

Pharmacodynamic Effects of a D-Amino Acid Oxidase Inhibitor Indicate a Spinal Site of Action in Rat Models of Neuropathic Pain

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ABSTRACT

Inhibition of D-amino acid oxidase (DAAO) activity is a potential target for the treatment of chronic pain. Here we characterized the effects of systemic administration of the DAAO inhibitor 4H-furo[3,2-b]pyrrole-5-carboxylic acid (SUN) in rat models of neuropathic and inflammatory pain. Oral administration of SUN dose dependently attenuated tactile allodynia induced by ligation of the L5 spinal nerve (SNL) and similarly reversed thermal hyperalgesia produced by chronic constriction injury. In addition, SUN was efficacious against complete Freund's adjuvant-induced thermal hyperalgesia. In these models, maximal reversal of pain-related behaviors corresponded with maximum rates of increase in brain and plasma D-serine concentrations, indicative of full inhibition of DAAO activity. To investigate the possible site(s) of action, we recorded spontaneous nerve activity

and mechanically evoked responses of central spinal cord dorsal horn neurons and compared these with spontaneous activity of peripheral dorsal root filaments in anesthetized SNL model animals. Oral SUN reduced spontaneous activity in both central and peripheral recordings at doses and pretreatment times that corresponded to reduced mechanical allodynia in behavioral experiments. After intravenous administration of SUN, the onset of action for this central effect was rapid (maximal effects within 30 minutes), but was abolished by severing afferent inputs to the dorsal horn. Overall, these results indicate that inhibition of DAAO in peripheral afferent spinal circuits reduced spontaneous neuronal activity to attenuate pain-related behaviors in rat models of neuropathic and inflammatory pain.

Introduction

D-Amino acid oxidase (DAAO, EC 1.4.3.3) is a flavin-dependent enzyme responsible for metabolism of endogenous D-serine in mammals. D-Serine is a neuromodulator important for the development of neural circuits and contributes to normal neuronal functioning of N-methyl-D-aspartate (NMDA) receptors. D-Serine is produced by the stereoconversion from L-serine via serine racemase in glial cells. DAAO activity, also in glial cells, is responsible for reducing D-serine concentrations and potentially reduces the occupancy of the glycine site in NMDA receptors. As such, DAAO inhibitors have been proposed as a therapeutic treatment of schizophrenia.

Neuropathic pain that arises after nerve injury is characterized by ongoing neurotransmission of pain signals through spinal circuits via the dorsal root ganglion and dorsal horn neurons. NMDA receptors are expressed in spinal cord neurons

and have been targets for candidate therapeutics discovered with preclinical animal models and tested clinically. DAAO is expressed spinally and its activity may contribute to NMDA-related neurotransmission. NMDA receptors play a key role in the development of ongoing pain states via central sensitization in the spinal cord. Therapeutically targeting the changes in synaptic strength that maintain ongoing neuropathic pain states with NMDA-related mechanisms of action may help to discover new analgesics. Given these notions of plasticity and pain states, we reasoned that DAAO inhibitors may alter the neurotransmission of pain signals under certain conditions by changing local D-serine concentrations proximal to NMDA receptors. Thus, during the course of a drug discovery and optimization campaign at Sunovion Pharmaceuticals, we discovered effects of DAAO inhibition in animal models of neuropathic pain (Fang et al., 2005, 2011; Dorsey et al., 2008; Heffernan et al., 2009). These observations, although counterintuitive to an understanding of the efficacy of NMDA receptor antagonists in models of pain, have also been described using sodium benzoate in

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ABBREVIATIONS: CCI, chronic constriction injury; CFA, complete Freund's adjuvant; DAAO, D-amino acid oxidase; NMDA, N-methyl-D-aspartate; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; SNL, spinal nerve ligation; SUN, 4H-furo[3,2-b]pyrrole-5-carboxylic acid; VFH, von Frey hair.

neuropathic models (Zhao et al., 2010). In addition, others (Zhao et al., 2010; Gong et al., 2011, 2012; Chen et al., 2012) reported on the effects of DAAO inhibition in a formalin model of tonic pain.

Here, we use the DAAO inhibitor SUN (4*H*-furo[3,2-*b*]pyrrole-5-carboxylic acid) to describe the efficacy observed in three animal models of neuropathic and inflammatory pain and relate this to the underlying pharmacodynamics of D-serine concentrations. We furthermore use these observations to identify a putative site of action at a key pain circuit that includes the spinal cord and dorsal root ganglion neurons.

Materials and Methods

Animals. Male Sprague-Dawley rats (180–350 g) were group housed in a temperature-controlled environment on a 12-hour light-dark cycle with food and water available ad libitum. Animals were allowed to acclimate to the facility for at least 2 days before in vivo testing. Separate groups of rats were used in pharmacokinetic-pharmacodynamic, behavioral, and electrophysiological studies. All procedures in this study were undertaken in compliance with the UK Animals (Scientific Procedures) Act 1986.

Pharmacokinetic-Pharmacodynamic Studies. Previous studies demonstrated low endogenous D-serine concentrations in the cerebellum compared with other brain regions in adult rats. Thus, the cerebellum was determined to be an ideal brain region in which to detect in vivo inhibition of DAAO, as evidenced by the resultant increase in endogenous D-serine concentrations. Rats were administered a single oral dose, from 3 to 30 mg/kg SUN, and killed at different time points after compound administration. For the dose-response and time-course studies, there was a minimum of $n = 3$ rats per time point. At death, trunk whole blood and cerebellum were collected, processed, and analyzed for D-serine concentrations. Whole blood samples were collected in tubes containing potassium ethylenediaminetetraacetic acid. These tubes were centrifuged, and the plasma was decanted. Plasma and cerebellum samples were stored at -80°C until analyzed by liquid chromatography-tandem mass spectrometry for D-serine concentrations.

Spinal Nerve Ligation Model. Rats were allowed to acclimatize to the experimental environment for 3 days by leaving them on a raised metal mesh for at least 40 minutes. The baseline paw withdrawal threshold (PWT) was determined using a series of graduated von Frey hairs (see below) for 3 consecutive days before surgery. After surgery, PWT were assessed again on days 7 to 8 and on days 11 to 14 before compound administration.

Rats underwent spinal nerve ligation surgery, as described by Kim and Chung (1992). Each rat was anesthetized with 5% isoflurane mixed with oxygen (2 l/min) followed by an intraperitoneal injection of sodium pentobarbitone at 50 mg/kg. The back was shaved and disinfected with 75% ethanol. The animal was placed in a prone position, and a paramedical incision was made on the skin covering the lumbar spinal cord at the L4 to L6 level. The L5 spinal nerve was carefully isolated and tightly ligated with 6-0 silk suture. The wound was then closed in layers after a complete hemostasis. A single dose of antibiotic [Amoxipen (amoxicillin; Intervet UK Ltd, Milton Keynes, UK), 15 mg/rat i.p.] was routinely given for prevention of infection after surgery. The animals were placed in a temperature-controlled recovery chamber until fully awake before being returned to their home cages.

Rats were placed in individual Perspex boxes on a raised metal mesh for at least 40 minutes before behavioral testing. Starting with the von Frey filament that generated the lowest mechanical force, each filament was applied perpendicularly to the center of the ventral surface of the paw until it was slightly bent for 6 seconds. If the animal withdrew or lifted the paw upon stimulation, then a hair with force immediately lower than that tested was evaluated next. If no response was observed, then a hair with force immediately higher

than the one tested was evaluated next. The lowest amount of force required to induce reliable withdrawal responses (positive in 3 out of 5 trials) was recorded as the value of the PWT. Only those animals that demonstrated significant mechanical allodynia, as defined by a $\text{PWT} \leq 3.5\text{g}$, were selected for compound-dosing experiments.

