Neurobiology of Disease

The Upregulation of Translocator Protein (18 kDa) Promotes Recovery from Neuropathic Pain in Rats

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At present, effective drug for treatment of neuropathic pain is still lacking. Recent studies have shown that the ligands of translocator protein (TSPO, 18 kDa), a peripheral receptor for benzodiazepine, modulate inflammatory pain. Here, we report that TSPO was upregulated in astrocytes and microglia in the ipsilateral spinal dorsal horn of rats following L5 spinal nerve ligation (L5 SNL), lasting until the vanishing of the behavioral signs of neuropathic pain (~50 d). Importantly, a single intrathecal injection of specific TSPO agonists Ro5-4864 or FGIN-1-27 at 7 and 21 d after L5 SNL depressed the established mechanical allodynia and thermal hyperalgesia dramatically, and the effect was abolished by pretreatment with AMG, a neurosteroid synthesis inhibitor. Mechanically, Ro5-4864 substantially inhibited spinal astrocytes but not microglia, and reduced the production of tumor necrosis factor-α (TNF-α) *in vivo* and *in vitro*. The anti-neuroinflammatory effect was also prevented by AMG. Interestingly, TSPO expression returned to control levels or decreased substantially, when neuropathic pain healed naturally or was reversed by Ro5-4864, suggesting that the role of TSPO upregulation might be to promote recovery from the neurological disorder. Finally, the neuropathic pain and the upregulation of TSPO by L5 SNL were prevented by pharmacological blockage of Toll-like receptor 4 (TLR4). These data suggested that TSPO might be a novel therapeutic target for the treatment of neuropathic pain.

Introduction

Chronic neuropathic pain, characterized by hyperalgesia (increase in response to noxious stimuli), allodynia (decrease in pain threshold), and spontaneous pain, has been long considered as a disease of the nervous system (Basbaum, 1999) and has been intensively studied for many years. Effective treatment for the disease, however, is still unmet in clinical practice.

The translocator protein (TSPO, 18 kDa), previously called peripheral benzodiazepine receptor, is localized predominantly on the outer mitochondrial membrane (Garnier et al., 1993; Culty et al., 1999). In the nervous system, TSPO is expressed in both glial cells and neurons (Veiga et al., 2005). Activation of TSPO is reported to be beneficial for several kinds of neurological diseases, such as injury of peripheral nerve or brain (Veiga et al., 2005), multiple sclerosis (Vowinckel et al., 1997), and Alzheimer's disease (Yasuno et al., 2008), by promoting neurosteroid synthesis (Rupprecht et al., 2010). TSPO agonist Ro5-4864 diminishes mechanical allodynia and thermal hyperalgesia induced by complete freund's adjuvant (Hernstadt et al., 2009) and prevents the first- and second-phase responses in the formalin test (DalBó et al., 2004). These data suggest that activation of TSPO is effective for treatment of inflammatory pain. However, the role of TSPO in neuropathic pain is still unclear.

An increasing body of evidence suggests that activation of glial cells in spinal dorsal horn plays an important role and that microglia and astrocytes may be involved in the initiation and maintenance of neuropathic pain, respectively (Watkins and Maier, 2003; Tsuda et al., 2005). The activated glial cells contribute to neuropathic pain by release of a variety of substances including tumor necrosis factor-α (TNF-α), interluklin-1β (IL-1β), IL-6, prostaglandins, and brain-derived neurotrophic factor (Clark et al., 2006; Zhuang et al., 2006; Hsieh et al., 2007; Zhang and Neufeld, 2007; Zhao et al., 2007; Zhou et al., 2011).

Toll-like receptor 4 (TLR4), a pattern recognition receptor, is related to immune and inflammatory diseases. Once activated by the endogenous or exogenous ligands, TLR4 induces the massive production of proinflammatory cytokines such as TNF-α via activation of nuclear factor kappaB (NF-κB) (Janeway and Medzhitov, 2002; Akira et al., 2006). Several lines of evidence have shown that TLR4 is expressed in astrocytes and microglial cells (Blanco and Guerri, 2007; Villalba et al., 2012). It has been shown that TNF-α causes an increase in TSPO mRNA (Rey et al., 2000); however, to date, no study has been conducted to investigate the role of TLR4 in the upregulation of TSPO in the spinal dorsal horn after peripheral nerve injury.

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To evaluate the role of TSPO in neuropathic pain, in the present study we first studied the time course of TSPO expression in L5 spinal dorsal horn following L5 SNL, and then investigated the effects of TSPO agonist Ro5-4864 and FGIN-1-27 on maintenance of neuropathic pain. The mechanisms of the TSPO upregulation and of the effect of TSPO agonist on neuropathic pain were also investigated *in vivo* and *in vitro*.

Materials and Methods

Animals. Male Sprague Dawley rats weighing 180–200 g were used. The rats were housed in separated cages and the room was kept at 24 ± 1°C temperature and 50–60% humidity, under a 12/12 h light/dark cycle and with *ad libitum* access to food and water. All experimental procedures were approved by the Local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animal (Zimmermann, 1983).

Drugs. Ro5-4864 (7-chloro-5–4-chlorophenyl)-1,3-dihydro-1-methyl-2-*H*-1,4-benzodiaze-pine-2), FGIN-1-27 (*N*,*N*-Dihexyl-2-(4-fluorophenyl) indole-3-acetamide 2-(4-fluorophenyl)-*N*), and AMG (*R*(+)-*p*-aminoglutethimide) were purchased from Sigma, and were dissolved in DMSO (dimethyl sulfoxide), stored as a stock solution of 0.1 M at −20°C, and diluted in sterile PBS to the appropriate concentration immediately before administration. Lipopolysaccharides (LPS) from *Escherichia coli* O127: B8 (Sigma) or the TLR4 antagonist LPS-RS (LPS Rhodobacter sphaeroides, Invitrogen) was reconstituted in sterile PBS to obtain a stock solution of 1 mg/ml or 5 mg/ml, respectively, aliquoted in small volumes, stored at −80°C, and diluted to final concentrations when used.

Surgical procedures and drug delivery. Intrathecal catheters were implanted according to the method described previously (Wei et al., 2007). Briefly, under sodium pentobarbital anesthesia (40 mg/kg, i.p.), a sterile PE-10 tube filled with saline was inserted through L5/L6 intervertebrae space, and the tip of the tube was placed at the spinal lumbar enlargement level. During the same surgery period, the L5 transverse process was removed to expose the L5 spinal nerve). The L5 spinal nerve was isolated carefully and ligated tightly with 6–0 silk thread 5–10 mm distal to the L5 DRG as described by Kim and Chung (1992). The animals in the sham operation group received the same operation except for ligation of the nerve. A complete hemostasis was confirmed and the wound was sutured in two layers. The rats with hindlimb paralysis or paresis after surgery were excluded. The successful catheterization was confirmed by bilateral hindlimb paralysis following injection of 2% lidocaine (7 μl) through the catheter within 30 s. Drugs or vehicle were administered in volumes of 10 μl followed by a flush of 7 μl of saline to ensure drugs delivered into the subarachnoid space.

Behavioral tests. Animals were habituated and basal pain sensitivity was tested before drug administration or surgery. Mechanical sensitivity was assessed with the up-down method described previously (Chaplan et al., 1994), using a set of von Frey hairs with logarithmically incremental stiffness from 0.6–15 g (0.6, 1, 1.4, 2, 4, 6, 8, 15 g). The 2 g stimulus, in the middle of the series, was applied first. In the event of paw withdrawal absence, the next stronger stimulus was chosen. On the contrary, a weaker stimulus was applied. Each stimulus consisted of a 6 to 8 s application of the von Frey hair to the sciatic innervation area of the hindpaws with a 5 min interval between stimuli. The quick withdrawal or licking on the paw in response to the stimulus was considered as a positive response. To test whether Ro5-4864 could affect the mechanical sensitivity in the sham rats, the incidence of paw withdrawal to von Frey hairs of four different strengths (2, 6, 15, and 26 g) was measured (Ringkamp et al., 1999; Eschenfelder et al., 2000). Testing always started with the lowest von Frey hair. Each von Frey hair was applied repetitively eight times with a frequency of ~1/s to the test area. Ten trials were performed on each hindpaw. For statistical analysis the incidence of positive responses, i.e., the percentage of positive trials, was recorded. Heat hypersensitivity was tested using a plantar test (7370, UgoBasile) according to the method described by Hargreaves et al. (1988). Briefly, a radiant heat source beneath a glass floor was aimed at the plantar surface of the hindpaw. Three measurements of latency were taken for each hindpaw in each test session. The hindpaw was tested alternately with >5 min intervals between consecutive tests. The three measurements of latency per side were averaged as the result of per test. Two persons performed the behavioral tests and only one knew the design of the study.

