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Original Reports

MAO-B Inhibitor, KDS2010, Alleviates Spinal Nerve Ligation-induced Neuropathic Pain in Rats Through Competitively Blocking the BDNF/TrkB/NR2B Signaling

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Abstract: **MAO-B inhibitors have been implicated to reverse neuropathic pain behaviors. Our previous study has demonstrated that KDS2010 (KDS), a newly developed reversible MAO-B inhibitor, could attenuate Paclitaxel (PTX)-induced tactile hypersensitivity in mice through suppressing reactive oxidant species (ROS)-decreased inhibitory GABA synaptic transmission in the spinal cord. In this study, we evaluated the analgesic effect of KDS under a new approach, in which KDS acts on dorsal horn sensory neurons to reduce excitatory transmission. Oral administration of KDS effectively enhanced mechanical thresholds in the spinal nerve ligation (SNL) induced neuropathic pain in rats. Moreover, we discovered that although treatment with KDS increased brain-derived neurotrophic factor (BDNF) levels, KDS inhibited Tropomyosin receptor kinase B (TrkB) receptor activation, suppressing increased p-NR2B-induced hyperexcitability in spinal dorsal horn sensory neurons after nerve injury. In addition, KDS showed its anti-inflammatory effects by reducing microgliosis and astrogliosis and the activation of MAPK and NF-κB inflammatory pathways in these glial cells. The levels of ROS production in the spinal cords after the SNL procedure were also decreased with KDS treatment. Taken together, our results suggest that KDS may represent a promising therapeutic option for treating neuropathic pain.**

Perspective: ***Our study provides evidence suggesting the mechanisms by which KDS, a novel MAO-B inhibitor, can be effective in pain relief. KDS, by targeting multiple mechanisms involved in BDNF/TrkB/NR2B-related excitatory transmission and neuroinflammation, may represent the next future of pain medicine.***

Key Words: *Neuropathic pain, spinal nerve ligation, MAO-B inhibitor, KDS2010, brain-derived neurotrophic factor.*

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MAO-B, an enzyme located in the mitochondrial outer membrane, together with its isoenzyme MAO-A, catalyzes the oxidative deamination of structurally diverse amines.[12,31] Due to its original function in monoamine metabolism, MAO has been linked to several psychiatric and neurological disorders that are associated with monoamine neurotransmitters.[24,29,35,39] MAO-B inhibitors have shown a significant anti-oxidant effect against neurotoxicity by decreasing the production of ROS.[2,17] In terms of pain research, an early study revealed significantly increased MAO-B activity in neuropathic mice compared with the sham mice, which was reversed by an MAO-B inhibitor treatment, hence resulting in an anti-allodynic effect.[53] However, the mechanisms by which MAO-B inhibitors can reverse neuropathic pain behaviors remain understudied. Notably, the involvement of oxidative stress has been regarded as an important mechanism in the pathogenesis of neuropathic pain.[48,51] In a line with this, our recent study has shown increased ROS levels contribute to decreased spinal GABA inhibition in a PTX-induced pain hypersensitivity mouse model.[46] KDS, as an MAO-B inhibitor, alleviated pain behaviors by suppressing ROS generation-decreased GABA release, theoretically from spinal astrocytes.

On the other hand, MAO-B inhibitors have been shown to increase BDNF levels not only in in vitro or in vivo experimental models of some neurodegenerative diseases,[32] but also in human patients with PD or severe refractory depression,[19,38] leading to neuroprotective effects of these drugs. It is well known that BDNF has a beneficial role in promoting neurogenesis against the progression of neurodegenerative disorders.[4,26,28] In terms of neuropathic pain, BDNF binding to its cognate high-affinity TrkB receptor is widely believed to be involved in the central sensitization and synaptic plasticity in the spinal cord.[42,49,50,52,57,62] BDNF/TrkB signaling has been shown to contribute to the development and maintenance of neuropathic pain by activation of the NR2B-containing NMDA receptors.[27,30,61] Moreover, the administration of TrkB phosphorylation inhibitor in the spinal cord has been demonstrated to be able to prevent the initiation and development of nerve injury-induced pain hypersensitivity in mice.[55] In the previous work, we have shown that KDS, an MAO-B inhibitor, exhibited its anti-allodynic effect in chemotherapy-induced peripheral neuropathy (CIPN) in mice.[46] This agrees with the early study which revealed Selegiline, a selective MAO-B inhibitor, could reverse partial sciatic nerve ligation-induced mechanical allodynia.[53] Notably, unlike the nerve injury-induced neuropathic pain model, the CIPN pain model does not present a significant contributing role of BDNF. To date, there is no report showing the link between BDNF and the pathogenesis of CIPN. We questioned how MAO-B inhibitors, here for KDS, work in other neuropathic pain conditions with the presence of nerve injury-induced BDNF/TrkB signaling activation? Is ROS-mediated GABA inhibition a general anti-allodynic effect of KDS or whether there is an additional contribution of BDNF/TrkB signaling? These questions prompted us to further study the relation between BDNF/TrkB and MAO-B inhibitors.

In the present study, we aimed to test the analgesic effect of KDS, a newly developed reversible MAO-B inhibitor, on SNL – induced neuropathic pain in rats. SNL model is a well-established pain model with the presence of nerve damage and neuroinflammation contributing to the onset of pain.[20] Among several reversible MAO-B inhibitors, KDS has been demonstrated in several previous studies to have excellent potency and drug ability with safety,[23,45] we reasoned that KDS, as an MAO-B inhibitor, may attenuate nerve injury-induced neuropathic pain by mechanisms involved in BDNF/Tribe/NR2B signaling. Given the previous report on the analgesic effect of KDS on neuropathic pain, our study provides, for the first time, a novel insight into the mechanisms by which KDS, a novel MAO-B inhibitor can be effective in pain relief.

Methods

Animals

Sprague-Dawley rats (6 weeks, 150–200 g) were purchased from Samtako Bio (Kyung-Gi-Do, Republic of Korea) and adapted to a new environment for at least 3 days prior to experiments. All animals were housed (3 rats per cage) in a room maintained at approximately 24 ± 1°C temperature and humidity of 55 ± 5% with 12-hours light/dark cycle and free access to food and water. These experiments were approved by the Animal Care and Use Committee at the Chungnam National University (CNUH-A017-0017) and prepared with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain.

Experimental Design

For behavioral tests, the rats, passing the baseline of at least 10-g filament in the von Frey tests, were randomly divided into 3 groups: the SHAM, the SNL-untreated (SNL), and the SNL-KDS-treated (KDS) groups for the behavior assessment in Fig 1B; or divided into 5 groups: the SHAM, the SNL, the SNL-ANA12, SNL-KDS, and the SNL-KDS-ANA12 treated groups for the behavior assessment in Fig 7D. The sham/SNL surgical procedure was conducted on the rats in the corresponding groups on the same day. The von Frey tests were performed to monitor the pain responses and were repeated in every experimental group to confirm behavior phenotypes. 3 days after the operation (post-operative day, POD), KDS was administered to the rats in the KDS-treated groups, daily, from POD3 to POD9 for the first cycle of treatment, and from POD14 to POD19 for the second cycle of treatment. The rats in the SNL groups received distilled water only.

The data obtained by Western blotting and immunohistochemistry were carried out in triplicates. A total of 88 male rats were used for 7D assessments: the SHAM group: 21; the SNL group: 22; the SNL-KDS group: 21; the SNL-ANA12 group: 12; the SNL-KDS-ANA12: 12. Following the behavior measurements, the spinal cords were harvested for Western blotting, or immunohistochemistry (n = 9 for the SHAM, SNL, and SNL-KDS groups; n = 6 for the SNL-ANA12 and SNL-KDS-ANA12

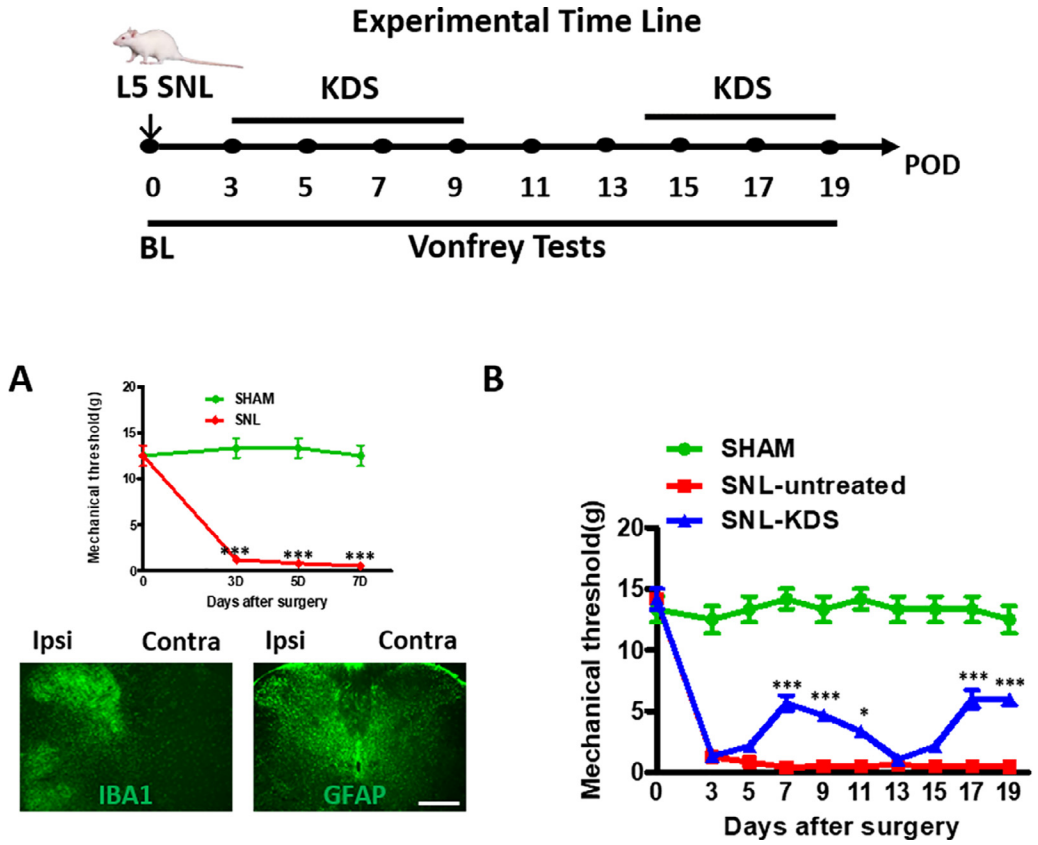

Figure 1. Oral administration of KDS attenuates mechanical allodynia following SNL-induced neuropathic pain in rats. (A) Before SNL surgery, rats were subjected to a von Frey filament test with only those passing a predefined baseline (>10 g) selected for L5 SNL surgery. Following surgery, the von Frey test was conducted on days 3, 5, and 7 post-injury to determine pain hypersensitivity. The establishment of neuropathic pain was also confirmed by the activation of microglia and astrocytes (assessed by immunostaining) in the ipsilateral side of the spinal dorsal horn (scale bar = 400 μm). Data are expressed as the mean ± SEM (two-way ANOVA, followed by Bonferroni post hoc test, ***$P < .001$: SNL vs SHAM). (B) KDS was orally administered to the SNL-induced rats at the indicated time points and the von Frey test was conducted on each group of the rats on POD 3, 5, 7, 9, 11, 13,15, 17, and 19. Data are expressed as the mean ± SEM (two-way ANOVA, followed by Bonferroni post hoc test, *$P < .05$, ***$P < .001$: KDS vs SNL, n = 12 per group).

groups), and electrophysiology recording (7–9 cells from 3–4 rats were recorded in each group of animals; n = 3 for the SHAM and SNL-KDS groups; n = 4 for the SNL group). For the assessment at POD19, 36 male rats were used: the SHAM group: 12; the SNL group: 12; and the SNL-KDS group: 12. The tissues were collected for Western blotting or immunohistochemistry (n = 6 per group). In the behavior data in Supplementary Fig 5: 18 female and 18 male rats (divided into the SHAM, SNL, and SNL-KDS groups, n = 6 per group) and in Supplementary Fig 6: 18 male rats (n = 6 per group) were tested.

