Journal of the Neurological Sciences 335 (2013) 174–181

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

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

Effects of the altered activity of δ-opioid receptor on the expression of glutamate transporter type 3 induced by chronic exposure to morphine ☆

Qiang Wu [a,1], Shuxuan Xia [a,1], Jing Lin [b], Dexiong Cao [a], Weiqiang Chen [a,c], Ling Liu [a], Yanni Fu [a], Jianjun Liang [a,*], Minghui Cao [a,**]

[a] Department of Anesthesiology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong, PR China
[b] Department of Operating Room, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong, PR China
[c] Department of Anesthesiology, Shantou Central Hospital, Shantou 515000, Guangdong, PR China

ARTICLE INFO

Article history:
Received 3 August 2013
Received in revised form 17 September 2013
Accepted 18 September 2013
Available online 25 September 2013

Keywords:
δ-opioid receptor
Morphine
Excitatory amino acid transporter
Conditioned place preference
C6 cells
Addiction

ABSTRACT

Altered δ-opioid receptor (DOR) activity can affect the activity and function of excitatory amino acid transporter 3 (EAAT3), but the effects of DOR on EAAT3 expression in morphine relapse remain unknown. In this study, a C6δ cell line and SD rats in a conditioned place preference (CPP) reinstatement model were used. Here, we show that EAAT3 protein levels in C6δ cells decreased significantly after chronic exposure to morphine (10 μM) for 48 h and returned to normal 12 h after drug withdrawal. When C6δ cells were re-exposed to 5 μM morphine for 4 h, EAAT3 protein levels again decreased significantly. The selective μ opioid receptor (MOR) specific agonist DAMGO had a similar effect as morphine, and CTOP, a specific MOR blocker, reversed the declined expression of EAAT3 protein triggered by morphine exposure. The selective DOR agonist [d-pen2, 5] enkephalin (DPDPE) significantly increased EAAT3 expression in C6δ cells and even reversed the decreased EAAT3 expression caused by chronic morphine exposure. The non specific antagonist naloxone, but not the DOR inhibitor Naltrindole (NTI), reversed the decreased EAAT3 expression in C6δ cells caused by chronic morphine exposure. In vivo, EAAT3 levels in the prefrontal cortex of rats with morphine-induced CPP reinstatement significantly decreased. Naloxone completely suppressed reinstatement and reversed the decrease in EAAT3 expression induced by morphine re-exposure. In contrast, NTI only weakened CPP reinstatement and exerted no influence on EAAT3 expression. These findings suggest that DOR can affect the expression of EAAT3. However, the morphine-induced down-regulation of EAAT3 in C6δ cells and in the prefrontal cortex of rats may not be mediated by DOR.

1. Introduction

In recent years, the involvement of neurotransmitters in drug addiction has drawn increasing attention. Glutamate, a primary excitatory neurotransmitter in the mammalian brain, has been confirmed to be involved in opioid addiction [1]. Several studies have demonstrated that the dopamine (DA) reward pathway from the ventral tegmental area (VTA) to the nucleus accumbens (NAC) is a common reward pathway stimulated by various addictive substances, through which the glutamate neurotransmitter system strengthens the rewarding effects of opioids [2,3].

The glutamate hydrolase system is insufficient in extracellular fluid. Therefore, glutamate released by nerve endings is mainly taken up into cells by excitatory amino acid transporters (EAATs) on neurons and glial cells to eliminate glutamate neurotransmission and to protect neurons from glutamate excitotoxicity. EAATs play an important role in maintaining the dynamic equilibrium of extracellular glutamate and in regulating glutamate neurotransmission. EAAT3 is the major amino acid transporter protein in neurons. EAAT3 is highly expressed in cerebral cortical and hippocampal neurons, with a transport capacity for glutamate ≤40% of the total capacity, and is one of the major glutamate transporters in the cerebral cortex and hippocampus [4]. EAAT3 in the hippocampus and cerebral cortex has been shown to have significant effects on the regulation of the rate of Glu uptake, function of the Glu receptor, GABA levels, and protection of neurons [5–8].