Mixed spinal glial cell cultures. Primary rat spinal cord mixed glial cells were prepared as described previously (Warf et al., 1991). Briefly, spinal cords from newborn Sprague Dawley rats were dissected and the meninges were removed. The tissue was broken into pieces with a pair of sclerotic scissors and suspended in HBSS (Invitrogen) containing 0.125% trypsin (Invitrogen) in the presence of DNase (100 μg/ml) for 10 min at 37°C. After adding trypsin inhibitor, the cells were triturated with a thin flame-polished glass pipette in DMEM-F12 medium containing 10% fetal calf serum (Hyclone) and subjected to centrifugation (5 min, 800 × *g*). The cells were resuspended in DMEM-F12 containing 10% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) and subsequently sieved through a 100 μm and a 30 μm mesh, respectively, to remove tissue clumps. The single spinal cord cell solution was plated at densities of either 2.5×10^5 cells /ml (high) in 6-well plates for Western blot experiments or 5×10^4 cells /ml (low) in 24-well plates for immunocytochemistry and incubated at 37°C in a humidified 95% air-5% CO_2. Non-adhering cells were removed 24 h after plating and the culture medium was replaced. Twelve to 14 d after plating, the mixed glial cultures were washed twice with warm DMEM and cultured in serum-free medium for at least 18 h before treatments. The ratio of microglia and astrocytes in the mixed cultures was determined by immunofluorescence, which showed that 70–80% of the cells were GFAP-positive and 10–15% Iba1-positive, indicating that majority of the cells was astrocytes. Cell viability was assessed by MTT assays. Briefly, cells (2×10^4 cells in 100 μl of medium per well) were seeded in 96-well microtiter plates and treated with various reagents for the indicated time periods. After various treatments, medium was removed and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma) was added followed by incubation at 37°C for 4 h in a CO_2 incubator to allow the conversion of MTT into formazan crystals. After a brief centrifugation, supernatants were carefully removed and DMSO was added. After insoluble crystals were completely dissolved, absorbance at 490 nm was measured using an iMark Microplate Reader (Bio-Rad).

Immunohistochemistry. Rats were perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4, 4°C. The L5 spinal cord segments were removed and postfixed in the same fixative for 3 h and then replaced with 30% sucrose overnight. Transverse free-floating spinal sections (25 μm) were cut in a cryostat (LEICA CM1900) and processed for immunohistochemistry following the method described previously (Wei et al., 2007). For immunocytochemistry, the mixed glial cells were fixed with 4% paraformaldehyde for 15 min and washed in PBS for three times (5 min each) at room temperature. Then the fixed cells were processed for immunostaining. All of the cryostat sections and fixed cells were blocked with 3% donkey serum in 0.3% Triton X-100 for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-TSPO antibody (1:500; Trevigen). The sections and cells were then incubated for 1 h at room temperature with Cy3- or FITC-conjugated secondary antibody (1:500; Jackson ImmunoResearch). To verify the specificity of TSPO antibody, the negative control sections were processed as above procedures but omitting the primary antibody. For double immunofluorescence staining, spinal sections were incubated with a mixture of rabbit anti-TSPO antibody and mouse monoclonal neuronal-specific nuclear protein (NeuN, neuronal marker, 1:200; Millipore Bioscience Research Reagents), glial fibrillary acidic protein (GFAP, astrocyte marker, 1:1000; Cell Signaling Technology), or a goat polyclonal anti-ionized calcium-binding adaptor molecule 1 (Iba1, microglia marker, 1:800; Abcam), while cells were incubated with a mixture of anti-rat TSPO antibody and GFAP, or Iba1 overnight at 4°C, all of the above sections and cells were treated by a mixture of FITC- and Cy3-conjugated secondary antibody for 1 h at room temperature. The stained sections or cells were examined with an Olympus IX71 (Olympus Optical) fluorescence microscope and images were captured with a CCD spot camera.

For quantification of immunofluorescence staining, the area of TSPO-IR per section was measured in spinal dorsal horn (laminae I–V)

using LEICA Qwin V3 digital-image processing system. A density threshold was set above background level first to identify positively stained structure. The area occupied by these structures was measured as positive area. In each spinal cord, every fifth section was picked from a series of consecutive spinal cord sections, and four to six sections for each condition were selected randomly. An average percentage of area of TSPO-IR relative to the total area of the spinal dorsal horn of the sections was obtained for each animal across the different tissue sections, and then the means ± SEM across animals was determined. Six rats were included for each group.

Western blotting. The dorsal quadrants of L5 spinal cord were harvested from different groups of rats (three rats at each group). The ipsilateral side were separated and put into liquid nitrogen immediately, followed by homogenization in 15 mM Tris buffer, pH 7.6 [250 mM sucrose, 1 mM MgCl, 1 mM DTT, 2.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, 1.25 μg/ml pepstatin, 2.5 μg/ml Aprotin, 2 mM sodium pyrophosphate, 0.1 mM $NaVO_4$, 0.5 mM PMSF, and protease inhibitor cocktail (Roche Molecular Biochemicals)]. The tissues were sonicated on ice, and then centrifuged at 13,000 × *g* for 15 min at 4°C to isolate the supernatant containing protein samples.

Proteins were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Bio-Rad). The blots were blocked with 5% w/v nonfat dry milk in TBST (20 mM Tris-base, pH 7.6, 137 mM NaCl and 0.1% Tween 20) for 1 h at room temperature and then incubated with primary antibodies, including goat polyclonal anti-TSPO antibody (1:200), mouse monoclonal anti-GFAP (1:1000), mouse monoclonal β-actin antibody (1:500), and goat polyclonal anti-Iba1 (1:500) antibody, overnight at 4°C with gentle shaking. The blots were washed three times for 10 min each with washing buffer (TBST) and then incubated with secondary antibody horseradish peroxidase (HRP)-conjugated rabbit anti-goat or goat anti-mouse IgG (1:8000 or 1:5000, Cell Signaling Technology) for 2 h at room temperature. After incubation with the secondary antibody, the membrane was washed again as above. The immune complex was detected by ECL (GE Healthcare) and exposed to x-ray film (Kodak). The band intensities on the film were analyzed by densitometry with a computer-assisted imaging analysis system (KONTRON IBAS 2.0).

ELISA. The dorsal quadrants of L5 spinal dorsal horn was rapidly harvested and homogenized in PBS followed by centrifugation at 4°C for 15 min at 13,000 × *g*. The supernatants, together with the glial cell culture medium, were used to measure the concentrations of TNF-α, using corresponding ELISA kits (R&D Systems). According to the manufacturer's instructions, the absorbance (A) was detected at 450 nm (A450) and standard curve was delineated based on the A of standards.

Quantification and statistics. All data were expressed as means ± SEM. The data of behavioral tests were analyzed using repeated measures two-way ANOVA with time and treatment as main effects. For the data of immunofluorescence, differences in changes of values over time were tested using one-way ANOVA followed by individual *post hoc* comparisons (Tukey *post hoc* tests). The relative densities of Western blots and TNF-α concentration measured by ELISA between different groups were compared using ANOVA with the least significant difference test (LSD-*t*). Statistical tests were performed with SPSS 10.0. $p < 0.05$ or less was considered significant.

Results

Upregulation of TSPO in the spinal dorsal horn is closely correlated with the behavioral signs of neuropathic pain following L5 SNL

Following L5 SNL the expression of TSPO changed with time. Compared with the sham group (Fig. 1*A*), the percentage of TSPO-IR-positive area in the ipsilateral L5 spinal dorsal horn increased significantly on day 4 (Fig. 1*C*,*I*, $F_{7, 32} = 80.58$, $p < 0.001$) but not on day 1 after L5 SNL (Fig. 1*B*,*J*, $p > 0.05$), reached a peak on day 14 (Fig. 1*E*,*I*, $p < 0.001$), remained at the high level for at least 35 d (Fig. 1*G*,*I*, $p < 0.001$), and then returned to baseline on day 50 (Fig. 1*H*,*I*, $p > 0.05$) after the surgery. Compared with the sham group, the area of TSPO-IR increased to 585.34 ± 30.25% in laminae I–II, to 344.40 ± 52.52% in laminae III–IV, and to 243.80 ± 20.06% in laminae V at 14 d after L5 SNL.

Interestingly, we found that the time course of behavioral signs of neuropathic pain was almost identical to that of TSPO expression following L5 SNL. As shown in Fig. 1*J*,*K*, significant decreases in the 50% paw withdrawal threshold (50% PWT, $F_{(7, 48)} = 10.83$, $p < 0.001$) and in paw withdrawal latency (PWL, $F_{(7, 48)} = 59.73$, $p < 0.001$) on the ipsilateral side were detected on day 4 but not on day 1 after L5 SNL. The behavioral signs of neuropathic pain vanished on day 50 after L5 SNL, when the expression of TSPO returned to control level (Fig. 1*H*). The percentage of TSPO-IR-positive area was negatively correlated to either 50% PWT ($r = -0.88$, $F_{(1, 7)} = 20.59$, $p < 0.01$, Fig. 1*L*) or PWL ($r = -0.86$, $F_{1, 7} = 17.97$, $p < 0.01$, Fig. 1*L*) in L5 SNL rats.

L5 SNL did not affect TSPO-IR in the contralateral L5 spinal dorsal horn (Fig. 1*I* $F_{7, 32} = 0.47$, $p > 0.05$), and no change in 50% PWT ($F_{(7, 48)} = 0.45$, $p > 0.05$) and in PWL (Fig. 1*J*,*K*, $F_{(7,48)} = 0.45$, $p > 0.05$) on the contralateral side was observed. No signal was detected in the negative control experiments, in which only secondary antibody but no primary antibody was added (data not shown).

