JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #204016
TITLE PAGE

PHARMACODYNAMIC EFFECTS OF A D-AMINO ACID OXIDASE INHIBITOR INDICATE A SPINAL SITE OF ACTION IN RAT MODELS OF NEUROPATHIC PAIN

Seth C. Hopkins, Fei-Yue Zhao, Carrie A. Bowen, Xin Fang, Haifeng Wei, Michele L. Heffernan, Kerry L. Spear, David C. Spanswick, Mark A. Varney, Thomas H. Large

Sunovion Pharmaceuticals Inc, Marlborough, Massachusetts (SCH, CAB, MLH, KLS, MAV, THL)
Neurosolutions Ltd., Coventry, UK (FYZ, XF, HW, DCS)
Department of Physiology, Monash University, Clayton, Victoria, Australia (DCS)
Warwick Medical School, University of Warwick, Coventry, UK (DCS)

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #204016
RUNNING TITLE PAGE

Running Title: DAAO inhibitor neuropathic pain

Corresponding author: Seth C. Hopkins, Sunovion Pharmaceuticals Inc, 84 Waterford Dr., Marlborough, MA 01752
tel: 508-357-7706
fax: 508-490-5454
email: seth.hopkins@sunovion.com

Text – 19 pages
Tables – 2 tables
Figures – 8 figures
References – 30 references
Abstract – 227 words
Introduction – 385 words
Discussion – 1,387 words

Abbreviations: D-amino acid oxidase (DAAO); chronic constriction injury (CCI); spinal nerve ligation (SNL); Complete Freund's Adjuvant (CFA); 4H-Furo[3,2-b]pyrrole-5-carboxylic acid (SUN); N-methyl-D-aspartate (NMDA); pharmacokinetic-pharmacodynamic (PK/PD); paw withdrawal threshold (PWT); wide dynamic range neurons (WDR); high-threshold mechanoceptive neurons (HTM)

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

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 4*H*-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 (CCI). In addition, SUN was efficacious against Complete Freund's Adjuvant (CFA)-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 to 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. Following i.v. 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 for schizophrenia.

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

Neuropathic pain that arises following 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 which 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 (Dorsey, et al., 2008;Fang, et al., 2005;Fang, et al., 2011;Heffernan, et al., 2009). These observations, although counterintuitive to an understanding of NMDA receptor antagonists' efficacy in models of pain, have also been described using sodium benzoate in neuropathic models (Zhao, et al., 2010). In addition, others (Gong, et al., 2011;Zhao, et al., 2010;Chen, et al., 2012;Gong, et al., 2012) reported on the effects of DAAO inhibition in a formalin model of tonic pain.

Here, we use the DAAO inhibitor SUN to describe the efficacy observed in 3 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.

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 PK/PD, behavioral and electrophysiological studies. All procedures in this study were undertaken in compliance with the UK Animals (Scientific Procedures) Act 1986.

PK/PD Studies

Previous studies demonstrated low endogenous D-serine concentrations in the cerebellum, compared to 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 sacrificed 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 sacrifice, trunk whole blood and cerebellum were collected, processed and analyzed for D-serine concentrations. Whole blood samples were collected in tubes containing potassium EDTA. These tubes were centrifuged and the plasma was decanted. Plasma and cerebellum samples were stored at -80°C until analyzed by LC/MS/MS for D-serine concentrations.

Spinal Nerve Ligation Model:

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

Rats underwent spinal nerve ligation surgery, as described by (Kim and Chung, 1992). Each rat was anaesthetized with 5% isoflurane mixed with oxygen (2 L per min) followed by an intraperitoneal (IP) 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, 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 min 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 PWT ≤ 3.5 g, were selected for compound-dosing experiments.

Rats that demonstrated significant mechanical allodynia after spinal nerve ligation were randomly divided into 5 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 8 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 following test article administration. The animals were returned to their home cages for a break (about 30 min) 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 min. 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 and re-assessed on the 7th day after surgery and on the 13th to 17th days, before compound dosing.

The chronic constriction injury (CCI) model was prepared following the standard protocols described previously (Bennett and Xie, 1988). Briefly, rats were anaesthetised with a 5% isofluorane/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 (s or sec) from the start of heat stimulation to the time the animal withdrew its hind limb. In the absence of a response, a cut-off latency of 20 sec was set to prevent tissue damage. The average PWL of 3 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 sec versus about 10.0 sec baseline) were selected for compound-dosing experiments.

Complete Freund's Adjuvant Model:

Rats were allowed to acclimatize to the experiment's environment for three 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 re-assessed 24 hours later, just before compound administration.

The animals were briefly anaesthetized with 5% isoflurane mixed with 95% oxygen.

CFA (0.05 ml; Sigma, St. Louis, USA) 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 hr after CFA injection. PWL was assessed at 1, 3, 6 and 24 hr following 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 PWL (i.e., ≤ 6.0 sec, versus a baseline of approximately 10.0 sec), were selected for compound-dosing experiments.

Electrophysiology experiments:

After behavioral validation of the presence of SNL-induced tactile allodynia, rats were anaesthetized 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 physiological range via a thermo-blanket system. The electrocardiogram (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 (RF) 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 15.6 grams, respectively, and squeeze using a standard artery clip applied to the RF) were examined to classify the neuron. In this study, only wide dynamic range neurons (WDR), which responded robustly to varied mechanical stimuli and exhibited force-dependent responding (i.e., a greater stimulation force resulted in a higher frequency discharge), or high-threshold mechanoceptive neurons (HTM), 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 15 grams. Neural activity (spontaneous discharge and mechanically-evoked responses) was amplified and monitored using standard electrophysiological techniques and recorded to a PC using CED Spike 2 software (Cambridge Electronics Design).

Analysis:

Behavioral data were analyzed using 2-way repeated-measures analysis of variance (RMANOVA; SigmaStat Version 3.5, Systat Software, Inc, Chicago, USA) 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 ± 1 SEM. 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 p.o.), and the second with a dose-response, each study having negative (vehicle) and postive control (gabapentin) treament groups.

In naive rats before surgery, the mean PWT was between 10 and 13 g (Figure 1). On day 7 after SNL, the mean PWT on the side ipsilateral to the ligated nerve was substantially lower (between 1 and 3 g, Figure 1), indicating that mechanical allodynia had developed. This allodynia was specific to the ipsilateral side, since the contralateral limbs continued to display more elevated PWT values between 7 and 10 g (grey symbols in Figure 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 14 days after SNL. As a negative control, vehicle administration had no effect on the PWT over the 24 hours of testing (Figure 1). As a positive control, gabapentin, administered at 100 mg/kg p.o., 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 (left and right panels,

Figure 1), and appeared to occur around 6 hours. The DAAO inhibitor SUN, administered at 10 mg/kg p.o., induced an increase in PWT of ipsilateral limbs, with effects increasing for 6 hours, and lasting over the 24 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 (right panel, Figure 1). Effects on PWT following 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, as 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, as there were no significant treatment-related effects observed in the contralateral limbs, for any treatment group of either experiment (grey symbols in Figure 1).

Effects of oral administration of SUN to CCI model rats

These effects of SUN on mechanical allodynia in the SNL model were compared to its effects on thermal hyperalgesia in the CCI model of neuropathic pain. As with the SNL model, 2 independent tests of SUN efficacy were performed.

In naive rats before surgery, the mean PWL was between 10 and 12 seconds (Figure 2). On day 7 after surgery, the mean PWL on the side ipsilateral to the injured nerve was substantially lower (between 3 and 6 seconds, Figure 2), indicating that thermal hyperalgesia had developed. This hyperalgesia was specific to the ipsilateral side, since the contralateral limbs continued to display more elevated PWL values between 9 and 12 seconds (grey symbols in Figure 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 14 days after CCI. As a negative control, vehicle administration had no effect on the PWL over the 24 hours of testing (Figure 2). As a positive control, gabapentin, administered at 100 mg/kg p.o., increased PWL on the ipsilateral side, with effects evident at the first observation (1 hour). This efficacy was observed in both experiments (left and right panels, Figure 2), and remained for 24 hours. The DAAO inhibitor SUN, administered at 10 mg/kg p.o., induced an increase in PWL of ipsilateral limbs for 6 hours, and maintained over the 24-hour observation period. These observations at 10 mg/kg were confirmed in a follow-up dose-response study (right panel, Figure 2). Effects on PWL following the 3 mg/kg dose were significantly greater than vehicle treatment, although reduced in magnitude relative to those observed following the 10 and 30 mg/kg doses. As observed with the SNL model, the effects of 10 mg/kg appeared to be maximal, as 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, as there were no significant treatment-related effects observed in the contralateral limbs at any time point following any treatment during either experiment (grey symbols in Figure 2).

Effects of oral administration of SUN to CFA model rats

The effects of SUN in models of neuropathic pain were compared to its effects on thermal hyperalgesia in a rat model of CFA-induced inflammatory pain. In naive rats prior to the CFA injection, mean PWL values were between 9 and 12 seconds. Following CFA administration, mean PWL 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 PWL values, whereas the positive control compound ibuprofen (50 mg/kg p.o.) increased PWL significantly ($P<0.05$) relative to vehicle at all time-points observed (Figure 3, left panel). The lowest dose of SUN (3 mg/kg p.o.) did not significantly increase PWL relative to vehicle. Similar to the effects observed in the neuropathic pain models, the effects on PWL following the 10 and 30 mg/kg dose levels were maximal. These anti-hyperalgesic effects were specific to the inflamed limbs, as PWL values in the contralateral limbs were unaffected by any treatments (Figure 3, right panel).

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 naïve rats treated with SUN at 3, 10, and 30 mg/kg p.o. (Figure 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 Figure 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 (Figure 4). SUN doses of 10 and 30 mg/kg produced a more rapid rate of increase in D-serine concentrations, such that levels of 4 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 Figure 4. However, the 24 hour time point remained elevated following administration of the higher dose (30 mg/kg, Figure 4). The pharmacodynamics of cerebellar D-serine concentrations was mimicked by the D-serine concentrations in plasma (Figure 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 (Figure 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 i.p. doses of D-serine resulted in cerebellar and plasma D-serine concentrations that overlapped those associated with oral SUN administration, but peak concentrations were observed at an earlier time point (1 hour post dose) and decreased over time (data not shown).

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 p.o., at 6 to 9 hours prior to electrophysiological measurements to correspond with the peak efficacy observed in the behavioral tests, or i.v. 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.

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1 summarizes the effects of 10 mg/kg SUN on dorsal horn neuronal activity at a time period following 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 (4.0 g, 15.6 g) ($P<0.05$).

