Neurosci Bull April 1, 2012, 28(2): 182−192. http://www.neurosci.cn
DOI: 10.1007/s12264-012-1216-8

·Original Article·

Disruption of δ-opioid receptor phosphorylation at Threonine 161 attenuates morphine tolerance in rats with CFA-induced inflammatory hypersensitivity

Hai-Jing Chen[1,*], Wei-Yan Xie[2,*], Fang Hu[1], Ying Zhang[1], Jun Wang[3], Yun Wang[1]

[1]*Neuroscience Research Institute, Peking University, Department of Neurobiology, School of Basic Medical Sciences, Peking University, Beijing 100191, China*
[2]*Beijing Neurosurgical Institute, Beijing Tiantan Hospital Affiliated to Capital Medical University, Beijing 100050, China*
[3]*Department of Anatomy and Histology, School of Basic Medical Sciences, Peking University, Beijing 100191, China*

© Shanghai Institutes for Biological Sciences, CAS and Springer-Verlag Berlin Heidelberg 2012

Abstract: Objective Our previous study identified Threonine 161 (Thr-161), located in the second intracellular loop of the δ-opioid receptor (DOR), as the only consensus phosphorylation site for cyclin-dependent kinase 5 (Cdk5). The aim of this study was to assess the function of DOR phosphorylation by Cdk5 in complete Freund's adjuvant (CFA)-induced inflammatory pain and morphine tolerance. **Methods** Dorsal root ganglion (DRG) neurons of rats with CFA-induced inflammatory pain were acutely dissociated and the biotinylation method was used to explore the membrane localization of phosphorylated DOR at Thr-161 (pThr-161-DOR), and paw withdrawal latency was measured after intrathecal delivery of drugs or Tat-peptide, using a radiant heat stimulator in rats with CFA-induced inflammatory pain. **Results** Both the total amount and the surface localization of pThr-161-DOR were significantly enhanced in the ipsilateral DRG following CFA injection. Intrathecal delivery of the engineered Tat fusion-interefering peptide corresponding to the second intracellular loop of DOR (Tat-DOR-2L) increased inflammatory hypersensitivity, and inhibited DOR- but not μ-opioid receptor-mediated spinal analgesia in CFA-treated rats. However, intrathecal delivery of Tat-DOR-2L postponed morphine antinociceptive tolerance in rats with CFA-induced inflammatory pain. **Conclusion** Phosphorylation of DOR at Thr-161 by Cdk5 attenuates hypersensitivity and potentiates morphine tolerance in rats with CFA-induced inflammatory pain, while disruption of the phosphorylation of DOR at Thr-161 attenuates morphine tolerance.

Keywords: inflammatory hypersensitivity; cyclin-dependent kinase 5; δ-opioid receptor; morphine tolerance

1 Introduction

Morphine and its three types of opioid receptors (μ, δ and κ) have confounded researchers for decades with their enigmatic mechanism of controlling pain with exceptional efficacy. However, morphine antinociceptive tolerance has largely limited its use. The mechanisms of morphine antinociceptive tolerance are poorly understood due to the complexity of the underlying pathways. Nonetheless, accumulating evidence suggested that the δ-opioid receptor (DOR) plays an important role in the development of this tolerance[1-4]. *DOR*$^{-/-}$ mice have no morphine tolerance[4,5]. Reduced cell surface insertion of DORs by protachykinin

*These authors contributed equally to this work.
Corresponding author: Yun Wang
Tel & Fax: +86-10-82801119
E-mail: wangy66@bjmu.edu.cn
Article ID: 1673-7067(2012)02-0182-11
Received date: 2012-01-15; Accepted date: 2012-03-12

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

A gene knock-out does not induce morphine tolerance[6]. Besides, μ-opioid receptor (MOR)-DOR heterodimerization has been indicated to play an important role in tolerance development[7,8].

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the cyclin-dependent kinase (CDK) family. The Cdk5/p35 complex plays a pivotal role in the nervous system. Furthermore, Cdk5 activity has recently been shown to potentiate morphine tolerance[9]. Our previous study identified Threonine 161 (Thr-161), located in the second intracellular loop of the DOR, as the only consensus phosphorylation site for Cdk5. Thr-161 is required for the cell-surface localization of DORs and the formation of DOR-MOR heterodimers, and then morphine tolerance. Interference with Thr-161 site phosphorylation significantly attenuates the development of morphine antinociceptive tolerance[10].

In the clinic, treatment of patients with inflammatory pain includes opioids acting outside the central nervous system[11]. Here, we chose a rat model of complete Freund's adjuvant (CFA)-induced inflammatory pain, and assessed the roles of phosphorylation of DORs by Cdk5 in CFA-induced inflammatory pain and morphine tolerance.

2 Materials and methods

2.1 Experimental animals Male Sprague-Dawley rats weighing 200–220 g were provided by the Animal Center of Peking University Health Science Center. Rats were individually housed at 24 ± 1°C in climate-controlled rooms under a 12 h: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 guidelines of Animal Care and Use Committee of Peking University, and were approved by this committee.

For intrathecal (i.t.) drug administration, i.t. cannulas were implanted following the method of Storkson *et al.*[12]. Briefly, rats were anesthetized with 10% chloral hydrate (0.3 g/kg, i.p.). The dorsal skin was incised and the spinal column was exposed. The intraspinal space between lumbar vertebrae 4 and 5 (L4 and L5) was chosen as the site of insertion of the needle. Slight movements of the tail indicated proper insertion of the needle into the subarachnoid space. A PE-10 polyethylene catheter (4.0 cm) was implanted using the catheter-through-needle technique to reach the lumbar enlargement. The outer end of the catheter was plugged and fixed onto the skin on closure of the wound. The rats were housed individually after surgery and allowed 5–6 days for recovery before being tested. Animals with neurological damage after catheter implantation were excluded. Nociceptive responses after i.t. injection or delivery of drugs were measured in a blinded manner.

