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Original Article

Effect of adenoviral delivery of prodynorphin gene on experimental inflammatory pain induced by formalin in rats

Xionggang Chen[1], Tingting Wang[2], Caizhu Lin[1], Baihong Chen[1]

[1]Department of Anesthesiology, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, P. R. China; [2]Department of Ophthalmology, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, P. R. China

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Abstract: Circumstantial evidences suggest that dynorphins and their common precursor prodynorphin (PDYN) are involved in antinociception and neuroendocrine signaling. DREAM knockout mice had increased levels of PDYN and dynorphin expression, and reduced sensitivity to painful stimuli. However, some data support the notion that the up-regulation of spinal dynorphin expression is a common critical feature in neuropathic pain. It is not clear whether the production of dynorphin A can be increased when more PDYN is present. In this study we investigated the changes in pain behaviors, spinal PDYN mRNA expression and dynorphin A production on formalin-induced pain in rats receiving the pretreatment of adenoviral delivery of PDYN. Our results showed that the adenoviral transfer of PDYN gene was sufficient to reduce pain behaviors resulting from formalin injection, and the antinociceptive effect after receiving the pretreatment of adenoviral delivery of PDYN was mediated at the level of the spinal cord via KOR.

Keywords: Adenoviral delivery, prodynorphin, dynorphin A, formalin, hyperalgesia, spinal dorsal horn

Introduction

It is currently accepted that the endogenous kappa-opioidergic system plays an important role in the endogenous pain control system [1-4]. The endogenous ligands for kappa-opioid receptor (KOR) consist of prodynorphin (PDYN), dynorphin A, dynorphin B, α-neo-endorphin and big dynorphin [5], whereas PDYN is the precursor of dynorphins [6-8]. Actions of these peptides are preferentially mediated through KOR and are critical for regulation of nociceptive transmission [9, 10]. Otherwise, the endogenous kappa-opioidergic system also includes downstream regulatory element antagonistic modulator (DREAM) which inhibits transcription of the PDYN gene [11, 12]. In DREAM knockout mice, there was increased expression of PDYN mRNA, but endogenous dynorphin increases might be limited to within physiological ranges [6]. Therefore, the expression of endogenous PDYN, dynorphins may be under the tight control of DREAM in central nerve system.

The endogenous kappa-opioidergic system also participates in facilitation of spinal nociceptive transmission [1, 13]. Several experimental models of chronic pain states show that a significant upregulation of spinal dynorphin A and its precursor peptide, PDYN, is a common consequence of nerve injury [14]. Previous observations showed that the mechanism of significant increase of PDYN expression in peripheral neuropathy was proposed to explain the paradox of dynorphin actions [15]. Otherwise, a study with PDYN knock-out mice that up-regulated spinal dynorphin was required for the maintenance of persistent neuropathic pain [16]. Recent findings implicate a direct excitatory action of dynorphin A at bradykinin receptors and NMDA receptors to promote hyperalgesia in nerve injured rats, and its upregulation may promote nociceptive input due to injury [17-22]. All these data support the notion that the upregulation of spinal dynorphin expression is a common critical feature of expression of neuropathic pain [23].

Previous study found that PDYN knockout mice almost didn't produce dynorphin [24], indicating that dynorphin cannot be produced without PDYN. Dynorphin A is an endogenous opioid neuropeptides derived from the PDYN gene [6, 25]. However, we are not aware whether the production of dynorphin A is obviously increased when more PDYN is present. To our knowledge, the involvement of the pretreatment of PDYN in inflammation-induced pain has not been previously investigated. Therefore in this study we investigated the behavior change of rats receiving the pretreatment of adenoviral delivery of PDYN, PDYN mRNA expression and dynorphin A production on Formalin-induced pain. We hypothesized that rats receiving the pretreatment of adenoviral delivery of PDYN by the tail vein would show a modulatory antinociceptive action and dynorphin A down-regulation in the spinal cord.

