

# Identification of a Novel Spinal Dorsal Horn Astroglial D-Amino Acid Oxidase–Hydrogen Peroxide Pathway Involved in Morphine Antinociceptive Tolerance

Nian Gong, B.S., Xin-Yan Li, Ph.D., M.D., Qi Xiao, M.S., Yong-Xiang Wang, Ph.D., M.D.

## ABSTRACT

**Background:** D-Amino acid oxidase (DAAO) is a flavin adenine dinucleotide-dependent peroxisomal flavoenzyme which is almost exclusively expressed within astrocytes in the spinal cord. DAAO catalyzes oxidation of D-amino acids to hydrogen peroxide, which is a stable and less active reactive oxygen species, and may represent a final form of reactive oxygen species. This study tested the hypothesis that the spinal astroglial DAAO–hydrogen peroxide pathway plays an important role in the development of morphine antinociceptive tolerance.

**Methods:** Rat and mouse formalin, hot-plate, and tail-flick tests were used, and spinal DAAO expression and hydrogen peroxide level were measured. Sample size of animals was six in each study group.

**Results:** Subcutaneous and intrathecal DAAO inhibitors, including 5-chloro-benzo[*d*]isoxazol-3-ol, AS057278, and sodium benzoate, completely prevented and reversed morphine antinociceptive tolerance in the formalin, hot-plate, and tail-immersion tests, with a positive correlation to their DAAO inhibitory activities. Intrathecal gene silencers, small interfering RNA/DAAO and small hairpin RNA/DAAO, almost completely prevented morphine tolerance. Intrathecal 5-chloro-benzo[*d*]isoxazol-3-ol and small interfering RNA/DAAO completely prevented increased spinal hydrogen peroxide levels after chronic morphine treatment. Intrathecal nonselective hydrogen peroxide scavenger phenyl-tert-*N*-butyl nitron and the specific hydrogen peroxide catalyst catalase also abolished established morphine tolerance. Spinal dorsal horn astrocytes specifically expressed DAAO was significantly up-regulated, accompanying astrocyte hypertrophy after chronic morphine treatment.

**Conclusions:** For the first time, the authors' result identify a novel spinal astroglial DAAO–hydrogen peroxide pathway that is critically involved in the initiation and maintenance of morphine antinociceptive tolerance, and suggest that this pathway is of potential utility for the management of morphine tolerance and chronic pain. (**ANESTHESIOLOGY 2014; 120:962-75**)

MORPHINE analgesic tolerance is associated with activation of glia.<sup>1–3</sup> For example, the glial metabolism inhibitor fluorocitrate when delivered in combination with chronic morphine treatment significantly attenuated the development of tolerance to morphine in rodent behavioral pain models.<sup>3</sup> Spinal astrocytes, in response to chronic morphine and injury or inflammation, produce proinflammatory cytokines such as tumor necrosis factor, interleukin-1, and interleukin-6.<sup>1</sup> These glia-derived substances produce numerous neuroexcitatory molecules, including nitric oxide, prostaglandins, excitatory amino acids, adenosine triphosphate, and reactive oxygen species (ROS) in particular.<sup>4</sup> ROS and neuroexcitatory molecules enhance pain *via* multiple effects on neurons of the pain pathway, resulting in neuroadaptations that may directly contribute to morphine analgesic tolerance.<sup>3,5</sup>

ROS, including superoxide anion, peroxyxynitrite, hydroxyl radical, and hydrogen peroxide, are critically involved in morphine analgesic tolerance.<sup>6–11</sup> Pharmacologic removal of these ROS by nonspecific ROS scavengers and antioxidants effectively prevent and reverse morphine antinociceptive tolerance.<sup>11</sup> However, the role of each specific ROS in morphine antinociceptive tolerance has not been determined mainly due

### What We Already Know about This Topic

- The activation of glia in the spinal cord is associated with analgesic tolerance to drugs such as morphine

### What This Article Tells Us That Is New

- The results of these studies suggest that reactive oxygen species such as hydrogen peroxide are generated by spinal astrocytes and are involved in tolerance
- The flavoprotein, D-amino acid oxidase, is an important astrocytic enzyme involved in this process

to the lack of specific measurements of individual ROS and selective agents to block synthesis or remove individual ROS. Among ROS, hydrogen peroxide is probably more important because it has been shown to be more stable and less reactive than other ROS and may represent a final form of ROS.<sup>12</sup>

D-Amino acid oxidase (DAAO) is a flavoprotein which was discovered more than 70 yr ago. In the central nervous system, DAAO activity is present only in astrocytes including Bergmann glial cells,<sup>13–16</sup> although the DAAO immunoreactivity is present in neurons as well.<sup>17,18</sup> With a strict stereospecificity and broad yet differential substrate specificity,

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DAAO catalyses oxidative deamination of neutral and polar D-amino acids, such as D-alanine, D-serine, D-proline, and D-arginine, leading to their corresponding hydroxyl  $\alpha$ -keto acids and hydrogen peroxide.<sup>12,16,19</sup>

Application of pharmacologic blockade and genetic ablation of the spinal astroglial DAAO would provide a useful means to study the specific role of hydrogen peroxide in the development of morphine antinociceptive tolerance. A series of compounds, such as 5-chloro-benzo[*d*]isoxazol-3-ol (CBIO), "Compound 8" (4*H*-thieno[3,2-*b*]pyrrole-5-carboxylic acid), SUN (4*H*-furo[3,2-*b*]pyrrole-5-carboxylic acid), AS057278 (5-methylpyrazole-3-carboxylic acid), and "Compound 2" (3-hydroxyquinolin-2-(1*H*)-one), with high potency and specificity for pharmacotherapy of schizophrenia and chronic pain in animal studies have been recently designed as novel inhibitors of DAAO.<sup>20–24</sup> Gene silencers targeting DAAO by small interference RNA technology, which takes advantage of high specificity, have emerged to knockdown DAAO gene expression in the mouse cerebellum and rat spinal cord.<sup>25–27</sup>

In this study, we tested the hypothesis that the spinal DAAO–hydrogen pathway plays an important role in the development of morphine antinociception tolerance *via* synthesis of hydrogen peroxide. We systemically studied a series of DAAO inhibitors and gene silencers targeting DAAO on morphine tolerance in the formalin, hot-plate, and tail-flick tests. We also directly measured hydrogen peroxide levels in the spinal cord after chronic morphine treatment and determined its causal relationship to morphine tolerance. We further tested the nonselective hydrogen peroxide scavenger phenyl-tert-*N*-butyl nitron (PBN)<sup>28–30</sup> and hydrogen peroxide decomposition enzyme catalase<sup>28,31</sup> on established morphine tolerance. We finally examined DAAO expression in the spinal cord in naïve and morphine-tolerant conditions. Our results identify a novel spinal DAAO–hydrogen peroxide pathway involved in the development of morphine antinociceptive tolerance, and suggest that the pathway is of potential for the management of morphine tolerance and chronic pain.

## Materials and Methods

### Animals

Adult male Swiss mice weighing 20–30 g and adult male Wistar rats weighing 200–250 g were purchased from Shanghai Laboratory Animal Center (Shanghai, China). After shipment, mice and rats were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle (lights on at 7:00 AM) for 3–7 days allowing acclimatization before experiment. Animals were given free access to food and water *ad libitum*. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy and followed the animal care guidelines of the National Institutes of Health. Efforts were made to reduce the number of animals used and to minimize their suffering. Six animals were assigned randomly in each

experimental study group, and the researchers were blind for behavior testing.

### Chemicals and Reagents

AS057278 and MK-801 were purchased from Sigma-Aldrich (St. Louis, MO), and sodium benzoate was from Sinopharm Group Chemical Reagent Co. (Shanghai, China), whereas CBIO and morphine hydrochloride injection were from Maybridge Chemicals (Cornwall, United Kingdom) and Shenyang First Pharmaceutical Co. (Shenyang, China), respectively. All drugs and reagents were freshly dissolved in sterile normal saline solution with the pH adjusted to 7.4 by adding 1 N NaOH solution as needed.