2.2 Tat-peptide, deltorphin I and anti-DOR-phosphothreonine-161 antibody (pDOR-Thr-161) construction The engineered Tat fusion-interefering peptide corresponding to the second intracellular loop of DOR (Tat-DOR-2L) (RKKRRQRRRVKALDFRTPAKAKL) and Tat-control (Tat-ctrl) (RKKRRQRRRRAAKVPKFLTLDKA) were synthesized and purified by the Chinese Peptide Co., Hangzhou, China. The mass and purity of the peptides were verified by HPLC. Peptides were dissolved in 0.9% NaCl to a concentration of 1 μg/μL.

Deltorphin I (Tyr-*D*-Ala-Phe-Asp-Val-Val-Gly-NH_2) was synthesized by GL Biochem (Shanghai) Ltd. Deltorphin I was dissolved in 0.9% NaCl to a concentration of 10 μg/12 μL.

Anti-pDOR-Thr-161 antibody was custom-made by 21st Century Biochemicals (Marlboro, MA, USA) as described in our previous paper[10]. Briefly, rabbits were immunized with the pDOR-Thr-161 peptide AcVKALDFR(pT)PAKAKLC-amide conjugated to keyhole limpet hemocyanin. Sera were obtained after five consecutive bleeds.

2.3 Western blot Rats were deeply anesthetized with 10% chloral hydrate (0.3 g/kg, i.p.) and then the L4–L6 DRGs and dorsal horn of the lumbar enlargement were removed and immediately homogenized in ice-chilled lysis buffer (containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mmol/L EGTA, 0.5 g/mL leupeptin, 1 mmol/L PMSF, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, and proteinase inhibitor cocktail). The homogenate was centrifuged at 12 000 g for 5 min at 4°C and the supernatant was analyzed. The con-

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

centration of protein was measured with a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). Then, equal amounts of sample (50 μg) were denatured and subjected to SDS-PAGE using 12% running gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk in TBST (containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibody. The blots were washed three times in TBST for 10 min. Then they were incubated with horseradish peroxidase-conjugated secondary antibody (1:2 000, goat anti-rabbit or goat anti-mouse; Bio-Rad Laboratories, Hercules, CA, USA.) for 1 h at room temperature. Finally, the blots were developed with a Lightning chemiluminescence kit (sc-2048; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4 Dissociated DRG cell surface biotinylation Rats were terminally anesthetized with 10% chloral hydrate (0.3 g/kg, i.p.), then the L4–L5 DRGs were removed, digested with collagenase type IA (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 50 min and then with 0.125% trypsin (Sigma-Aldrich) for 10 min at 37°C. Fetal bovine serum (FBS) was added to terminate the enzymatic treatment, followed by centrifugation at 500 rpm for 5 min. Then the cell pellets were resuspended with DMEM containing 10% FBS. The dissociated cells (150 μL) were plated onto poly-*D*-Lysine-coated (100 μg/mL; Sigma-Aldrich) glass cover-slips in 35-mm culture dishes and incubated for 4 h at 37°C in an incubator with 5% CO_2 and 95% air.

Dissociated DRG cells were incubated with 500 μg/mL sulfo-N-hydroxysuccinimide-biotin (Pierce Biotechnology) in PBS for 45 min at 4°C to biotinylate surface proteins. After terminating the reaction, cells were lysed in buffer containing 0.1% Triton X-100, 150 mmol/L NaCl, 25 mmol/L KCl, 10 mmol/L Tris-HCl, pH 7.4, 1 μmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 12 000 g for 5 min at 4°C. Biotinylated proteins were bound overnight at 4°C on immobilized NeutrAvidin protein (Pierce Biotechnology), and nonspecifically bound material was removed by washing six times in PBS containing 1% Triton X-100 and 0.1% SDS. Washed beads were eluted with SDS sample buffer, and the eluate was analyzed by SDS-PAGE and Western blotting using rabbit anti-pDOR-Thr-161 antibody.

2.5 Assessment of hypersensitivity Thermal hypersensitivity was assessed in unrestrained rats according to a previous report[13]. Briefly, the animals were placed onto the surface of a 2-mm-thick covered glass and were allowed 20 min for acclimation to the environment before testing. Paw withdrawal latency (PWL) in response to radiant heat was recorded by a stimulator. The PWL was averaged over four trials at 5-min intervals. To prevent tissue injury, the cut-off time was set at 30 s.

2.6 Acute morphine antinociceptive tolerance Rats received six consecutive injections of morphine (5 mg/kg, s.c.; Qinghai Pharmaceutical Factory, Qinghai, China) at 2-h intervals. Nociception was assessed 30 min after each injection by the radiant heat stimulator.

2.7 Delivery of drugs to the DRG After 5–6 days of recovery from surgery for placement of the intrathecal catheter, rats were subjected to i.t. injection of Tat-ctrl or Tat-DOR-2L. The basal PWL was measured, and rats were subjected to i.t. injection of the peptides. After injection through the catheter, the needle remained *in situ* for 2 min before being withdrawn. Thirty minutes later, 100 μL 25% CFA was injected into the plantar surface of the left hindpaw. Nociceptive responses were again measured at 1 h, 2 h, 6 h, 1 day, and 3 days after CFA administration.

For the acute morphine tolerance test, the basal PWL was measured, and CFA was injected into the plantar surface of the left hindpaw. One day after injection of CFA, PWL was measured and rats were subjected to i.t. injection of Tat-ctrl or Tat-DOR-2L (3 μL). These drugs were injected slowly over 2 min. After injection through the catheter, the needle remained *in situ* for 2 min before being withdrawn. Thirty minutes after injection of drugs, acute morphine antinociceptive tolerance was induced.

2.8 Statistical analysis All data are presented as mean ± SEM. Differences between groups were compared using the unpaired *t*-test. The criterion for statistical significance was $P < 0.05$.

