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Inhibition of Neuropathic Pain by a Single Intraperitoneal Injection of Diazepam in the Rat: Possible Role of Neurosteroids

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Abstract

Diazepam binds with the same high affinity to the central benzodiazepine receptor (CBR) and the peripheral benzodiazepine receptor, which has been renamed translocator protein (TSPO). Both receptors could promote neurosteroid synthesis. In the present study, we investigated whether a single dose of diazepam could inhibit neuropathic pain induced by L5 spinal nerve ligation (L5 SNL), and whether CBR and TSPO mediated this effect. We found that a single intraperitoneal injection of diazepam 9 d after L5 SNL significantly depressed the established mechanical allodynia and thermal hyperalgesia, which persisted until the end of the experiments. Furthermore, the effects were mimicked by a single intraperitoneal injection of Ro5-4864, a specific TSPO agonist and pregnenolone, a neurosteroid precursor. In addition, we found that the inhibitory effect of diazepam was also completely blocked by pretreatment with a specific CBR antagonist, flumazenil. The effects of diazepam or Ro5-4864 on neuropathic pain were completely blocked by pretreatment with a neurosteroid synthesis inhibitor, aminoglutethimide (AMG). Finally, any one of the three drugs, diazepam, Ro5-4864 and pregnenolone, could reduce the activation of astrocytes and the production of interleukin-1beta (IL-1β) in the L5 spinal dorsal horn 14 d after L5 SNL. These results suggest that in addition to exerting effects on CBR, diazepam may inhibit neuropathic pain *via* TSPO, which promotes neurosteroid formation, subsequently reducing the activation of astrocytes and production of cytokines.

Key Words: diazepam, interleukin, neuropathic pain, pregnenolone, spinal cord, TSPO

Introduction

Neuropathic pain is characterized by exaggerated response to noxious stimuli (hyperalgesia), pain response to normally innocuous stimuli (allodynia) and spontaneous pain (48, 49). Neuropathic pain has long been considered as a disease of the nervous system (2) and has been intensively studied for many years. However, effective treatment for the disease is still unmet.

A previous study has shown that intrathecal injection of diazepam reduced inflammatory heat hyperalgesia, as well as chronic constriction injury (CCI)-induced heat hyperalgesia, cold allodynia and mechanical sensitization in mice (19). Our recent work has also shown that diazepam depresses early as well as late phase long-term potential (LTP) of C-fiber-evoked field potentials (12), which is proposed as a synaptic model of hyperalgesia (13, 38). These results suggest that diazepam applied locally could inhibit neuropathic pain, but whether similar antinociceptive effects can also be achieved by a single intraperitoneal administration and the mechanisms are still unclear.

There are two types of receptors for diazepam, the central benzodiazepine receptor (CBR) and the translocator protein (TSPO). CBR is coupled to type A γ-aminobutyric acid (GABAA) receptor, and is present exclusively in the central nervous system (CNS) being localized on neurons (51). Benzodiazepines bind

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to a domain that regulates chloride flux by modulating GABA binding to GABAA receptors (30). It has been reported that the CBR agonist, octadecaneuropeptide (ODN), can stimulate neurosteroids biosynthesis through activation of CBR (8, 9). TSPO, which was once named the peripheral benzodiazepine receptor (31), is present in many tissues including endocrine steroidogenic tissues (32), cells of the immune system (52) and neurons and glia cells (43). TSPO could also promote the synthesis of neurosteroids, including pregnenolone, progesterone, allopregnanolone and dehydroepiandrosterone, which play important roles in pain modulation (25, 26). Some studies have shown that acute diazepam treatments (10 mg/kg or 20 mg/kg) but not long-term diazepam treatment (10 mg/kg) reduced carrageenan-induced paw edema volume, and that this effect was attributed to the presence of TSPO in the adrenal and immune cells (21, 22). Our previous study has shown that TSPO was up-regulated after L5 SNL and neuropathic pain was depressed by its agonists, suggesting that TSPO may be induced to counteract the hyperexcitability in neuropathic pain states (43). Diazepam has been reported to show similar high affinity to the CBR and TSPO (40). The results have led us to speculate that diazepam, which acts on CBR, as well as TSPO, may exert protective effects against neuropathic pain by promoting the synthesis of neurosteroids.

Activation of astrocytes may be involved in the maintenance of neuropathic pain by releasing proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β and interleukin-6 (IL-6) (41). It has also been shown that treatment with diazepam decreases interleukin release from lymphocytes (35), and that neurosteroid progesterone can reduce the expression of TNF-α and IL-1β in cultured microglia (14). However, whether intraperitoneal injection of diazepam could inhibit the production of IL-1β in the spinal dorsal horn by promoting neurosteroid synthesis is still unknown.

In the present study, we first investigated whether a single intraperitoneal injection of diazepam (5 mg/kg) or Ro5-4864 (5 mg/kg) could inhibit neuropathic pain. We then investigated whether CBR mediated the effect of diazepam on neuropathic pain. Next, we investigated whether a neurosteroid synthesis inhibitor could block the effects of diazepam and Ro5-4864. Further experiments were also done to test whether a single intraperitoneal injection of pregnenolone (5 mg/kg), a neuroactive steroid precursor, could inhibit neuropathic pain. Lastly, whether intraperitoneal injection of diazepam, Ro5-4864 or pregnenolone could inhibit the activation of astrocytes and subsequent production of IL-1β in L5 spinal dorsal horn induced by L5 SNL was investigated.

Materials and Methods

Animals

Adult male Sprague Dawley rats (Guangzhou, PRC) weighing 200-220 g were housed in separated cages under a 12/12 light/dark cycle with access to food and water *ad libitum*. The room temperature was controlled at 24 ± 1°C and 50-60% humidity. The studies were approved by the Institutional Animal Care and Use Committee at the Sun Yat-Sen University, and all experimental procedures were performed in accordance with the guideline of the National Institutes of Health on animal care and the ethical guidelines for study of pain (53).

Drugs Administration

Diazepam, Ro5-4864 (7-chloro-5-4-chlorophenyl)-1,3-dihydro-1-methyl-2-H-1,4-benzodiaze-pine-2), and AMG (R(+)-p-aminoglutethimide) (Sigma, St. Louis, MO, USA) were dissolved and stored as a 0.1 M stock solution at -20°C, and was diluted in sterile PBS to the appropriate concentration immediately before administration. The drug vehicles, propylene glycol (for diazepam), dimethyl sulfoxide (DMSO) for Ro5-4864 and AMG were used as controls, which had no discernable effects on the behavioral responses when injected. Pregnenolone (LKT LABs, St. Paul, MN, USA) was dissolved in minimal Tween 80, and diluted in sterile PBS to the appropriate concentration immediately before administration. Flumazenil (Sigma) was suspended in physiological saline containing 9% Tween 80 and was injected intraperitoneally (i.p.) in a total volume of 200 μl.

Surgical Procedures

The L5 transverse process was removed to expose the L5 spinal nerve, which was then isolated carefully and ligated tightly with 6-0 silk thread 5-10 mm distal to the L5 DRG as described previously (18). 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. Rats with hind limb paralysis or paresis after surgery were excluded.

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 (5), 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 absence of paw withdrawal, 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 hind paws 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.

Heat hypersensitivity was tested using the plantar test (7370, UgoBasile, Comeria, Italy). Briefly, a radiant heat source beneath a glass floor was aimed at the plantar surface of the hind paw. Three measurements of latency were taken for each hind paw in each test session. The hind paw was tested alternately with longer than 5 min intervals. The three measurements of latency per side were averaged as the result of per test.