Rats that demonstrated significant mechanical allodynia after spinal nerve ligation were randomly divided into five experimental groups: vehicle (negative control, isotonic 50 mM phosphate buffer), gabapentin (positive control, 100 mg/kg PO), and three doses of SUN (3, 10 and 30 mg/kg PO). Each group had eight animals. Test article administration and behavioral testing were carried out 11 to 14 days after surgery. One experimenter dosed the rats, and a second experimenter, who was blinded to the treatment of individual animals, determined the PWT at 1, 3, 6 and 24 hours after test article administration. The animals were returned to their home cages (about 30 minutes) between time points.

Chronic Constriction Injury Model. Rats were allowed to acclimatize to the experimental environment for three days by leaving them on a raised glass plate for 10–20 minutes. The baseline paw withdrawal latency (PWL) was determined using a radiant light source (modified Hargreave's test, see below) for 3 consecutive days before surgery. After surgery, PWL were assessed again on day 7 and on days 13 to 17 before compound administration.

The chronic constriction injury (CCI) model was prepared following the standard protocols described previously (Bennett and Xie, 1988). Briefly, rats were anesthetized with a 5% isoflurane/95% oxygen gas mixture for induction followed by an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). The lateral side of the left thigh was shaved and sterilized with 75% ethanol. A small incision of about 1 cm was made parallel to the femur. The muscle was carefully separated to expose the sciatic nerve. Four loose ligatures were placed on the sciatic nerve with 4-0 suture silk thread at about 1-mm intervals. The wound was closed in layers with suture silk, and the animals were placed in a recovery chamber with the temperature controlled at 30°C . The animals were placed back in their home cages after complete return of consciousness and free movement. A single dose of antibiotic (Amoxipen, 15 mg/rat i.p.) was routinely given for prevention of infection after surgery.

Thermal hyperalgesia was assessed by determination of PWL to a noxious heat stimulus using a beam of radiant light (modified Hargreave's apparatus). Rats were placed in individual plastic boxes on top of a glass plate for about 10–20 minutes before the experiment. The noxious heat stimulus was focused through the glass onto the plantar surface of a hind paw until the animal withdrew its paw. The PWL was defined as the time in seconds from the start of heat stimulation to the time the animal withdrew its hindlimb. In the absence of a response, a cut-off latency of 20 seconds was set to prevent tissue damage. The average PWL of three trials from each hind paw was calculated. PWL was tested at 1, 3, 6, and 24 hours after a single oral dose of a vehicle, a reference compound, or a test compound at a volume of 3 ml/kg. Only those animals with significantly reduced PWL (≤ 6.0 second versus about 10.0 second baseline) were selected for compound-dosing experiments.

Complete Freund's Adjuvant Model. Rats were allowed to acclimatize to the experiment's environment for 3 days by leaving them on a raised glass plate for 10–20 minutes. The baseline PWL was determined using a radiant light source (modified Hargreave's test, as described above) for 3 consecutive days before complete Freund's adjuvant (CFA) injection and reassessed 24 hours later, just before compound administration.

The animals were briefly anesthetized with 5% isoflurane mixed with 95% oxygen. CFA (0.05 ml; Sigma-Aldrich, St. Louis, MO) emulsion in saline (CFA: saline = 1:1, vol/vol) was injected subcutaneously into the plantar surface of the left hind paw. After CFA injection, the animals were returned to their home cages. Hourly observations were carried out up to 4 hours after CFA injection to monitor the condition of the animals. Compounds were administered 24 hours after CFA injection. PWL was assessed at 1, 3, 6, and 24

hours after a single oral dose of a vehicle, a reference compound, or a test compound at a volume of 3 ml/kg. Only those animals with thermal hyperalgesia, defined as a significantly reduced PWT (i.e., ≤ 6.0 second, versus a baseline of approximately 10.0 second), were selected for compound-dosing experiments.

Electrophysiology Experiments. After behavioral validation of the presence of spinal nerve ligation (SNL)-induced tactile allodynia, rats were anesthetized with urethane (1.2–1.6 g/kg i.p.). The right carotid artery and jugular vein were cannulated separately to monitor blood pressure and permit drug administration, respectively. The body temperature was monitored and controlled within a physiologic range via a thermo-blanket system. The ECG was routinely monitored through a pair of stainless steel needles inserted into the left forepaw and right hind paw. A laminectomy was carried out to expose the L2–L6 segments of the spinal cord. In some experiments, spinalization at the C2–C3 level or section of the L4–L6 dorsal roots of the left side at the site near the root entry zone was performed within 1–2 hours after oral dosing with the vehicle or SUN. An oil pool was formed by clipping the skin flaps onto the metal frame and filling the space with warm mineral oil to cover the spinal cord and the sectioned roots. Extracellular unitary recordings from the spinal dorsal horn were achieved by using a tungsten or carbon microelectrode. The peripheral receptive field was located and the responses of the neurons to mechanical stimulation (i.e., brush, von Frey hairs 4.17, 4.56, 5.18 representing forces of 1.15, 4.0, and 15.6g, respectively, and squeeze using a standard artery clip applied to the receptive field) were examined to classify the neuron. In this study, only wide dynamic range neurons, which responded robustly to varied mechanical stimuli and exhibited a force-dependent response (i.e., a greater stimulation force resulted in a higher frequency discharge), or high-threshold mechanoreceptive neurons, which responded robustly only to high force mechanical stimulation, were recorded. In rats with dorsal roots sectioned, only spontaneous activity was recorded. For recording of dorsal root filaments, left L4 dorsal root was sectioned and repeatedly teased into small bundles. A small bundle (filament) teased from the L4 dorsal root was looped on to a unipolar silver wire recording electrode with an indifference electrode connected to nearby connective tissues, allowing spontaneous activity to be recorded. When a filament displaying spontaneous activity was found, its peripheral receptive field(s) on the hind paw plantar surface was examined using three von Frey hairs representing the forces of 1, 6, and 15g. Neural activity (spontaneous discharge and mechanically-evoked responses) was amplified and monitored using standard

electrophysiological techniques and recorded to a personal computer using CED Spike 2 software (Cambridge Electronics Design, Cambridge, UK).

Analysis. Behavioral data were analyzed using two-way repeated-measures analysis of variance (SigmaStat version 3.5; Systat Software, Inc, Chicago, IL) with treatment as the between-groups factor and time as the within-groups factor. For SNL model data, nonparametric analyses were conducted on ranks of paw withdrawal threshold values. For CCI and CFA model data, parametric analyses were conducted on paw withdrawal latency values. Comparisons were made at the criterion of $P < 0.05$ for statistical significance, and multiple comparisons were conducted using the Holm-Sidak method. Data from electrophysiological experiments were grouped based on treatment and expressed as mean \pm 1 S.E.M. Non-paired Student's t tests were conducted to compare treatment groups. The significance level was set at $P < 0.05$.

Results

Effects of Oral Administration of SUN to SNL Model Rats. The effects of SUN were investigated using the SNL model of neuropathic pain. Two independent tests of SUN efficacy were performed, the first with a single dose level (10 mg/kg PO) and the second with a dose-response, each study having negative (vehicle) and positive (gabapentin) control treatment groups.

In naive rats before surgery, the mean PWT was between 10 and 13g (Fig. 1). On day 7 after SNL, the mean PWT on the side ipsilateral to the ligated nerve was substantially lower (between 1 and 3g; Fig. 1), indicating that mechanical allodynia had developed. This allodynia was specific to the ipsilateral side, because the contralateral limbs continued to display more elevated PWT values between 7 and 10g (gray symbols in Fig. 1). Behaviorally, the animals showed some degree of disuse of the affected limb or limping. However, the general appearance of the animals was not remarkably different from their naive counterparts.

