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Title Page

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

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Running Title Page

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CNS, central nervous system; DAO, D-amino acid oxidase; H_2O_2, hydrogen peroxide; NMDA, N-methyl-daspartate; PCR, polymerase chain reaction.

Abstract

D-amino acid oxidase (DAO) is an enzyme catalyzing oxidative deamination of neutral and polar D-amino acids and 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. Following L5/L6 spinal nerve ligation, the messager RNA expression (measured by real-time quantitative PCR) 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). Intraperitoneal 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, directly 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 pro-nociceptive (rather than anti-nociceptive) role and might be a target molecule for the treatment of chronic pain of neuropathic origin.

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Introduction

D-amino acid oxidase (DAO) is a peroxisomal flavoprotein that catalyzes with strict stereospecificity the oxidative deamination of neutral and polar D-amino acids to α-keto acids, NH_3 and hydrogen peroxide (H_2O_2) (Angermüller et al., 2009). In the central nervous system (CNS), DAO is restricted to the lower brainstem, cerebellum and spinal cord, decreasing levels in the midbrain, the cortex and hippocampus (Horiike et al., 2001; Kapoor and Kapoor, 1997; Yoshikawa et al., 2004). Recently, we found that DAO gene deletion/mutation by using mutant ddY/DAO$^{-/-}$ mice (compared to ddY/DAO$^{+/+}$ mice) and intravenous administration of the DAO inhibitor sodium benzoate significantly blocked the late phase flinch response (hyperalgesia) of the mouse formalin test. Intravenous administration of sodium benzoate did not affect the acute pain transmission such as in the early phase flinch response in the formalin test, or thermal nociceptive responses in the tail flick test or hot-plate test in mice (Zhao et al., 2008). In agreement with our studies, another non-specific DAO inhibitor chlorpromazine (Yagi et al., 1956) was also reported to block formalin-induced hyperalgesia without affecting the early phase acute pain response (Li et al., 2000).

Subcutaneous injection of formalin produces biphasic behavioral effects in rats, with the early phase reflecting an acute nociceptive state followed by the late phase reflecting a state of persistent hyperalgesia which involves central sensitization (Coderre et al., 1993; Jett et al., 1997). Central sensitization is an increase in

excitability of spinal and brain neurons following persistent nociceptive stimulation. The results with sodium benzoate suggest that DAO may contribute to central sensitization-mediated pain transmission. Neuropathic pain remains one of the most challenging of all neurological conditions and presents a large unmet need for improved therapies. Central sensitization is fundamental to the development of neuropathic pain (Hulsebosch et al., 2009). It is thus possible that DAO is involved in neuropathic pain which shares the common mechanism of central sensitization with formalin-induced hyperalgesia.

The aim of this present study is 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) following L5/L6 spinal nerve ligation; 2) testing the analgesic actions of the competitive DAO inhibitor sodium benzoate by systemic injection in neuropathic rats; 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 to the rat acute nociceptive model in this study. The preliminary result of

this study was presented in the 1st International Conference of D-Amino Acid Research (in Japan) in an abstract form (Wang and Zhao, 2009).

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 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 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 & 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 5-7 days before entering the study. Experimental study groups were assigned in random and the researcher was blind for behavior testing.

The rat model of neuropathic pain. The unilateral ligation of two spinal nerves (L5/L6) was performed under intraperitoneal pentobarbital anesthetized (50 mg/kg) rats as described earlier (Kim and Chung, 1992; Wei et al., 2007). Briefly, left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread. Following 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 (hindlimb withdrawal thresholds in the operated side < 8 g) and with no major motor impairment were selected for further studies.