To determine in which types of cells TSPO expression may be changed, double immunofluorescence staining of TSPO with three cell-specific markers: NeuN (a marker for neuron), GFAP (a marker for astrocyte), and Iba1 (a marker for microglia) was performed on L5 spinal cord sections from sham-operated and L5 SNL rats. As shown in Figure 2, the shapes of glial cells become hypertrophic and the numbers of GFAP- and Iba1-positive cells increased in L5 SNL rats, indicating that the glial cells were activated. Compared with the sham group, the percentages of astrocytes ($F_{(3,16)} = 165.39$, $p < 0.001$) and microglia ($F_{(3,16)} = 32.07$, $p < 0.001$) that expressed TSPO-IR increased gradually from day 4 to 14 after L5 SNL, while the percentage of neurons ($F_{(3,16)} = 0.97$, $p > 0.05$) that expressed TSPO-IR did not change with time (Fig. 2*G*), suggesting that the expression of TSPO may increase in glial cells but not in neurons.

Activation of TSPO reverses both neuropathic pain and upregulation of TSPO, and the effects are blocked by neurosteroid synthesis inhibitor

Having demonstrated that the upregulation of TSPO in the spinal dorsal horn was correlated with abnormal pain behaviors following L5 SNL, we next tested whether activation of TSPO could affect neuropathic pain. To do so, a single intrathecal injection of TSPO agonist Ro5-4864 was performed at the seventh day after L5 SNL and 50% PWT was tested before and after the treatment. The results showed that Ro5-4864 suppressed mechanical allodynia in a dose-dependent manner. Ro5-4864 at 1.5 μg completely reversed the decrease in 50% PWT (Fig. 3*A*, main effect of drug $F_{(3,23)} = 78.86$, $p < 0.001$, main effect of time $F_{(10,220)} = 22.37$, $p < 0.001$, interaction $F_{(30,220)} = 7.39$, $p < 0.001$, $n = 6$/group), and the effect persisted until the end of experiment (18 d after injection); at 0.6 μg it also had a similar effect ($p > 0.05$ vs before L5 SNL, $p < 0.001$ vs predrug), but persisting for a short period of time (~10 d), whereas Ro5-4864 at 0.3 μg did not affect 50% PWT, which was comparable to the DMSO-treated group (Fig. 3*A*, $p > 0.05$). Ro5-4864 injection had no effect on the 50% PWT in the contralateral side (data not shown). To test whether TSPO agonist also affect thermal hyperalgesia, Ro5-4864 at 1.5 μg was injected intrathecally in another group of rats, and the results showed that the decrease in PWL induced by L5 SNL was reversed (main effect of drug $F_{(1,10)} = 44.40$, $p < 0.001$, main effect of time $F_{(5,54)} = 19.56$, $p < 0.001$, interaction $F_{(5,54)} =$

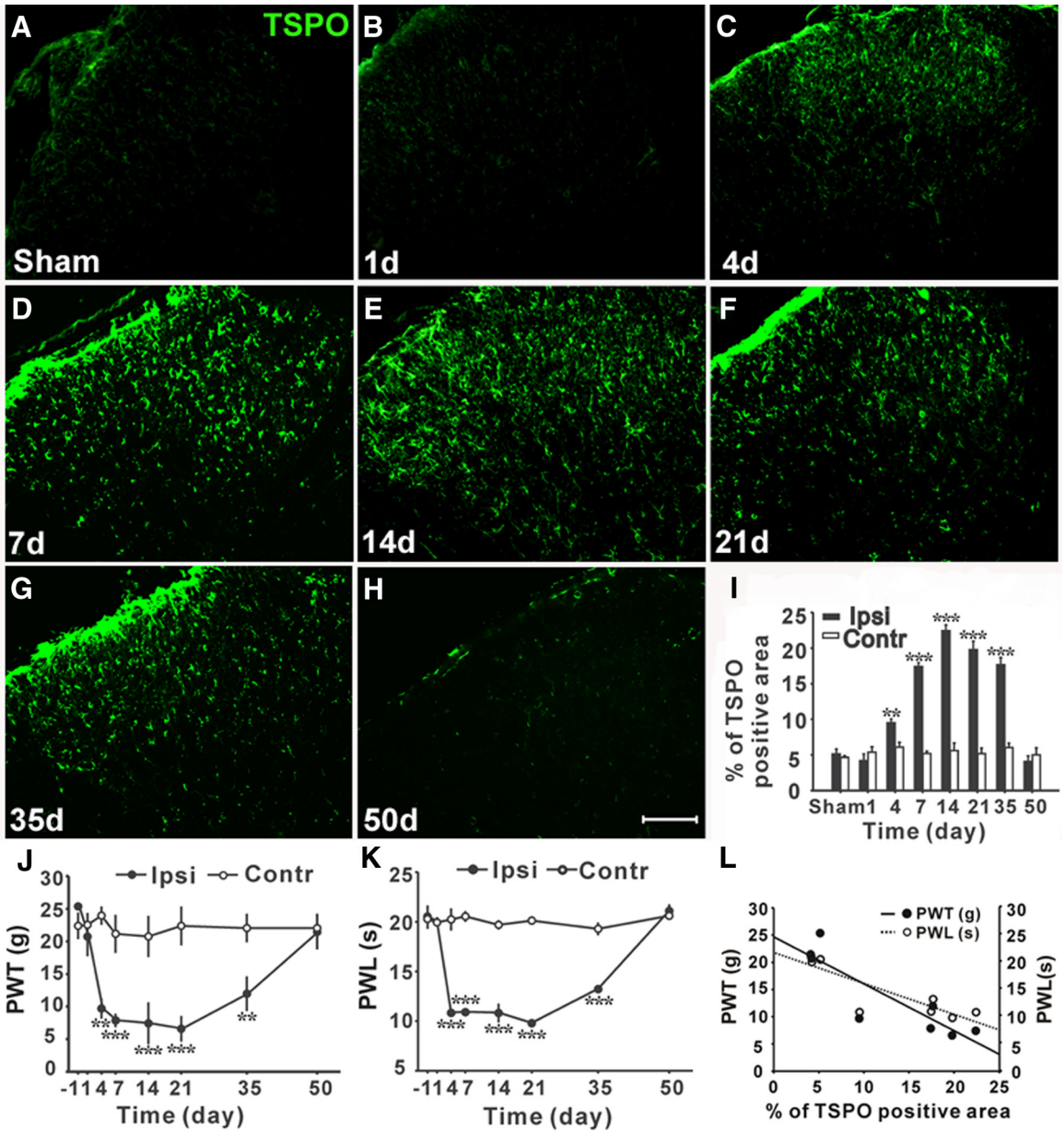

Figure 1. The expression of TSPO in spinal dorsal horn is correlated with mechanical allodynia and thermal hyperalgesia following L5 SNL. ***A–H***, Representative experiments show the change of TSPO-IR in the ipsilateral L5 spinal dorsal horn from sham-operated rats and rats that received L5 SNL at different time points, as indicated. ***I***, Quantification of TSPO-IR-positive area in the ipsilateral L5 spinal dorsal horn in sham and L5 SNL rats ($n = 5$/group). ***J***, ***K***, The time course of changes in 50% PWT and in PWL ($n = 7$) on bilateral hindpaws produced by L5 SNL. ***L***, The percentage of TSPO-IR-positive area was negatively correlated with either 50% PWT ($r = -0.88$, $p = 0.004$) or PWL ($r = -0.86$, $p = 0.005$) in L5 SNL rats. Scale bar, 100 μm. $^{**}p < 0.01$; $^{***}p < 0.001$ versus sham group or baseline.

14.15, $p < 0.001$, $n = 5–6$/group). A single intrathecal injection of Ro5-4864 (1.5 μg) was also capable of depressing the mechanical allodynia and thermal hyperalgesia, when applied at 21 d after L5 SNL. As shown in Fig. 3, *D* and *E*, both 50% PWT (main effect of drug $F_{(1,16)} = 167.7$, $p < 0.001$, main effect of time $F_{(6,105)} = 30.03$, $p < 0.001$, interaction $F_{(6,105)} = 13.75$, $p < 0.001$, $n = 8–9$/group) and PWL (main effect of drug $F_{(1,16)} = 60.03$, $p < 0.001$, main effect of time $F_{(6,105)} = 45.85$, $p < 0.001$, interaction $F_{(6,105)} = 16.47$, $p < 0.001$) increased significantly after Ro5-4864 injection, and 50% PWT was no longer different from that measured before L5 SNL ($p > 0.05$), while PWL was still lower than that before L5 SNL ($p < 0.01$). In DMSO-treated L5 SNL rats, no change was detected (Fig. 3*A–E*, $p > 0.05$ vs predrug). To test whether the effect of Ro5-4864 is specific, another TSPO agonist FGIN-1-27, which is different from Ro5-4864 in chemical structure, was tested with the same experimental procedure. We found FGIN-1-27 was also able to reverse the mechanical allodynia (Fig. 3*C*, main effect of drug $F_{(1,11)} = 232.44$, $p < 0.001$, main effect of time $F_{(10,110)} = 15.43$, $p < 0.001$, interaction $F_{(10,110)} = 10.72$, $p < 0.001$, $n = 6$/group), when applied 7 d after L5 SNL.

To test whether TSPO ligand may affect physiological pain, paw withdrawal incidences to repetitive application of four different von Frey hairs were measured before and after intrathecal injection of Ro5-4864 in the sham-operated rats. As shown in Figure 3*F*, the paw withdrawal incidence enhanced with increasing force, but no difference between the DMSO- and Ro5-4864- (1.5 μg) treated groups was detected ($p > 0.05$).