Drug Administration

KDS2010 was kindly provided by Prof. C. Justin Lee (Center for Cognition and Sociality, Institute for Basic Science, Daejeon, Korea). The drug was dissolved in distilled water at the concentration of 15 mM and was then delivered orally (25 mg/kg, BID, at 9 AM and 6 PM daily) to the neuropathic rats for the indicated treatment periods as described. ANA12 (Sigma Aldrich, St. Louis, MO) was prepared in PBS containing 1% DMSO at the concentration of 0.3 mM and was injected (i.p) at 0.5 mg/kg. The drug solutions were prepared freshly every experimental day.

Induction of Neuropathic Pain by L5 Spinal Nerve Ligation

The neuropathic pain model induced by L5 SNL in rats is a well-established model of neuropathic pain.[20] Before surgery, we performed the von Frey test, and the animals responding to ≥ 10-g filaments were selected for experiments. The L5 SNL surgical procedure was constructed in rats according to a previous study.[20] Briefly, rats were anesthetized using Avertin (Sigma Aldrich, St. Louis, MO) (250 mg/kg, i.p), and were then put on their dorsal surface under microscopy in a prone position. An incision from L4 to S2 level was made on the back of the left side. The subcutaneous tissue was removed, the paraspinal muscles were exposed toward the left side, and were then separated using a 2-cm long skin incision. The transverse process was carefully removed to visually identify the L4 and L5 spinal nerves. They were carefully isolated from each other without damage and the L5 spinal nerve was tightly ligated 3 times using a 3 to 0 silk thread (Ethilion, Somerville, NJ). Complete

hemostasis was confirmed, and the wound was sutured. A surgical procedure for the sham group was performed by using the same technique except ligation of nerves.

Pain Behavior Test With von Frey Filaments

The pain behavior test was performed according to the previous report,[47] using von Frey filaments (Touch-Test Sensory Evaluator; North Coast Medical Inc., Morgan Hill, CA). Pain behavior was tested 2 hours after drug administration and the tests were conducted on the rats in all groups prior to surgery (baseline), and at 3, 5, 7, 9, 11, 13, 15, 17, and 19 days post-sham and -SNL surgery or at the indicated time points as described. The withdrawal threshold for each paw was measured by accurately applying von Frey hairs to the plantar surface of the hind paws 3 times for a duration of 2 to 3 second. A positive response was recognized when at least 2 paw withdrawals were obtained upon 3 stimuli on each hind paw at approximately 3-minute intervals The measurement procedure started with a 10g filament and the next filaments were determined using the up-and-down testing paradigm.[7] If a positive response occurred, the next-smallest von Frey hair was used; if a negative response was observed, the next-highest force was applied. The threshold of each animal was determined as the smallest filament that the animal positively responds to. Threshold $\geq$ 10g was used to set the baseline (cutoff value is 15g to avoid tissue damage). Following SNL surgery, if the rats have thresholds of $\leq$ 4 g, they are considered to have developed neuropathic pain.

Western Blotting

Spinal dorsal horn tissues (L4–L6 segments, 1cm) from the rats were harvested using the RIPA buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitor (Sigma Aldrich, St. Louis, MO). Following the quantitation of cell lysates with the BCA assay (Sigma Aldrich, St. Louis, MO), 30 μg of each lysate was separated on an 8% or 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore). The blots were blocked with 3% bovine serum albumin in Tris buffered saline (TBS)-Tween 20 (Sigma Aldrich, St. Louis, MO) for 1 hour at room temperature. After the blocking step, the blots were probed at 4°C with primary antibodies anti-MAO-B antibody (1:1000, Abcam), anti-BDNF (1:1000, Abcam), anti-p-TrkB antibody (1:1000, Biorbyt, Cambridge, UK), anti-p-p65 (1;1000 Cell Signaling Technology, Danvers, MA), anti-p-p38 (1:1000, Cell Signaling Technology, Danvers, MA), anti-IκB-α antibody (1:1000, ThermoFisher Scientific, Waltham, MO), anti-NR2B (1:1000, Millipore, Darmstadt, Wetzlar, Germany), anti-p-NR2B (1:1000, Cell Signaling Technology, Danvers, MA), anti-p-Akt (1:1000, Cell Signaling Technology, Danvers, MA) and anti-β-actin (1:3000, ThermoFisher Scientific, Waltham, MO), followed by secondary antibodies conjugated with horseradish peroxidase (HRP; anti-rabbit-HRP or anti-mouse-HRP; KOMA, Seoul, Republic of Korea). Following incubation, signals were detected using the X-ray film imaging system (Bio-Rad) with an enhanced chemiluminescence detection kit (ECL; Pierce). The levels of these protein expression were then normalized to those of β-actin using Image J software (NIH, Bethesda, MD). The person who conducted these analyses was blinded to experimental groups.

Immunohistochemistry and Detection of ROS Generation

Animals were anesthetized with Avertin (Sigma Aldrich, St. Louis, MO, 250 mg/kg, i.p), perfused with phosphate-buffered saline (PBS, pH 7.4). The perfusion was followed by 4% paraformaldehyde (Merck, Darmstadt, Wetzlar, Germany) in PBS for 10 min using a peristaltic pump at a rate of 20 ml/min. The lumbar region (L4–L6) of the spinal cord was immediately isolated and bathed overnight in the same fixative at 4°C, followed by immersion in a series of sucrose solutions in PBS (10% –30%) for cryoprotection. After 2 days, the lumbar region (L4–L6) of a spinal cord was obtained and the fragments were cut transversely at 30-μm thickness using a cryostat (CM1950; Leica Microsystems, Wetzlar, Germany) and kept at -20°C in a storage buffer (30% glycerol, 30% ethylene glycol in PBS).

For immunofluorescence staining, tissues were blocked with a blocking buffer (10% normal serum, 0.1% Triton X-100 in PBS) for 2 hours at room temperature. Afterward, the tissues were incubated with a mixture of primary antibodies (IBA1, 1:500, Wako, Richmond, VA; GFAP, 1:1000, Abcam; NEUN, 1:500, Millipore, Darmstadt, Wetzlar, Germany; p-p65, 1:500, Cell Signaling Technology, Danvers, MA; p-p38, 1:500, Cell Signaling Technology, Danvers, MA; MAO-B, 1:200, Abcam; BDNF, 1:200, Abcam; p-NR2B, 1:200, Cell Signaling Technology, Danvers, MA) diluted in the blocking buffer overnight at 4°C, followed by a mixture of secondary antibodies conjugated with either Alexa Fluor 488 (1:1000; Thermo Fisher Scientific, Waltham, MA), or Cy[3] (1:1000; Jackson ImmunoResearch, West Grove, PA) diluted in the same blocking buffer. After 3 time washing in PBS, the sections were then mounted on glass slides with a gel-mounting medium containing DAPI (Thermo Fisher Scientific, Waltham, MA). To detect the generation of ROS, the fresh spinal sections (30 μm) were incubated with 1 μM DHE (Thermo Fisher Scientific, Waltham, MA) at room temperature for 5 minutes and mounted on slides. Images were captured with an Axiophot microscope (Carl Zeiss, Germany) at 20X objective and magnification of 200X. The images in 1 set of staining were taken using constant illumination settings and used for additional data analysis. Immunodensity was quantified by Image J program software (NIH, Bethesda, MD). Briefly, the IBA1, GFAP, MAO-B, p-p38, and p-p65 immunoreactivity (IR) in single staining images was analyzed through a morphometric approach and expressed as a proportional area (percentage of positive elements relative to the scanned area). The percentages of double staining including MAO-B$^+$ IBA1$^+$/ MAO-B$^+$, MAO-B$^+$ GFAP$^+$/ MAO-B$^+$, MAO-B$^+$ NEUN$^+$/ MAO-B$^+$, p-p38$^+$ IBA1$^+$ / IBA1$^+$, p-p65$^+$ GFAP$^+$/

GFAP+, BDNF+ GFAP+/GFAP+, BDNF+ NEUN+/NEUN+ were calculated by counting the numbers of positive cells per area. The images were taken using constant illumination settings in each channel. The green and red colocalization appear in yellow. These yellow-labeled IR were determined using the colocalization threshold plugin applied to the green and red labeled images.

To quantify the numbers of MAO-B+, IBA+, GFAP+, and NEUN+ cells in single-channel images, or the green-red IR co-expressing cells in double-channel images, the same area of the region of interest was outlined and cropped from each raw image in one staining set. The pictures were converted into 8- bit using ImageJ, and a threshold was applied to render the staining as a binary image in black and empty spaces. The threshold was determined by setting a value cutoff in which every pixel larger in comparison to the cutoff value refers to 1 class and every pixel smaller in comparison to the cutoff value refers to the other class, accompanied by the morphology of the cells. The analyze particles plugin was then applied to the area occupied by the desired staining to count the cells. Averages were obtained from 3 randomly selected spinal cord sections for each animal. The person who conducted these analyses was blinded to experimental groups.