Spontaneous activity of dorsal horn neurons was elevated in SNL animals (243 ± 50 impulses/min) compared to 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 to gabapentin treatment. Overall the effects of SUN and gabapentin were comparable in SNL rats.

After sectioning the dorsal roots (DR sectioned, in Table 1) of SNL rats, 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 Chung animals. 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 Chung 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 following oral administration, we next sought to follow their onset using acute i.v. administration. Figure 6 shows spontaneous and mechanically-evoked activity of dorsal horn 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 i.v. administration (Figure 6A). The magnitude of this effect, evident by the large reduction in spontaneous activity was consistent with the efficacy observed 6 – 9 hours following 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 (Figure 6A), which was consistent with what was observed for the DR 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 (Figure 6A), further supporting the notion that higher level brain activity is not a requirement for SUN efficacy.

The effects of acute administrations of SUN on mechanically-evoked dorsal horn activity were also apparent within 30 minutes of i.v. administration (Figure 6B, C, D). As observed with the animals pre-dosed orally, the effects of SUN were comparable to those observed following 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 acivity 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 Figure 7. Table 2 summarizes these peripheral recordings. Spontanous 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 reduced significantly 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 i.v. administration of SUN and gabapentin. The effects of SUN and gabapentin on spontaneous firing of dorsal root filaments are shown in Figure 8A. In contrast to the large and prompt effects of acute i.v. treatments on spontaneous activity of dorsal horn neurons (Figure 6A), SUN and gabapentin produced

smaller and slower decreases in the spontaneous firing activity in dorsal root filaments (Figure 8A). Gabapentin produced no detectable effects on the responses of dorsal root filaments to mechanical stimulation by von Frey Hairs (Figure 8B). In contrast, there was a small, but statistically significant, effect of acute i.v. SUN administration on mechanically-evoked responses (Figure 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 with 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 p.o. 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) following 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 (Kappor and Kapoor, 1997;Wang and Zhu, 2003), this brain region exibits 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 (Duplantier, et al., 2009;Sparey, et al., 2008;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 to the activity of control animals. Following 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 to 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 againstneuropathic 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 of SUN on dorsal horn spontaneous activity. In fact, in SNL animals with sectioned dorsal roots, the dramatic effect of acute i.v. 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. Following oral administration of SUN at 10 mg/kg 6 – 9 hours prior to recordings, spontaneous and mechanically-evoked activity were reduced significantly, compared to vehicle treatment. Following i.v. administration, SUN yielded relatively modest and slow inhibitory effects on spontaneous and mechanically-evoked activity of dorsal root filaments. In comparison, i.v. administration 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) suggests 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 contributes 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 (Gong, et al., 2011;Zhao, et al., 2008;Zhao, et al., 2010) 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). Though 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;Oliet and Mothet, 2009;Panatier, et al., 2006;Heffernan, et al., 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, in spite of 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 (Guo, et al., 2006;Kolhekar, et al., 1994). 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. This spatiotemporal reach may be fundamentally altered under the conditions of nerve injury, as (Zhao, et al., 2010) reported marked increases in spinal DAAO expression and enzyme activity that was 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 μM against a panel of over 173 enzyme and receptor targets (Sunovion Pharmaceuticals, unpublished data), including pain-related targets. The possibility of a non-DAAO mechanism also is 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 discovery program (Dorsey, et al., 2008;Fang, et al., 2007;Fang, et al., 2011;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, and the down-regulation 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 SUN 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.

ACKNOWLEDGEMENTS

The authors performed this work as employees of their respective organizations, and otherwise have no conflict of interests to declare.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hopkins, Zhao, Bowen, Heffernan, Spanswick, 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, Spanswick.

REFRENCES

Reference List

Bennett GJ (2000) Update on the neurophysiology of pain transmission and modulation: focus on the NMDA-receptor. *J Pain Symptom Manage* **19**:S2-S6.

Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**:87-107.

Burnet PW, Anderson PN, Chen L, Nikiforova N, Harrison PJ and Wood MJ (2011) D-amino acid oxidase knockdown in the mouse cerebellum reduces NR2A mRNA. *Mol Cell Neurosci* **46**:167-175.

Chen XL, Li XY, Qian SB, Wang YC, Zhang PZ, Zhou XJ and Wang YX (2012) Down-regulation of spinal D-amino acid oxidase expression blocks formalin-induced tonic pain. *Biochem Biophys Res Commun* **421**:501-507.

Dorsey J, Hopkins S, Foglesong R, heffernan M, Jones M, Perales J, Fang Q, Ogbu C, Jones S, Soukri M, Varney M and Spear K. FUSED HETEROCYCLIC INHIBITORS OF D-AMINO ACID OXIDASE. (WO2008/005456 A2). 1-10-2008. 7-2-2007.
Ref Type: Patent

Duplantier AJ, Becker SL, Bohanon MJ, Borzilleri KA, Chrunyk BA, Downs JT, Hu LY, El-Kattan A, James LC, Liu S, Lu J, Maklad N, Mansour MN, Mente S, Piotrowski MA, Sakya SM, Sheehan S, Steyn SJ, Strick CA, Williams VA and Zhang L (2009) Discovery, SAR, and pharmacokinetics of a novel 3-hydroxyquinolin-2(1H)-one series of potent D-amino acid oxidase (DAAO) inhibitors. *J Med Chem* **52**:3576-3585.

Fang Q, Hopkins S, Heffernan M and Chytil M. PYRROLE AND PYRAZOLE DAAO INHIBITORS. Sepracor Inc. 12/566,990(US 7,893,098 B2). 2-22-2011. 9-25-2009.
Ref Type: Patent

Fang Q, Hopkins S, Heffernan M, Chytil M and Wipf P. BENZO[D]ISOXAZOL-3-OL DAAO INHIBITORS. Sepracor Inc. 11/024,151(US 7,166,725 B2). 1-23-2007. 12-28-2004.
Ref Type: Patent

Fang Q, Hopkins S and Jones S. Pyrrole and Pyrazole DAAO Inhibitors. (2005/0143443 A1). 6-30-2005.
Ref Type: Patent

Fisher K, Coderre TJ and Hagen NA (2000) Targeting the N-methyl-D-aspartate receptor for chronic pain management. Preclinical animal studies, recent clinical experience and future research directions. *J Pain Symptom Manage* **20**:358-373.

Gong N, Gao ZY, Wang YC, Li XY, Huang JL, Hashimoto K and Wang YX (2011) A series of D-amino acid oxidase inhibitors specifically prevents and reverses formalin-induced tonic pain in rats. *J Pharmacol Exp Ther* **336**:282-293.

Gong N, Wang YC, Wang HL, Ma AN, Hashimoto K and Wang YX (2012) Interactions of the potent D-amino acid oxidase inhibitor CBIO with morphine in pain and tolerance to analgesia. *Neuropharmacology* **63**:460-468.

Guo JD, Wang H, Zhang YQ and Zhao ZQ (2006) Distinct effects of D-serine on spinal nociceptive responses in normal and carrageenan-injected rats. *Biochem Biophys Res Commun* **343**:401-406.

Hashimoto A, Nishikawa T, Konno R, Niwa A, Yasumura Y, Oka T and Takahashi K (1993) Free D-serine, D-aspartate and D-alanine in central nervous system and serum in mutant mice lacking D-amino acid oxidase. *Neurosci Lett* **152**:33-36.

Heffernan M, Dorsey J, Fang Q, Foglesong R, Hopkins S, Ogbu C, Soukri M and Spear K. FUSED HETEROCYLES. Sepracor Inc. (US 7,579,370 B2). 8-25-2009. 8-3-2007. Ref Type: Patent

Kappor R and Kapoor V (1997) Distribution of D-amino acid oxidase (DAO) activity in the medulla and thoracic spinal cord of the rat: implications for a role for D-serine in autonomic function. *Brain Res* **771**:351-355.

Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**:355-363.

Kolhekar R, Meller ST and Gebhart GF (1994) N-methyl-D-aspartate receptor-mediated changes in thermal nociception: allosteric modulation at glycine and polyamine recognition sites. Neuroscience **63**:925-936.

Lu JM, Gong N, Wang YC and Wang YX (2012) D-Amino acid oxidase-mediated increase in spinal hydrogen peroxide is mainly responsible for formalin-induced tonic pain. *Br J Pharmacol* **165**:1941-1955.

Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA and Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A* **97**:4926-4931.

Naur P, Hansen KB, Kristensen AS, Dravid SM, Pickering DS, Olsen L, Vestergaard B, Egebjerg J, Gajhede M, Traynelis SF and Kastrup JS (2007) Ionotropic glutamate-like receptor delta2 binds D-serine and glycine. *Proc Natl Acad Sci U S A* **104**:14116-14121.

Oliet SH and Mothet JP (2009) Regulation of N-methyl-D-aspartate receptors by astrocytic D-serine. *Neuroscience* **158**:275-283.

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA and Oliet SH (2006) Glia-derived D-serine controls NMDA receptor activity and synaptic memory. *Cell* **125**:775-784.

Papouin T, Ladepeche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP and Oliet SH (2012) Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell* **150**:633-646.

Schell MJ (2004) The N-methyl D-aspartate receptor glycine site and D-serine metabolism: an evolutionary perspective. *Philos Trans R Soc Lond B Biol Sci* **359**:943-964.

Sparey T, Abeywickrema P, Almond S, Brandon N, Byrne N, Campbell A, Hutson PH, Jacobson M, Jones B, Munshi S, Pascarella D, Pike A, Prasad GS, Sachs N, Sakatis M, Sardana V, Venkatraman S and Young MB (2008) The discovery of fused pyrrole carboxylic acids as novel, potent D-amino acid oxidase (DAO) inhibitors. *Bioorg Med Chem Lett* **18**:3386-3391.

Strick CA, Li C, Scott L, Harvey B, Hajos M, Steyn SJ, Piotrowski MA, James LC, Downs JT, Rago B, Becker SL, El-Kattan A, Xu Y, Ganong AH, Tingley FD, III, Ramirez AD, Seymour PA, Guanowsky V, Majchrzak MJ, Fox CB, Schmidt CJ and Duplantier AJ (2011) Modulation of NMDA receptor function by inhibition of D-amino acid oxidase in rodent brain. *Neuropharmacology* **61**:1001-1015.

Wake K, Yamazaki H, Hanzawa S, Konno R, Sakio H, Niwa A and Hori Y (2001) Exaggerated responses to chronic nociceptive stimuli and enhancement of N-methyl-D-aspartate receptor-mediated synaptic transmission in mutant mice lacking D-amino-acid oxidase. *Neurosci Lett* **297**:25-28.