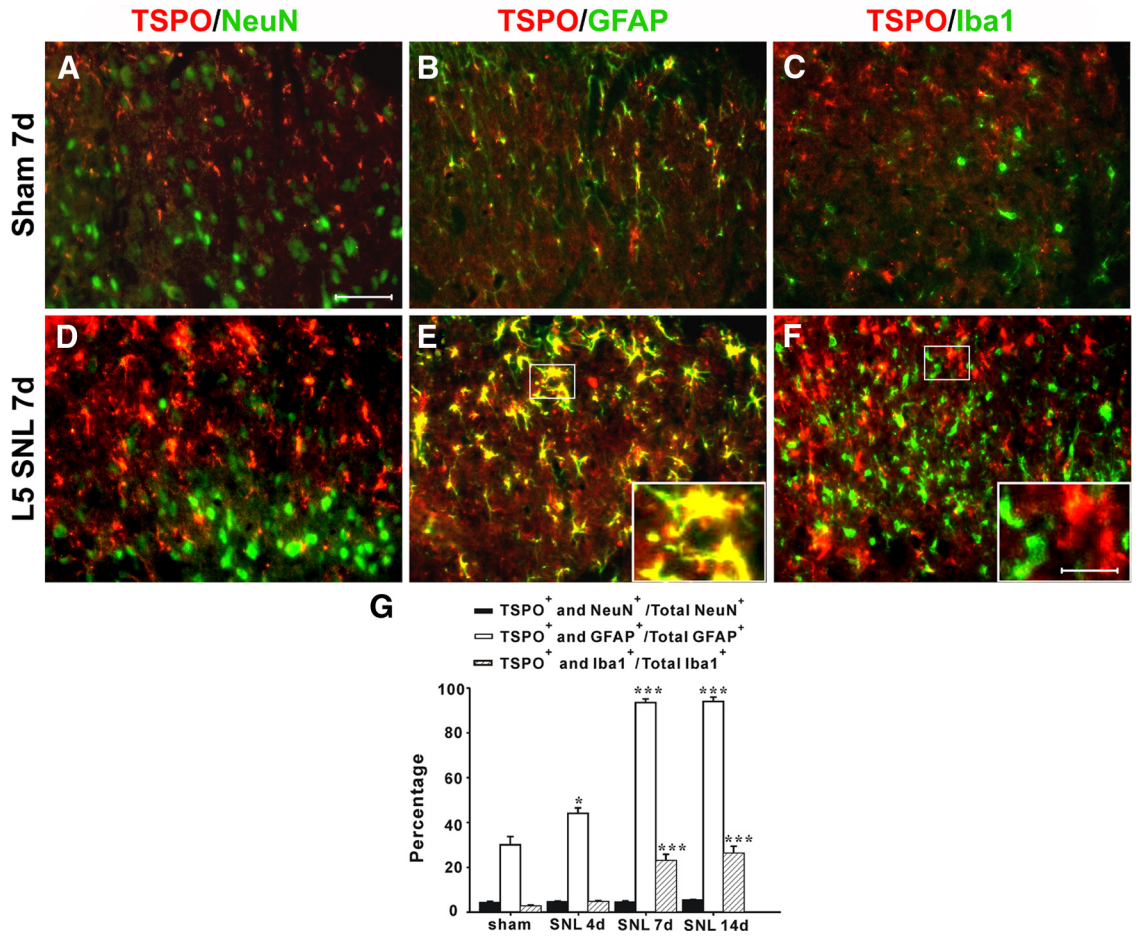

Figure 2. TSPO in spinal glial cells but not in neurons is upregulated following L5 SNL. ***A***, Double immunofluorescence staining of the ipsilateral L5 spinal dorsal horn sections shows that TSPO-IR (***A–F***, red) was mainly colocated with GFAP (a marker for astrocyte, ***B*** and ***E***, green) and to a lesser extent with NeuN (a marker for neuron, ***A*** and ***D***, green) and Iba1 (a marker for microglia, ***C*** and ***F***, green) in both sham and L5 SNL rats. ***G***, The percentages of astrocytes, microglia, and neurons that express TSPO-IR in the spinal dorsal horn 4, 7, and 14 d after L5 SNL and sham-operation are shown. Scale bars: (in ***A***) ***A–F***, 50 μm; (in ***F*** insert) ***E***, ***F*** insert, 16.7 μm. *$p < 0.05$, ***$p < 0.001$ versus sham group.

How could TSPO agonists reverse behavioral signs of neuropathic pain? It has been shown that TSPO functions via promotion of neurosteroidogenesis (Papadopoulos et al., 2006; Schüle et al., 2011; Nothdurfter et al., 2012). To test whether synthesis of neurosteroid is also involved in the effect of TSPO agonists on neuropathic pain, a neurosteroid synthesis inhibitor (AMG, 10 mg/kg, i.p.) was injected intraperitoneally 30 min before intrathecal injection of Ro5-4864 in the rats 7 d after L5 SNL. As shown in Figure 4, in AMG-treated group, 50% PWT (main effect of drug $F_{(1,10)} = 110.99$, $p < 0.001$, main effect of time $F_{(5,54)} = 52.06$, $p < 0.001$, interaction: $F_{(5,54)} = 31.86$, $p < 0.001$, $n = 5–6$/group) and PWL (main effect of drug $F_{(1,10)} = 25.17$, $p < 0.001$, main effect of time $F_{(5,54)} = 15.22$, $p < 0.001$, interaction $F_{(5,54)} = 8.99$, $p < 0.001$) in the ipsilateral side did not change following intrathecal injection of Ro5-4864 ($p > 0.05$ vs predrug), while in rats pretreated with DMSO, both 50% PWT and PWL increased significantly ($p < 0.05$ vs predrug) to the levels that were not different from those measured before L5 SNL ($p > 0.05$). The results indicated that the inhibitory effect of Ro5-4864 on neuropathic pain might depend on neurosteroid synthesis.

Ro5-4864 downregulates TSPO *in vivo* and in cultured glial cells, and the effects are prevented by AMG

Considering the close association of TSPO upregulation with the behavioral signs of neuropathic pain following L5 SNL and the potent inhibitory effect of TSPO agonist on the behavioral signs of neuropathic pain, we assumed that the role of TSPO upregulation might be to promote recovery from neuropathic pain. If this is true, the increased TSPO expression should be downregulated, when neuropathic pain was completely reversed by TSPO agonist. To test this, we measured TSPO expression in the ipsilateral L5 spinal dorsal horn with Western blot 7 d after a single intrathecal injection of Ro5-4864 in L5 SNL rats. As shown in Figure 5*B*, the expression of TSPO in Ro5-4864-treated group was significantly lower, compared with the DMSO-treated group (Fig. 5*B*, $F_{(3,8)} = 59.898$, $p < 0.001$). Interestingly, the effect was again prevented by AMG (Fig. 5*B*, $p < 0.001$ vs Ro5-4864 alone).

To confirm the effect of Ro5-4864 and AMG on TSPO expression *in vivo*, we tested whether the drugs could affect the upregulation of TSPO in mixed cultured spinal glial cells, which contain astrocytes and microglia (Fig. 5*H*,*I*). We found that LPS (100 ng/ml) increased TSPO expression without affecting cell survival. Western blot revealed that TSPO expression was significantly higher in the cultures treated with LPS than that treated with DMSO (Fig. 5*C*, $F_{(3,8)} = 18.189$, $p = 0.001$). The effect of LPS on TSPO expression was strongly inhibited by Ro5-4864 (10^{-6} M) applied 24 h after LPS and the effect of Ro5-4864 was blocked by AMG (10^{-6} M) applied 1 h before (Fig. 5*C*, $p < 0.01$). Interestingly, we found that Ro5-4864 reduced the upregulation of TSPO