Spinal Cord Slice Preparation and Electrophysiological Recording

Spinal cord slices were obtained from rats as previously described.[10] Briefly, rats were anesthetized with Avertin (250 mg/kg), spinal cord was quickly removed from the vertebral canal by hydraulic extrusion and placed in ice-cold artificial cerebrospinal fluid (aCSF), containing 126 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 10 mM glucose, 2.4 mM $CaCl_2$; pH 7.3 - 7.4 and saturated with 95% O_2 and 5% CO_2. Transverse spinal cord slices (300 μm thick) from L4 to L6 were cut in the ice-cold aCSF, using vibrotome (VT 1200s, Leica, Germany), and then incubated for 1 hour in aCSF at 32 °C before they were transferred to the recording chamber. For patch clamp recording, patch pipettes were filled with internal solution containing (in mM): Cesium chloride 135, KCl 10, HEPES 10, Mg^{2+}ATP 5, $MgCl_2$ 0.9, and EGTA 10. In the whole-cell configuration, tonic NMDA currents were recorded (at +40 mV) from spinal superficial dorsal horn neurons in the presence of picrotoxin (PIC;100μM, Tocris), and 0.5 μM Strychnine (Sigma) to block GABA and glycine receptors respectively. Recordings were obtained using Multiclamp 700B (Molecular devices). Data were filtered at 1 kHz and digitized at 10 kHz (Digidata 1440A) and acquired using pClamp 11 software. The series resistance was monitored at the beginning and end of the experiments and data were excluded if changes >20% were observed. The NMDA receptor-mediated tonic current was estimated by holding current difference before and after the application of NMDA receptors antagonists; ifenprodil (IFN; 30 μM, Tocris) and DL-2-Amino-5-phosphonopentanoic acid (DL-AP5; 100 μM, Tocris) as in our previous reports.[41]

Cell Culture

To confirm the effects of KDS and ANA12 on BDNF-induced TrkB activation and signaling, we employed the human neuroblastoma cell line SH-SY5Y, in which TrkB is induced by treatment with retinoic acid.[13] This cell line has been used in previous studies for investigating BDNF/TrkB signaling.[9] SH-SY5Y cell line was kindly provided by Prof. Kang Min Hur (Department of Pharmacology, Chungnam National University). Cells were maintained under 20 passages and were cultured as has been described previously. Briefly, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA) and 1 % penicillin/streptomycin (Sigma Aldrich, St. Louis, MO) at 37 °C and 5% CO_2. Cells were seeded at a density of 3×10^4 cells per 6-well plate. 24 hours after seeding, the serum-reduced medium containing all-trans Retinoic acid (RA 10 μM, Sigma-Aldrich, St Louis, MO, USA) was replaced to ensure TrkB receptor expression in the cells. For the experimental groups containing BDNF, 3 days following RA treatment, BDNF was added to the media to reach the concentration of 20 ng/mL for 12 hours before being harvested. ANA12 (100 nM), and KDS (100 nM) were added to the cells when needed 30 minutes prior to BDNF treatment.

Statistical Analysis

The data are expressed as mean ± Standard Error of Mean (SEM). Statistical significance between multiple groups in the repeated measurements of the behavior data obtained by the von Frey tests was compared by two-way analysis of variance (ANOVA). Statistical significance between multiple groups in other investigations was compared by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P*-values < .05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

Results

Oral Administration of KDS Attenuates Mechanical Allodynia Following SNL-Induced Neuropathic Pain in Rats

A neuropathic pain model was established by the tight ligation of L5 spinal nerve in rats. Following the surgical procedure, the pain behaviors were determined at the indicated time points by the von Frey filament tests conducted on the ipsilateral hind paws of the rats. The pain hypersensitivity developed early on POD 3 in the SNL-induced rats, compared to the sham controls, exhibited by a decrease in the mechanical thresholds (Fig 1A). This sign of mechanical allodynia in the SNL rats was maintained stably after the operation. The

successful neuropathic pain establishment was confirmed by the staining of the SNL spinal cord sections with anti-IBA1/GFAP antibodies showing robustly increased reactive gliosis in the ipsilateral dorsal horns (Ipsi), compared to respective contralateral sites (Contra) (Fig 1A).[40,54] KDS was next administered orally (25 mg/kg, BID) to the SNL rats to test its novel analgesic effect (Fig 1B). Based on the previous studies which investigated the pharmacokinetics and toxicokinetics of orally administered KDS,[23] and our primary data on the analgesic effect of KDS on pain models,[46] the dose of 25mg/kg was selected for the treatment due to its effectiveness and limited side effects on the body weight and other essential brain functions (the rats in the KDS-treated group had similar growth in weight, compared to the rats in the SNL-untreated groups). The first round of treatment was conducted from POD3 to POD9. KDS enhanced mechanical thresholds, with significant increase on POD7 and 9, in the SNL KDS-treated rats, compared to the SNL-untreated rats. The analgesic effect was still significant on POD11, 2 days after KDS administration had been stopped, and was diminished completely on POD13. The second round of treatment was repeated from POD14 to POD19 with the same dose as the first cycle treatment. A similar analgesic effect was observed in the SNL KDS-treated group with the peak on POD17 and POD19. These data indicate that oral administration of KDS significantly improved mechanical allodynia in neuropathic rats without drug resistance.

Cellular Location and Expression of MAO-B After KDS Administration

First, the regulation of MAO-B expression in the spinal dorsal horn after KDS treatment was determined at POD7, and POD19 when the analgesic effects peaked. No significant changes in the protein expression of MAO-B among the groups were detected (Fig 2A). In Fig 2B, the spinal cord sections from the sham, the SNL-untreated, and SNL-KDS treated groups on POD7 were co-immunostained with anti-MAO-B and anti-IBA1/GFAP/ or NEUN (microglial, astrocytic and neuronal markers, respectively) antibodies. The results not only confirmed that MAO-B expression was not altered but also show the cellular location of MAO-B. MAO-B was distributed mainly in GFAP and NEUN positive cells (Fig 2B, Supplementary Fig 1). More interestingly, most GFAP and MAO-B co-expressing cells were located in the superficial layers while MAO-B expressing neurons were found mainly in deeper layers. These findings are in good agreement with the previous report showing increased MAO-B activity, not expression was observed in neuropathic mice[53] and also indicate that KDS may have affected the activities of superficial astrocytes and deep layer neurons. Moreover, KDS, as a newly developed reversible MAO-B inhibitor has been shown to limit adverse effects on inhibiting new MAO-B enzyme synthesis, which may cause compensatory mechanisms.

KDS Reduces Astrogliosis and Microgliosis in the Spinal Dorsal Horn After Nerve Injury

It has been reported that KDS treatment dramatically reduces astrocytic and microglial reactivity in the brains of AD[1] and stroke mice.[37] Microglia and astrocytes are robustly activated in the spinal dorsal horn in response to nerve injury. To test whether the KDS-induced analgesic effect on neuropathic rats is associated with neuroinflammation, a well-known pathology in neuropathic pain, we performed and compared immunohistochemical staining with antibodies against GFAP and IBA1 (Fig 3A) and quantitative data for immunodensities are shown in Fig 3B. Staining with DAPI-labeled nuclei is shown in *Supplementary Fig 2*. We found robust immunoreactivities of GFAP and IBA1 in the spinal dorsal horns of the SNL-untreated rats on POD7 and POD19, with a peak on POD7, which were significantly reduced by KDS. These results indicate that KDS is effective in reducing neuroinflammation, a critical mechanism for the initiation and development of neuropathic pain.

Impaired Activation of NF-κB and MAPK Pathways in Spinal Astrocytes and Microglia Following Treatment With KDS

The activation of glial cells with their inflammatory process is of great significance in the development of neuropathic pain. In response to nerve injury, glial cells start proliferation and multiple intracellular signaling are altered, contributing to the manifestation of neuropathic pain including the accumulation and recruitment of inflammatory cytokines, chemokines, as well as the modulation of extracellular proteins, changes in transmembrane receptor expression and so on. MAPK and NF-κB are among the earliest events triggering the expression of inflammatory mediators and recruiting immune cells to the damaged nerve. We found the protein expression of both NF-κB phosphorylated-p65 (p-p65) and MAPK phosphorylated-p38 (p-p38) was significantly increased in the spinal dorsal horns of the SNL-induced rats on POD7 (Fig 4A). Consistent with the increased active molecules p-p65, the depletion of IκB-α, one of the 2 NF-κB inhibitors, was detected. In contrast, the activation of these inflammatory pathways was significantly abolished in the SNL KDS-treated rats, exhibited by lower levels of p-p65 and p-p38 and higher expression of IκB-α. Activation of NF-κB leads to the transcription of proinflammatory cytokines. We also examined the protein expression of IL-1β and consistently we found IL-1β was increased in the neuropathic rats on POD7, but was reduced by KDS (Fig 4A).

By immunohistochemistry, we confirmed that activation of NF-κB p-p65 was predominantly in GFAP-positive cells while MAPK p-p38 was mainly activated in microglia on POD7 (Fig. 4B and 4C). These staining results also confirm again KDS reduced microgliosis and astrogliosis in the spinal dorsal horns, thus, reduced activation of p-p65 and p-p38 was observed in the KDS-treated rats on POD7, compared to the SNL-untreated rats (Fig 4D). Similar results on the expression of NF-κB p-p65 were

