Spinal d-Amino Acid Oxidase Contributes to Neuropathic Pain in Rats

Wen-Juan Zhao, Zhen-Yu Gao, Hong Wei, Hui-Zhen Nie, Qian Zhao, Xiang-Jun Zhou, and Yong-Xiang Wang

King’s Laboratory, School of Pharmacy, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

d-Amino acid oxidase (DAO) is an enzyme catalyzing oxidative deamination of neutral and polar d-amino acids and is expressed in the kidneys, liver, and central nervous system (CNS) including the spinal cord. We have previously demonstrated that DAO gene deletion/mutation by using mutant ddY/DAO(−/−) mice and systemic administration of the DAO inhibitor sodium benzoate blocked formalin-induced hyperalgesia in mice. In this study, we further investigated the potential role of DAO in neuropathic pain in a rat model of tight L5/L6 spinal nerve ligation. After L5/L6 spinal nerve ligation, the mRNA expression (measured by real-time quantitative polymerase chain reaction) and enzyme activity (measured by a colorimetric method) of DAO in the lumbar spinal cord were markedly increased, in agreement with the development of neuropathic pain (mechanical allodynia). Intrapertoneal injection of sodium benzoate (400 mg/kg) specifically blocked mechanical allodynia in neuropathic rats and formalin-induced hyperalgesia but did not suppress acute pain responses in the tail-flick test or formalin test. Systemic injection of sodium benzoate also inhibited DAO activity in the lumbar spinal cord of rats. Furthermore, direct intrathecal (spinal cord) injection of benzoate (30 μg/rat) specifically blocked spinal nerve ligation-induced mechanical allodynia in neuropathic rats and formalin-induced hyperalgesia (but not acute pain) in the formalin test. Based on the above results, we conclude that spinal DAO plays a pronociceptive (rather than an antinociceptive) role and might be a target molecule for the treatment of chronic pain of neuropathic origin.

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ABBREVIATIONS: DAO, d-amino acid oxidase; CNS, central nervous system; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, cycle threshold; ANOVA, analysis of variance; AS057278, 5-methylpyrazole-3-carboxylic acid; NMDA, N-methyl-d-aspartate.
pathic pain, which shares the common mechanism of central sensitization with formalin-induced hyperalgesia.

The aim of this present study was to determine the potential role of DAO in neuropathic pain in a rat model of tight L5/L6 spinal nerve ligation. Tight ligation of L5 and L6 spinal nerves in rats produces mechanical allodynia and heat hyperalgesia mediated by central sensitization, representing characteristic neuropathic pain syndromes in humans (Kim and Chung, 1992; Jett et al., 1997). This study included the following procedures: 1) examining expression and activity changes of the spinal DAO, as well as development of neuropathic pain (mechanical allodynia) after L5/L6 spinal nerve ligation; 2) testing the analgesic actions of the competitive DAO inhibitor sodium benzoate by systemic injection in neuropathic rats; and 3) further determining whether systemic administration of sodium benzoate exerts its analgesic effect through inhibition of spinal DAO activity by measuring spinal DAO activity and testing the analgesic effect of intrathecally injected sodium benzoate. The inhibitory effect of sodium benzoate was also confirmed in the rat formalin test and compared with the effect in the rat acute nociceptive model in this study. The preliminary result of this study was presented in abstract form at the 1st International Conference of D-Amino Acid Research (in Japan) (Wang and Zhao, 2009).

Materials and Methods

Drugs and Reagents. Sodium benzoate and formalin were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Morphine was obtained from Qinghai Pharmaceutical Factory Co. Ltd. (Qinghai, China). All of these drugs and reagents were freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) for administration.

Animals. Male Wistar rats (180–250 g) were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle (lights on at 6:00 AM). Food and water were freely available. The research protocol was approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University School of Pharmacy and followed the animal care guidelines of the National Institutes of Health. Animals were acclimated to the laboratory environment for 5 to 7 days before entering the study. Experimental study groups were assigned in random, and the researcher was blind for behavior testing.

