Biochemical and Biophysical Research Communications 421 (2012) 501–507

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Down-regulation of spinal D-amino acid oxidase expression blocks formalin-induced tonic pain

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ARTICLE INFO

Article history:
Received 30 March 2012
Available online 10 April 2012

Keywords:
D-Amino acid oxidase (DAAO)
Spinal cord
RNA interfere
Chronic pain
Gene silencing
Formalin test

ABSTRACT

A series of inhibitors of D-amino acid oxidase (DAAO) are specific in blocking chronic pain, including formalin-induced tonic pain, neuropathic pain and bone cancer pain. This study used RNA interference technology to further validate the notion that spinal DAAO mediates formalin-induced pain. To target DAAO, a siRNA/DAAO formulated in polyetherimide (PEI) complexation and a shRNA/DAAO (shDAAO, with the same sequence as siRNA/DAAO after intracellular processing) expressed in recombinant adenoviral vectors were designed. The siRNA/DAAO was effective in blocking DAAO expression in NRK-52E rat kidney tubule epithelial cells, compared to the nonspecific oligonucleotides. Furthermore, multiple-daily intrathecal injections of both siRNA/DAAO and Ad-shDAAO for 7 days significantly inhibited spinal DAAO expression by 50–80% as measured by real-time quantitative PCR and Western blot, and blocked spinal DAAO enzymatic activity by approximately 60%. Meanwhile, both siRNA/DAAO and Ad-shDAAO prevented formalin-induced tonic phase pain by approximately 60%. Multiple-daily intrathecal injections of siRNA/DAAO and Ad-shDAAO also blocked more than 30% spinal expression of GFAP, a biomarker for the activation of astrocytes. These results further suggest that down-regulation of spinal DAAO expression and enzymatic activity leads to analgesia with its mechanism potentially related to activation of astrocytes in the spinal cord.

1. Introduction

D-Amino acid oxidase (DAAO) is a flavoprotein that catalyzes the oxidative deamination of neutral and polar D-amino acids to α-keto acids, NH_3, and hydrogen peroxide [1,2]. Located within peroxisomes, DAAO is selectively distributed in the kidneys, liver and the central nervous system, as well as in leukocytes, small intestine, epididymis, and in the preputial and adrenal glands in nearly all mammals including humans [2]. Within the central nervous system, DAAO expression is restricted to the lower brainstem, cerebellum and spinal cord [3].

We have recently demonstrated that spinal D-amino acid oxidase (DAAO) contributes to the development of central sensitization-mediated chronic pain and may be a potential target molecule for the treatment of chronic pain [4–7]. Mutation of DAAO gene in ddy/DAAO$^{-/-}$ mice [4] efficaciously relieved formalin-induced tonic phase pain but not acute phase nociception. Intrathecal injection of a series of DAAO inhibitors including CBIO (5-chloro-benzo[d]isoxazol-3-ol), "Compound 8" (4H-thieno[3,2-b]pyrrole-5-carboxylic acid), AS057278 (5-methylpyrazole-3-carboxylic acid) and sodium benzoate all specifically prevented and reversed formalin-induced tonic phase pain by 60% but not acute phase nociception, with a significant positive correlation between the potencies of analgesia and inhibition of spinal DAAO enzymatic activity [6]. Given systemically, DAAO inhibitors produced analgesia in chronic pain including neuropathic pain [5,8] and bone cancer pain [10], but not in acute nociception [4–6]. In addition, intrathecal application of the enzyme protein of DAAO did not alter formalin-induced pain [10].

However, other studies have suggested that DAAO expression de novo was an anti-nociceptive factor by reducing the level of the gliotransmitter D-serine that contributes to *N*-methyl-D-aspartate (NMDA)-dependent dorsal horn central sensitization. Mutation of DAAO potentiated formalin-induced central sensitization and pain, and NMDA receptor mediated excitatory postsynaptic currents recorded from the spinal cord dorsal horn [11]. Inhibition of D-serine with DAAO reduced tetanically sciatic stimulation induced mechanical allodynia [12] and strychnine-induced allodynia [13]. DAAO was also consistently shown to reduce NMDA receptor dependent activity by oxidation and reducing D-serine [14].

In order to further illustrate the role of DAAO in pain transmission, this study employed RNA interfere technology to test whether knockdown of spinal DAAO expression blocked formalin-induced

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http://dx.doi.org/10.1016/j.bbrc.2012.04.030

pain. By targeting DAAO, we first designed a siRNA/DAAO and an Ad-shRNA/DAAO (with the same sequence as siRNA/DAAO after intracellular processing) expressed in recombinant adenoviral vectors. We further evaluated their inhibitory effects on DAAO expression in NRK-52E rat kidney tubule epithelial cells. Afterwards, we investigated the inhibitory effects of multiple-daily intrathecal injections of both siRNA/DAAO and Ad-shRNA/DAAO on spinal DAAO expression and enzymatic activity, as well as formalin-induced pain in rats, a well-characterized and widely-used pain model. Lastly, we tested whether the analgesic effects of both siRNA/DAAO and Ad-shRNA/DAAO were related to activation of spinal astrocytes.