Wang LZ and Zhu XZ (2003) Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. *Acta Pharmacol Sin* **24**:965-974.

Zhao W, Konno R, Zhou XJ, Yin M and Wang YX (2008) Inhibition of D-amino-Acid oxidase activity induces pain relief in mice. *Cell Mol Neurobiol* **28**:581-591.
Zhao WJ, Gao ZY, Wei H, Nie HZ, Zhao Q, Zhou XJ and Wang YX (2010) Spinal D-amino acid oxidase contributes to neuropathic pain in rats. *J Pharmacol Exp Ther* **332**:248-254.

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

Figure 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 (grey). Panels from 2 independent experiments: single dose level (left panel) and a dose-response (right panel). P: pre-surgery baseline. B: day 7 post-surgery. 0: pre-dosing control on day of compound administration (14 days after surgery). Vehicle: Isotonic 50 mM phosphate buffer, p.o.; gabapentin: 100 mg/kg, p.o.; SUN: 3, 10, and 30 mg/kg, p.o. n=9 in each group. *P<0.05, compared to the same time point in the vehicle group, 2-way RMANOVA followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

Figure 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 (grey). Panels from 2 independent experiments: single dose level (left panel) and a dose-response (right panel). P: pre-surgery baseline. B: day 7 post-surgery. 0: pre-dosing control on day of compound administration (14 days after surgery). Vehicle: Isotonic 50 mM phosphate buffer, p.o.; gabapentin: 100 mg/kg, p.o.; SUN: 3, 10, and 30 mg/kg, p.o. n=9 in each group. *P<0.05, compared to the same time point in the vehicle group, 2-way RMANOVA followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

Figure 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 (left panel) and contralateral limbs (right panel). P: naive rats pre-CFA.

0: pre-dosing control on day of compound administration. Vehicle: Isotonic 50 mM phosphate buffer, p.o.; ibuprofen: 50 mg/kg, p.o.; SUN: 3, 10, and 30 mg/kg, p.o. n=8 in each group. *P<0.05, compared to the same time point in the vehicle group, 2-way RMANOVA followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

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

Figure 5. D-Serine administration (i.p.) is inactive on PWT in ipsilateral limbs of SNL model rats. P: pre-surgery baseline. B: day 7 post-surgery. 0: pre-dosing control on day of compound administration. Vehicle: Isotonic 50 mM phosphate buffer, p.o.; gabapentin: 100 mg/kg, p.o.; D-Serine 30, 100, 300 mg/kg, i.p. *P<0.05, compared to the same time point in the vehicle group, 2-way RMANOVA followed by the Holm-Sidak method for pairwise multiple comparisons versus a control group.

Figure 6. Effects of intravenous vehicle, SUN, and gabapentin administration on spontaneous activity and mechanically-evoked responses of 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) ± S.E.M. Levels of activity following vehicle administration (Veh in figure) were recorded 20 minutes prior to compound administration (Cmp in figure). Data collected from 7 neurons recorded from 7 different rats, for each treatment group.

Figure 7. Example traces from dorsal root filaments that demonstrated spontaneous activity (left panel) and no spontaneous activity (right panel) along with the corresponding mechanically-evoked responses to challenges with von Frey hairs. The black arrows indicate the application of von Frey hairs. VFH1, VFH2 and VFH3: von Frey hairs 1 (1 g), 2 (6 g) and 3 (15 g).

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

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Effects[A] 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.

					von Frey Hair (g pressure)		
Rat model	Treatment[B]	Spontaneous	Brush	Squeeze	1.15	4.0	15.6
Control[C]	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[D]	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[E]	*33 ± 15***	*125 ± 15***	*306 ± 25***	*62 ± 10***	*123 ± 16***	*299 ± 36***
DR sectioned[F]	vehicle	209 ± 45					
	SUN	*91 ± 29**					
Spinalized[G]	vehicle	437 ± 62	179 ± 17	403 ± 25	146 ± 17	273 ± 32	552 ± 50
	SUN	*189 ± 34***	*161 ± 18*	*314 ± 22**	*108 ± 15*	*193 ± 21**	*429 ± 36**

[A]Neuronal activity as mean ± SEM; spontaneous activity in impulses/min, mechanically-evoked responses in impulses/10 trials.[B]All treatments p.o., 6-9 hours prior to recordings. [C]A total of 49 neurons were recorded (40 WDR 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. [D]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 [E]gabapentin. [F]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. [G]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.

TABLES

JPET Fast Forward. Published on March 21, 2013 as DOI: 10.1124/jpet.113.204016
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Effects[A] of SUN on electrophysiological recordings of dorsal root filaments in SNL rats

			von Frey Hair (g pressure)		
Rat model	Treatment[B]	Spontaneous	1	6	15
Chung	vehicle[C]	1121 ± 107	62 ± 8	239 ± 23	345 ± 21
	SUN[D]	*351 ± 72***	*30 ± 7.2***	*140 ± 17***	*221 ± 21***

[A]Firing activity mean ± SEM; spontaneous activity, impulses/min, mechanically-evoked responses in impulses/10 trials.[B]All treatments p.o., 6-9 hours prior to recordings. [C]A total of 88 filaments were recorded from a total of 12 SNL rats (pre-dosed with vehicle, p.o.), and [D]122 filaments from 9 SNL rats (pre-dosed with SUN, 10 mg/kg p.o.). ** $P < 0.001$, compared to vehicle, unpaired Student's *t*-test.

Figure 1 (left panel)

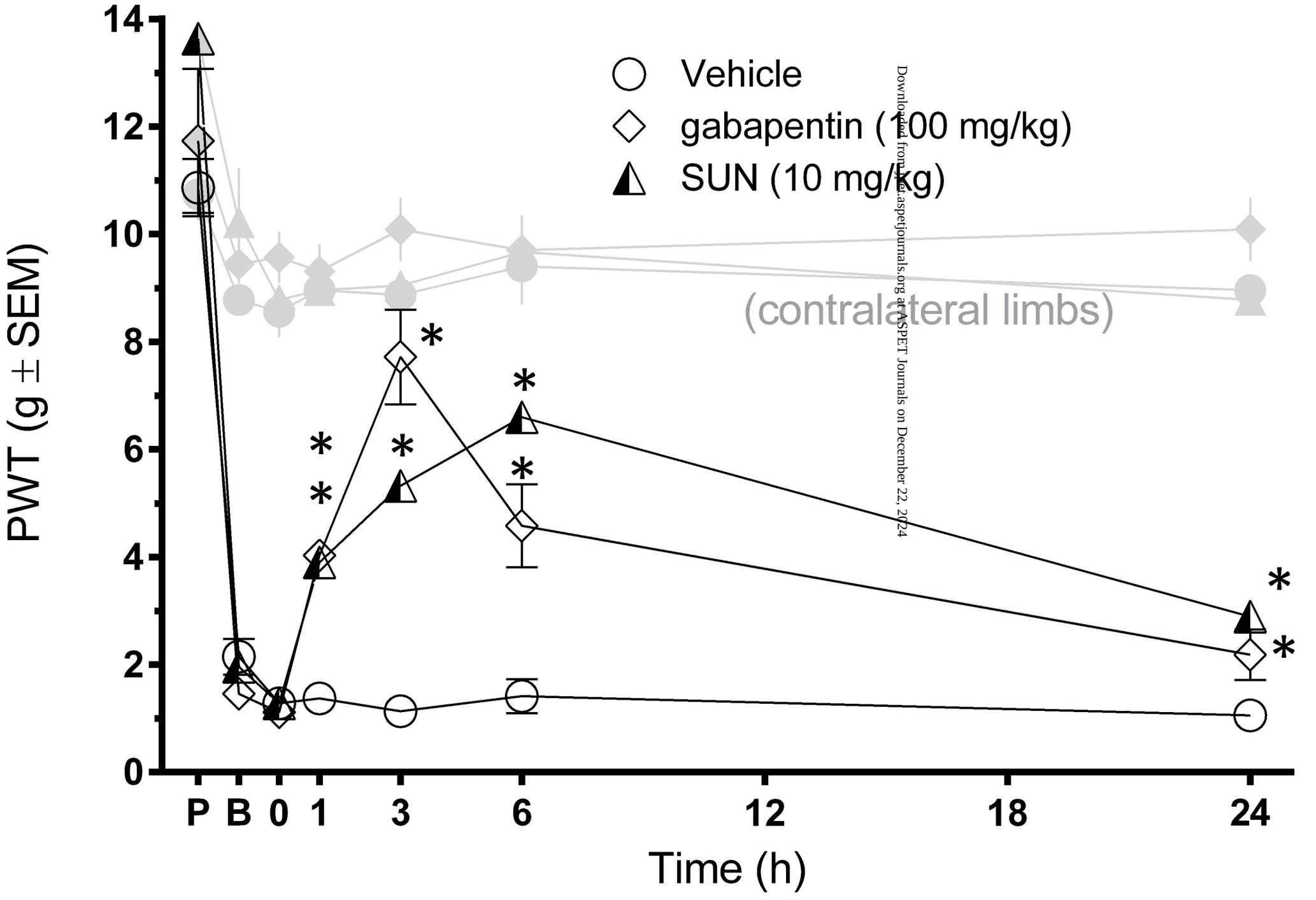

Figure 1 (right panel)