The effects of test compound were assessed 11 to 14 days after SNL. As a negative control, vehicle administration had no effect on the PWT over the 24 hours of testing (Fig. 1). As

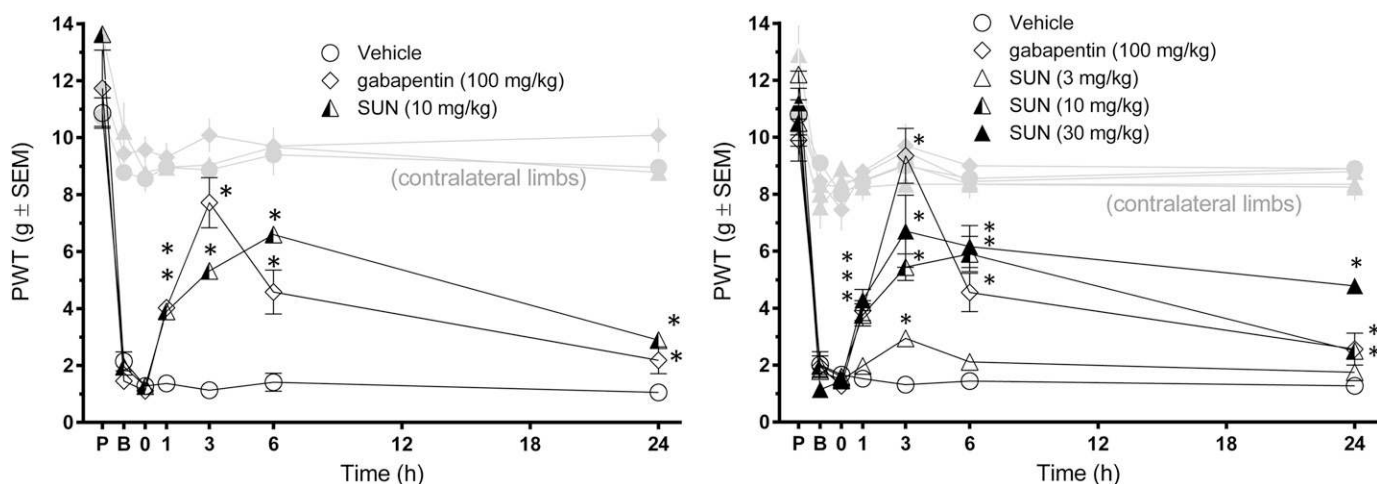


Fig. 1. Effect of a single oral administration of vehicle, gabapentin, and SUN on mechanical allodynia in SNL model rats. Paw withdrawal threshold (PWT) determined on the ipsilateral limbs (black) and contralateral limbs (gray). Panels from two independent experiments: single dose level (left panel) and a dose response (right panel). P, presurgery baseline. B, day 7 postsurgery. 0, Predosing control on day of compound administration (11 to 14 days after surgery). Vehicle, isotonic 50 mM phosphate buffer PO; gabapentin: 100 mg/kg PO; SUN: 3, 10, and 30 mg/kg PO, $n = 9$ in each group. * $P < 0.05$, compared with the same time point in the vehicle group, two-way repeated-measures analysis of variance (RMANOVA) followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

a positive control, gabapentin, administered at 100 mg/kg PO, increased PWT on the ipsilateral side, with effects evident at the first observation (1 hour) and peak effects at 3 hours. This peak in efficacy was observed in both experiments (Fig. 1, left and right panels) and appeared to subside by 6 hours. The DAAO inhibitor SUN, administered at 10 mg/kg PO, induced an increase in PWT of ipsilateral limbs, with effects increasing for 6 hours and lasting over the 24-hour observation period. These observations with 10 mg/kg were similar to those observed at the same dose as part of the follow up dose-response study (Fig. 1, right panel). Effects on PWT after the 3 mg/kg dose were evident, although reduced relative to the 10 and 30 mg/kg doses and returned to baseline by 24 hours. The effects of 10 mg/kg appeared to be maximal, because the higher 30 mg/kg dose did not produce a further increase in efficacy over the 6-hour observation period. However, at 24 hours, the 30 mg/kg effect was higher than the 3 and 10 mg/kg effects. These effects were specific to the ipsilateral side, because there were no significant treatment-related effects observed in the contralateral limbs, for any treatment group of either experiment (gray symbols in Fig. 1).

Effects of Oral Administration of SUN to CCI Model Rats. These effects of SUN on mechanical allodynia in the SNL model were compared with its effects on thermal hyperalgesia in the CCI model of neuropathic pain. As with the SNL model, two independent tests of SUN efficacy were performed.

In naive rats before surgery, the mean PWT was between 10 and 12 seconds (Fig. 2). On day 7 after surgery, the mean PWT on the side ipsilateral to the injured nerve was substantially lower (between 3 and 6 seconds; Fig. 2), indicating that thermal hyperalgesia had developed. This hyperalgesia was specific to the ipsilateral side, since the contralateral limbs continued to display more elevated PWT values between 9 and 12 seconds (gray symbols in Fig. 2). Like the SNL model animals, these animals showed some degree of disuse of the affected limb or limping, but the general appearance of the animals was not remarkably different from their naive counterparts.

The effects of test compound were assessed 13 to 17 days after CCI. As a negative control, vehicle administration had no effect on the PWT over the 24 hours of testing (Fig. 2). As a positive control, gabapentin, administered at 100 mg/kg PO, increased PWT on the ipsilateral side, with effects evident at the first observation (1 hour). This efficacy was observed in both experiments (Fig. 2, left and right panels) and remained for 24 hours. The DAAO inhibitor SUN, administered at 10 mg/kg PO, induced an increase in PWT of ipsilateral limbs for 6 hours that was maintained over the 24-hour observation period. These observations at 10 mg/kg were confirmed in a follow up dose-response study (Fig. 2, right panel). Effects on PWT after the 3 mg/kg dose were significantly greater than vehicle treatment, although reduced in magnitude relative to those observed after the 10 and 30 mg/kg doses. As observed with the SNL model, the effects of 10 mg/kg appeared to be maximal, because the higher 30 mg/kg dose did not produce a further increase in efficacy over the 6-hour observation period. However, at 24 hours, the 30 mg/kg effect appeared greater than the 3 and 10 mg/kg effects. These effects were specific to the ipsilateral injured side, because there were no significant treatment-related effects observed in the contralateral limbs at any time point after any treatment during either experiment (gray symbols in Fig. 2).

Effects of Oral Administration of SUN to CFA Model Rats. The effects of SUN in models of neuropathic pain were compared with its effects on thermal hyperalgesia in a rat model of CFA-induced inflammatory pain. In naive rats prior to the CFA injection, mean PWT values were between 9 and 12 seconds. After CFA administration, mean PWT values decreased to 5 seconds, indicating that thermal hyperalgesia had developed. This effect was specific to the inflammatory insult, as latencies for limbs contralateral to CFA injection did not decrease. Vehicle administration did not affect PWT values, whereas the positive control compound ibuprofen (50 mg/kg PO) increased PWT significantly ($P < 0.05$) relative to vehicle at all time points observed (Fig. 3). The lowest dose of SUN (3 mg/kg PO) did not significantly increase PWT relative to vehicle. Similar to the effects observed in the neuropathic