2. Materials and methods

2.1. Cells and synthesis and delivery of double-stranded RNA

The small interfering double-stranded RNA (siRNA) targeting the cDNA sequence of the rat DAAO (GeneBank Accession No. NM_001917.4) was designed and subjected to a Blast-Search (http://blast.ncbi.nlm.nih.gov) to ensure that only one gene was being targeted. The nonspecific oligonucleotide was also designed. Their sequences were the following: siRNA/DAAO sense: 5′-GGAG UGAAGUUCAUCCAUCUU-3′; siRNA/DAAO antisense: 5′-GAUGGAU GAACUUCACUCCUU-3′; nonspecific oligonucleotide sense: 5′-UUC UCCGAACGUGUCACGUUU-3′; nonspecific oligonucleotide antisense: 5′-ACGUGACACGUUCGGAGAAUU-3′. The 19 nucleotide duplex and 2 unpaired nucleotides overhang of 3′end were chemically synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). To formulate siRNAs, linear polyetherimide (PEI) (Poly-Science, Niles Illinois, USA) was dissolved in 5% dextrose in water (2 mg/ml, pH 7.0). 1 mg RNA was dissolved in 1.5 mg PEI in a PEI: RNA ratio of six equivalents of PEI nitrogen per RNA phosphate to form RNA–polymer complexes at room temperature for 10 min.

For the recombinant adenovirus generation, small hairpin RNA (shRNA) against DAAO (shDAAO) and nonspecific oligonucleotide shRNA (shControl) were designed according to the above sequences and were chemically synthesized by Sangon Biotech Co., (Shanghai, China) with the following sequences: shDAAO: 5′-GATCCGGAGTG AAGTTCATCCATCTTCAAGAGAGATGGATGAACTTCACTCCTTTTTTA-3′; shControl: 5′-GATCCGCCAGCTGATACTAACTCCTTCAAGAGAGGAGT TAGTATCAGCTGGCTTTTTTA-3′. The sequences form a loop by hydrogen bonding of AT or GC (Fig. 1A). The oligonucleotides were subcloned into the BamH I and HindIII sites of the recombinant pDC316-EGFP-U6 adenoviral vector (Fig. 1B). Ad-shDAAO and Ad-shControl plasmids were then generated by homologous recombination in HEK293 package cells (American Type Culture Collection, Manassas, Virginia, USA), with respective titers of virus of 6.3×10^{10} and 7.5×10^{10}. The sequences of the cloned Ad-shDAAO and Ad-shControl plasmids were confirmed by digestion analysis through restriction endonuclease and DNA sequencing.

NRK-52E rat kidney tubule epithelial cells, purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) at 37 °C in 95% O_2/5% CO_2. A 0.25% trypsin/EDTA solution was used to detach the cells from the culture flask for planting and passing the cells.

2.2. Real-time quantitative PCR, Western blot and measurement of spinal DAAO enzymatic activity

For real-time PCR analysis, the spinal lumbar enlargements were collected and mechanically homogenized using electronic microhomogenizer at 10,000 rpm for 30 s in TRIzol on ice. According to the manufacturer's instruction, total RNA of the spinal lumbar enlargements was purified from individual sample by use of the TRIzol reagent. Real-time quantitative PCR was carried out with Mastercycler® ep realplex (Eppendorf AG, Hamburg, Germany) using RealmasterMix (SYBR Green I). The primer sequences used were: DAAO: 5′-CCC TTT CTG GAA AAG CAC AG-3′ (sense) and 5′-CTC CTC TCA CCA CCT CTT CG-3′ (antisense); GFAP: 5′-ACA TCG AGA TCG CCA CCT AC-3′ (sense) and 5′-ACA TCA CAT CCT TGT GCT CC-3′ (antisense); GAPDH (glyceraldehyde-3-phosphate dehydrogenase): 5'-CGG CAA GTT CAA CGG CAC AG-3' (sense) and 5'-AGA CGC CAG TAG ACT CCA CGA C-3' (antisense). All primers were chemically synthesized by United Gene Company (Shanghai, China). Amplification of the housekeeping gene GAPDH was measured for each sample as an internal control for sample loading and normalization [5].

For Western blot, the spinal lumbar enlargements were homogenized and lysed with the ratio of 1:5 (m/v) in a lysis buffer RIPA with PMSF. The homogenate was centrifuged for 10 min at 12,000 rpm at 4 °C. The proteins were separated by SDS–PAGE (12%) and then transferred to a PVDF membrane by an electrophoretic method. The membrane was blocked in 5% skim milk powder in PBST at room temperature for one hour, and incubated with sheep polyclonal primary antibody raised against DAAO (Abcam, England) with a dilution ratio of 1:1000, mouse monoclonal antibody against GFAP (Millipore Corporation, Billerica, USA) or rabbit polyclonal antibody against β-actin (Abcam, England) with a dilution ratio of 1:2500 at 4 °C for 24 h. Antibody binding was visualized using a horseradish peroxidase-conjugated secondary antibody and a DAB detection system. The bands were scanned using an image scanning densitometer (Tanon Science & Technology Co., Shanghai, China).

