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Translocator protein agonist Ro5-4864 alleviates neuropathic pain and promotes remyelination in the sciatic nerve

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Abstract

Our previous study reported the translocator protein to play a *critical* role in neuropathic pain and the possible mechanisms in the spinal cord. However, its mechanism in the peripheral nervous system is poorly understood. This study was undertaken to explore the distribution of translocator protein in the dorsal root ganglion and the possible mechanisms in peripheral nervous system in a rat model of spared nerve injury. Our results showed that translocator protein was activated in dorsal root ganglion after spared nerve injury. The translocator protein signals were primarily colocalized with neurons in dorsal root ganglion. A single intrathecal (i.t.) injection of translocator protein agonist (7-chloro-5–4-chlorophenyl)-1,3-dihydro-1-methyl-2-H-1,4-benzodiaze-pine-2) (Ro5-4864) exerted remarkable analgesic effect compared with the spared nerve injury group ($P < 0.01$). After i.t. administration of 2 μg Ro5-4864 on day 3, the expression of translocator protein in ipsilateral dorsal root ganglion was significantly increased on day 7($P < 0.01$) but decreased on day 14 ($P < 0.05$) compared with the same point in time in the control group. The duration of translocator protein activation in dorsal root ganglion was remarkably shortened. Ro5-4864 also inhibited the activation of phospho-extracellular signal-regulated kinase 1(p-ERK1) ($P < 0.01$), p-ERK2 (D7: $P < 0.01$, D14: $P < 0.05$), and brain-derived neurotrophic factor ($P < 0.05$) in dorsal root ganglion. Meanwhile, i.t. administration of 2 μg Ro5-4864 on day 3 further accelerated the expression of myelin protein zero(P0) and peripheral myelin protein 22 (PMP22). Our results suggested Ro5-4864 could alleviate neuropathic pain and attenuate p-ERK and brain-derived neurotrophic factor activation in dorsal root ganglion. Furthermore, Ro5-4864 stimulated the expression of myelin regeneration proteins which may also be an important factor against neuropathic pain development. Translocator protein may present a novel target for the treatment of neuropathic pain both in the central and peripheral nervous systems.

Keywords

Neuropathic pain, translocator protein, remyelination, p-ERK, brain-derived neurotrophic factor

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Introduction

Neuropathic pain (NP), which is characterized by hyperalgesia, allodynia, and spontaneous pain, is considered as a major clinical and social problem. The refractory pain negatively impacts quality-of-life and leads to economic burden to patients. Despite the availability of various kinds of medications, such as non-steroidal anti-inflammatory, opioids, anticonvulsant, and antidepressant medicines, for clinical treatment, the general effects are not satisfactory.[1,2]

The translocator protein (TSPO, 18 kDa), which was first introduced as peripheral benzodiazepine receptor, is expressed throughout the whole body. It mediates the translocation of cholesterol from the outer to the inner mitochondrial membrane and represents an important rate-limiting step of the synthesis of neurosteroids. TSPO has received increased attention due to its

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involvement in chronic inflammation[3] and neurological disorders.[4] It is expressed in both neurons and glial cells in the nervous system.[5] Recently, neuroprotection of TSPO is reported in diabetic neuropathy,[6] inflammatory pain,[7] and NP.[5,8] Our previous study indicated that TSPO agonist (7-chloro-5–4-chlorophenyl)-1,3-dihydro-1-methyl-2-H-1,4-benzodiaze-pine-2) (Ro5-4864) elicited potent analgesic effects against NP, which may be attributed to inhibiting CXCL1-CXCR2-dependent astrocyte-to-neuron signaling and central sensitization in the spinal cord.[8] However, as most studies are based on the function of TSPO in spinal cord, its mechanism in the peripheral nervous system (PNS) is poorly understood. In the development of NP, dorsal root ganglia (DRGs), which metabolically supports physiologic functions between the PNS and the central nervous system, is a mediator involved in peripheral processes that lead to NP. Accordingly, we aimed to study TSPO in DRG.

When the peripheral nerve is injured, a complex response occurs, including axonopathy and demyelination. The demyelination is involved in the pathogenesis of mechanical pain hypersensitivity by disrupting the precise molecular and structural signature, including abnormal insertion of ion channels.[9,10] For example, the demyelination induces the abnormal expression and trafficking of Na^+ channels to increase cellular excitability.[11,12] A previous study indicated that after nerve injury, TSPO expression was increased and returned to normal level when regeneration was completed, suggesting a role for TSPO in nerve regeneration.[13] TSPO ligand SSR180575 increased the survival of motor neurons and the regeneration of peripheral nerves after facial nerve freeze injury.[14] Ro5-4864 also accelerated the regeneration of peripheral nerves.[15] Therefore, we wanted to explore the effects of TSPO agonist Ro5-4864 on the morphology of the sciatic nerve after spared nerve injury (SNI).

Myelin protein zero (P0) is a major protein component of peripheral nerve myelin proteins, which represents between 50% and 70% of the total protein. P0 is a 28-kDa integral membrane glycoprotein, which is appeared to be expressed by myelinating Schwann cells, but neither by the non-myelinating Schwann cells nor by the central glia cells. Peripheral myelin protein 22 (PMP22) is a smaller 22-kDa glycoprotein that was first purified and characterized from bovine peripheral nerve myelin. It represents 2–5% of the total protein. Therefore, our present study aimed to (1) study the antinociceptive effect of TSPO agonist Ro5-4864 in a rat SNI model, (2) observe the distribution and expression of TSPO in DRG, and (3) evaluate the effect of TSPO agonist on myelin regeneration in the injured sciatic nerve.

Materials and methods

Animals

Experiments were carried out using adult male Sprague–Dawley rats (body weight, 200 ± 20 g), which were provided by the Animal Center of Xinhua Hospital in Shanghai. All work corresponded with guidelines of the International Association for the Study of Pain. The animals were maintained in a temperature-controlled room ($23 \pm 1^{\circ}$C) with a 12-h light–dark cycle at the animal center of Xinhua Hospital for seven days before surgery.

