Neurochem Res (2007) 32:1592–1599
DOI 10.1007/s11064-007-9364-3

ORIGINAL PAPER

# Distribution of Downstream Regulatory Element Antagonist Modulator (DREAM) in Rat Spinal Cord and Upregulation of Its Expression during Inflammatory Pain

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Accepted: 25 April 2007 / Published online: 12 June 2007


**Abstract** A previous knockout study revealed the critical role of downstream regulatory element antagonist modulator (DREAM) in pain processing in the spinal cord by transcriptional regulation of prodynorphin (PPD) gene. Here, we report that, in contrast to the nuclear localization of other transcription factors, DREAM showed a punctate staining pattern in rat spinal dorsal horn in immunofluorescent analysis, with a membrane localization profile in some neurons and its expression accumulated in the inner zone of lamina II. In an inflammatory pain model induced by complete Freund's adjuvant (CFA) injection, we used Western blot analysis and detected transient upregulation of DREAM in the nuclear fraction of ipsilateral spinal dorsal horn at 2 h and 6 h post-injection, and a slow upregulation in the membrane fraction for 7 days. These studies suggest that DREAM might have other roles in pain modulation in the spinal cord in addition to its well-known role as a transcriptional repressor.

**Keywords** Complete Freund's adjuvant · Immunofluorescence · Nociception · Spinal dorsal horn · Transcription factor

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## Introduction

Downstream regulatory element antagonist modulator (DREAM) was originally identified in a study of transcriptional regulation of the human prodynorphin (PPD) gene in 1999 [1]. It binds as a tetramer downstream to the promoter via an intragenic sequence termed the downstream regulatory element (DRE), blocking transcription [2]. Structural analysis of DREAM reveals the presence of four $Ca^{2+}$-binding domains known as EF-hand domains. DREAM belongs to the neuronal calcium sensor (NCS) family and it represents the first known $Ca^{2+}$-binding protein to function as a DNA binding transcription regulator. Calcium binding blocks its capability to associate with DNA. It is known that DREAM can repress the gene expression of, for example, PPD, *c-fos*, apoptotic gene Hrk, genes engaged in circadian rhythms arylalkylamine N-acetyltransferase (AA-NAT), thyroglobulin and thyroid-specific transcription factors Pax8 and TTF-1, 2, $Na^{+}$ and $Ca^{2+}$ exchangers NCX3 and cytokine interleukin-2 [1, 3–8]. Recently, it has been reported that the repressor DREAM can act as a transcriptional activator on vitamin D and retinoic acid response elements [9]. DREAM also has other functions. It was initially known as calsenilin [10], which interacts with calcium and presenilin (PS)-1 and -2, and later as Kv channel-interacting protein 3 (KChIP3) [11], which binds to the cytoplasmic amino termini of the Kv4 $\alpha$ subunit. Calsenilin can regulate the levels of a proteolytic product of PS2. In cells expressing amyloid precursor protein, transfection of calsenilin can increase A$\beta$42 production [12]. KChIP3 has a profound effect on the trafficking [13], subunit assembly [14], and channel gating of Kv4 channels [11]. DREAM might play pleiotropic roles through interactions with diverse proteins in different cell compartments, functioning as a PS-interacting protein,

a transcription repressor, and a Kv4 channel modulator [15].

Previous studies have reported high expression of DREAM mRNA in brain [1, 10, and 11], as well as in non-neuronal tissues such as thymus, thyroid gland, pineal gland, and pituitary [4–6, 8, 16]. As ongoing analgesia was observed in DREAM knockout mice, DREAM appears to be a critical molecule in pain processing in the spinal cord by transcriptional regulation of the PPD gene [17]. Thus, the hypothesis ''no DREAM, no pain'' was developed [18]. However, there is a lack of detailed information on the expression of DREAM in the spinal cord.

In the present study, we determined the distribution of DREAM in the spinal cord by immunofluoresence analysis. Furthermore, in the rat inflammatory pain models by intraplantar injection of complete Freund's adjuvant (CFA), we examined changes of DREAM in the nuclear and membrane fractions of ipsilateral spinal dorsal horn with Western blot analysis. This study will provide some insights into the other roles of DREAM in the modulation of pain besides its role in gene expression.