The small interfering RNA (siRNA) targeting the rat DAAO and its nonspecific oligonucleotide were synthesized by GenePharma Co. (Shanghai, China), according to the designation of Chen *et al.*<sup>26</sup> The 19 nucleotide duplex and 2 unpaired nucleotide overhanging 3' end were as follows: DAAO: 5'-GGA GUG AAG UUC AUC CAU CUU-3'/5'-GAU GGA UGA ACU UCA CUC CUU-3'; nonspecific control: 5'-UUC UCC GAA CGU GUC ACG UUU-3'/5'-ACG UGA CAC GUU CGG AGA AUU-3'. To formulate siRNAs, linear polyetherimide of 25 kDa (Polysciences, Niles Illinois, PA) was dissolved in 5% dextrose diethyl pyrocarbonate-treated water (pH 7.4). One milligram of RNA was dissolved in 1.5 mg polyetherimide in a polyetherimide:RNA ratio of six equivalents of polyetherimide nitrogen per RNA phosphate to form RNA–polymer complexes at room temperature for 20 min.<sup>26</sup>

For the generation of recombinant adenovirus, small hairpin RNA targeting DAAO (shDAAO) and nonspecific oligonucleotide small hairpin RNA (shControl) were designed according to the above sequences and were chemically synthesized by Sangon Biotech Co. (Shanghai, China) with the following sequences: shDAAO: 5'-GATCCGGAGTGAAGTTCATCCATCTTCAAGAGAGATGGATGAAGTTCATCCCTTTT-3'; shControl: 5'-GATCCGCCAGCTGATACTAACTCCTTC AAGAGAGGAGTTAGTATCAGCTGGCTTTT-3'. The sequences form a loop by hydrogen bonding of AT or GC. The oligonucleotides were subcloned into the BamH-I and HindIII sites of the recombinant pDC316-EGFP-U6 adenoviral vector.<sup>26</sup>

### Induction and Expression of Morphine Tolerance to Antinociception

Morphine was given twice daily at 12-h intervals, subcutaneously at 10 mg/kg for 7 days<sup>32</sup> or intrathecally at 50  $\mu$ g per rat for 5 or 7 days<sup>33</sup> as indicated, to establish systemic or spinal antinociceptive tolerance. To evaluate the expression of morphine antinociceptive tolerance, morphine antinociception to chemical or thermal stimuli was assessed 20 min after its acute dose (5 mg/kg or 5  $\mu$ g per rat) given subcutaneously or intrathecally, and the antinociceptive effects of morphine from each group were examined.

### **Behavioral Nociceptive Tests in Rats and Mice**

Mice and rats were acclimated individually to the observation cage for 30 min before the formalin, tail-flick, and hot-plate tests, which were simple, sensitive, and conventionally used behavioral models for determination of morphine antinociceptive tolerance. In addition to mice, rats were used owing to their reliable intrathecal catheterization and injection.

**The Rat and Mouse Formalin Test.** The formalin test was performed as previously described<sup>20,32,34</sup> by injecting 50  $\mu$ l (for rats) or 10  $\mu$ l (for mice) of 5% formalin in 0.9% saline subcutaneously on the dorsal side of the right hind paw, and the animal was immediately placed in a transparent polycarbonate box. For rats, nociceptive behavior was manually quantified by counting the number of the formalin-injected paw flinches in 1-min epochs, and measurements were taken at 10-min intervals beginning immediately after formalin injection and ending 90 min later. For mice, the duration of nociceptive behaviors (lick/biting) was manually quantified in the pooled durations at 0–5 min and 20–40 min, which were considered as the acute nociception and tonic pain, respectively.

**The Mouse Tail-flick and Hot-plate Tests.** Two tests were subsequently performed with the same animals according to the previous description with modifications.<sup>32,35</sup> For the tail-flick test, the model SSY-H digital display thermostatic water bath (Shanghai Sanshen Medical Instrument Co., Shanghai, China) was used to maintain a constant water temperature of  $50 \pm 0.5^\circ\text{C}$ . Although the mice were placed in a tubular restrainer, its tail was immersed 3.5 cm in the water bath. The nociceptive threshold was defined as the time required for eliciting a flick of the tail. To minimize the tissue injury, the cutoff time was set 30 s for tail-flick measurement.

For the hot-plate test, pain reflexes in response to thermal stimulus were measured by using YLS-6B Intelligence Hot Plate Analgesia Meter (Shandong Academy of Medical Sciences Device Co., Shandong, China). The surface of the hot plate was heated to a constant temperature at  $55 \pm 0.1^\circ\text{C}$ , as measured by a built-in digital thermometer with an accuracy of  $0.1^\circ\text{C}$  and verified by a surface thermometer. Mice were placed on the hot plate, which was surrounded by a clear acrylic cage, and the start or stop button on a timer was activated. The latency to respond with hind paw lick (mainly), hind paw flick, or jump (whichever came first) was measured to the nearest 0.1 s by deactivating the timer when the response was observed. To avoid tissue damage, trials were terminated if the animals did not respond within the 40-s cutoff period.

### **Rat Intrathecal Catheterization**

An 18-cm polyethylene catheter (polyethylene tubing-10: 0.28 mm in inner diameter and 0.61 mm in outside diameter; Clay Adams, Parsippany, NJ) with a volume of 13  $\mu$ l was inserted into the rat lumbar level of the spinal cord as described elsewhere<sup>20</sup> under inhaled isoflurane anesthesia (4% for induction and 1% for maintenance) run by

an anesthesiometer (Ugo Basile Gas Anesthesia System, Comerio, Italy). Two days after recovery from anesthesia, the correct intrathecal cannula placement was verified by administering 4% lidocaine (10  $\mu$ l followed by 15  $\mu$ l of saline for flushing) with a 50- $\mu$ l micro injector (Shanghai Anting Micro-Injector Factory, Shanghai, China). Only rats that had no motor impairment after intrathecal catheterization were considered for the study; only rats that developed immediate bilateral paralysis of hind limbs after intrathecal administration of lidocaine were selected for the study.

### **Hydrogen Peroxide Detection Assay**

Rats were killed by decapitation, and their spinal lumbar enlargements were taken (1 g per 5 ml) and homogenized (4,000 rpm) for 10 s by using a homogenizer (Fluko Equipment Co., Shanghai, China) in 10 mM of Tris-HCl (pH, 7.4) and centrifuged (4,000 rpm) at  $4^\circ\text{C}$  for 15 min. Hydrogen peroxide concentrations in the spinal cord homogenates were determined using a commercial fluorescence Amplex Red Hydrogen Peroxide assay kit (Invitrogen, Shanghai, China), which is a sensitive and chemically stable assay with selectivity for hydrogen peroxide.<sup>36</sup> In brief, the supernatant was mixed with the reaction solution containing Amplex Red (100  $\mu$ mol/l) and horseradish peroxidase (0.2 U/ml) and was incubated at room temperature for 10 min. Fluorescence values were read at excitation and emission wavelength of 560 and 590 nm, respectively, on a Varioskan Flash spectral scanning multimode reader (Thermo Labsystems, Vantaa, Finland). The hydrogen peroxide concentration was calculated by using a standard curve and was normalized to tissue protein as measured by a standard bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Jiangsu, China).

### **Immunofluorescence Staining and Quantification of DAAO-Immunopositive Cell Profiles**

Double immunofluorescence labeling of DAAO and astrocytes, neurons, or microglia were performed on spinal cord sections and observed using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Rats ( $n = 6$  in each group) were anesthetized with pentobarbital injection (50 mg/kg) and perfused intracardially with 500 ml normal saline followed by 300 ml 4% paraformaldehyde (wt/vol) in phosphate buffered saline.<sup>37</sup> Spinal lumbar enlargements were collected and fixed in 4% buffered paraformaldehyde at  $4^\circ\text{C}$  for 12 h and cut into 20- $\mu$ m thick coronal frozen sections, which were incubated with the anti-DAAO antibody (1:400, sheep polyclonal, ab24200; Abcam, Cambridge, MA) and other appropriate primary antibodies at  $4^\circ\text{C}$  for 24 h. Identification of DAAO-positive cells was performed using the following marker antibodies: neuronal nuclei (NeuN, 1:60, mouse polyclonal, MAB377; Millipore, Billerica, MA) for mature neurons, OX42 (CD11b/c or integrin  $\alpha$ M, 1:100, mouse polyclonal, ab1211; Abcam) for microglia, and glial fibrillary acidic protein (GFAP, 1:100, mouse polyclonal, MAB360; Millipore) for astrocytes. DAAO was

visualized with the Alexa 555–conjugated goat anti-sheep secondary antibody (1:200; Invitrogen), whereas other marker antibodies were detected with the Alexa 488–conjugated goat anti-mouse secondary antibody (1:200; Invitrogen). All antibodies were tested for the optimal dilution, the absence of cross-reactivity, and nonspecific staining.

For quantity measurement of immunofluorescent intensity, photomicrographs of the medial three quarters of the superficial dorsal horn (laminae I–V) were captured under a  $\times 5$  magnification. Positively stained surface area was measured using a computer-assisted image analysis program (Image J Software; National Institutes of Health, Bethesda, MD) after low and high thresholds were set to exclude background fluorescence and include immunofluorescent intensity measurements only from positively stained cell surfaces. Colocalization analysis was performed using Image J software with a Colocalization Finder to generate images in which colocalized pixels appeared as white. Same configuration set up was used to measure all surface areas in each experimental group at the same time. The measured areas were transferred to Excel automatically and calibrated by standardization. Three nonadjacent sections through each lumbar spinal cord were randomly selected; DAAO-, GFAP-, and double DAAO/GFAP-immunolabeled surface areas were measured in laminae I–V of the spinal dorsal horn, respectively, by a researcher blinded to the experimental conditions. The average percentage of immunolabeled surface area was the fraction of the positive immunofluorescent surface area of total measured picture area from three sections of each spinal cord. Data were then calculated from six animals of each group.