Drugs and administration

Ro5-4864 was purchased from Sigma-Aldrich (St. Louis, MO), which was dissolved in dimethyl sulfoxide (DMSO). The TSPO agonist Ro5-4864 was dissolved in 100% DMSO and stored in sterile aliquots at -20°C. Immediately prior to administration, aliquots were thawed and diluted to the final concentrations of 0.5 μg/μl in 20% DMSO. Drugs or vehicle solutions were injected intrathecally. Animals in the SNI and sham group received 20% DMSO (2 μL/100 g). Spinal cord puncture was made with a 30-G needle between the L5 and L6 levels to deliver the reagents to the cerebral spinal fluid for i.t. injection.

SNI model

Rats were randomly assigned into one of the three groups: (1) the sham group, (2) the SNI (control) group, and (3) the Ro group. NP was established using the method as described previously.[16] In brief, rats were anesthetized by 10% chloral hydrate (400 mg/kg, intraperitoneally). The left common peroneal and tibial nerves were tightly ligated with 5.0 silk sutures and sectioned distal to the ligation with removal of 2–4 mm of the nerve stump, leaving the sural nerve intact. The animals in the sham group experienced the same operation except for nerve injury. The day on which SNI was performed was defined as day 1. In the Ro group, 2 μg of Ro5-4864 was injected intrathecally on day 3. Vehicles (4 μl) were injected intrathecally on day 3 in the sham and control groups. DRGs and sciatic nerves were collected on days 7 and 14, separately ($n = 3$ at each point of time in every group).

Assessment of mechanical allodynia

Animals were acclimated and basal pain sensitivity was tested before surgery (day 0). Behavioral testing was performed in all animals on days 3, 7, and 14 after SNI surgery or sham operation. The day of surgery was

referred as day 1. For mechanical withdrawal threshold, rats were placed in the testing chamber individually, which is consisted of a $30 \times 30 \times 30$ cm plastic boxes with a clear plastic floor containing 0.5 cm diameter holes. Each rat was allowed to acclimate for 30 min before testing. The withdrawal threshold was determined by "up-down" method[17] and expressed as the mean withdrawal threshold. The first filament applied was a force of 2 g. Each filament was applied three times at intervals of 5 s. The next filament of lesser force was applied if a positive response was observed. Otherwise, the next filament of greater force was used. The quick withdrawal or licking on the paw in response to the stimulus was considered as a positive response. The range of score was from 0.26 g to 26 g possibly.

Western blotting

The DRGs and the left common peroneal and tibial nerves distal to the ligation were harvested from different groups of rats (n = 3 at each point of time in every group). The ipsilateral sides were separated and immediately put into liquid nitrogen. After quantifications of the protein samples using BCA protein assay kit (Pierce Biotechonology, Rockford, IL), 40 μg of proteins was electrophoresed through a 15% sodium dodecyl sulfate-polyacrylamide gel loaded for each lane and then transferred to 0.4 μm polyvinylidene fluoride membranes for the proteins. The blots were incubated overnight at 4°C with antibodies (TSPO: 1:1000, rabbit, Santa Cruz Biotechnology, USA; brain-derived neurotrophic factor (BDNF): 1:1000, rabbit, Santa Cruz Biotechnology; phosphor-extracellular signal-regulated kinase (p-ERK): 1:1000, rabbit, Cell Signaling Technology; P0: 1:1000, rabbit, Abcam, UK; PMP22: 1:1000, rabbit, Abcam; Actin: 1:1000, mouse, Beyotime Biotechnology, China) and then incubated with a goat anti-rabbit or anti-mouse horseradish peroxides–conjugated secondary antibody (1:1000, Beyotime Biotechnology) for 1 h at room temperature. The immune complex was detected by enhanced chemiluminescence (Millipore) and exposed to x-ray film (Kodak). We used the ImageJ analysis system (National Institutes of Health (NIH), Bethesda, MD) to analyze specific bands.

Immunofluorescence

Rats (n = 3 at each point of time in every group) were deeply anesthetized and were perfused through the ascending aorta with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2–7.4, 4°C) . The L4 and L5 DRGs were then removed, postfixed in the same fixative at 4°C overnight, and then kept in 30% sucrose in 0.1 M PB overnight. Dissected tissue was mounted in optical coherence tomography compound and frozen at −80°C. DRGs (10 μm) were cut by a cryostat (LEICA CM1900 UV) and processed for immunofluorescence. The sections were briefly rinsed in phosphate-buffered saline (PBS) and blocked with the solution containing 5% normal donkey serum (Jackson) and 0.3% Triton X-100 for 1 h at room temperature. Free-floating tissue sections were then incubated overnight at 4°C on a rocker with the following primary antibodies: TSPO: 1:100, rabbit, Santa Cruz Biotechnology; NF200: mouse, 1:1000, Sigma Aldrich; Calcitonin Gene Related Peptide (CGRP): mouse, 1:1000, Sigma Aldrich; Isolectin GS-IB4 (IB4): 1:200, Invitrogen, USA. The sections were then washed three times with PBS for 5 min each and incubated with Alexa 488-conjugated anti-rabbit antibody or Alexa 594-conjugated anti-mouse antibody (1:300, Invitrogen) for 1 h at room temperature in the dark. Sections were washed three times with PBS for 5 min and mounted with antifade mounting medium (Beyotime, Haimen, Jiangsu Province, China). Images were acquired using a Leica microscope (Leica DMI300B). We used the ImageJ analysis system ((NIH), Bethesda, MD) to analyze the fluorescent density specific bands.

Morphological investigations and electron microscopy

Rats (n = 3 at each point of time in every group) were deeply anesthetized and were perfused through the ascending aorta with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PB (pH 7.2–7.4, 4°C) on days 7 and 14. The left common peroneal and tibial nerves distal to the ligation were rapidly removed, cut in small segments (1–2 mm length), and fixed by immersion in 2.5% glutaraldehyde in 0.1 M PB (pH 7.2–7.4, 4°C) for 24 h. Tissue samples were washed three times with PBS for 15 min each, post-fixed for 2 h in 1% osmic acid. And tissue samples were firstly dehydrated through an ascending series of alcohol (50, 70, and 90), 90% alcohol +90% acetone of 15 min each at 4°C, then through an ascending series of acetone (90% and 100%) three times for 15 min each. The samples were oriented longitudinally and embedded in Epon. Nerves were sectioned transversely using a LKB-I Ultra-cut microtome. Semi-thin sections (1 μm) stained with toluidine blue were prepared for evaluation under a light microscope. Ultrathin (50–60 nm) sections were stained with 3% uranyl acetate-lead citrate and then observed under PHILIPS CM-120 transmission electron microscope and photographed.