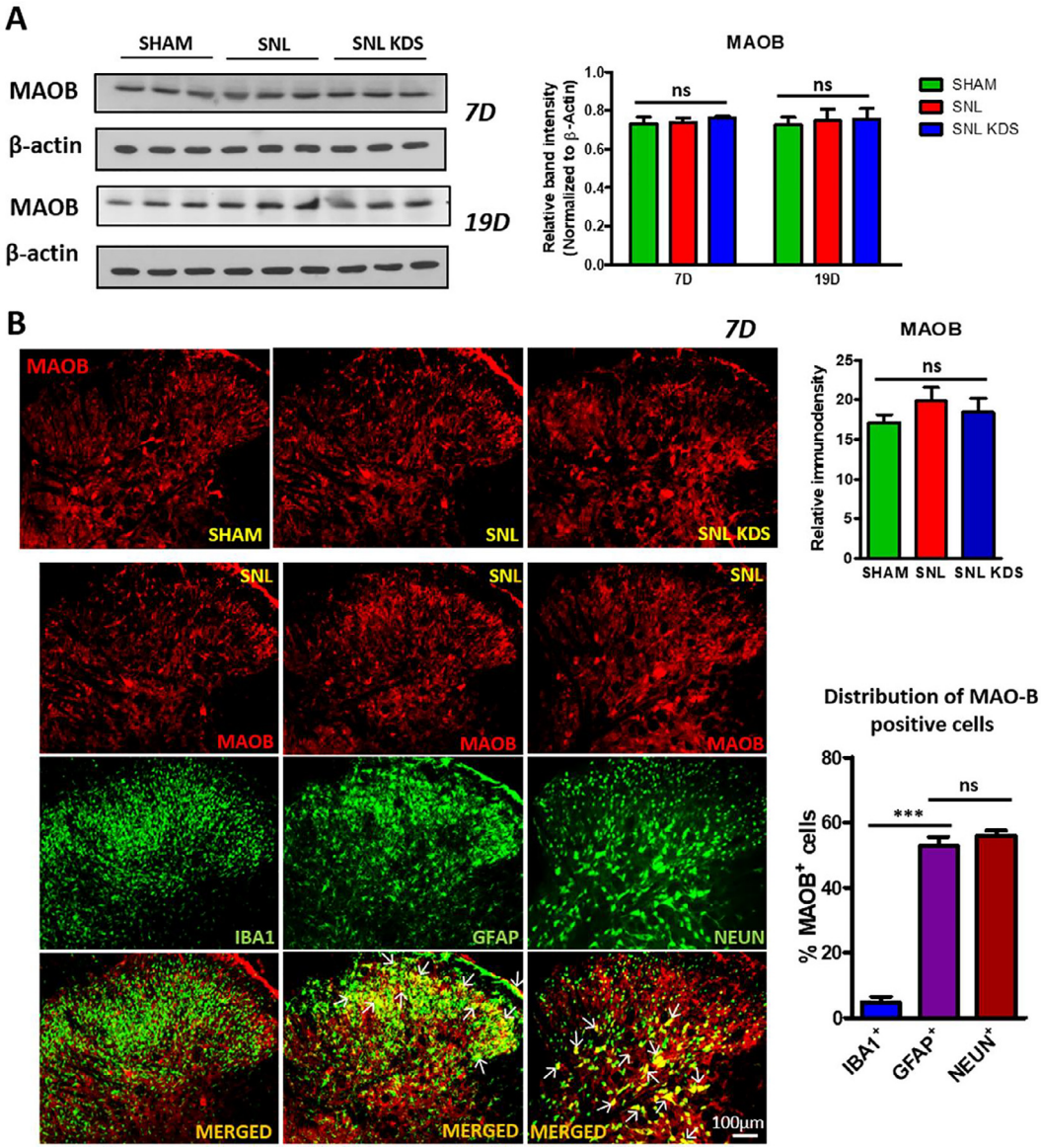

Figure 2. Cellular location and expression of MAO-B after KDS administration. (A) The western blot and blot quantification results showing the protein expression levels of MAO-B in the spinal dorsal horns after KDS treatment on POD 7 and 19. Data are expressed as the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test, ns (not significant) SNL, KDS vs SHAM. (B) On POD 7, the cellular location and expression of MAO-B in the spinal cords were assessed by immunostaining and were confirmed by immunodensity quantification in the graphs. Arrowheads indicate the co-localization between red and green IR. Scare bars = 100 μm. Data are expressed as the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test, ns: SNL, KDS vs SHAM, ***P< .001: IBA1$^+$ vs GFAP$^+$, ns: GFAP$^+$ vs NEUN$^+$, n = 6 per group).

obtained with the tissues on POD19 (*Supplementary Fig 3*). KDS reduced microgliosis, astrogliosis as well as activation of NF-κB in astrocytes. MAPK p-p38, however, was not detected even in the spinal cords of the SNL-induced rats on POD19 (*Supplementary Fig 3*). This correlates with the previous reports showing the activation of MAPK pathway occurs in the early phase, within 2 weeks after nerve injury.[25]

KDS Reduces Oxidative Stress in the Spinal Dorsal Horn After Nerve Injury

Oxidative stress caused by nerve injury has been implicated as a contributing mechanism in the pathogenesis of neuropathic pain. Neuroinflammation induced by oxidative stress-mediated activation of NF-κB is a key player in various persistent pain conditions.[22,60] As consequence, many antioxidant agents have been tested for the treatment of pain.[14] These trials in both animal experiments and human patients have brought about positive results.[5] In this study, we also examined whether the analgesic effect of KDS was involved in oxidative stress conditions in the spinal cord. The levels of ROS production were detected by Dihydroethidium (DHE) staining within the spinal dorsal horns of the rats (Fig 5). In good agreement with previous studies on pain, the SNL-induced rats showed robustly increased levels of cytosolic ROS generation in their spinal cords, compared to the sham rats at both POD7 and POD19 (Fig.5A). Whereas, treatment with KDS effectively reduced ROS production

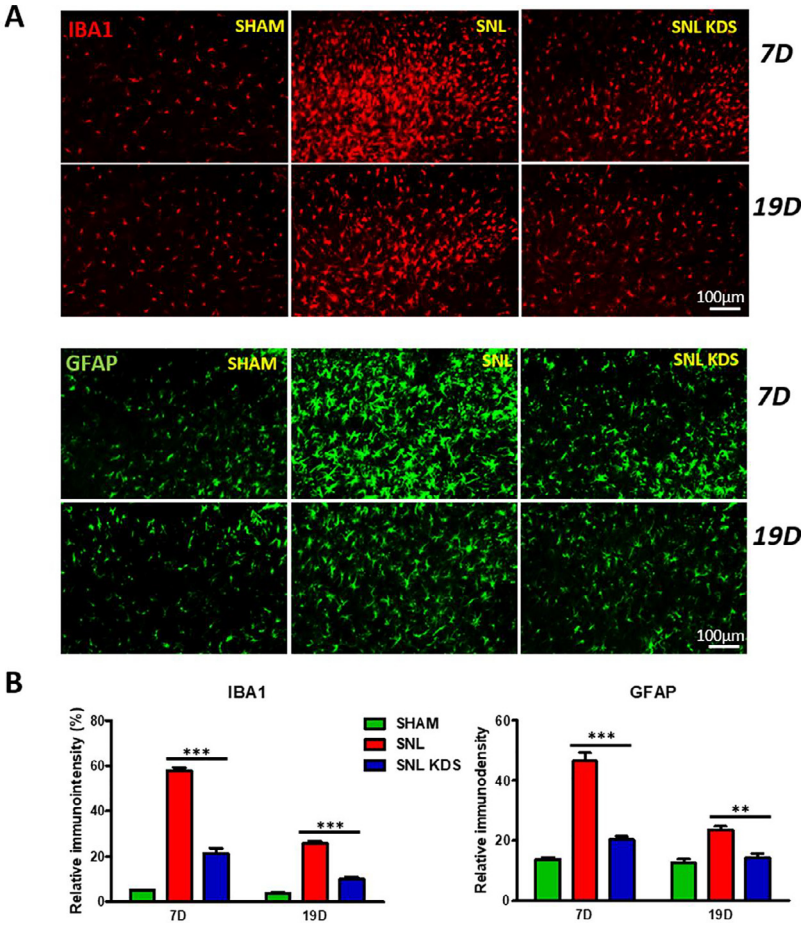

Figure 3. KDS reduces astrogliosis and microgliosis in the spinal dorsal horn after nerve injury. (A) On POD7 and POD19, microgliosis and astrogliosis in the spinal cords of the rats in the SHAM, the SNL untreated (SNL), and the SNL KDS-treated (KDS) groups were assessed by immunostaining. Scale bar = 100 μm. (B) The relative immunodensities of IBA1 and GFAP in Fig 3A were quantified. Data are presented as the mean ± SEM (One-way ANOVA followed by Tukey's post hoc test, ***$P < 0.001$, **$P < .01$: KDS vs SNL, n = 6–9 per group).

at both indicated time points. Of note, the data also indicates that the increased ROS levels in the neuropathic rats were higher at early state (POD7) than those at later state (POD19), suggesting the contribution of ROS production to the initiation of pain (Fig 5B).

Increased Level of BDNF in the Spinal Dorsal Horn Following Treatment With KDS

We further investigated the mechanisms by which KDS could reduce neuroinflammation and pain behaviors in SNL rats. MAO-B inhibitors have been shown to increase the secretion of BDNF in both in vitro and in vivo experiments.[19,32] Several lines of evidence have revealed upregulation of endogenous BDNF promotes either neuroinflammation or synaptic plasticity, contributing to neuropathic pain.[11,15] This seems to be opposite to our behavior results in which we observed KDS reduced pain hypersensitivity. That raised the question how KDS, as an MAO-B inhibitor, could play an anti-nociceptive role while upregulating BDNF? To investigate this, we first examined the expression of BDNF. The western blot results show that KDS significantly enhanced the levels of BDNF in the spinal dorsal horns of the KDS-treated rats at both POD7 and POD19, compared to the SNL-untreated rats (Fig 6A). The data also reveal BDNF was upregulated in the SNL-induced rats, compared with the sham controls at POD7, but not at the later time point POD19. This suggests that endogenous BDNF is transiently upregulated to a certain level within the early phase in response to SNL-induced nerve injury. It has been documented that the function of BDNF is largely mediated by TrkB receptors. Indeed, we found the active phosphorylated form of the TrkB receptor was increased in the spinal dorsal horn tissue of the SNL-induced rats, compared to the sham, in response to the increased levels of BDNF. Unexpectedly, although BDNF levels were higher in the SNL-KDS treated rats, we observed decreased p-TrkB in these rats, compared to the SNL untreated rats. (Fig 6A). Moreover, we also examined the cell types of the spinal cord, in which BDNF was increased. The spinal sections on POD7 were co-immunostained with anti-BDNF and GFAP or NEUN. The results reveal BDNF was more excessively increased in the KDS treated group, compared to the SNL untreated, and co-localized mainly with NEUN and less with GFAP (Fig. 6B and 6C). This correlates with the results in Fig 2B, showing the expression of MAO-B in astrocytes and neurons. Notably, KDS-induced increased BDNF levels were distributed largely in NEUN-positive cells (Fig 6D).

The observation of elevated BDNF was not well supported by behavioral data demonstrating KDS's anti-nociceptive effect. BDNF/TrkB signaling in neurons may be involved in the action of KDS, the mechanisms by which and how, however, were elusive.