Western Blotting

The dorsal quadrants of L5 spinal cord were harvested from different groups of rats (six rats at each group). The ipsilateral sides 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 NaVO4, 0.5 mM PMSF, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany)]. 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, Hercules, CA, USA). 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 rabbit polyclonal anti-IL-1β antibody (1:1000, Abcam, Bristol, UK) and rabbit polyclonal anti-mouse β-actin antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) 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 a secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit 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, Piscataway, NJ, USA) and exposed to x-ray film. The band intensities on the film were analyzed by densitometry with a computer-assisted imaging analysis system (KONTRON IBAS 2.0, Eching, Germany).

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, at 4°C. The L5 spinal cord segments were removed and post-fixed in the same fixative for 3 h and then replaced with 30% of sucrose overnight. Transverse free-floating spinal sections (25 μm) were cut in a cryostat (Leica CM 1900, Heidelberg, Germany) and processed for immunostaining with immunofluorescence according to the method described previously (45). All of the cryostat sections 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 mouse monoclonal glial fibrillary acidic protein (GFAP), an astrocyte marker (1:300; Chemicon, Temecula, CA, USA). The sections were then incubated for 1 h at room temperature with FITC-conjugated secondary antibody (1:400; Jackson ImmunoResearch, West Grove, PA, USA). The stained sections were examined with an Olympus IX71 (Olympus Optical, Tokyo, Japan) fluorescence microscope and images were captured with a CCD spot camera.

Quantification and Statistics

For quantification of the immunofluorescence staining, the area of GFAP-immunoreactivity (IR) per section was measured using a Leica Qwin V3 digital image processing system (Leica, Wetzlar, Germany). A density threshold was first set above the background level to identify positively-stained structure. The area occupied by these structures was measured as a positive area. In each rat, every fifth section was picked from a series of consecutive sections; and four to six sections at each time point were randomly selected. An average percentage of area of GFAP-IR relative to the total area of the sections was obtained for each animal across the different tissue sections, and was normalized to the control values. Six rats were included for each group for quantification of immunohistochemistry results.

All analysis was done in a blinded fashion with the same criterion. All data were expressed as means ± SEM. For the data of behavioral tests, nonparametric tests were employed in comparison between various testing days. For immunofluorescence data, 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 between different groups were compared using ANOVA with the least significant difference test (LSD-t). Statistical tests were taken

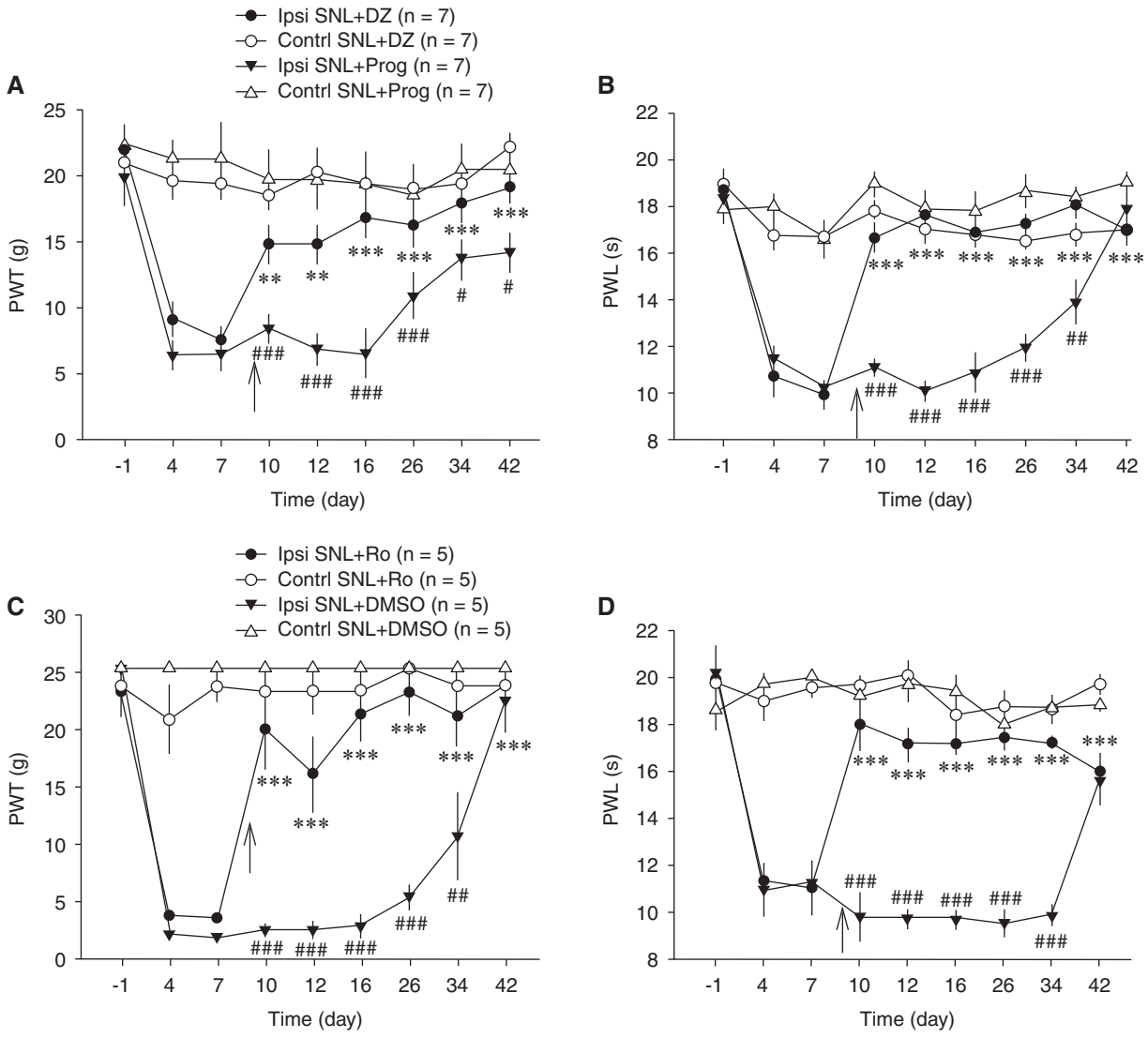

Fig. 1. A single intraperitoneal injection of diazepam or Ro5-4864 9 d after L5 SNL inhibited the mechanical allodynia and thermal hyperalgesia. (A and B) Intraperitoneal injection of diazepam (DZ, 5 mg/kg) 9 d after L5 SNL depressed the decrease of 50% paw withdrawal threshold (PWT) (A) and paw withdrawal latency (PWL) (B) in the ipsilateral (Ipsi) side (n = 7), whereas injection of vehicle propylene glycol (Prog) had no effect (n = 7). (C and D) Intraperitoneal injection of Ro5-4864 (Ro, 5 mg/kg) but not DMSO 9 d after L5 SNL inhibited the decrease of 50% PWT (C) and PWL (D) in the Ipsi side (n = 5 per group). The arrows indicate injection of DZ, Ro or vehicle. $^{**}P < 0.01$, $^{***}P < 0.001$ versus predrug; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus before L5 SNL. Contrl, control.

with SPSS 10.0 (SPSS Inc., Chicago, IL, USA). A difference was accepted as significant if the *P* value is less than 0.05.