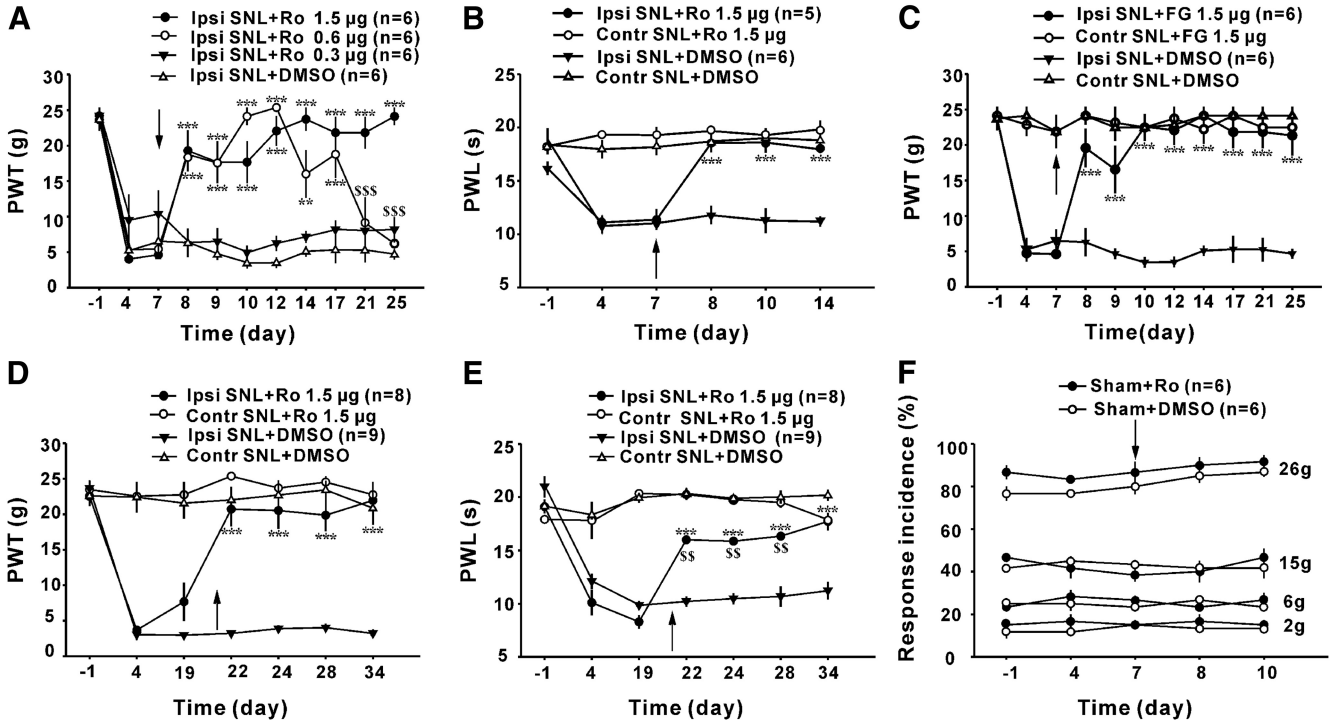

Figure 3. TSPO agonists depress the mechanical allodynia and thermal hyperalgesia induced by L5 SNL. ***A***, A single intrathecal injection of TSPO agonist Ro5-4864 7 d after L5 SNL reversed the decrease of 50% PWT in the ipsilateral side in a dose-dependent manner ($n = 6$/group). ***B***, A single intrathecal injection of Ro5-4864 (1.5 μg) significantly reduced the decrease of PWL induced by L5 SNL ($n = 5–6$/group). ***C***, A single intrathecal injection of another TSPO agonist FGIN-1-27 (1.5 μg) 7 d after L5 SNL also significantly suppressed the abnormal pain behaviors ($n = 6$/group). ***D***, ***E***, A single intrathecal injection of 1.5 μg Ro5-4864 reversed mechanical allodynia completely, and strongly inhibited thermal hyperalgesia when applied at 21st day after L5 SNL ($n = 8–9$/group). ***F***, Ro5-4864 at 1.5 μg did not affect the paw withdrawal incidence evoked by four different forces of von Frey hairs in the sham-operated rats. The arrows indicate the time of intrathecal injection of Ro5-4864 (Ro) or FGIN-1-27 (FG). $^{**}p < 0.01$, $^{***}p < 0.001$ versus predrug; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus before L5 SNL.

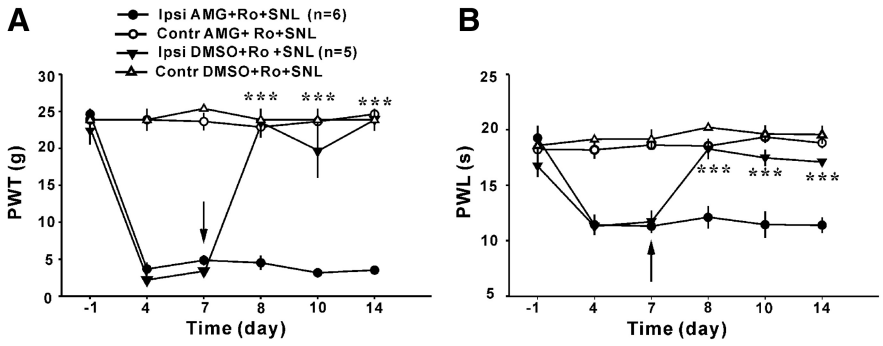

Figure 4. The effect of Ro5-4864 on neuropathic pain induced by L5 SNL is prevented by pretreatment with AMG in rats. ***A***, ***B***, Application of AMG (10 mg/kg, i.p) 30 min before intrathecal injection of Ro5-4864 (1.5 μg) abolished the effects of Ro5-4864 on the decrease of 50% PWT (***A***) and PWL (***B***) in the ipsilateral side induced by L5 SNL ($n = 5–6$/group). The arrows indicate the time of intrathecal injection of Ro5-4864. $^{***}p < 0.001$ versus before Ro5-4864 (Ro).

in the GFAP-positive astrocytes but not in the Iba1-positive microglia (Fig. 5*E–G*, also see Fig. 6).

Ro5-4864 selectively inhibits activated astrocytes and reduces the production of TNF-α in the spinal dorsal horn

It has been well established that activation of astrocytes in the spinal cord is important for the maintenance of neuropathic pain (Watkins and Maier, 2003; Tsuda et al., 2005). In the present study, we found that Ro5-4864 selectively inhibited the activated astrocytes without affecting activated microglia in L5 SNL rats. Immunostaining experiments showed that the shapes of both GFAP-positive and Iba1-positive cells become hypertrophic (Fig. 6*Ab*,*f*) and Western blot revealed that the expression of both GFAP and Iba1 in the ipsilateral L5 spinal dorsal horn was significantly higher 14 d after L5 SNL, compared with that in the sham-operated rats (Fig. 6*B*,*C*, $F_{(3,12)} = 65.226$, $p < 0.001$), suggesting that both astrocytes and microglia were activated. Importantly, compared with DMSO-treated group, the expression of GFAP was significantly lower in the rats receiving Ro5-4864 injection 7 d before experiment (14 d after L5 SNL) (Fig. 6*Ac*,*B*,*C*, $p < 0.001$), but that of Iba1 was not different (Fig. 6*Ag*,*B*,*C*, $p > 0.05$). Furthermore, the expression of GFAP in rats treated with both AMG and Ro5-4864 was significantly higher than that treated with Ro5-4864 alone ($p < 0.001$), and was not different from that treated with DMSO ($p > 0.05$), indicating that the inhibitory effect of Ro5-4864 on astrocyte may also depend on neurosteroid synthesis.

Activated astrocytes may contribute to neuropathic pain by releasing proinflammatory cytokines (Kawasaki et al., 2008). To test whether TSPO agonist may affect the production of cytokines, TNF-α, a leading proinflammatory cytokine, was measured *in vivo* and *in vitro* with use of ELISA. As reported in our previous work in spared nerve injury model (Chen et al., 2011), 14 d after L5 SNL, TNF-α increased in the ipsilateral spinal dorsal horn (Fig. 7*A*, $F_{(3,8)} = 18.358$, $p < 0.001$, sham vs DMSO-treated L5 SNL group). The increased TNF-α produced by L5 SNL was suppressed by a single intrathecal injection of Ro5-4864 7 d be-

