulation of BDNF. Consistent with what was found, in the SNI model, microglia were activated, BDNF/TrkB expression was increased and minocycline significantly inhibited the activation of microglia.[15] We envision a feedback interaction between the HIV-associated neuropathic and Wnt/β-catenin/BDNF signal axis: hyperactivation of spinal microglia under pathological pain conditions would stimulate Wnt/β-catenin signaling and BDNF secretion which, in turn, promote pain expression. This feedback interaction may provide a mechanism for the maintenance of persistent pain. Recent studies suggest that BDNF, secreted by primary sensory neurons, plays a key role in mediating the transition from acute to chronic pain.[17] These studies indicate that BDNF is a potential target for pain treatment.

In summary, our results suggest that in the NP model induced by i.t. of gp120, Wnt/β-catenin signaling regulates BDNF release from spinal microglia (Figure 7). In addition, these results help to elucidate the mechanism of the Wnt/β-catenin/BDNF signaling axis in HNP genesis and provide a theoretical basis for the development of drugs for the treatment of early pain.

Author Contributions

XXZ and LT contributed to the experiments equally and are listed as co-first authors, and XXZ is taking this study forward. WPZ conceived and coordinated the study and directed the experiments. XXZ, MRZ, EO, and WPZ wrote and edited the article. WPZ, SJW, and DW provided technical support for the immunohistochemistry, intrathecal injection, and biochemical experiments. All authors reviewed the results and approved the final version of the manuscript.

Declaration of Conflicting Interests

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