Ram Model of Neuropathic Pain. The unilateral ligation of two spinal nerves (L5/L6) was performed in intraperitoneal pentobarbital anesthetized (50 mg/kg) rats as described earlier (Kim and Chung, 1992; Wei et al., 2007). In brief, left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread. After ligation, the wound was sutured, and the rats were allowed to recover. In sham-operated rats the same surgical procedure except for spinal nerve isolation and ligation was performed. Of the nerve-ligated rats, only those with marked unilateral allodynia to mechanical stimulation (hind limb withdrawal thresholds in the operated side <8 g) and with no major motor impairment were selected for further studies.

Behavioral Assessment of Mechanical Alldynia. For assessment of mechanical allodynia, the hind limb withdrawal threshold evoked by stimulation of the hind paw with a series of calibrated monofilaments (von Frey hairs, 2290 CE, IITC Life Science Inc., Woodland Hills, CA) was determined while the rat stood on a metal grid. The monofilaments, which produced forces ranging from 0.1 to 65 g, were applied to the foot pad with increasing force until the rat suddenly withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold. The threshold in each time point was based on three separate measurements, and the mean of these threshold values for each hind paw at each time point was used.

Real-Time Quantitative PCR Analysis. The rats were sacrificed by decapitation, and the lumbar enlargements of the spinal cord were collected at days −1, 1, 2, 7, or 14 after L5/L6 spinal nerve ligation or sham operation. Total RNA was purified by use of TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse transcription reaction was performed on 0.5 to 1 μg of total RNA using a ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed with Rotor-Gene 3000 (Corbett Robotics Pty Ltd, Brisbane, Australia) using RealMasterMix (SYBR Green I) (Tiangen, Beijing, China) and running 40 cycles of the following protocol: 2-min predenaturation at 94°C, 30-s denaturation at 94°C, and 30-s annealing at 58°C followed by a 40-s extension at 68°C. The upstream primers were CCC TTT CTA GAA AAC CAG AC (da5) and CGG CAA GTT CAA CGG CAC AG (gapdh); the downstream primers were CTC TCA CCA CCT CTT CG (da6) and AGA CGC CAG TAG ACT CCA CGA C (gapdh). All primers were synthesized by Sangon (Shanghai, China). Quantitative expression of DAO was normalized using an internal control (GAPDH), and the fold change in expression of DAO relative to GAPDH at various time points was calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001), where ΔΔCt = (Ct, Target − Ct, Gapdh)Time x − (Ct, Target − Ct, Gapdh)Time 0.

Measurement of Spinal DAO Activity. The rats were sacrificed by decapitation, and the lumbar enlargements of the spinal cord were quickly removed on ice and weighed. The tissues were homogenized in 0.1 M Tri-HCl (pH 8.2) and centrifuged (4°C, 12,000 rpm, 30 min). Next 200 μl of t-alumine (0.1 M) dissolved in 0.1 M Tri-HCl buffer, pH 8.2) was added to 200 μl of supernatant and incubated at 37°C for 30 min. Then 25% trichloroacetic acid (200 μl) was added to the assay mixture, and the mixture was vortexed and centrifuged (14,000 rpm, 5 min). The supernatant (200 μl) was mixed with 200 μl of 2,4-dinitrophenylhydrazine (1 mM in 1 M HCl) and incubated (37°C, 10 min). Finally, 400 μl of sodium hydroxide (1.5 M) was added, and the mixture was vortexed and incubated (37°C, 10 min). The absorbance was read at 450 nm on a Bio-Tek ELx800 Universal Microplate Reader (Cole-Parmer Instrument Co., IL). The enzyme activity was expressed as pyruvate production per gram of total protein of the lumbar enlargements of the spinal cord.

Rat Formalin Test. Rats were acclimated individually to the observation cage for 30 min before testing. The formalin test was performed by injecting 100 μl of 5% formalin (in 0.9% saline) subcutaneously on the dorsal side of the left hind paw as described previously (Wang et al., 2000). The nociceptive threshold was defined as the time required to elicit a flick of the tail. The cutoff time was previously (Wang et al., 2000). The nociceptive behavior was quantified by counting numbers of formalin-injected paw flinches in 1-min epochs. Measurements were taken at 10-min intervals beginning immediately after formalin injection and ending 90 min later.