Materials and methods

Experimental animals

Adult male SPF grade Sprague-Dawley rats weighing between 250 and 300 g were housed at a constant ambient temperature of 24°C ± 1°C, humidity of 40%-70% under a 12 h light/dark cycle and given food and water ad libitum. The rats were individually housed in plastic cages with wood-chip bedding for at least 1 day before surgery. All experimental procedures were approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Fujian Medical University and were in accordance with the guidelines for the use of laboratory animals [26]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Recombinant adenoviral vectors

Construction of the recombinant adenoviruses was determined as described previously [27]. Briefly, PDYN gene (GenBank accession number. NM 019374) primer sets (Sence: 5'-GACATGGCGTGGTCCAGGCTGATG-3'; Antisence: 5'-GTCTCAAACATCTAAATCT TCAGAATAGGTATTGGG-3') were designed via PDYN cDNA was cloned by PCR technique, and then inserted into the cloning vector pUC57. The reconstructed plasmid with PDYN gene was detected by the electrophoresis and the sequence analysis [28]. Plasmid pUC57-PDYN and pDC316-LacZ-a (Microbix Biosystems, Inc.) were all digested with Hind III and EcoR I, and the digested fragment s were re-collected from the low melt agarose gel and fast ligated directly by T4 DNA ligase. The reaction product was transformed into E. coli (DH5α), and the positive bacterial colony was selected. The recombinant eukaryotic expression vector pDC316-PDYN was identified by using restriction endonucleases digestive reaction and DNA sequencing. The HEK 293 cells were cotransfected by the shuttle plasmid of pDC316-PDYN and the skeleton plasmid of pBHG which was adenoviruses type 5 with deletions in E1 and E3, and the recombinant plasmid of Ad5-PDYN was obtained. The expression of the transfected genes was evaluated by PCR and immunocytochemical staining. Virus was purified by double CsCl centrifugation and subsequently dialyzed as described previously [29, 30]. Final yields as assessed by plaque assays on 293 cells were approximately 1×10^{12} plaque forming units (pfu)/ml [31].

Recombinant adenoviral injection

Group Ad5 received respectively Ad5, with 1×10^{11} pfu in 100 μl of PBS by the tail vein in day 1 and day 7; group Ad5-PDYN received respectively Ad5-PDYN, with 1×10^{11} pfu in 100 μl of PBS by the tail vein in day 1 and day 7, while group C were injected with normal saline as control. Infusions of the recombinant adenoviruses into the tail vein of rats anesthetized with pentobarbital sodium (50 mg/kg, i.p.) were performed as reported previously [32].

Experiment 1: The SD rats were randomly divided into three groups (n = 10): group C, group Ad5 and group Ad5-PDYN to assess whether adenoviral delivery of prodynorphin inhibits experimental inflammatory pain induced by formalin in rats.

Experiment 2: The SD rats were randomly divided into group Ad5 (n = 5) and group Ad5-PDYN (n = 10) to determine whether the possible role of nor-binaltorphimine (nor-BNI) after receiving the pretreatment of adenoviral delivery of PDYN, and nor-BNI was dissolved in artificial cerebrospinal fluid (aCSF) [33]. Rats in the control group received aCSF infusions. We used the method of intrathecal injection to infuse Nor-BNI or aCSF [34]. Intrathecal injection (i.t.) was given in a volume of 5 μl through an intervertebral space at the level of the 5th or 6th lumbar vertebra using a microsyringe.

Formalin-induced nociceptive behaviors and pain intensity scoring

On day 6 after second virus injection, the rats were placed in a transparent glass trough and were allowed to get habituated for about 30 min. Then formalin (50 μl, 5%) was injected subcutaneously (s.c.) into the large lateral footpad on the plantar surface of the right hind paw by 100 μl microsyringe. Each rat was measured 12 times and Formalin-induced nociceptive behaviors of rats were observed by trained individuals who were blinded to experimental conditions, and continued for the next 60 min [35]. Pain intensity scoring (PIS) of nociceptive behavior was recorded per 5 min by assigning weights to the following categories: 0 = the injected paw is no abnormalities; 1 = no or little weight is placed on the injected paw (weight = 1); 2 = the injected paw is elevated and not in contact with any other surface (weight = 2); 3 = the injected paw is licked, bitten or shaken (weight = 3) [36]. PIS for each animal was calculated by the total test session period (300 s) using the following formula. PIS = (TI + 2 × T2 + 3 × T3)/300 T1, T2, T3 represents the time an animal spent in each behavioral category [37].