## Materials and methods

### Animals

Male Sprague-Dawley rats weighing 200–250 g were supplied by the Animal Center of Peking University Health Science Center. They were housed in constant temperature rooms at 23 ± 1°C, on a 12 h light-dark cycle with free access to food and water. The animals were acclimated for 5 days prior to the start of any experimental procedures. All experimental procedures conformed to the Animal Care and Use Committee of Peking University guidelines and all efforts were made to minimize discomfort to the animals.

### CFA-induced inflammatory pain model and behavioral test

About 100 μl CFA (Sigma-Aldrich, St Louis, MO, USA) was injected into the plantar surface of the left hind paw of rats under ether anesthesia [19]. Classical signs of acute inflammation including edema, redness, and heat were observed. Thermal hyperalgesia was evaluated using radiant heat testing. Briefly, the animals were allowed to become accustomed to the environment for 20 min before testing. Then, the plantar surface of a hind paw was exposed to a beam of radiant heat through a transparent Perspex surface. A cut-off time of 30 s was allowed to prevent tissue damage. The paw withdrawal latency was recorded and averaged over three trials at 5 min intervals.

### Tissue preparation and immunofluorescence

After anesthesia with chlorate hydrate (300 mg/kg, i.p.), rats were perfused transcardially with 200 ml saline, followed by 200 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lumbar enlargement of the spinal cord was removed. Frozen tissue sections were then cut coronally in a cryostat at a thickness of 10 μm. Briefly, sections were blocked for 1 h at room temperature with 10% normal goat serum, from the species in which the second antibody was raised. Subsequently, sections were incubated overnight at 4°C with primary antibody, including rabbit DREAM antibody, 1:100, (Santa Cruz Biotechnology, CA, USA), monoclonal NeuN antibody, 1:200, (Chemicon, Temecula, CA, USA), FITC-labeled isolectin B4 (IB4) from *Griffonia simplicifolia*, 1:50, (Sigma, USA) and monoclonal calcitonin gene-related peptide (CGRP) antibody, 1:1000, (Sigma, USA). For double labeling, sections were simultaneously incubated with a mixture of two primary antibodies. The sections were then washed and incubated for 1–2 h with a mixture of fluorescence-labeled secondary antibodies (goat against rabbit or mouse) at room temperature. Green and red fluorophores were FITC and TRITC, respectively. The stained sections were examined with a Leica fluorescence microscope (Heidelberg, Germany) and captured using a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI, USA).

### Immunohistochemistry

After pretreatment with 0.3% hydrogen peroxide and blocking, sections were immunostained according to the Avidin Biotin Complex (ABC) method. Briefly, the sections were incubated sequentially with rabbit DREAM antibody (1:500) overnight at 4°C, biotinylated goat anti-rabbit secondary antibody, and avidin-biotin peroxidase complex for 30 min at room temperature. Bound peroxidase was visualized after incubation with 0.05% diaminobenzidine (Sigma, USA) and 0.003% hydrogen peroxide in phosphate buffer (PB, pH 7.4) and enhanced by nickel.

### Protein extraction

After anesthesia, lumbar enlargement of the spinal dorsal horn of naïve rats and rats 2 h, 6 h, 1day, 3 days, 5 days, or 7 days post CFA-injection was dissected out, frozen in liquid nitrogen, and stored at –80°C until analysis.

### Extraction of nuclear protein

The samples were homogenized in ice-cold buffer A [10 mM Hepes-NaOH (pH 7.8), 15 mM KCl, 1 mM

$MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/μl leupeptin], and vortexed for 10 s with 10% NP-40 (the volume of which was 10% of buffer A). The homogenate was then centrifuged at 10,000 g for 20 s at 4°C. The resulting pellet was dissolved in ice-cold buffer B [20 mM Hepes-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol (v/v), 0.5 mM DTT, 1 mM PMSF, 1 μg/μl leupeptin], and centrifuged at 12,000*g* for 4 min at 4°C. The resulting supernatant was the nuclear fraction.