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

3 Results

3.1 Phosphorylated DOR at Thr-161 (pThr-161-DOR) in DRG neurons increased after CFA injection Our previous study indicated that Cdk5 phosphorylates Thr-161 in the second loop of the DOR, and this phosphorylation contributes to Cdk5-mediated morphine antinociceptive tolerance[10]. In the present study, we aimed to study the functions of phosphorylation of the DOR by Cdk5 in CFA-induced inflammatory pain and morphine tolerance. Based on our previous results, CFA-induced inflammatory hypersensitivity reached a peak and showed good reproducibility in the repeated experiments at 1 day after CFA and was maintained for more than 1 week in the ipsilateral paw[14]. Thus we first tested whether Thr-161-DOR could be phosphorylated at 1 day after CFA injection by using acutely dissociated DRG neurons from the ipsilateral inflammatory side. Western blot (Fig. 1A) and surface biotinylation (Fig. 1B) results showed that both the total amount and the surface-localized pThr-161-DOR increased 1 day after CFA injection compared to the naïve group (n = 4, P <0.05). Together, these results indicate that the phosphorylation of Thr-161-DOR might be involved in CFA-induced hypersensitivity.

3.2 Intrathecal delivery of Tat-DOR-2L enhanced pain hypersensitivity Having shown that the phosphorylation of membrane Thr-161-DOR increased during CFA-induced hypersensitivity, we hypothesized that the second intracellular loop of the DOR, in which Thr-161 is located, may be involved in CFA-induced inflammatory pain and morphine tolerance. To test this, we engineered a Tat

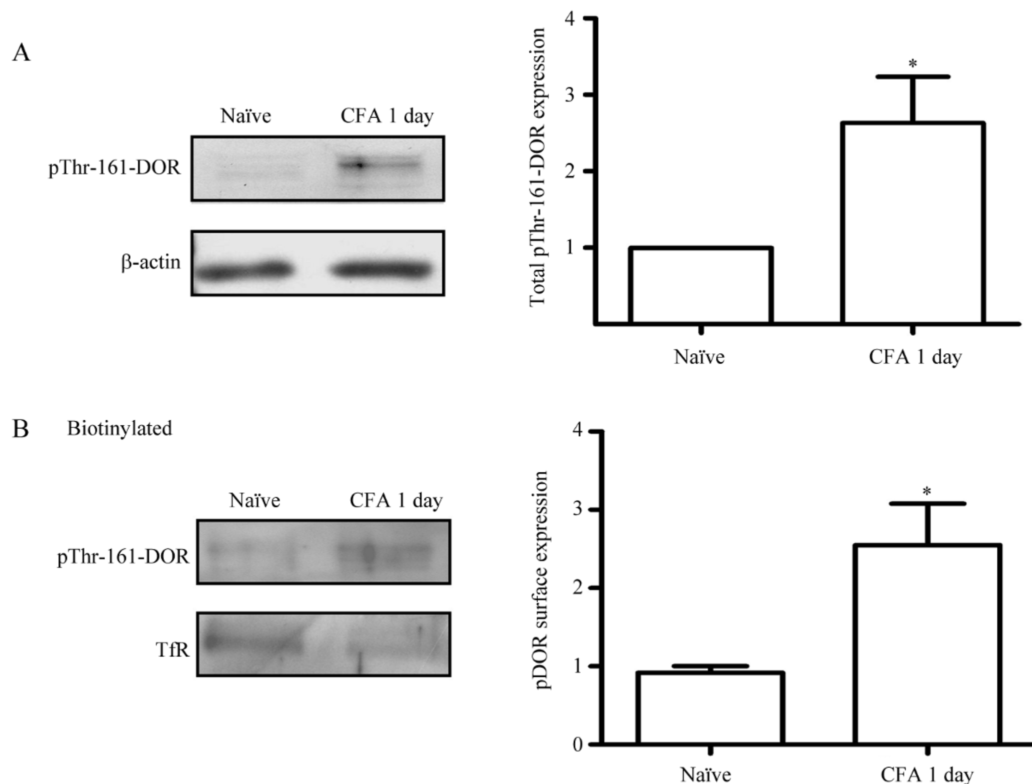

Fig. 1. The δ-opioid receptor (DOR) Thr-161 phosphorylation level increased in DRG cells at 1 day after CFA injection. A: Total level of phosphorylated DOR at Thr-161 (pThr-161-DOR) in DRG neurons in Western blots (left panel). The β-actin bands are the loading controls. Quantitative analysis showed that total pThr-161-DOR expression increased in DRG neurons at 1 day after CFA, compared to the naïve group (right panel). Mean ± SEM. *P <0.05, n = 4, two-tailed paired t test. B: DOR Thr-161 phosphorylation level on the DRG cell surface detected by the biotinylation assay (left panel). The TfR antibody bands are the loading controls. Quantitative analysis showed that surface-biotinylated pThr-161-DOR at the cell surface increased in DRG neurons at 1 day after CFA compared to the naïve group (right panel). Mean ± SEM. *P <0.05, n = 4, two-tailed paired t test.

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

fusion-interfering peptide corresponding to the second intracellular loop of the DOR (Tat-DOR-2L), including the TPAK sequence[10]. To study the role of Tat-DOR-2L in CFA-induced hypersensitivity, the basal PWL of rats was measured after 5 days of recovery from surgery. Rats were pretreated with 3 μg Tat-DOR-2L or Tat-ctrl, and 30 min later with CFA. Nociceptive responses were measured at 1 h, 2 h, 6 h, 1 day, or 3 days after CFA administration. Compared with Tat-ctrl, pretreatment with Tat-DOR-2L enhanced the CFA-induced heat hypersensitivity, and the effect was maintained until 1 day after inflammation (Fig. 2). These results indicate that phosphorylation of DOR at Thr-161 attenuates CFA-induced heat hypersensitivity.