### Data Analysis and Statistical Evaluation

For dose–response curve analysis, the parameters, *i.e.*, minimum effect ( $E_{\min}$ ), maximum effect ( $E_{\max}$ ), half-effective dose ( $ED_{50}$ ), and Hill coefficient ( $n$ ), were calculated from individual dose–response curves. To determine the parameters of dose–response or concentration–response curves, values of response ( $Y$ ) were fitted by nonlinear least-squares curves to the relation  $Y = a + bx$ , where  $x = [D]^n / (ED_{50}^n + [D]^n)$ , to give the value of  $ED_{50}$  and  $b$  ( $E_{\max}$ ) yielding a minimum residual sum of squares of deviations from the theoretical curve.<sup>38,39</sup> For the correlation between potencies of DAAO inhibitors on DAAO enzymatic activity and morphine antinociceptive tolerance, lineal correlation coefficient was calculated.

The results are expressed as mean  $\pm$  SEM, and there were no data missing. Statistical significance was evaluated by an unpaired Student  $t$  test or a one-way or repeated measures two-way ANOVA by using Prism (version 5.01; GraphPad Software Inc., San Diego, CA). *Post hoc* Student–Newman–Keuls test was followed when a statistically significant drug (dose) effect (one-way ANOVA, factor is drug [dose]) or the drug (dose)  $\times$  time interaction (repeated measures two-way ANOVA, factors are drug [dose], time and their interaction) was observed. Probability values were two-tailed, and the statistical significance criterion  $P$  value was 0.05.

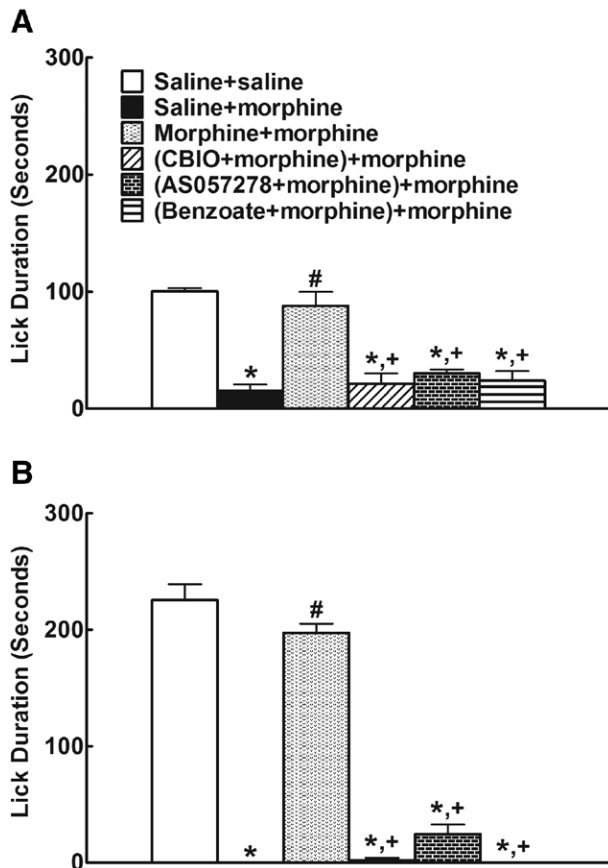
## Results

### Pharmacologic Blockade and Genetic Ablation of DAAO Prevent Morphine Antinociceptive Tolerance

We tested the hypothesis that blockade of DAAO would lead to attenuation or prevention of the development of antinociceptive tolerance after chronic morphine administration. The DAAO inhibitors with different chemical structures (CBIO, AS057278, and sodium benzoate) were used in the mouse formalin test. Six groups of mice ( $n = 6$  in each group) received multiple bi-daily subcutaneous injections of saline (10 ml/kg), morphine (10 mg/kg), and the combination of morphine (10 mg/kg) and CBIO (10 mg/kg), AS057278 (40 mg/kg), or benzoate (400 mg/kg), respectively, for 7 days. On the eighth day, single subcutaneous injection of saline (10 ml/kg) or morphine (10 mg/kg) was performed in these mice 20 min before formalin challenge. Formalin produced an acute phase nociception (fig. 1A) and tonic phase pain (fig. 1B) in mice that received a 7-day saline. Single subcutaneous injection of morphine (5 mg/kg) completely suppressed formalin-induced nociceptive responses, whereas multiple bi-daily injection of morphine induced tolerance to antinociception. Compared with morphine-tolerant mice, multiple bi-daily coadministration of CBIO, AS057278, and sodium benzoate completely prevented morphine tolerance ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test). It should be noted that DAAO inhibitors were not effective in reducing formalin-induced acute nociception,<sup>20</sup> and that their antinociceptive effects in chronic pain lasted less than 4 h.<sup>27,32</sup> These negative effects excluded the possibility that prevention of morphine tolerance by DAAO inhibitors was due to their acute antinociceptive effect.

To further validate the role of DAAO in mediating morphine antinociceptive tolerance by a method distinct from the chemical biology technology, we used siRNA technique to knockdown spinal DAAO gene expression. Five groups of intrathecally cannulated rats ( $n = 6$  in each group) received the combination of multiple bi-daily subcutaneous injections of saline (1 ml/kg) or morphine (10 mg/kg) and multi-daily intrathecal injections of polyetherimide (7.5  $\mu$ g), the nonspecific oligonucleotide (5  $\mu$ g) or siRNA/DAAO (5  $\mu$ g) in polyetherimide complexation for 7 days before formalin challenge. Two more groups of intrathecally cannulated rats ( $n = 6$  in each group) also received the combination of the abovementioned multiple bi-daily injections of morphine with a single intrathecal injection of shControl or shDAAO expressed in adenoviral vectors ( $1.9 \times 10^{12}$ ) just before multiple morphine treatments. On the eighth day, rats received a single subcutaneous injection of saline (1 ml/kg) or morphine (5 mg/kg) 20 min before the formalin test. Intrathecal injections of siRNA/DAAO and shDAAO have been previously shown to effectively block gene transcription, protein expression, and enzymatic activity of DAAO by 50–80%.<sup>26,27</sup>

As shown in figure 2A, subcutaneous injection of formalin in control rats receiving multi-daily intrathecal injections of saline or polyetherimide produced characteristic



**Fig. 1.** Preventive effects of subcutaneous injection of D-amino acid oxidase inhibitors on morphine antinociception tolerance in formalin-induced acute nociception (A) and tonic pain (B). Mice received multiple bi-daily subcutaneous injections of saline (10 ml/kg), morphine (10 mg/kg), and the combination of morphine (10 mg/kg) and 5-chloro-benzo[d]isoxazol-3-ol (CBIO; 10 mg/kg), AS057278 (40 mg/kg), or sodium benzoate (400 mg/kg), respectively, for 7 days. The single injection of saline (10 ml/kg) or morphine (5 mg/kg) was made on the eighth day at 20 min before formalin challenge. Cumulated biting duration from 0 to 5 min and 20 to 40 min after formalin injection represented acute phase nociception and tonic phase pain, respectively. Data are presented as means  $\pm$  SEM ( $n = 6$  in each group). \*, #, + Denote statistically significant difference from the saline + saline group, saline + morphine group, and morphine + morphine group, respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