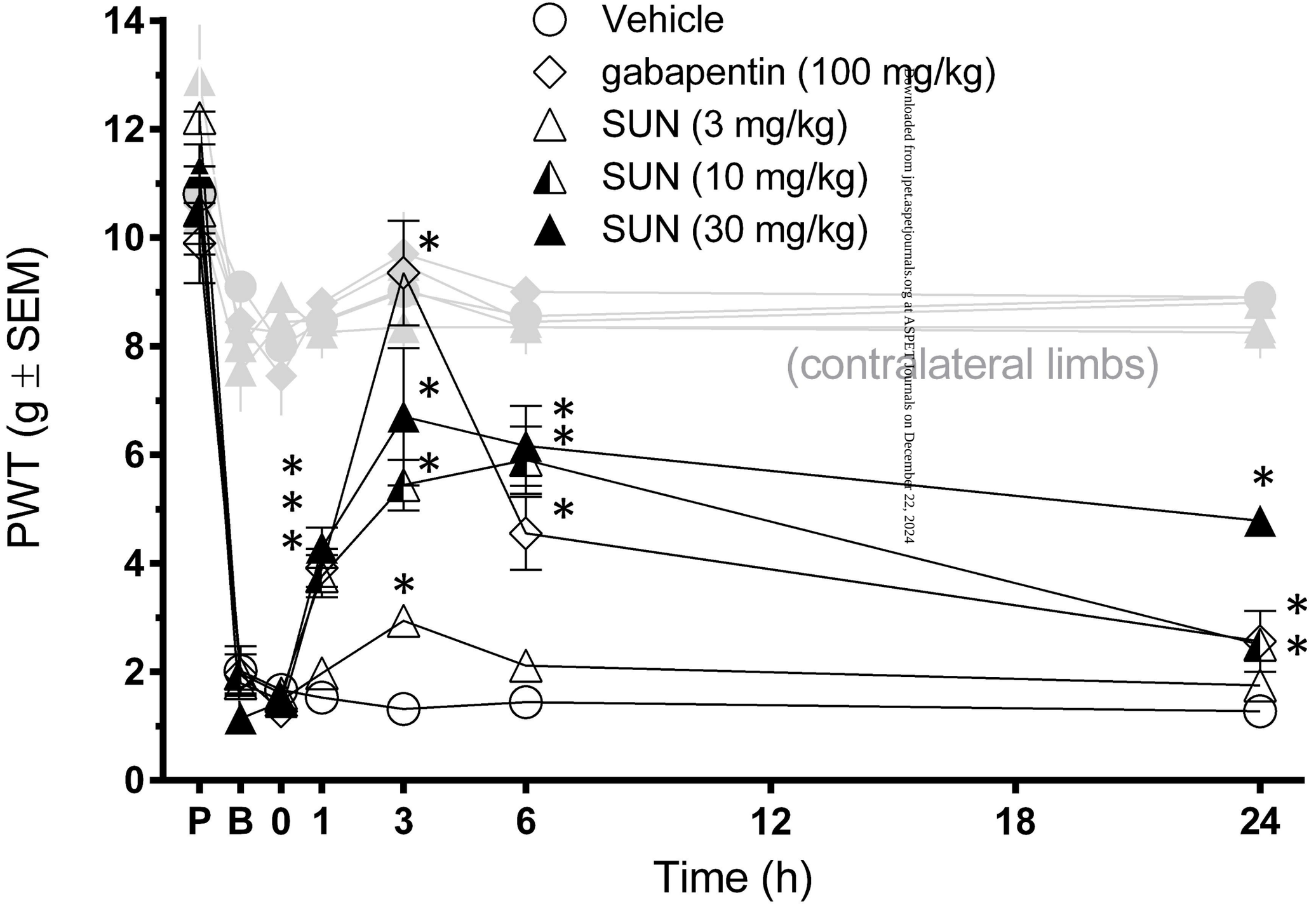

Figure 2 (left panel)

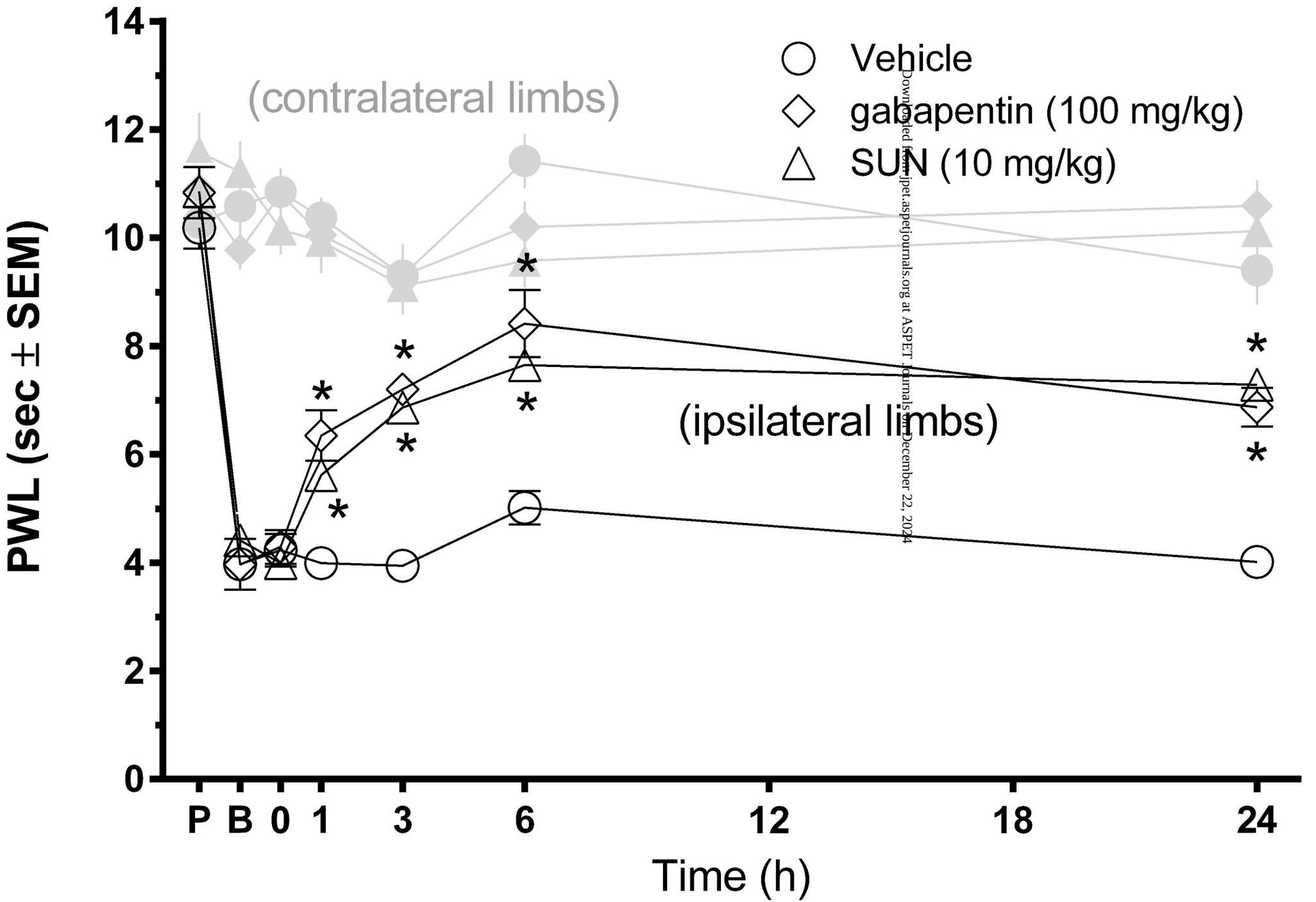

Figure 2 (right panel)

