

Expression and localization of cannabinoid receptor 1 in rats' brain treated with acute and repeated morphine

Lingjing Jin^{1#}, Lizhen Pan¹, Yan Guo¹, Yuguo Zheng³, Zhiyu Nie¹, and Rongrong Zhu^{1,2*#}

[#]Authors contributed equally to the work

¹Department of Neurology, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai, China, *Email: rrzhu@tongji.edu.cn; ²School of Life Science and Technology, Tongji University, Shanghai, China; ³Department of Biomedicine, China National Center for Biotechnology Development, Beijing, China

Morphine induces adaptive changes in gene expression throughout the reward circuitry of brain. Recent research has proven the functional interactions between opioid and endogenous cannabinoid system in the central nervous system (CNS). The cannabinoid receptor 1 (CB₁-R) is one of the receptors that mediate the actions of cannabinoids and endocannabinoids in the CNS. Here, we investigated the expression of CB₁-R in mRNA and protein levels in the brains of rats treated with acute and repeated morphine. Three groups of rats received intraperitoneal injections (ip injections) of saline, acute morphine (10 mg/kg) and repeated morphine (10 mg/kg, twice daily for 12 consecutive days), and the mRNA levels and protein expressions of CB₁-R were examined. RT-PCR and western blot analyses supported that both mRNA and protein levels of CB₁-R in cortex, cerebellum and hippocampus were increased by repeated morphine treatment. However, the mRNA level in cerebellum was down-regulated only after acute morphine treatment and would returned to basal levels later. We used immunohistochemistry techniques to determine the functional expression of CB₁-R in morphine treated rat's brain. Enzyme-Linked Immunosorbent Assay (ELISA) revealed the significant increase of cytokine (IL-1 β , IL-6) levels in the repeated morphine treatment rats' cortex and hippocampus regions, which are both addiction-related brain areas. In addition, the results from RT-PCR and western blot assay indicated that the expression of CB₁-R was directly increased by morphine treatment *in vitro*. All the results indicated that the CB₁-R expression could be changed by morphine exposure and it might be involved in neural immune function, which provided a potential target for neurogenic disease treatment.

Key words: acute and repeated morphine treatment, cannabinoid receptor 1, IL-1 β , IL-6, immune function

INTRODUCTION

Opioid analgesics remain the mainstay in the management of moderate-to-severe pain. However, prolonged or repeated administration of opioids induces dependence, which has been a major limitation of their clinical applications. Opioid dependence is considered to be associated with adaptive changes initiated by the activations of μ -opioid receptor and other receptors (Wang et al. 1999, Zelek-Molik et al. 2010, Mickiewicz and Napier 2011). Although the activation of μ -opioid receptor is mainly responsible for opioid dependence induced by morphine (Zelek-Molik et al. 2010), the

adaptation of *k*-opioid receptor system is also involved in the opioid dependence (De Vries and Shippenberg 2002). Actually, the dysregulation of endogenous *k*-opioid receptor system is a consequence of repeated morphine treatment and affects morphine dependence. Moreover, that the CB₁ receptor and the opioid receptor have a common intracellular signaling pathway (Lopez-Moreno et al. 2010).

The mesocorticolimbic dopamine system contains many dopamine neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc), amygdala, hippocampus and prefrontal cortex (Carlezon and Wise 1996, Kelley and Berridge 2002). The NAc is a complex forebrain region (Jongenrelo et al. 1994) which directly relates to cocaine and morphine addiction (Lee et al. 1999). Moreover, drug-induced neuron

Correspondence should be addressed to R. Zhu,
Email: rrzhu@tongji.edu.cn

Received 28 November 2013, accepted 06 July 2014

plasticity in the NAc projections leads to addiction by strengthening the reward-driven behavior (Kauer 2004). The midbrain and the brainstem are interesting since both have been involved in drug addiction. It's worth mentioning that some specific nuclei have also participated in drug addiction, such as locus coeruleus, which plays a role in many somatic and neurovegetative signs during drug withdrawal.

The endocannabinoid (eCB) system contains at least two G-protein coupled receptors which are named CB1 and CB2 receptors, and many endogenous ligands including N-arachidonoyl-ethanolamide (anandamide) and 2-arachidonoyl glycerol-anandamide (2-AG) (Castle and Ames 1996, Wilson and Nicoll 2002, Heifets and Castillo 2009). The implication of this system in rewards is well documented and the pharmacological, behavioral and genetic approaches all indicate its instrumental role in both acute and prolonged effects of drug abuse (Mackie 2007). The eCB system also controls the synaptic activity by modulating the release of many neurotransmitters, including GABA, glutamate, dopamine (DA) (Schlicker and Kathmann 2001) and so on. The CB₁-R expresses mostly in the central nervous system (CNS), especially in the mesocorticolimbic system (Herkenham 1992, Mailleux and Vanderhaeghen 1992), while CB₂-R principally expresses in immune cells and were considered as peripheral CB₂-R. Considering the wide expressions of eCB system and their cellular functions in the brain (Mackie 2008), it is not surprising that the CB₁-R participates in nervous system and immune system functions (Klein and Cabral 2006, Tanasescu and Constantinescu 2010).