Spinal DAAO activity was measured using the "keto-acid method" and was expressed as pyruvate production (μmol) per milligram protein per minute.

2.3. Animals, intrathecal catheterization and tat formalin test

Male Sprague–Dawley rats (weighing 180–250 g) were provided by the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China), and were housed 2–3 per cage at room temperature of 22 ± 2 °C, with food and water ad libitum and a 12-h light–dark cycle with lights on at 7:00 am. Animals were put in the laboratory environment for 5–7 days for acclimation before experiments. The research protocol, approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University School of Pharmacy, followed the animal care guidelines of the National Institutes of Health.

Intrathecal catheterization and the rat formalin test were described in detail elsewhere [8].

2.4. Data analysis and statistics

The results are expressed as mean ± SEM and statistical significance was evaluated by a one-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test. The statistical significance criterion P value was 0.05.

3. Results

3.1. Silencing DAAO transgene by Ad-shDAAO in NRK-52E rat kidney tubule epithelial cells

DAAO is localized within the peroxisomes of renal tubular epithelial cells [16,17]. We employed NRK-52E rat kidney tubule epithelial cells to test the efficacy of Ad-shDAAO to knock down DAAO

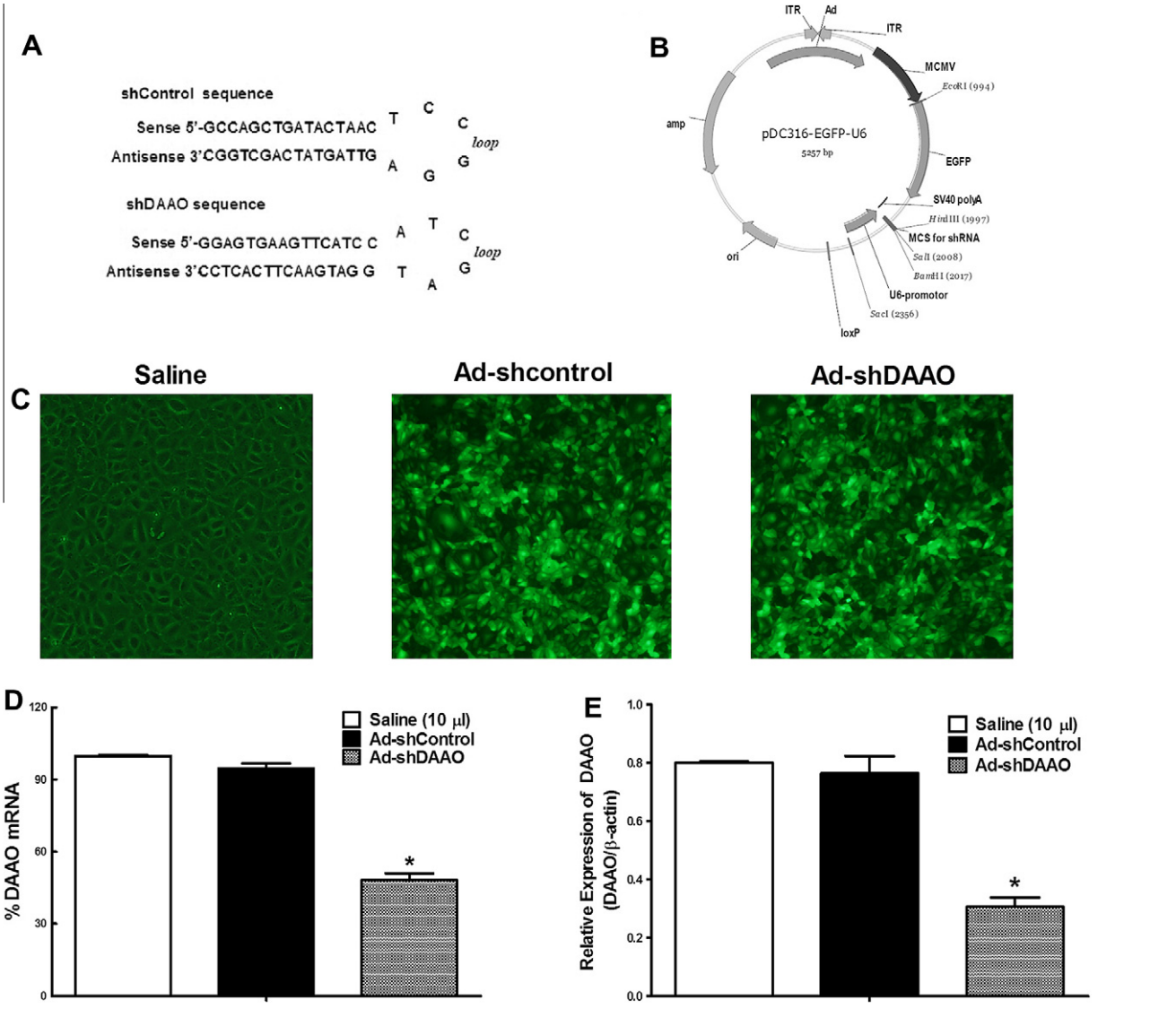

Fig. 1. Sequences of shRNA against D-amino acid oxidase (shDAAO) and nonspecific oligonucleotide shRNA (shControl) (A) and the structure of the recombinant DC316-EGFP-U6 adenoviral shuttle vector (B). Effects of transfection with Ad-shDAAO (6.3×10^{10}/ml) on green fluorescence emitted by enhanced green fluorescent protein (EGFP) observed by an inverted fluorescence microscope (scale bar of 100 μm) (C), DAAO mRNA expression measured by real-time PCR (D) and DAAO protein expression measured by Western blotting (E) in NRK-52E rat kidney tubule epithelial cells. Data are presented as means ± S.E.M. ($n = 3$ in each group). *Denotes statistically significant difference ($P < 0.05$ by a one-way ANOVA followed by post hoc Student–Newman–Keuls test) compared to the saline or Ad-shControl group.

expression. 24 h after transfection, cells treated with Ad-shDAAO (6.3×10^{10}/ml) and Ad-shControl (6.3×10^{10}/ml), but not saline, showed clear green fluorescence emitted by enhanced green fluorescent protein (EGFP) under a fluorescence microscope, indicating the high transfection efficiency of adenoviral vectors in cells (Fig. 1C). Expressions of mRNA and protein of DAAO were tested at 24 and 48 h after transfection, respectively. As shown in Fig. 1D, transfection with Ad-shDAAO significantly inhibited DAAO mRNA by 51.7% or 49.1%, respectively, compared to the saline or nonspecific Ad-shControl group ($P < 0.05$ by a one-way ANOVA followed by post hoc Student–Newman–Keuls test). Furthermore, Ad-shDAAO significantly inhibited DAAO protein expression by 50.2% or 51.2%, respectively, compared to the saline or nonspecific Ad-shControl group ($P < 0.05$) (Fig. 1E).