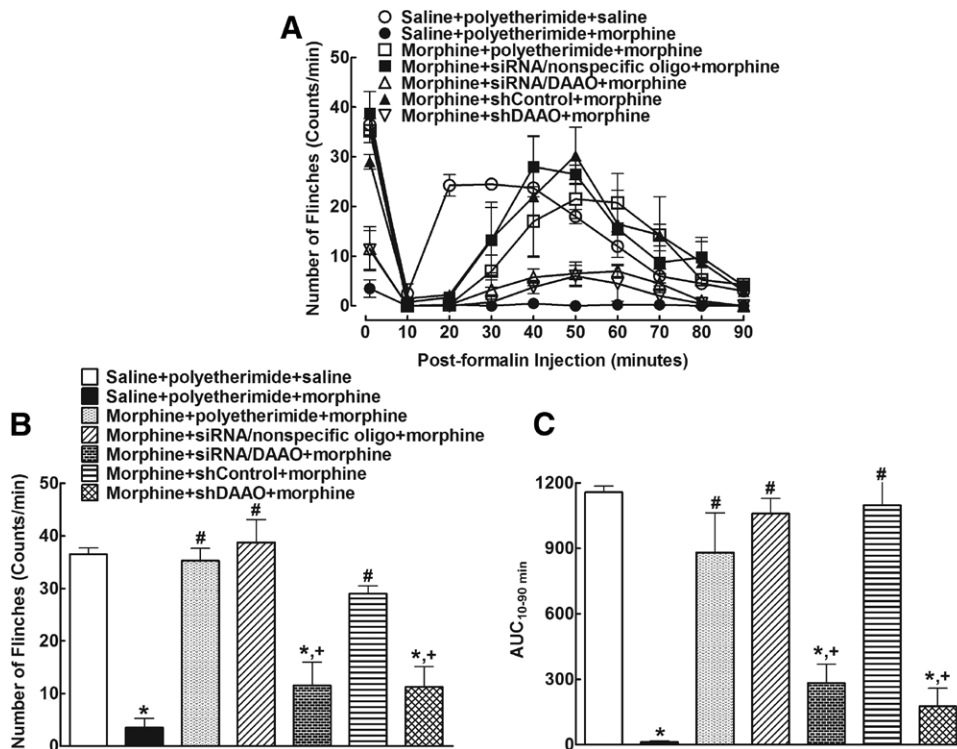
acute and tonic flinching responses, and single subcutaneous injection of morphine (5 mg/kg) completely inhibited both bi-phasic flinching responses. In contrast, multiple bi-daily injections of morphine induced tolerance to antinociception. Compared with morphine-tolerant rats cotreated with saline, intrathecal injection of polyetherimide, nonspecific oligonucleotide, or shControl was not effective in altering morphine tolerance. In contrast, intrathecal injections of both siRNA/DAAO and shDAAO markedly prevented morphine tolerance to antinociception. Measured by area under curve (AUC) over 10–90 min, the protective rates

of siRNA/DAAO and shDAAO were 68.5 and 69.2% in acute nociception (fig. 2B) and 75.6 and 84.9% in tonic pain (fig. 2C), respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test). It was also noted that the DAAO gene silencers were not effective in reducing formalin-induced acute nociception,<sup>26</sup> and thus their blockade of morphine tolerance was not due to antinociceptive effects.

### Pharmacologic Blockade of DAAO Reverses Established Morphine Tolerance

To test whether DAAO inhibition reversed established morphine antinociceptive tolerance, we examined systemic injection of the DAAO inhibitors in the hot-plate and tail-flick tests. Thirteen groups of mice ( $n = 6$  in each group) received subcutaneous injections of saline (10 ml/kg) or morphine (10 mg/kg) twice daily at 12-h intervals for 7 days. On day 8, mice received a single subcutaneous administration of saline (10 ml/kg), CBIO (1, 3, or 10 mg/kg), AS057278 (3, 10, or 30 mg/kg), or sodium benzoate (10, 30, 100, or 300 mg/kg) 20 min before saline (10 ml/kg) or morphine (5 mg/kg) challenge. The latency for tail-flick or paw-lick response was measured before, 0.5, 1, and 2 h after saline or morphine injection. Compared with the vehicle group, single subcutaneous injection of morphine reversibly increased the latency for tail-flick (fig. 3, A–C) or paw-lick (mainly) response (fig. 3, D–F), with peak effects at 0.5 h postinjection in multiple saline-treated naïve mice, whereas multiple bi-daily injections of morphine induced tolerance to antinociception. Single subcutaneous injection of CBIO (fig. 3, A and D), AS057278 (fig. 3, B and E), and sodium benzoate (fig. 3, C and F) dose-dependently restored morphine antinociceptive response in morphine-tolerant mice. AUCs over 2 h were calculated for dose–response analysis. Maximum reversal rates of these inhibitors were approximately 100%, and the  $ED_{50}$  values for CBIO, AS057278, and sodium benzoate were 1.0, 3.2, and 44.9 mg/kg in the tail-flick test (fig. 3G) and 1.0, 6.4, and 44.4 mg/kg in the hot-plate test, respectively (fig. 3H). DAAO inhibitors have not been shown to block thermally evoked acute nociception.<sup>20,28,32</sup> Hence, their blockade of morphine tolerance was not due to antinociceptive effects.

We previously demonstrated that CBIO, AS057278, and sodium benzoate inhibited rat spinal cord–derived DAAO enzymatic activity in a concentration-dependent manner, with maximal inhibition of 100%, and their  $IC_{50}$  values of 0.09, 15.4, and 75.4  $\mu$ M, respectively.<sup>20</sup> The correlation analysis of CBIO, AS057278, and sodium benzoate was calculated between these DAAO inhibitory values and reversal rates of morphine tolerance. As shown, the  $IC_{50}$  values of these compounds on enzymatic activities were positively correlated to their  $ED_{50}$  values in morphine tolerance measured in the tail-flick test ( $r^2 = 0.98$ ;  $P = 0.09$  by unpaired and two-tailed Student *t* test; fig. 3I) and hot-plate test



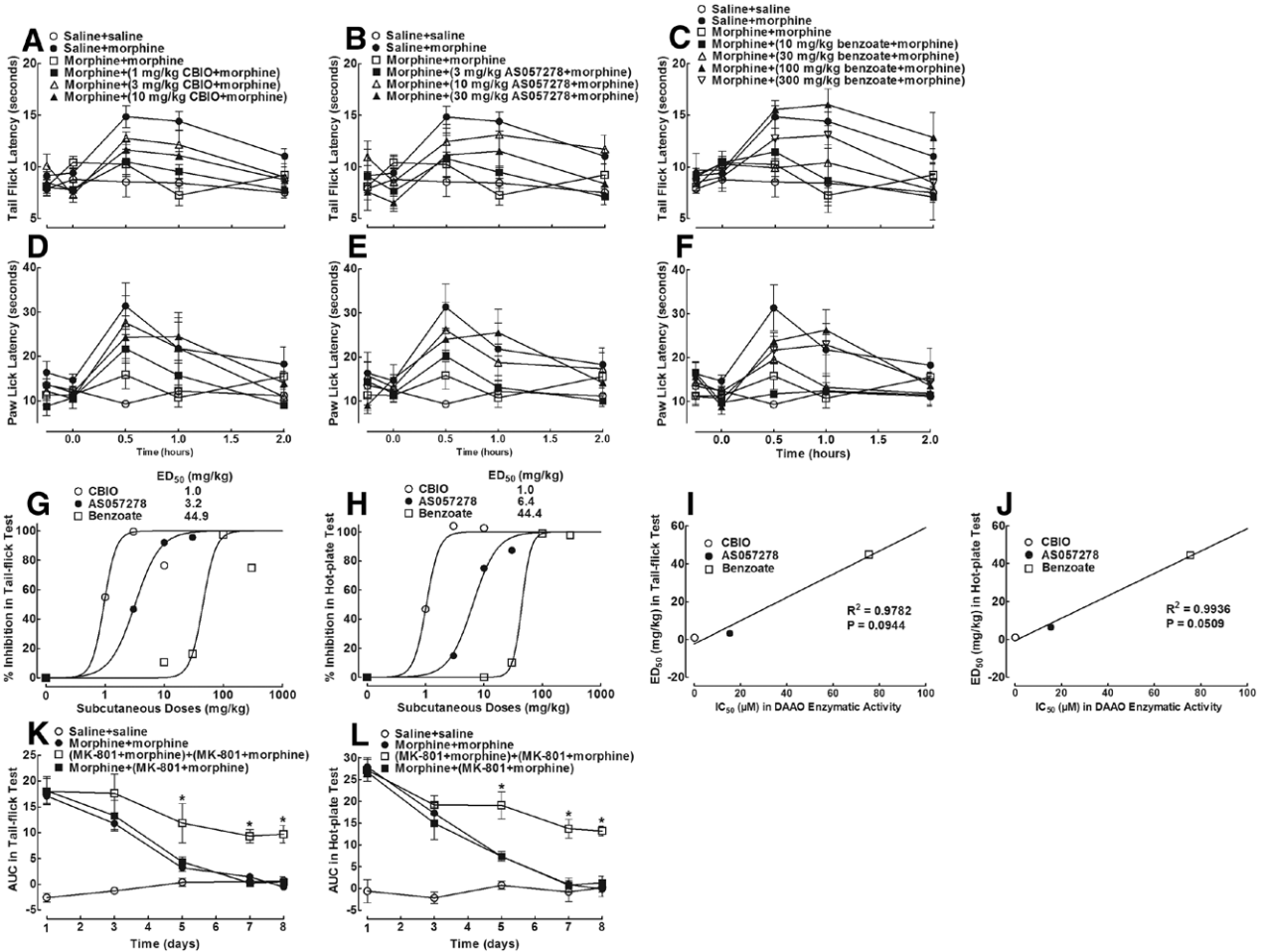
**Fig. 2.** Preventive RNA targeting effects of intrathecal injection of D-amino acid oxidase (DAAO) gene silencers, small interfering (siRNA)/DAAO, and small hairpin DAAO (shDAAO), on morphine antinociception tolerance in the rat formalin test (A). Rats received the combination of multiple bi-daily subcutaneous injections of saline (1 ml/kg) or morphine (10 mg/kg) and multi-daily intrathecal injections of the nonspecific oligonucleotide (5  $\mu$ g) or siRNA/DAAO (5  $\mu$ g) in polyetherimide complexation continuously for 7 days before formalin challenge. Two other groups of rats also received the combination of the abovementioned multiple bi-daily injections of morphine with a single intrathecal injection of the small hairpin control (shControl) or shDAAO expressed in adenoviral vectors ( $1.9 \times 10^{12}$ ) just before multiple morphine treatments. On the eighth day, rats received a single subcutaneous injection of saline (1 ml/kg) or morphine (5 mg/kg) 20 min before 5% formalin challenge. Nociceptive behavior was quantified by counting the number of formalin-injected paw flinches in 1-min epochs. (B and C) Represent acute phase nociception and tonic phase pain measured by areas under curve over 10–90 min ( $AUC_{10-90 \text{ min}}$ ), respectively. Data are presented as means  $\pm$  SEM ( $n = 6$  in each group). \*, #, + Denote statistically significant difference from the saline + polyetherimide + saline group, saline + polyetherimide + morphine group, and morphine + polyetherimide + morphine group, respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