### Extraction of membrane protein

The samples were homogenized in ice-cold RIPA buffer [50 mM Tris (pH 7.5), 250 mM NaCl, 10 mM EDTA, 0.5% NP-40, 1 μg/ml leupeptin, 1 mM PMSF, 4 mM NaF], and the homogenate was centrifuged at 500 g for 10 min at 4°C. The resulting pellet was removed and the supernatant (total protein extract) was further centrifuged at 25,000*g* for 60 min. The resulting pellet was dissolved in ice-cold RIPA buffer containing 0.1% (v/v) Triton X-100 and the solution was the membrane fraction. The protein concentration was measured with a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA).

### Western blotting

Equivalent amounts (50 μg) of protein preparations for each sample were denatured, subjected to SDS-PAGE using 12% running gels, and transferred to nitrocellulose membranes. After blocking with TBST [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] containing 5% non-fat dried milk, for 1 h at room temperature, the membranes were incubated with DREAM antibody (1:500) or monoclonal $\beta$-actin antibody, 1:2000, (Sigma, USA) overnight at 4°C. The membranes were then incubated with HRP-conjugated goat anti-rabbit or mouse secondary antibody, 1:2000, (Jackson Laboratories; West Grove, PA, USA) for 1 h at room temperature. Between incubations, the sections were washed three times in TBST for 10 min. Finally, the blots were examined using a chemiluminescence kit (Santa Cruz, USA).

### Statistical analysis

For Western blot analysis, the films were scanned and the optical density of specific bands was measured with TotalLab software (Newcastle, UK) and normalized with $\beta$-actin. Data are expressed as the mean ± SEM. Differences among different time points were analyzed by one-way ANOVA, followed by the Newman-Keuls Multiple Comparison Test. Significance was determined as $P < 0.05$.

## Results

### Distribution of DREAM in rat spinal cord

The commercial DREAM antibody was raised against a recombinant protein corresponding to amino acids 1–214 representing full-length DREAM of human origin. Our preliminary results of immunofluorescent staining and Western blot using this antibody revealed high expression of DREAM in hippocampus and cerebellum (data not shown), consistent with previous reports [20].

We explored the expression pattern of DREAM in the spinal cord, which is the critical relay center for the transmission of nociception. Immunohistochemical analysis indicated that DREAM-immunoreactive (IR) staining was present throughout the dorsal horn, with a tendency to accumulate in the superficial laminae (Fig. 1A), but no expression was observed in the motor neurons of the ventral horn. To our surprise, no obvious cell profiles were seen on the high-magnification image (Fig. 1B) and the staining revealed a punctate pattern. Then we performed double-labeling immunofluorescence study to examine the localization of DREAM in spinal cord. As shown in Fig. 1D, IB4-positive afferents projected predominantly to the inner zone of lamina II (lamina IIi), whereas CGRP-positive peptidergic afferents predominantly terminated in lamina I and the outer zone of lamina II (lamina IIo). Figures 1G and E show that DREAM exhibited striking co-localization with IB4 in lamina IIi and only faint co-localization with CGRP was observed (Fig. 1H). Altogether, the above studies showed that DREAM expression was greater in lamina IIi, with weaker expression in laminae I, IIo and the deeper laminae of the spinal dorsal horn.

To further determine the subcellular localization of DREAM, we performed double-labeling immunofluorescence study of DREAM and NeuN, a neuron-specific nucleus marker. No obvious co-localization of DREAM and NeuN were observed (Fig. 2C) and as mentioned above, DREAM exhibited a punctate staining pattern in the dorsal horn (Fig. 2A). In the high magnification image, we observed a membrane localization profile of DREAM in some neurons (Fig. 2D). It seems that the ''holes''seen in slide staining for DREAM were filled with NeuN staining (Fig. 2F).

Next we isolated the cytoplasm and membrane fractions from spinal dorsal horn and carried out Western blot experiments. The ~30 kDa major band of DREAM was detected in both fractions, but the expression was higher in the membrane fraction (Fig. 4A). The results confirmed that DREAM could localize in the membrane of spinal cord cells.