3.2. Effects of Ad-shDAAO given by multiple-daily intrathecal injections on formalin-induced pain and DAAO expression

The effects of multiple-daily intrathecal injections of Ad-shDAAO were tested on pain and spinal DAAO expression in rats. Three groups of rats ($n = 4$ in each group) received multiple-daily intrathecal injections of saline (30 μl/day), Ad-shControl (1.9×10^{12} vectors/day) and Ad-shDAAO (1.9×10^{12} vectors/day) for 7 days prior to the formalin challenge. On the 8th day, subcutaneous injection of formalin produced a characteristic bi-phasic flinching response in saline-treated rats, consisting of an initial rapidly decaying acute phase followed by a slowly rising and long-lived tonic phase. Ad-shControl did not significantly affect formalin-induced pain compared to the saline group. Multiple-daily intrathecal injections of Ad-shDAAO, however, did significantly reduce formalin-tonic phase pain but not acute phase nociception by 60.9% or 62.1%, respectively, compared to the saline group or Ad-shControl group ($P < 0.05$ by a one-way ANOVA followed by post hoc Student–Newman–Keuls test) (Fig. 2A).

The rats were immediately sacrificed after the completion of the formalin test and the homogenates of the spinal cord enlargements were used for DAAO gene and protein analysis. In a preliminary experiment with the same procedure, frozen spinal cord enlargement slides showed clear green fluorescence emitted by EGFP under the fluorescence microscope in Ad-shDAAO and Ad-shControl,

but not saline-treated rats, confirming the high in vivo transfection efficiency of Ad-shRNA in the spinal cord. Compared to the saline or Ad-shControl group, multiple-daily injections of Ad-shDAAO effectively reduced DAAO mRNA expression in the spinal cord by 49.5% or 51.4%, respectively ($P < 0.05$ by ANOVA followed by post hoc Student–Newman–Keuls test) (Fig. 2B). In addition, Ad-shDAAO effectively blocked spinal DAAO protein expression by 59.8% or 68.5%, respectively, compared to the saline or Ad-shControl group ($P < 0.05$) (Fig. 2C).

3.3. Effects of siRNA/DAAO given by multiple-daily intrathecal injections on formalin-induced pain and DAAO expression

The effectiveness of siRNA/DAAO in PEI complexation on formalin-induced pain and DAAO was further studied. Three groups of rats ($n = 4–5$ in each group) received multiple-daily injections of PEI (7.5 μg/10 μl/day), or nonspecific oligonucleotide (5 μg/day) and siRNA/DAAO (5 μg/day) for 7 days before formalin injection.