Reverse transcription-polymerase chain reaction

After PIS testing at day 13, animals were rapidly sacrificed by decapitation. The L4-6 lumber spinal cord (n = 6 per group) was rapidly removed, and immediately deep-frozen in liquid nitrogen and stored at -80°C for further analysis. RT-PCR was used to determine effects of recombinant adenoviral vector on PDYN mRNA expression [38]. The total RNA was extracted from L4-6 lumber spinal cord using Trizol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/2 μg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Both concentration and purity of the total RNA were determined through measuring the absorbance at 260 and 280 nm by spectrophotometry [39]. The cDNA sequences of rat PDYN (No. NM_019374) and β-actin (No. NM_031144) were obtained from GenBank at the National Center for Biotechnology Information (NCBI). Specific primer sequences for PDYN (sense: 5'-GCTCACAGAACTGCCATAGG-3'; reverse: 5'-CCCATACCCACCATCACACC-3') and β-actin (sense: 5'-CACCCGCGAGTACAACCTTC-3'; antisense: 5'-CCCATACCCACCATCACACC-3') were designed and synthesized by Invitrogen. PCR was performed after reverse transcription at 42°C for 60 min and initial denaturation at 95°C for 10 min. The temperature cycles (Roche, Branchburg, NJ) were 94°C/30 s (denaturing), 58°C/30 s (annealing), and 72°C/30 s (extension). A total of 32 cycles and a final 5-min extension at 72°C were conducted. The PCR amplified fragments, 239 bp for PDYN and 207 bp for β-actin, were separated on 3% ethidium bromide stained agarose gel (Invitrogen, Carlsbad, CA). The PCR gel image was captured and analyzed by a gel documentation system (BIO-RAD, USA). The positive PCR bands were purified and sequenced, and the resulting sequences were identical to the targeted cDNA sequences. Each experiment was run three times and each sample was run in triplicate.

Radioimmunoassay of dynorphin A content

The spinal tissue was collected in the same way as the procedure of RT-PCR. Radioimmunoassay was performed for quantitative determination of dynorphin A at the protein level in the spinal tissue [40]. The tissue homogenization was prepared as described previously [41]. Briefly, tissues were homogenized, and samples were spun at 3000 × g for 30 min at 4°C. The aliquot supernatant was stored at -80°C for the subsequent protein quantification. Dynorphin A was eluted and analyzed by radioimmunoassay [42, 43]. Rabbit anti-dynorphin A antibodies were generated against peptide C-terminus and demonstrated 100% molar cross-reactivity with dynorphin A. Results were expressed and analyzed as fmol/mg wet tissue weight, and the protein concentrations were determined by the Bradford assay [44].

Immunohistochemistry

After PIS testing at day 13, the animals (n = 4) while deeply anesthetized with pentobarbital (50 mg/kg, i.p.), were perfused transcardially through the ascending aorta with normal saline, immediately followed by 4% paraformaldehyde (0.1 mol/L phosphate buffer, pH 7.4). For histopathological analysis of the spina1 cords, the L4-6 spinal cord segments were removed and post-fixed in 4% paraformaldehyde at 4°C, to remove water in the tissue. Paraffined sections

were selected for further immunohistochemical processing. A half of sections were tested by Hematoxylin and Eosin (H&E) histology [45]. The sections were examined using a light microscope and were photographed.

Nor-BNI challenge

To determine whether the analgesic mechanism after receiving the pretreatment of adenoviral delivery of PDYN in experiment 2, we injected nor-BNI (3 mg/kg) [33, 46], a specific kappa-opioid receptor antagonist (Tocris Bioscience, Ellisville, MO, USA), to five rats in group Ad5-PDYN before 2 h of PIS testing, while another five rats in group Ad5-PDYN were injected with aCSF as control. Pain behavior in formalin test was measured. In order to rule out non-specific effects on pain processing of the antagonist, rats in group Ad5 were also injected with the same dose of nor-BNI.

Data analysis

Investigators were blinded to all treatments in all tests. Animals were randomly assigned to each group. SPSS 11.5 for windows (Chicago, IL, USA) was used for all data analyses. All data were presented as mean ± standard error of mean (SEM). Changes within each group over time were analyzed by using one-way ANOVA, and followed by post hoc comparison (Student-New-man–Keuls test). Significant differences between treatment groups were detected by two-way ANOVA. $P < 0.05$ is set as the level of statistical significance.

Results

Physiological functions

In the entire observation period, group Ad5-PDYN consumed similar amount of food and fluid compared with group C, group Ad5, respectively ($P > 0.05$). No significant difference was observed in terms of body weight, rectal temperature, and respiratory rate among group C, group Ad5 and group Ad5-prodynorphin ($P > 0.05$).