Several studies have shown that the expression of or functional changes in EAATs are closely related to the development and symptoms of addiction [9,10]. EAAT1 and EAAT2 have always been the focus of such studies. In recent years, attention has been paid to the effect of EAAT3 on drug addiction. Lim et al. found that intrathecal administration of the μ opioid receptor (MOR) agonist morphine (10 μg) twice daily for 6 days significantly down-regulated EAAT3 expression in the spinal cords of mice [11]. Tai et al. had the same finding using similar methods [12]. Our previous studies showed that during morphine addiction and relapse, EAAT3 protein expression in the frontal cortex

☆ This study was supported by a grant from the National Natural Science Foundation of China (No.81272892/H1622).
* Corresponding author. Tel.: +86 2081332283.
** Corresponding author. Tel.: +86 2081332282; fax: +86 80332027.
E-mail addresses: Xafony@qq.com (J. Liang), caofox5188@126.com (M. Cao).
[1] Q. Wu and S. Xia contributed equally to this work.

0022-510X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jns.2013.09.026

is significantly decreased in Sprague–Dawley (SD) rats, suggesting that EAAT3 may be involved in opioid addiction and relapse.

EAAT3 is a membrane protein that can be regulated by various pathways [11–15]. Altered δ-opioid receptor (DOR) activity is closely related to the development of drug addiction [16,17], and DOR is the only opioid receptor with neuroprotective properties [18]. Pei et al. found that DOR expression can down-regulate EAAT3 activity via protein–protein interactions, whereas the activation of DOR can decrease this inhibition, increase EAAT3 activity, and enhance Glu uptake via EAAT3 [14]. Yang et al. also discovered that activated DOR can rapidly increase Glu uptake via EAAT3 and decrease the levels of Glu in the synaptic cleft [19]. However, the effect of DOR activity on EAAT3 expression during chronic exposure to morphine has not been investigated.

Functional interactions between DOR and MOR have been described. DOR and MOR heterodimerization has been reported [20], and there is evidence that the DOR subtype may correspond to a MOR–DOR complex [21,22]. Repeated administration of MOR agonists increases DOR cell surface expression in the brain and spinal cord [23,24]. In addition, chronic morphine exposure changes DOR function, and aberrant DOR activity may contribute to the behavioral dysregulation produced by repeated MOR activation.

In the present study, C6 cells and SD rats were used for simulating morphine addiction, withdrawal and relapse. These experiments were performed to explore the effects of chronic exposure to morphine on EAAT3 protein expression in both C6 cells and in the prefrontal cortex of rats as well as the regulation of EAAT3 by DOR.

2. Materials and methods

2.1. Cell lines and animals

Rat glioma cell lines (C6 cells) were purchased from American Type Culture Collection (Manassas, VA, USA). Forty adult Sprague–Dawley rats (200–250 g) were used (provided by Guangdong Province Medicine Experimental Center). Following the IASP guidelines for addiction research in animals, all animal studies were approved by the Animal Care and Use Committee at Sun Yat-Sen University and were in accordance with the University's guidelines for the care and use of laboratory animals. The animals were habituated for 3 days at the Sun Yat-Sen University animal center and kept under standard housing conditions in a temperature-controlled room with a 12-h light–dark cycle and with free access to food and water.

Forty adult male SD rats were randomly divided into 4 groups: control group (rats were injected with the same volume of 0.9% normal saline (NS) rather than drugs); NS + Mor (morphine relapse group, in which the conditioned place preference (CPP)-extinction-reinstatement model was used and established with a low-dose morphine injection); NAL + Mor (NAL + morphine relapse group, in which rats received 5 mg/kg naloxone via intraperitoneal injection before morphine-induced CPP reinstatement); and NTI + Mor (NTI + morphine relapse group, in which rats received 3 mg/kg Naltrindole, a DOR blocker, via intraperitoneal injection before morphine-induced CPP presented). Each group contained 10 rats. Behavioral tests were performed 15 min after intraperitoneal injection of the low dose of morphine.