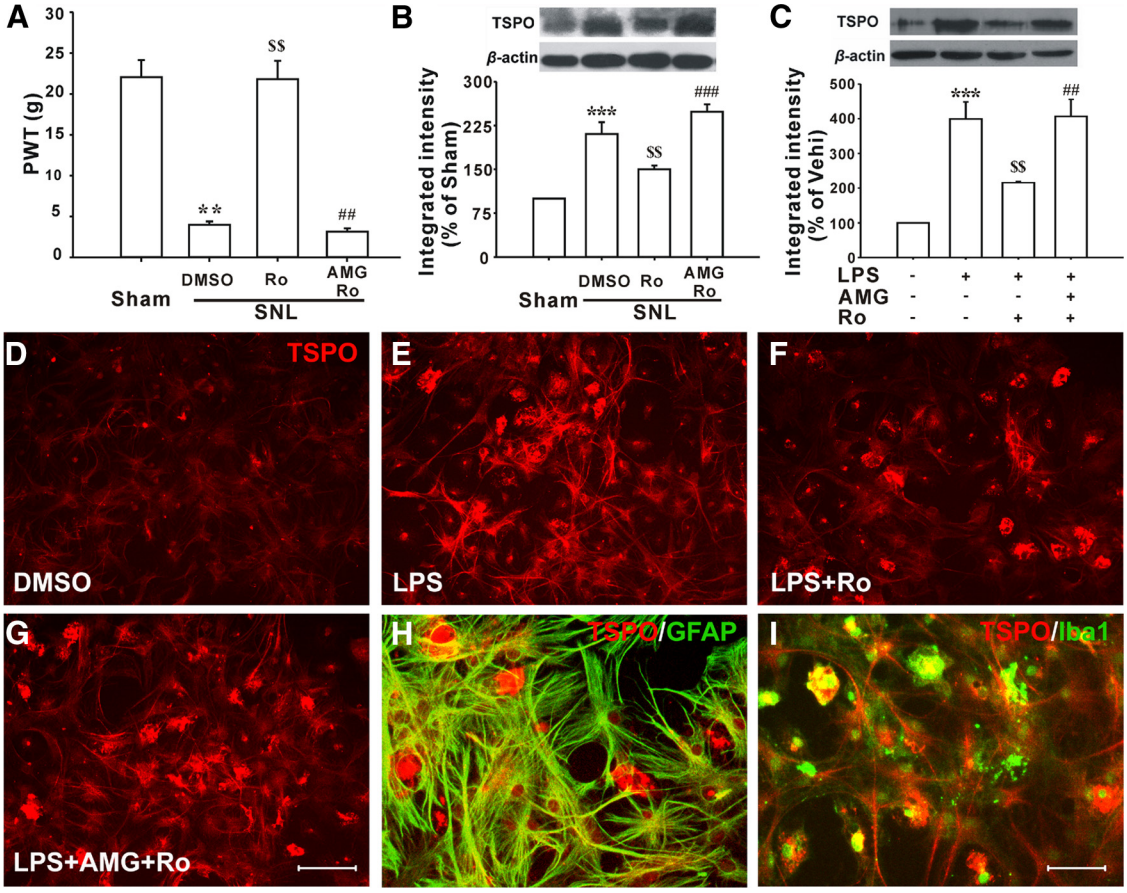

Figure 5. Ro5-4864 downregulates TSPO *in vivo* and in cultured glial cells, and this effect is prevented by AMG. ***A***, The 50% PWTs of four groups of the rats, from which L5 spinal cord tissue was harvested for Western blot, are shown. The samples were collected 14 d after sham-operation (sham) or L5 SNL. Ro5-4864 (Ro) or DMSO was injected intrathecally 7 d after L5 SNL and AMG was injected intraperitoneally 30 min before Ro5-4864 (AMG + Ro). ***B***, The bands show the expression of TSPO and β-actin in the above groups ($n = 3$/group). ***C***, Immunoblots of TSPO and β-actin in mixed cultured spinal glial cells in different groups, as indicated. In ***A*** and ***B***, $^{**}p < 0.01$, $^{***}p < 0.001$ versus sham group; $^{\$\$}p < 0.01$ versus DMSO-treated group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus Ro5-4864-treated group. In ***C***, $^{***}p < 0.001$ versus DMSO-treated group; $^{\$\$}p < 0.01$ versus LPS-treated group; $^{\#\#}p < 0.01$ versus LPS and Ro5-4864-treated group. ***D–G***, Representative photographs show immunostaining of TSPO in cultured glial cells treated with DMSO, LPS, LPS + Ro5-4864 or LPS + AMG + Ro5-4864. Ro5-4864 was added 24 h after LPS and AMG was applied 1 h before Ro5-4864. The photographs were taken 48 h after DMSO or LPS application. (***H***, ***I***) TSPO is colocated with GFAP and Iba1 48 h after LPS stimulation. Scale bars: (in ***G***) ***D–G***, 100 μm; (in ***I***) ***H***, ***I***, 50 μm.

fore ($p < 0.01$, Ro5-4864 group vs DMSO group). The effect of Ro5-4864 on TNF-α was completely blocked by AMG (Fig. 7*A*, $p < 0.01$, AMG plus Ro5-4864 group vs Ro5-4864 group; $p > 0.05$, AMG plus Ro5-4864 group vs DMSO group). In cultured spinal glial cells, LPS enhanced TNF-α concentration in the culture media (Fig. 7*B*, $F_{(3,8)} = 70.015$, $p < 0.001$ vs DMSO alone). Consistent with our *in vivo* results, Ro5-4864 applied 30 min after LPS decreased TNF-α release induced by LPS (Fig. 7*B*, $p < 0.001$, Ro5-4864 group vs LPS group) and the effect was also prevented by AMG (Fig. 7*B*, $p < 0.01$, AMG plus Ro5-4864 group vs Ro5-4864 group; $p > 0.05$, AMG plus Ro5-4864 group vs DMSO group).

Blockage of TLR4 prevents neuropathic pain, the upregulation of TSPO and the increase in TNF-α

TLR4 is important for initiating the immune response via recognizing substances from invaded organisms or from damaged tissues in the case of nerve injury (Bettoni et al., 2008). To evaluate the role of TLR4 in the upregulation of TSPO and development of neuropathic pain produced by L5 SNL, a TLR4 antagonist lipopolysaccharide Rhodobacter sphaeroides (LPS-RS) was tested in L5 SNL rats. Intrathecal injection of LPS-RS (20 μg) at 1 h, 6 d, 9 d, and 12 d after L5 SNL completely blocked the decrease in 50% PWT tested on day 4, 7, and 10 (Fig. 8*A*, main effect of drug $F_{(1,15)} = 392.33$, $p < 0.001$, main effect of time $F_{(3,56)} = 34.57$, $p < 0.001$, interaction $F_{(3,56)} = 30.22$, $p < 0.001$, $n = 8$/group) and significantly attenuated the decrease in PWL on day 4 and 7 (main effect of drug $F_{(1,15)} = 33.25$, $p < 0.001$, main effect of time $F_{(3,56)} = 59.62$, $p < 0.001$, interaction $F_{(3,56)} = 16.84$, $p < 0.001$, $n = 8$/group) but not on day 10. The results of Western blot and ELISA with the ipsilateral spinal cord tissue of the same L5 SNL rats revealed that the expression of TSPO (Fig. 8*C*, $F_{(2,6)} = 87.876$, $p < 0.001$) and the concentration of TNF-α (Fig. 8*D*, $F_{(2,6)} = 11.888$, $p < 0.01$) was significantly lower in the rats treated with LPS-RS, compared with that treated with PBS. The results indicated that activation of TLR4 might be critical for the initiation of neuropathic pain, the upregulation of TSPO and increase in TNF-α. To verify the *in vivo* results, we tested TLR4 antagonist in cultured spinal glial cells. As shown in Figure 9, TSPO upregulation induced by LPS was completely prevented by LPS-RS (100 ng/ml), which was added 1 h before LPS, as the TSPO level in the cells treated with LPS-RS and LPS was significantly lower than that treated with LPS alone (Fig. 9*E*, also see

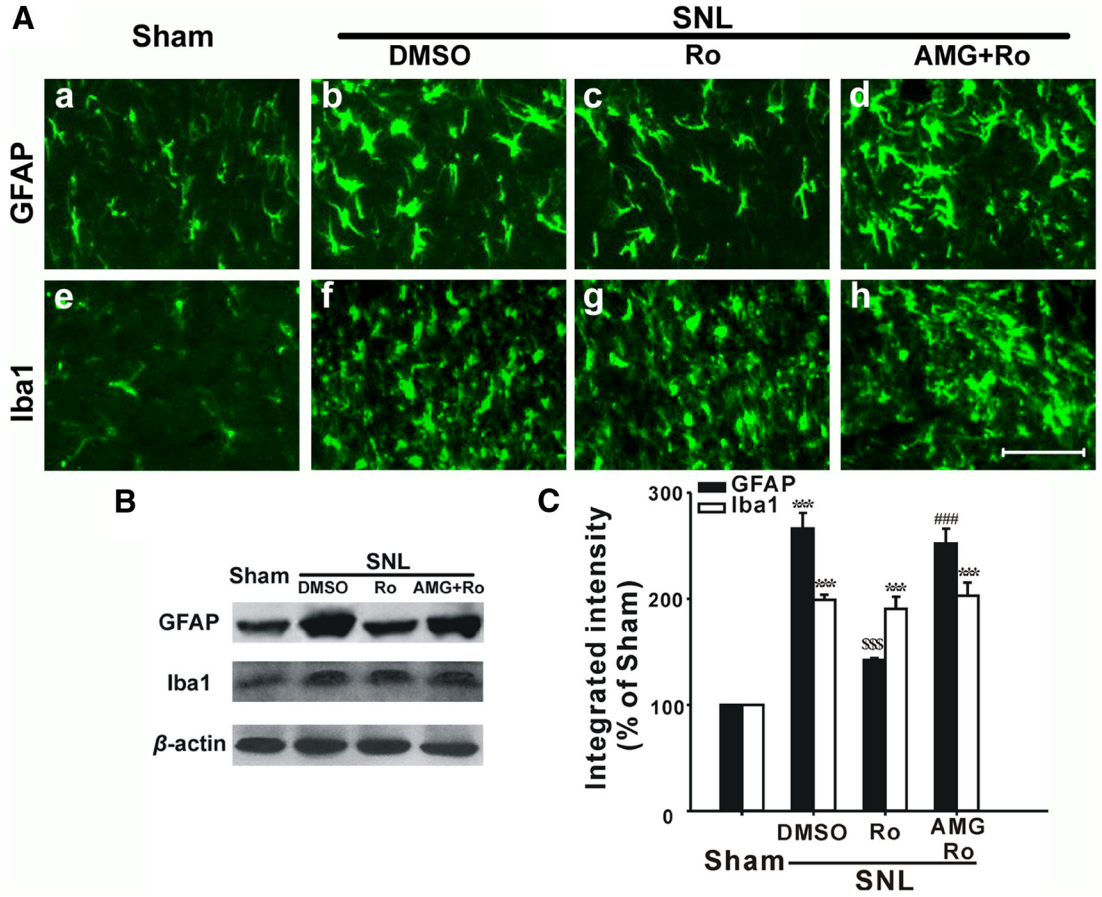

Figure 6. Ro5-4864 selectively inhibits activation of astrocytes but not of microglia in L5 SNL rats, and the effect is prevented by AMG. ***A***, Representative photographs of GFAP and Iba1 immunostaining are shown in four different groups as indicated. Tissues were collected 14 d after sham-operation or L5 SNL ($n = 6$/group). ***B***, Western blot shows the expression of GFAP and Iba1 in the ipsilateral L5 spinal dorsal horn in four different groups. ***C***, The histogram shows the quantification of GFAP or Iba1 normalized by β-actin ($n = 4$/group or 3/group). $^{***}p < 0.001$ versus sham group. $^{\$\$\$}p < 0.001$ versus DMSO-treated group. $^{\#\#\#}p < 0.001$ versus the Ro5-4864-treated group. Scale bar: ***Aa–h***, 50 μm.