Ad5-PDYN reduced pain behavior in the late phase of formalin test

After the right intraplantar injection of formalin, rats of three groups all evoked a biphasic agitation response when the response was monitored. Phase 1 of the agitation response was transient and lasted for approximately 10 min, and no significant difference in pain intensity scoring (PIS) was found among group C, group Ad5 and group Ad5-prodynorphin ($P > 0.05$). As

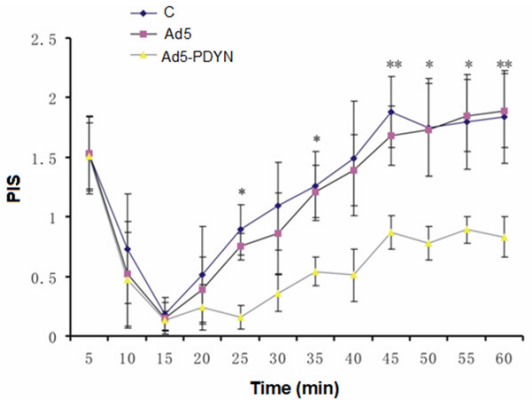

Figure 1. Pain intensity scoring (PIS) of three groups (n = 10 per group) in the formalin test. The formalin tests were performed after the injection of Ad5-PDYN or the control viruses (Ad) twice by tail vein. After injection of Ad5-PDYN, there was a significant reduction of the nociceptive response in the second phase of the formalin test. *$P < 0.05$, **$P < 0.01$ versus group Ad5.

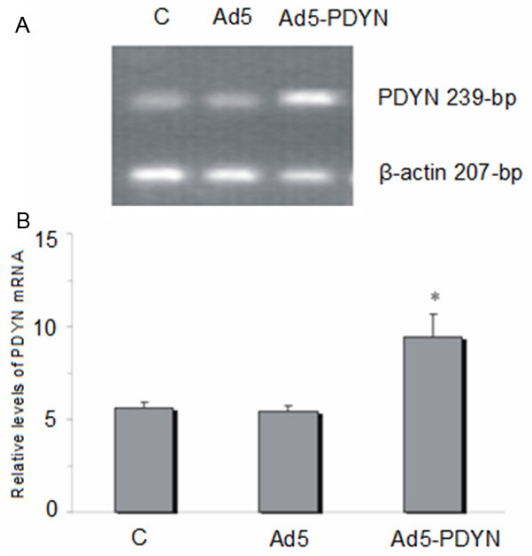

Figure 2. Effect of the pretreatment of adenoviral delivery of PDYN on Formalin test (n = 6 per group). A. An example of agarose gel electrophoresis of PCR products. β-actin PCR (a specific 207-bp segment of cDNA) was used as an internal control. B. Quantification of relative levels of spinal PDYN mRNA expression. The PDYN mRNA levels of spinal cord in group Ad5-PDYN were markedly higher than those of spinal cord in group Ad5. *$P < 0.01$ versus group Ad5.

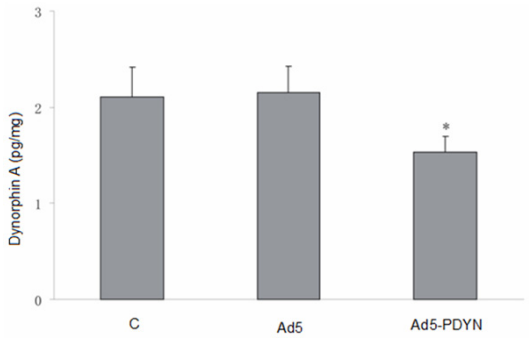

Figure 3. Decreased dynorphin A protein in the spinal cord of rats treated with Ad5-PDYN. Dynorphin A protein of spinal cord in group Ad5-PDYN was significantly lower than those of spinal cord in group Ad5. *$P < 0.05$ versus group Ad5.

shown in **Figure 1**, phase 2 was sustained and lasted 50 min, PIS of group Ad5-prodynorphin was significantly lower than that of group Ad5 (*P < 0.05, **P < 0.01), while no significant difference in PIS was found between group C and group Ad5 (P > 0.05).

Increased PDYN mRNA in the spinal cord of rats treated with Ad5-PDYN

Rats treated with Ad5-PDYN displayed significant up-regulation in the expression of PDYN mRNA (9.47 ± 1.21) compared with group Ad5 (5.46 ± 0.29; *P < 0.01) (**Figure 2**), while no significant difference in the expression of PDYN mRNA was found between group C and group Ad5 (P > 0.05).