Behavioral assessment of mechanical allodynia. For assessment of mechanical allodynia, the hindlimb withdrawal threshold evoked by stimulation of the hind paw with a series of calibrated monofilaments (von Frey hairs, 2290 CE, IITC, CA, USA) 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 hindlimb. 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 days after L5/L6 spinal nerve ligation or SHAM-operation. Total RNA was purified by use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was performed on 0.5-1 μg of total RNA using ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd. Osaka, Japan). Real-time PCR was performed with Rotor-Gene 3000 (Corbett Robotics, Australia) using RealmasterMix (SYBR Green I) (Tiangen, Beijing, China) and running 40 cycles of the following protocol: 2-minute predenaturation at 94 ºC, 30-second denaturation at 94 ºC, 30-second annealing at 58 ºC followed by a 40-second extension at 68 ºC. The upstream primers were CCC

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TTT CTG GAA AAG CAC AG (DAO) and CGG CAA GTT CAA CGG CAC AG (GAPDH); the downstream primers were CTC CTC TCA CCA CCT CTT CG (DAO) 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) where $\Delta\Delta Ct = (Ct,_{Target} - Ct,_{Gapdh})_{Time\ x} - (Ct,_{Target} - Ct,_{Gapdh})_{Time\ 0}$.

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 Tris-HC1 (pH 8.2) and centrifuged (4 ºC, 12,000 rpm, 30 min). 200 µl D-alanine (0.1 M dissolved in 0.1 M Tris-HC1 buffer, pH 8.2) was added to 200 µl of supernatant and incubated at 37 ºC for 30 minutes. 25% trichloroacetic acid (200 µl) was then added to the assay mixture, vortexed, and centrifuged (14,000 rpm, 5 min). The supernatant (200 µl) was mixed with 200 µl 2,4-dinitrophenylhydrazine (1 mM in 1 M HCl) and incubated (37 ºC, 10 min). Finally, 400 µl sodium hydroxide (1.5 M) was added, vortexed, and incubated (37 ºC, 10 min). The absorbance was read at 450 nm on an ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., USA). The enzyme activity was expressed as pyruvate production per gram total protein of the lumbar enlargements of the spinal cord.

The rat formalin test. Rats were acclimated individually to the observation cage for 30 min prior to 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 previously described (Wang et al., 2000) and the rat was immediately placed in a 23 cm × 35 cm × 19 cm transparent polycarbonate box. 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 minutes later.

The rat tail flick test. The model SSY-H digital display thermostatic water-bath (Shanghai Sanshen Medical Instrument CO., Ltd., Shanghai, China) was used to maintain a constant water temperature of 50 ± 0.5 ºC. While being placed in a tubular restrainer, the tail of the rat was immersed 5 cm in the water bath as previously described (Wang et al., 2000). The nociceptive threshold was defined as the time required to elicit a flick of the tail. The cut-off time was 30 seconds for tail flick measurement to minimize tissue injury.

Intrathecal catheterization. A polyethylene catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d., Clay Adams, Parsippany, NJ, USA) was inserted into the rat lumbar level of the spinal cord under pentobarbital (50 mg/kg, intraperitoneal injection) anesthesia as described elsewhere (Wei et al., 2007). Following recovery from anesthesia, the placing of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μl followed by a 10 μl of saline for flushing) with a 50-μl micro injector (Shanghai Anting Miro-injector Factory, Shanghai, China). The lidocaine

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test was performed approximately 7 days prior to the start of the drug testing sessions. Only those rats that had no motor impairment before lidocaine injection but had a transit bilateral paralysis of hind limbs following intrathecal administration of lidocaine were selected for the study. For intrathecal administration, the control and test drugs were microinjected with a 50 μl micro injector in a volume of 5 μl followed by a normal saline flush in a volume of 15 μl.

Statistical analysis. The results were expressed as mean ± S.E.M. and statistical significance was evaluated by one-way ANOVA or two-tailed Student's t-test. The statistical significance criterion *P* value was 0.05.