( $r^2 = 0.99$ ;  $P = 0.05$  by unpaired and two-tailed Student  $t$  test; fig. 3J), although the number of compounds was relatively small with less degree of freedom.

The effects of the *N*-methyl-D-aspartic acid (NMDA) receptor noncompetitive antagonist MK-801 on morphine tolerance were also studied to make a comparison with DAAO inhibition. Four groups of mice ( $n = 6$  in each group) received subcutaneous injections of saline (10 ml/kg), morphine (10 mg/kg), MK-801 (0.1 mg/kg) + morphine (10 mg/kg), or morphine (10 mg/kg), twice daily at 12-h intervals for 7 days. On day 8, mice received a single subcutaneous administration of saline (10 ml/kg) or MK-801 (0.1 mg/kg) 20 min before saline (10 ml/kg) or morphine (5 mg/kg) challenge. The latency for tail-flick or paw-lick response was measured before, 0.5, 1, and 2 h after saline or morphine injection on day 1, 3, 5, 7, and 8, and  $AUC_{0-2 \text{ h}}$  were calculated. As shown in figure 3, K and L, multiple bi-daily subcutaneous injections of morphine induced progressive and complete antinociceptive tolerance both in the

hot-plate and tail-flick tests during the 7-day administration; coadministration of MK-801 significantly prevented the development of morphine tolerance ( $P < 0.05$  by two-way ANOVA followed by *post hoc* Student–Newman–Keuls test). In contrast, single injection of MK-801 did not reverse established morphine tolerance on day 8.

We further evaluated spinal DAAO inhibition on reversal of morphine antinociceptive tolerance in the rat formalin test. Six groups of intrathecally cannulated rats ( $n = 6$  in each group) received intrathecal injections of saline (10  $\mu$ l) or morphine (50  $\mu$ g) twice daily for 5 days. On the sixth day, rats were challenged by a single intrathecal injection of saline (10  $\mu$ l) or morphine (5  $\mu$ g) 20 min before formalin test. CBIO (30  $\mu$ g), AS057278 (100  $\mu$ g), or sodium benzoate (300  $\mu$ g) was injected 12 h earlier before morphine challenge to avoid their acute antinociceptive effects. As shown in figure 4A, single intrathecal injection of morphine suppressed acute and tonic flinching responses, whereas multiple bi-daily intrathecal injections of morphine induced



**Fig. 3.** Reversal effects of single subcutaneous injection of D-amino acid oxidase (DAAO) inhibitors on established morphine antinociceptive tolerance in the mouse tail-flick test (A–C) and hot-plate test (D–F). Mice received subcutaneous injections of saline (10 ml/kg) or morphine (10 mg/kg) twice daily for 7 days. On day 8, mice received subcutaneous administration of 10 ml/kg saline, 5-chloro-benzo[d]isoxazol-3-ol (CBIO; 1, 3, or 10 mg/kg, A and D), AS057278 (3, 10, or 30 mg/kg, B and E), or sodium benzoate (10, 30, 100, or 300 mg/kg, C and F) 20 min before saline (10 ml/kg) or morphine (5 mg/kg) challenge. Dose–response analysis in the mouse tail-flick test (G) and hot-plate test (H). The correlation of DAAO inhibitors between their reversal of established morphine tolerance and DAAO enzymatic inhibition (I and J). Effects of the noncompetitive antagonist MK-801 of N-methyl-D-aspartic acid receptors on morphine antinociceptive tolerance in the mouse tail-flick test (K) and hot-plate test (L), measured by areas under curve (AUC) over 2 h, respectively. Mice received subcutaneous injections of saline (10 ml/kg), morphine (10 mg/kg), MK-801 (0.1 mg/kg) + morphine (10 mg/kg), or morphine (10 mg/kg), twice daily at 12-h intervals for 7 days. On day 8, mice received a single-bolus subcutaneous administration of saline or 0.1 mg/kg MK-801 20 min before saline or morphine (5 mg/kg) challenge. Data are presented as means ± SEM (n = 6 in each group). Correlation coefficient was calculated and statistically tested by unpaired and two-tailed Student *t* test. \*Denotes statistically significant difference from morphine-tolerant mice (*P* < 0.05 by repeated measures two-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

tolerance to antinociception. Single intrathecal injection of CBIO, AS057278, and sodium benzoate nearly completely abolished established morphine tolerance, measured in either the acute nociception (fig. 4B) or tonic pain (fig. 4C; *P* < 0.05 by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

**CBIO and siRNA/DAAO Prevent Chronic Morphine Treatment–increased Spinal Hydrogen Peroxide Level**

To test the hypothesis that DAAO-mediated hydrogen peroxide synthesis is involved in morphine antinociceptive tolerance, we examined whether chronic morphine treatment

induced spinal hydrogen peroxide production, and whether intrathecal CBIO and siRNA/DAAO prevented the induction. Four groups of intrathecally cannulated rats (n = 6 in each group) received multiple bi-daily intrathecal injections of saline (10 μl), morphine (50 μg), the combination of morphine (50 μg) and bi-daily CBIO (10 μg), or daily siRNA/DAAO (5 μg) for 7 days. On the eighth day, the rats were killed and the spinal hydrogen peroxide levels were measured *via* a fluorescence Amplex Red assay. Compared with the basic level in the saline control group, multiple bi-daily injections of morphine significantly increased spinal hydrogen peroxide level by 22.0%. Intrathecal CBIO and

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siRNA/DAAO nearly completely prevented the increase ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test; fig. 5).

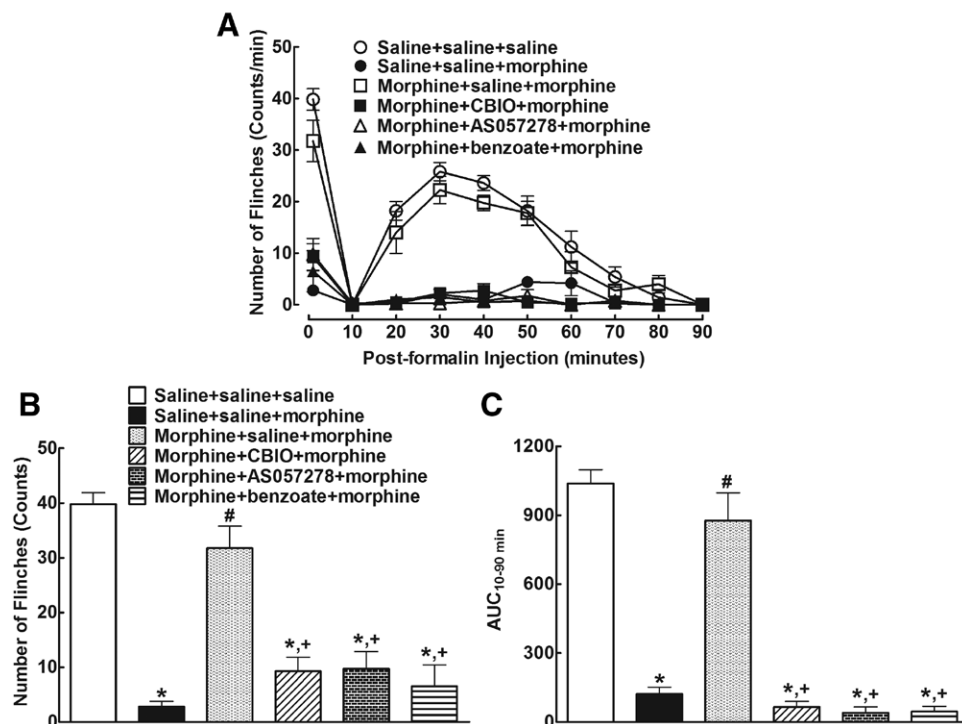
### PBN and Catalase Reverse Established Morphine Tolerance