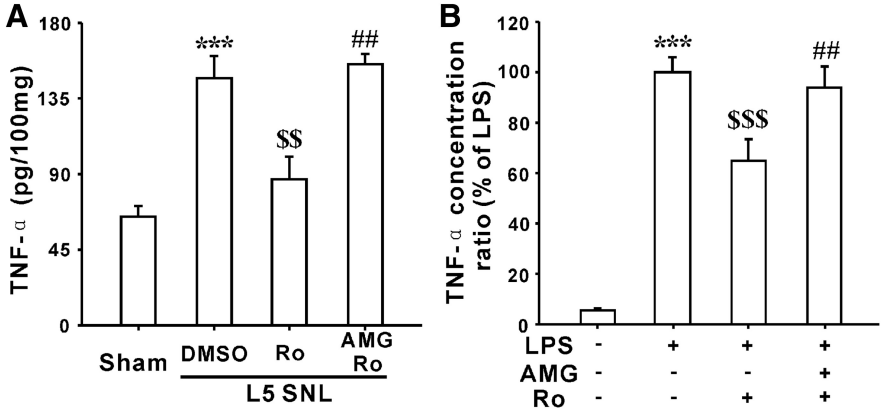

Figure 7. Ro5-4864 inhibits the increase in TNF-α in L5 SNL rats and in cultured glial cells, and the effect is prevented by AMG. ***A***, TNF-α levels in the ipsilateral spinal cord tissue measured with ELISA in 4 groups of rats are shown ($n = 3$/group). The samples were collected 14 d after sham-operation (sham), 7 d after (14 d after L5 SNL) treatment with Ro5-4864 (Ro), with DMSO, or with both AMG and Ro5-4864 (AMG + Ro). AMG was applied 30 min before Ro5-4864. $^{***}p < 0.001$ versus sham group or control; $^{\$\$}p < 0.01$ versus DMSO-treated group; $^{\#\#}p < 0.01$ versus Ro5-4864-treated group. ***B***, TNF-α levels in culture media of glial cells in different conditions, as indicated, are shown. Ro5-4864 was applied 24 h after LPS and AMG applied 1 h before Ro5-4864 application. $^{***}p < 0.001$ versus DMSO-treated group, $^{\$\$\$}p < 0.001$ versus LPS-treated group, $^{\#\#}p < 0.01$ versus LPS and Ro5-4864-treated group.

A–C; $F_{(2,6)} = 79.626$, $p < 0.001$) and was not different from that treated with vehicle ($p > 0.05$). The increase of TNF-α in the culture media induced by LPS was also significantly attenuated by LPS-RS (Fig. 9*F*, $F_{(2,6)} = 200.370$, $p < 0.001$).

Discussion

In the present study, we showed that the upregulation of TSPO in spinal astrocytes and microglia was closely associated with the behavioral signs of neuropathic pain following L5 SNL. Importantly, a single intrathecal injection of TSPO agonists Ro5-4864 or FGIN-1-27 depressed the behavioral signs of neuropathic pain 7 and 21 d after L5 SNL. Ro5-4864 inhibited the activation of astrocytes, the release of TNF-α, and the upregulation of TSPO in L5 SNL rats and in cultured glial cells. All the effects of Ro5-4864 were prevented by AMG, a neurosteroid synthesis inhibitor. Furthermore, both neuropathic pain and upregulation of TSPO were prevented by TLR4 antagonist LPS-RS. These results suggested that both upregulation of TSPO and neuropathic pain behaviors might be initiated by activation of TLR4. The role of TSPO upregulation might be to promote recovery from the neuropathic pain state.

Previously, the inhibitory effect of Ro5-4864 on inflammatory pain has been demonstrated in CFA injection model (Hernstadt

et al., 2009) and in formalin test (DalBó et al., 2004). TRO19622, another TSPO ligand, has been reported to reverse neuropathic pain behaviors in the experimental models of diabetic and chemotherapy-induced neuropathies, while the compound has no effect on inflammatory pain induced by formalin injection and neuropathic pain by constriction injury of the sciatic nerve (Bordet et al., 2008). As the affinity of TRO19622 for TSPO (Ki: 100 nM) (Bordet et al., 2007) is much lower than that of Ro5-4864 (6.0 nM) (French and Matlib, 1988), TRO19622 may have a small impact on cholesterol metabolism or steroidogenesis (Bordet et al., 2007). However, the compound has been shown to bind to voltage-dependent anion channels in mitochondrial membrane, which is critically involved in the apoptosis processes (Shoshan-Barmatz and Ben-Hail, 2012). Since apoptosis is important for diabetic and chemotherapy-induced neuropathies (Srinivasan et al., 2000; Scuteri et al., 2009), it is possible that TRO19622 exert neuroprotective effect by anti-apoptosis but not by promotion of neurosteroids (Bordet et al., 2008). This may explain the different effects of TRO19622 and Ro5-4864 on different neuropathic pain models.

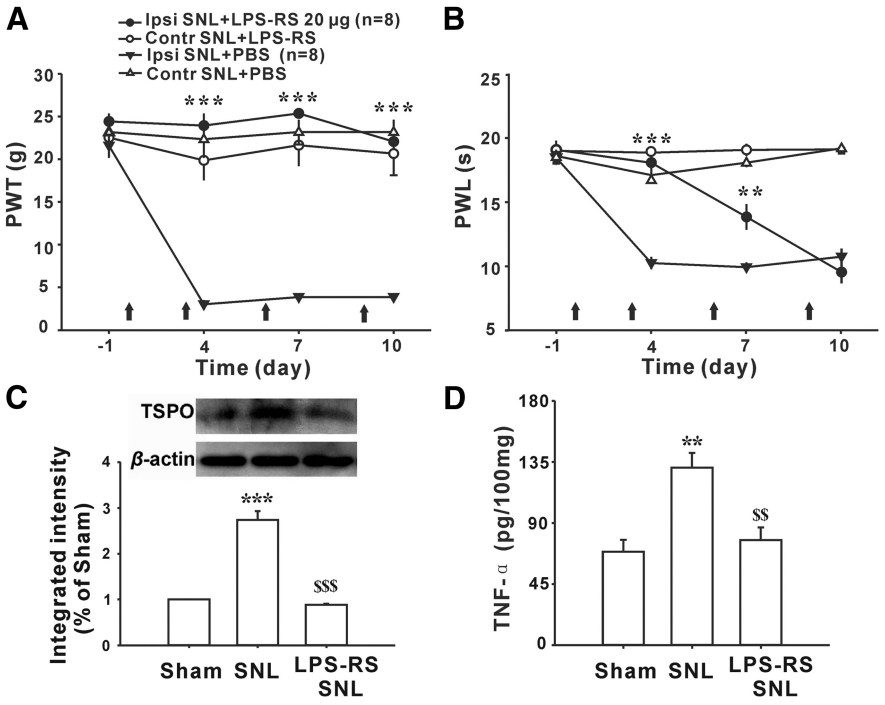

Figure 8. TLR4 antagonist LPS-RS inhibits neuropathic pain, the upregulation of TSPO and the increase in TNF-α in L5 SNL rats. ***A***, ***B***, Effects of intrathecal injection of LPS-RS, a TLR4 antagonist, 1 h, 3 d, 6 d, and 9 d after L5 SNL (arrows) on the 50% PWT (n = 8/group) and PWL are shown. ***C***, ***D***, Representative experiments of Western blot and ELISA show TSPO expression and TNF-α concentration in the ipsilateral L5 spinal dorsal horn from different groups 10 d after operation. In ***A*** and ***B***, $^{**}p < 0.01$; $^{***}p < 0.001$ versus PBS-treated group. In ***C*** and ***D***, $^{**}p < 0.01$; $^{***}p < 0.001$ versus sham group; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ versus L5 SNL group.

The upregulation of TSPO is protective against neuronal damage

Many proteins are upregulated following peripheral nerve injury (Niederberger and Geisslinger, 2008). Some of them contribute directly to neuropathic pain, for example, the upregulation of sodium channels leading to ectopic discharges (Cummins et al., 2007). In the present study, we found that TSPO was upregulated in spinal dorsal horn following L5 SNL and that a single intrathecal injection of TSPO agonists (either Ro5-4864 or FGIN-1-27) inhibited neuropathic pain dramatically. Interestingly, the upregulated TSPO returned to control level or was significantly downregulated, when the behavioral signs of neuropathic pain healed naturally or were reversed by Ro5-4864. These results suggested that the role of TSPO upregulation might be to promote recovery from neuropathic pain state. We found that Ro5-4864 increased the 50% paw withdrawal threshold and thermal latency measured at the painful but not the pain-free hindpaw, and that Ro5-4864 had no effect on the paw withdrawal incidence of the sham operated rats, suggesting that TSPO may selectively depress neuropathic pain, but leaving physiological pain intact. The protective effects of TSPO has also been reported previously, for example, activation of TSPO by olesoxime rescued motor neurons from axotomy-induced cell death in neonatal rats and promoted sciatic nerve regeneration following crush injury (Bordet et al., 2007); Ro5-4864 prevents hippocampal neuronal death and reactive gliosis produced by kainic acid (Veiga et al., 2005).

Up to now, majority of drugs are designed to target at the upregulated proteins that directly causes disease, for example sodium channel blocker for neuropathic pain. As the channel is distributed in all excitable cells, side effect is inevitable. A novel strategy may be to develop agents that target at the upregulated proteins, which exert a protective effect against the diseases. We assumed that natural cure may occur, when endogenous agonists for such kinds of proteins are sufficient, but in majority of cases they are probably deficient, exogenous ones are needed for recovery.