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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) were performed SHAM operation or tight L5/L6 spinal nerve ligation. Tactile sensitivity of each rat's hindpaw (ipsilateral side of the ligated spinal nerves) was tested by measuring withdrawal threshold in response to von Frey monofilaments prior to surgery (-1 day) and 1, 2, 7, 14 days post-surgery. The paw withdrawal threshold for SHAM animals was approximately 55 g similar to the pre-surgical level, while spinal nerve ligated rats showed mechanical allodynia with threshold ≤ 8 g following surgery. The distinct mechanical allodynia on the ipsilateral hindpaw occurred one day or earlier (4 hours 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 enlargement of the spinal cords were excised from either SHAM or L5/L6 spinal nerve ligated rats (n = 6 in each time point) on days of -1, 1, 2, 7 or 14 day after surgery. Expression and enzymatic activity of DAO were then examined by real-time quantitative RT-PCR and the colorimetric method, respectively. As shown in Fig. 1B and 1C, spinal mRNA expression and enzymatic activity of DAO from neuropathic rats were significantly elevated by approxiamately 9-fold and 2-fold respectively, and maitained for at least 14 days, in contrast to SHAM-operated rats. These increases of spinal DAO

expression and activity (to a less 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 10-14 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. 400 mg/kg sodium benzoate 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 ED_{50} of 436 mg/kg (Zhao et al., 2009); 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-D-arginine in rats (Xin et al., 2005, 2007). Following intraperitoneal injection of normal saline, mechanical allodynia remained unchanged during the 180-minutes 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 the onset of 30 minutes or earlier and the duration of at least 60 minutes. No apparent motor side effects of sodium benzoate at this dose were observed 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 injection of normal saline (4 ml/kg), sodium benzoate (400 mg/kg), and morphine (10 mg/kg), respectively. Subcutaneous injection of 5% formalin (100 µl) was then received 30 minutes later. Subcutaneous injection of formalin in normal saline-treated control rats produced a characteristic biphasic flinch responses consisting of an early phase (within 10 minutes 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 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 phase.

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 minutes 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 inhibiting 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 minutes 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 D-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 minutes 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 5-7 days after surgery of spinal nerve ligation. 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 ED_{50} value of approximately 10 μg/rat derived from a dose-response curve on formalin-induced hyperalgesia in rats (Gao et al., unpublished data, 2009).

Withdrawal thresholds of the neuropathic hindlimb were recorded before, and 15, 30, 60, 90, 120, 150, 180 and 240 minutes after intrathecal administration of control and test drugs. Intrathecal treatment with either sodium benzoate or morphine but not normal saline produced significant and time-related elevation of thresholds of mechanical allodynia, with the onset of 15 minutes or earlier and the duration of at least 150 minutes (Fig. 4A). No apparent motor side effects of sodium benzoate were observed during the observation period.

In the formalin test, three groups of rats (n = 8 in each group) chronically implanted with intrathecal cannulas received intrathecal injection of normal saline (5 μl/rat), sodium benzoate (30 μg/rat) or morphine (10 μg/rat) 10 minutes before formalin injection. Compared with the normal saline control, intrathecal injections of both sodium benzoate and morphine produced significant inhibition of formalin-induced hyperalgesia in the late phase but not the flinch response (acute nociception) in the early phase (Fig. 4B).

Discussion

The present study for the first time provides evidence that spinal DAO is a pro-nociceptive factor in neuropathic pain in rats. First, both DAO mRNA expression and enzymatic activity in the lumbar enlargement of the spinal cord were markedly increased following 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. Lastly, both systemic and intrathecal administrations of sodium benzoate blocked formalin-induced hyperalgesia, in a similar inhibitory degree with 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; Coderre et al., 1993; Jett et al., 1997). Together with the literature that revealed that DAO measured by histochemical detection was localized predominantly in the grey 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 where 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 (Gao et al., unpublished data, 2009). 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 to ddY/DAO+/+ mice (Zhao et al., 2008). However, different results were also presented from other laboratories. Formalin-induced hyperalgesia (tonic phase) were reported to be exaggerated in ddY/DAO-/- mice compared to ddY/DAO+/+ mice (Wake et al., 2001). Moreover, intrathecal (spinal cord) administration of exogenous enzyme of DAO blocked mechanical allodynia in the rat model of tetanically sciatic stimulation (Ying et al., 2006). The reason for these opposing results is not known. We have thus employed a series of methods including the DAO gene deletion technique and the DAO inhibition technique by using 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), is highly suggestive that DAO in the spinal cord is a pro-nociceptive (rather than anti-nociceptive) 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 following 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 suggest that DAO is involved in central (spinal) sensitization in pain, as both employed pain models (spinal nerve ligation-induced neuropathic pain and formalin-induced hyperalgesia) are generally believed to be mediated by central sensitization (Kim and Chung, 1992; Coderre et al., 1993; Jett et al., 1997). The mechanism for this is not known. It is well noted that certain level of D-Serine is present in the CNS and D-Serine binds to and activates strychnine-insensitive glycine sites associated with N-methyl-daspartate (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-maintained pain that involve 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 (Wake et al., 2001). These results suggested that DAO exerted a neuromodulatory function by controlling the