Rat Tail-Flick Test. The model SSY-H digital display thermo-static water-bath (Shanghai Sanshen Medical Instrument Co., Ltd., Shanghai, China) was used to maintain a constant water temperature of 50 ± 0.5°C. While the rat was being placed in a tabular restrainer, its tail was immersed 5 cm in the water bath as described previously (Wang et al., 2000), and the rat was immediately placed in a 23 cm × 35 cm × 19 cm transparent polycarbonate box. Noxious behavior was quantified by counting numbers of formalin-injected paw flinches in 1-min epochs. Measurements were taken at 10-min intervals beginning immediately after formalin injection and ending 90 min later.

Intrathecal Catheterization. A polyelectrolyte catheter (PE-10, 0.28 mm i.d. and 0.61 mm o.d.; Clay Adams, Parsippany, NJ) was inserted into the rat at the lumbar level of the spinal cord under pentobarbital (50 mg/kg i.p.) anesthesia as described elsewhere (Wei et al., 2007). After recovery from anesthesia, placement of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μl followed by 10 μl of saline for flushing) with a 50-μl microinjector (Shanghai Antiing Miro-injector Factory, Shanghai, China). The lidocaine test was performed approximately 7 days before the start of the drug testing sessions. Only those rats that had no motor impair-
ment before lidocaine injection but had a transient bilateral paralysis of hind limbs after intrathecal administration of lidocaine were selected for the study. For intrathecal administration, the control and test drugs were microinjected with a 50-μl microinjector in a volume of 5 μl followed by a normal saline flush in a volume of 15 μl.

**Statistical Analysis.** Results are expressed as means ± S.E.M., and statistical significance was evaluated by one-way analysis of variance (ANOVA) or two-tailed Student’s *t* test. The statistical significance criterion *P* value was 0.05.

**Results**

**Effects of L5/L6 Spinal Nerve Ligation on Spinal DAO mRNA Expression, Enzymatic Activity, and the Development of Mechanical Allodynia.** Two groups of rats (*n* = 12 in each group) underwent sham operation or tight L5/L6 spinal nerve ligation. Tactile sensitivity of each rat’s hind paw (ipsilateral side of the ligated spinal nerves) was tested by measuring withdrawal threshold in response to von Frey monofilaments before surgery (−1 day) and 1, 2, 7, and 14 days after surgery. The paw withdrawal threshold for sham animals was approximately 55 g, similar to the presurgical level, whereas spinal nerve-ligated rats showed mechanical allodynia with a threshold of ≤8 g after surgery. The distinct mechanical allodynia on the ipsilateral hind paw occurred 1 day or earlier (4 h in another study, data not shown) after surgery and lasted for at least 14 days (Fig. 1A) or at least 28 days in another study (data not shown). In addition, the lumbar enlargements of the spinal cords were excised from either sham or L5/L6 spinal nerve-ligated rats (*n* = 6 in each time point) on days −1, 1, 2, 7, or 14 after surgery. Expression and enzymatic activity of DAO were then examined by real-time quantitative reverse-transcriptase PCR and the colorimetric method, respectively. As shown in Fig. 1, B and C, spinal mRNA expression and enzymatic activity of DAO from neuropathic rats were significantly elevated by approximately 9- and 2-fold, respectively, and were maintained for at least 14 days in contrast to results in sham-operated rats. These increases in spinal DAO expression and activity (to a lesser degree) were in agreement with the time course of mechanical allodynia development (Fig. 1).

**Effects of Intraperitoneal Injection of Sodium Benzoate on Neuropathic Pain, Hyperalgesia, and Nociceptive Pain.** The effect of sodium benzoate on mechanical allodynia was examined in L5/L6 spinal nerve-ligated rats 10 to 14 days after surgery. Three groups of neuropathic rats (*n* = 8 in each group) received intraperitoneal injections of normal saline (4 ml/kg), sodium benzoate (400 mg/kg), and morphine (10 mg/kg), respectively. The 400 mg/kg sodium benzoate dose was selected based on 1) our unpublished results that intraperitoneal injection of sodium benzoate blocked formalin-induced hyperalgesia in a dose-dependent manner in rats with an ED50 of 436 mg/kg (W.-J. Zhao and Y.-X. Wang, unpublished data) and 2) our previous reports that systemic administration of 400 mg/kg sodium benzoate completely inhibited kidney DAO activity and in vivo chiral inversion of N^{G}-nitro-l-arginine in rats (Xin et al., 2005, 2007). After intraperitoneal injection of normal saline, mechanical allodynia remained unchanged during the 180-min observation period. Sodium benzoate by intraperitoneal administration produced significant elevation of the thresholds of mechanical allodynia. The effect of sodium benzoate was time-dependent, with onset at 30 min or earlier and duration