It was reported that deltorphin I, an agonist of the DOR, induces DOR-mediated spinal analgesia[29]. Here, we confirmed this result (Fig. 3). After 5–6 days of recovery from surgery for placement of the i.t. catheter, we measured the basal PWL, and then CFA was injected into the

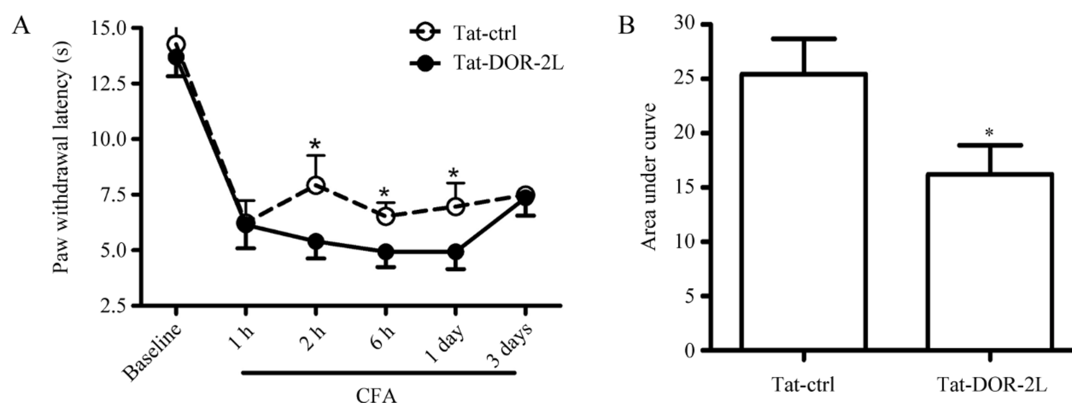

Fig. 2. Pretreatment with Tat-DOR-2L enhanced CFA-induced heat hypersensitivity. A: Time course of the paw withdrawal latency of the ipsilateral hindpaw measured by radiant heat stimuli before and after intrathecal injection of Tat-control (Tat-ctrl) or Tat-DOR-2L (3 μg). Mean ± SEM. *n* = 11–13. Two-tailed unpaired *t* test, **P* <0.05 compared to Tat-DOR-2L. B: Area under the curve of the time course (from 1 h to 1 day) of the paw withdrawal latency in A. Two-tailed unpaired *t* test. **P* <0.05, compared with Tat-ctrl.

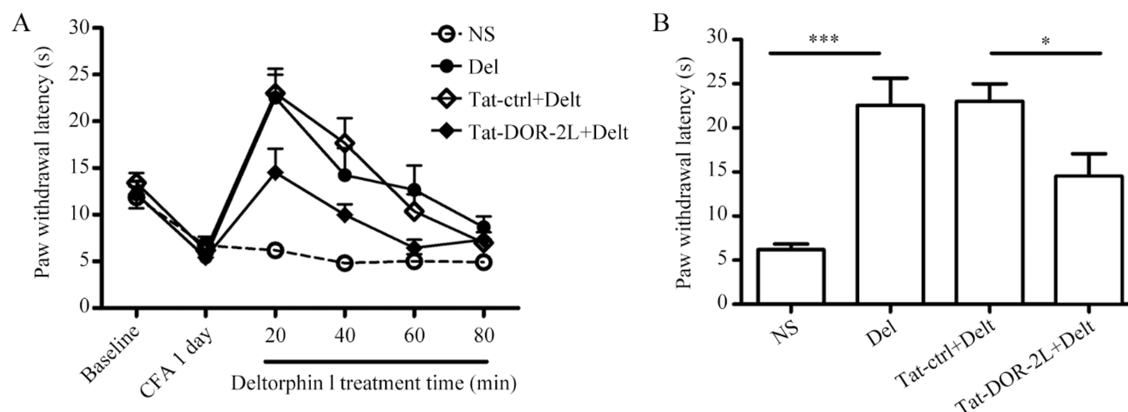

Fig. 3. Tat-DOR-2L inhibited DOR-mediated spinal analgesia in CFA-treated rats. A: Intrathecal administration of deltorphin I (Delt), an agonist of the DOR, reduced CFA-induced hypersensitivity. Co-delivery of Tat-DOR-2L and deltorphin I inhibited deltorphin I-mediated analgesia compared with Tat-control (Tat-ctrl). The values represent the paw withdrawal latency (s) to heat stimuli, and are presented as mean ± SEM. *n* = 8–10. B: Paw withdrawal latency of rats with CFA-induced inflammatory pain to a radiant heat stimulator 20 min after administration of deltorphin I. Data were analyzed by ANOVA followed by the Newman-Keuls multiple comparison test. **P* <0.05, ****P* <0.001, compared with normal saline (NS) or Tat-ctrl.