2.2. Primary reagents

Dulbecco's modified Eagle's medium (DMEM) dry powder, fetal bovine serum, penicillin–streptomycin, and trypsin (418) were purchased from Gibco (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA), δ-opioid receptor expression plasmid (pcDNA3.1 + DOR) (Land, New York, NY, USA), cationic liposomal transfection reagent (Lipofectamine™ 2000, Invitrogen), and DPDPE (Tocris Bioscience, Minneapolis MN, USA) were also obtained. Naltrindol (NTI; Tocris Bioscience), DAMGO (Tocris Bioscience, UK), CTOP (Tocris Bioscience, UK), naloxone (Jiangsu Nhwa Pharmaceutical Co., Ltd., Shenzen, China), a protein assay kit (BioRad), anti-δ-opioid receptor monoclonal antibodies (Millipore, Billerica, MA, USA), rabbit anti-EAAT3 antibodies (Alpha Diagnostic International, San Antonio, TX, USA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (Amersham, Uppsala, Sweden), ECL-plus chemiluminescent agent (Amersham, Buckinghamshire, UK), photographic film (Kodak, Tokyo, Japan) and TRIzol (Invitrogen) were also purchased. Chloroform, anhydrous ethanol, isopropanol, TRIS, lissamine rhodamine B, sulforhodamine B (SRB) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich. δ-opioid receptor primers (Invitrogen), reverse transcription kit (Promega, Fitchburg, WI, USA) and a q-PCR kit (TOYOBO) were also obtained.

2.3. Experimental methods

2.3.1. Construction of a C6 glioma cell line (C6δ) for the stable expression of DOR

C6 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin, placed in a 37 °C chamber containing 5% CO_2, incubated for 2–3 days, and passaged once. After performing a kill curve, 800 μg/mL G418 was used to select cells stably expressing DOR. Cells were seeded in 24-well plates at 1×10^5 cells/well, and the growth medium was replaced with serum-free medium at approximately 80% confluence. A mixture of Lipofectamine 2000 and plasmid was added for transfection, and the medium was replaced after 4–6 h. DOR over-expression in cells was confirmed by western blotting and reverse transcription-polymerase chain reaction (RT-PCR).

2.3.2. Change in EAAT3 protein expression in C6δ cells during chronic morphine exposure, withdrawal and re-exposure

After chronic morphine exposure, C6δ cells were incubated in fresh medium (morphine withdrawal) and re-exposed to morphine at a lower concentration to simulate in vivo morphine addiction, withdrawal and relapse. Changes in EAAT3 protein expression in C6δ cells were evaluated.

2.3.3. Regulation of the activity of the MOR and its effect on EAAT3 in C6δ cells during chronic morphine exposure

Fifteen minutes before chronic exposure to morphine, 10 μM of the selective MOR agonist DAMGO, or 10 μM the selective MOR inhibitor CTOP was used to change the activity of the MOR in cells. The control group was given normal saline (NS) of the same volume. Regulation of changed MOR activity on expression change of EAAT3 in C6δ cells induced by chronic morphine exposure was studied.

2.3.4. Regulation of DOR activity and its effect on EAAT3 in C6δ cells during chronic morphine exposure

Fifteen minutes before chronic exposure to morphine, 1 μM of the non-selective opioid receptor antagonist naloxone, the selective DOR agonist DPDPE and the selective DOR inhibitor NTI were used to alter the activity of DOR in cells. The control group was given the same volume of NS. The effects of altered DOR activity on EAAT3 expression in C6δ cells induced by chronic morphine exposure were studied.

2.3.5. Sulforhodamine B (SRB)

To exclude the interference of physiological factors in experimental results, the effects of each treatment on the survival and growth of cells were determined using SRB.

Cells were seeded in 96-well plates and treated with drugs. Then, the medium was removed, and 100 μL of ice-cold 10% TCA (10% final concentration) was added to each well. The plates were incubated in a 4 °C refrigerator for 1 h. The solution was decanted, and the cells were washed with water, spin-dried and air-dried. A volume of 100 μL of 0.4% SRB was added to each well and incubated with the cells at room temperature for 30 min. Free dye was washed away with 1% glacial acetic acid. The plates were spin-dried and air-dried.