To further support the role of spinal hydrogen peroxide in morphine antinociceptive tolerance, we investigated whether PBN, a “spin-trap” and nonselective scavenger of ROS including hydrogen peroxide,<sup>28–30</sup> and catalase, an enzyme for specific decomposition of hydrogen peroxide,<sup>28,40</sup> were able to reverse morphine tolerance. Five groups of intrathecally cannulated rats ( $n = 6$  in each group) received multiple bi-daily injections of morphine (50  $\mu\text{g}$ ) at 12-h intervals for 5 days. On the sixth day, rats were challenged by a single intrathecal injection of saline (10  $\mu\text{l}$ ) or morphine (5  $\mu\text{g}$ ) 20 min before formalin test. A single intrathecal injection of PBN (1 mg)<sup>41</sup> and catalase (500 units)<sup>28,42</sup> were made 12 h before morphine challenge to avoid their possible acute antinociceptive effects or interactions. As shown in figure 6A, single intrathecal injection of morphine produced marked antinociception in multiple bi-daily saline-treated naïve rats

but not in rats that received 5-day intrathecal morphine. Single intrathecal injection of both PBN and catalase markedly reversed established morphine tolerance. Measured by AUCs, the inhibition of PBN and catalase were 77.5 and 85.6% in acute nociception (fig. 6B) and 80.7 and 88.8% in tonic pain (fig. 6C), respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

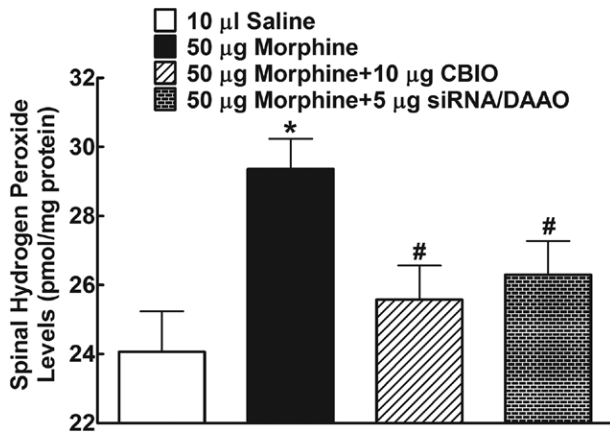
### DAAO Expressed in Astrocytes in the Spinal Dorsal Horn Is Up-regulated after Chronic Morphine Injection

To examine whether chronic morphine treatment up-regulates DAAO expression and clarify which spinal cells specifically express DAAO in both naïve and morphine-tolerant conditions, we performed double immunofluorescence staining in the spinal cord sections. Two groups of rats ( $n = 6$  in each group) received subcutaneous injections of saline (10 ml/kg) or morphine (10 mg/kg) twice daily at 12-h intervals for 7 days. In the naïve rats, DAAO immunofluorescence staining was widely expressed throughout the white and grey matters of the spinal cord. In the ventral horn, DAAO immunofluorescence was predominantly in processes surrounding relatively bigger motor neurons,



**Fig. 4.** Reversal effects of single intrathecal injection of D-amino acid oxidase inhibitors on established morphine antinociceptive tolerance in the rat formalin test (A). Rats received intrathecal injections of saline (10  $\mu\text{l}$ ) or morphine (50  $\mu\text{g}$ ) twice daily for 5 days. On the sixth day, rats were challenged by a single-bolus intrathecal injection of saline (10  $\mu\text{l}$ ) or morphine (5  $\mu\text{g}$ ) 20 min before formalin test. 5-Chloro-benzo[d]isoxazol-3-ol (CBIO; 30  $\mu\text{g}$ ), AS057278 (100  $\mu\text{g}$ ), and sodium benzoate (300  $\mu\text{g}$ ) were injected 12 h earlier before morphine challenge to avoid their acute antinociceptive effects. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches in 1-min epochs. (B and C) Represent acute phase nociception and tonic phase pain measured by areas under curve over 10–90 min ( $\text{AUC}_{10-90 \text{ min}}$ ), respectively. Data are presented as means  $\pm$  SEM ( $n = 6$  in each group). \*, #, + Denote statistically significant difference from the saline + saline + saline group, saline + saline + morphine group, and morphine + saline + morphine group, respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).





**Fig. 5.** Inhibitory effects of intrathecal injection of the D-amino acid oxidase (DAAO) inhibitor 5-chloro-benzo[d]isoxazol-3-ol (CBIO) and gene silencer small interfering (siRNA)/DAAO on spinal hydrogen peroxide levels after chronic morphine treatment in rats. Rats received multiple bi-daily intrathecal injections of saline (10 µl), morphine (50 µg), the combination of morphine (50 µg) and CBIO (10 µg), or multiple daily siRNA/DAAO (5 µg/day) for 7 days. On the eighth day, the rats were killed and the spinal hydrogen peroxide levels were measured by an Amplex Red method. Data are presented as means ± SEM (n = 6 in each group). \*, # Denote statistically significant difference from the saline control group and morphine group, respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

restrictively double-labeled with the astrocytic marker GFAP but not the mature neuronal marker NeuN or microglial marker OX42 (data not shown). In the dorsal horn, immunofluorescence DAAO-positive cells were mostly present in relatively smaller, stellate, and fibrous-shaped cells widespread in the I–V laminae. As shown ( $\times 40$  magnification) in representative photomicrographs from laminae I–III of the superficial dorsal horn, identified DAAO-positive cells colocalized with GFAP labeling (fig. 7A). However, few or none DAAO-positive cells were colabeled with OX42 (fig. 7B) or NeuN (fig. 7C). Furthermore, glial hypertrophy was noted after chronic morphine treatment. This glial activation was also demonstrated by an enhanced immunofluorescent labeling of astrocytes with GFAP (fig. 7D) and microglia with OX42 (fig. 7E). The pattern of specific expression of DAAO in astrocytes (but not in microglia or neurons; fig. 7F) remained the same after chronic morphine treatment, and the DAAO-immunoreactive fluorescence staining appeared to be up-regulated accompanying astrocyte hypertrophy (fig. 7D).

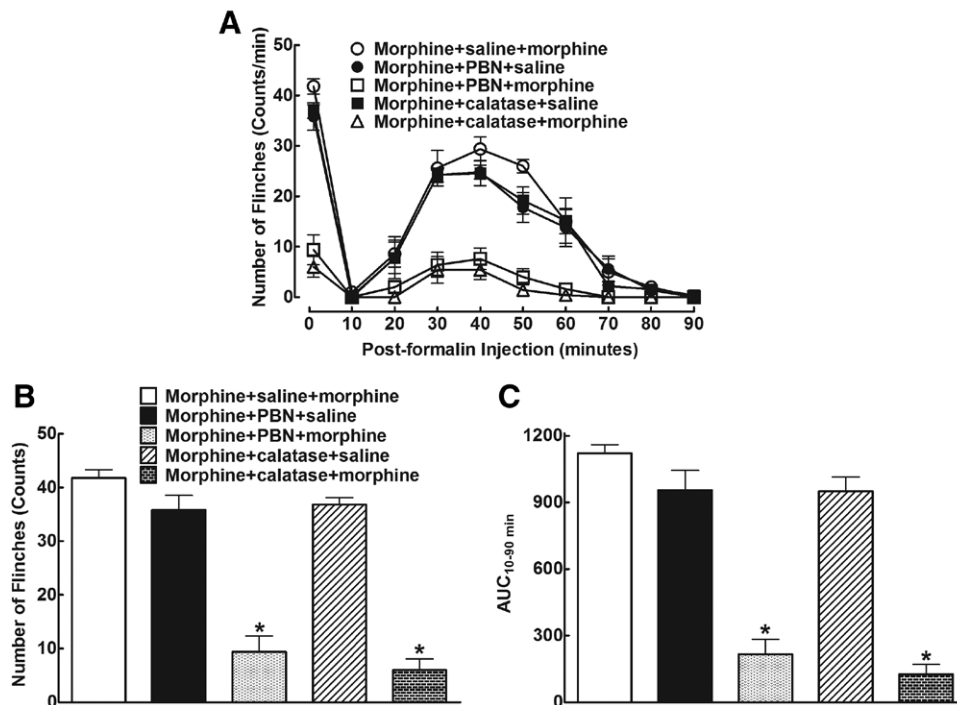
A smaller  $\times 5$  magnification was used to further quantify the up-regulation of spinal dorsal horn astroglial DAAO expression. The average percentage of immunolabeled surface area of GFAP and DAAO from six animals was significantly increased by 215% (fig. 8A) and 97.7% (fig. 8B), respectively ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test) after chronic morphine administration. In addition, chronic morphine

administration also markedly increased double GFAP/DAAO immunofluorescence staining by 239% (fig. 8C;  $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test). Figure 8, D–I, is representing spinal dorsal horn slides from naïve and morphine-tolerant rats.