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

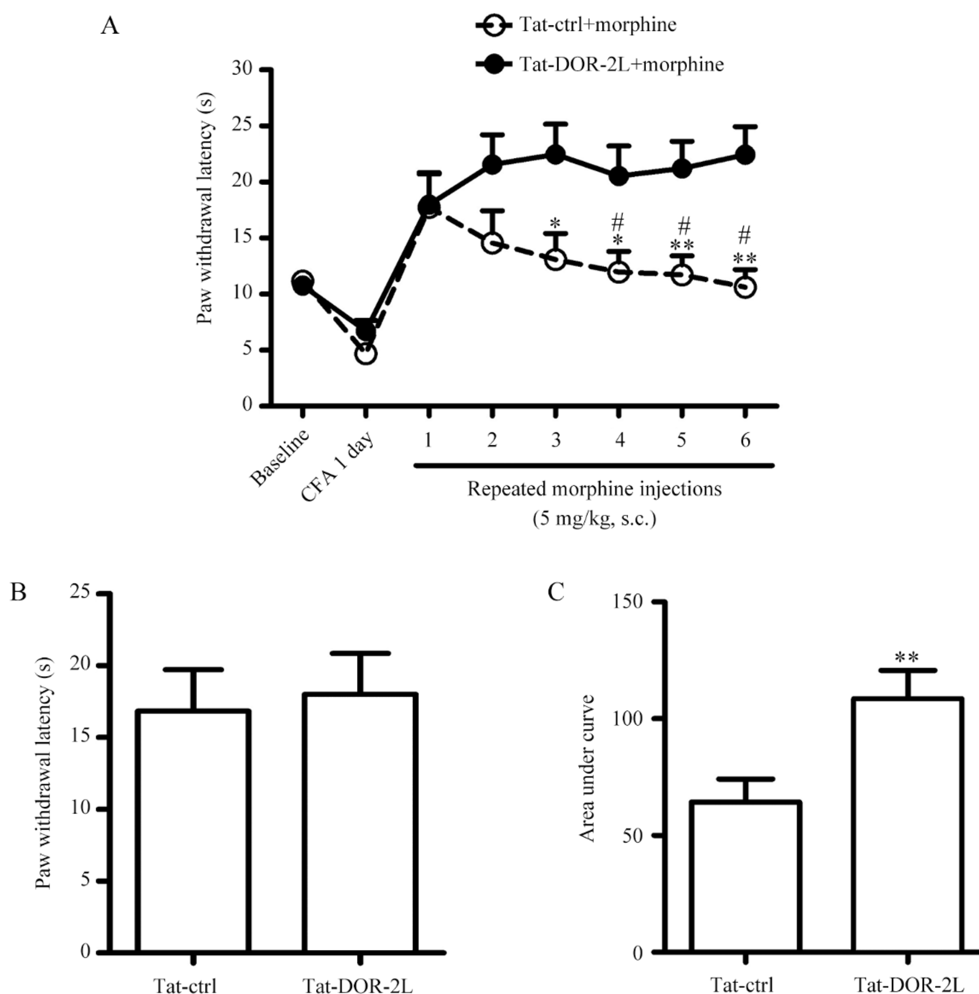

Fig. 4. Tat-DOR-2L inhibited acute morphine tolerance in CFA-treated rats. A: Intrathecal pretreatment with Tat-DOR-2L for 30 min at 3 μg caused profoundly slower development of morphine antinociceptive tolerance compared to Tat-control (Tat-ctrl). The values represent the paw withdrawal latency (s) to heat stimuli, and are presented as mean ± SEM. *n* = 13. Two-tailed paired *t* test. **P* <0.05, ***P* <0.01, compared to Tat-ctrl; #*P* <0.05 compared to the first injection of morphine in Tat-ctrl. B: Pretreatment with Tat-DOR-2L did not affect the paw withdrawl latency (PWL) to the first injection of morphine. C: Area under the curve of the time course (from 2nd to 6th morphine injections) of the PWL in A. Two-tailed paired *t*-test. ***P* <0.01, compared with Tat-ctrl.

plantar surface of the left hindpaw. On day 1 after CFA injection, we measured the PWL, and then rats were injected i.t. with normal saline (NS), deltorphin I, Tat-ctrl plus deltorphin I (at a 30-min interval, that is, Tat-ctrl first and 30 min later deltorphin I), or Tat-DOR-2L plus deltorphin I (at a 30-min interval, that is, Tat-DOR-2L first and 30 min later deltorphin I). Nociceptive responses were again measured at 20, 40, 60, and 80 min after i.t. injection. Deltorphin I significantly reduced the CFA-induced hypersensitivity at 20 min after injection compared with the NS group (Fig. 3A). In addition, at 20 min after deltorphin I injection, Tat-DOR-2L inhibited DOR-mediated spinal analgesia, while Tat-ctrl did not (Fig. 3A, B). We concluded that disruption of Thr-161-DOR phosphorylation specifically reduced DOR-mediated spinal analgesia. Taken together, the phosphorylation of Thr-161-DOR by Cdk5 was important for heat hypersensitivity, and inhibition of this phosphorylation enhanced CFA-induced inflammatory

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

hypersensitivity.

3.3 Intrathecal delivery of Tat-DOR-2L postponed morphine tolerance in rats with CFA-induced inflammation Having shown that Tat-DOR-2L enhanced CFA-induced hypersensitivity, we next assessed the role of Tat-DOR-2L in morphine tolerance in CFA-treated rats. After 5–6 days of recovery from surgery, the basal PWL was measured. Then CFA was injected into the plantar surface of the left hindpaw, and PWL was measured 1 day later. The rats were further treated with 3 μg Tat-DOR-2L or Tat-ctrl. Thirty minutes later, the acute morphine antinociceptive tolerance model was induced by six consecutive injections of morphine. Significant differences of prolonged PWL were noted in rats receiving Tat-DOR-2L compared with Tat-ctrl (Fig. 4A). Pretreatment with Tat-DOR-2L did not affect the PWL response to the first injection of morphine (Fig. 4B), but significantly attenuated acute morphine tolerance in rats with CFA-induced heat hypersensitivity. These results suggest that phosphorylation of Thr-161-DOR plays a vital role in the development of acute morphine tolerance, while disruption of the phosphorylation postpones morphine tolerance without affecting the analgesic effect.

4 Discussion

4.1 DORs play an important role in inflammatory hypersensitivity The present results revealed that phosphorylation of Thr-161-DOR attenuated CFA-induced heat hypersensitivity. Disruption of Thr-161-DOR phosphorylation enhanced CFA-induced heat hypersensitivity, postponed morphine tolerance, but did not affect its analgesic effect.

Preclinical studies have shown that selective DOR agonists increase antinociceptive potency during peripheral inflammatory hypersensitivity[15,16], although MOR agonists are particularly used in the management of inflammatory pain. DOR ligands also have less abuse potential than morphine, and reduced respiratory gastrointestinal impairments than those produced by MOR agonists[17,18]. Thus, strategies that increase the antinociceptive potency of DORs are promising targets for inflammatory pain treatment.