**Fig. 1.** Time courses of mechanical allodynia development, increased spinal DAO gene expression, and increased enzymatic activity in rats with L5/L6 spinal nerve ligation (SNL). A, ipsilateral hind paw withdrawal thresholds in sham and L5/L6 spinal nerve-ligated rats (*n* = 12 in each group). B, spinal DAO mRNA expression measured by real-time quantitative PCR in sham and L5/L6 spinal nerve-ligated rats (*n* = 6 in each time point). C, spinal DAO activity measured by keto acid method in sham and L5/L6 spinal nerve-ligated rats (*n* = 6 in each time point). Data are presented as means ± S.E.M., *p*, statistically significant difference (*P* < 0.05) compared with sham-operated rats at the same time point; one-way ANOVA.

of at least 60 min. No apparent motor side effects of sodium benzoate at this dose were seen during the observation period. Intraperitoneal injection of morphine also blocked mechanical allodynia as expected (Fig. 2A).

The effect of sodium benzoate on hyperalgesia was tested in the rat formalin test. Three groups of rats (*n* = 8) received intraperitoneal injections of normal saline (4 ml/kg), sodium benzoate (400 mg/kg), and morphine (10 mg/kg), respectively. A subcutaneous injection of 5% formalin (100 μl) was then received 30 min later. Subcutaneous injection of formalin in normal saline-treated control rats produced characteristic...
biphasic flinch responses consisting of an early phase (within 10 min after formalin injection), followed by a slowly rising and long-lasting (10–90 min) late phase (i.e., hyperalgesia). As shown in Fig. 2B, intraperitoneal injection of sodium benzoate did not significantly inhibit flinch responses in the early phase. In contrast, sodium benzoate produced a significant inhibition of the hyperalgesic response in the late phase. No apparent motor side effects of sodium benzoate at this dose or even up to 1000 mg/kg (data not shown) were observed during the observation period. Morphine by intraperitoneal injection significantly blocked flinch responses in both the early phase and late phases.

The effect of sodium benzoate on acute nociceptive pain was also observed in the rat tail-flick test. The thresholds of tail flick were measured before and 30, 60, 90, 120, 150, and 180 min after intraperitoneal injection of normal saline (4 ml/kg), sodium benzoate (400 mg/kg), or morphine (10 mg/kg). Thermal nociceptive thresholds in normal saline-treated rats remained stable throughout the testing period. Intraperitoneal injection of morphine blocked nociceptive responses in a time-dependent manner. On the other hand, intraperitoneal injection of sodium benzoate did not affect thermal nociceptive thresholds or produce motor side effects (Fig. 2C).

Inhibition of Systemic Sodium Benzoate on Spinal DAO Activity. DAO activity in the lumbar enlargement of the spinal cord was measured to determine whether systemic administration of sodium benzoate exerted its analgesic effect through inhibition of DAO activity in the spinal cord. Rats (n = 6 in each group) were sacrificed, and the lumbar enlargements of the spinal cord were prepared 60 and 120 min after intraperitoneal injection of normal saline (4 ml/kg) or sodium benzoate (400 mg/kg). The enzymatic activity was assayed by measuring pyruvate production from DAO-catalyzed α-alanine oxidation. Intraperitoneal administration of sodium benzoate at 400 mg/kg significantly inhibited DAO activity in the lumbar spinal cord measured at both 60 and 120 min after drug administration (Fig. 3).