Similar to the siRNA study, multiple-daily injections of siRNA/DAAO effectively blocked formalin-induced tonic phase pain by 48.8% or 42.9%, respectively, compared to the PEI group or nonspecific oligonucleotide group ($P < 0.05$ by a one-way ANOVA followed by post hoc Student–Newman–Keuls test) (Fig. 3A). The rats were immediately sacrificed after the completion of the formalin test. siRNA/DAAO significantly blocked spinal DAAO gene expression by 40.2% or 42.6% (Fig. 3B) and DAAO protein expression by 80.3% or 81.0%, respectively, compared to the PEI group or nonspecific oligonucleotide group, respectively ($P < 0.05$) (Fig. 3C). The enzymatic activity of spinal DAAO was also determined and siRNA/DAAO significantly reduced spinal DAAO enzymatic activity by 64.1% or 53.1%, respectively, compared to the PEI group or nonspecific oligonucleotide group ($P < 0.05$) (Fig. 3D).

3.4. Inhibitory effects of multiple-daily intrathecal injections of siRNA/DAAO and shDAAO on astrocyte activation

In the spinal cord, DAAO is almost restrictively found in astrocytes [18,19]. This study tested whether intrathecal administration of siRNA/DAAO or Ad-shDAAO blocked activation of astrocytes marked by GFAP (glial fibrillary acidic protein) in the spinal cord. Spinal cord samples were obtained immediately after completion of the formalin test from rats treated with multiple-daily saline, PEI, nonspecific oligonucleotide or Ad-shControl, or siRNA/DAAO or Ad-shDAAO. Multiple-daily intrathecal injections of siRNA/DAAO (5 μg/kg/day) but not nonspecific oligonucleotide for 7 days significantly reduced GFAP gene expression by approximately 40.6%, compared to PEI-treated rats ($P < 0.05$ by a one-way ANOVA followed by post hoc Student–Newman–Keuls test) (Fig. 4A). siRNA/DAAO, but not nonspecific oligonucleotide, significantly reduced GFAP protein expression by approximately 32.0%, compared to the PEI-treated rats ($P < 0.05$) (Fig. 4C). The similar inhibitory effect on GFAP expression of astrocytes was confirmed by application of Ad-shDAAO (Fig. 4B and D).

4. Discussion

Chemical biology and gene knockdown/knockout/mutation technologies have been widely used to identify and validate potential target molecules, each with advantages and shortcomings. RNA interfere technology can be controlled both temporally and spatially particularly in vivo, avoiding the complications from constitutive gene deletion/mutation in systems other than those of interest as well as gene redundancy during development leading to early postnatal lethality [2,18]. These complications usually happen in mutant and knockout animals. For example, ddy/DAAO$^{-/-}$