concentration of D-Serine in the CNS (Wake et al., 2001). However, our combined results as mentioned above do not support this hypothesis. Alternative putative mechanism for the role of DAO in pain may be due to generation of high amount of H_2O_2 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 H_2O_2 has been reported to be involved in central sensitization-mediated pain states including neuropathic pain (Hacimuftuoglu et al., 2006; Kim et al., 2004, 2006; Lee et al., 2007; Viggiano et al., 2005). Further studies are warranted to explore exact mechanisms for algesic 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 in order 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 reporting non-specific related biological effects (Moses et al., 1996; Williams and Lock, 2005; Wu et al., 2006; Xin et al., 2005, 2007), due to its low potency (mM 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 non-specific effect

due to high doses. However, this is not the case in our analgesic findings as 1) systemically administration of sodium benzoate was effective only in central sensitization-maintained 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; 4) intrathecal injection of the more potent DAO inhibitor AS057278 as little as 0.3-1 μg significantly blocked formalin-induced hyperalgesia (Gao et al., unpublished data). Further performance to test the specific effect of DAO by both siRNA 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 arena. The present study indicates that spinal DAO contributes significantly to the development of central sensitization-mediated pain and suggests that DAO might be an important molecular target for the treatment of chronic pain of neuropathic origin.

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Footnotes

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Legends for Figures

Fig. 1. Time-courses of mechanical allodynia development, increased spinal D-amino acid oxidase (DAO) gene expression, and increased enzymatic activity in rats with L5/L6 spinal nerve ligation (SNL). **A.** Ipsilateral hindpaw 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. * denotes statistically significant difference ($P < 0.05$) compared with SHAM-operated rats at the same time point; one-way ANOVA.

Fig. 2. Effects of intraperitoneal injection of the D-amino acid oxidase (DAO) inhibitor sodium benzoate (400 mg/kg) on neuropathic pain, hyperalgesia and nociceptive pain in rats (n = 8 in each group). **A.** Mechanical allodynia in L5/L6 spinal nerve ligated rats. The ipsilateral hindlimb withdrawal thresholds to mechanical stimuli were recorded at different time points. **B.** Hyperalgesia in the rat formalin test. Control and test drugs were given 30 minutes before subcutaneous injection of formalin. Flinch measurements were taken after a 1-min observation period. **C.** Acute nociceptive pain in the rat tail flick test. The thresholds of tail flick were measured at the hot-water bath (50 ± 0.5 °C). Data are presented as

means ± S.E.M. * denotes statistically significant difference ($P < 0.05$) compared with normal saline control rats; one-way ANOVA.

Fig. 3. Inhibition of D-amino acid oxidase (DAO) activity in the rat lumber enlargements of the spinal cord by intraperitoneal injection of sodium benzoate (400 mg/kg). The DAO enzymatic activity was expressed utilizing pyruvate production as a read-out per gram total protein. N = 6 in each group. Data are presented as means ± S.E.M. * denotes statistically significant difference ($P < 0.05$) compared with normal saline controls; two-tailed Student's t-test.

Fig. 4. Effects of intrathecal injection of sodium benzoate (30 μg/rat) on neuropathic pain, hyperalgesia, and nociceptive pain in rats (n = 8 in each group). **A.** Mechanical allodynia in the rat L5/L6 spinal nerve ligation model of neuropathic pain. The ipsilateral hindlimb withdrawal thresholds to mechanical stimuli in L5/L6 spinal nerve legated rats were recorded at different time points. **B.** Acute nociceptive pain and hyperalgesia in the rat formalin test. Control and test drugs were intrathecally injected 10 minutes before subcutaneous injection of formalin. Flinch measurements were taken after a 1-min observation period. Data are presented as means ± S.E.M. * denotes statistically significant difference ($P < 0.05$) compared with normal saline controls; one-way ANOVA.