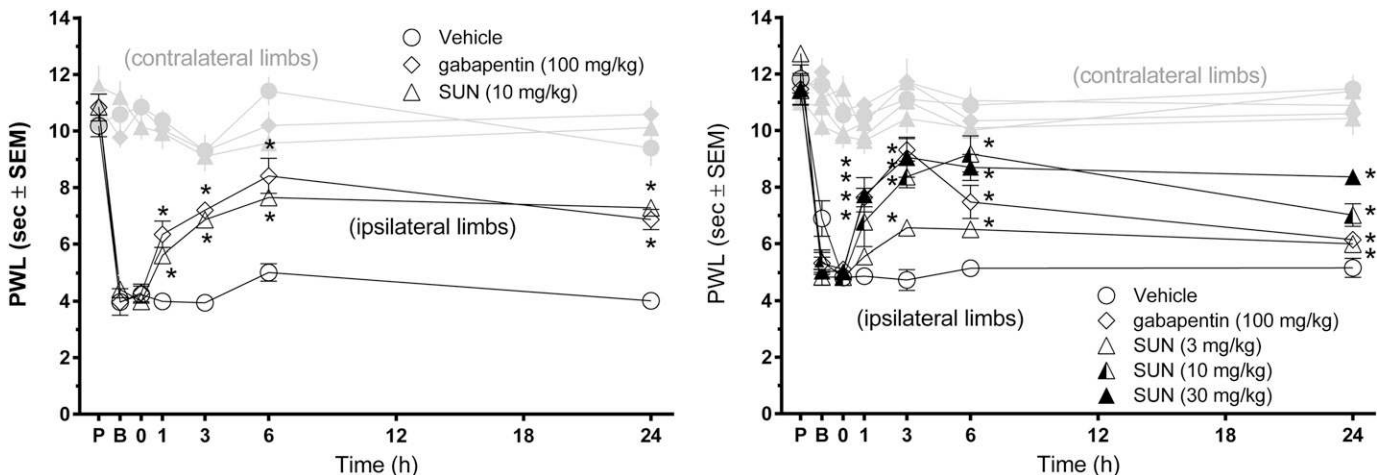


Fig. 2. Effect of a single oral administration of vehicle, gabapentin, and SUN on thermal hyperalgesia in CCI model rats. Paw withdrawal latency (PWL) determined on the ipsilateral limbs (black) and contralateral limbs (gray). Panels from two independent experiments: single dose level (left panel) and a dose-response (right panel). P, presurgery baseline. B, day 7 postsurgery. 0, Predosing control on day of compound administration (13 to 17 days after surgery). Vehicle, isotonic 50 mM phosphate buffer PO; gabapentin: 100 mg/kg PO; SUN: 3, 10, and 30 mg/kg PO, $n = 9$ in each group. * $P < 0.05$, compared with the same time point in the vehicle group, two-way repeated-measures analysis of variance (RMANOVA) followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

pain models, the effects on PWL after the 10 and 30 mg/kg dose levels were maximal. These antihyperalgesic effects were specific to the inflamed limbs, because PWL values in the contralateral limbs were unaffected by any treatments (Fig. 3).

D-Serine Pharmacodynamics in Rats. To correlate the pharmacodynamics of central DAAO inhibition with the effects observed on these pain-related behaviors, D-serine concentrations were determined in the cerebellum and plasma of separate groups of naive rats treated with SUN at 3, 10, and 30 mg/kg PO (Fig. 4). Consistent with a high level of DAAO activity in this brain area, D-serine concentrations in vehicle-treated and naive rats were relatively low and stable at 4.97 ± 1.2 nmol/g cerebellum ($N = 88$; horizontal line in Fig. 4). Consistent with inhibition of central DAAO, administration of 3 mg/kg SUN resulted in a transient increase in D-serine concentrations that lasted for at least 6 hours and returned to baseline levels by 24 hours (Fig. 4). SUN doses of 10 and 30 mg/kg produced a more rapid rate of increase in D-serine concentrations, such that levels of four times baseline were achieved within 6 hours (20 nmol/g cerebellum and 8 nmol/ml plasma). Similar to the observations in pain-related behaviors, the 10 and 30 mg/kg doses did not further increase D-serine concentrations, at least for the 6 hours of measurements in Fig. 4. However, the 24-hour time point remained elevated after administration of the higher dose (30 mg/kg; Fig. 4). The pharmacodynamics of cerebellar D-serine concentrations was mimicked by the D-serine concentrations in plasma (Fig. 4) for each dose of SUN.

To investigate if the effects on neuropathic pain-related behaviors resulted from increased D-serine concentrations, irrespective of DAAO activity, we administered D-serine to SNL model rats (Fig. 5) at 30, 100, and 300 mg/kg i.p. in the absence of inhibitor and saw no significant effects on tactile allodynia. In a separate group of rats, it was confirmed that exogenous administration of these intraperitoneal doses of D-serine resulted in cerebellar and plasma D-serine concentrations that overlapped those associated with oral SUN

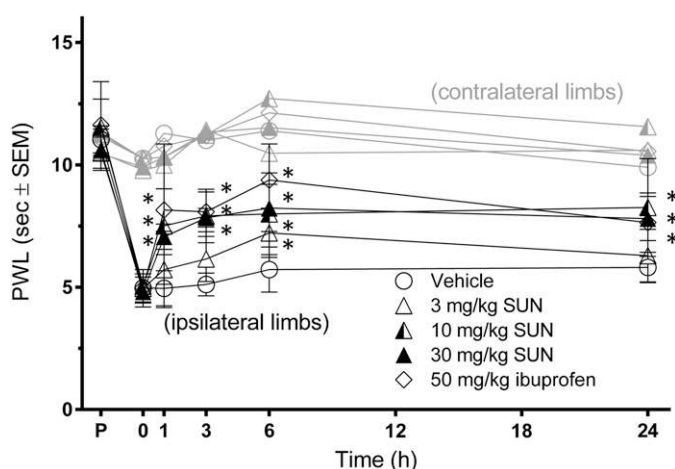


Fig. 3. Effect of a single oral administration of vehicle, ibuprofen, and SUN on thermal hyperalgesia in CFA model rats. Paw withdrawal latency (PWL) determined on the ipsilateral limbs (black) and contralateral limbs (gray). P, naive rats pre-CFA. 0, predosing control on day of compound administration. Vehicle, isotonic 50 mM phosphate buffer PO; ibuprofen: 50 mg/kg PO; SUN, 3, 10, and 30 mg/kg PO, $n = 8$ in each group. * $P < 0.05$, compared with the same time point in the vehicle group, two-way repeated-measures analysis of variance (RMANOVA) followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

administration, but peak concentrations were observed at an earlier time point (1-hour post dose) and decreased over time (unpublished data).

Electrophysiological Recordings from Dorsal Horn Neurons in Anesthetized Rats. Dorsal horn neuronal activity was recorded in anesthetized control and SNL model rats to see if the efficacy of SUN in behavioral tests included a spinal site of action. Compounds were administered either orally, at 6 to 9 hours prior to electrophysiological measurements to correspond with the peak efficacy observed in the behavioral tests, or intravenously at the time of measurements to examine the onset of action. Spontaneous neuronal activity was recorded in addition to that evoked by mechanical stimuli such as brush, squeeze, and 3 levels of von Frey Hair (VFH) stimulation.

Table 1 summarizes the effects of 10 mg/kg SUN on dorsal horn neuronal activity at a time period after oral administration that corresponds to the peak efficacy observed in behavioral experiments. In control rats that did not undergo SNL surgeries, spontaneous activity was low in both vehicle and SUN treatment groups. SUN treatment did not appear to alter activity evoked by brush, squeeze, or the lowest VFH stimulus (1.15g). SUN modestly but significantly reduced the neuronal activity evoked by the higher VFH stimuli (4.0g, 15.6g; $P < 0.05$).

Spontaneous activity of dorsal horn neurons was elevated in SNL animals (243 ± 50 impulses/min) compared with control (8 ± 4 impulses/min; Table 1), indicative of ongoing activity as a result of nerve injury. SUN treatment, like gabapentin, reduced this spontaneous activity almost to control levels. For mechanically evoked responses, the effects of SUN treatments in reducing neuronal activity were comparable with gabapentin treatment. Overall the effects of SUN and gabapentin were comparable in SNL rats.

After the dorsal roots of SNL rats were sectioned (Table 1) in an attempt to remove the contribution of peripheral afferent inputs to these dorsal horn recordings, the spontaneous activity of vehicle-treated animals was still elevated (209 ± 45 impulses/min) and similar to the intact SNL model rats. Here, SUN treatment still reduced spontaneous activity (91 ± 29 impulses/min), although not to the low levels observed with SUN treatment of animals with dorsal roots intact, suggesting the possibility that full efficacy of SUN treatment required intact peripheral inputs to the dorsal horn.