Increasing evidence indicates that DORs play an important role in inflammatory hypersensitivity. Intraplantar injection of CFA or capsaicin promotes the trafficking of DORs to the plasma membrane of DRG neurons. Direct exposure of DRG neurons to prostaglandin E2, bradykinin, or capsaicin also promotes the trafficking of DORs to the plasma membrane[19]. Under conditions of inflammatory injury, the levels of DOR and MOR mRNA and protein are increased in the ipsilateral dorsal horn[20-23]. Visceral inflammation also similarly increases the levels of DOR mRNA in the dorsal horn, but the levels of protein are not significantly increased[24]. In DOR mutants, the increased hypersensitivity response seems to occur in both early and late phases after formalin injection, however, in MOR mutants this only happens in the early phase with no effects in the late phase[25]. These results indicate that both DORs and MORs are involved in responses to irritative noxious stimulation, while DORs are mainly implicated in the inflammatory phase. In a model of peripheral inflammation, DOR-selective agonists were shown to have robust antihyperalgesic effects[23,26-32]. The present study provides evidence that phosphorylation of Thr-161-DOR attenuates CFA-induced heat hypersensitivity.

4.2 Phosphorylation of the DOR by protein kinases modulates its function It is still unclear whether the adaptations in cellular signaling evoked by repeated morphine administration lead to the development of tolerance. Evidence shows that intrathecal administration of roscovitine, an inhibitor of Cdk5, has an antinociceptive effect, while co-administration of roscovitine and morphine enhances the antinociceptive effect of morphine in tolerant rats[33]. Parkitna and Przewlocki also reported that when injections of morphine are preceded by i.t. administration of roscovitine, the development of tolerance to morphine analgesia is completely abolished. Besides, a single i.t. injection of roscovitine restores the analgesic effect of morphine in a dose-dependent manner in tolerant rats. These workers supposed that chronic morphine treatment activates a highly efficient pathway by means of which Cdk5 regulates glycogen synthase kinase 3β activity[34]. The DOR has been conclusively shown to be involved in morphine antinoci-

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

ceptive tolerance. In order to clarify this idea, numerous studies have shown that inhibition of DORs attenuates this tolerance[1-4,35]. Recently, our group reported that mutation of the DOR at Thr-161 impairs acute morphine antinociceptive tolerance *in vivo,* which might be regulated by Cdk5. Our previous results indicate that Cdk5 promotes this tolerance by phosphorylating Thr-161 of the DOR, and inhibition of this phosphorylation might destroy the function of DORs by reducing their surface expression and the formation of DOR-MOR heterodimers[10]. In the present study, we found that the phosphorylation level of Thr-161-DOR increased both in DRG neurons and on the membrane surface during CFA-induced inflammatory pain. We supposed that Cdk5 might regulate the membrane trafficking of DORs through phosphorylation at Thr-161 to play a role in CFA-induced hypersensitivity.

As noted above, Cdk5 can phosphorylate Thr-161-DOR, and indeed, the phosphorylation of an opioid receptor is the first step in its activation during the processes of desensitization and trafficking. As a classical G-protein-coupled receptor (GPCR), the phosphorylation state of an opioid receptor can be divided into two parts: basal (constitutive) phosphorylation, and agonist-induced phosphorylation. Desensitization of GPCR signaling involves agonist-mediated receptor phosphorylation, followed by the recruitment of arrestins and sequestration of the arrestin-bound receptors into agonist-inaccessible cellular compartments[36]. The enzymes activated by phosphorylated opioid receptors include second messenger-dependent protein kinases [protein kinase C (PKC), cyclic AMP-dependent protein kinase II (CaMKII)], and G-protein kinases (MAPKs), which play important roles in the regulation of opioid signal transduction[37]. But, the information about the kinases for phosphorylating and modulating DOR function is limited.

4.3 Hypothesis of the development of morphine antinociceptive tolerance Receptor trafficking is considered to be a key process in the regulation of receptor signaling. In order to study DOR internalization, Pradhan and Kieffer created knock-in mice expressing fluorescent DOR (DOR-eGFP) in place of the endogenous receptor. The results of the study showed that DOR-eGFP internalization is strongly correlated with receptor phosphorylation and uncoupling from G-proteins[38]. Chronic morphine treatment produces adaptive changes at the β-arrestin 1 level, which in turn attenuate the agonist-mediated desensitization and internalization of GPCRs[39]. Patwardhan *et al*. have shown that peripheral activation of primary afferent nociceptors with bradykinin[40] or trypsin[41] enhances both the targeting of DORs to the cell surface and receptor competence. Gendron *et al.* found a similar effect on DOR trafficking in small DRG neurons following injection of capsaicin into rat hindpaw[42]. Constitutive receptor activity has been reported to increase in animals treated chronically with morphine. Translocation of DORs from intracellular compartments to neuronal plasma membranes is induced by chronic morphine treatment, and then increases the numbers of functional receptors[19]. Another hypothesis about the development of morphine antinociceptive tolerance is the heterodimerization of MORs and DORs[43]. This idea is further supported by findings that MORs and DORs are co-expressed in the DRG[44-46] and some dorsal horn neurons[47,48], suggesting that physical interactions between MORs and DORs are possible *in vivo.* Considerable evidence demonstrates the formation of heterodimers between MORs and DORs *in vitro* and *in vivo*[49-51]. The membrane density of DORs was shown to increase 72 h after the induction of inflammation, an effect abolished in MOR-knockout (KO) mice[52]. Intrathecal administration of the DOR-selective agonist deltorphin II fails to have antihyperalgesic effects in MOR-KO mice[53]. Here, we demonstrated that interference with the phosphorylation of DORs attenuated morphine tolerance, without affecting the antinociception.

Disruption of DOR phosphorylation can attenuate morphine tolerance during CFA-induced hypersensitivity, which may potentially have important clinical advantages. In the present study, we first confirmed that DORs, phosphorylated by Cdk5, were involved in CFA-induced inflammatory hypersensitivity and morphine tolerance. However, the mechanisms controlling this process are still undefined. Admitting that the analgesic effects and involvement in morphine tolerance of DORs are directly

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

related to the level of DORs at the membrane[10,19,54], our results suggest the hypothesis that phosphorylation of DORs by Cdk5 is important for its trafficking and distribution.