The mechanisms underlying the inhibitory effect of TSPO ligands on neuropathic pain

Multiple mechanisms may be involved in the inhibitory effect of TSPO ligand on neuropathic pain. It has been demonstrated that activation of glial cells in spinal dorsal horn, infiltration of macrophages and lymphocytes into peripheral and CNS play important roles in the development of neuropathic pain (Watkins and Maier, 2003; Tsuda et al., 2005; Wei et al., 2007). TSPO ligands have been shown to modulate chemotaxis and phagocytosis in peripheral monocytes and neutrophils (Ruff et al., 1985; Cosentino et al., 2000; Marino et al., 2001), and to regulate the viability and functions of immune cells, including lymphocytes and macrophages (Sakai et al., 2006). TSPO ligands also inhibit the production of reactive oxygen species, which is important for neuropathic pain (Siniscalco et al., 2007). In the present study, we found that in the spinal dorsal horn TSPO was upregulated in astrocytes and microglia but not in neurons after L5 SNL. Ro5-4864 selectively inhibited activated astrocytes but not activated microglia. The results suggested that inhibition of spinal astrocytes might contribute to the effect of Ro5-4864 on neuropathic pain. The notion is also consistent with the fact that microglia are critical for initiation and astrocytes for the maintenance of neuropathic pain (Jin et al., 2003; Zhuang et al., 2005; Romero-Sandoval et al., 2008a, b).

How could TSPO ligand inhibit activated astrocytes and reduce production of TNF-α? TSPO promotes neurosteroidogen-

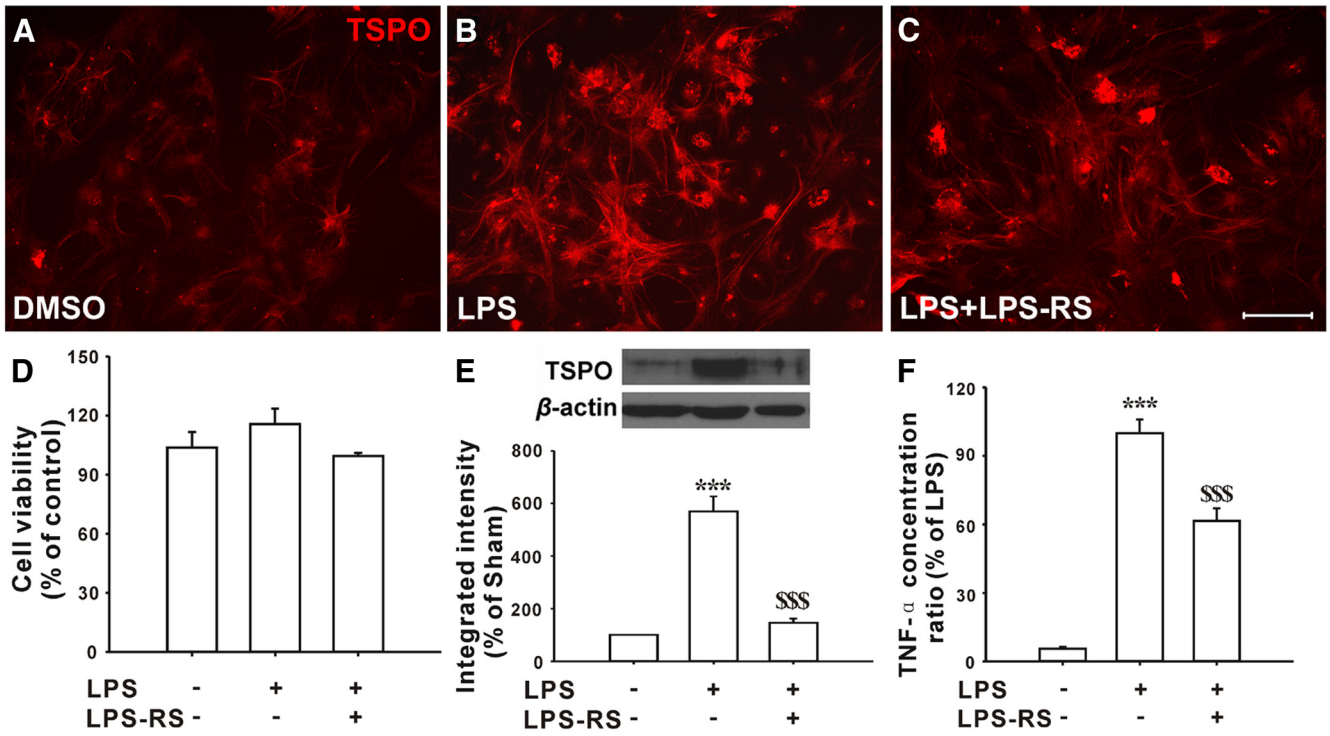

Figure 9. TLR4 antagonist inhibits LPS-induced upregulation of TSPO and the increase in TNF-α in cultured glial cells. ***A–C***, Representative immunostaining experiments show the expression of TSPO in different culture conditions. The glia cells were cultured in the media that contains either DMSO (<0.1‰) or LPS (100 ng/ml) for 48 h and LPS-RS was added into the media 1 h before LPS. ***D***, Cell viability in different culture conditions is shown. ***E***, ***F***, Both the upregulation of TSPO and the increase in TNF-α induced by LPS were inhibited by TLR4 antagonist LPS-RS. $^{***}p < 0.001$ versus DMSO-treated group; $^{\$\$\$}p < 0.001$ versus LPS-treated group.

esis by allowing the translocation of cholesterol across the inner mitochondrial membrane (Rupprecht et al., 2010). Recently, it has been shown that concentrations of neurosteroids in brain and sciatic nerve can be affected by selective TSPO activation (Giatti et al., 2009). TSPO ligand can regulate synaptic function by promoting neurosteroids synthesis in glial cells (Malayev et al., 2002; Papadopoulos et al., 2006, Eser et al., 2008). Progesterone, one of the neurosteroids, can reduce the expression of TNF-α and IL-1β in cultured microglia (Jiang et al., 2011). In the present study, we showed that pretreatment with AMG that specifically blocks P450 side-chain cleavage, which transforms cholesterol into pregnenolone (the precursor for the biosynthesis of all steroid hormone), reversed the inhibitory effect of Ro5-4864 on astrocytes and TNF-α release both *in vivo* and *in vitro*. Therefore, Ro5-4864 may depress the activation of astrocytes and reduce the production of TNF-α by increasing endogenous neurosteroid. It has been shown that neurosteroids regulate transcription factor cAMP-responsive element binding protein and NF-κB (Charalampopoulos et al., 2006), which are critically involved in the maintenance of neuropathic pain (Hoeger-Bement and Sluka, 2003). Thus, TSPO agonist may inhibit neuropathic pain via regulation of gene expression by neurosteroids. In addition, it has been shown that TSPO ligand etifoxine potentiates $GABA_A$ receptor function by promoting the production of 3α-reduced neurosteroids (Charlet et al., 2008; Aouad et al., 2009). Therefore, TSPO in glial cells may cooperate with neurons to counteract hyperexcitability in neuropathic pain states.

As TSPO is found in many subcellular regions, including mitochondrial membranes, in plasma membrane, nuclear membrane, Golgi apparatus, lysosomes, and peroxisomes (Basile and Skolnick, 1986; O'Beirne et al., 1990; Oke et al., 1992; Hardwick et al., 1999; Marino et al., 2001), TSPO agonists may function in multiple subcellular structures. Further studies are needed to fully understand the mechanisms underlying the effects of TSPO ligand.

The possible mechanisms underlying the upregulation of TSPO

In the present study, we showed that neuropathic pain was prevented by the TLR4 antagonist, which is in consistent with a recent report (Bettoni et al., 2008). Interestingly, we found that LPS-RS also blocked the upregulation of TSPO and the production of TNF-α *in vivo* and *in vitro*. It has been well established that TLR4 can recognize not only the substances from pathogens but also from damaged tissue. Activation of TLR4 upregulates varieties of proinflammatory cytokines (Denkers et al., 2004; Buchanan et al., 2009). Previous studies have shown that in pancreatic islet cells, the proinflammatory cytokines TNF-α, IFN-γ, and IL-1β cause an increase in TSPO mRNA and [3H]-PK11195 binding in a transcription-dependent fashion (Rey et al., 2000). In cultured astrocytes, TNF-α and IL-1β also increase [3H]-PK11195 binding (Oh et al., 1992). In experimental autoimmune encephalitis, TNF-α and IL-6 expression profiles are correlated with increase in spinal [3H]-PK11195 binding (Agnello et al., 2000). Thus, it is possible that activation of TLR4 may upregulate TSPO by increase of the cytokines. Whether TLR4 can directly upregulate TSPO in the spinal dorsal horn remains elusive.

In summary, following peripheral nerve injury, TLR4 was activated by the endogenous ligands released from damaged tissue, leading to neuropathic pain and upregulation of TSPO in spinal dorsal horn by activation of spinal glial cells and overproduction of inflammatory cytokines. Activation of upregulated TSPO may inhibit the activated astrocytes and reduce production of inflam-

matory cytokines by promotion of neurosteroid synthesis, leading to recover from neuropathic pain and the downregulation of TSPO.

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