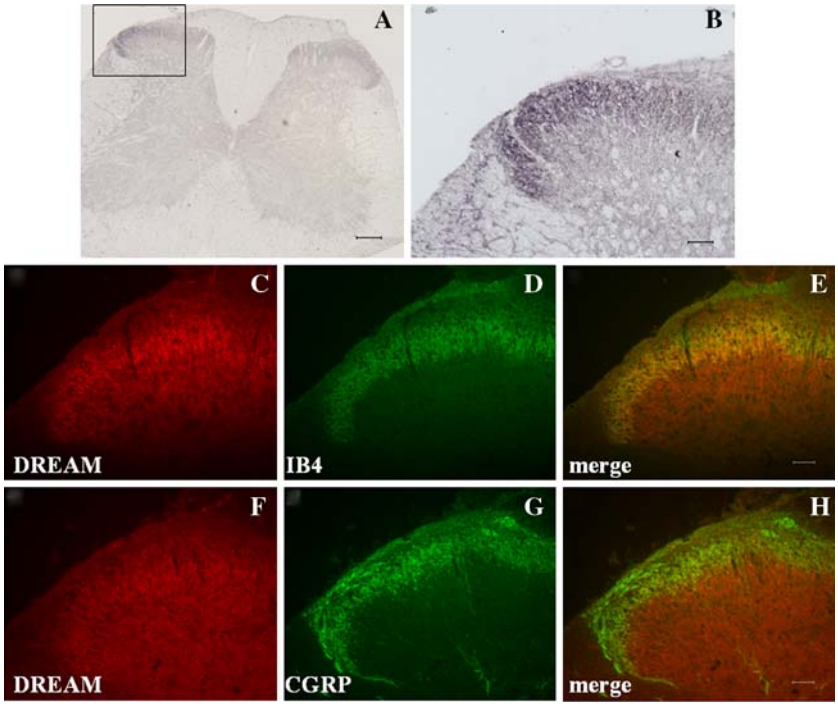


**Fig. 1** Distribution of DREAM in spinal cord. (**A**) Immunohistochemistry image showing the expression pattern of DREAM in spinal cord. (**B**) Enlargement of boxed area in A. (C, D and E) Immunofluorescence double-labeling of DREAM (**C**) and IB4 (**D**). (**F**, **G** and **H**) Immunofluorescence double-labeling of DREAM (**F**) and CGRP (**G**). (**E**) and (**H**) are superimpositions of images in the left and center panels, respectively. The figures show representative images of at least three independent experiments. Scale bar = 200 μm in A, 50 μm in **B**, **E**, and **H**. The scale bars in **E** and **H** also can be applied in **C** and **D**, **F** and **G**, respectively

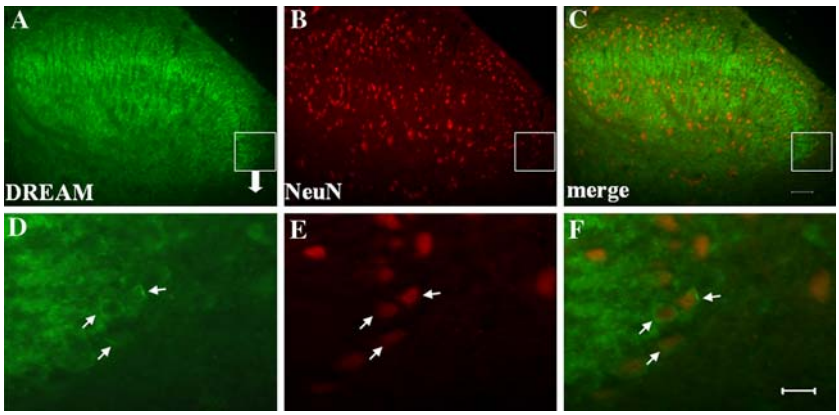


**Fig. 2** Localization DREAM in the cytomembrane of spinal dorsal horn. (**A**, **B** and **C**) Double staining of DREAM (**A**) and NeuN (**B**). (**D**, **E** and **F**) Enlargement of boxed area in **A**, **B** and **C**, respectively. (**C**) and (**F**) are superimpositions of images in the left and center panels, respectively. Arrows indicate characteristic cells. Three separate experiments were performed and similar results were obtained. Scale bar = 50 μm in **C**, 10 μm in **F**. The scale bars in **C** and **F** also can be applied in **A** and **B**, **D** and **E**, respectively

## Transient upregulation of DREAM expression in the nuclear extracts of ipsilateral spinal dorsal horn in inflammatory pain

As DREAM has been reported to be a transcriptional repressor for pain modulation of the spinal cord, we examined whether its expression in the nuclei changed under peripheral inflammation. We injected 100 μl CFA into the rat plantar surface of the left hindpaw under ether anesthesia. This produced an area of localized swelling and erythema associated with hypersensitivity to mechanical and thermal stimuli. Heat hyperalgesia was detected at 2 h post injection and reached a peak at 6 h, manifested by a significantly lower paw withdrawal latency, and had recovered slightly from 1 to 7 days (Fig. 3A). Heat hyperalgesia was not observed on the contralateral side.