Previous reports have illustrated that chronic drug administration changed the expressions of CB1 and μ -opioid (MOP) receptors in several regions of the CNS. In reward-related brain areas, both CB₁ and MOP receptors are up-regulated after chronic heroin exposure (Fattore et al. 2007). Moreover, chronic morphine administration enhances the expressions of CB₁ and CB₂ receptors in the dorsal horn of the spinal cord (Fattore et al. 2007).

Although the regulatory role of eCB system on morphine addiction has received intense research interest, the expression and function of CB₁-R in the CNS system is not thoroughly understood. To the best of our knowledge, few researches have been reported

about the CB₁-R expression modulation in immune cells from morphine treated rats. The aim of this study was to highlight the role of CB1-R in morphine addiction. In the acute and repeated morphine administration rat model, we explored whether the expression of CB1-R would be changed after morphine exposure which would be involved in neural immune function. Our findings may serve as a basis for the interpretation of the mechanisms by which the expression and distribution of CB₁-R was changed after morphine exposure.

METHODS

Animal experiments and samples

The male Wistar rats weighing 200–250 g were supplied by the Tongji University. Animals were housed in cages with constantly controlled photoperiod (08:00 AM to 08:00 PM light), temperature, and humidity. All the animals had free access to food and water. The experimental protocol was approved through the University Institutional Animal Care and Use Committee.

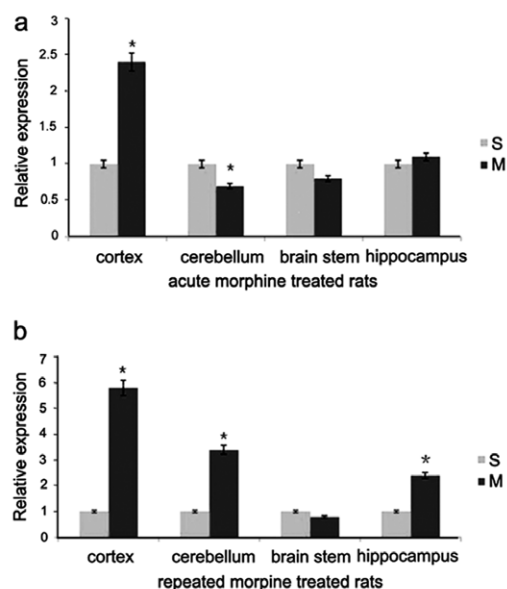


Fig. 1. The relative expression of CB1-R in CNS system of rats treated with acute (a), repeated (b) morphine administration ($n=8$ for each subgroup) and saline administration ($n=8$ for each subgroup) as revealed by real-time PCR. The data are presented as mean \pm SEM ($n=8$ animals per treatment group). (M) morphine; (S) saline. * $P<0.05$, significant difference compared to the saline control.

Drug treatment and tissue preparation

Morphine was dissolved in 0.9% saline. Morphine (10 mg/kg, sc) was administered daily. All injections were administered in volume of 0.01 mL/1 g of body weight. The animals were chronically treated with morphine (10 mg/kg, sc) twice per day at 12 h intervals for 12 days as described previously (Pu et al. 2002). Rats treated with a single morphine injection (10 mg/kg, sc) were demonstrated as the acute morphine addiction rats. Rats received the saline vehicle were set as control. In the repeated control group, saline solution was injected 2× daily for 12 days. In the acute control group, saline solution was injected once a day. All rats were sacrificed by decapitation 24 hours after the last injection of morphine or saline vehicle. Sacrificed animals were perfused with phosphate buffer solution (PBS). The brains were removed within 45–60 s after decapitation and immediately chilled in liquid nitrogen. The cortex, cerebellum, brainstem and the hippocampus were dissected out from the brain.

Real-time RT-PCR

Total RNA was extracted from different brain regions using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by absorbance at 260 nm, and RNA quality was analyzed by agarose gel electrophoresis and 260:280 nm absorbance ratios. First-strand cDNA was synthesized using Oligo dT primers and M-MLV RTase (Takara,

Dalian, China). The primers for CB1-R (forward, 5'-CCATTTCAAGCAAGGAGCAC-3', reverse, 5'-GTCATTTCGAGCCCACGTAGA-3') were synthesized. The primers for actin were: forward, 5'-TCTGTGTGGATTGGTGGCTCTA-3', reverse, 5'-CTGCTTGCTGATCCACATCTG-3'. The PCR reaction process was first incubated at 95°C for 3 min, followed by 40 cycles of thermal cycling at 95°C for 15 s and 60°C for 30 s. The Real-time PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Relative quantitation values were calculated using the $2^{-\Delta\Delta Ct}$ method with b-actin as an internal control.