Results

A Single Intraperitoneal Injection of Diazepam or Ro5-4864 Reversed Neuropathic Pain Induced by L5 SNL

A significant decrease in 50% paw withdrawal threshold (50% PWT, Fig. 1A) and paw withdrawal latency (PWL, Fig. 1B) on the ipsilateral side was detected 4 d after L5 SNL, compared with pre-operative baseline, showing that prominent mechanical allodynia and thermal hyperalgesia was established. The changes were maintained for 4-5 weeks after operation, which were consistent with our previous work (18). To investigate the effect of diazepam on the behavioral signs of neuropathic pain, a single intraperitoneal injection of diazepam (5 mg/kg) was done 9 d after L5 SNL, and the 50% PWT and PWL were tested before and after the treatment. One day after diazepam treatment, the 50% PWT ($P < 0.01$ *vs*. predrug) and PWL ($P < 0.001$ *vs*. predrug) were already significantly increased, almost to the normal range characteristic of sham animals, and the effects persist until the end of

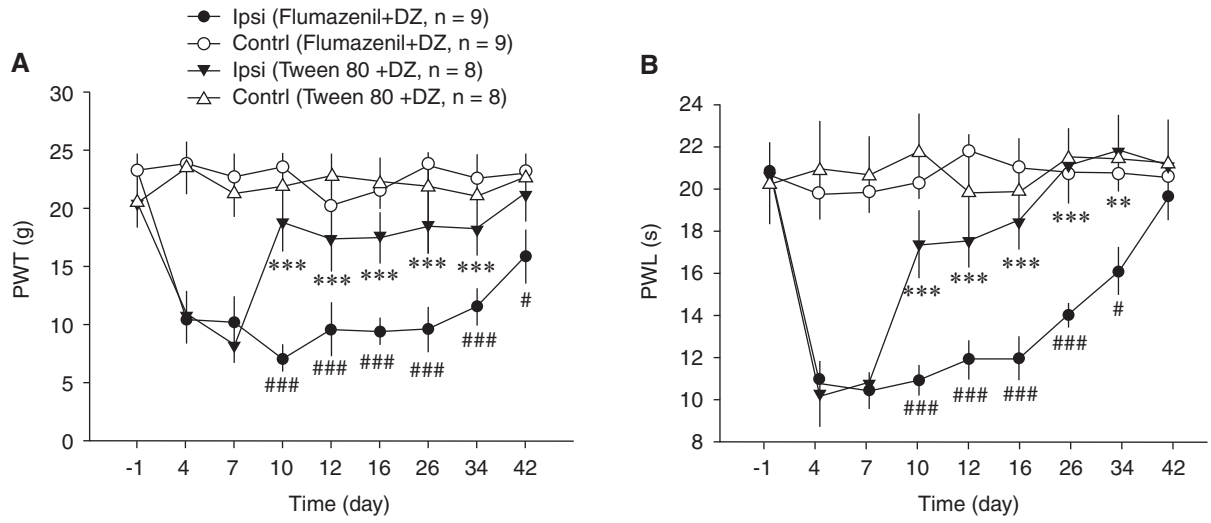

Fig. 2. Effects of diazepam on neuropathic pain induced by L5 SNL was prevented by intraperitoneal injection of flumazenil in rats. (A and B) Intraperitoneal injection of flumazenil (5 mg/kg, i.p.) but not its vehicle 30 min before abolished the effects of diazepam (5 mg/kg) on the decrease of 50% PWT (A) and PWL (B) in the ipsilateral (Ipsi) side induced by L5 SNL (n = 8-9 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ versus before diazepam (DZ); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus before L5 SNL. Contrl, control.

the experiment (33 d after the drug injection, that is 42 d after L5 SNL) (Fig. 1, A and B). In contrast, in propylene glycol (which was used to dissolve diazepam)-treated L5 SNL rats, no change was detected ($P > 0.05$ *vs*. predrug, n = 7) (Fig. 1, A and B).

To test whether TSPO, a diazepam receptor, participates in neuropathic pain, a TSPO agonist, Ro5-4864 (5 mg/kg) was injected intraperitoneally 9 d after L5 SNL. The results showed that a single injection of Ro5-4864 significantly inhibited the decrease of 50% PWT (Fig. 1C, $P < 0.001$ *vs*. predrug) and the decrease in PWL (Fig. 1D, $P < 0.001$ *vs*. predrug) produced by L5 SNL, and the effect also persisted until the end of the experiment. Treatment with the vehicle DMSO had no effect on the decrease of 50% PWT and PWL (Fig. 1, C and D, n = 5).

Pretreatment with CBR Antagonist Flumazenil Blocked the Effects of Diazepam on Neuropathic Pain

To investigate whether CBR is involved in the effects of diazepam on neuropathic pain, a specific CBR antagonist, flumazenil (5 mg/kg, i.p.) was injected 30 min before intraperitoneal injection of diazepam at day 9 after L5 SNL. Flumazenil pretreatment could completely prevent the increase in 50% PWT and PWL in the ipsilateral side following intraperitoneal injection of diazepam (Fig. 2, A and B, $P > 0.05$ *vs*. predrug), while vehicle pretreatment had no effect on the increase in 50% PWT and PWL ($P < 0.01$ *vs*. predrug), which increased significantly to the levels that were not different from those measured before L5 SNL ($P > 0.05$).

Pretreatment with Neurosteroid Synthesis Inhibitor AMG Blocked the Effects of Diazepam and Ro5-4864 on Neuropathic Pain

It has been shown that both CBR and TSPO function *via* promotion of neurosteroidogenesis (8, 9, 29). To investigate whether synthesis of neurosteroid is also involved in the effects of diazepam and Ro5-4864 on neuropathic pain, a neurosteroid synthesis inhibitor, AMG (10 mg/kg, i.p.), was injected intraperitoneally 30 min before intraperitoneal injection of diazepam or Ro5-4864. In the AMG-treated group, 50% PWT and PWL in the ipsilateral side did not change following intraperitoneal injection of either diazepam (Fig. 3, A and B, $P > 0.05$ *vs*. predrug) or Ro5-4864 (Fig. 3, C and D, $P > 0.05$ *vs*. predrug), while in rats pretreated with DMSO, which was used to dissolve AMG, both 50% PWT and PWL increased significantly ($P < 0.01$ *vs*. predrug) to the levels that were not different from those measured before L5 SNL ($P > 0.05$).

A Single Intraperitoneal Injection of Pregnenolone Reversed Neuropathic Pain Induced by L5 SNL

To further test whether neurosteroids participate in neuropathic pain, a neurosteroid precursor, pregnenolone, which is also the most abundant neurosteroid produced in the neural tissue of rats and humans (3, 24), was injected intraperitoneally (5 mg/kg) 9 d after L5 SNL. The results showed that a single injection of pregnenolone also significantly inhibited the decrease in 50% PWT (Fig. 4A) and PWL (Fig. 4B)