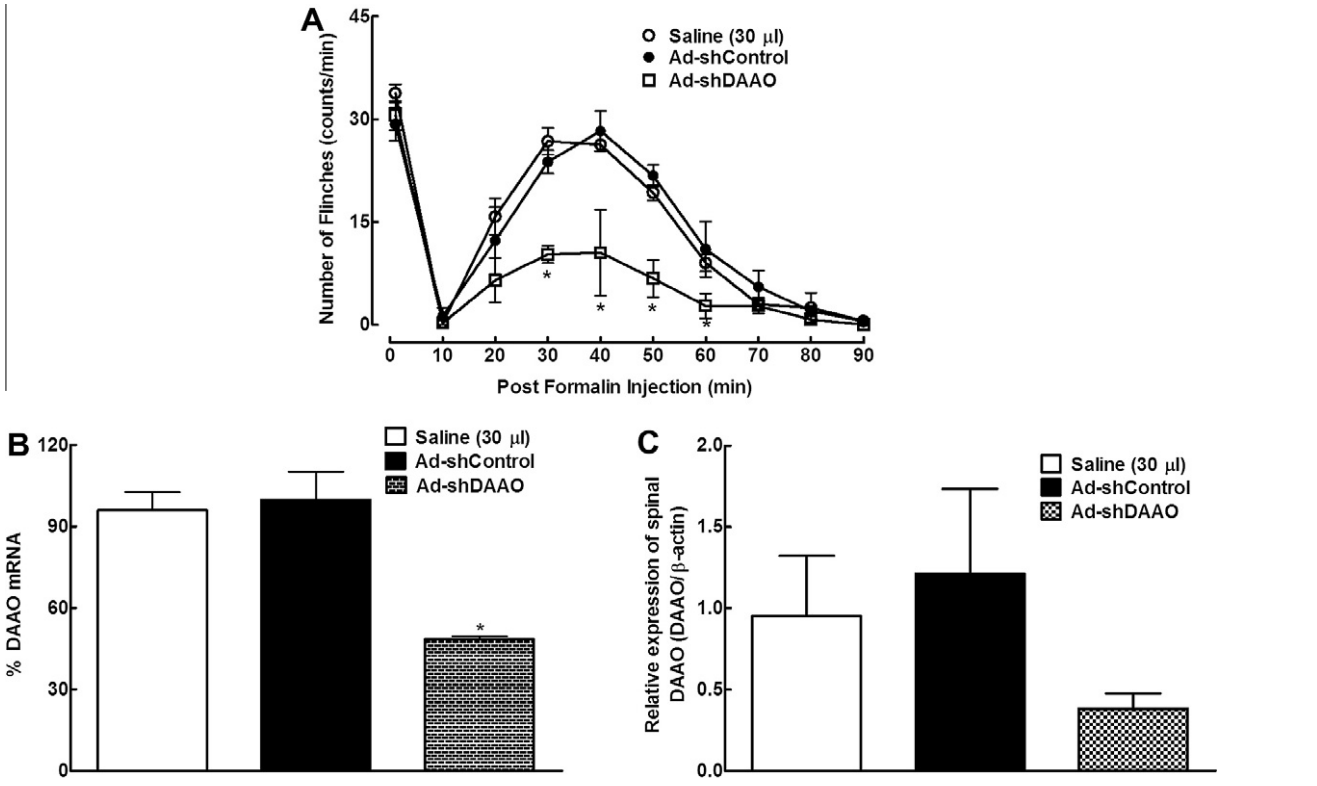

Fig. 2. Inhibitory effects of multiple-daily intrathecal injections of shRNA against D-amino acid oxidase (shDAAO) in recombinant adenoviral vectors on formalin-induced pain (A), spinal DAAO mRNA expression (B) and protein expression (C) in rats. Data are presented as means ± S.E.M. ($n = 4$ in each group). *Denotes statistically significant difference ($P < 0.05$ by a one-way ANOVA followed by post-hoc Student–Newman–Keuls test), compared to the saline or Ad-shControl group.

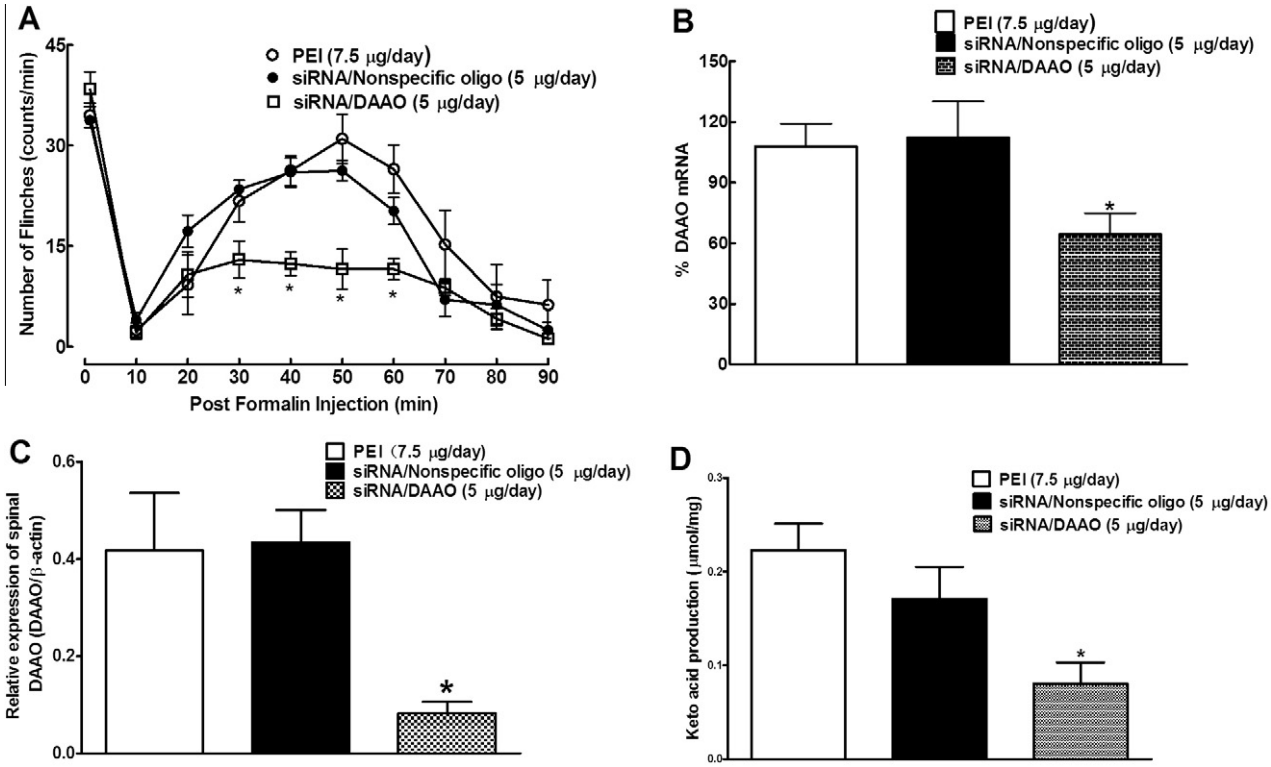

Fig. 3. Inhibitory effects of multiple-daily intrathecal injections of siRNA/DAAO (D-amino acid oxidase) in PEI complexation on formalin-induced pain (A), spinal DAAO mRNA expression (B), protein expression (C) and DAAO enzymatic activity (D) in rats. Data are presented as means ± S.E.M. (n = 4–5 in each group). *Denotes statistically significant difference (P < 0.05 by a one-way ANOVA followed by post-hoc Student–Newman–Keuls test) compared with the PEI or nonspecific oligonucleotide group.