Western blot analysis

The protein levels was analyzed by western blot. Equal amount of protein (50 µg) obtained from the brains of morphine addiction and control rats were extracted. Total proteins were electrophoresed in 8% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The membrane was washed and blocked with 5% non-fat milk in Tris-buffered saline (TBS) overnight at 4°C. After blocking, the membrane was incubated in TBS with anti-CB 1 primary antibodies (1:250, Sigma-Aldrich, St. Louis, MO, USA) for 6 h at room temperature (RT). β -actin was used as an inner reference. Subsequently, the blots were incubated for 2 h at RT containing the secondary antibody (1:5000, peroxidase-conjugated goat anti-rabbit). After the secondary incubation and washing, the membrane was placed on a piece of plastic wrap. The bands were visualized in ECL solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). The relative density of the staining was analyzed by software Bandscan 5.0 (Glyko Inc., Novato, CA, USA).

Immunohistochemistry

The CB1-R location was analyzed by Immunohistochemistry stain. The brain regions were dissected, postfixed in the same fixative solution for 2 h at RT, equilibrated with 30% sucrose in phosphate buffer (PB; 0.1 M, pH 7.4) at 4°C and then cut into 20–40 µm sections by a cryostat. The sections were dried at room temperature (RT) and

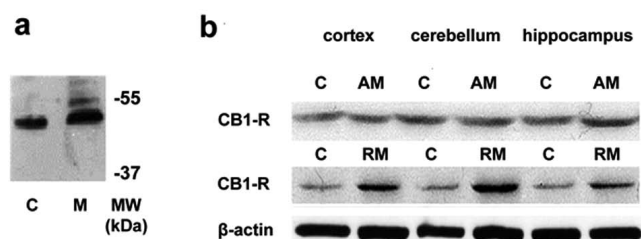


Fig. 2. Characterization of CB1-R protein expression. (a) Western analyses of CB1-R proteins expression on SDS-PAGE gels along with size standards Sigma antibody. (b) The CB1-R protein expression was estimated in both control group and repeated morphine treated group including with cortex, cerebellum and hippocampus. (M) morphine; (S) saline.

then fixed in precooled acetone for 15 min. Floating sections were incubated in 0.3% H₂O₂ in PBS at RT for 10 min to block endogenous peroxidase activity. The sections were incubated in 2% bovine plasma albumin in PBS for 1 h at RT, incubated in primary CB1 antibody (Sigma-Aldrich), diluted 1:100 in PBS for 4 h at RT. After washing with PBS, the slides were incubated with biotinylated goat anti-rabbit secondary antibody for 1 h at RT. The sections were washed, incubated with diaminobenzidine (DAB) solution and then counterstained with hematoxylin. The sections were viewed and photographed using a Leitz microscope.

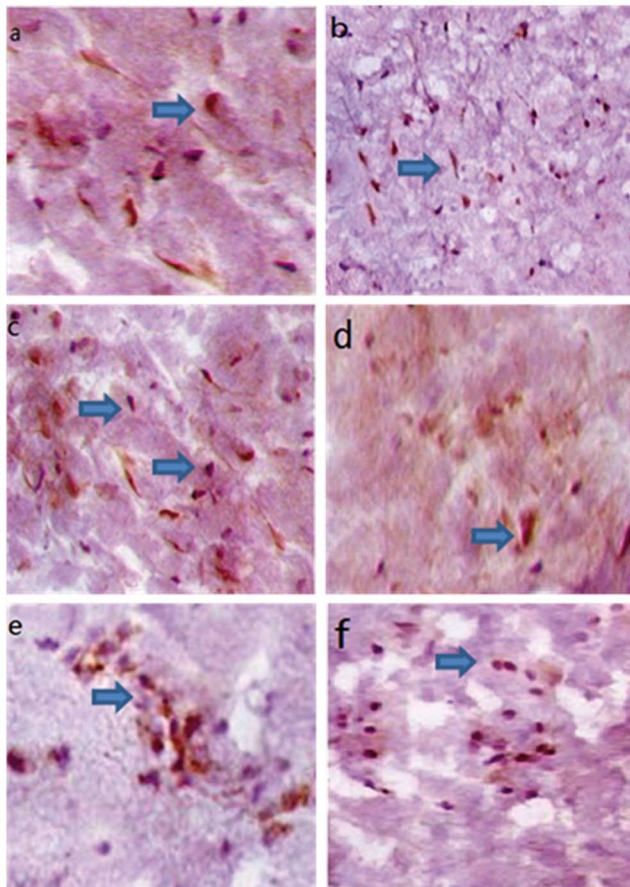


Fig. 3. The immunochemistry analyzed the CB1-R on the central nervous system tissues of repeated morphine treated rats ($n=8$ for each subgroup) and control rats ($n=8$ for each subgroup). (a) Cortex of repeated morphine treated rats, (b) cortex of control rats, (c) cerebellum of repeated morphine treated rats, (d) cerebellum of control rats, (e) brain stem of repeated morphine treated rats, and (f) brain stem of control rats. (a, c–f) magnification $\times 200$; (b) magnification $\times 100$. Arrows indicate CB1-R positive cells.