Effects of Intrathecal Injection of Sodium Benzoate on Neuropathic Pain, Hyperalgesia, and Nociceptive Pain. The analgesic effects of sodium benzoate were further studied on hyperalgesia and neuropathic pain via direct spinal cord administration. In the model of spinal nerve ligation, neuropathic rats were chronically implanted with intrathecal cannulas 5 to 7 days after spinal nerve ligation surgery. Seven days later, three groups of spinal nerve-ligated rats (n = 8 in each group) received intrathecal injections of normal saline (5 μl/rat), sodium benzoate (30 μg/rat), or morphine (10 μg/rat). The dose of sodium benzoate was selected based on its ED50 value of approximately 10 μg/rat derived from a dose-response curve on formalin-induced hyperalgesia in rats (Z.-Y. Gao and Y.-X. Wang, unpublished data). With-
markedly increased after L5/L6 spinal nerve ligation, with a time course in agreement with the development of neuropathic pain (mechanical allodynia). Second, systemic injection of the DAO inhibitor sodium benzoate specifically blocked neuropathic pain (but not thermal stimuli- or formalin-induced nociceptive pain) via inhibition of DAO activity in the lumbar enlargement of the spinal cord. Third, direct intrathecal (spinal cord) injection of sodium benzoate specifically reduced spinal nerve ligation-induced neuropathic pain but not formalin-induced nociception in a dose of 30 μg/rat.

Last, both systemic and intrathecal administrations of sodium benzoate blocked formalin-induced hyperalgesia, in an inhibitory degree similar to that for spinal nerve ligation-induced neuropathic pain. It is known that both formalin-induced hyperalgesia and spinal nerve ligation-induced neuropathic pain share a common mechanism of central sensitization for pain states (Kim and Chung, 1992; Codere et al., 1993; Jett et al., 1997). Together with the literature revealing that DAO measured by histochemical detection was localized predominantly in the gray matter of the medulla and spinal cord (Kapoor and Kapoor, 1997), our results suggest that spinal DAO significantly contributes to neuropathic pain and is a potential target molecule for the treatment of neuropathic pain. Indeed, SEP-227900, a DAO inhibitor of unknown structure, was reportedly in early stage clinical investigation for the treatment of neuropathic pain (Williams, 2009).

The present findings confirmed our and other laboratories’ reports in which DAO inhibitors blocked formalin-induced hyperalgesia in rats (Li et al., 2000) and mice (Zhao et al., 2008). In addition, we recently expanded the finding by intrathecal administration of a more potent and selective DAO inhibitor AS057278 that blocked formalin-induced hyperalgesia in a dose-dependent manner in rats (Z.-Y. Gao and Y.-X. Wang, unpublished data). The current results are also in agreement with our previous DAO gene mutation/deletion study, in which nociceptive responses in the acetic acid test and the tonic phase of formalin flinch responses were markedly reduced in ddY/DAO(−/−) mice compared with those in ddY/DAO(+/+) mice (Zhao et al., 2008). However, different results from other laboratories were also presented. Formalin-induced hyperalgesia (tonic phase) was reported to be exaggerated in ddY/DAO(−/−) mice compared with that in ddY/DAO(+/+) mice (Wake et al., 2001). Moreover, intrathecal (spinal cord) administration of the exogenous enzyme DAO blocked mechanical allodynesia in the rat model of tetanically sciatic stimulation (Ying et al., 2006). The reason for these opposing results is not known. We have thus used a series of methods including the DAO gene deletion technique and the DAO inhibition technique with at least two DAO inhibitors in both rats and mice, in addition to the measurement of spinal cord DAO expression and activity, to clarify these discrepancies. Our consistent results by systemic approaches, supplemented by the fact that the DAO inhibitor has been in the early clinical stage for investigational treatment of neuropathic pain (Williams, 2009), highly suggests that DAO in the spinal cord is a pronociceptive (rather than antinociceptive) factor for pain states, particularly for neuropathic pain and chronic pain mediated by central (spinal) sensitization.