Figure 1

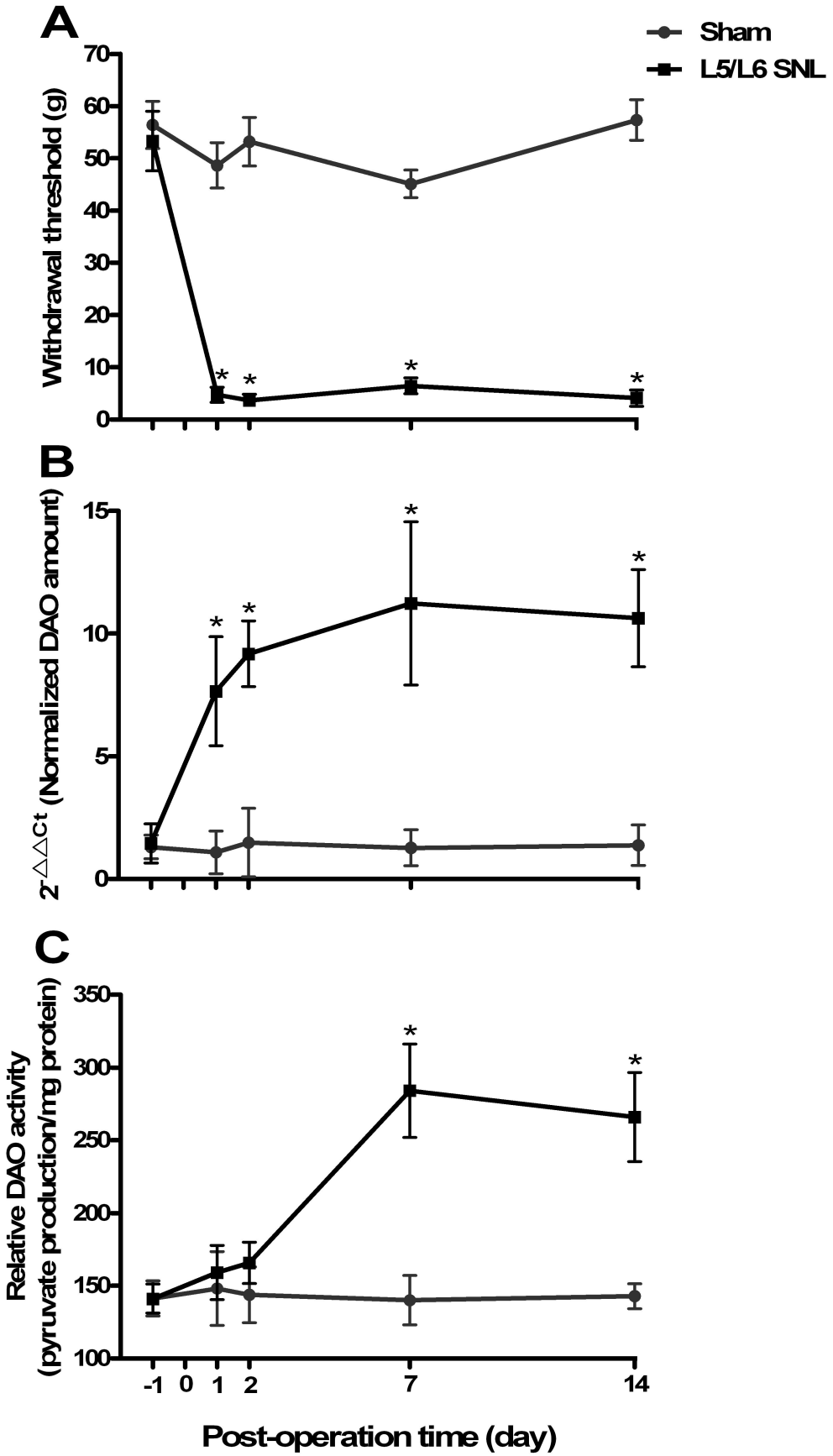

A
Sham
L5/L6 SNL
Withdrawal threshold (g)
70
60
50
40
30
20
10
0
*
*
*
*
B
2^-△△Ct (Normalized DAO amount)
15
10
5
0
*
*
*
*
C
Relative DAO activity
(pyruvate production/mg protein)
350
300
250
200
150
100
*
*
-1
0
1
2
7
14
Post-operation time (day)