KDS Acts on TrkB to Reduce the Phosphorylation State of NR2B, Suppressing BDNF-enhanced Tonic NMDAR Excitation in Dorsal Horn Sensory Neurons

Given the previous studies demonstrating BDNF-TrkB pathway mediates NMDA receptor NR2B subunit phosphorylation leading to long-term synaptic plasticity in different pathological conditions,[3,6,43] we questioned how KDS reduced mechanical hypersensitivity while increasing BDNF levels? Particularly, a recent study has revealed that increased levels of p-NR2B and the interaction between p-NR2B and postsynaptic density-95 protein restricted in dorsal horn sensory neurons contribute to neuropathic pain.[8] We hypothesized that KDS may act in competition with BDNF at the TrkB receptor site to prevent the BDNF/TrkB activation-induced downstream cascades. To test this, we first compared the levels of p-NR2B among the groups. In line with previous studies, we detected higher levels of both NR2B and p-NR2B at both POD7 and POD19 in the SNL-induced rats, compared to the sham rats, and

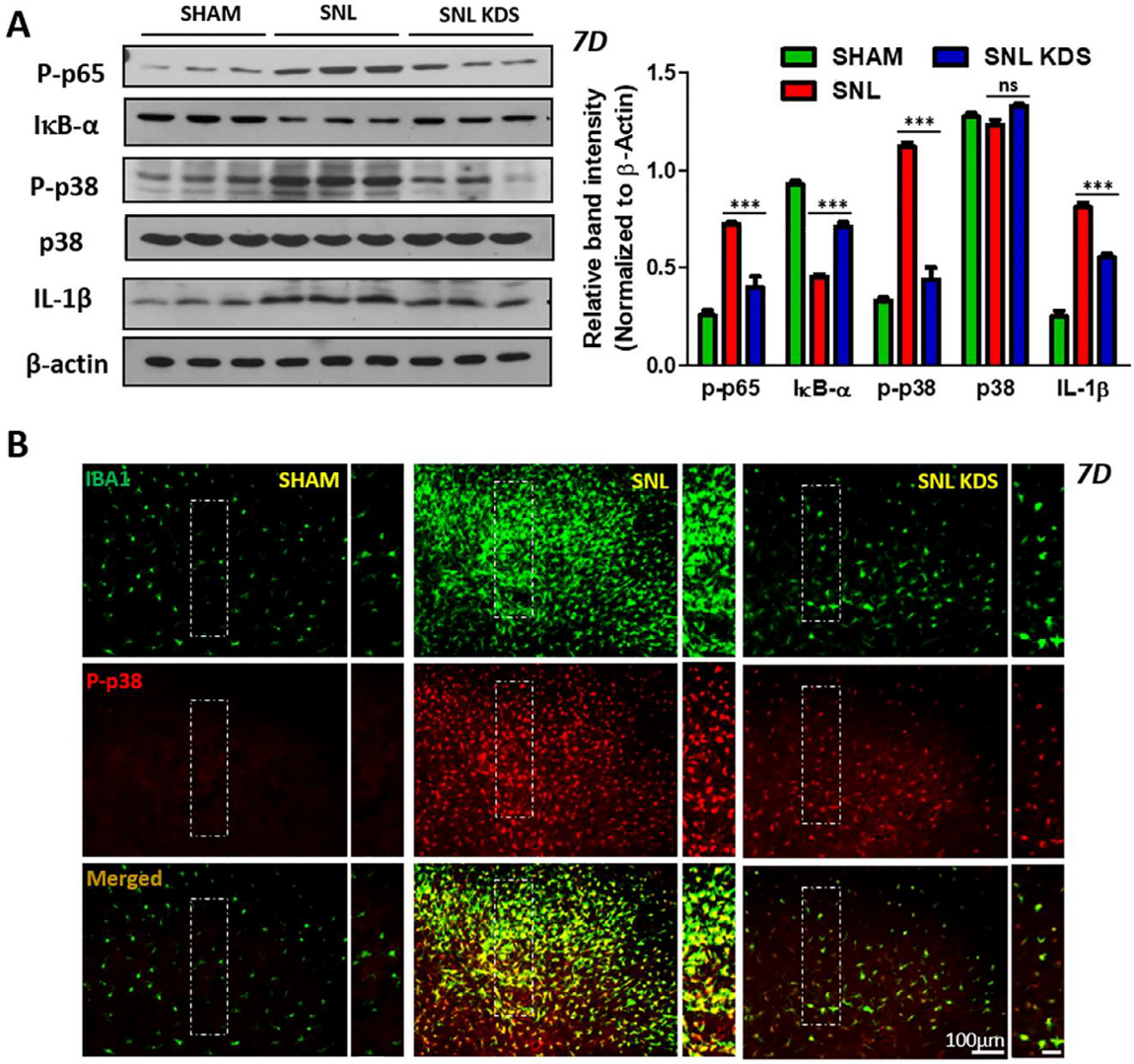

Figure 4. Impaired activation of NF-κB and MAPK pathways in spinal astrocytes and microglia following treatment with KDS. (A) The protein expression of p-p65, IκB-α, p-p38, p-38, and IL-1β in the spinal dorsal horns of the SHAM, the SNL-untreated, and the SNL KDS-treated rats was assessed on POD7. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ***$P < .001$, ns: KDS vs SNL). (B) The spinal cord sections of the sham, the SNL-untreated, and the SNL KDS-treated rats were co-stained with anti-p-p38 and anti-IBA1 antibodies. The right panels represent the images in the white dot frame with higher magnification. Scare bars = 100 μm (left) and 25 μm (right). (C) The spinal cord sections of the SHAM, the SNL untreated and the SNL KDS-treated rats were co-stained with anti-p-p65 and anti-GFAP antibodies. The right panels represent the images in the white dot frame with higher magnification. Scare bars = 100 μm (left) and 25 μm (right). (D) The immunodensities of total p-p38 or p-p65 and p-p38$^+$ IBA1$^+$/IBA1$^+$ or p-p65$^+$ GFAP$^+$/GFAP$^+$ were quantified in the graphs. Data are presented as the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test, ***$P < .001$: p-p38, p-p65 immunodensity: SNL vs SHAM, KDS vs SNL, ns, **$P < .01$, ***$P < .001$: p-p38$^+$ IBA$^+$/IBA1$^+$ or p-p65$^+$ GFAP$^+$/GFAP$^+$: SNL vs SHAM, KDS vs SNL, n = 6 per group).

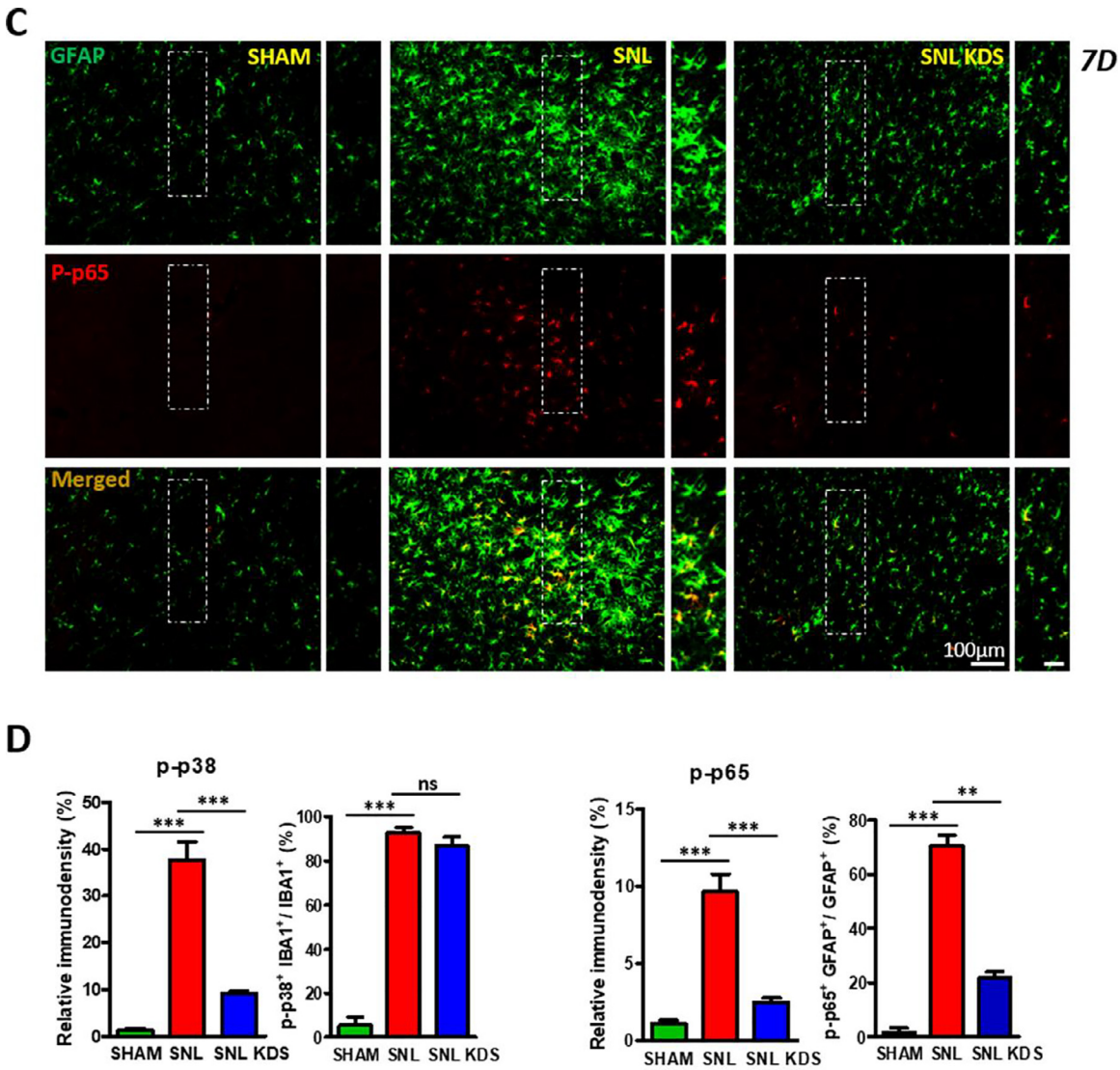

Figure 4. Continued

KDS reduced elevated p-NR2B levels only, not NR2B levels (Fig 7A).

To determine the functional activity of enhanced NR2B and p-NR2B observed in the neuropathic rats, we recorded the NR2B-sensitive tonic NMDA currents (at + 40 mv) from dorsal horn neurons in the acute spinal cord slices at POD7 (Fig 7B). Bath application of ifenprodil (an NR2B receptor antagonist) caused significantly larger holding shift ($I_{ifenprodil}$) in the SNL rats compared to the sham rats. Interestingly, KDS2010 treatment in the SNL rats significantly restored the NR2B-sensitive tonic NMDA currents to the sham levels (Fig 7B). The tonic NMDA currents mediated by additional application of AP5 (on the top of ifenprodil) were not different among the sham (3.3 ± 1.4 pA), and SNL (2.9 ± .9 pA) and SNL-KDS treated rats (3. ± 1.5 pA; one-way ANOVA, $P = .81$). Of note, our western blot results show that there was no significant difference in total NR2B protein levels between the SNL-induced and the SNL-KDS treated rats, but only the phosphorylated form of NR2B. These data indicate that tonic activation of NR2B containing NMDARs by p-NR2B plays a potential role in the SNL-induced neuronal excitability, and KDS treatment significantly suppresses NR2B containing NMDAR activity in the SNL rats.