The purity of nuclear isolate from the tissue extracts was confirmed by the presence of nucleoporin p62, a protein that is part of the nuclear pore complex (data not shown). Only a ~70 kDa band, the dimers of DREAM, was detected in the nuclear fraction with polyclonal DREAM antibody and similar results were acquired with the monoclonal DREAM antibody (data not shown), implicating that dimers might be the stable and functional form of DREAM in

the nucleus of spinal dorsal horn. As shown in Fig. 3B, expression of DREAM in nuclear extracts was relatively low in normal rats, which is consistent with the results of the immunofluorescent analysis. Peripheral inflammation induced an upregulation of DREAM expression in the ipsilateral spinal dorsal horn, which peaked at 2 and 6 h post-CFA injection and declined rapidly. According to the quantification analysis, DREAM expression was upregulated about seven-folds at 2 h and 6 h after injection ($P < 0.05$) (Fig. 3C). Interestingly, the time course of heat hyperalgesia matched with the upregulation of DREAM expression in the nuclear fraction. A significant negative correlation was found between them ($R^2 = 0.6215$, $P < 0.05$) (Fig. 3D).

### Persistent upregulation of DREAM expression in the membrane fraction of ipsilateral spinal dorsal horn in inflammatory pain

Upon observing DREAM-IR presence mainly in the cytomembrane of dorsal horn neurons, but not in the nucleus, we extracted the membrane fraction of the ipsilateral spinal dorsal horn of the lumbar enlargement. A slow but persistent upregulation of DREAM expression was detected until 7 days post-injection (Fig. 4B) and as shown by the quantification analysis, the upregulation was about two-folds at 7 days after injection ($P < 0.05$) (Fig. 4C).

## Discussion

The main findings of this study are as follows (1) There was a punctate staining pattern of DREAM in normal rat spinal dorsal horn and the staining accumulated in lamina IIi, exhibiting intense co-localization with IB4-positive afferent fibers. DREAM in normal rat dorsal horn neurons was concentrated on the cytomembrane (2) CFA injection induced transient upregulation of DREAM expression in nucleus fraction isolated from the ipsilateral spinal dorsal horn, followed by a slow but persistent upregulation in the cytomembrane fraction.

### Basal expression of DREAM in spinal cord

The present immunofluorescent study revealed a punctate staining pattern of DREAM in the spinal dorsal horn and intense staining in lamina IIi, a pattern similar to that of NCS1, another member of the NCS family [21]. Lamina II (the substantia gelatinosa) of the spinal cord plays an important role in the processing of nociceptive information from primary afferent nerves. The lamina II neurons targeted by IB4-positive non-peptidergic afferents contact lamina V projection neurons. These neurons then predominantly project to the amygdala, the hypothalamus, the bed nucleus of the stria terminalis and the globus pallidus, an independent nociceptive pathway