Enzyme-linked immunosorbent assay (ELISA)

ELISA revealed the cytokine (IL-1 β , IL-6) levels in rats. The protein concentrations were determined by the Bradford method. A rat interleukin-1 β (rat IL-1 β) ELISA kit was used for the quantitative determination of rat IL-1 β concentration in rats brain regions. The IL-6 concentration was determined using a rat IL-6 ELISA kit. The assays were performed according to the manufacturer's instructions. IL-1 β and IL-6 concentrations (pg/mg total protein) in the samples were calculated based on standard IL-1 β and IL-6 concentration-absorbance curves using the Graph Pad Prism 4.0 software (San Diego, CA).

Cell culture and experiments

The human SH SY5Y neuroblastoma cells and human Jurkat T lymphocytes were used as models of CNS nerve cells and immune cells for the morphine treatment *in vitro*. The cells were cultured in RPMI 1640 medium. The media were supplemented with 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid buffer, L-glutamine, 10% fetal bovine plasma (GIBCO, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were cultured in 25 cm culturing flasks, removed, and then cultured in 24-well plates for morphine treatments at different concentrations (0 , 5×10^{-7} M, 5×10^{-6} M, and 5×10^{-5} M) for 3 h. The

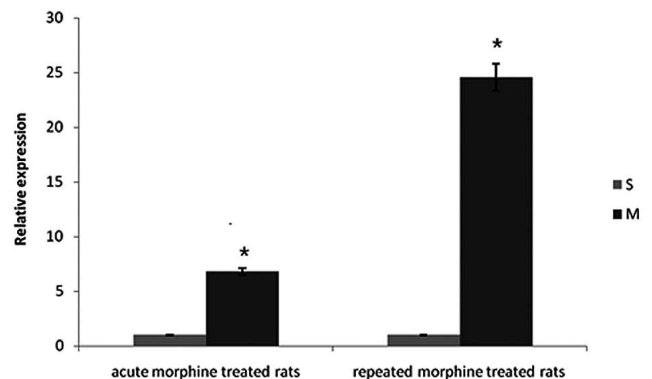


Fig. 4. The expression of CB1-R in rats periphery blood mononuclear cells (PBMCs) treated with acute, repeated morphine and saline injection ($n=8$ for each subgroup). (M) morphine; (S) saline. The data are presented as mean \pm SEM ($n=8$ animals per treatment group). * $P<0.05$, as compared with the saline control.

cells treated with PBS were used as the control. All of the cells were harvested on time and used for total protein extraction for western blotting.

Statistical analysis

The data were expressed as the mean \pm SEM. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). The level of statistical significance was defined as $P < 0.05$.

RESULTS

Involvement of CB₁-R expression in acute and repeated morphine treated rats

Firstly, we determined the CB₁-R expression in CNS system of normal rats, including cortex, cerebellum, brain stem and hippocampus. The RT-PCR results

showed that CB₁-R expressed abundantly in all the CNS regions, meanwhile, CB₁-R mRNA expression showed a higher density in cortex than in other regions (data not shown).

The CB₁-R mRNA expression levels were determined in acute and repeated morphine treated rats by RT-PCR, including the regions of cortex, brain stem, cerebellum and hippocampus. The CB₁-R mRNA expressions in cortex, cerebellum and hippocampus were increased after repeated treatment with morphine (Fig. 1b). In the rats subjected to acute morphine, there was a significant reduction of CB₁ gene expression in the cerebellum, whereas no change of CB₁ gene expression in hippocampus and brainstem regions was observed (Fig. 1a). These results showed that CB₁-R presented significantly different expressions of mRNA levels in CNS regions between control group rats and repeated /acute morphine treated group.

Distribution of CB₁-R in the CNS system after repeated morphine treatment

To determine the expression and localization of CB₁-R in CNS regions, we used both western blotting and immunohistochemical staining methods. Firstly, we analyzed the protein expression of CB₁-R in the CNS regions subjected to acute or repeated morphine. Our data resulted from the western blotting analyses showed a major CB₁-R band of approximately 52 kDa (Fig. 2a). The CB₁-R protein expression was estimated in both control group and repeated morphine treated group. Densitometric analyses illustrated that CB₁ protein expression was significantly increased in the cortex, cerebellum and hippocampus by the repeated morphine treatment (Fig. 2b). There was no apparent change of CB₁-R protein levels in cerebellum and cortex regions of acute morphine treated rats, and there was an up-regulation of CB₁ protein expression in the hippocampus (Fig. 2b).

We then performed immunohistochemical analysis on the brain regions of the control and repeated morphine treated rats (Fig. 3). CB₁-R immunoreactivity (IR) was obviously observed in the cerebral cortex. Apical dendrites and cell bodies of pyramidal neurons of rat cerebral cortex were moderately to heavily immunolabeled for CB₁-R (Fig. 3a,b). We found that the scattered fibers in the rat's cerebral cortex also showed CB₁-R positive (Fig. 3a). A moderate to heavy CB₁-R immunostaining was observed in pyramidal neuron of cerebellum and brain stem (Fig. 3c–f).