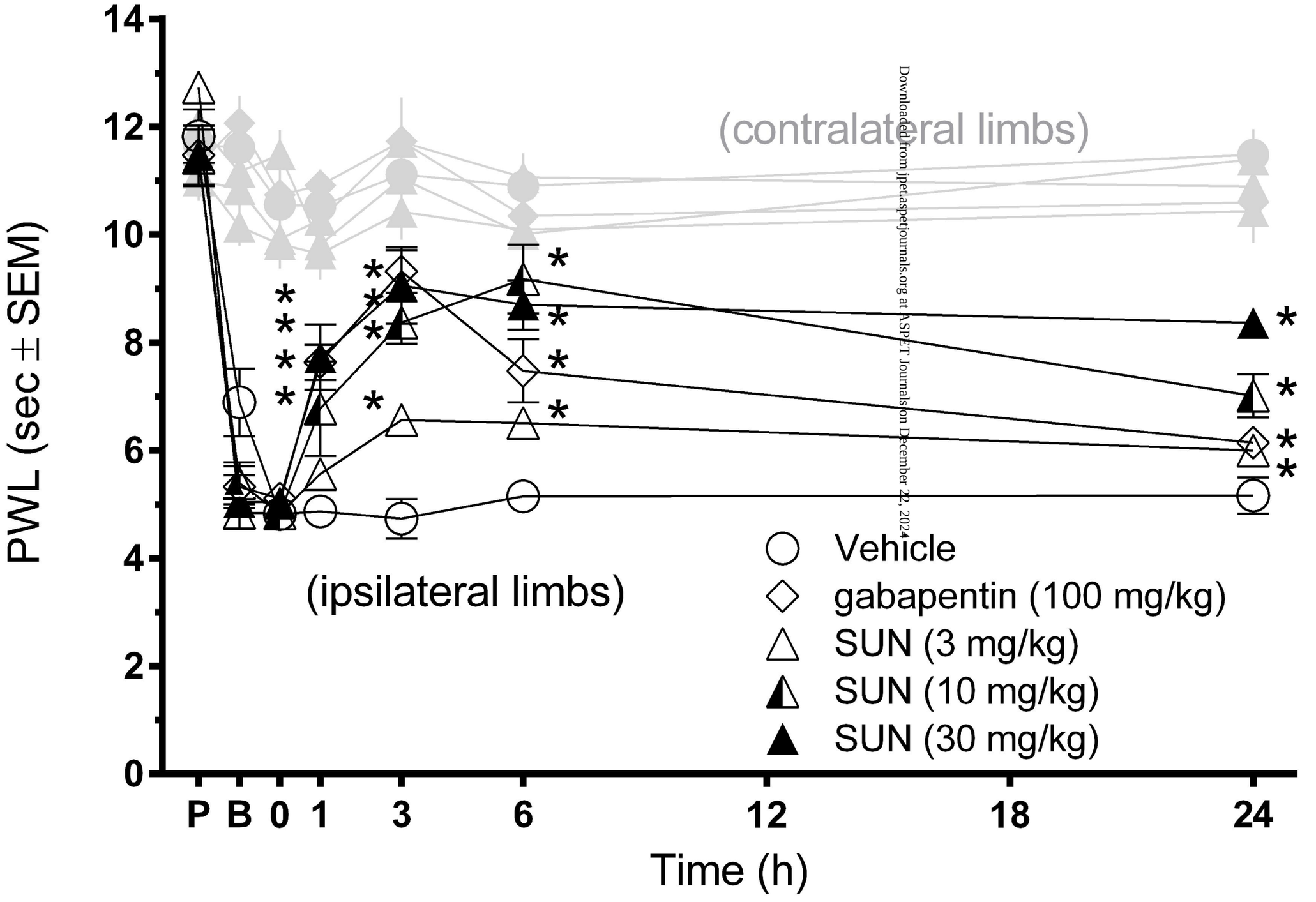

Figure 3

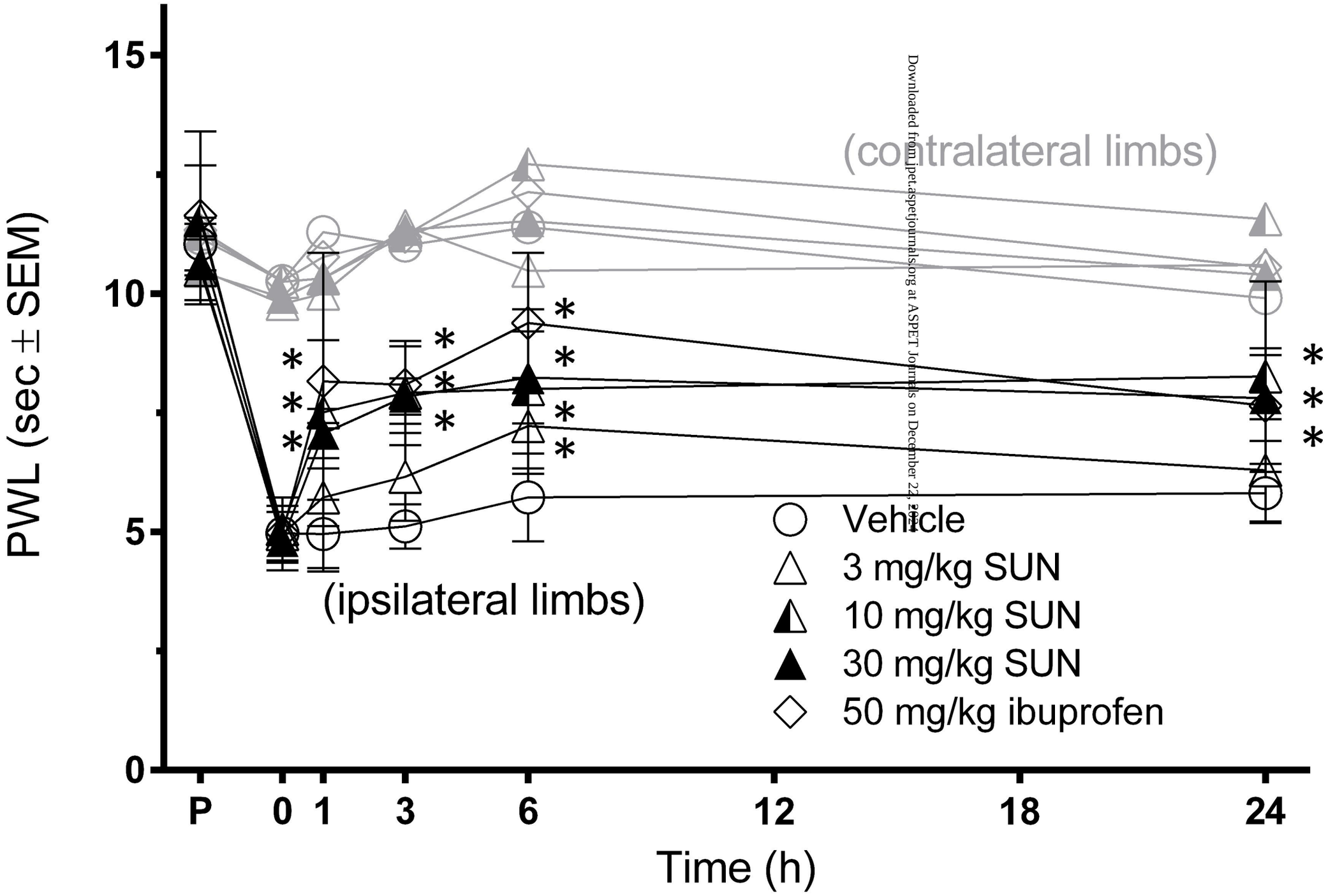

FIGURE 4 (left panel)

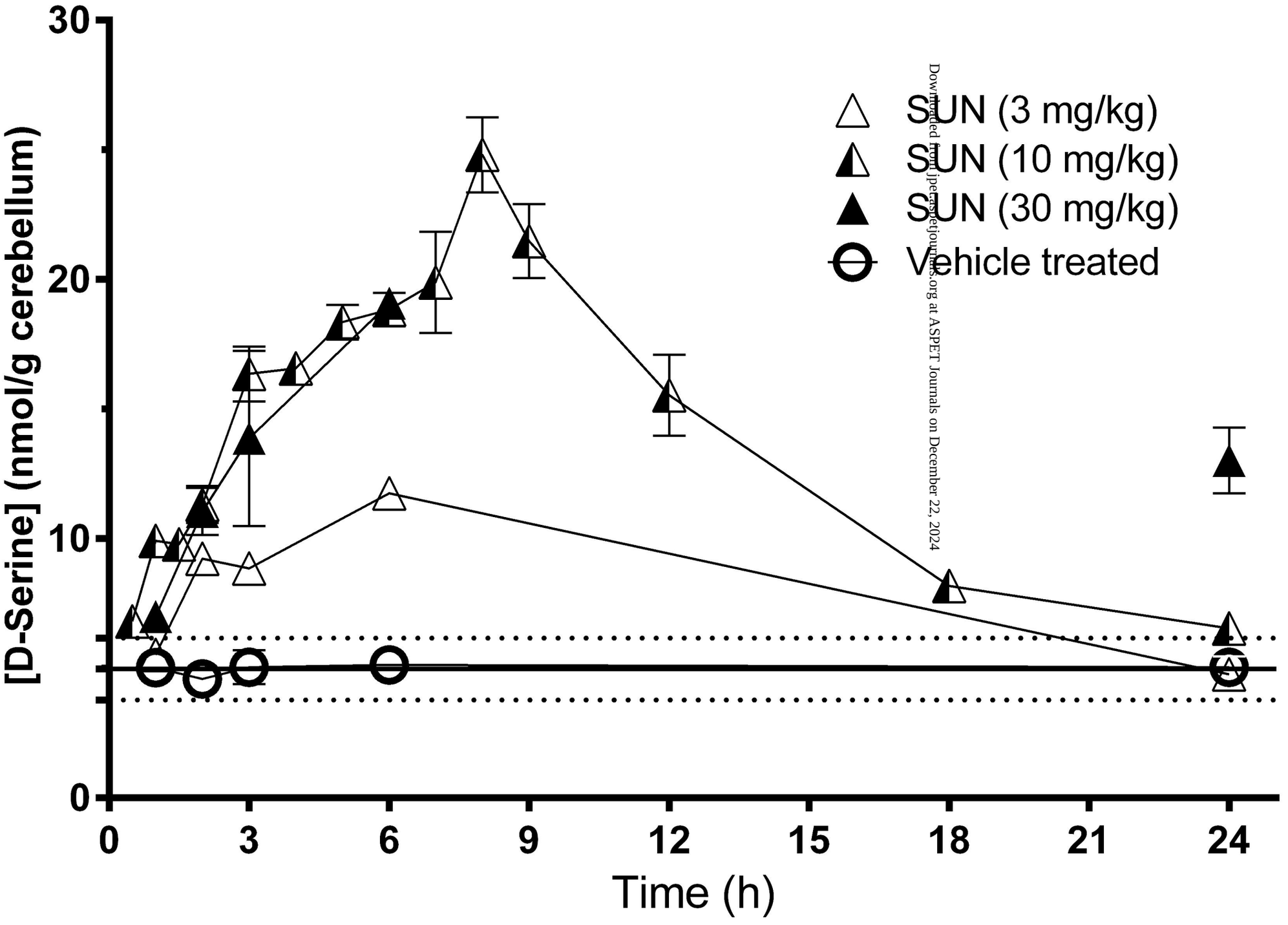

FIGURE 4 (right panel)