To further investigate whether the decrease in p-NR2B in the SNL-KDS treated rats was attributed to KDS-TrkB signaling, we employed ANA12, a selective TrkBR full antagonist. ANA12 was systemically applied during the KDS treatment period from POD3 to POD7. The von Frey tests show ANA12 almost completely blocked the analgesic effects of KDS in the SNL-KDS-ANA12 treated group (Fig 7D). Corresponding well with the behavioral tests, the decreased protein levels and expression of p-NR2B in the KDS-treated group were abrogated by the co-treatment of ANA12 (Fig. 7E and 7F). Meanwhile, the protein levels of BDNF were not altered by ANA12. The phosphatidylinositol 3-kinase (PI3-K)/Akt pathway is one of the key signaling cascades downstream BDNF/TrkB and is believed to regulate NR2BR-mediated synaptic plasticity.[36] The results obtained in the protein expression of p-Akt show a similar fashion to the expression of p-NR2B (Fig 7E). These data suggest that KDS may have acted via TrkB receptors to suppress the BDNF/TrkB/Akt-induced phosphorylation of NR2B in the KDS treated

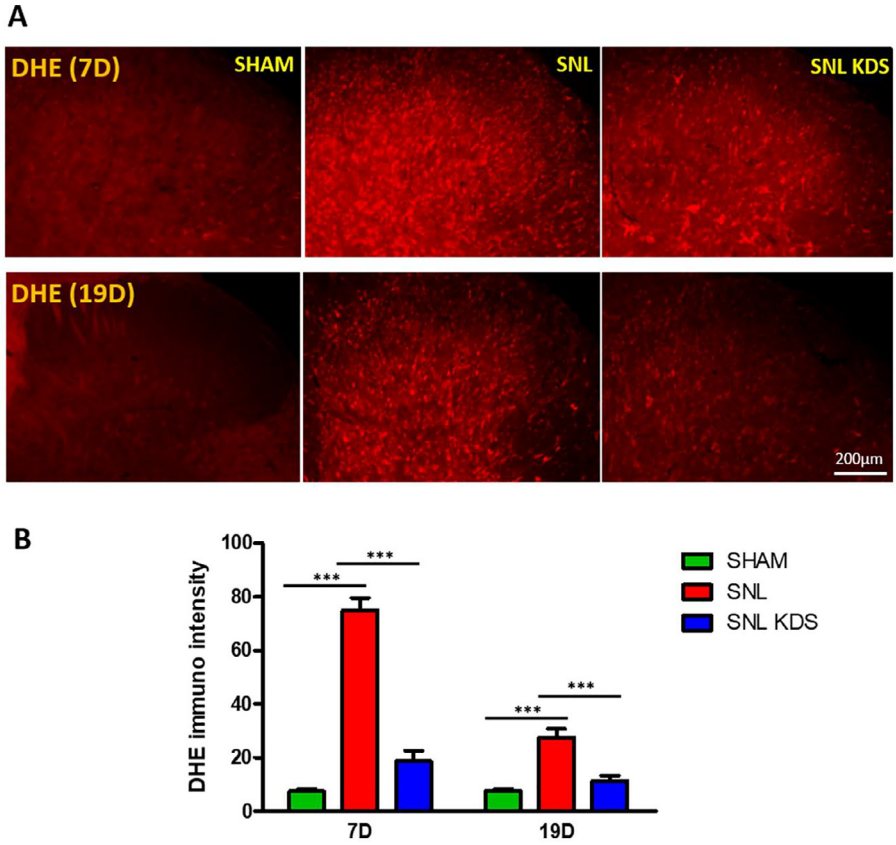

Figure 5. KDS reduces oxidative stress in the spinal dorsal horn after nerve injury. (A, B) On POD7 and POD19, the levels of ROS production were detected with DHE staining. The immunodensities of DHE fluorescence were quantified in the graphs. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ***$P < .001$: 7D, 19D: SNL vs SHAM, KDS vs SNL, n = 6 per group).

group. Alternatively, KDS may work competitively with BDNF at TrkBR to alter the downstream cascades.

In addition, we also checked the effect of ANA12 alone in the SNL-induced rats. Interestingly, treatment of ANA12 alone partially enhanced the mechanical thresholds of the SNL-induced rats, compared with the sham-operated rats (Fig 7D). As shown in Fig 4A, the levels of BDNF were upregulated in the spinal dorsal horns of the SNL-operated rats on POD7 after nerve injury and disappeared on POD19. However, KDS blocked BDNF-induced increased p-NR2B, resulting in pain relief and co-treatment of KDS and ANA12 reversed this analgesic effect. We also examined whether the anti-inflammatory effect of KDS was prevented by ANA12. We found that the reduced states of microgliosis and astrogliosis in the spinal dorsal horns were blocked when KDS was co-treated with ANA12 (Supplementary Fig 4). These findings suggest that BDNF/TrkB signaling may also contribute to the activation of glial cells in the spinal cord after nerve injury.

KDS Suppresses BDNF/TrkB Signaling-induced p-NR2B Upregulation in the SH-SY5Y Cultured Cells

We then evaluated the function of KDS on BDNF/TrkB in the SH-SY5Y neuronal cell line. The cells were treated with BDNF in the presence or absence of KDS and/or ANA12. BDNF induced TrkB activation via upregulation of p-TrkB followed by its downstream cascades p-Akt and p-NR2B increase in the BDNF-treated groups (Fig. 8A, and 8B). Either KDS (100 nM) or ANA12 (100 nM) blocked the BDNF-induced downstream signaling in the cells pre-treated with KDS/ANA12 independently. However, interestingly, co-treatment of KDS and ANA12 did not bring about synergistic effects. In contrast, a combination of KDS and ANA12 resulted in diminishing the inhibitory effect of each drug. This suggests that KDS and ANA12 may competitively antagonize each other at TrkB, in the presence of BDNF, allowing BDNF to bind to the receptors. Whereas, in the absence of BDNF, neither KDS nor ANA12 showed any effects. These data suggest that KDS may properly act, in competition with BDNF/ANA12, at TrkB.

Finally, we investigated whether sex is an important variable in the effects of KDS since there is clear sexual dimorphism in pain mechanisms that have been discovered thus far. Although the BDNF/TrkB/NR2B signaling investigated in the study was not found to be sex-hormone dependent,[33] we observed less pain reduction in female neuropathic rats in response to KDS treatment compared to male rats (Supplementary Fig 5). In the present study, we only studied male animals, however, both genders play a fundamental role and the mechanisms underlying the sex-dependent difference in the effects of KDS should be studied concurrently in future studies.

Discussion

We investigated, for the first time, the involvement of BDNF/TrkB signaling in the mechanisms by which MAO-B inhibitors can reduce pain behaviors in neuropathic animals. A previous study has shown the analgesic effect of Selegiline in a mouse pain model.[53] In good agreement, the anti-allodynic effect of KDS, a newly developed MAO-B inhibitor, via ROS-GABA inhibition, has been revealed in our previous study (unpublished data). The study focused on the effect of KDS on suppressing astrocytic MAO-B/ROS production-decreased GABA inhibition in PTX-induced neuropathic pain in mice. The CIPN pain model presents major contributing roles of reactive astrocytes and oxidative stress to pain processes. But these mechanisms may not be sufficient to explain for the analgesic effect of MAO-B inhibitors in the nerve injury-induced neuropathic pain models, in which nerve injury-induced neuronal hyperexcitability and central sensitization are driving forces. It has been investigated that glia cells including astrocytes and microglia are both strongly activated in the spinal dorsal horn following nerve injury and interact with neurons, resulting in long-term changes in microenvironment and synaptic transmission. Many studies support the pro-nociceptive role of neuronal-derived BDNF, in response to nerve injury, contributing to synaptic plasticity and pain development. As a sequence, blockage of BDNF/TrkB signaling by deletion of BDNF or inhibition of TrkBR is critical and sufficient to prevent or reverse neuropathic pain after nerve damage.[42,50] KDS, as a MAO-B inhibitor, can alleviate PTX-induced tactile hypersensitivity in a ROS/GABA dependesnt manner. MAO-B inhibitors have also been shown to reduce neuropathic pain after nerve injury, however, the mechanisms remain undetermined. On the other hand, early studies revealed that MAO-B inhibitors increase BDNF levels in both in vitro and in vivo after treatment.[32,38] In this view, the anti-nociceptive effects of MAO-B inhibitors are not matched with increased BDNF levels found in animals/cells with MAO-B inhibitor treatment.