Statistical analyses

SPSS 19.0 was used to perform statistical analyses. For behavioral assessment, data were analyzed using

a repeated measures two-way analysis of variance (ANOVA). For Western blotting, data were analyzed by a one-way ANOVA followed by the least significant difference (LSD) test. For the analysis of immunoreactivity, six sections from the L4 or L5 DRGs were randomly selected in every animal (n = 3 per group). An image in a rectangle on the DRGs was captured with a numerical value of intensity and then using a computer-assisted imaging analysis system (Image J) to calculate. Data were analyzed by one-way ANOVA followed by the LSD test. For quantifying the myelin debris, new myelin sheaths, and wrapped axons, six semi-thin sections were randomly selected in every animal (n = 3 per group), five squares were randomly selected in every section and then we used a computer-assisted imaging analysis system (Image J) to calculate related data. Data were analyzed by one-way ANOVA followed by the LSD test. All data were presented as mean ± SD. In all statistical comparisons, $P < 0.05$ was considered as the criterion for statistical significance.

Results

Phase I: SNI-induced NP and TSPO activation in L4-L5 DRG

Behavioral testing was performed in all animals at baseline (day 0) and on days 3, 7, and 14 after SNI. The ipsilateral paw 50% withdrawal threshold significantly decreased in the SNI group during the whole period compared with the sham group ($P < 0.01$, Figure 1(a)). There was no difference between the two groups on the contralateral 50% paw withdrawal threshold (PWT) (Figure 1(a)). The expression of TSPO in L4-L5 DRG after SNI surgery was examined using immunofluorescence. The intensity of TSPO was relatively low at baseline, but significantly increased on days 7 and 14 (D7: $P < 0.05$, D14: $P < 0.01$, Figure 1(b) to (e)).

Double staining of TSPO using different cell markers was then performed to define the cellular distribution of TSPO. The TSPO signals were partly colocalized with

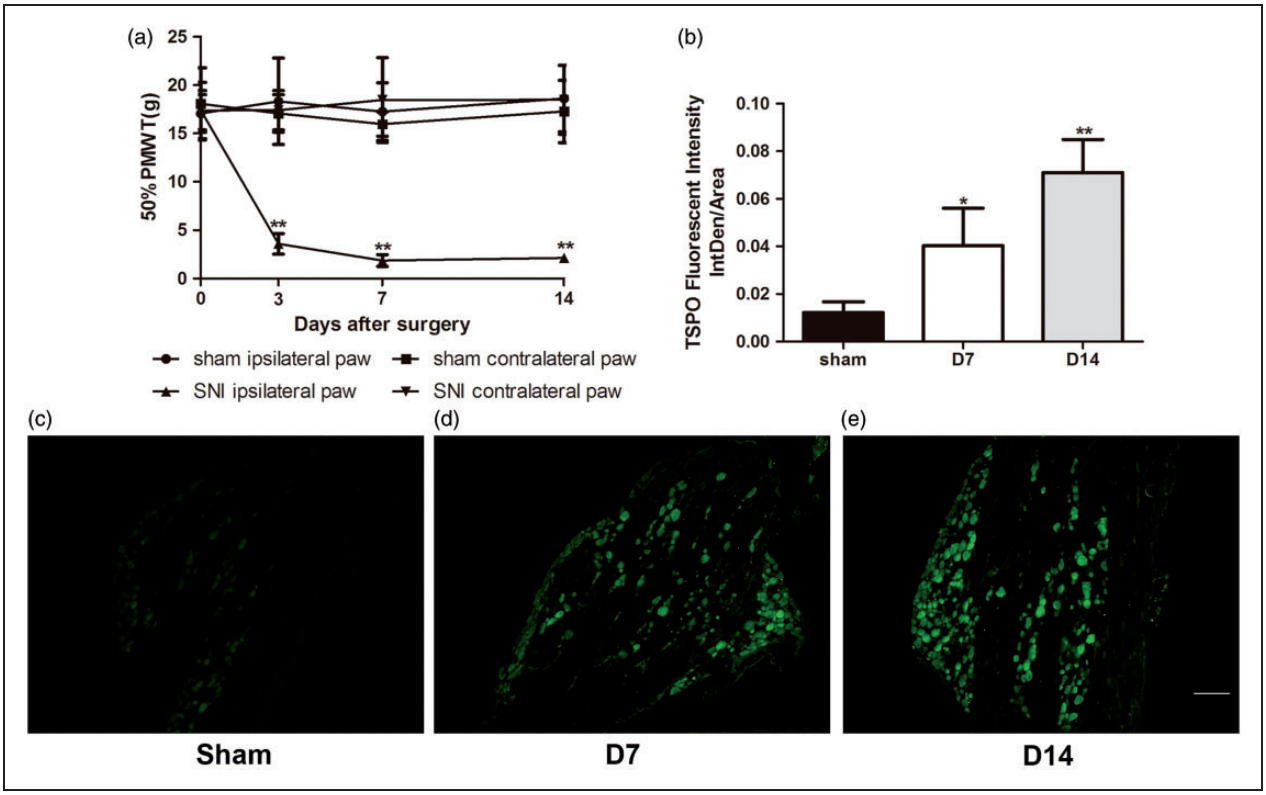

Figure 1. SNI induces mechanical allodynia and TSPO upregulation in DRG. (a) Paw mechanical withdraw threshold (PMWT) after SNI. Behavioral test was performed on days 3, 7, and 14. The results showed that PWTs of the ipsilateral paws were significantly reduced after SNI ($P < 0.01$). *$P < 0.05$ vs. sham group; **$P < 0.01$ vs. sham group. Repeated-measures two-way analysis of variance (ANOVA) were used to analyze PWT. Data are presented as mean ± SD (n = 6). (b) TSPO fluorescent intensity of the sham and SNI (D7, D14) group. Data were analyzed by a one-way ANOVA followed by the LSD test. (c–e) TSPO expression in DRG of the rats. Rats in the SNI group were heart perfused to remove L4-L5 DRG on days 7 and 14, the frozen sections by immunofluorescence showed that TSPO signals increased significantly after SNI. Magnification ×50, scale represented 200 μm. SNI: spared nerve injury.

the NF200 (47%), the IB4 (31%), and the CGRP (20%) but hardly with S100 (marker of satellite glial cells, 2%) (Figure 2). Therefore, the TSPO signals were primary colocalized with neurons in DRG.