Activity of dorsal horn neurons in SNL rats was also recorded after spinalization (Table 1) in an attempt to remove modulation by higher level brain activity. Spontaneous activity in these rats was elevated over intact SNL animals (437 ± 62 impulses/min). SUN treatment still reduced this spontaneous activity (189 ± 34), although not to the baseline levels seen with intact animals. SUN treatments also achieved modest reductions in the mechanically evoked responses to squeeze and the higher force von Frey Hair pressures (Table 1). The effects of SUN on brush and VFH 1.15g were not statistically different from vehicle ($P > 0.05$). Overall, these results in spinalized rats suggest that the efficacy of SUN is still apparent at the level of the dorsal horn neurons, even without the contribution of higher brain centers.

Since the effects summarized in Table 1 were observed 6–9 hours after oral administration, we next sought to follow their onset using acute intravenous administration. Figure 6 shows spontaneous and mechanically evoked activity of dorsal horn

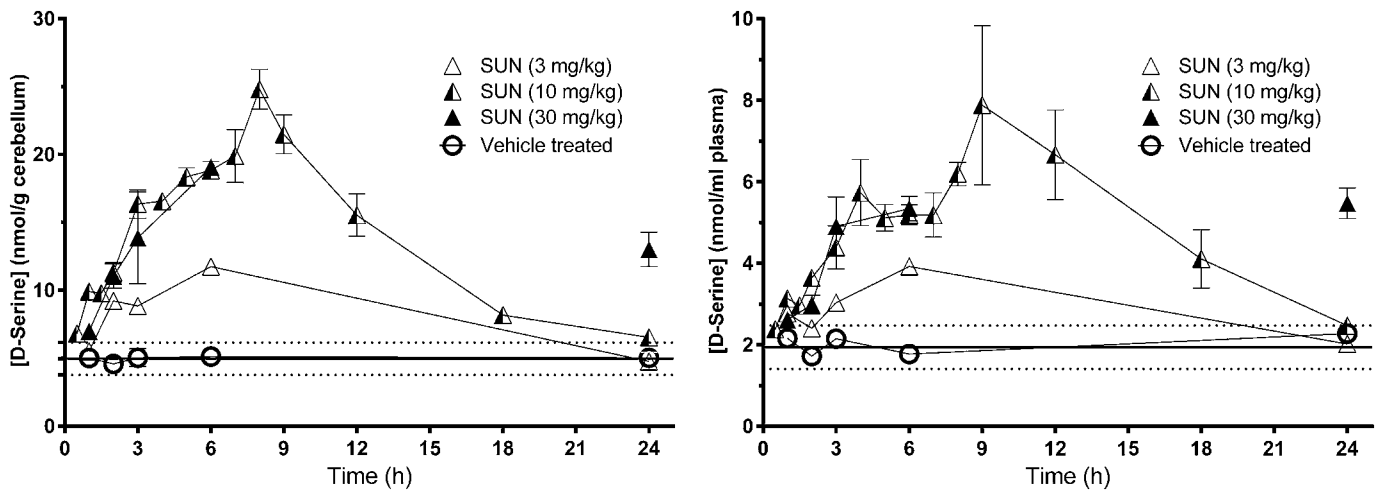


Fig. 4. Dose-response and time course of SUN on D-serine concentrations (mean \pm S.E.M.) in cerebellum (left panel) and plasma (right panel) of corresponding rats. SUN, 3, 10, 30 mg/kg PO. Vehicle solid line is mean D-serine concentration \pm 1 S.D. calculated from the $N = 88$ rats represented at the time points indicated with open circles.

neurons from SNL rats monitored before and after SUN (10 mg/kg i.v.) or gabapentin (100 mg/kg i.v.) administration. Both SUN and gabapentin induced a rapid reduction in the spontaneous activity, with peak effects apparent by 30 minutes post-intravenous administration (Fig. 6A). The magnitude of this effect, evident by the large reduction in spontaneous activity, was consistent with the efficacy observed 6–9 hours after oral administration, indicating that SUN effects on reducing spontaneous activity were prompt and long lasting. However, the efficacy of SUN was completely lost in animals that had their dorsal roots sectioned (Fig. 6A), which was consistent with what was observed for the dorsal root-sectioned animals reported in Table 1 and provides further support to the notion that SUN efficacy requires intact afferent inputs to the dorsal horn. On the other hand, spinalized animals retained the full effects of SUN treatment (Fig. 6A), further supporting the notion that higher level brain activity is not a requirement for SUN efficacy.

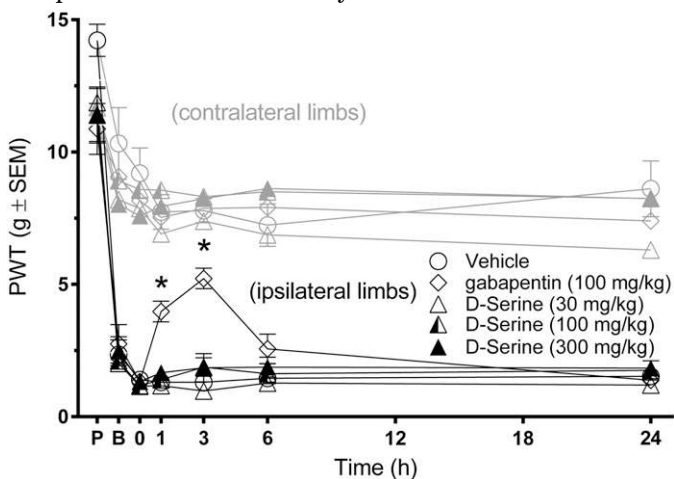


Fig. 5. D-Serine administration (i.p.) is inactive on PWT in ipsilateral limbs of SNL model rats. P, presurgery baseline. B, day 7 postsurgery. 0, Predosing control on day of compound administration. Vehicle, isotonic 50 mM phosphate buffer PO; gabapentin, 100 mg/kg PO; D-serine, 30, 100, 300 mg/kg i.p. * $P < 0.05$, compared with the same time point in the vehicle group, two-way repeated-measures analysis of variance (RMANOVA) followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

The effects of acute administrations of SUN on mechanically evoked dorsal horn activity were also apparent within 30 minutes of intravenous administration (Fig. 6, B, C, and D). As observed with the animals predosed orally, the effects of SUN were comparable to those observed after gabapentin administration. In the case of mechanically evoked activity, spinalization did not appear to have any influence. (Mechanical stimuli did not evoke dorsal horn neuronal activity under these conditions of sectioned dorsal roots.)

Electrophysiological Recordings from (Peripheral) Dorsal Root Filaments in Anesthetized Rats. Since the efficacy of SUN appeared to require intact dorsal roots, we next examined the effects of SUN treatment on the firing activity of dorsal root filaments peripheral to the spinal cord. In recordings from dorsal root filaments of SNL rats, the frequency of spontaneous activity varied considerably from filament to filament. Those units with no obvious peripheral receptive field or those that only responded to the passive movement of the ankle (flexion and extension, twisting) were excluded. However, those filaments that did not display any spontaneous activity but still responded to touch or von Frey hair stimulation at the receptive field on the hind paw surface were included. Two typical units, representing spontaneously active and silent filaments, respectively, are shown in Fig. 7. Table 2 summarizes these peripheral recordings. Spontaneous firing in SNL dorsal root filaments was elevated (1121 ± 107 impulses/min; Table 2) and reduced significantly (351 ± 72 impulses/min) by oral pretreatment with SUN. Oral pretreatment with SUN also significantly reduced mechanically evoked firing activity of dorsal roots at each level of von Frey Hair pressure (Table 2). These results indicate that the effects of SUN treatment reduced peripheral afferent input to spinal cord neurons.