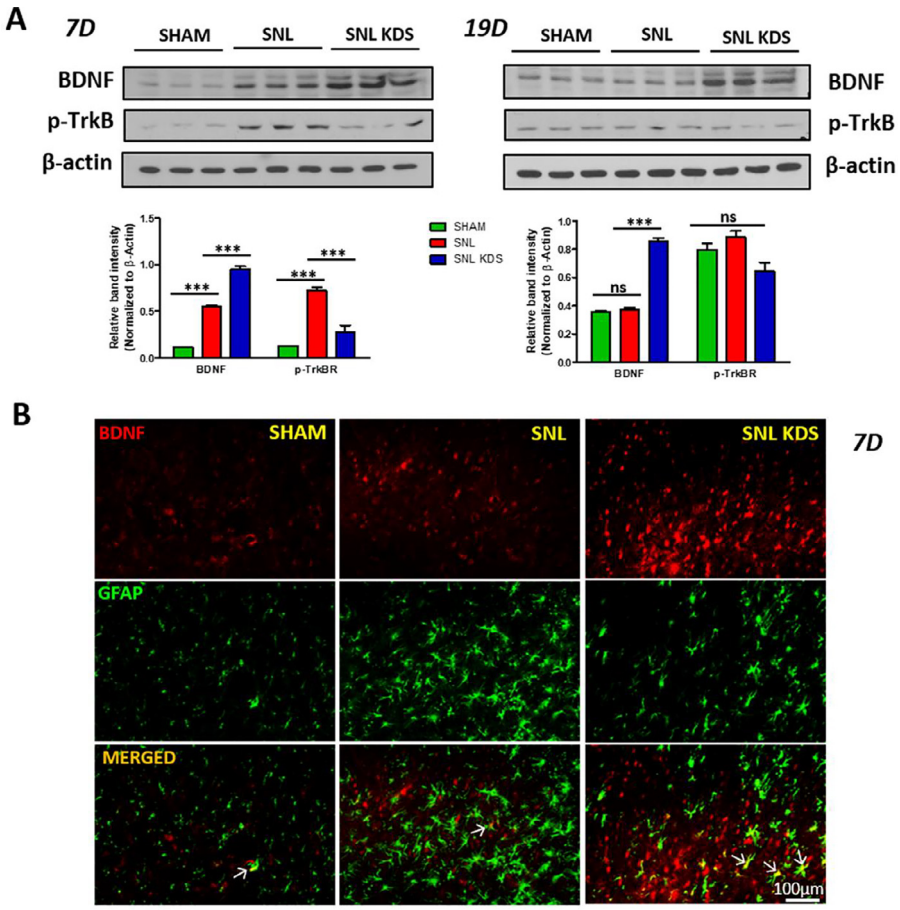

Figure 6. Increased level of mature BDNF in the spinal dorsal horn following treatment with KDS. (A) The protein levels of BDNF and p-TrkB receptors in the spinal dorsal horns were assessed and compared among the SHAM, the SNL untreated, and the SNL KDS-treated on POD7 and POD19. The relative blot intensities are shown in the graphs. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ***P < .001: 7D: SNL vs SHAM, KDS vs SNL, ns: 19D: SNL vs SHAM, ***P < .001: 19D: KDS vs SNL) (B, C) The immunostaining of spinal cord sections on POD 7 showing BDNF was more excessively increased in the SNL KDStreated group, and co-localized with both GFAP and NEUN (arrowheads indicate the co-localization between red and green IR). Scale bars = 100 μm. (D) Immunodensities of BDNF expression were quantified. Data are presented as the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test **P < .01: SNL vs SHAM, ***P< .001: KDS vs SNL). (E) The percentages of BDNF$^+$ cells in GFAP$^+$ or NEUN$^+$ cells were calculated. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ns: GFAP$^{+:}$ SNL vs SHAM, **P < .01: GFAP$^+$: KDS vs SNL, ***P< .001: NEUN$^+$: SNL vs SHAM, KDS vs SNL, n = 6 per group).

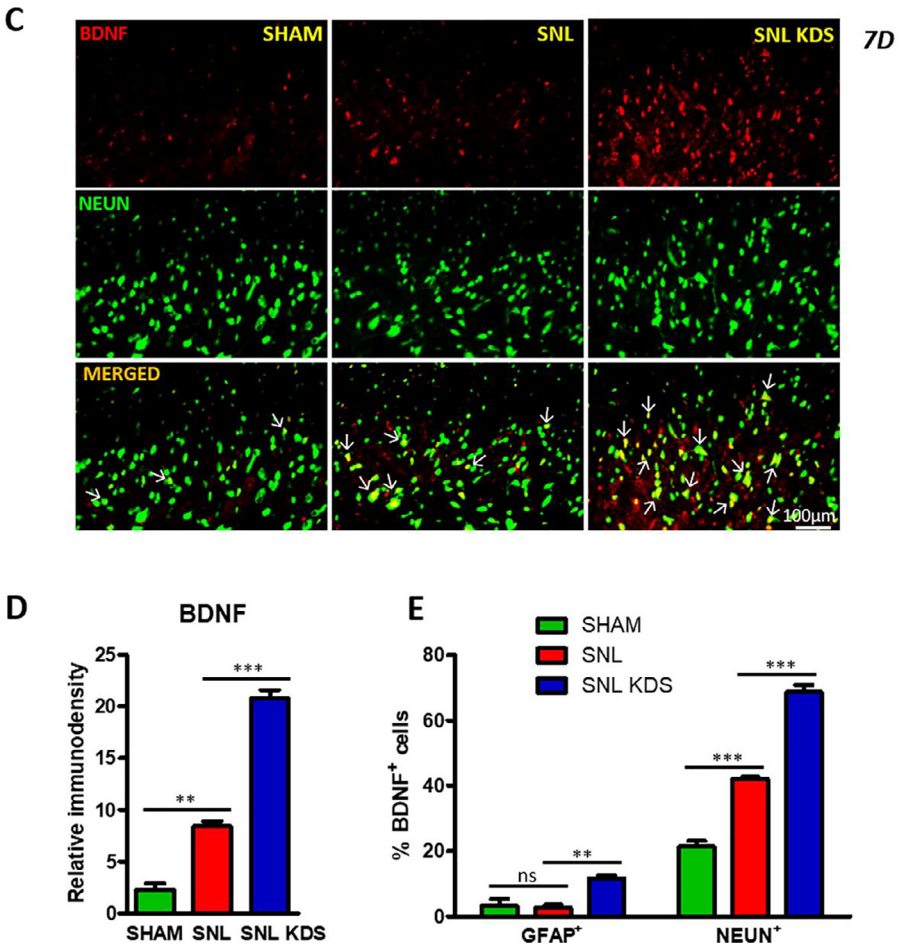

Figure 6. Continued

In good agreement with previous studies, our results show BDNF expression was upregulated in the spinal dorsal horns of the SNL-induced rats at early stage, within 7 days after surgical procedure and diminished on POD19. Particularly, administration of TrkB specific inhibitor ANA12 significantly enhanced mechanical thresholds in the SNL rats, suggesting the pro-nociceptive effect of increased endogenous BDNF in response to nerve injury. On the other hand, we also observed higher levels of BDNF in the SNL-KDS treated group. Interestingly, unlike the anti-allodynic effect of ANA12 in the SNL rats, co-treatment of ANA12 with KDS almost completely blocked KDS-induced pain relief. There are 2 questions arisen here: first, how KDS reduced pain hypersensitivity while increasing BDNF, and second, how ANA12 acted in different cases, alone and in combination with KDS treatment? To address the first question, we examined the BDNF/TrkB signaling cascades. It has been demonstrated that BDNF/TrkB pathway exerts its function by activating the NR2B containing NMDAR, which is widely accepted to be a crucial factor for the spinal nociceptive synaptic transmission and for the development of long-lasting spinal hyperexcitability after nerve injury.[27,30,61] Consistently, we found both NR2B and active p-NR2B were increased in the spinal dorsal horns of the SNL-induced rats, following BDNF/TrkB activation, confirming the contribution of BDNF/TrkB/NR2B to the development of pain. In the KDS-treated neuropathic rats, BDNF levels were significantly higher, compared to the SNL-untreated rats, however, the levels of p-TrkB and p-NR2B were conversely lower with no significant change in total NR2B protein levels. In addition, p-Akt, a key downstream BDNF/TrkB that is believed to be involved in the phosphorylation process of NR2B,[36] was activated in the SNL rats and was also suppressed by the KDS. These data suggest that KDS may have acted at TrkBR level to suppress or block BDNF/TrkB activation-induced signaling cascades downstream response. KDS may possibly serve as a TrkB inverse agonist or a partial antagonist. In the presence of KDS, BDNF was unable to bind to its receptor TrkB, or the BDNF/TrkB complex was inactivated due to competition with KDS. Eventually, the final product p-NR2B was reduced in the SNL-KDS treated rats.

In addition to the NR2B and p-NR2B protein levels, the functional changes of the NR2B-containing NMDAR were also confirmed by dorsal horn neuron recordings. Several pain studies have shown that NR2B containing NMDAR-mediated excitatory post synaptic currents contribute to the imbalance between inhibition and excitation in the spinal cord, leading to long-term synaptic plasticity and pain hypersensitivity.[21,56] The present study investigated, for the first time, how extrasynaptic NR2B subunit contributes to persistent pain via mediating tonic