Phase II: Effects of Ro5-4864 on NP and TSPO expression in DRG

A single-dose of TSPO agonist Ro5-4864 was administered intrathecally to investigate the analgesic effect against SNI. Our results showed that a single-dose i.t. administration of 2 μg Ro5-4864 increased the 50% PWT of the ipsilateral paw significantly ($P < 0.01$, Figure 3(a)). Western blots revealed that the TSPO protein content was remarkably increased after i.t. administration of Ro5-4864 on day 7 ($P < 0.01$), but decreased on day 14 ($P < 0.05$, Figure 3(b)).

According to the results of Western blots, the expression of BDNF was higher than the sham group ($P < 0.05$). After i.t. injection of Ro5-4864, the expression of BDNF was decreased ($P < 0.05$, Figure 4(a)). Similarly, compared with the sham group, p-ERK1 ($P < 0.01$) and p-ERK2 (D7: $P < 0.01$, D14: $P < 0.05$) were increased after SNI and the administration of Ro5-4864 reversed the upregulation (Figure 4(b)).

Phase III: Effects of Ro5-4864 on remyelination in the sciatic nerve

Light microscopy showed that there were severe degeneration of myelinated fibers and loss of myelinated axons in the distal nerves near the ligation spot on days 7 (Figure 5(b)) and 14 (Figure 5(e)). Compared with the sham group, the number of myelin debris significantly increased in the SNI and Ro group ($P < 0.01$, Figure 5(g)). The morphological changes following i.t. administration of Ro5-4864 in the nerves were illustrated in Figure 5(c) and (f). More new myelin sheaths wrapping axons appeared after the administration compared to the control group on day 14 ($P < 0.01$, Figure 5(h) and (i)).

Electron microscopy further revealed the loss of myelinated axons and degenerating myelinated fibers near the ligatures (Figure 6). Degenerated axon profiles (suggested by inflated axons and accumulated mitochondria) and demyelinated axons (with disrupted myelin lamellae and Schwann cell cytoplasm containing myelin debris) predominated on day 7. A few axons possessing particular thin myelin sheaths for their axoplasmic areas were present on day 14, possibly indicating early stages of remyelination. Compared with the control group, the lesions in the Ro group presented more proliferous Schwann cells and thicker myelin sheaths, probably indicating that i.t. administration of Ro5-4864 may accelerate remyelination.

For assessing the regenerated effects of TSPO agonist Ro5-4864 against demyelination following SNI in the distal nerves near the ligation, we then detected P0 and PMP22 expression using Western blotting analysis. Figure 7(a) indicated significant downregulation of P0 expression after SNI compared to the sham group ($P < 0.01$). The downregulation was reversed by the administration of Ro5-4864 on day 14 ($P < 0.01$, Figure 7(a)). The expression of PMP22 was found significantly downregulated compared to the sham animals ($P < 0.01$), which is similar to P0. Western blot analysis also showed that i.t. injection of Ro5-4864 significantly increased the PMP22 expression level, but the increase was present on day 7 ($P < 0.01$, Figure 7(b)), which was earlier than P0.

Discussion

Our present analyses demonstrated that TSPO was upregulated in DRG after nerve injury. A single i.t. injection of TSPO agonist Ro5-4864 on day 3 alleviated the allodynia by SNI. Furthermore, Ro5-4864 inhibited the activation of p-ERK and BDNF in DRG. In the sciatic nerve, SNI produces severe degeneration. The administration of Ro5-4864 accelerated the remyelination of the injured sciatic nerve and increased the expression of P0 and PMP22. These results proposed that TSPO might regulate P0/PMP22 to promote the remyelination in the sciatic nerve after SNI.

TSPO expression in DRG

Nerve injury leads to upregulation of many proteins, some of them are harmful and directly contribute to NP, such as sodium channels.[18] While others are self-defensive, such as TSPO or Liver X receptors,[19] which has been verified to promote recovery. The proteins promoting recovery are promising drug targets for NP treatment. In our study, the expression of TSPO in DRG increased after peripheral nerve injury, i.t. injection of 2 μg Ro5-4864 on day 3 induced a significant increase of TSPO on day 7 while inhibited the TSPO activation on day 14 compared with the control group. The probable mechanisms are as follows. Many scholars believe that the upregulation of TSPO expression is the endogenous protective response under stress stimulus. It decreased to normal levels when the nerves repair completed.[5] We previously assumed that TSPO serves as an intrinsic shield, which is usually in a resting state physiologically. When ''attacked by external enemies'' (e.g., suffered from injury or oxidative stress), the shield is activated as one kind of self-defense or self-protection. And the shield will return to inactive state once the recovery process completed. If the shield is tough enough, the natural restoration and cure may occur by