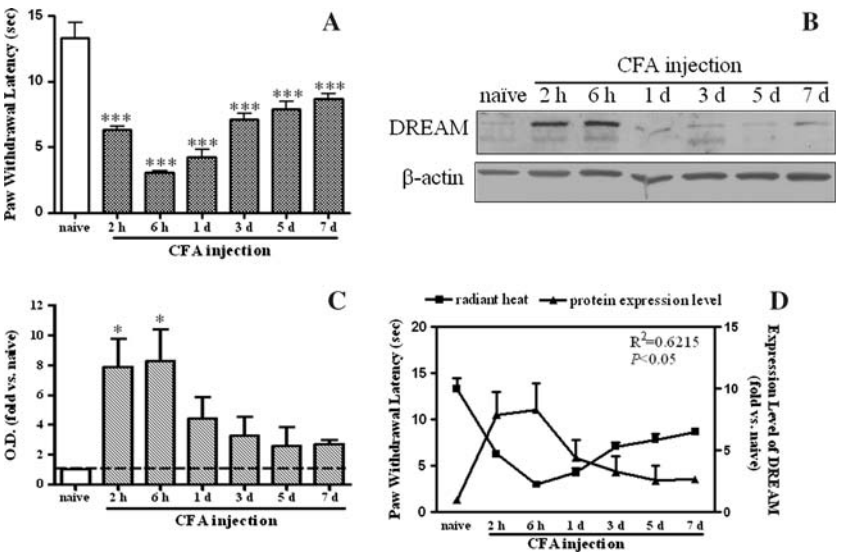


**Fig. 3** Transient upregulation of DREAM expression in the nuclear fraction from spinal dorsal horn following peripheral inflammation. (**A**) Paw withdrawal latencies detected by radiant heat test following CFA injection. ***$P < 0.001$, compared with naïve group, $n$=3–4. (**B**) Representative Western blot analysis results for the nuclear fraction from ipsilateral spinal dorsal horn. The lower panel shows the loading control. Three separate experiments were performed and similar results were obtained. (**C**) Quantification analysis of the optical density of these bands. Fold represents comparative levels over naïve group after normalization by the loading control. *$P < 0.05$, compared with naïve group. (**D**) Correlation between the decrease of paw withdrawal latency and upregulation of DREAM expression in the nuclear fraction ($R^2 = 0.6215$, $P < 0.05$). The left Y-axis shows paw withdrawal latency and the right Y axis shows the expression level of DREAM in the nuclei (■ radiant heat; ▲ DREAM protein expression level). Mean ± SEM, one-way ANOVA followed by the Newman-Keuls Multiple Comparison Test

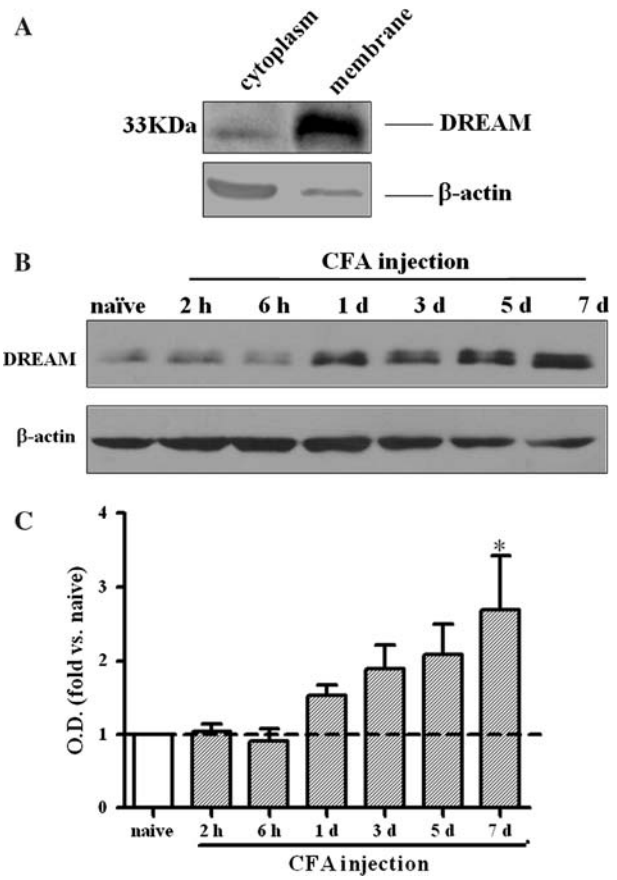


**Fig. 4** Persistent upregulation of DREAM expression in the membrane fraction from ipsilateral spinal dorsal horn following peripheral inflammation. (**A**) Western blot analysis showing the membrane localization of DREAM in the dorsal horn. (**B**) Representative Western blot analysis results for the membrane fraction from ipsilateral spinal dorsal horn. The lower panel shows the loading control. Three separate experiments were performed and similar results were obtained. (**C**) Quantification analysis of the optical density of these bands. The values were normalized by the loading control and compared with the naïve group. The significance was determined for 7 days after injection. $*P < 0.05$, compared with the naïve group

parallel to the lamina I-based ascending pathway [22, 23].