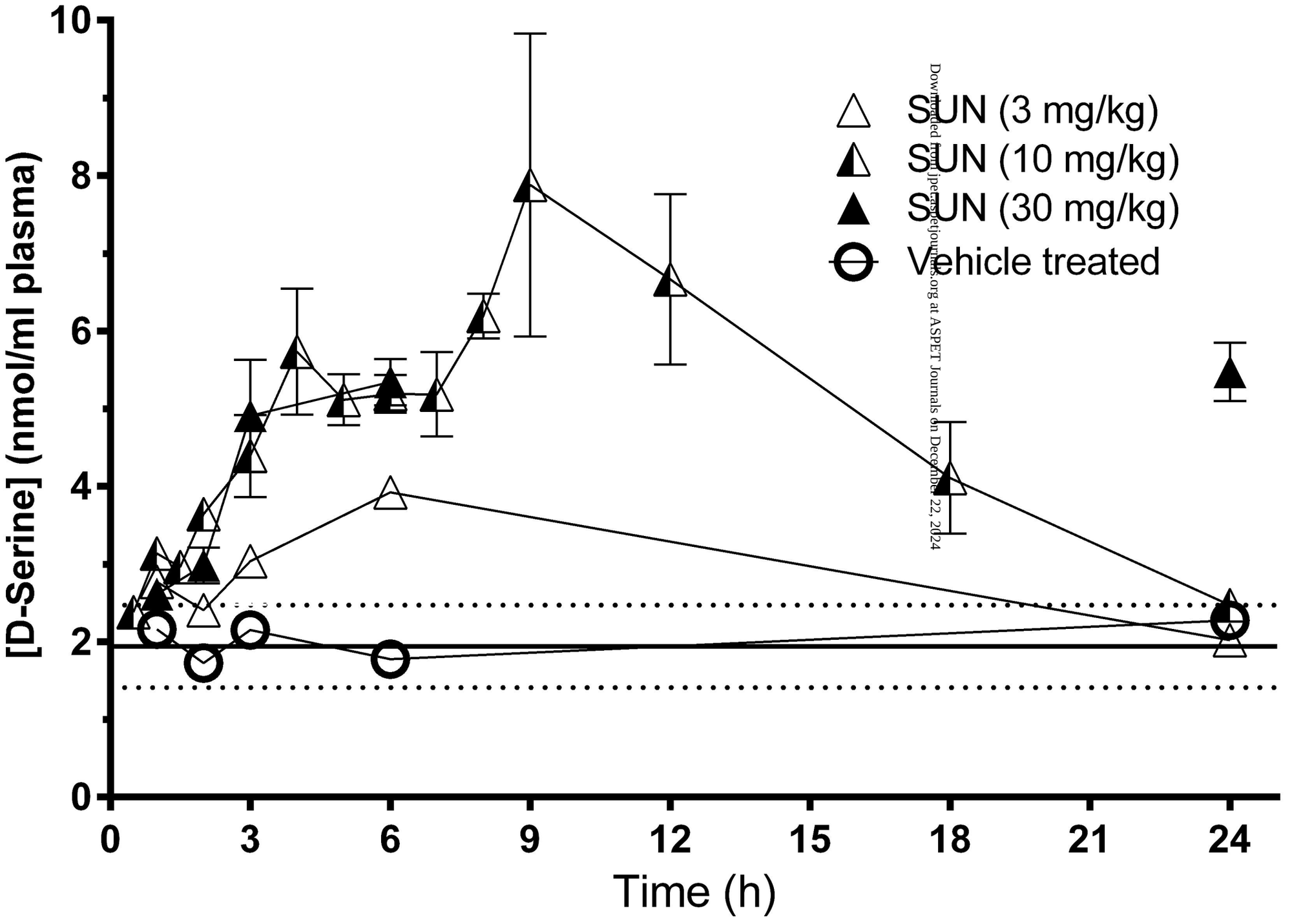

Figure 5

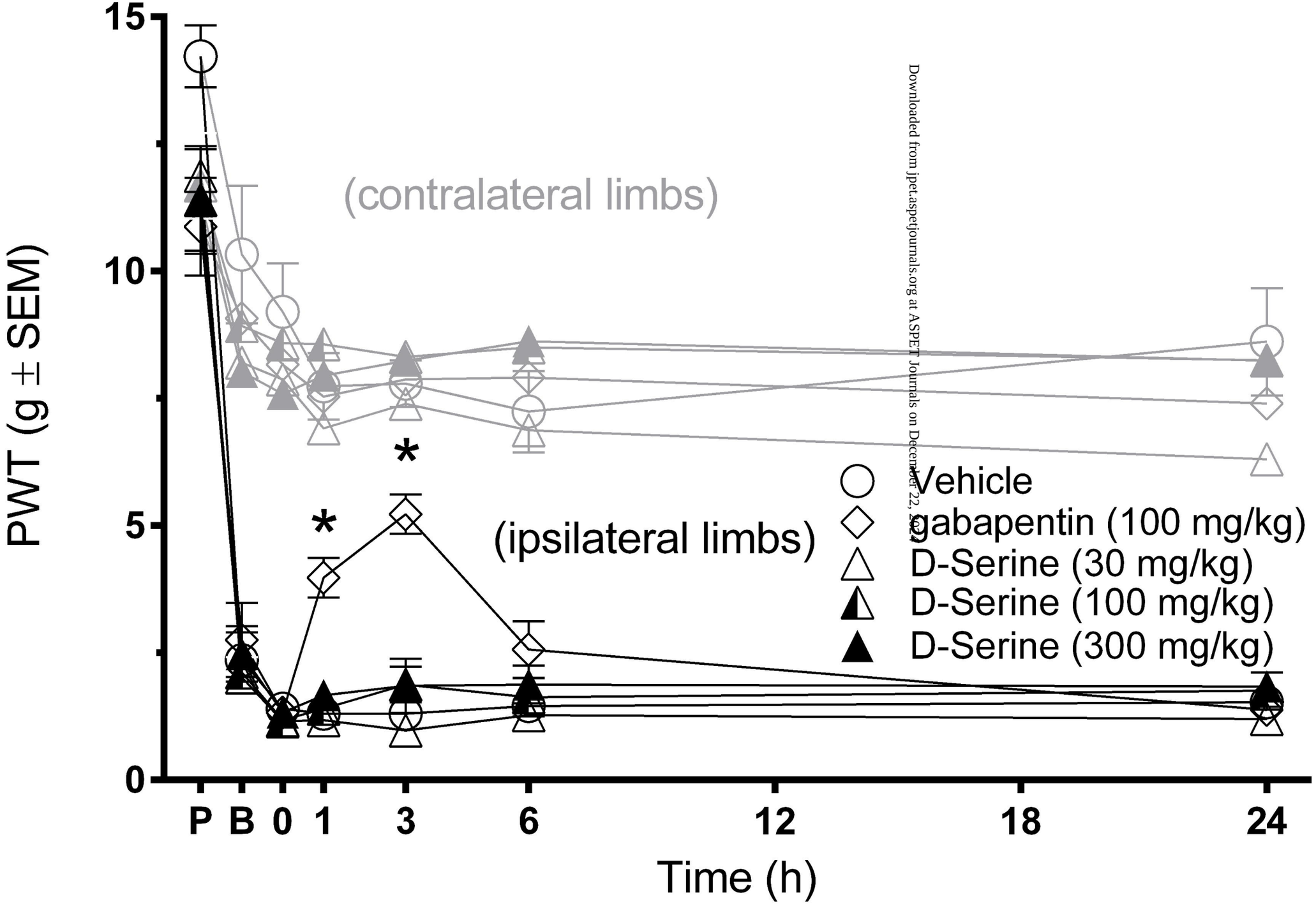

Figure 6A

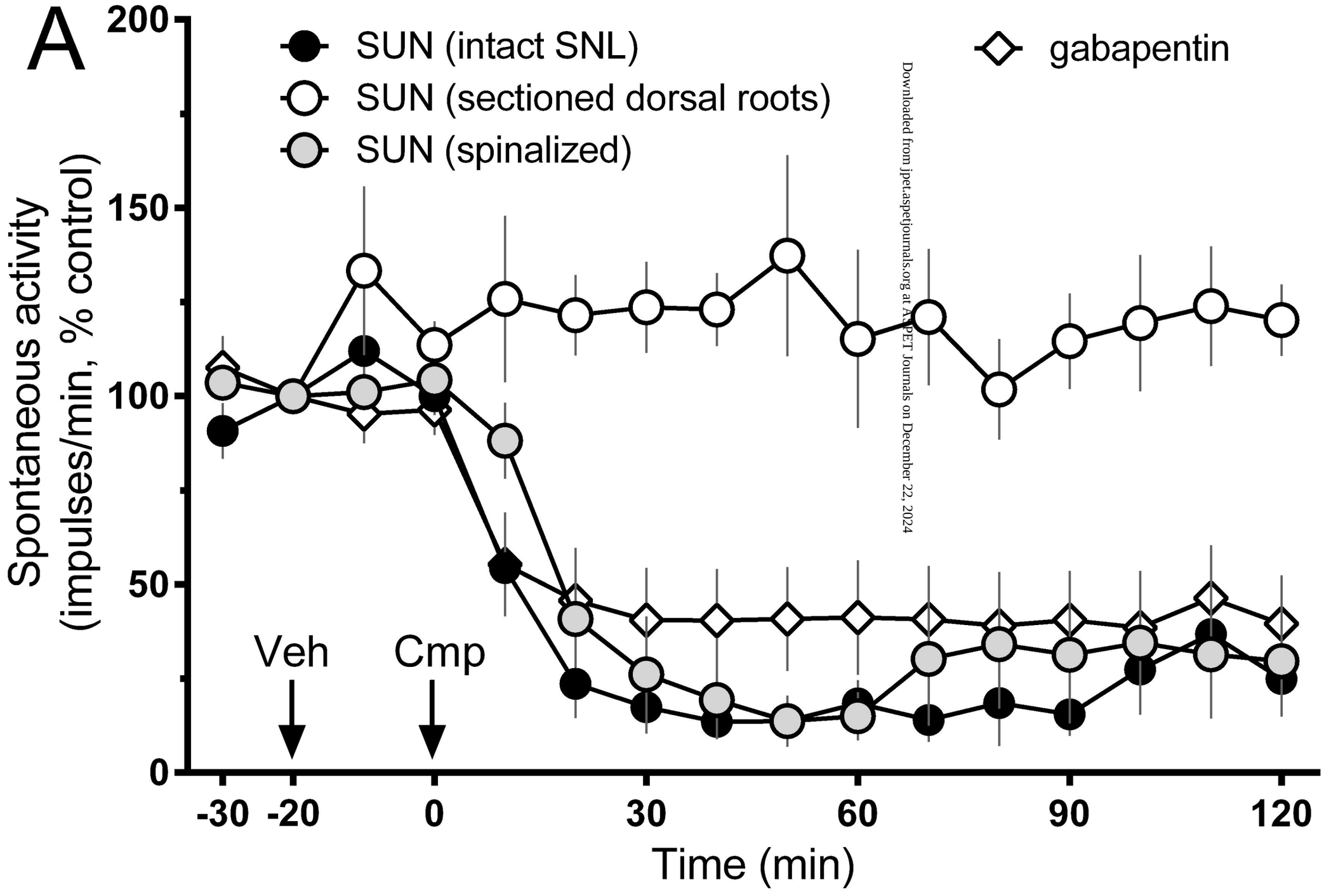

Figure 6B