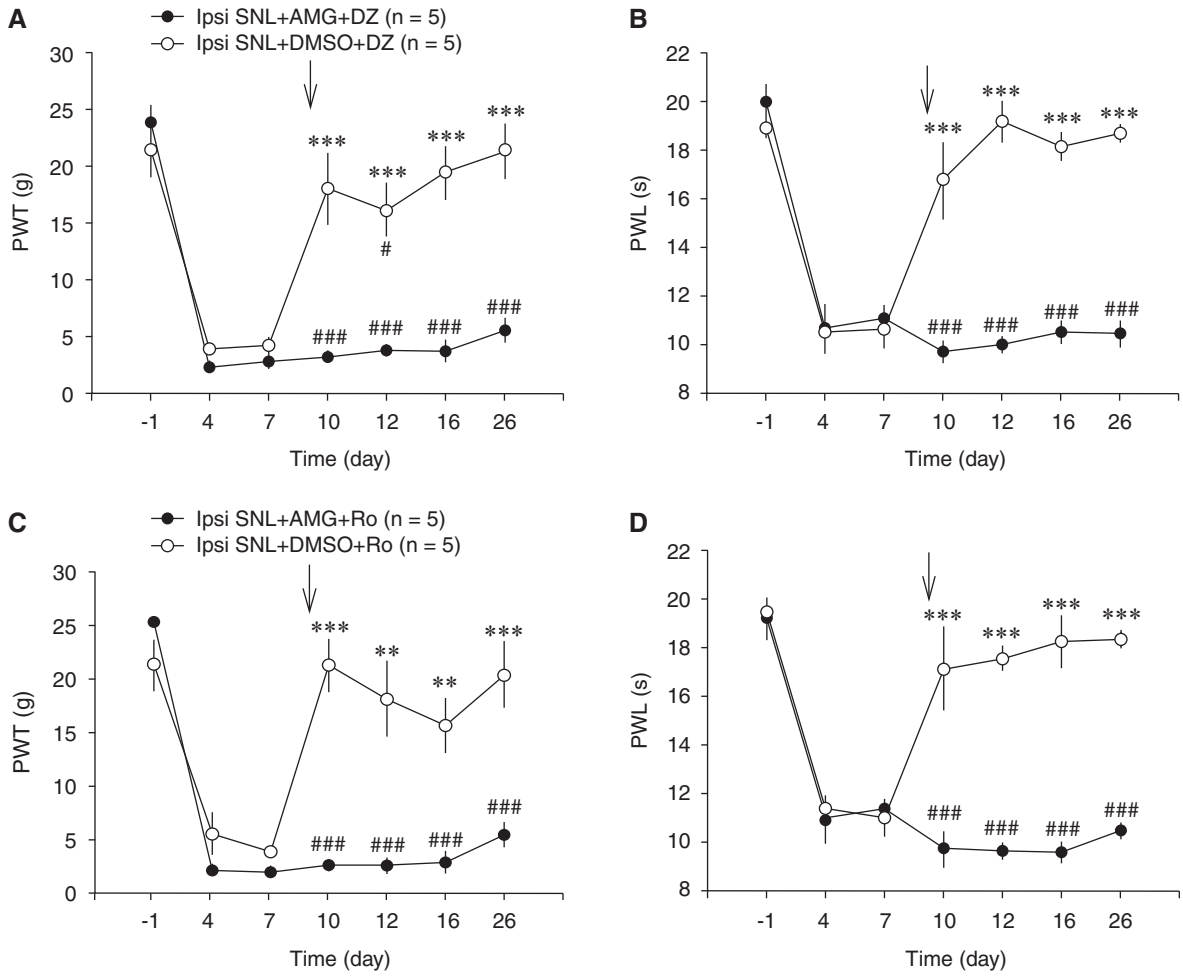

Fig. 3. Effects of diazepam and Ro5-4864 on neuropathic pain induced by L5 SNL was prevented by pretreatment with AMG in rats. (A and B) Application of AMG (10 mg/kg, i.p) but not its vehicle DMSO 30 min before intraperitoneal injection of diazepam (5 mg/kg) abolished its effects on the decrease of 50% PWT (A) and PWL (B) in the ipsilateral (Ipsi) side induced by L5 SNL (n = 5 per group). (C and D) Application of AMG (10 mg/kg, i.p) but not DMSO 30 min before intraperitoneal injection of Ro5-4864 (5 mg/kg) abolished its effects on the decrease of 50% PWT (C) and PWL (D) in the ipsilateral side induced by L5 SNL (n = 5 per group). The arrows indicate the time of intraperitoneal injection of diazepam or Ro5-4864. $^{*}P < 0.01$, $^{***}P < 0.001$ versus before diazepam (DZ) or Ro5-4864 (Ro). $^{\#\#\#}P < 0.001$ versus before L5 SNL.

induced by L5 SNL, and the effect persisted until the end of experiment, which was comparable to diazepam and Ro5-4864. Treatment with the vehicle Tween-80 had no effect on the established mechanical allodynia (Fig. 4A) and thermal hyperalgesia (Fig. 4B).

A Single Intraperitoneal Injection of Diazepam, Ro5-4864 or Pregnenolone Inhibited the Activation of Astrocytes in the L5 Spinal Dorsal Horn

Activation of astrocytes in the spinal cord is important for the maintenance of neuropathic pain (41). In the present study, we next investigated whether a single intraperitoneal of diazepam, Ro5-4864 or pregnenolone affect the activation of astrocytes in the ipsilateral L5 spinal dorsal horn induced by L5 SNL with use of immunohistochemistry. Compared with the sham group (Fig. 5, A, D and G) 14 d after L5 SNL, astrocytes were significantly activated in the ipsilateral L5 spinal dorsal horn, manifesting as over-expression of GFAP (Fig. 5, B, E and H, $P < 0.001$, vehicle-treated L5 SNL group *vs*. sham). The activation of astrocytes produced by L5 SNL was suppressed by diazepam (Fig. 5, C and J), Ro5-4864 (Fig. 5, F and K) or pregnenolone (Fig. 5, I and L) administrated 5 d before (14 d after L5 SNL, $P < 0.001$, drug treated group *vs*. vehicle treated group).

A Single Intraperitoneal Injection of Diazepam, Ro5-4864 or Pregnenolone Reduced the Production of IL-1β in the L5 Spinal Dorsal Horn

IL-1β, which has a long lasting effect (44) than TNFα (45), may contribute to the development and

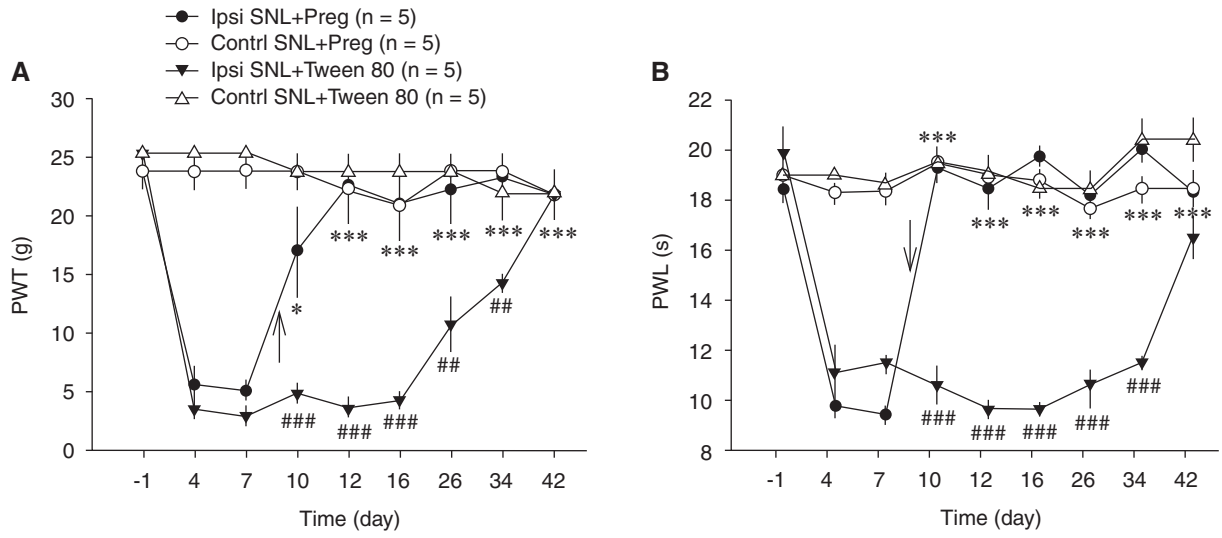

Fig. 4. A single intraperitoneal injection of pregnenolone 9 d after L5 SNL inhibited the mechanical allodynia and thermal hyperalgesia. (A and B) Intraperitoneal injection of pregnenolone (5 mg/kg) 9 d after L5 SNL depressed the decrease of 50% PWT (A) and PWL (B) in the ipsilateral (Ipsi) side (n = 5), whereas injection of vehicle Tween 80 had no effect (n = 5). The arrows indicate the time of intraperitoneal injection of pregnenolone. $^{*}P < 0.05$, $^{***}P < 0.001$ versus before pregnenolone (Preg); $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus before L5 SNL. Contrl, control.