Ad5-PDYN reduced the production of dynorphin A

Rats receiving Ad5 injection were served as the control. A group of three rats were sacrificed in the same time period and the spinal cord was obtained for dynorphin A radioimmunoassay. As shown in **Figure 3**, dynorphin A protein of spinal cord in group Ad5-PDYN (1.53 ± 0.17) was significantly lower than those of spinal cord in group Ad5 (2.15 ± 0.28) (*P < 0.05), while no significant difference in dynorphin A was found between group C and group Ad5 (P > 0.05).

Ad5-PDYN reduced inflammation in the spinal cord

H-E staining was performed in spinal cord tissues of Ad5-treated and Ad5-PDYN-treated mice. Formalin-induced evident vasodilatation and hyperemia were observed in spinal grey matter of Ad5-treated rats, but was not detected in that of Ad5-PDYN-treated rats (**Figure 4**).

Nor-BNI challenge

In experiment 2, the anti-nociceptive effects of group Ad5-PDYN were antagonized by injection of Nor-BNI before 2 h of PIS testing, while no effect occurred with aCSF (**Figure 5**). No animal showed abnormal sensory or motor function after administration of Nor-BNI in group Ad5.

Discussion

In our behavioral studies we found that the adenoviral transfer of the PDYN gene into tail vein was sufficient to reduce pain behavior resulting from formalin test. The application of adenoviral vectors had previously been used to produce analgesia [47, 48] but these studies differed from the present study in two key aspects. Firstly, in the current study gene transfer of PDYN by recombinant adenovirus attenuated inflammatory pain in rats; and secondly, the antinociceptive effect after receiving the pretreatment of adenoviral delivery of PDYN was mediated at the level of the spinal cord via kappa-opioid receptor.

We observed a significant antinociceptive effect of Ad5-PDYN treatment on pain in the late phase, but not the early phase, of the rat formalin test, suggesting that Ad5-PDYN treatment might more predominately reduce pain caused by inflammatory response than acute pain in the early phase. As the late phase of formalin test was related to primary afferent fiber-mediated central sensitization [35, 49], it seemed that Ad5-PDYN treatment might effectively inhibit inflammatory-induced hyperactivity or central sensitization.

Gene transfer to nociceptive neurons of the spinal cord is a promising approach to dissect mechanisms of pain in rodents, and is a potential therapeutic strategy for the treatment of persistent pain disorders such as neuropathic pain. A number of studies have demonstrated transduction of the central nervous system using adenovirus by tail vein [32, 50], oral administration [51] and injection directly [48]. Recombinant adenovirus serotype 5 in the nervous system currently have several advantages over other vectors, including stable and safe

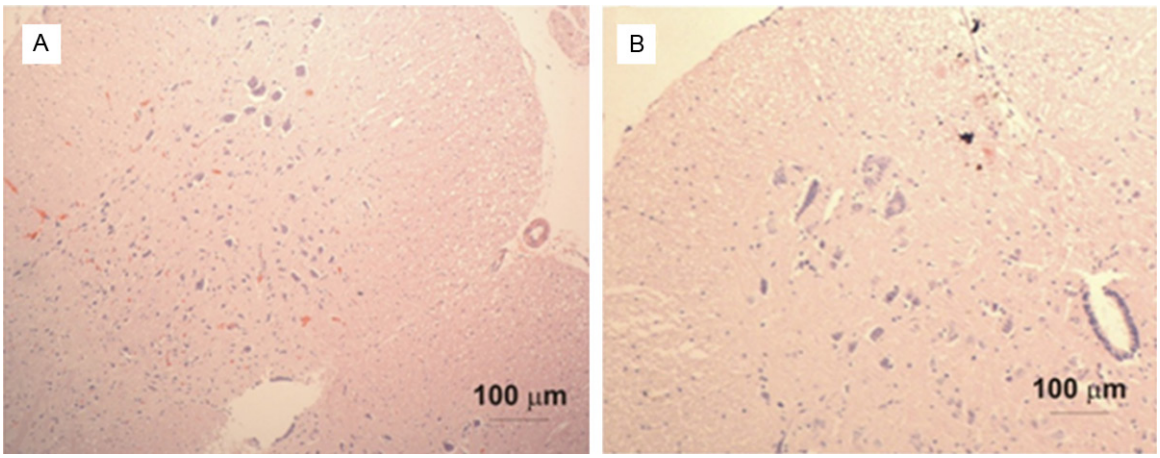

Figure 4. Histology in spinal cord tissues of Ad5-treated and Ad5-PDYN-treated mice. Formalin-induced evident vasodilatation and hyperemia were seen in spinal grey matter of Ad5-treated mice (A), but wasn't detected in that of Ad5-PDYN-treated mice (B). H-E staining (Scale bar: 100 μm).