## Discussion

Our initial observations showed that subcutaneous injection of the DAAO inhibitor (CBIO) blocked morphine tolerance to antinociception in normal mice<sup>32</sup> and in rats with bone cancer pain.<sup>27</sup> To exclude the possibility that CBIO-induced blockade of morphine tolerance was due to its actions unrelated to DAAO inhibition, we have further systemically examined the preventive and reversal effects of two more DAAO inhibitors with chemical structures different from CBIO in the current study. We have also used small interference RNA technology for gene abruption of spinal DAAO. At the behavioral level, pharmacologic blockade by a series of DAAO inhibitors, including CBIO, AS057278, and sodium benzoate, dose-dependently and completely prevented and abolished the initiation and development of morphine antinociceptive tolerance. The potencies of inhibition of DAAO were positively correlated to those of their blockade of morphine tolerance. Meanwhile, genetic ablation of spinal DAAO delivered by both siRNA/DAAO in polyetherimide complexation and small hairpin RNA/DAAO expressed in adenoviral vectors also almost completely prevented morphine tolerance. Our findings are in contrast to a recently published report by Chen *et al.*<sup>26</sup> These authors showed that intrathecal injection of exogenous DAAO partially attenuated the development of morphine tolerance by using tail-flick test after twice-daily morphine (10 mg/kg) injections for 5 days. The reason for this discrepancy is not understood, although it might be related to the difference between intracellular effects of DAAO inhibitors and possible extracellular effect of exogenous DAAO. Nevertheless, our results demonstrate that spinal DAAO functionally mediates the initiation and development of morphine tolerance and validate spinal DAAO as a potential novel target molecule for the treatment of morphine tolerance to antinociception.

Compelling evidence has shown that activation of astrocytes is involved in morphine tolerance.<sup>1–3</sup> Although spinal DAAO is generally described as an astroglial enzyme, based on previous histochemical studies,<sup>13–16</sup> there were recent reports showing that the DAAO immunoreactivity was also present in neurons in the brain.<sup>17,18</sup> By using double immunofluorescence technology specifically targeting the spinal DAAO protein, we demonstrate that DAAO is expressed primarily in astrocytes and not in either microglia or neurons. Our results, supported by a recent publication using double immunofluorescence staining technique as well,<sup>43</sup> indicate that DAAO is exclusively (nearly if not completely) expressed in astrocytes in the spinal dorsal horn. We further demonstrate that immunofluorescence GFAP-positive cells and OX42-positive cells display a hypertrophic morphology.

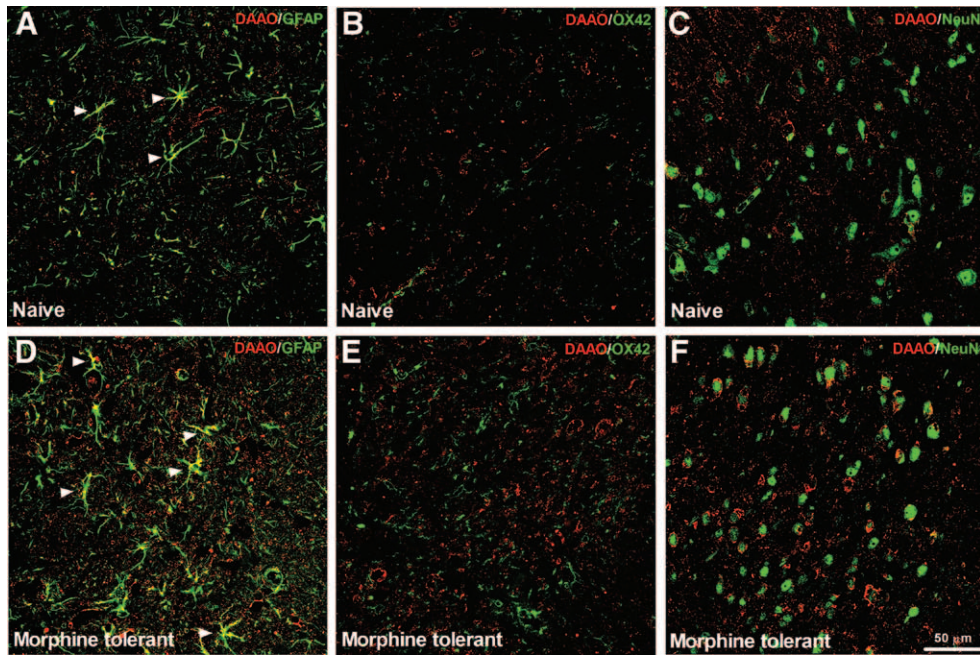


**Fig. 6.** Reversal effects of single intrathecal injection of phenyl-tert-*N*-butyl nitron (PBN) and catalase on established morphine tolerance to antinociception in the rat formalin test (A). Rats received intrathecal injections of morphine (50  $\mu$ g) twice daily for 5 days. On the sixth day, rats were challenged by a single intrathecal injection of saline (10  $\mu$ l) or morphine (5  $\mu$ g) 20 min before formalin challenge. PBN (1 mg) or catalase (500 units) was injected 12 h earlier before morphine bolus injection to avoid their acute antinociceptive effects. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches in 1-min epochs. (B and C) Represent acute phase nociception and tonic phase pain measured by areas under curve over 10–90 min (AUC<sub>10–90 min</sub>), respectively. Data are presented as means  $\pm$  SEM ( $n = 6$  in each group). \*Denotes statistically significant difference from the morphine + saline + morphine group ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test).

Our finding of glial activation after chronic morphine administration is consistent with previously published data in literature.<sup>44,45</sup> Furthermore, our study extends these findings by showing enhanced immunofluorescent labeling of DAAO that colocalizes with astrocytes after the development of morphine antinociceptive tolerance.

Astrocyte-derived DAAO synthesizes hydrogen peroxide by oxidatively deaminating D-amino acids.<sup>12,19,46</sup> We for the first time demonstrate that spinal astroglial DAAO-mediated synthesis of hydrogen peroxide is critically involved in morphine antinociceptive tolerance. (1) Chronic morphine injection significantly up-regulated spinal astrocyte activation and DAAO expression by approximately 100–200%. As well, spinal hydrogen peroxide level was also increased by approximately 20% accompanying morphine antinociceptive tolerance. These results are consistent with previous findings that spinal nerve ligation and bone inoculation of cancer cells stimulated spinal DAAO expression.<sup>27,47</sup> In addition, paw formalin injection and bone cancer cell implantation increased spinal hydrogen peroxide levels by 20–30% in synchronous with the development of chronic pain.<sup>27,28</sup> (2) Concurrent intrathecal injection of CBIO and siRNA/DAAO significantly inhibited *in vivo* DAAO enzymatic activity and expression.<sup>20,26</sup> At the same time, they completely prevented chronic morphine-induced increase in

spinal hydrogen peroxide level and antinociceptive tolerance. These results are in line with our previous data that formalin-increased (but not the basic) level of hydrogen peroxide was completely blocked by systemic CBIO treatment.<sup>27,28</sup> (3) Single intrathecal administration of the specific hydrogen peroxide catalyst catalase<sup>28,40</sup> and the nonselective hydrogen peroxide scavenger PBN (which is demonstrated to have no DAAO inhibitory activity)<sup>28–30</sup> nearly completely abolished morphine antinociceptive tolerance. PBN and catalase have been shown to completely prevent formalin-increased hydrogen peroxide level in the spinal cord.<sup>28</sup> Our findings of the specific role of hydrogen peroxide in morphine tolerance are supported by a recent publication.<sup>8</sup> The development of morphine-induced hyperalgesia and antinociceptive tolerance was associated with an increased activation of nicotinamide adenine dinucleotide phosphate oxidase and superoxide release; both morphine tolerance and superoxide release were effectively blocked by nicotinamide adenine dinucleotide phosphate oxidase inhibitors. In addition, peroxynitrite scavengers and decomposition catalysts have been extensively reported to attenuate opioid antinociceptive tolerance.<sup>6,7,9–11</sup> Taken together, these results support the critical role of reactive oxygen and nitrogen species in morphine nociceptive tolerance while advancing our knowledge of their biomolecular sources.