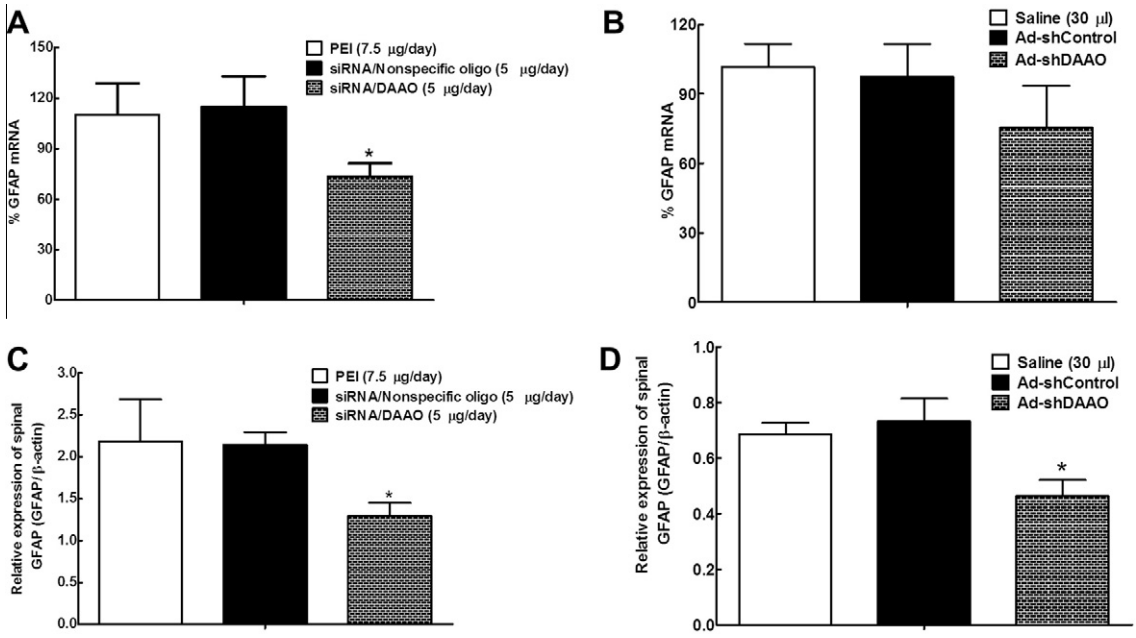

Fig. 4. Inhibitory effects of multiple-daily intrathecal injections of siRNA/DAAO (D-amino acid oxidase) in PEI complexation and expressed in recombinant adenoviral vectors on spinal GFAP (glial fibrillary acidic protein) gene expression (A and C) and protein expression (B and D) in formalin-challenged rats. Data are presented as mean ± SEM (n = 4–5 in each group). *Denotes statistically significant difference (P < 0.05 by a one-way ANOVA followed by post-hoc Student–Newman–Keuls test), compared with saline-, PEI-nonspecific oligonucleotide- or Ad-shControl-treated rats.

mice have less locomotor activity [19]. On the other hand, although siRNA is specifically designed to target the gene of interest and blasted to ensure it does not bind to other gene, off-target effects often occur [20]. Small molecule probe agents are easy to apply, however, their possible nonspecific binding and biological effects make conclusions less unreliable. The nonspecific effects of small molecule probe agents are more common if the compound is less potent. For example, sodium benzoate is a less potent DAAO

inhibitor and as a result, as high as 400 mg/kg was needed to apply in vivo for inhibition of DAAO [15,21]. Given the pros and cons of each of these methods, it would make sense to combine two or more technologies to validate potential target molecules.