Figure 2

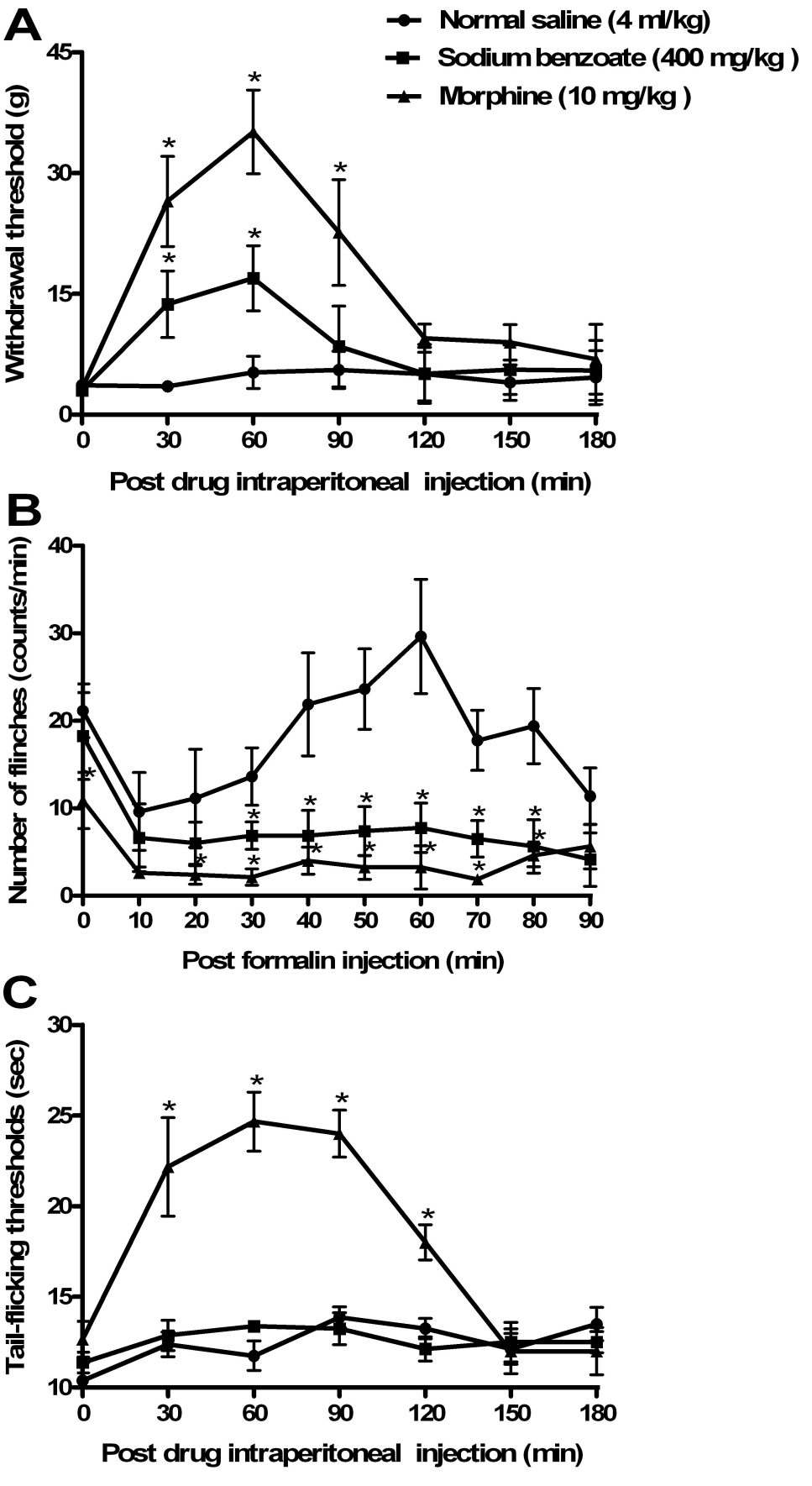

A
Normal saline (4 ml/kg)
Sodium benzoate (400 mg/kg)
Morphine (10 mg/kg)
Withdrawal threshold (g)
45
30
15
0
0
30
60
90
120
150
180
Post drug intraperitoneal injection (min)
B
Number of flinches (counts/min)
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Post formalin injection (min)
C
Tail-flicking thresholds (sec)
30
25
20
15
10
0
30
60
90
120
150
180
Post drug intraperitoneal injection (min)