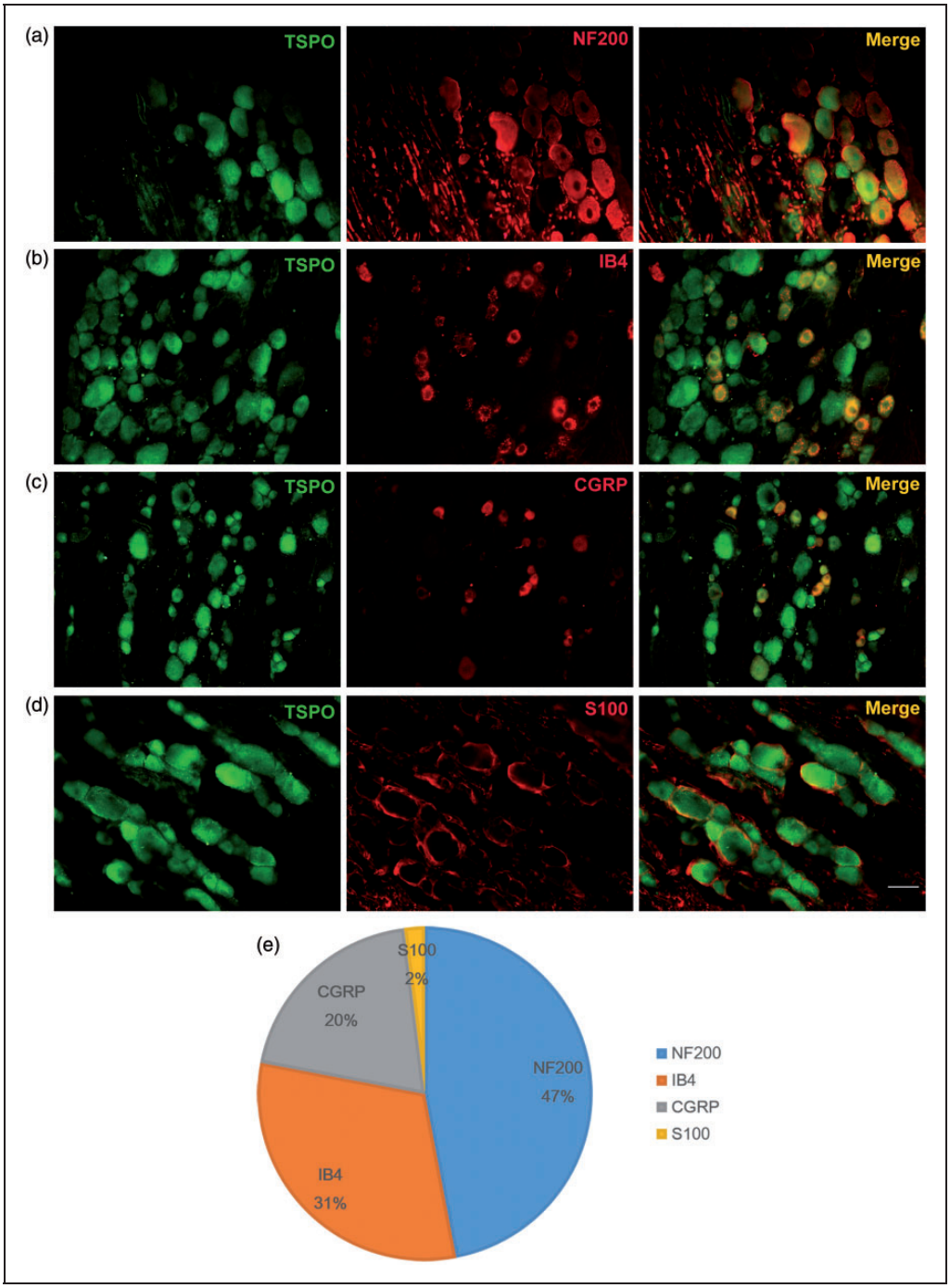

Figure 2. Distribution of TSPO in DRG. Rats in the SNI group were heart perfused to remove L4-L5 DRG on day 7, the frozen sections by double staining of immunofluorescence (a–d) showed that TSPO was colocalized with NF200, IB4, Calcitonin Gene Related Peptide (CGRP), and S-100. Magnification ×200, scale represented 50 μm. (e) Percentage of TSPO distribution in DRG. TSPO: translocator protein.

itself. Early administration of exogenous TSPO agonists may help to reinforce the "shield" and promote the recovery.[8] According to our result, the duration for TSPO activation was shortened by Ro5-4864 compared with the control group, which implied that the recovery process may be accelerated by TSPO agonist after SNI injury.

TSPO inhibiting the activation of BDNF and p-ERK

After peripheral nerve injury, the mRNA and protein level of BDNF in DRG were peaked on the first day, then decreased, and kept higher than the normal level until 14 days after surgery.[20] In our study, the level of BDNF and p-ERK increased after SNI and was

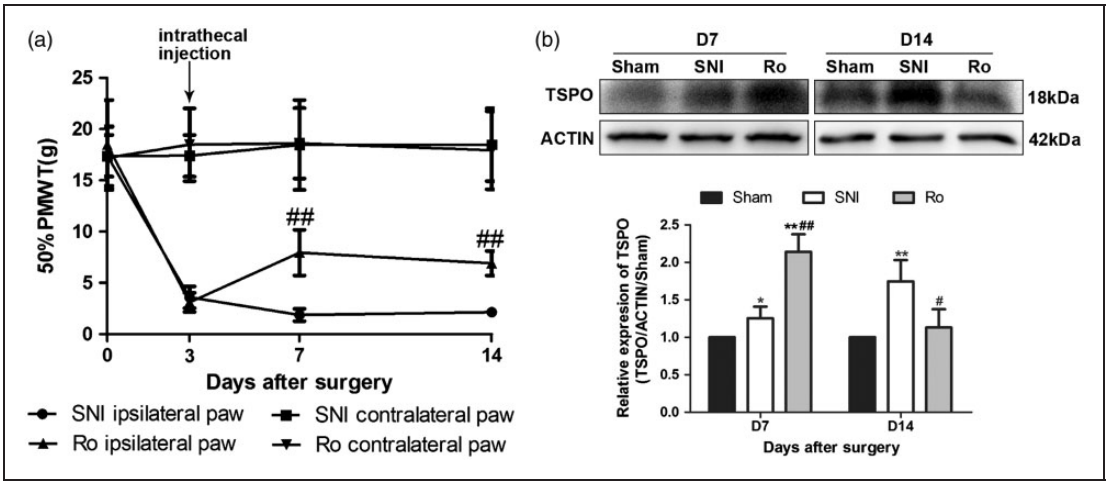

Figure 3. Intrathecal injection of Ro5-4864 alleviates mechanical allodynia and regulates TSPO expression in DRG. (a) Analgesic effects of Ro5-4864. After behavioral testing was performed in the Ro group, Ro5-4864 was injected intrathecally on day 3. Arrow indicates the time of intrathecal injection. Repeated-measures two-way analysis of variance (ANOVA) were used to analyze PWT. Data are presented as mean ± SD (n = 6). (b) TSPO expression in the DRG after intrathecal injection of Ro5-4864. Western blots revealed the expression of TSPO on days 7 and 14. One-way analysis of variance followed by LSD tests was used at each time, and n = 3 for each group at each time. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SNI group. TSPO: translocator protein; PMWT: paw mechanical withdraw threshold; SNI: spared nerve injury.