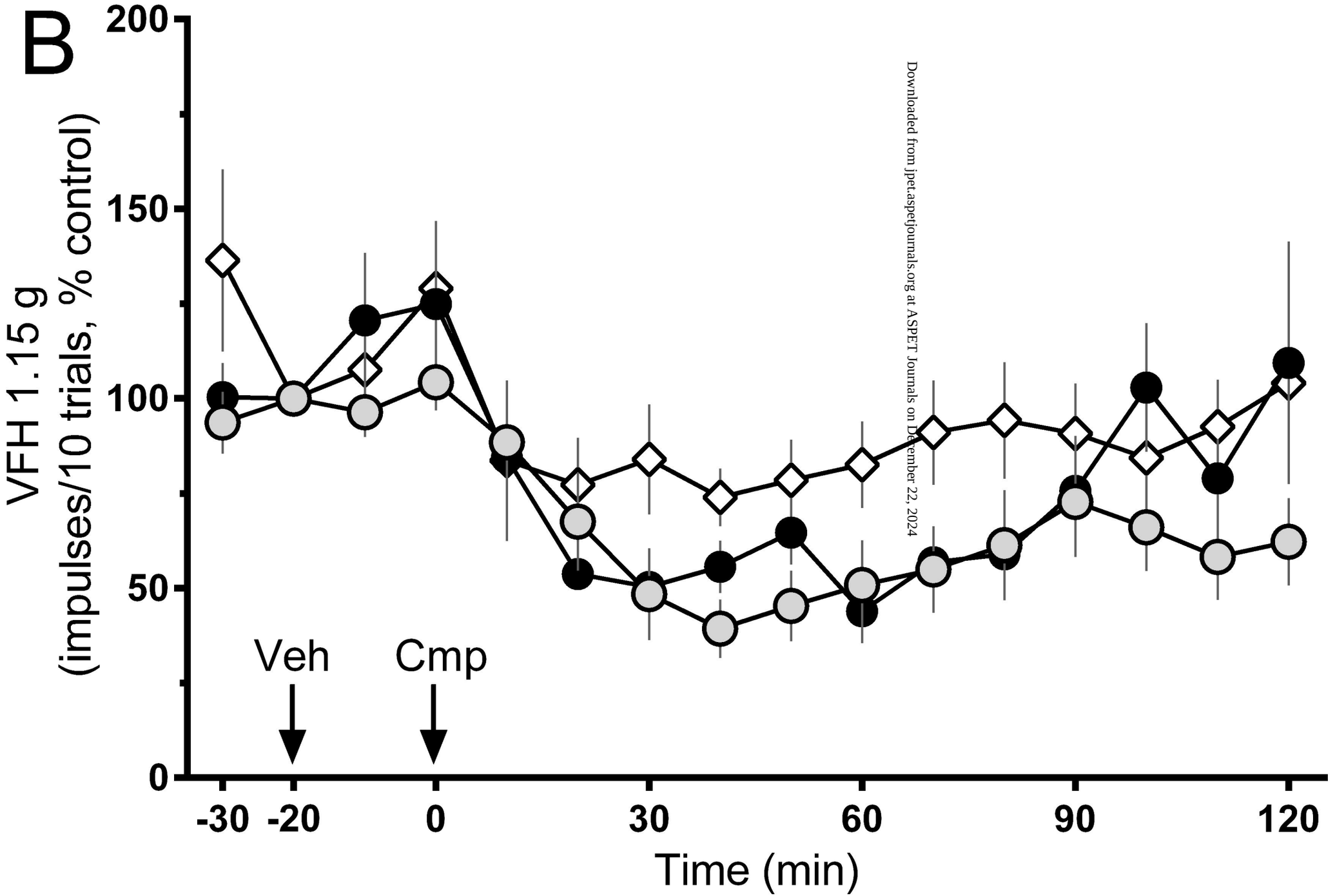

Figure 6C

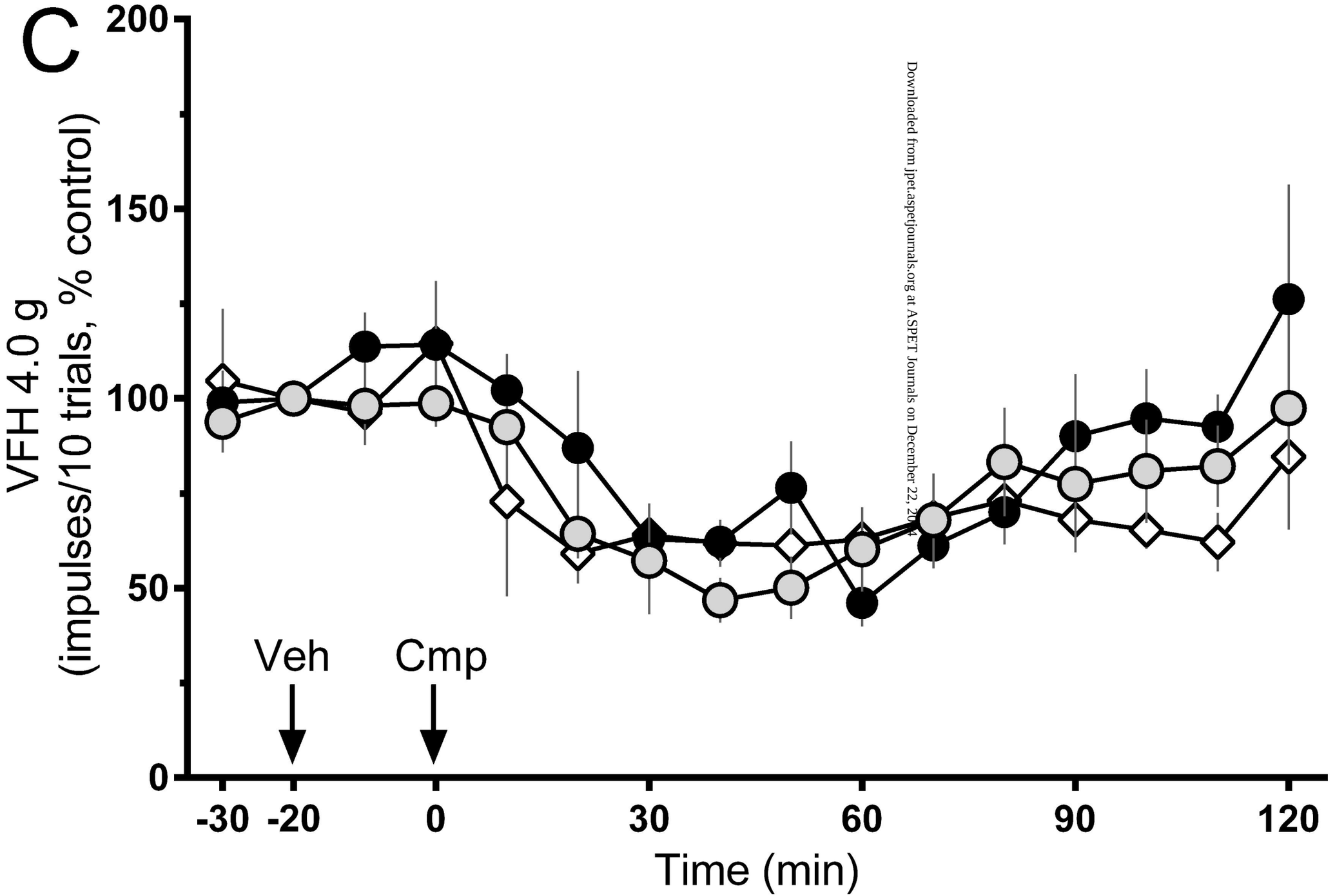

Figure 6D

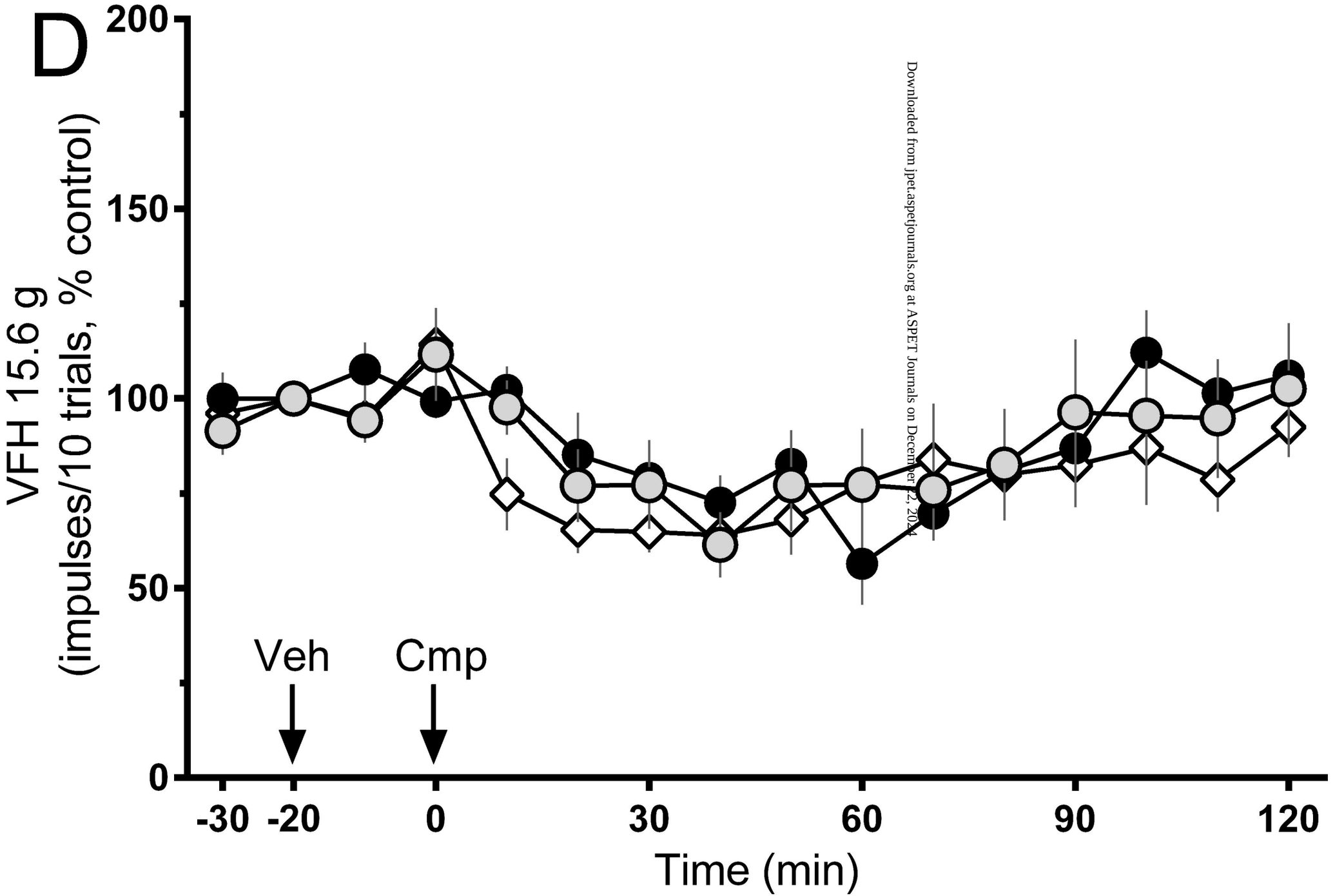

FIGURE 7 left panel