maintenance of neuropathic pain by multiple mechanisms (17). We next investigated by western blot whether a single intraperitoneal injection of diazepam, Ro5-4864 or pregnenolone affect the production of IL-1β in the ipsilateral L5 spinal dorsal horn. Compared with the sham group, 14 d after L5 SNL, IL-1β increased significantly in the ipsilateral L5 spinal dorsal horn (Fig. 6, A, B and C, $P < 0.001$, vehicle-treated L5 SNL group *vs*. sham). The increased IL-1β produced by L5 SNL was suppressed by diazepam, Ro5-4864 or pregnenolone administrated 5 d before ($P < 0.001$, drug treated group *vs*. vehicle treated group).

Discussion

In the present study, we showed that a single intraperitoneal injection of diazepam significantly inhibited the established mechanical allodynia and thermal hyperalgesia induced by L5 SNL for at least 3 weeks. Flumazenil, a specific antagonist, could completely prevent the inhibitory effect of diazepam on neuropathic pain. A single intraperitoneal injection of the TSPO agonist Ro5-4864 or the neurosteroid precursor, pregnenolone, could mimic the effects of diazepam on neuropathic pain. Importantly, pretreatment with AMG, a neurosteroid synthesis inhibitor, significantly abolished the effects of diazepam and Ro5-4864. Further more, each of the three drugs, diazepam, Ro5-4864 and pregnenolone, reduced activation of astrocytes and the production of an important cytokine, IL-1β, in the L5 spinal dorsal horn induced by L5 SNL. These results suggest that diazepam might inhibit neuropathic pain *via* activation of CBR and TSPO, which then inhibited the activation of astrocytes and the production of IL-1β in the spinal dorsal horn by promoting the synthesis of neurosteroids.

Diazepam May Inhibit Neuropathic Pain via *CBR- and TSPO-Mediated Neurosteroid Synthesis*

In the present study, we found that a single intraperineal injection of diazepam could inhibit the neuropathic pain even 3 weeks after the injection, when diazepam injected had been eliminated from the circulation in the rats. One possibility why diazepam had such a lasting effect is that it induced a persistent synthesis of chemicals, thus changing the residing environment that of the neurons.

It has been reported that activation of CBR can stimulate neurosteroids biosynthesis (8, 9). In the present study, we found that pretreatment with flumazenil, a specific CBR antagonist, completely prevented the inhibitory effect of diazepam on neuropathic pain induced by L5 SNL. Thus, diazepam may also promote the synthesis of neurosteroids *via* CBR. Diazepam also binds to TSPO with high affinity (40). Our previous study has demonstrated that TSPO is upregulated in spinal dorsal horn following L5 SNL, and that a single intrathecal injection of TSPO-specific agonists (either Ro5-4864 or FGIN-1-27) clearly inhibited neuropathic pain (43). In the present study, we also found that a single intraperineal injection of Ro5-4864 inhibited L5 SNL induced neuropathic pain,

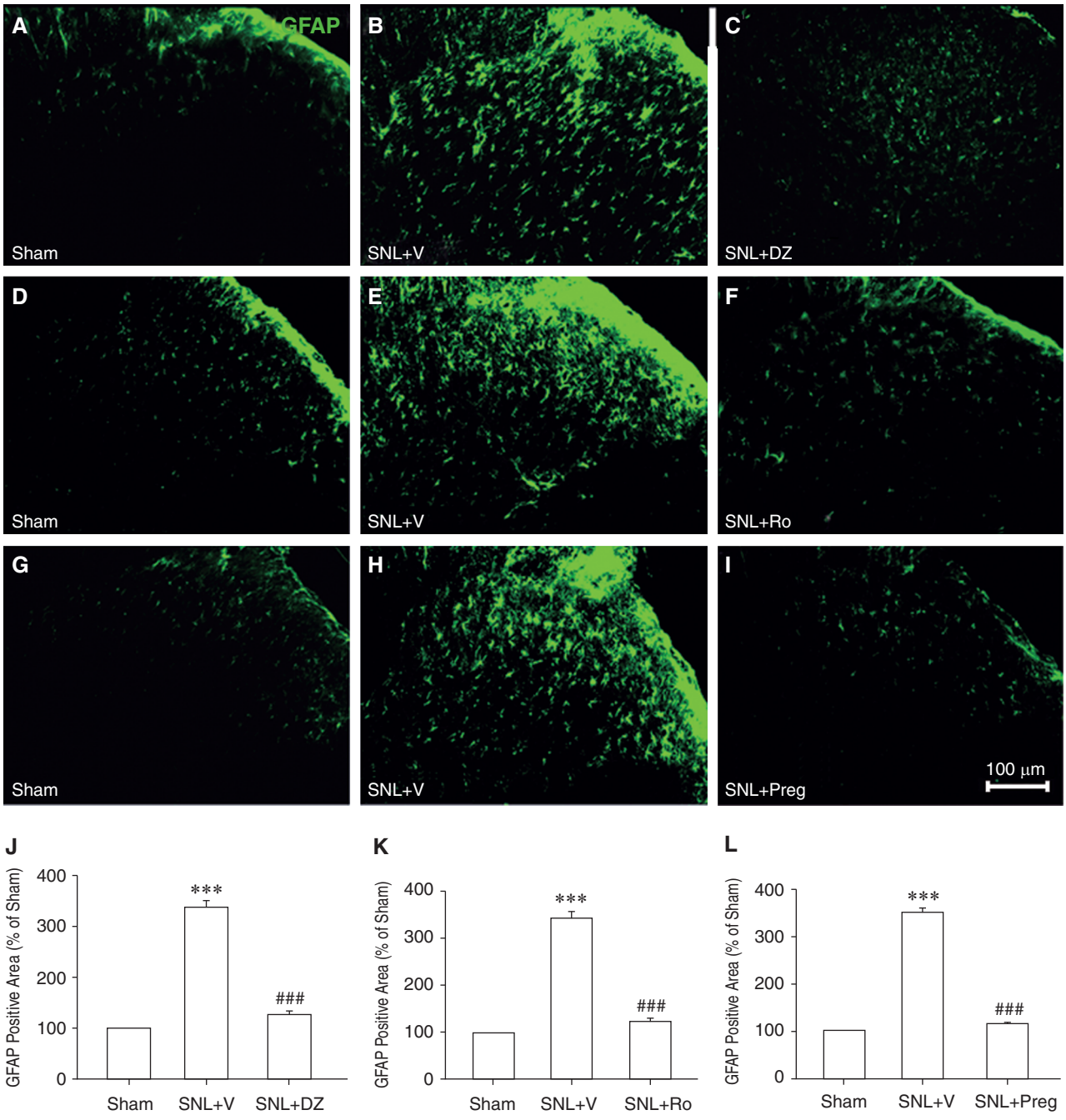

Fig. 5. A single intraperitoneal injection of diazepam, Ro5-4864 or pregnenolone 9 d after L5 SNL inhibited the activation of astrocytes in the L5 spinal dorsal horn. (A to I) Representative experiments showing the change of GFAP-IR in the ipsilateral L5 spinal dorsal horn from different group, as indicated. $^{***}P < 0.001$ versus sham group, $^{###}P < 0.001$ versus vehicle (V)-treated SNL group. J-L, Quantification of GFAP-IR-positive area in the ipsilateral L5 spinal dorsal horn in sham, V-treated, and diazepam [Ro5-4864 (Ro), or pregnenolone (Preg)]-treated L5 SNL rats (n = 6/per group).

and the effects was comparable to diazepam, suggesting that TSPO may also contribue to the beneficial effects of a single injection of diazepam on neuropathic pain. This is consistent with previous studies showing that acute diazepam treatments (10 or 20 mg/kg) reduce carrageenan-induced paw edema volume, and that this effect is attributed to an action of diazepam on the TSPO (21, 22). Thus, the neurosteroid synthesis following CBR and TSPO activation is necessary for the long lasting effect of diazepam on neuropathic pain (Fig. 7).