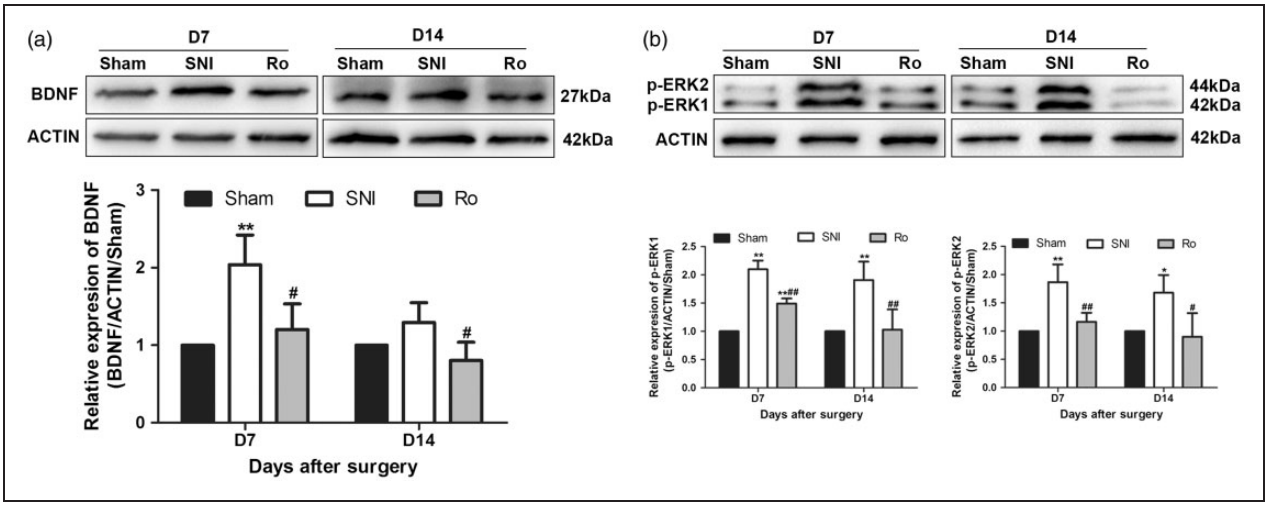

Figure 4. Ro5-4864 down-regulates BDNF and p-ERK expression. (a) Effects of Ro5-4864 on brain-derived neurotrophic factor (BDNF) in DRG. One-way analysis of variance followed by least significant difference (LSD) tests was used at each time, and n = 3 for each group at each time. (b) Effects of intrathecal injection of Ro5-4864 on phospho-extracellular signal-regulated kinase (p-ERK) activation in DRG. One-way analysis of variance followed by LSD tests was used at each time, and n = 3 for each group at each time. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group at the same point in time; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SNI group at the same point in time. SNI: spared nerve injury.

decreased after the i.t. injection of Ro5-4864. TSPO can regulate the synthesis of steroid hormones. BDNF levels were increased after brain injury, and the levels of Pro-BDNF and mature BDNF were decreased after treatment with steroid hormone progesterone (PROG).[19] In our study, the level of BDNF increased after SNI and was decreased after the i.t. injection of Ro5-4864. Thus, we hypothesized that TSPO receptor agonists in DRG may decrease BDNF levels by modulating the synthesis of steroid hormones. The activation of ERK upregulates BDNF in DRG neurons in peripheral inflammation and NP.[21] On the other hand, BDNF could promote ERK phosphorylated in neurons.[22] Our previous study also found that TSPO might be indirect through inhibition

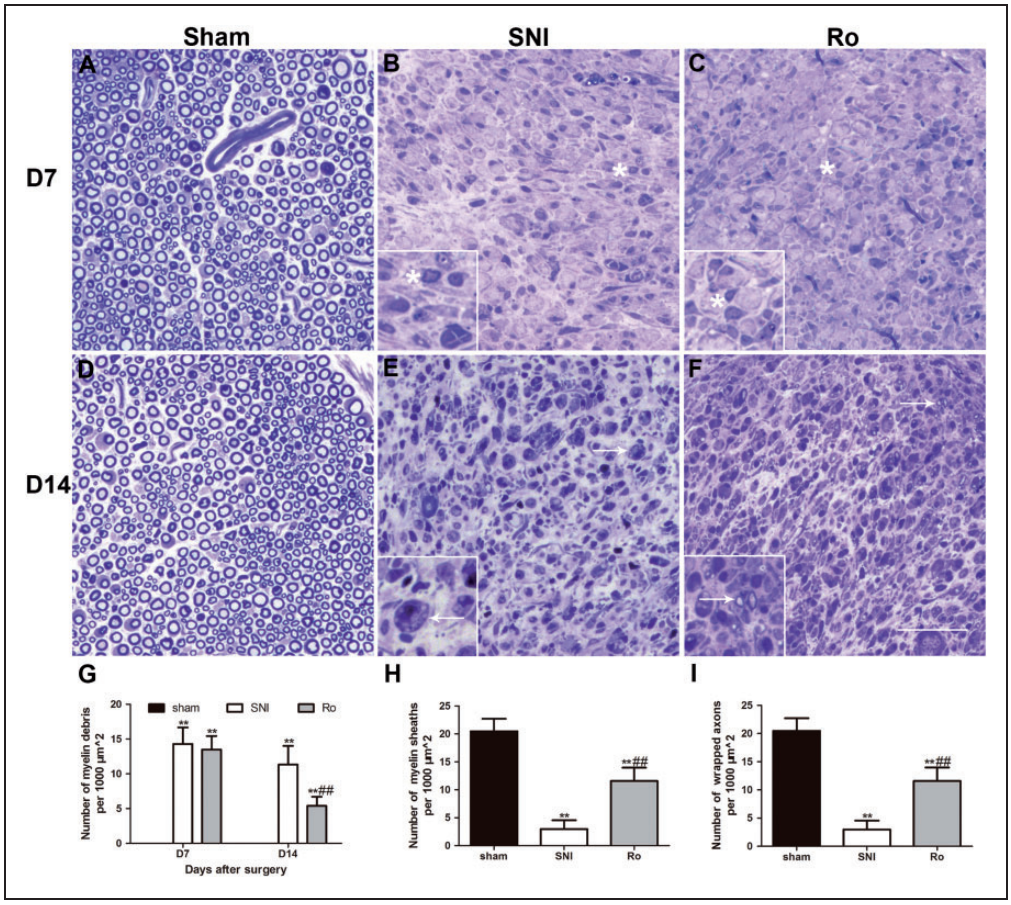

Figure 5. Samples are semi-thin sections with toluidine blue staining in the sciatic nerve. (a) The morphology of the sciatic nerve was normal, the myelin sheath was round or oval and there was no obvious axonal degeneration and myelin loss. (b)The normal morphology disappeared and severe degeneration was apparent, including degeneration of axons, demyelination, and myelin debris. (c) The morphology was similar to (b), its structure was denser and there were more Schwann cells. Myelin debris was shown by asterisks. (d) The morphology was similar to (a). (e) The degeneration was still serious, but a little of new myelin sheaths wrapping axons appeared. (f) The degeneration still existed, but some new myelin sheaths wrapping axons appeared. Myelin debris was shown by asterisks. The arrows showed the new myelin sheaths wrapping axons, the new myelin sheath was small and the sheath was thin. Original magnification, ×200, scale bars correspond to 50 μm. (g) Quantification for the number of myelin debris in the three groups on D7 and D14. (h) Quantification for the number of myelin sheaths in the three groups on D14. (i) Quantification for the number of wrapped axons in the three groups on D14. Data were analyzed by one-way ANOVA followed by the LSD test. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group at the same point in time; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SNI group at the same point in time. SNI: spared nerve injury.