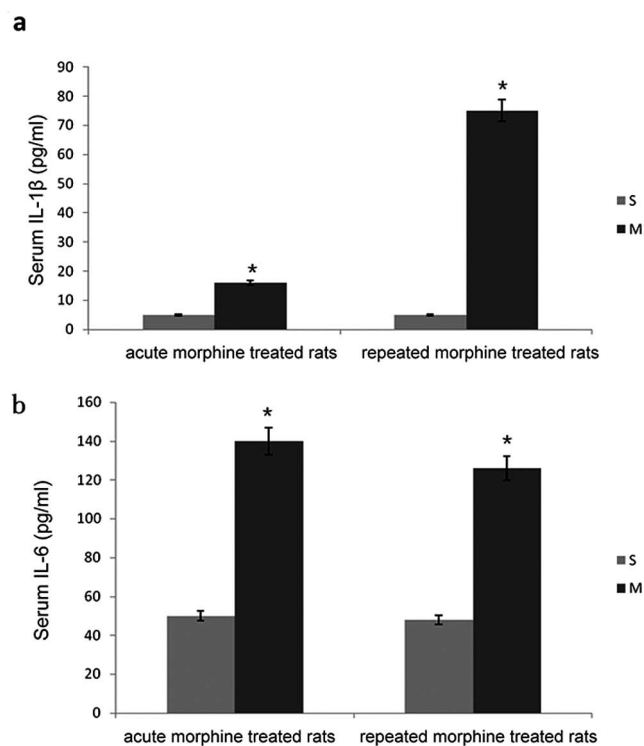


Fig. 5. The serum cytokine IL-1 β (a) and IL-6 (b) levels measured in the acute, repeated morphine treated and saline injected ($n=8$ for each subgroup) rats. The serum IL-1 β and IL-6 levels are significantly increased in repeated morphine treated rats. (M) morphine; (S) saline. The data are presented as mean \pm SEM ($n=8$ animals per treatment group). * $P < 0.05$, as compared with the saline control.

Cannabinoid receptor 1 expression in morphine exposure rat peripheral blood mononucleated cells (PBMCs)

Repeated exposure to morphine has been proved to be capable of modulating the expression and function of CB₁-R in the brains of rodents. However, it was not clear whether similar changes would be seen in the immune cells of repeated morphine treated rats PBMCs. To explore this, we examined the expression of CB₁-R in rat PBMCs. We investigated CB₁-R expressions in the eight blood samples from rats exposed to repeated morphine treatment, eight blood samples from rats exposed to acute morphine treatment and the five blood samples from control rats *via* Q-RT-PCR. The mRNA expressions of CB₁-R were increased significantly in the PBMCs from both acute and repeated morphine treated rats compared to that from untreated ones (Fig. 4). We also found that the mRNA expression of CB₁-R in repeated morphine treated rats was much higher than acute morphine treated rats.

Chronic morphine-induced pro-inflammatory cytokine expression

IL-1 β and IL-6 are immune inflammation factors participated in the regulations of immune system functions. The serum IL-1 β (Fig. 5a) and IL-6 (Fig. 5b) levels were significantly increased in repeated morphine treated rats, which implied the possibility of morphine induced proinflammatory cytokines. Compared to the untreated rats, the cortex and hippocampus expressions of the pro-inflammatory cytokines IL-1 β (Fig. 6a) and IL-6 (Fig. 6b) were significantly higher in the CNS regions of repeated morphine treated rats.

Comparison of CB₁-R expression in cells treated with or without morphine

The expression of CB₁-R was examined *via* Q-RT-PCR in cultured cells after various concentrations of morphine stimulation. We found that morphine affected the expression of CB₁-R not only in nervous system-derived cells, but also in immune system derived cells. In nervous system-derived cells (SH SY5Y), the expression of the CB₁-R was observed. The CB₁-R protein (Fig. 7) levels were upregulated after morphine stimulation. There was only a little CB₁-R expressed in naive Jurkat cells (T lymphocytes cell line). The

expression of the CB₁-R was upregulated after morphine treatment, and the protein (Fig. 8) levels were upregulated in a dose depending matter. Thus, our results demonstrated that the activation of Jurkat cells was resulted from the induction of functional CB₁-R expression.

DISCUSSION

Both opioids and cannabinoids are related with analgetic, psychotropic, and immunomodulatory effects. Moreover, co-administered morphine and cannabinoids had systemically potentiated antinociception (Cichewicz and McCarthy 2003). The mechanism of this effect was still not clear and published researches on the expression and function sites of cannabinoid receptors were still contradictory.