The synthesis of neuroactive steroids, including pregnenolone, progesterone, allopregnanolone, and dehydroepiandrosterone, could be promoted by TSPO

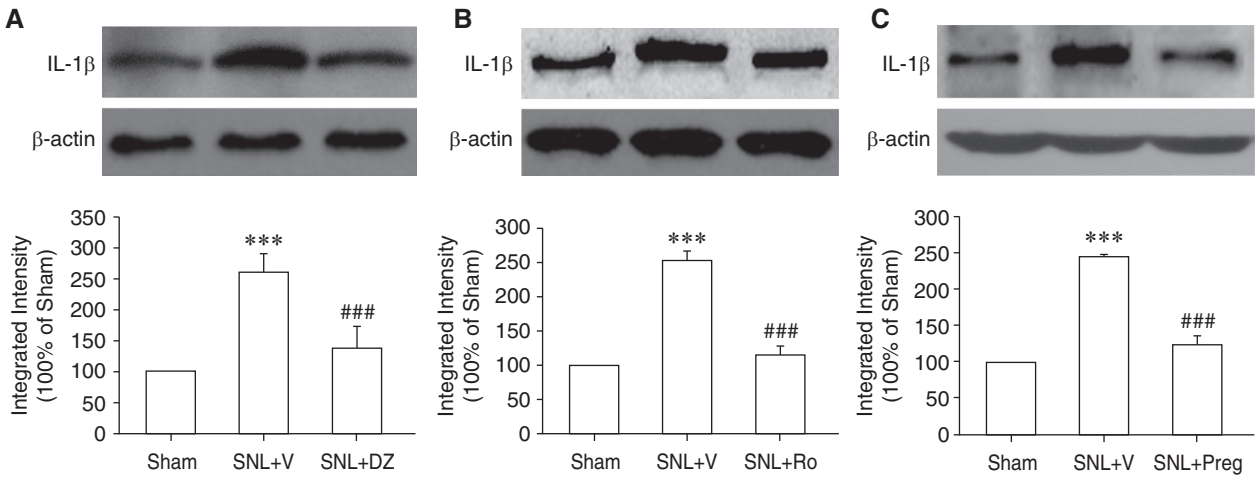

Fig. 6. A single intraperitoneal injection of diazepam, Ro5-4864 or pregnenolone 9 d after L5 SNL inhibited the upregulation of IL-1β in the L5 spinal dorsal horn. (A to C) The bands show the expression of IL-1β and β-actin in the ipsilateral L5 spinal dorsal horn in different groups. The histogram shows the quantification of IL-1β normalized by β-actin (n = 6 per group). $^{***}P < 0.001$ versus sham group, $^{\#\#\#}P < 0.001$ versus vehicle (V) - treated SNL group. DZ, diazepam; Ro, Ro-4864; Preg, Pregnenolone.

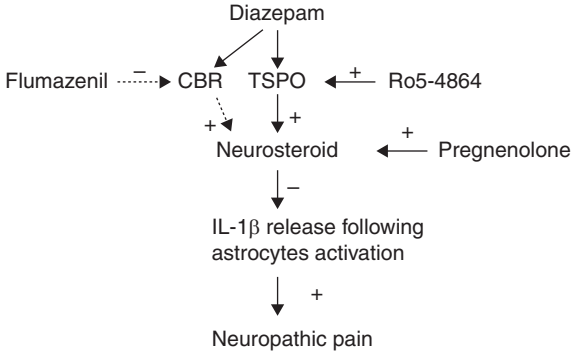

Fig. 7. Schematic illustration of mechanisms by which diazepam inhibits L5 SNL- induced neuropathic pain. IL-1β release following astrocyte activation plays an important role in the maintenance of neuropathic pain. Diazepam activates CBR and TSPO, leads to increased expression of neurosteroid, downregulates the IL-1β expression, and eventually inhibits neuropathic pain. CBR antagonist flumazenil could prevent the effect of diazepam on neuropathic pain. TSPO-specific agonist Ro5-4864 and the neurosteroid precursor pregnenolone can also inhibit neuropathic pain by inhibiting IL-1β.

(37). Pregnenolone, as a precursor of all steroids, may lead to substantial synthesis of steroid metabolites. Previous data have indicated that a substantial part of the steroid metabolites such as progesterone may be also synthesized in the CNS from the steroid precursors, or directly transported through blood brain barrier (BBB) from the periphery, modulating the function of neurons (15). In the present study, we found that diazepam, Ro5-4864 or pregnenolone each had similar effects on neuropathic pain, astrocytes activation and IL-1β expression. Furthermore, the neurosteroid synthesis inhibitor AMG that specifically blocks P450 side-chain cleavage, which converts cholesterol into pregnenolone (the precursor for the biosynthesis of all steroid hormone), prevented the effects of diazepam or Ro5-4864 on neuropathic pain. These results suggest that these drugs inhibit neuropathic pain by inducing the same targets, that is, neurosteroids.

Multiple mechanisms may be involved in the inhibitory effects of neurosteroids on neuropathic pain. Neuropathic pain has been associated with reduced spinal GABA-ergic inhibitory function. Activation of spinal GABA receptors attenuates chronic central neuropathic pain after spinal cord injury (10). Previous studies have revealed that neurosteroids interact with an allosterically coupled binding complex of the GABAA chloride ionophore. This coupling augments the GABA-initiated opening of the channel. At higher concentrations, these neurosteroids directly activate the GABAA receptor channel complex (4). It has also been shown that progesterone prevents the injury-induced upregulation of N-methyl-D-aspartate receptor subunits and protein kinase C gamma RNA (6), and that allopregnanolone attenuates neuropathic pain by blocking T-type calcium channels (33, 34). Neurosteroids have also been shown to interact with glycine receptor mediated chloride currents (27, 28, 46).

Diazepam May Inhibit Neuropathic Pain by Inhibiting Neuroinflammation

It has been demonstrated that cytokines, includ-

ing TNF-α, IL-1β and IL-6, play important roles in neuropathic pain (7). Our previous studies have shown that application of recombinant rat TNF-α or IL-1β to the healthy sciatic nerve could induce mechanical allodynia (44, 45), and that inhibition either of TNF-α or IL-6 could alleviate neuropathic pain following L5 ventral root transection (42, 50). In the present study, we found that a single intraperitoneal injection of diazepam 9 d after L5 SNL inhibited the production of IL-1β, one of the important cytokines, and the effects were mimicked by Ro5-4864 or pregnenolone, suggesting that TSPO- mediated anti-inflammatory effects may contribute to the effects of diazepam on neuropathic pain (Fig. 7). These results are also consistent with previous studies showing that diazepam could inhibit the release of the inflammatory molecules nitric oxide (NO) and TNF-α in cell culture supernatants of primary rat microglia (47), and that progesterone, one of the neurosteroids, can reduce the expression of TNF-α and IL-1β in cultured microglia (14).