Acknowledgements: This work was supported by grants from the National Natural Science Foundation of China (30830044, 30925015, 30800330, and 81161120497), Beijing Natural Science Foundation (7092061) and Specialized Research Fund for Doctoral Program of Higher Education Grants, China (200800011028 and 20060001121).

References:

[1] Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE. Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. J Pharmacol Exp Ther 1991, 258: 299–303.

[2] Fundytus ME, Schiller PW, Shapiro M, Weltrowska G, Coderre TJ. Attenuation of morphine tolerance and dependence with the highly selective δ-opioid receptor antagonist TIPP[ψ]. Eur J Pharmacol 1995, 286: 105–108.

[3] Kest B, Lee CE, McLemore GL, Inturrisi CE. An antisense oligodeoxynucleotide to the delta opioid receptor (DOR-1) inhibits morphine tolerance and acute dependence in mice. Brain Res Bull 1996, 39(3): 185–188.

[4] Zhu Y, King MA, Schuller AG, Nitsche JF, Reidl M, Elde RP, *et al*. Retention of supraspinal delta-like analgesia and loss of morphine tolerance in δ opioid receptor knockout mice. Neuron 1999, 24: 243–252.

[5] Nitsche JF, Schuller AG, King MA, Zengh M, Pasternak GW, Pintar JE. Genetic dissociation of opiate tolerance and physical dependence in δ-opioid receptor-1 and preproenkephalin knock-out mice. J Neurosci 2002, 22: 10906–10913.

[6] Guan JS, Xu ZZ, Gao H, He SQ, Ma GQ, Sun T, *et al*. Interaction with vesicle luminal protachykinin regulates surface expression of δ-opioid receptors and opioid analgesia. Cell 2005, 122: 619–631.

[7] Rozenfeld R, Devi LA. Receptor heterodimerization leads to a switch in signaling: β-arrestin2-mediated ERK activation by μ-δ opioid receptor heterodimers. FASEB J 2007, 21: 2455–2465.

[8] He SQ, Zhang ZN, Guan JS, Liu HR, Zhao B, Wang HB, *et al*. Facilitation of μ-opioid receptor activity by preventing δ-opioid receptor-mediated codegradation. Neuron 2011, 69: 120–131.

[9] Pareek TK, Kulkarni AB. Cdk5: a new player in pain signaling. Cell Cycle 2006, 5: 585–588.

[10] Xie WY, He Y, Yang YR, Li YF, Kang K, Xing BM, *et al*. Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the δ-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. J Neurosci 2009, 29: 3551–3564.

[11] Stein C, Schafer M, Machelska H. Attacking pain at its source: new perspectives on opioids. Nat Med 2003, 9: 1003–1008.

[12] Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. J Neurosci Methods 1996, 65: 167–172.

[13] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hypersensitivity. Pain 1988, 32: 77–88.

[14] Yang YR, He Y, Zhang Y, Li Y, Li Y, Han Y, *et al*. Activation of cyclin-dependent kinase 5 (Cdk5) in primary sensory and dorsal horn neurons by peripheral inflammation contributes to heat hyperalgesia. Pain 2007, 127: 109–120.

[15] Stein C. The control of pain in peripheral tissue by opioids. N Engl J Med 1995, 332: 1685–1690.

[16] Leanez S, Hervera A, Pol O. Peripheral antinociceptive effects of μ- and δ-opioid receptor agonists in NOS2 and NOS1 knockout mice during chronic inflammatory pain. Eur J Pharmacol 2009, 602: 41–49.

[17] Stein C, Millan MJ, Shippenberg TS, Peter K, Herz A. Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. J Pharmacol Exp Ther 1989, 248: 1269–1275.

[18] Pol O, Ferrer I, Puig MM. Diarrhea associated with intestinal inflammation increases the potency of mu and delta opioids on the inhibition of gastrointestinal transit in mice. J Pharmacol Exp Ther 1994, 270: 386–391.

[19] Cahill CM, Holdridge SV, Morinville A. Trafficking of δ-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. Trends Pharmacol Sci 2007, 28: 23–31.

[20] Ji RR, Zhang Q, Law PY, Low HH, Elde R, Hokfelt T. Expression of μ-, δ-, and κ-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. J Neurosci 1995, 15: 8156–8166.

[21] Maekawa K, Minami M, Masuda T, Satoh M. Expression of μ- and κ-, but not δ-, opioid receptor mRNAs is enhanced in the spinal dorsal horn of the arthritic rats. Pain 1996, 64: 365–371.

[22] Goff JR, Burkey AR, Goff DJ, Jasmin L. Reorganization of the spinal dorsal horn in models of chronic pain: correlation with behaviour. Neuroscience 1998, 82: 559–574.

[23] Cahill CM, Morinville A, Hoffert C, O'Donnell D, Beaudet A. Up-

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

regulation and trafficking of δ opioid receptor in a model of chronic inflammation: implications for pain control. Pain 2003, 101: 199–208.

[24] Pol O, Palacio JR, Puig MM. The expression of δ- and κ-opioid receptor is enhanced during intestinal inflammation in mice. J Pharmacol Exp Ther 2003, 306: 455–462.

[25] Martin M, Matifas A, Maldonado R, Kieffer BL. Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones. Eur J Neurosci 2003, 17: 701–708.

[26] Desmeules JA, Kayser V, Gacel G, Guilbaud G, Roques BP. The highly selective δ agonist BUBU induces an analgesic effect in normal and arthritic rat and this action is not affected by repeated administration of low doses of morphine. Brain Res 1993, 611: 243–248.

[27] Stewart PE, Hammond DL. Activation of spinal delta-1 or delta-2 opioid receptors reduces carrageenan-induced hypersensitivity in the rat. J Pharmacol Exp Ther 1994, 268: 701–708.