In this study, we demonstrated that multiple-daily intrathecal injections of siRNA/DAAO formulated in PEI complexation or expressed in adenoviral vectors markedly inhibited spinal DAAO gene and protein expressions, DAAO enzymatic activity, and formalin-induced tonic phase pain by 50–80% but not acute phase nociception. Intrathecal injections of siRNA/DAAO also produced a long-lasting blockade of mechanical allodynia in the rat bone cancer pain model (Huang et al., unpublished data, 2012). These findings are consistent with our previous results that mutation of DAAO in ddy/DAAO$^{-/-}$ mice [4] and application of a series of structure-unrelated DAAO inhibitors were effective in blockade of formalin-induced tonic phase pain [4,6,7], spinal nerve ligation-induced neuropathic pain [5], and bone cancer pain [9]. Other studies have also reported that intraperitoneal injection of DAAO inhibitors such as 4-[2-(4-chlorophenyl)-ethyl]-lH-pyrrole-2-carboxylic acid blocked mechanical allodynia in neuropathic rats by unilateral L5/L6 spinal nerve ligation [8]. All of these studies, by taking advantage of chemical biology, gene mutation, and gene silencing technologies, systemically demonstrate that down-regulation of spinal DAAO expression and enzymatic activity leads to analgesia, suggesting that spinal DAAO may be a potential target molecule for the treatment of chronic pain. Indeed, the DAAO inhibitor SEP-227900 has been under clinical investigation for the management of chronic neuropathic pain [1].

The reasons for the contradictory results of DAAO in pain [11–13] are not clear, particularly noting that ddy/DAAO$^{-/-}$ mice employed by both studies [4,11] were from the same laboratory [22]. However, it may not be a valid assumption that reduction of D-serine by DAAO via oxidative deamination is the underlying mechanism for the possible analgesic action of DAAO [11–13]. Indeed, chronic pain is partly due to central sensitization, which is highly dependent upon NMDA receptor activation [23]. Cumulative evidence suggests that D-serine acts as an endogenous agonist on the glycine binding B site of the NMDA receptors and modulates glutamate-mediated receptor activation [25]. DAAO has consistently been shown to reduce NMDA receptor dependent activity in vitro [14]. On the other hand, it has been shown that the DAAO inhibitor CBIO given alone does not increase endogenous D-serine level in the brain [24] or spinal cord [7] in vivo. Even if DAAO inhibitors could increase endogenous spinal D-serine content, the possible raised level might not be sufficient to activate spinal NMDA receptors or produce algesia. We have demonstrated that intrathecal injection of exogenous D-serine did not alter formalin-induced tonic phase pain or CBIO's analgesia [7].

In contrast, it has been suggested that reactive oxygen species (ROS) are involved in NMDA receptor activation and thus contribute to neuropathic and capsaicin-induced pain [26]. Hydrogen peroxide is an active but stable ROS and can be produced from oxidation of D-amino acids by DAAO [1,2]. We have recently found that both formalin-induced tonic phase pain and spinal hydrogen peroxide level were blocked specifically by the DAAO inhibitor CBIO, catalase, and ROS scavenger PBN (phenyl-*N*-tert-butylnitrone). These results suggest that spinal DAAO produces algesia via production of hydrogen peroxide [7]. It is evidenced that DAAO is almost exclusively localized in astrocytes but not in other glial cell types or neurons [1]. We have shown that intrathecal pretreatment with the selective astrocyte metabolic poison inhibitor fluorocitrate specifically prevented formalin-induced increase of spinal hydrogen peroxide and tonic phase pain [7]. Thus, spinal hydrogen peroxide is most likely derived from spinal astrocytes. The present study further supports this conclusion. Multiple-daily intrathecal injections of siRNA/DAAO formulated in PEI complexation or expressed in adenoviral vectors blocked activation of astrocytes in the spinal cord marked by GFAP expression. The inhibition of GFAP expression by siRNA/DAAO and Ad-shDAAO is not due to their direct silencing GFAP gene because CBIO effectively blocked GFAP expression in the spinal cord (Huang et al., unpublished data, 2012).

In vivo delivery of siRNAs into the central nervous system is complicated by the fact that oligonucleotides do not efficiently cross the blood–brain barrier. Many studies have applied siRNAs locally into the spinal cord, such as continuous infusion via a mini-pump, formulation with delivery agents like Intra-i-Fect^{TM} or PEI, and viral vectors. Long-lasting and stable expression of viral vectors are especially important when exploring the therapeutic opportunities for the use of RNA interfere technology to knock down the expression of genes contributing to persistent conditions such as chronic pain. It was reported that shRNA/NMDA receptor (NR1) expressed in recombinant associate adenoviral vectors produced prolonged knockdown of NR1 subunit of NMDA receptors for as long as 10 months after intrathecal administration [18]. We thus expect the analgesia from DAAO inhibition to be long lasting. In addition, shRNA viral plasmids are transcribed to their respective shRNAs by the transcriptase in the host cell and the shRNAs containing the sense and antisense sequences from the target gene connected by a loop are transported from the nucleus into the cytoplasm where the Dicer processes them into siRNAs [27]. Thus Ad-shDAAO would be expected to produce a siRNA with the same sequence as that of the siRNA/DAAO we designed. Multiple-daily intrathecal injections of siRNA/DAAO formulated in PEI complexation for 7 days did effectively inhibit spinal DAAO expression and enzymatic activity, as well as formalin-induced tonic phase pain, with the same inhibitory rate as Ad-shDAAO.

Acknowledgments

This work was funded by National Natural Science Foundation of China (No. 81072623) and Shanghai Innovation Project (No. 11ZR1416400).

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