Previous studies have demonstrated that IL-1β can enhance AMPA- or NMDA-induced currents, and suppress GABA- and glycine-induced currents (17). As IL-1β is expressed by both astrocytes and microglia (1), and TSPO is upregulated in astrocytes and microglia (43), diazepam may first bind with TSPO located in glial cells, inhibiting their cytokines release, then modulated the function of neurons. A previous study has reported that after peripheral nerve injury, microglia cells are activated within 24 h after injury in the spinal dorsal horn, whereas astrocyte activation occurs later than microglial, around 4 days post-injury (36), suggesting that microglia cells are critical for initiation and astrocytes for the maintenance of neuropathic pain. In the present study, diazepam, Ro5-4864 or pregnenolone was injected 9 d after L5 SNL, when the neuropathcic pain had been established. These drugs may have first influenced astrocytes, then modulated the function of neurons. Direct effects on neurons could not be excluded, as TSPO is also located in neurons, although it is not up-regulated after L5 SNL (43).

Previous reports have shown that TSPO and their endogenous ligands, the diazepam-binding inhibitor derived-peptides, are present in Schwann cells in the peripheral nervous system. This expression is increased after nerve lesion, and returns to normal pattern when regeneration is complete (20). In the present study, though we did not evaluate the changes in the peripheral system after intraperitoneal injection of diazepam, TSPO located in damaged nerves and dorsal root ganglia may also be involved in facilitating nerve remyelination and regeneration *via* neurosteroid synthesis, thus resulted in a long-term protection of the nerve, and relieved the neuropathic pain induced by L5 SNL. Previous studies have reported that TSPO level in the brain was increased in patients with chronic low back pain (23) and that diazepam can readily be distributed into the different regions of brain tissues (16), it is possible that diazepam also exerts effects via binding to recepors in the brain.

Thus, the effects of a single injection of diazepam on neuropathic pain might be a consequence of the TSPO-dependent increased neurosteroids levels, which then inhibits the production of cytokines and at last modulates the function of GABAA, AMPA, NMDA and receptors. In conclusion, in addition to CBR, TSPO-mediated neurosteroid synthesis may play an important role in the effects of diazepam on neuropathic pain.

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References

1. Bao, L., Zhu, Y., Elhassan, A.M., Wu. Q., Xiao, B., Zhu, J. and Lindgren, J.U. Adjuvant-induced arthritis: IL-1 beta, IL-6 and TNF-alpha are up-regulated in the spinal cord. *Neuroreport* 12: 3905-3908, 2001.
2. Basbaum, A.I. Distinct neurochemical features of acute and persistent pain. *Proc. Natl. Acad. Sci. USA* 96: 7739-7743, 1999.
3. Baulieu, E.E., Robel, P. and Schumacher, M. Neurosteroids: beginning of the story. *Int. Rev. Neurobiol.* 46: 1-32, 2001.
4. Callachan, H., Cottrell, G.A., Hather, N.Y., Lambert, J.J., Nooney, J.M. and Peters, J.A. Modulation of the GABAA receptor by progesterone metabolites. *Proc. R. Soc. Lond. B. Biol. Sci.* 231: 359-369, 1987.
5. Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M. and Yaksh, T.L. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* 53: 55-63, 1994.
6. Coronel, M.F., Labombarda, F., Roig, P., Villar, M.J., De Nicola, A.F. and González, S.L. Progesterone prevents nerve injury-induced allodynia and spinal NMDA receptor upregulation in rats. *Pain Med.* 12: 1249-1261, 2011.
7. DeLeo, J.A., Colburn, R.W. and Rickman, A.J. Cytokine and growth factor immunohistochemical spinal profiles in two animal models of mononeuropathy. *Brain Res.* 759: 50-57, 1997.
8. Do-Rego, J.L., Mensah-Nyagan, A.G., Beaujean, D., Leprince, J., Tonon, M.C., Luu-The, V., Pelletier, G. and Vaudry, H. The octadecaneuropeptide ODN stimulates neurosteroid biosynthesis through activation of central-type benzodiazepine receptors. *J. Neurochem.* 76: 128-138, 2001.
9. Garnier, M., Boujrad, N., Oke, B.O., Brown, S., Riond, J., Ferrara, P., Shoyab, M., Suarez-Quian, A. and Papadopoulos, V. Diazepam binding inhibitor is a paracrine/autocrine regulator of Leydig cell proliferation and steroidogenesis: action *via* peripheral-type benzodiazepine receptor and independent mechanisms. *Endocrinology* 132: 444-458, 1993.