[28] Fraser GL, Gaudreau GA, Clarke PB, Menard DP, Perkins MN. Antihyperalgesic effects of δ opioid agonists in a rat model of chronic inflammation. Br J Pharmacol 2000, 129: 1668–1672.

[29] Hurley RW, Hammond DL. The analgesic effects of supraspinal μ and δ opioid receptor agonists are potentiated during persistent inflammation. J Neurosci 2000, 20: 1249–1259.

[30] Qiu C, Sora I, Ren K, Uhl G, Dubner R. Enhanced δ-opioid receptor-mediated antinociception in μ-opioid receptor-deficient mice. Eur J Pharmacol 2000, 387: 163–169.

[31] Brandt MR, Furness MS, Mello NK, Rice KC, Negus SS. Antinociceptive effects of δ-opioid agonists in Rhesus monkeys: effects on chemically induced thermal hypersensitivity. J Pharmacol Exp Ther 2001, 296: 939–946.

[32] Petrillo P, Angelici O, Bingham S, Ficalora G, Garnier M, Zaratin PF, *et al*. Evidence for a selective role of the δ-opioid agonist [8R-(4bS*,8aα,8aβ, 12bβ)]7,10-Dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]isoquinoli ne hydrochloride (SB-235863) in blocking hypersensitivity associated with inflammatory and neuropathic pain responses. J Pharmacol Exp Ther 2003, 307: 1079–1089.

[33] Wang CH, Lee TH, Tsai YJ, Liu JK, Chen YJ, Yang LC, *et al*. Intrathecal cdk5 inhibitor, roscovitine, attenuates morphine antinociceptive tolerance in rats. Acta Pharmacol Sin 2004, 25: 1027–1030.

[34] Parkitna JR, Obara I, Wawrzczak-Bargiela A, Makuch W, Przewlocka B, Przewlocki R. Effects of glycogen synthase kinase 3β and cyclin-dependent kinase 5 inhibitors on morphine-induced analgesia and tolerance in rats. J Pharmacol Exp Ther 2006, 319: 832–839.

[35] Roy S, Guo X, Kelschenbach J, Liu Y, Loh HH. *In vivo* activation of a mutant μ-opioid receptor by naltrexone produces a potent analgesic effect but no tolerance: role of μ-receptor activation and δ-receptor blockade in morphine tolerance. J Neurosci 2005, 25: 3229–3233.

[36] Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 1998, 38: 289–319.

[37] Liu JG, Anand KJ. Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. Brain Res Brain Res Rev 2001, 38: 1–19.

[38] Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, *et al*. *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. PLoS One 2009, 4: e5425.

[39] Eisinger DA, Ammer H, Schulz R. Chronic morphine treatment inhibits opioid receptor desensitization and internalization. J Neurosci 2002, 22: 10192–10200.

[40] Patwardhan AM, Berg KA, Akopain AN, Jeske NA, Gamper N, Clarke WP, *et al.* Bradykinin-induced functional competence and trafficking of the δ-opioid receptor in trigeminal nociceptors. J Neurosci 2005, 25: 8825–8832.

[41] Patwardhan AM, Diogenes A, Berg KA, Fehrenbacher JC, Clarke WP, Akopian AN, *et al.* PAR-2 agonists activate trigeminal nociceptors and induce functional competence in the delta opioid receptor. Pain 2006, 125: 114–124.

[42] Gendron L, Lucido AL, Mennicken F, O'Donnell D, Vincent JP, Stroh T, *et al.* Morphine and pain-related stimuli enhance cell surface availability of somatic δ-opioid receptors in rat dorsal root ganglia. J Neurosci 2006, 26: 953–962.

[43] Rozenfeld R, Abul-Husn NS, Gomez I, Devi LA. An emerging role for the delta opioid receptor in the regulation of mu opioid receptor function. ScientificWorldJournal 2007, 7: 64–73.

[44] Fields HL, Emson PC, Leigh BK, Gilbert RF, Iversen LL. Multiple opiate receptor sites on primary afferent fibres. Nature 1980, 284: 351–353.

[45] Egan TM, North RA. Both mu and delta opiate receptors exist on the same neuron. Science 1981, 214: 923–924.

[46] Zieglgansberger W, French ED, Mercuri N, Pelayo F, Williams JT. Multiple opiate receptors on neurons of the mammalian central nervous system. *In vivo* and *in vitro* studies. Life Sci 1982, 31: 2343–2346.

[47] Arvidsson U, Dado RJ, Riedl M, Lee JH, Law PY, Loh HH, *et al.* δ-Opioid receptor immunoreactivity: distribution in brainstem and

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

spinal cord, and relationship to biogenic amines and enkephalin. J Neurosci 1995, 15: 1215–1235.

[48] Cheng PY, Liu-Chen LY, Pickel VM. Dual ultrastructural immunocytochemical labeling of mu and delta opioid receptors in the superficial layers of the rat cervical spinal cord. Brain Res 1997, 778: 367–380.

[49] George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, *et al.* Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 2000, 275: 26128–26135.

[50] Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA. Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. J Neurosci 2000, 20: RC110.

[51] Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, Devi LA. A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. Proc Natl Acad Sci U S A 2004, 101: 5135–5139.

[52] Morinville A, Cahill CM, Kieffer B, Collier B, Beaudet A. Mu-opioid receptor knockout prevents changes in delta-opioid receptor trafficking induced by chronic inflammatory pain. Pain 2004, 109: 266–273.

[53] Gendron L, Pintar JE, Chavkin C. Essential role of mu opioid receptor in the regulation of delta opioid receptor-mediated antihyperalgesia. Neuroscience 2007, 150: 807–817.

[54] Zhang X, Bao L, Guan JS. Role of delivery and trafficking of delta-opioid peptide receptors in opioid analgesia and tolerance. Trends Pharmacol Sci 2006, 27: 324–329.

© 1994-2012 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net