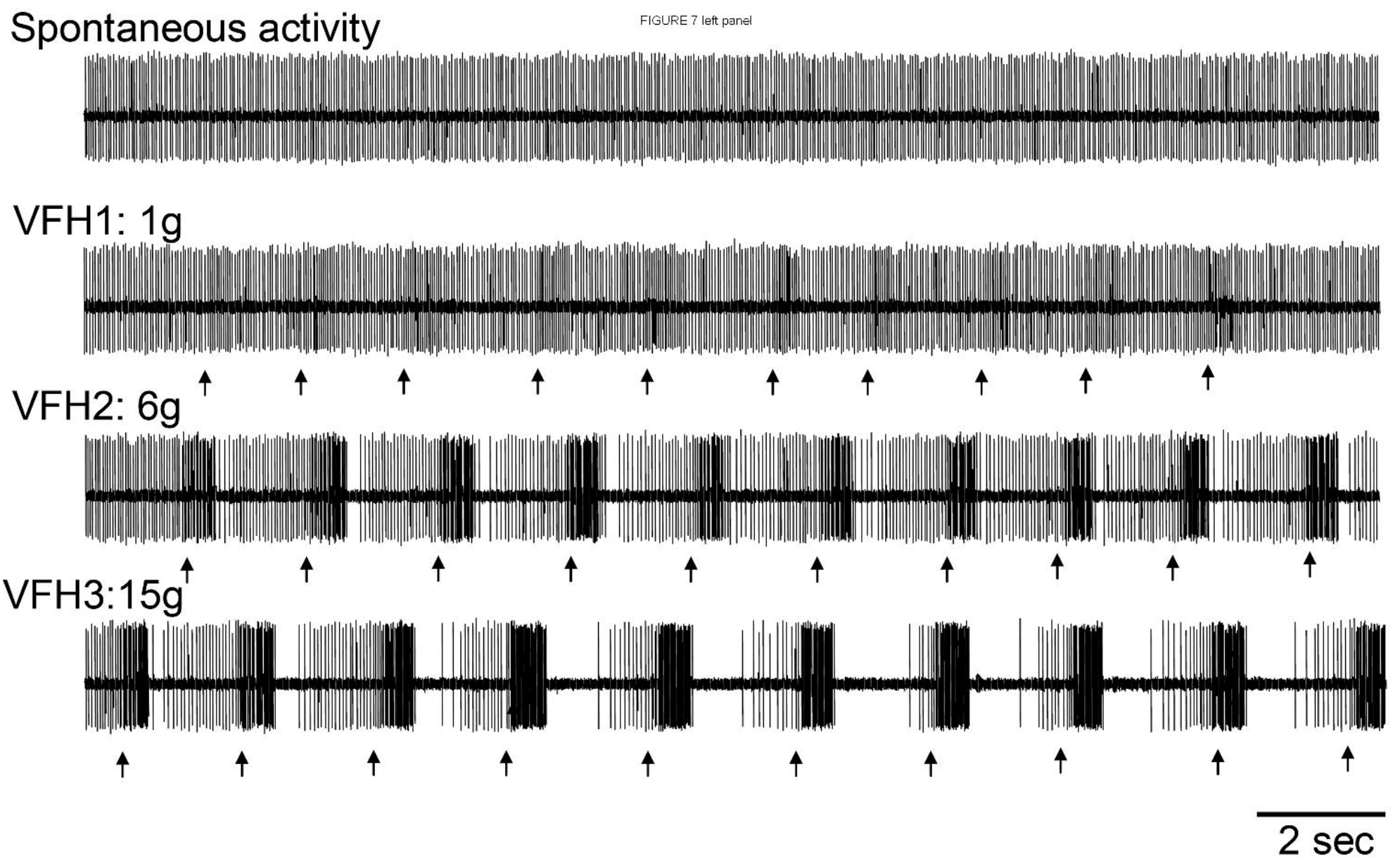

FIGURE 7 right panel

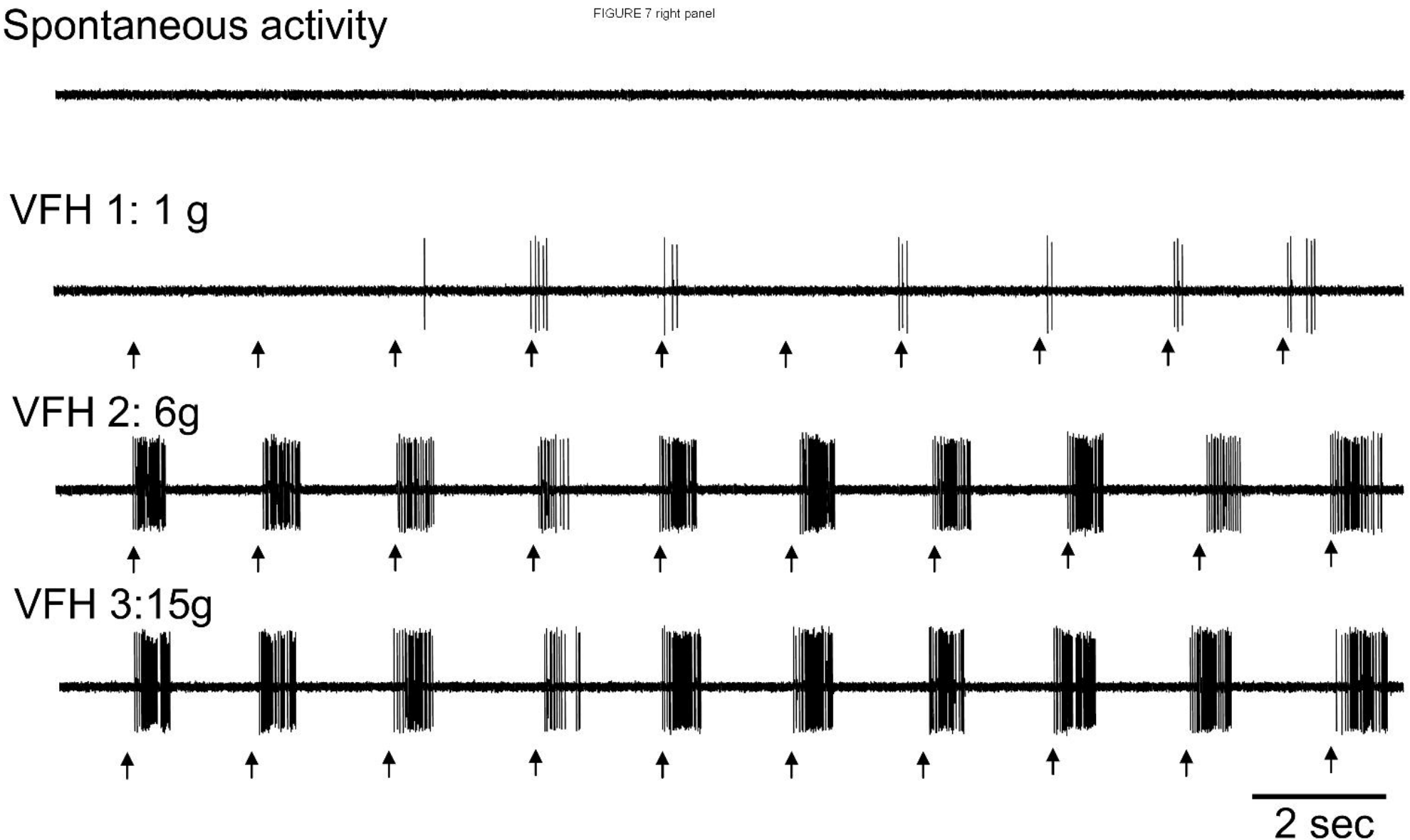

Figure 8A

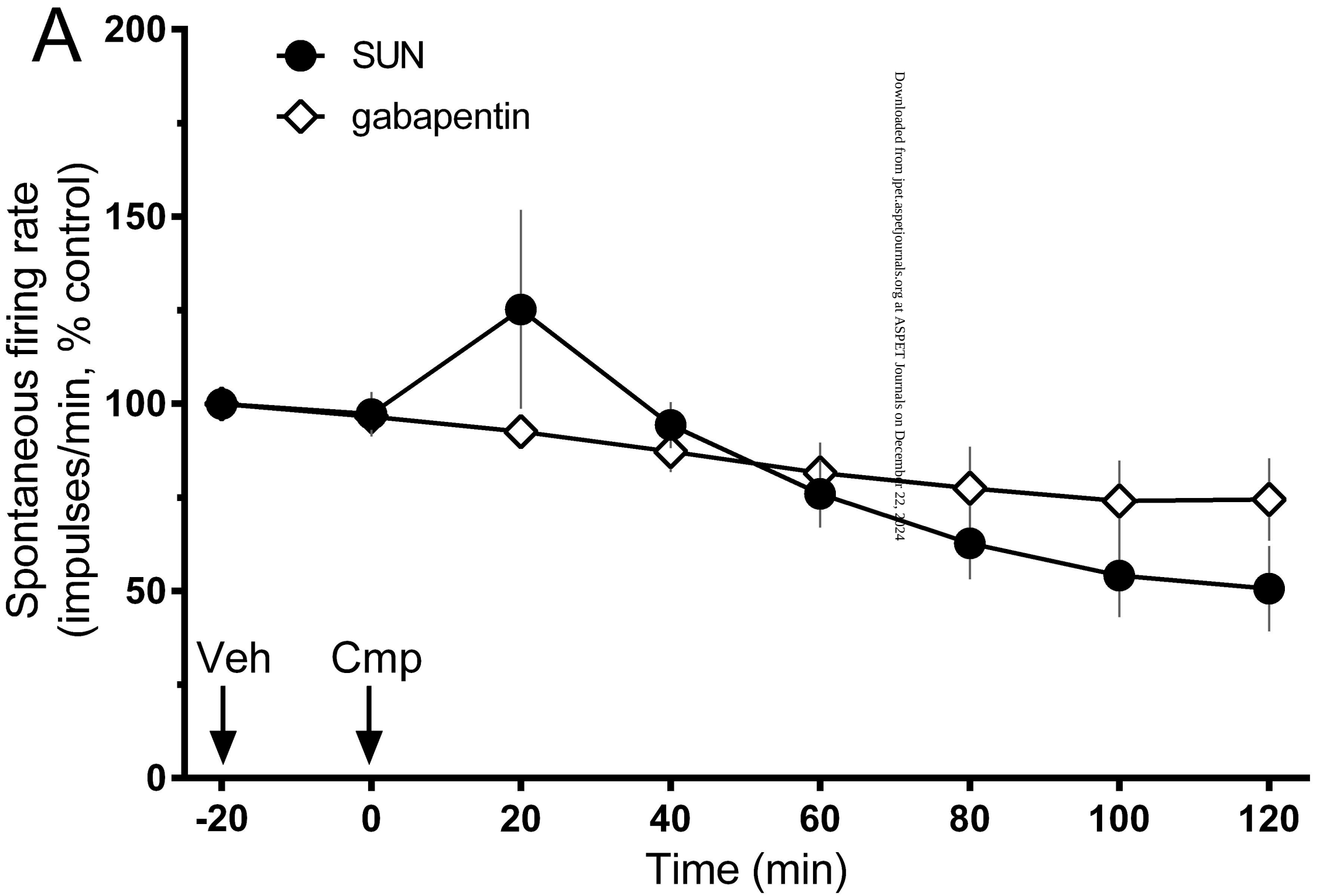

Figure 8B