10. Gwak, Y.S., Tan, H.Y., Nam. T.S., Paik, K.S., Hulsebosch, C.E. and Leem, J.W. Activation of spinal GABA receptors attenuates chronic central neuropathic pain after spinal cord injury. *J. Neurotrauma* 23: 1111-1124, 2006.
11. Hargreaves, K., Dubner, R., Brown. F., Flores, C. and Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32: 77-88, 1988.
12. Hu, X.D., Ge, Y.X., Hu, N.W., Zhang, H.M., Zhou, L.J., Zhang, T., Li, W.M., Han, Y.F. and Liu, X.G. Diazepam inhibits the induction and maintenance of LTP of C-fiber evoked field potentials in spinal dorsal horn of rats. *Neuropharmacology* 50: 238-244, 2006.
13. Ji, R.R., Kohno, T., Moore, K.A. and Woolf, C.J. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci.* 26: 696-705, 2003.
14. Jiang, C., Cui, K., Wang, J. and He, Y. Microglia and cyclooxygenase-2: possible therapeutic targets of progesterone for stroke. *Int. Immunopharmacol.* 11: 1925-1931, 2011.
15. Kancheva, R., Hill, M., Novak, Z., Chrastina, J., Kancheva, L. and Stárka, L. Neuroactive steroids in periphery and cerebrospinal fluid. *Neuroscience* 191: 22-27, 2011.
16. Kaur, P. and Kim, K. Pharmacokinetics and brain uptake of diazepam after intravenous and intranasal administration in rats and rabbits. *Int. J. Pharm.* 364: 27-35, 2008.
17. Kawasaki, Y., Zhang, L., Cheng, J.K. and Ji, R.R. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J. Neurosci.* 28: 5189-5194, 2008.
18. Kim, S.H. and Chung, J.M. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50: 355-363, 1992.
19. Knabl, J., Witschi, R., Hösl, K., Reinold, H., Zeilhofer, U.B., Ahmadi, S., Brockhaus, J., Sergejeva, M., Hess, A., Brune, K., Fritschy, J.M., Rudolph, U., Möhler, H. and Zeilhofer, H.U. Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature 4*51: 330-334, 2008.
20. Lacor, P., Benavides, J. and Ferzaz, B. Enhanced expression of the peripheral benzodiazepine receptor (PBR) and its endogenous ligand octadecaneuropeptide (ODN) in the regenerating adult rat sciatic nerve. *Neurosci. Lett.* 220: 61-65, 1996.
21. Lazzarini, R., Malucelli, B.E., Muscará, M.N., de Nucci, G. and Palermo-Neto, J. Reduction of inflammation in rats by diazepam: tolerance development. *Life Sci.* 72: 2361-2368, 2003.
22. Lazzarini, R., Malucelli, B.E. and Palermo-Neto, J. Reduction of acute inflammation in rats by diazepam: role of peripheral benzodiazepine receptors and corticosterone. *Immunopharmacol. Immunotoxicol.* 23: 253-265, 2001.
23. Loggia, M.L., Chonde, D.B., Akeju, O., Arabasz, G., Catana, C., Edwards, R.R., Hill, E., Hsu, S., Izquierdo-Garcia, D., Ji, R.R., Riley, M., Wasan, A.D., Zurcher, N.R., Albrecht, D.S., Vangel, M.G., Rosen, B.R., Napadow, V. and Hooker, J.M. Evidence for brain glial activation in chronic pain patients. *Brain* 138: 604-615, 2015.
24. Mellon, S.H. Neurosteroid regulation of central nervous system development. *Pharmacol. Ther.* 116: 107-124, 2007.
25. Mensah-Nyagan, A.G., Meyer, L., Schaeffer., V. Kibaly, C. and Patte-Mensah, C. Evidence for a key role of steroids in the modulation of pain. *Psychoneuroendocrinology* 34 (Suppl 1): S169-S177, 2009.
26. Meyer, L., Patte-Mensah, C., Taleb, O. and Mensah-Nyagan, A.G. Allopregnanolone prevents and suppresses oxaliplatin-evoked painful neuropathy: multi-parametric assessment and direct evidence. *Pain* 152: 170-181, 2011.
27. Mitchell, E.A., Gentet, L.J., Dempster, J. and Belelli, D. GABAA and glycine receptor-mediated transmission in rat lamina II neurons: relevance to the analgesic actions of neuroactive steroids. *J. Physiol.* 583: 1021-1040, 2007.
28. Mitchell, E.A., Herd, M.B., Gunn, B.G., Lambert, J.J. and Belelli, D. Neurosteroid modulation of GABAA receptors: molecular determinants and significance in health and disease. *Neurochem. Int.* 52: 588-595, 2008.
29. Nothdurfter, C., Baghai, T.C., Schule, C. and Rupprecht, R. Translocator protein (18 kDa) (TSPO) as a therapeutic target for anxiety and neurologic disorders. *Eur. Arch. Psychiatry Clin. Neurosci.* 262 (Suppl 2): S107-S112, 2012.
30. Olsen, R.W., McCabe, R.T. and Wamsley, J.K. GABAA receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. *J. Chem. Neuroanat.* 3: 59-76, 1990.
31. Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapere, J.J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.R. and Gavish, M. Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol. Sci.* 27: 402-409, 2006.
32. Papadopoulos, V., Mukhin, A.G., Costa, E. and Krueger, K.E. The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J. Biol. Chem.* 265: 3772-3779, 1990.
33. Pathirathna, S., Brimelow, B.C., Jagodic, M.M., Krishnan, K., Jiang, X., Zorumski, C.F., Mennerick, S., Covey, D.F., Todorovic, S.M. and Jevtovic-Todorovic, V. New evidence that both T-type calcium channels and GABAA channels are responsible for the potent peripheral analgesic effects of 5alpha-reduced neuroactive steroids. *Pain* 114: 429-443, 2005.
34. Pathirathna, S., Todorovic, S.M., Covey, D.F. and Jevtovic-Todorovic, V. 5alpha-reduced neuroactive steroids alleviate thermal and mechanical hyperalgesia in rats with neuropathic pain. *Pain* 117: 326-339, 2005.
35. Ramseier, H., Lichtensteiger, W. and Schlumpf, M. *In vitro* inhibition of cellular immune responses by benzodiazepines and PK 11195. Effects on mitogen- and alloantigen-driven lymphocyte proliferation and on IL-1, IL-2 synthesis and IL-2 receptor expression. *Immunopharmacol. Immunotoxicol.* 15: 557-582, 1993.
36. Romero-Sandoval, A., Chai, N., Nutile-McMenemy, N. and Deleo, J.A. A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res*. 1219: 116-126, 2008.
37. Rupprecht, R., Papadopoulos, V., Rammes, G., Baghai, T.C., Fan, J., Akula, N., Groyer, G., Adams, D. and Schumacher, M. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nat. Rev. Drug Discov.* 9: 971-988, 2010.
38. Sandkuhler, J. Understanding LTP in pain pathways. *Mol. Pain* 3: 9, 2007.
39. Smith, H.S. Activated microglia in nociception. *Pain Physician* 13: 295-304, 2010.
40. Snyder, S.H., Verma, A. and Trifiletti, R.R. The peripheral-type benzodiazepine receptor: a protein of mitochondrial outer membranes utilizing porphyrins as endogenous ligands. *FASEB J*. 1: 282-288, 1987.
41. Watkins, L.R. and Maier, S.F. Glia: a novel drug discovery target for clinical pain. *Nat. Rev. Drug Discov.* 2: 973-985, 2003.
42. Wei, X.H., Na, X.D., Liao, G.J., Chen, Q.Y., Cui, Y., Chen, F.Y., Li, Y.Y., Zang, Y. and Liu, X.G. The up-regulation of IL-6 in DRG and spinal dorsal horn contributes to neuropathic pain following L5 ventral root transection. *Exp. Neurol.* 241: 159-168, 2013.
43. Wei, X.H., Wei, X., Chen, F.Y., Zang, Y., Xin, W.J., Pang, R.P., Chen, Y., Wang, J., Li, Y.Y., Shen, K.F., Zhou, L.J. and Liu, X.G. The upregulation of translocator protein (18 kDa) promotes recovery from neuropathic pain in rats. *J. Neurosci.* 33: 1540-1551, 2013.
44. Wei, X.H., Yang, T., Wu, Q., Xin, W.J., Wu, J.L., Wang, Y.Q., Zang, Y., Wang, J., Li, Y.Y. and Liu, X.G. Peri-sciatic adminis-

tration of recombinant rat IL-1beta induces mechanical allodynia by activation of src-family kinases in spinal microglia in rats. *Exp. Neurol.* 234: 389-397, 2012.

45. Wei, X.H., Zang, Y., Wu, C.Y., Xu, J.T., Xin, W.J. and Liu, X.G. Peri-sciatic administration of recombinant rat TNF-alpha induces mechanical allodynia *via* upregulation of TNF-alpha in dorsal root ganglia and in spinal dorsal horn: the role of NF-kappa B pathway. *Exp. Neurol.* 205: 471-484, 2007.
46. Weir, C.J., Ling, A.T., Belelli, D., Wildsmith, J.A., Peters, J.A. and Lambert, J.J. The interaction of anaesthetic steroids with recombinant glycine and GABAA receptors. *Brit. J. Anaesth.* 92: 704-711, 2004.
47. Wilms, H., Claasen, J., Rohl, C., Sievers, J., Deuschl, G. and Lucius, R. Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells *in vitro*. *Neurobiol. Dis.* 14: 417-424, 2003.
48. Woolf, C.J. and Mannion, R.J. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 353: 1959-1964, 1999.
49. Woolf, C.J. and Salter, M.W. Neuronal plasticity: increasing the gain in pain. *Science* 288: 1765-1769, 2000.
50. Xu, J.T., Xin, W.J., Zang, Y., Wu, C.Y. and Liu, X.G. The role of tumor necrosis factor-alpha in the neuropathic pain induced by Lumbar 5 ventral root transection in rat. *Pain* 123: 306-321, 2006.
51. Young, W.S.III. and Kuhar, M.J. Radiohistochemical localization of benzodiazepine receptors in rat brain. *J. Pharmacol. Exp. Ther.* 212: 337-346, 1980.
52. Zavala, F. Benzodiazepines, anxiety and immunity. *Pharmacol. Ther.* 75: 199-216, 1997.
53. Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16: 109-110, 1983.