of CXCL1 and other chemokines such as interleukin-8 to decrease the level of p-ERK in the spinal cord.[8]

TSPO alleviating NP

Our previous studies showed that TSPO agonist inhibit astrocyte activation in the spinal cord and further inhibited p-JNK1-CXCL1-CXCR2 signaling pathway and central sensitization to alleviate hyperpathia.[8] In steroid-derived cells, TSPO mediates the translocation of cholesterol from the outer to the inner mitochondrial membrane, which is a rate-limiting step for the synthesis of steroid hormones and neurosteroids.[23] The neurosteroids modulate the receptors by autocrine or paracrine in DRG, further regulate the role of DRG. Many steroids have analgesic effects. For example, PROG is metabolized to 5α-DHT and 3α, 5α-THP. 3α, 5α-THP, whose concentration regulates the excitability of sensory neurons in DRG, can produce analgesic effects by the T-type calcium channel and modulate the $GABA_A$ receptor.[24] Therefore, TSPO in DRG may produce analgesic effects through the regulation of steroid hormones. BDNF is synthesized in DRG neurons and transported to the nerve terminals of the spinal dorsal horn. In the spinal dorsal horn, BDNF promotes central sensitization by regulating neurotransmitters to produce NP.[25] On the other hand, BDNF activates astrocytes and microglial cells to regulate NP.[26] Direct injection of exogenous

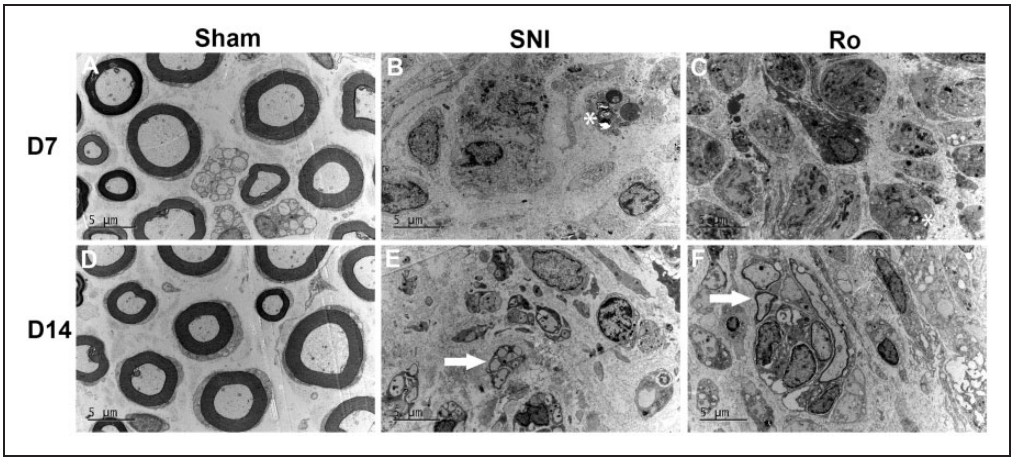

Figure 6. Observing the morphology of the sciatic nerve by transmission electron microscopy. (a) The morphology of the sciatic nerve was normal, the myelin sheath was round or oval, the myelin sheaths arranged dense and there was no obvious axonal degeneration and myelin loss. (b) The normal morphology disappeared, axonal degeneration (including the expansion of axons, accumulation of mitochondria and myeloid), demyelination and myelin debris were apparent. (c) The morphology was similar to (b), its structure was denser and there were more Schwann cells. (d) The morphology was similar to (a). (e) Many Schwann cells were wrapping the axons, the myelin sheaths were extremely thin for their axoplasmic areas or absent, possibly indicating early stages of remyelination. (f) Piles of Schwann cells formed particularly thin myelin sheaths to wrap nerve axons. Myelin debris was shown by asterisks. The arrows showed Schwann cells were forming the new myelin sheaths to wrap the regenerated axons. Original magnification, $\times$1680, scale bars correspond to 5 μm. SNI: spared nerve injury.