As with the recordings in the spinal cord, we next sought to follow the onset of action for these peripheral effects using acute intravenous administration of SUN and gabapentin. The effects of SUN and gabapentin on spontaneous firing of dorsal root filaments are shown in Fig. 8A. In contrast to the large and prompt effects of acute intravenous treatments on spontaneous activity of dorsal horn neurons (Fig. 6A), SUN and gabapentin produced smaller and slower decreases in the

TABLE 1

Effects on electrophysiological recordings of dorsal horn neurons in SNL model rats 6 to 9 hours after oral administration of SUN (10 mg/kg), gabapentin (100 mg/kg), or vehicle

Neuronal activity as mean \pm S.E.M.; spontaneous activity in impulses/min, mechanically evoked responses in impulses/10 trials.

Rat Model	Treatment ^a	Spontaneous	Brush	Squeeze	von Frey Hair		
					1.15	4.0	15.6
<i>g pressure</i>							
Control ^b	Vehicle	8 ± 4	170 ± 22	532 ± 38	92 ± 12	245 ± 27	659 ± 53
	SUN	24 ± 14	137 ± 16	453 ± 36	83 ± 10	175 ± 19*	500 ± 46*
SNL ^c	Vehicle	243 ± 50	242 ± 22	673 ± 52	184 ± 25	368 ± 44	750 ± 71
	SUN	20 ± 6**	130 ± 18**	381 ± 38**	76 ± 14**	156 ± 25**	373 ± 40**
	GBP	33 ± 15**	125 ± 15**	306 ± 25**	62 ± 10**	123 ± 16**	299 ± 36**
DR sectioned ^d	Vehicle	209 ± 45					
	SUN	91 ± 29*					
Spinalized ^e	Vehicle	437 ± 62	179 ± 17	403 ± 25	—	273 ± 32	552 ± 50
	SUN	189 ± 34**	161 ± 18	314 ± 22*	108 ± 15	193 ± 21*	429 ± 36*

DR, dorsal root; GBP, gabapentin; HTM, high-threshold mechanoreceptive neurons.

^a All treatments PO, 6–9 hours prior to recordings.

^b A total of 49 neurons was recorded [40 WDR (wide dynamic range neurons) and 9 HTM] from 6 control (non-SNL) rats dosed with vehicle; 51 neurons (38 WDR and 13 HTM) from 6 control rats dosed with SUN.

^c 52 neurons (48 WDR and 4 HTM) recorded from 6 SNL model rats dosed with vehicle; 50 neurons (35 WDR and 15 HTM) from 6 SNL model rats dosed with SUN; 52 neurons (48 WDR and 4 HTM) from 6 SNL model rats dosed with gabapentin.

^d L4–L6 dorsal roots (DR) were sectioned 1–2 hours after dosing in 3 SNL model rats dosed with vehicle; 30 neurons recorded, and 4 SNL model rats dosed with SUN; 30 neurons; recordings with apparent muscle spindles were excluded.

^e Spinalization at the C2–C3 level in 6 SNL model rats for each treatment group; vehicle: 50 neurons (40 WDR and 10 HTM) and SUN: 49 neurons (42 WDR and 7 HTM).

* $P < 0.05$; ** $P < 0.001$, non-paired Student's t test versus vehicle treatment.

spontaneous firing activity in dorsal root filaments (Fig. 8A). Gabapentin produced no detectable effects on the responses of dorsal root filaments to mechanical stimulation by von Frey Hairs (Fig. 8B). In contrast, there was a small, but statistically significant, effect of acute intravenous SUN administration on mechanically evoked responses (Fig. 8B) that reached a peak effect after 60 minutes.

Discussion

Here we correlated the pharmacodynamics of DAAO inhibition with efficacies in rat models of neuropathic and inflammatory pain. The effects on pain behavior corresponded to a reduction in electrophysiological activity in spinal cord dorsal horn neurons and peripheral afferent inputs.

Under the conditions used in these studies, SUN demonstrated reliable and repeatable effects on mechanical allodynia in the SNL model and on thermal hyperalgesia in the CCI and CFA models. SUN inhibited DAAO activity in vivo, as evidenced by the resulting increases in D-serine concentrations. The effects of SUN at 10 mg/kg PO, in SNL and CCI models were repeated in the course of separate independent experiments and were comparable in magnitude to gabapentin positive control conditions. These effects of SUN on pain-related behaviors displayed a pharmacodynamic relationship to the increases in cerebellar and plasma D-serine concentrations in the following ways: 1) the effects on behavior and D-serine levels both increased to a maximum over 6–9 hours post-oral administration of the 10 mg/kg dose; 2) the initial rates of increase of D-serine concentrations saturated between the 10 and 30 mg/kg doses, just as the effects on pain-related behaviors saturated in this dose range; 3) the 3 mg/kg dose engendered less-than-maximal effects on both behavior and rates of increase in D-serine levels; and 4) longer-lasting effects were observed (24 hours) for the 30 mg/kg dose for both D-serine increases and behavior.

D-Serine increased both centrally (cerebellum tissue) and peripherally (plasma) after SUN administration, suggesting

that DAAO inhibition was occurring across the blood-brain barrier and, therefore, likely also at the spinal cord level. Since DAAO has been reported to be expressed highly in the cerebellum (Wang and Zhu, 2003), this brain region exhibits lower baseline levels of D-serine (Hashimoto et al., 1993; Wang and Zhu, 2003). This inverse relationship between local tissue D-serine concentrations and tissue DAAO activity has been demonstrated ontologically and phylogenetically (Schell, 2004). Increases in cerebellum D-serine concentrations have also been reported after cerebellar DAAO activity was inhibited by RNAi (Burnet et al., 2011). Therefore, cerebellum tissue has been used as an indicator of central DAAO inhibition (Sparey et al., 2008; Duplantier et al., 2009; Strick et al., 2011). DAAO also has been reported to be expressed in the spinal cord of rats (Kappor and Kapoor, 1997), with localization including the dorsal horn. From these relationships, and an additional assumption that maximal rates of increase in D-serine concentrations correspond to 100% inhibition of DAAO, maximal efficacy on neuropathic and inflammatory pain-related behaviors required near full inhibition of DAAO activity.

In addition to pain-related behaviors, DAAO inhibition also affected neuronal activity of spinal cord dorsal horn and peripheral afferents in SNL model animals. Ligation of the spinal nerve elicited a large increase in spontaneous activity of dorsal horn neurons compared with the activity of control animals. After oral administration of SUN at 10 mg/kg 6–9 hours prior to recordings, dorsal horn spontaneous and mechanically evoked neuronal activity were reduced significantly compared with vehicle treatment. Such a response may reflect a reduction in the transmission of ongoing, spontaneous nerve-related pain signals to higher order brain centers. Indeed, gabapentin, a drug with clinical efficacy against neuropathic pain, also significantly reduced dorsal horn spontaneous and mechanically evoked activity. However, SUN efficacy appeared to require an intact connection between spinal cord dorsal horn and dorsal root ganglion neurons, since severing the dorsal roots diminished the effects