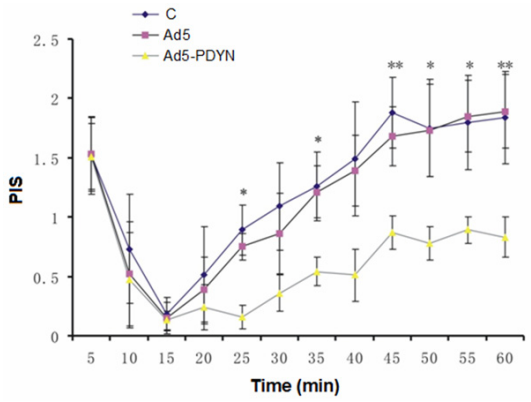

Figure 5. Effects of Nor-BNI on group Ad5-PDYN. Values are expressed as mean ± SEM (five rats in each group). $P < 0.05$ as compared to the animals injected with aCSF.

gene expression [52, 53]. In the pre-experiment, we found PDYN mRNA up-regulation in the spinal cord after Ad5-PDYN injection by tail vein twice in normal rats. Previous finding showed that gene expression remained stable up to 5~8 days after infusion of the recombinant adenovirus serotype 5 [54, 55]. The present study confirmed that the PDYN mRNA levels of group Ad5-PDYN were markedly higher than those of group Ad5 in spinal cord on day 6 after second virus injection, suggesting the recombinant adenovirus successfully transferred the PDYN gene into the spinal cord, consistent with previous reports [56, 57].

Here we showed that dynorphin A protein of spinal cord in group Ad5-PDYN was significantly lower than those of spinal cord in group Ad5, and the PDYN mRNA levels in group Ad5-PDYN were markedly higher than those in group Ad5, also suggesting dynorphin A down-regulation and PDYN up-regulation, consistent with previous reports [58]. There are possible reasons for the observed low level of dynorphin A protein when exogenous PDYN was applied. One is that the function of pro-protein convertases [10] from PDYN to dynorphin A may be down-regulated via both lysosome and proteasome systems [59] after the pretreatment of adenoviral delivery of PDYN; the other is that the production of dynorphin A may be decreased during the observations when Ad5-PDYN treatment reduces the nociceptive effect induced by formalin, since spinal dynorphin A up-regulation is a consequence of nociceptive pain [14, 60, 61]. Our data provided clear evidence that the antinociceptive effect after receiving the pretreatment of adenoviral delivery of PDYN is mediated at spinal cord via KOR, only suggesting that PDYN-derived peptides acting on KOR are important in the control of inflammatory pain, because PDYN obviously may be processed to multiple peptides that could elicit a behavioral effect. Consistent with this, a pronounced impact of endogenous PDYN-derived peptides on nociception was mediated via KOR [5, 9], showed the same effects.

Dynorphin A, an endogenous opioid neuropeptides derived from the PDYN gene [6], is thought to play a role in activity-dependent processes,

including synaptic plasticity and learning and memory [62, 63]. As the transcriptional repressor, DREAM regulates a number of genes including PDYN [64, 65], and DREAM knockout (KO) mice showed significantly enhanced PDYN mRNA expression and memory 24 hours after training as measured by percent freezing, suggesting that dynorphin A didn't obviously upregulated [58]; while PDYN knockout mice demonstrated diminished age-associated impairment in spatial water maze performance, suggesting dynorphin A expression significantly downregulated [43]. These opposing effects of endogenous kappa-opioidergic system suggest that control of DREAM-PDYN-dynorphin message may be very important between dynorphin A expression and pathological processes. This has led to the notion that PDYN and dynorphin A were involved in antinociception when dynorphin A increases were be limited to within physiological ranges [6]; otherwise, endogenous dynorphin A appeared to be involved in chronic allodynia and hyperalgesia when dynorphin A increases were in the supraphysiological ranges [66]. We are not aware whether DREAM inhibits or promotes the production of dynorphin A when more PDYN is present. Further studies to delineate the interaction of spinal DREAM-PDYN-dynorphin message in pain states might lead to better strategies for the prevention of inflammation-induced pain.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Tingting Wang, Department of Ophthalmology, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, P. R. China. Tel: +86 0591 87981990; E-mail: wangtit163@163.com

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