Central sensitization reflects an increase in excitability of spinal and brain neurons after persistent nociceptive stimulation resulting from nerve injuries and/or inflammation.
(Cook et al., 1987; Woolf et al., 1994). Our observed specific analgesia produced by sodium benzoate suggests that DAO is involved in central (spinal) sensitization in pain, as both pain models used (spinal nerve ligation-induced neuropathic pain and formalin-induced hyperalgesia) are generally believed to be mediated by central sensitization (Kim and Chung, 1992; Codere et al., 1993; Jett et al., 1997). The mechanism for this is not known. It is well noted that a certain level of D-serine is present in the CNS and that D-serine binds to and activates strychnine-insensitive glycine sites associated with N-methyl-D-aspartate (NMDA) receptors (Schell et al., 1995; Ying-Luan et al., 2007). Activation of NMDA receptors is considered to be a major contributor to central sensitization-mediated pain that involves inflammation and/or nerve injury (Petrenko et al., 2003; Ying-Luan et al., 2007). Hashimoto et al. (1993) showed that the concentration of D-serine was higher in the CNS in ddY/DAO(−/−) mice lacking DAO activity. Furthermore, NMDA receptor-mediated synaptic transmission and formalin-induced hyperalgesia were exaggerated in ddY/DAO(−/−) mice lacking DAO activity. Therefore, the combined results as mentioned above do not support this hypothesis. An alternative putative mechanism for the role of DAO in pain may be the generation of high amounts of H2O2 in the spinal cord after its oxidation of neutral and polar D-amino acids such as D-serine (Pollegioni et al., 2007), D-alanine (Moreno et al., 1999), and glycine (De Marchi and Johnston, 1969). Spinal H2O2 has been reported to be involved in central sensitization-mediated pain states including neuropathic pain (Kim et al., 2004, 2006; Viggiano et al., 2005; Hacimuftuoglu et al., 2006; Lee et al., 2007). Further studies are warranted to explore the exact mechanisms for the algiesia action of spinal DAO.

Known inhibitors of DAO include benzoic acid (sodium benzoate), chlorpromazine, pyrrole, and potassium sorbate. Sodium benzoate is a common food preservative and has been used in the treatment of patients with inborn errors of urea synthesis to facilitate the alternative pathway of nitrogen waste excretion (Kubota and Ishizaki, 1991). It has also been shown to be a competitive inhibitor of DAO in vitro and can efficiently attenuate DAO-mediated functions in vivo (Moses et al., 1996; Williams and Lock, 2005; Wu et al., 2006; Xin et al., 2007). Sodium benzoate is widely given in high doses (such as 100–1000 mg/kg) by systemic administration for blockade of DAO activity without reports of nonspecific related biological effects (Moses et al., 1996; Williams and Lock, 2005; Xin et al., 2005, 2007; Wu et al., 2006), because of its low potency (millimolar concentrations in IC50 values) and rapid excretion in urine (Kubota and Ishizaki, 1991; MacArthur et al., 2004; Xin et al., 2007). It is reasonable to speculate that the analgesic action of sodium benzoate may result from its possible nonspecific effect due to high doses. However, this is not the case in our analgesic findings because of the following: 1) systemic administration of sodium benzoate was effective only in central sensitization-mediated neuropathic pain and hyperalgesia but not in acute pain responses with the same observation period; 2) no motor side effects were observed during systemic administration of sodium benzoate; 3) intrathecal injection of sodium benzoate at 30 μg/rat significantly blocked formalin-induced hyperalgesia; and 4) intrathecal injection of as little as 0.3 to 1 μg of the more potent DAO inhibitor AS057278 significantly blocked formalin-induced hyperalgesia (Z.-Y. Gao and Y.-X. Wang, unpublished data). Additional experiments to test the specific effect of DAO by both small interfering RNA to silence DAO gene expression and highly potent DAO inhibitors such as AS057278 are under way.

Neuropathic pain and chronic pain in humans often incapacitate patients and are resistant to conventional narcotic therapies. The search for novel treatments for this class of pain syndromes characterized by central sensitization has stimulated numerous investigations in both the basic science and clinical arenas. The present study indicates that spinal DAO contributes significantly to the development of central sensitization-mediated pain and suggests that DAO may be an important molecular target for the treatment of chronic pain of neuropathic origin.

References
Viggiano A, Monda M, Viggiano D, Viggiano R, Chiefari M, Aurilio C, and


Address correspondence to: Dr. Yong-Xiang Wang, King’s Laboratory, School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Rd., Shanghai 200240, China. E-mail: yxwang@sjtu.edu.cn