Figure 3

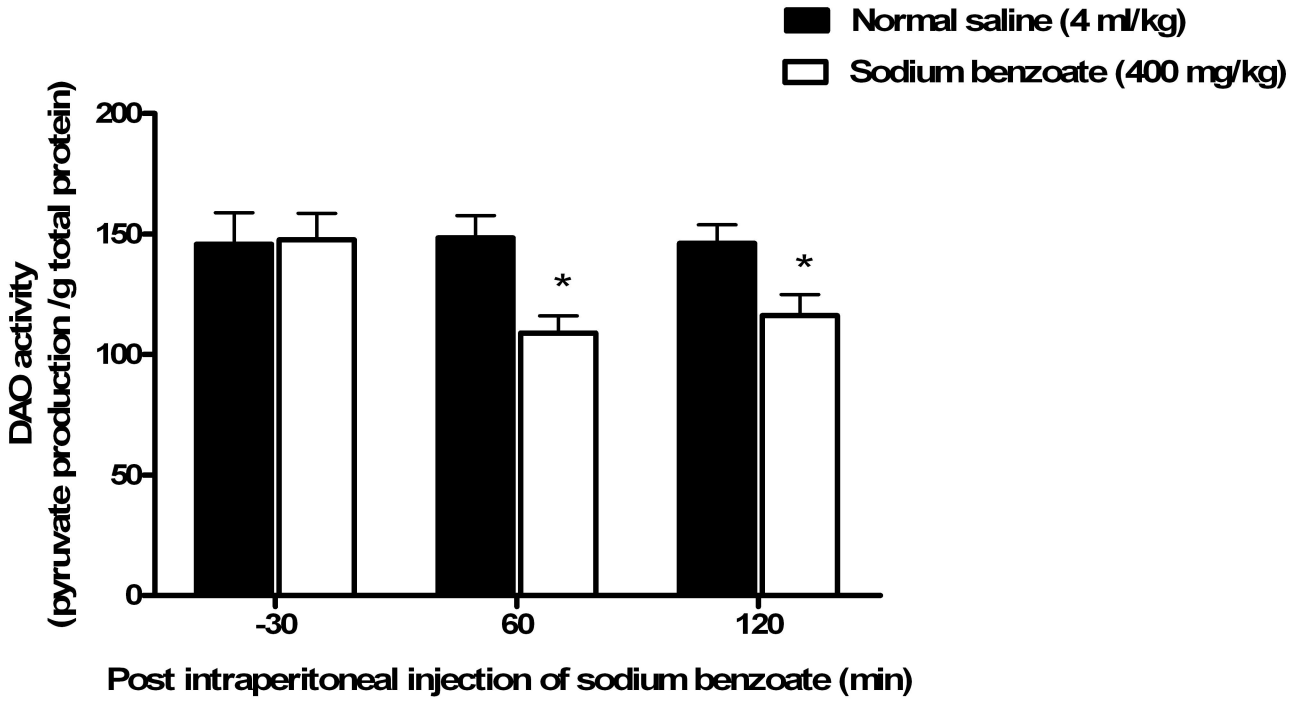
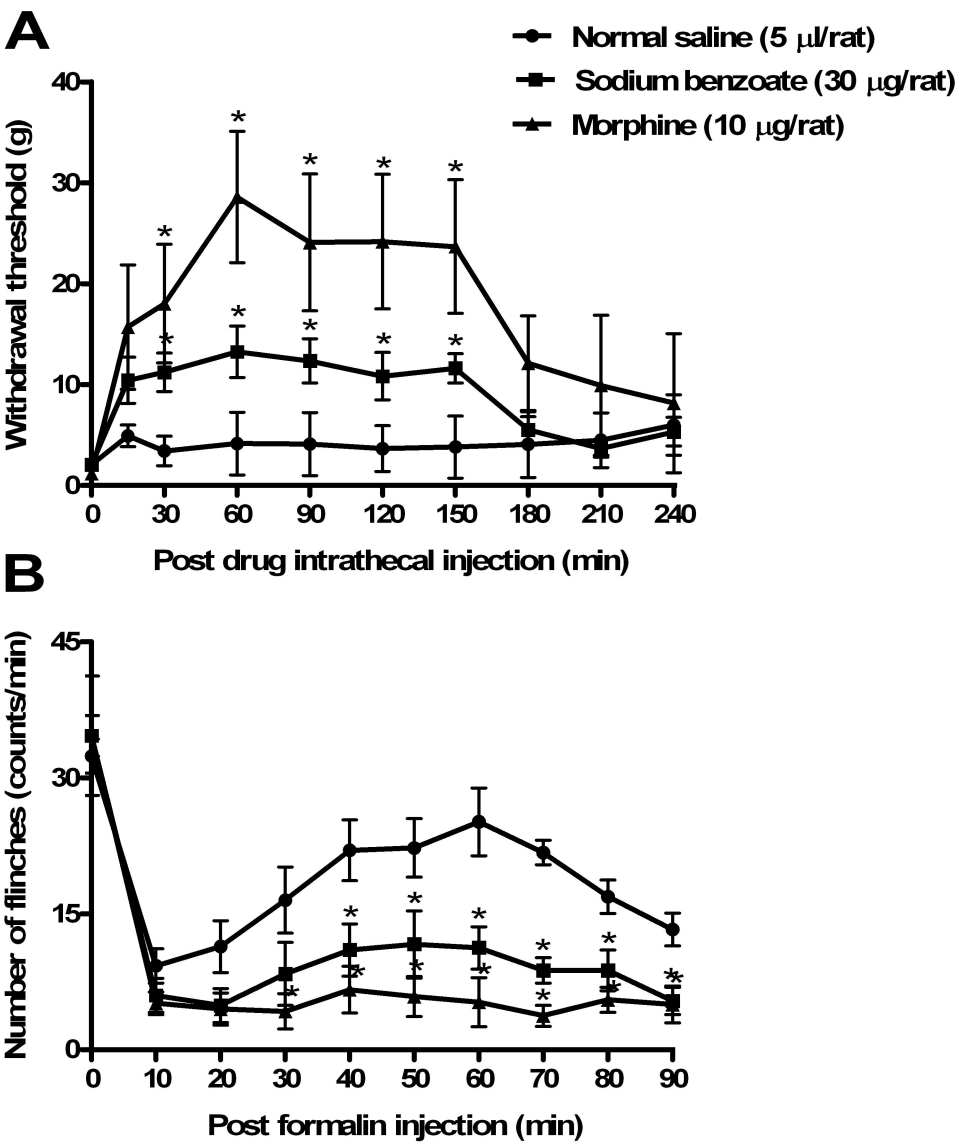

Normal saline (4 ml/kg)
Sodium benzoate (400 mg/kg)
DAO activity
(pyruvate production /g total protein)
200
150
100
50
0
*
*
-30
60
120
Post intraperitoneal injection of sodium benzoate (min)

Figure 4


A
Normal saline (5 μl/rat)
Sodium benzoate (30 μg/rat)
Morphine (10 μg/rat)
Withdrawal threshold (g)
40
30
20
10
0
0
30
60
90
120
150
180
210
240
Post drug intrathecal injection (min)
B
Number of flinches (counts/min)
45
30
15
0
0
10
20
30
40
50
60
70
80
90
Post formalin injection (min)