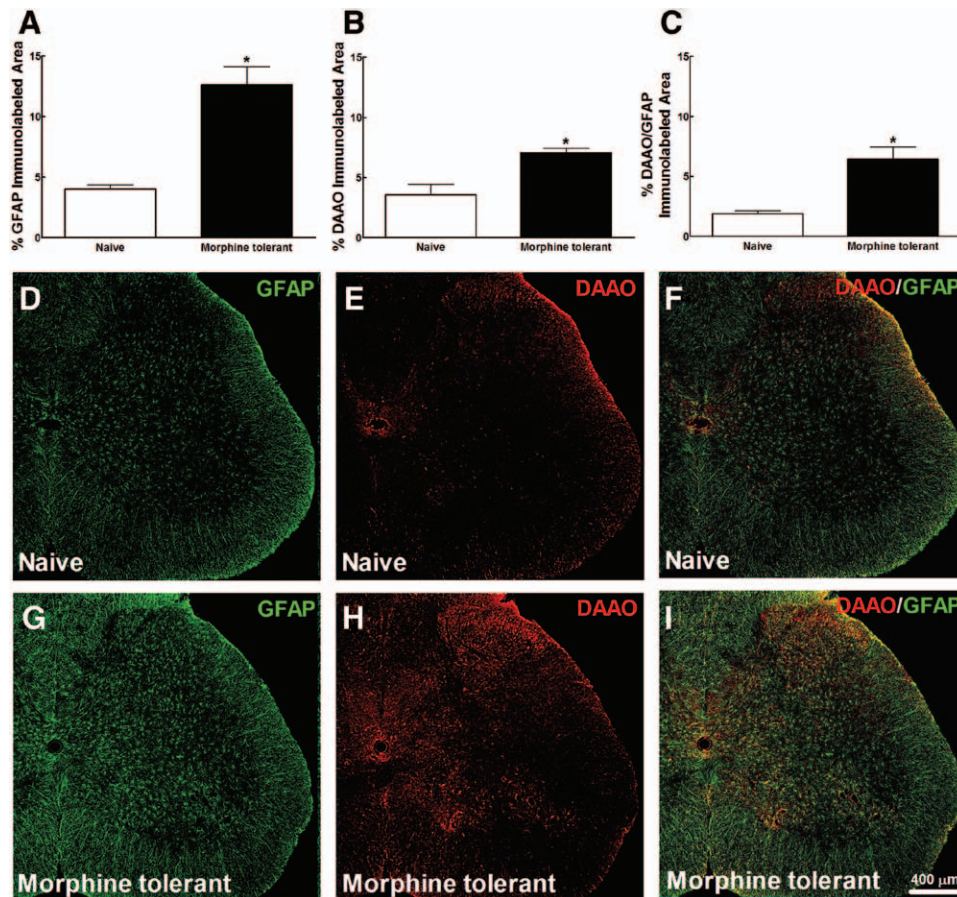
**Fig. 7.** Representative photomicrographs of D-amino acid oxidase (DAAO) double labeling with the astrocytic marker, glial fibrillary acidic protein (GFAP, A and D), mature neuronal marker, neuronal nuclei (NeuN, C and F), and microglial marker, OX42 (B and E). Photomicrographs were taken from the superficial dorsal horn (laminae I–III) in the naive (A–C) and morphine-tolerant rat (D–F). Animals ( $n = 6$  in each group) were injected twice daily for 7 days either with morphine (10 mg/kg) or equal volume of saline. Arrows indicate double labeling of DAAO in astrocytes (A, D), but not in neurons or microglia. Note increased intensity of labeling of GFAP, DAAO, and OX42 after chronic morphine administration. Scale bar: 50  $\mu\text{m}$ .

There are several features for the spinal astroglial DAAO–hydrogen peroxide pathway in mediating morphine antinociceptive tolerance. First, the DAAO–hydrogen peroxide pathway induces morphine antinociceptive tolerance regardless of whether acute nociception models or chronic pain models are used. Pharmacologic and genetic inhibitions of DAAO were roughly equal in reducing morphine tolerance in formalin-induced acute nociception, and hot-plate and tail-immersion tests, as well as formalin-induced tonic pain and bone cancer–induced mechanical allodynia<sup>(27,32 and current study)</sup>. This feature implies that blockade of DAAO and its subsequent hydrogen peroxide production would have a broad application in clinical management of morphine tolerance.

Second, pharmacologic blockade of DAAO not only prevents but also reverses morphine analgesic tolerance. Indeed, single subcutaneous and intrathecal injection of DAAO inhibitors nearly completely abolished established morphine tolerance in the formalin, hot-plate, and tail-flick tests. However, the important role of spinal NMDA receptors has been extensively evidenced in the development of morphine tolerance.<sup>48</sup> We confirmed the previous findings<sup>49,50</sup> that coinjection of the NMDA receptor antagonist MK-801 significantly prevented morphine antinociceptive tolerance in the hot-plate and tail-flick tests. However, single injection of MK-801 did not reverse established tolerance. The results suggest separate roles of NMDA receptors and DAAO in the initiation and maintenance of morphine tolerance. The results also imply that DAAO inhibitors might be superior to

NMDA receptor antagonists in the blockade and reversal of morphine antinociceptive tolerance. In addition, our head-to-head comparison between DAAO inhibitors and MK-801 may be helpful to illustrate the possible role of D-serine in the development of DAAO-mediated morphine tolerance. DAAO is implicated in the degradation of D-serine,<sup>12,16,19,28</sup> which is an endogenous ligand at the strychnine-insensitive glycine site of NMDA receptors, and controls NMDA receptor activity and synaptic plasticity.<sup>51–53</sup> It could be postulated that increased D-serine in the central nervous system after DAAO inhibition<sup>21,22</sup> has initiated morphine tolerance by activating NMDA receptors. Instead, our results conclude that inhibition of DAAO blocks both initiation and maintenance of morphine antinociceptive tolerance.

Third, DAAO–hydrogen peroxide pathway–mediated morphine antinociceptive tolerance appears indistinguishable from its antinociception. Blockade of spinal DAAO enzymatic activity and knockdown (or mutation) of DAAO gene have been extensively demonstrated to produce specific antinociception in chronic pain, including formalin-induced tonic pain, painful peripheral neuropathy, and bone cancer–induced mechanical allodynia.<sup>20,21,26–28,32,35,47</sup> Between these two effects, DAAO inhibitors blocked morphine tolerance and relieved pain *via* the same spinal cord site (rather than the brain),<sup>20,21,27,28,47</sup> in agreement with that the DAAO activity is restricted to the cerebellum, lower brainstem, and spinal cord.<sup>14–16</sup> In addition, the potencies for DAAO inhibitors to block morphine tolerance were roughly equal



**Fig. 8.** Up-regulation of D-amino acid oxidase (DAAO) immunostaining expressed in astrocytes in the spinal cord after morphine tolerance. Rats were injected twice daily for 7 days either with morphine (10 mg/kg) or equal volume of saline. The spinal cords (L3–5 segments) were obtained, and double immunofluorescence was labeled with DAAO and the astroglial marker fibrillary acidic protein (GFAP). The measurement of GFAP (A)-, DAAO (B)-, and double DAAO/GFAP (C)-immunolabeled surface areas was quantified in the spinal dorsal horn (laminae I–V) using Image J computer program (National Institutes of Health, Bethesda, MD). The average percentage of immunolabeled surface area was the fraction of the positive immunofluorescent surface area of total measured area in the picture from three sections of each spinal cord (six animals in each group). Data are presented as means  $\pm$  SEM. \*Denotes statistically significant difference from the saline-treated group ( $P < 0.05$  by one-way ANOVA followed by *post hoc* Student–Newman–Keuls test). Representative photomicrographs of GFAP (D, G)-, DAAO (E, H)-, and double GFAP/DAAO (F, I)-immunofluorescent staining of the left spinal cord were obtained from the naïve and morphine-tolerant rat. Scale bar: 400  $\mu$ m.

to those to produce antinociception. For example, the  $ED_{50}$  value for subcutaneous injection of CBIO was 1.0 mg/kg in morphine tolerance, whereas the antinociceptive  $ED_{50}$  was 0.8–0.9 mg/kg in the formalin test.<sup>28,32</sup> Furthermore, DAAO inhibitors exhibited positive correlation of their DAAO inhibitory activity to both the blockade of morphine tolerance and antinociception.<sup>20</sup> Hence, our results support the notion that morphine tolerance shares a common mechanism of central sensitization with chronic pain such as formalin-induced tonic pain, painful peripheral neuropathy, and pain associated with cancer.<sup>5,54,55</sup> Given all the facts, the unique combination of antinociception and blockade of morphine tolerance would make DAAO inhibitors feasible to be developed as novel medicines for the treatment of chronic pain by administration alone or in combination with morphine. This is further supported by the fact that

concurrent injection of CBIO and morphine produced apparent additive antinociception.<sup>27,32</sup>

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## Competing Interests

The authors declare no competing interests.

## Correspondence

Address correspondence to Dr. Wang: King's Lab, Shanghai Jiao Tong University School of Pharmacy, 800 Dongchuan Road, Shanghai 200240, China. yxwang@sjtu.edu.cn. Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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