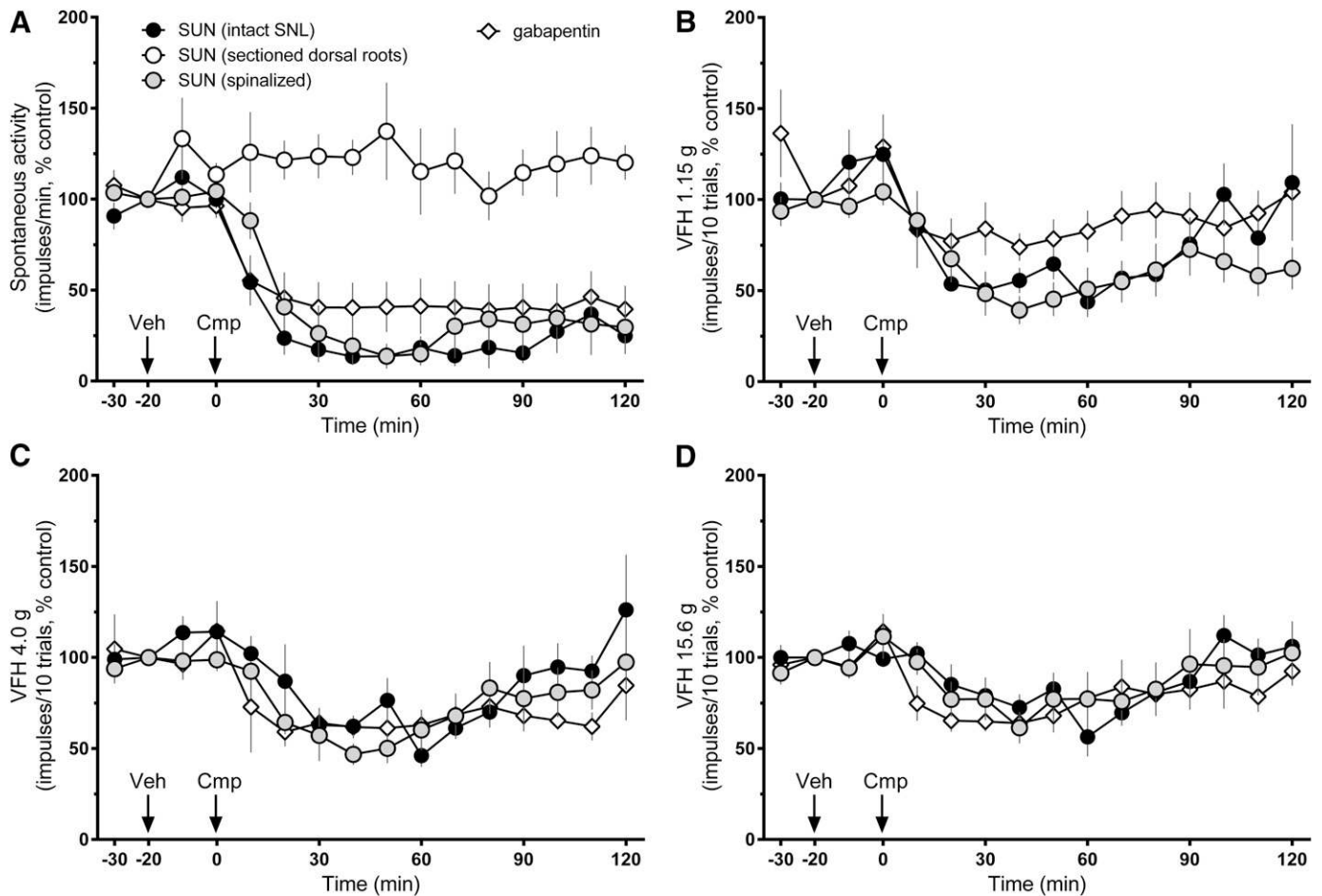


Fig. 6. Effects of intravenous vehicle, SUN, and gabapentin administration on spontaneous activity (A) and mechanically evoked responses (B, C, and D) of wide dynamic range neurons (WDR) neurons in the dorsal horn in SNL model rats. The data are expressed as a percentage of the baseline response (prior to vehicle administration) \pm S.E.M. Levels of activity after vehicle administration (Veh) were recorded 20 minutes prior to compound administration (Cmp). Data collected from seven neurons recorded from seven different rats for each treatment group.

of SUN on dorsal horn spontaneous activity. In fact, in SNL animals with sectioned dorsal roots, the dramatic effect of acute intravenous administration of SUN on spontaneous activity in dorsal horn neurons was abolished. The peripheral effects of SUN also were evaluated by recording the activity of isolated dorsal root filaments. After oral administration of

SUN at 10 mg/kg 6–9 hours prior to recordings, spontaneous and mechanically evoked activity were reduced significantly compared with vehicle treatment. After intravenous administration, SUN yielded relatively modest and slow inhibitory effects on spontaneous and mechanically evoked activity of dorsal root filaments. In comparison, intravenous administration

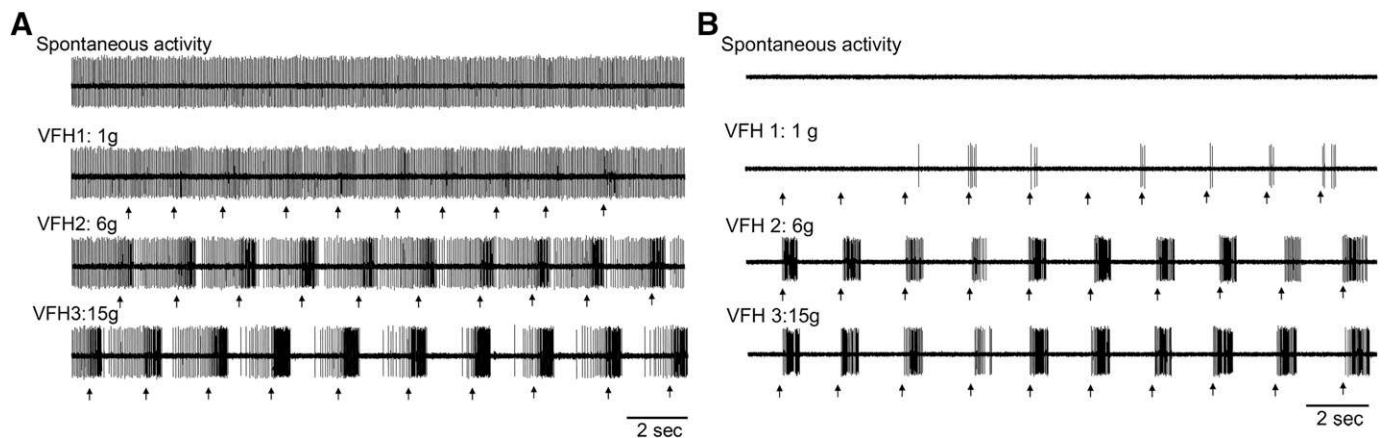


Fig. 7. Example traces from dorsal root filaments that demonstrated spontaneous activity (A) and no spontaneous activity (B) along with the corresponding mechanically evoked responses to challenges with von Frey hairs. Arrows indicate the application of von Frey hairs. VFH1, VFH2, and VFH3: von Frey hairs 1 (1g), 2 (6g), and 3 (15g).

TABLE 2

Effects of SUN on electrophysiological recordings of dorsal root filaments in SNL rats

Firing activity mean \pm S.E.M.; spontaneous activity, impulses/min, mechanically-evoked responses in impulses/10 trials.

Treatment ^a	Spontaneous	von Frey Hair		
		1	6	15
		<i>g pressure</i>		
Vehicle ^b	1121 \pm 107	62 \pm 8	239 \pm 23	345 \pm 21
SUN ^c	351 \pm 72**	30 \pm 7.2**	140 \pm 17**	221 \pm 21**

^a All treatments PO, 6–9 hours prior to recordings.

^b A total of 88 filaments was recorded from a total of 12 SNL rats (predosed with vehicle PO).

^c 122 filaments from 9 SNL rats (predosed with SUN, 10 mg/kg PO).