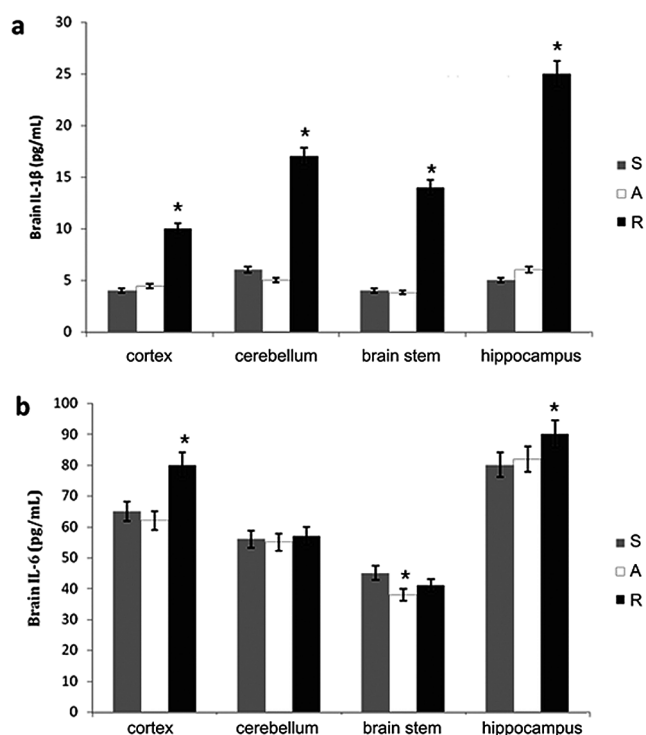


Fig. 6. The brain cytokine IL-1 β (a) and IL-6 (b) levels of the acute, repeated morphine treated and saline injected ($n=8$ for each subgroup) rats. Compared to the saline injected rats, the expressions of the pro-inflammatory cytokines IL-1 β (a) and IL-6 (b) levels in cortex and hippocampus were significantly higher in the CNS regions of repeated morphine treated rats. (A) acute morphine administration; (R) repeated morphine administration; (S) saline. The data are presented as mean \pm SEM ($n=8$ animals per treatment group). * $P<0.05$, as compared with the saline control.

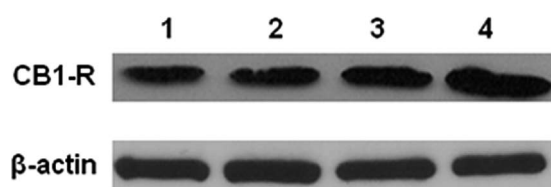


Fig. 7. Western blot analysis of the CB₁-R expression on SH SY5Y cells with or without morphine stimulation. The morphine concentrations were set as 1 (0); 2 (5×10^{-7} M); 3 (5×10^{-6} M); and 4: (5×10^{-5} M).

The past research results provide evidence for the involvement of cannabinoid CB₁ receptors in the central antinociception induced by activation of μ -opioid receptors by the agonist morphine. The release of endocannabinoids appears not to be involved in central antinociception induced by activation of κ - and δ -opioid receptors (Pacheco et al. 2009). The CB₁-R was known to be mainly presynaptic in the CNS system, and endocannabinoids emerged as one of the classes of retrograde messengers involved in the regulation of synaptic transmission.

The morphine-induced the CB₁-R immunoreactivity could be activated via enhanced translation or increased stability of protein, however, neither of these mechanisms has been elaborated until now (Vigano et al. 2003). The absence of CB₁ mRNA in CB₁-R deficient mice and presence in wild type controls has been demonstrated by other studies. We found differential modification of CB₁-R expression in the CNS regions of acute or repeated morphine treated rats. We also confirmed that the cortex had more CB₁ gene transcripts compared to the other regions. Moreover, previous researches had demonstrated that using two types of CB₁ antibodies, similar staining patterns in both the mice and rat cerebellum were observed. Western blot analyses revealed that specific bands were identified using CB₁ antibodies and were absent when the CB₁ antibodies were pre-adsorbed with the immunizing peptide. It confirmed the CB₁-R immunoreactivity detected in the rat brain. The alterations of the protein expression in the cerebellum, hippocampus and cortex were coincident with the changes of mRNA expression. But the mRNA level of CB₁-R was not consistent with the protein level in brain stem of the repeated morphine treated rats. This phenomenon might due to the quick degradation of cannabinoid receptor protein and the acceleration of the mRNA transcription through the feedback regulation. These results indicated that

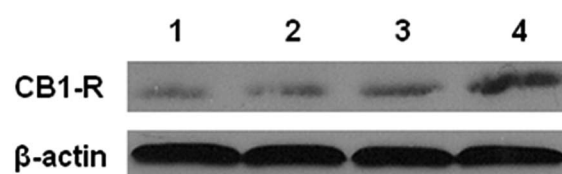


Fig. 8. Western blot analysis of the CB₁-R expression on Jurkat cells with or without morphine stimulation. The morphine concentrations were set as 1 (0); 2 (5×10^{-7} M); 3 (5×10^{-6} M); and 4 (5×10^{-5} M).

repeated morphine exposure affected the expression of CB₁-R in both mRNA and protein levels. The increased expressions of CB₁-R in the cerebellum and hippocampus regions might be the indicative of an involvement of these receptors in the specific processes of morphine addiction, either as a part of the addictive phenomenon or as a response to morphine. The results obtained from the pharmacological activation or blockade of CB₁-R in morphine-dependent animals, or from the CB₁-R gene deleted mice might support these findings (Vaseghi et al. 2012). However, the reported data was contradictory, so more studies would be required.