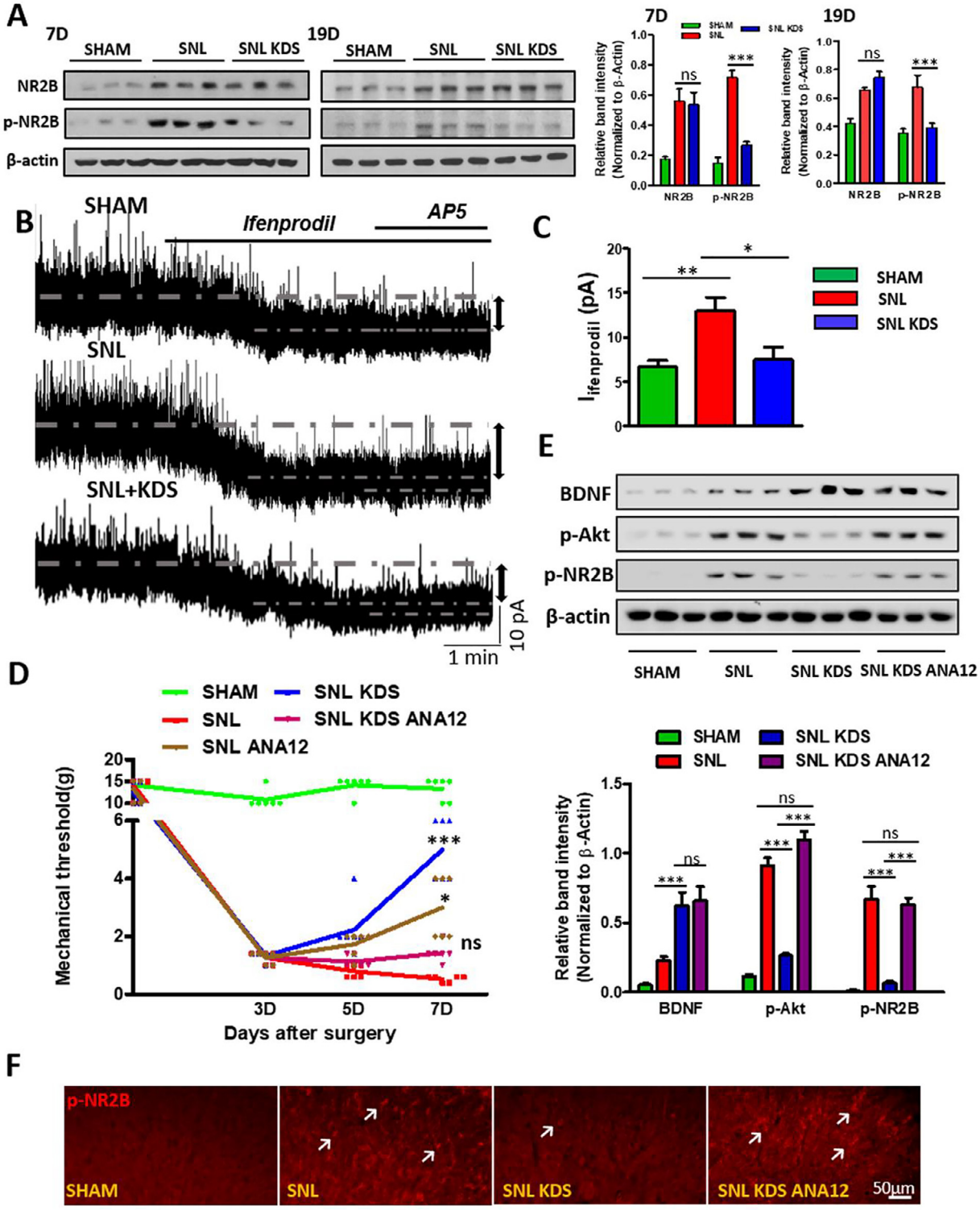

Figure 7. KDS acts on TrkB to reduce the phosphorylation state of NR2B, suppressing BDNF-enhanced tonic NMDAR excitation in dorsal horn sensory neurons. (A) The protein levels of NR2B anrereceptorseptor in the spinal dorsal horns were assessed and compared among the SHAM, the SNL-untreated, and the SNL KDS-treated on POD7 and POD19. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ***P < .001: NR2B: SNL vs SHAM, ns: NR2B: KDS vs SNL, ***P < .001: p-NR2B: SNL vs SHAM, KDS vs SNL). (B) The representative current traces and current amplitude (C) recorded from the spinal dorsal horn neurons at POD7 were shown and compared among the groups (n = 7–9 cells from 3–4 animals per group). Data are presented as the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test **P < .01: SNL vs SHAM, *P< .05: KDS vs SNL). (D) ANA12 was systemically administered during KDS treatment period from POD3 to POD7. The von Frey tests were conducted on each group of the rats on POD3, 5, and 7 to determine mechanical thresholds. Data are expressed as the mean ± SEM (two-way ANOVA, followed by Bonferroni post hoc test, ***P < .001: KDS vs SNL, *P < .05: SNL ANA12 vs SNL, ns: SNL KDS ANA12 vs SNL). (E) The protein levels of BDNF, p-NR2B, and p-Akt in the spinal dorsal horns were assessed and compared among the SHAM, the SNL untreated, the SNL KDS-treated, and the SNL KDS ANA12-treated rats on POD7. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, ***P < .001: BDNF: KDS vs SNL, ns: BDNF: KDS ANA12 vs KDS, *** P < .001: pNR2B:

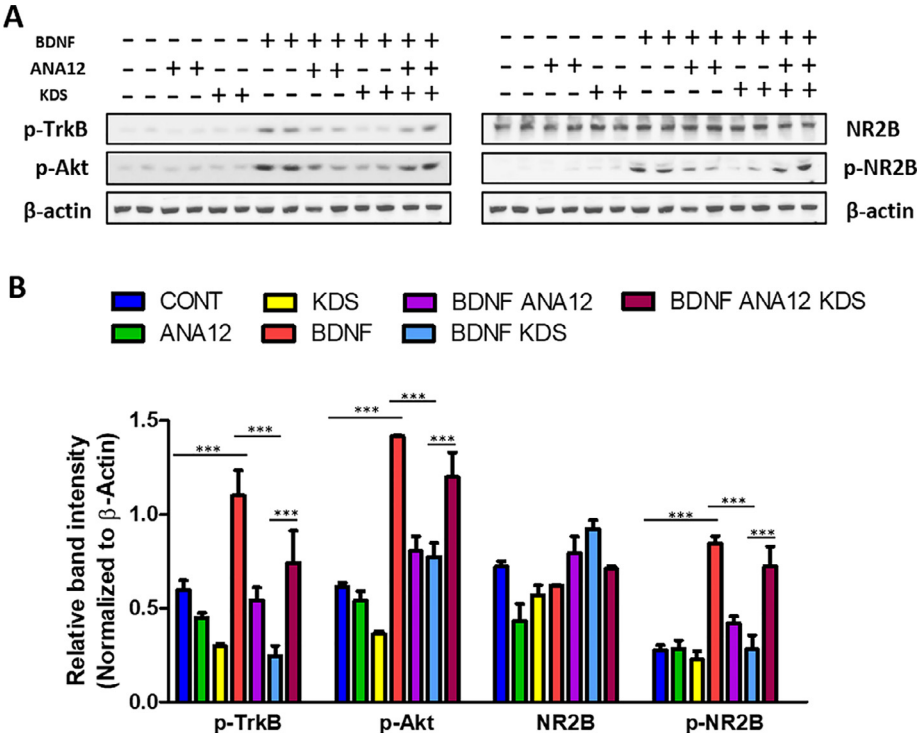

Figure 8. KDS suppresses BDNF/TrkB signaling-induced p-NR2B upregulation in the SH-SY5Y cultured cells. (A) SH-SY5Y cells were cultured and stimulated with BDNF in the presence or absence of ANA12 and/or KDS. The protein levels of p-TrkB, p-Akt, NR2B, and p-NR2B in the cells were assessed and compared among the groups. (B) The blot densities were quantified in the graph. Data are presented as the mean ± SEM (two-way ANOVA followed by Bonferroni post hoc test, *** $P < .001$: p-TrkB: BDNF vs CONT, *** $P < .001$: p-TrkB: BDNF KDS vs BDNF, *** $P < .001$: p-TrkB: BDNF ANA12 KDS vs KDS, *** $P < .001$: p-Akt: BDNF vs CONT, *** $P < .001$: p-Akt: BDNF KDS vs BDNF, *** $P < .001$: p-Akt: BDNF ANA12 KDS vs KDS, *** $P < .001$: p-NR2B: BDNF vs CONT, *** $P < .001$: p-NR2B: BDNF KDS vs BDNF, ** $P < .01$: p-NR2B: BDNF ANA12 KDS vs KDS, n = 6 per group).

NMDAR currents. The NR2B subunit-mediated tonic excitatory currents were enhanced in the dorsal horn neurons following SNL procedure, but were reduced by KDS in the neuropathic rats with KDS treatment. Without significant difference in total NR2B protein levels between the SNL-untreated and the SNL-KDS treated rats, the functional changes in NR2B-mediated neuronal excitability are attributed to the active p-NR2B alteration. This result serves as strong evidence not only supporting the contributing role of NR2B in persistent pain response but also confirming the effect of KDS on repressing tonic NR2B containing NMDAR activation.

ANA12 acts as a selective antagonist of TrkB. With this function, ANA12 has been employed to block BDNF/TrkB signaling, resulting in pain reduction. Our behavioral results show a similar response upon ANA12 treatment alone in the SNL-induced rats. However, when ANA12 was introduced together with KDS, the analgesic effects of both drugs disappeared. We also observed the p-NR2B levels as well as the activation state of the glial cells in the SNL-KDS ANA12 treated rats were not significantly altered, compared to the SNL-untreated rats. Supporting of this notion, co-treatment of ANA12 with KDS prevented KDS decreased p-NR2B in response to BDNF stimulation in the SH-SY5Y cultured cells. These results indicate that KDS and ANA12 competitively antagonize each other. However, our limitation is that we were unable to further investigate how KDS and ANA12 worked at TrkB, and the mechanism by which KDS increased BDNF while reducing p-TrkB is not clear yet. Further studies are required to address these questions.

Of particular relevance, it has been demonstrated that, in the SNL pain model, the maximal enhancement of BDNF expression occurs at very early stage after nerve injury and is followed by increased p-NR2B.[16] BDNF has also been shown to regulate the activation of glial cells.[11] BDNF mediates phosphorylation of the NR2B containing NMDARs and glial interaction, which in turn modulate the development and maintenance of neuropathic pain. It is likely that BDNF plays a central role in the initiation and transition of acute pain to chronic neuropathic pain after nerve injury. In consistency with these observations, the present study demonstrated that blockage of BDNF/TrkB signaling by KDS not only reduced p-NR2B but also suppressed microgliosis and astrogliosis. Both of these effects were impaired when KDS was applied with ANA12, possibly due to a competitive mechanism. Further research is warranted to elucidate the hypothesis as well as the interaction between these ligands at TrkB.

In addition to modulating neuronal activity, KDS also reduced the levels of ROS production in the spinal cord

KDS vs SNL, *** $P < .001$: p-NR2B: KDS ANA12 vs KDS, ns: p-NR2B: KDS ANA12 vs SNL, *** $P < .001$: p-Akt: KDS vs SNL, *** $P < .001$: p-Akt: KDS ANA12 vs KDS, ns: p-Akt: KDS ANA12 vs SNL, n = 6 per group). (F) The spinal cord sections of the SHAM, the SNL untreated, the SNL KDS-treated, and the SNL KDS ANA12-treated rats were stained with anti-p-NR2B antibody. Arrowheads indicate the positive IR. Scare bar = 50 μm.

after nerve injury. KDS, as an MAO-B inhibitor, inhibits MAO-B enzyme activities, which has been shown to result in the production of by-products, particularly ROS.[18] Indeed, this is one of the major mechanisms of action of MAO-B inhibitors in PD.[34,58] This mechanism also works in CIPN pain model, in which we showed that KDS suppresses ROS-decreased inhibitory GABA synaptic transmission. This may serve as a general mechanism of action of MAO-B inhibitors in relieving pain. In neuropathic pain with nerve injury, an increase in BDNF level occurs at very early stage and is likely to mediate the subsequent development and maintenance of pain. Whereas, ROS have been implicated in the development of persistent pain states.[44,59] Likely, the anti-allodynic effect of KDS in the first round of treatment might be largely dependent on BDNF/TrkB while the second round might result from decreased levels of p-NR2B and ROS production.

Neuropathic pain is a very complex disease with multiple molecular pathways and mechanisms involved in the pathogenesis. It is extremely difficult to stop the pain if the treatment only targets its individual character. Current clinically available drugs have shown disappointing efficacy due to their generalized nature and action on temporal pain symptoms rather than to treat the mechanisms underlying neuropathic pain. MAO-B inhibitors with their effectiveness by targeting multiple mechanisms on neuronal activity, inflammation, and oxidative stress in the spinal cord after nerve injury can be suitable solutions for treating refractory neuropathic pain. Indeed, Selegiline, an irreversible MAO-B inhibitor, also shows an analgesic effect as revealed by our data (Supplementary Fig 6), indicating the general therapeutic potential of MAO-B inhibitors in pain management. Most notably, due to the important roles of BDNF/TrkB in regulating neuronal circuit and central sensitization, a driving force of chronic neuropathic pain over time, targeting this signaling may open a new molecular method for pain management. However, future studies are needed to better elucidate the precise interaction between KDS and BDNF/TrkB. This may help develop new generations of drugs directly targeting BDNF/TrkB or to modify currently available MAO-B inhibitors for clinical pain treatment.

Author's Contribution

TLP and CN performed the experiments and analyzed the data, prepare the figures. CN performed electrophysiology. RS, HJS performed behavioral tests. KDP, CJL, HWK, SYL, and JBP designed the experiments and discussed the results. JBP supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jpain.2022.07.010.

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