Previous studies have confirmed the repressor function of DREAM in PPD gene expression. High expression of PPD mRNA was seen in the spinal cord of DREAM knockout mice [17]. However, the present immunofluorescent analysis indicates that the expression of DREAM in dorsal horn neurons was focused in the cytomembrane, as also observed by the antigen-retrieval method, which enhances staining sensitivity (data not shown). Our Western blot analysis revealed that the basal levels of DREAM in nuclei were comparatively low. Besides DREAM, many other transcription factors, such as CREB, *c-fos* and *c-Jun*, participate in the transcriptional regulation of PPD [24]. CREB-IR is exclusively present in the nuclei of dorsal horn neurons [25]. Non-phosphorylated CREB may repress PPD gene expression in the basal state. Thus the other transcription factors may also be involved in repressing PPD expression.

The membrane localization of DREAM raised the possibility of its role in channel modulation. It is well known that the A-type Kv current formed by Kv4 family α subunits plays a critical role in determining excitatory responses in neuronal cell bodies and dendrites [26, 27]. Kv4.2 and Kv4.3 are specifically expressed in lamina II excitatory interneurons and are co-localized with pain-modulating molecules [28]. Genetic elimination of Kv4.2 reduces A-type currents and increases the excitability of dorsal horn neurons, resulting in enhanced sensitivity to tactile and thermal stimuli [29]. KChIPs are integral components of native Kv4 channel complexes [11, 30]. KChIP3, namely DREAM, has a profound effect on the biochemical and electrophysiological properties of these channels. The anatomical localization of DREAM and Kv4 in spinal dorsal horn neurons makes it possible for DREAM to function as a modulator of Kv4 channels, in addition to its role as a transcriptional repressor.

## Temporal and spatial changes of DREAM expression after peripheral inflammation

Rapid and prominent upregulation of DREAM in the nuclei extracts of ipsilateral spinal dorsal horn was observed at 2 h and 6 h post CFA injection. It has been reported that the increased levels of DREAM in the nucleus may be mediated by changes in intracellular calcium [31]. Peripheral inflammation have been reported to activate spinal N-methyl-D-aspartate (NMDA) receptors, neurokinin 1 (NK1) receptors, and T-type voltage-gated calcium channels, synergistically triggering a rise in the cytosolic free $Ca^{2+}$ concentration of spinal projection neurons [32]. Upregulation of DREAM in the nucleus may be the result of calcium influx. In addition, binding of DREAM to the DRE site can be terminated either by entry of calcium into the nucleus and direct binding between DREAM and calcium or phosphorylated cAMP-responsive element modulator (CREM), both of which are likely to occur after neuron activation. The reduced binding of DREAM may lead to its upregulation in the nucleus driven by the autoregulatory feedback mechanism. Thus, in the early phase of inflammatory pain, the repressor function of DREAM may be strengthened, repressing target genes expression.

Interestingly, peripheral inflammation resulted in a slow but persistent upregulation of DREAM in membrane extracts of the ipsilateral spinal dorsal horn at 7 days after CFA injection. As Kv4.2 mediates most of the A-type currents in dorsal horn neurons, DREAM may augment A-type currents via increased surface channel density,

slower inactivation and faster recovery from inactivation of Kv4 channels [11]. Expression of DREAM also induced a dramatic redistribution of Kv4.2, from intrinsic endoplasmic reticulum to the cell surface [13]. Upregulation of DREAM in the cytomembrane by inflammatory pain may reduce the excitability of dorsal horn neurons by affecting the biophysical and molecular characteristics of Kv4. From this point of view, DREAM may inhibit the hyperactivity of dorsal horn neurons during inflammatory pain and seemed analgesic, inconsistent with the hypothesis ''no DREAM, no pain''.

In summary, our studies implied that DREAM may be a multi-functional protein in pain modulation in the spinal cord. In addition to its well-known role as a transcriptional repressor, it may have other functions in the spinal cord. Further studies are needed to clarify the physiological role of DREAM in vivo.

**Acknowledgements** This work was supported by grants from the National Natural Science Foundation of China (30330026 and 30470550); a grant from the Beijing Natural Science Foundation (5052019), the grant for Outstanding Young Teacher of Higher Academic School to YW from the Ministry of Education, China [2001-182] and a grant from State Administration of Traditional Chinese Medicine of the People's Republic of China (04-05JP04) to YW.

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