Furthermore, abundant CB₁-R immunoreactivity in CNS regions of repeated morphine treated rats was detected in our study. Our results confirmed that the CB₁-R immunoreactivity was detected in CNS system of repeated morphine treated rats and this phenomenon was supported by several reports of the identification of neuronal CB₁-R in the brain stem involved in emesis (Van Sickle et al. 2001, 2003, Sharkey et al. 2007, Ray et al. 2009). The previous reports identified that functional expression of CB₁-R was immunolabeling in the CNS including somatodendritic (Abrams et al. 2011), axonal/terminal (Tsou et al. 1998) and astrocytic (Mukhopadhyay et al. 2010). The autoradiography, in situ hybridization and immunohistochemistry results identified that CB₁-R and μ -opioid peptide (MOP) receptors were co-expressed in the rats' brain (Commons et al. 2000, Abrams et al. 2011). Both CB₁-R and MOP receptors were G-protein coupled receptors that had directly effects on Gi/Go proteins (Connor and Christie 1999, Howlett et al. 2002). Activation of both CB₁ and MOP receptors inhibited adenylyl cyclase, which caused decreased accumulation of cyclic AMP (cAMP) (Meng and Johansen 2004). It's worth noting that adenylyl cyclase regulation also implicated in the processes of morphine addiction. The previous findings also demonstrated

the widely co-localization of the CB₁-R and MOP receptors in CNS system which was significantly related with morphine dependence. Moreover, these results were consistent with the previous study which indicated that CB₁-R and MOP receptors co-localized in somatodendritic profiles of the spinal cord (Pugh et al. 1996). Thus, all the data demonstrated the functional expression of CB₁-R in CNS system, which may provide potential target for the acute and chronic morphine addiction.

Morphine is best known for its effect on the function of nervous system. In addition to neuronal effects, morphine generates immunosuppression effects on T lymphocytes. But the way by which morphine affects the immune system remains unknown. The CB₁-R distributed in PBMCs could be related with the modulation of immune cell activity. It was reported that the cannabinoid receptors belonged to the group of GPCR, and the activation of cannabinoid receptors in the immune system inhibited the MEK/ERK signal pathway (Gertsch et al. 2004). This inhibition always led to immune cell apoptosis, T helper cell development, monocytes and neutrophil migration, and cytokine release. In this study, we determined that the CB₁-R was significantly upregulated in the PBMCs of rats exposed to morphine compared with untreated ones. Therefore, the elevation of CB₁-R expression in PBMCs might be related with abnormal immune functions after morphine exposure. Our results suggested that at least a part of the immunomodulatory activities of the morphine was mediated through CB₁-R.

In previous study, the cytokine disorder was observed in the immunosuppression of the morphine misused animal model (Wang et al. 2002). In this study, our findings supported that morphine synergistically stimulated the productions of some cytokines in the peripheral and central nervous systems *in vivo*. We further found that IL-1 β and IL-6 levels were significantly increased in the peripheral system of repeated morphine treated rats. IL-1 β and IL-6 levels were found increased in the morphine addiction-related CNS regions. These findings implied that the alterations of cytokines could be attributed to the morphine exposure.

In addition, an important issue was whether the morphine exposure would directly stimulate the expression of cannabinoid receptors. So we treated nervous system-derived cells and Jurkat T lymphocytes culture cells with morphine *in vitro*. The expres-

sion of the CB₁-R was increased in both nervous system- and immune system-derived cells after morphine stimulation.

In the present study, the expression of the CB₁-R was altered in both nervous system- and immune system-derived cells after morphine stimulation. Thus, we speculated that morphine abuse could directly affect the expression of CB₁-R and its signal pathways, and then influence the immune functions. In addition, morphine could directly act on immune system apart from mediating the neuroendocrine system.

CONCLUSION

In conclusion, morphine exposure would lead to the alteration of CB₁-R expression on nervous and immune system-derived cells both *in vitro* and *in vivo*. The alteration of CB₁-R expression in nerve and immune cells may be involved in abnormal neurological and immune functions caused by morphine dependence. Indeed our study provided the evidence for the effects of morphine on the expression of CB₁-R in nervous and immune system of rats. These findings were of importance as it opened new areas of researches and approaches in understanding morphine addiction.

ACKNOWLEDGMENT

This study was supported by the Natural Science Foundation of China (81000481) and the Fundamental Research Funds for the Central Universities (2000219063 and 1508219048).

REFERENCES

- Abrams DI, Couey P, Shade SB, Kelly ME, Benowitz NL (2011) Cannabinoid-opioid interaction in chronic pain. *Clin Pharmacol Ther* 90: 844–851.
- Carlezon WA, Wise RA (1996) Microinjections of phencyclidine (PCP) and related drugs into nucleus accumbens shell potentiate medial forebrain bundle brain stimulation reward. *Psychopharmacology* 128: 413–420.
- Castle DJ, Ames FR (1996) Cannabis and the brain. *Aust N Z J Psychiat* 30: 179–183.
- Cichewicz DL, McCarthy EA (2003) Antinociceptive synergy between Delta(9)-tetrahydrocannabinol and opioids after oral administration. *J Pharmacol Exp Ther* 304: 1010–1015.