** $P < 0.001$, compared with vehicle, unpaired Student's *t* test.

of gabapentin had effects similar to SUN on spontaneous activity but no detectable effects on mechanically evoked activity of dorsal root filaments. The contrasting effects of SUN observed during central dorsal horn recordings (rapid and full effects) and peripheral dorsal root filament recordings (slow and partial effects) suggest that DAAO inhibition, under conditions of nerve injury, requires local circuit-level components that span the peripheral nerves synapsing at the level of spinal cord dorsal horn neurons for full effects. Given the involvement of glial cells in the regulation of D-serine concentrations as well as in the development and maintenance of neuropathic pain, neuronal components might combine with astrocytic, microglial, or even inhibitory circuits in a way that D-serine and DAAO activity contribute to pain circuits under conditions of nerve injury. As reported with formalin-induced pain (Lu et al., 2012), conditions of nerve injury may even include changes in local concentrations of reactive oxygen species, such as hydrogen peroxide, a product of DAAO activity itself. Thus, the mechanism of DAAO inhibition in neuropathic pain models may include reduction of local spinal concentrations of reactive oxygen species.

The molecular mechanism of DAAO inhibition in neuropathic pain models may relate to the activation of NMDA

receptors via endogenously produced and released D-serine. Although an initial study reporting enhanced formalin-induced tonic pain-related behavior in DAAO mutant mice suggested that the activity of DAAO may contribute to the transmission of ongoing pain via central sensitization and D-serine (Wake et al., 2001), further investigations (Zhao, et al. 2008, 2010; Gong et al., 2011) have demonstrated efficacy of DAAO inhibitors in models of tonic and chronic pain. The latter may seem incongruous with the efficacy of NMDA receptor antagonists in pain models (Fisher et al., 2000) and the role of NMDA receptor activation in central sensitization (Bennett, 2000). Although D-serine occupancy of the glycine site on NR1 subunits serves to increase NMDA receptor activation, this increase would occur only in neuronal regions where this site is not already saturated by glycine or D-serine (Mothet et al., 2000; Panatier et al., 2006; Heffernan et al., 2009; Oliet and Mothet, 2009; Papouin et al., 2012). Regardless of the mechanism of action for SUN in the neuropathic pain models, the efficacy was not recapitulated by exogenously administered D-serine. Like with other neurotransmitter systems, simply increasing D-serine levels by exogenous administration, in the absence of DAAO inhibition, was not sufficient to replicate the effects on pain-related behavior, despite the resulting elevation in D-serine concentrations in central and peripheral tissues. In fact, reports with intrathecal administration would suggest pronociceptive effects of spinal D-serine (Kolhekar et al., 1994; Guo et al., 2006). This may indicate that the spatiotemporal reach of exogenously applied D-serine is limited by DAAO activity itself, and only by inhibiting DAAO will endogenous D-serine reach NR1 subunits. Under the conditions of nerve injury, Zhao et al. (2010) reported marked increases in spinal DAAO expression and enzyme activity consistent with the onset of mechanical allodynia in SNL model rats.

Alternatively, SUN may have been acting via an unrecognized molecular target, although SUN was inactive when tested at 30 μ M against a panel of over 173 enzyme and receptor targets (unpublished data), including pain-related targets. The possibility of a non-DAAO mechanism is also disfavored when considering the structural diversity of DAAO inhibitors with established efficacy in animal models of neuropathic pain, as we observed over the course of a drug

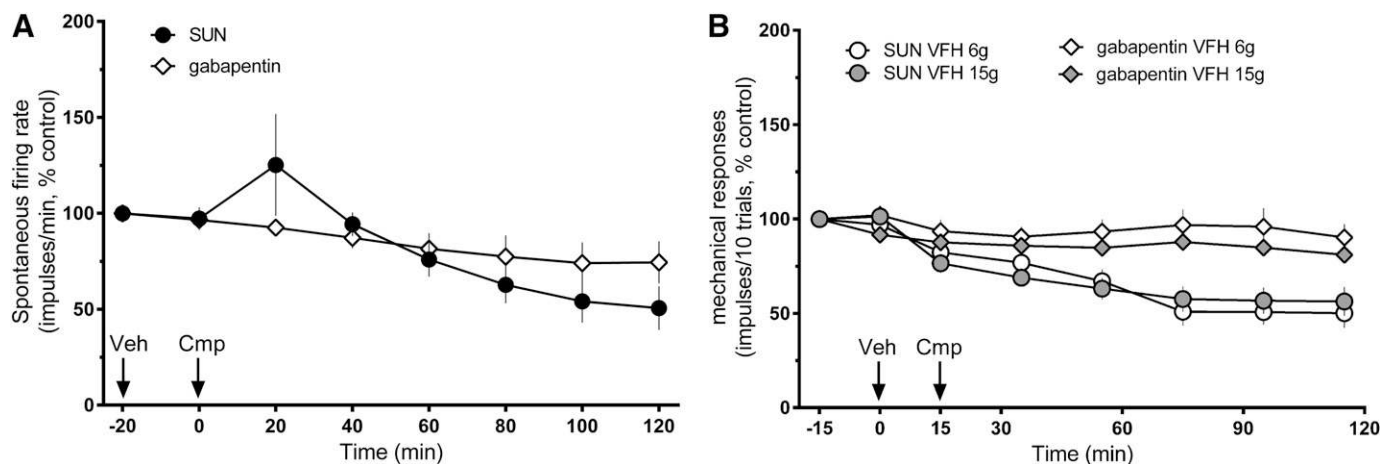


Fig. 8. Spontaneous firing rates derived from electrophysiological recordings of dorsal root filaments. (A) Gabapentin intravenous treatment: recordings from eight dorsal root filaments of SNL rats, the baseline (mean \pm S.E.M.) spontaneous firing rate before vehicle injections was 1540 \pm 157 impulses/min; mechanically evoked responses were 315 \pm 36 (VFH 6g) and 381 \pm 22 (VFH 15g) impulses/10 trials, before vehicle injections. (B) SUN intravenous treatment: recordings from 11 dorsal root filaments of SNL rats with baseline spontaneous firing at 1283 \pm 296 impulses/min; mechanically evoked baselines were 284 \pm 37 (VFH 6g) and 379 \pm 49 (VFH 15g) impulses/10 trials.

discovery program (Fang et al., 2007, 2011; Dorsey et al., 2008; Heffernan et al., 2009). In similar work, Gong et al. (2011) described the close correlation between in vitro DAAO inhibitory potencies and intrathecal doses required for efficacy of structurally diverse DAAO inhibitors on formalin-induced tonic pain consistent with a downregulation of spinal DAAO blocked formalin-induced tonic pain (Chen et al., 2012). An alternative to the NMDA receptor mechanism, still consistent with DAAO inhibition, is the possibility that D-serine itself might be acting via a non-NMDA receptor target. Consistent with this idea, D-serine was recognized to have effects on another glutamate receptor, iGluRdelta (Naur et al., 2007). In this possibility, the mechanism of action for SUN would again be primarily to increase D-serine concentrations proximal to its final molecular target.

Further work is required to determine the contribution of NMDA receptors to the mechanism of action of this DAAO inhibitor in neuropathic and inflammatory pain models.

Conclusions

Overall, these results demonstrated a reliable and robust effect of the DAAO inhibitor SUN on neuropathic and inflammatory pain-related behaviors. This efficacy was likely linked to the effects of SUN on ongoing (spontaneous) and (mechanically) evoked neuronal activity at the spinal cord level. These effects appeared to require intact circuits spanning dorsal root ganglion and spinal cord neurons for their full expression. Low clinical response rates with the established treatments motivate the discovery and clinical development of therapeutic agents with novel mechanisms of action to treat ongoing pain and human suffering related to nerve injury. These results suggest that DAAO inhibitors may be useful in treating pain states induced by nerve injury and warrant further investigation of DAAO inhibitors for the treatment of neuropathic pain in humans.

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Authorship Contributions

Participated in research design: Hopkins, Zhao, Bowen, Heffernan, Spanwick, Varney, Large.

Conducted experiments: Zhao, Fang, Wei, Bowen.

Contributed new reagents or analytic tools: Heffernan, Large, Spear.

Performed data analysis: Bowen, Zhao, Fang, Wei, Hopkins.

Wrote or contributed to the writing of the manuscript: Hopkins, Zhao, Bowen, Spanwick.

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