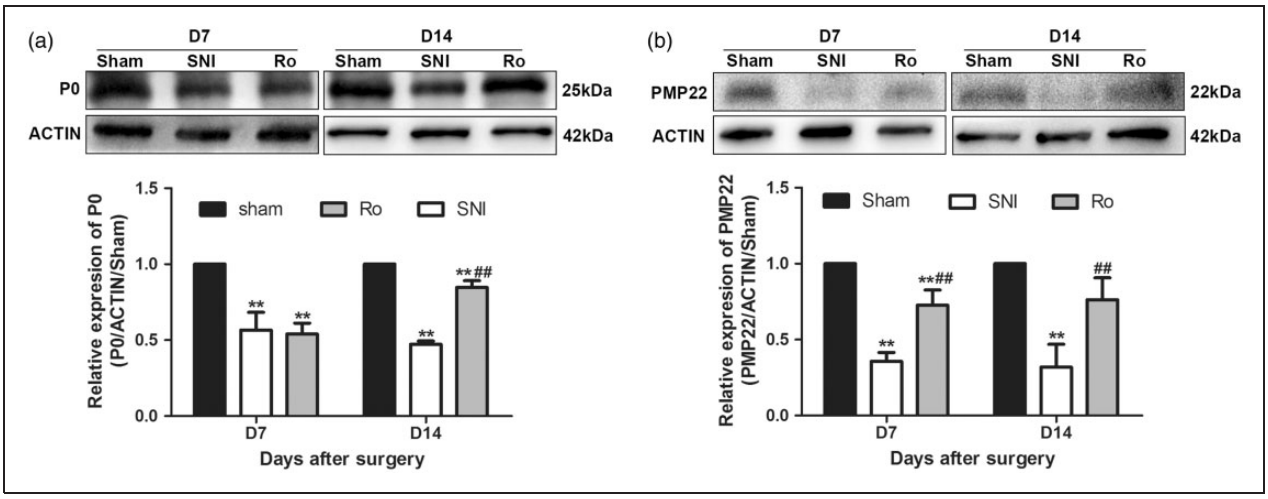

Figure 7. Effects of Ro5-4864 on the expression of P0, PMP22 in the sciatic nerve. (a) Effects of Ro5-4865 on P0 expression in the sciatic nerve. One-way analysis of variance followed by LSD tests was used at each time, and n = 3 for each group at each time. (b) Effects of intrathecal injection of Ro5-4864 on PMP22 activation in the sciatic nerve in. One-way analysis of variance followed by LSD tests was used at each time, and n = 3 for each group at each time. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SNI group. SNI: spared nerve injury; PMP: peripheral myelin protein.

BDNF can cause mechanical hyperpathia. Increased BDNF in DRG after nerve injury may reduce large conductance K^+ channel l activity through Tyrosine kinase B receptors, resulting in the hyperexcitability of primary afferent neurons and increasing the transmission of nociceptive information in the spinal cord, leading to NP.[27] The activation of ERK upregulates BDNF in DRG neurons in peripheral inflammation and NP.[21] Moreover, in NP, the phosphorylation of ERK in DRG upregulates Neuropeptide Y (NPY) in damaged neurons. NPY in DRG could alter the excitability of neurons and regulate pain.[21] In the model of chronic compression of DRG(CCD), CCD inhibits A-type rapidly inactivated potassium channels by activating ERK

to raise the excitability of DRG neurons.[28] Therefore, the mechanism of TSPO regulating NP may also due to the downregulation of p-ERK and BDNF.

TSPO promoting remyelination

Our study observed that i.t. administration of Ro5-4864 may promote remyelination. Compared with the control group, the samples in the Ro group morphologically presented more Schwann cells and thicker regenerated myelin sheaths. Schwann cells not only produce and retain the myelin sheath but also maintain the developmental plasticity to break down myelin in response to injury, secrete molecules to support axonal survival, and then eventually remyelinate axons in regenerating nerves[29–31]; however, their ability to survive and support axon growth declines within 8 weeks of denervation because of apoptotic cell death.[32,33] TSPO, as a major component of MPTP, plays an important role in mitochondrial functions. TSPO ligand can reduce membrane potential consumption, reduce the loss of mitochondrial Ca^{+} and release of apoptosis-inducing factors, further inhibit mitochondrial permeability and reduce the release of cytochrome c to reduce apoptosis.[34] Thus, the anti-apoptotic properties of TSPO ligand may have an important role in the survival of Schwann cells, which further accelerates the regeneration.[30]

We observed the regeneration effects of TSPO by evaluating the levels of P0 and PMP22. In this study, we demonstrated that SNI, which produced severe demyelination, significantly decreased the P0 and PMP22 levels in the sciatic nerve. The downregulation of P0 produced by SNI was greatly reversed by Ro5-4864 on day 14, when electron microscopy revealed the remyelination began. These findings suggested that the administration of Ro5-4864 promoted the remyelination of the myelin sheath. In the PNS, some TSPO ligands are shown to display neuroprotective properties in models of toxic and diabetic-induced neuropathies.[6,35] Ro5-4864 is able to exert beneficial effects on aging-associated myelin degeneration of rat sciatic nerve.[36] In vivo, Ro5-4864 accelerated the regeneration of facial nerve.[15] TSPO mediates the conversion of cholesterol to pregnenolone and its steroid derivatives. Most importantly, it has been shown that PROG and its metabolites not only promote the viability and regeneration of neurons but also act on the Schwann cells in the PNS and play an important role in promoting myelin formation.[37,38] For example, PROG and its derivatives can modulate the expression of myelin proteins in the PNS, such as P0 and PMP22,[39,40] and stimulate the expression of their gene promoters in cultured rat Schwann cells.[40] Neuroactive steroids have been reported to motivate the expression of P0 through classical PROG receptor and the expression of PMP22 through GABA (A) receptor in Schwann cell cultures.[38] Therefore, we supposed that TSPO may regulate P0/PMP22 through the synthesis of neurosteroids to accelerate the remyelination.

However, there are some limitations in our study. First, we observed TSPO was primarily colocalized with the NF 200-positive neurons, nearly 47 % among all $TSPO^{+}$ areas. Other studies found that TSPO appeared in small- and medium-sized neurons after sciatic nerve transaction or axotomy in rat[41] and mouse.[15] Under physiological state, small- and medium-sized DRG neurons are believed to mediate nociceptive behavioral responses to painful stimuli, while large-sized DRG neurons (NF 200-positive neurons) are believed to translate non-nociceptive signals. However, some studies indicated that hypersensitivity (ectopic discharge) in large-sized DRG neurons, especially A-beta fiber subtype, may be a crucial driver to mechanical allodynia in NP.[42–44] So we assume TSPO may inhibit hypersensitivity in NF200-positive neurons to alleviate NP, but more research is needed to evaluate the hypothesis. Second, regeneration was appeared on day 10 after nerve injury. Many axons possessing extremely thin myelin sheaths for their axoplasmic areas were present and continuing at least through 42 days, the number, size, and myelin thickness of myelinated axons increased with the time.[45] In our study, we preliminarily observe the morphological changes of sciatic nerve after SNI until day 14, the later time after injury was not explored. Third, we found i.t. injection of TSPO agonist Ro5-4864 could exert remarkable analgesic effect in NP and promote the regeneration of injured sciatic nerve. However, direct or indirect cooperation with regeneration to hyperalgesia behaviors in NP state was not explored. Further studies are needed to figure out these problems.

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Author Contributions

Bingjie Ma was responsible for study design, conduct of the study, data collection, data analysis and manuscript preparation. Xiaoming Liu was responsible for study design, conduct of the study, data analysis and manuscript preparation. Xuehua Huang participated in conduct of the study and data analysis. Yun Ji participated in conduct of the study and data collection. Tian Jin participated in conduct of the study and data collection. Ke Ma Was responsible for study design, conduct of the study, data analysis and manuscript preparation.

Declaration of Conflicting Interests

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