- Commons KG, Aicher SA, Kow LM, Pfaff DW (2000) Presynaptic and postsynaptic relations of mu-opioid receptors to gamma-aminobutyric acid-immunoreactive and medullary-projecting periaqueductal gray neurons. *J Comp Neurol* 419: 532–542.
- Connor M, Christie MD (1999) Opioid receptor signalling mechanisms. *Clin Exp Pharmacol Physiol* 26: 493–499.
- De Vries TJ, Shippenberg TS (2002) Neural systems underlying opiate addiction. *J Neurosci* 22: 3321–3325.
- Fattore L, Vigano D, Fadda P, Rubino T, Fratta W, Parolaro D (2007) Bidirectional regulation of mu-opioid and CB1-cannabinoid receptor in rats self-administering heroin or WIN 55,212-2. *Eur J Neurosci* 25: 2191–2200.
- Gertsch J, Schoop R, Kuenzle U, Suter A (2004) Echinacea alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Lett* 577: 563–569.
- Heifets BD, Castillo PE (2009) Endocannabinoid signaling and long-term synaptic plasticity. *Annu Rev Physiol* 71: 283–306.
- Herkenham M (1992) Cannabinoid receptor localization in brain: relationship to motor and reward systems. *Ann NY Acad Sci* 654: 19–32.
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 54: 161–202.
- Jongenrelo AL, Voorn P, Groenewegen HJ (1994) Immunohistochemical characterization of the shell and core territories of the nucleus-accumbens in the rat. *Eur J Neurosci* 6: 1255–1264.
- Kauer JA (2004) Learning mechanisms in addiction: Synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. *Annu Rev Physiol* 66: 447–475.
- Kelley AE, Berridge KC (2002) The neuroscience of natural rewards: Relevance to addictive drugs. *J Neurosci* 22: 3306–3311.
- Klein TW, Cabral GA (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. *J Neuroimmune Pharmacol* 1: 50–64.
- Lee RS, Criado JR, Koob GF, Henriksen SJ (1999) Cellular responses of nucleus accumbens neurons to opiate-seeking behavior: I. Sustained responding during heroin self-administration. *Synapse* 33: 49–58.
- Lopez-Moreno JA, Lopez-Jimenez A, Gorriti MA, de Fonseca FR (2010) Functional Interactions between Endogenous Cannabinoid and Opioid Systems: Focus on Alcohol, Genetics and Drug-Addicted Behaviors. *Curr Drug Targets* 11: 406–428.
- Mackie K (2007) Understanding cannabinoid psychoactivity with mouse genetic models. *Plos Biol* 5: 2106–2108.
- Mackie K (2008) Cannabinoid receptors: Where they are and what they do. *J Neuroendocrinol* 20: 10–14.
- Mailleux P, Vanderhaeghen JJ (1992) Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. *Neuroscience* 48: 655–668.
- Meng ID, Johansen JP (2004) Antinociception and modulation of rostral ventromedial medulla neuronal activity by local microinfusion of a cannabinoid receptor agonist. *Neuroscience* 124: 685–693.
- Mickiewicz AL, Napier TC (2011) Repeated exposure to morphine alters surface expression of AMPA receptors in the rat medial prefrontal cortex. *Eur J Neurosci* 33: 259–265.
- Mukhopadhyay P, Pan H, Rajesh M, Batkai S, Patel V, Harvey-White J, Mukhopadhyay B, Hasko G, Gao B, Mackie K, Pacher P (2010) CB1 cannabinoid receptors promote oxidative/nitrosative stress, inflammation and cell death in a murine nephropathy model. *Br J Pharmacol* 160: 657–668.
- Pacheco DF, Klein A, Perez AC, Pacheco CM, de Francischi JN, Reis GM, Duarte ID (2009) Central antinociception induced by mu-opioid receptor agonist morphine, but not delta- or kappa-, is mediated by cannabinoid CB1 receptor. *Br J Pharmacol* 158: 225–231.
- Pugh G, Jr., Smith PB, Dombrowski DS, Welch SP (1996) The role of endogenous opioids in enhancing the antinociception produced by the combination of delta 9-tetrahydrocannabinol and morphine in the spinal cord. *J Pharmacol Exp Ther* 279: 608–616.
- Pu L, Bao GB, Xu NJ, Ma L, Pei G (2002) Hippocampal long-term potentiation is reduced by chronic opiate treatment and can be restored by re-exposure to opiates. *J Neurosci* 22: 1914–1921.
- Ray AP, Griggs L, Darmani NA (2009) Delta(9)-Tetrahydrocannabinol suppresses vomiting behavior and Fos expression in both acute and delayed phases of cisplatin-induced emesis in the least shrew. *Behav Brain Res* 196: 30–36.
- Schlicker E, Kathmann M (2001) Modulation of transmitter release via presynaptic cannabinoid receptors. *Trends Pharmacol Sci* 22: 565–572.
- Sharkey KA, Cristino L, Oland LD, Van Sickle MD, Starowicz K, Pittman QJ, Guglielmotti V, Davison JS, Di Marzo V (2007) Arvanil, anandamide and N-arachidonoyl-

- dopamine (NADA) inhibit emesis through cannabinoid CB1 and vanilloid TRPV1 receptors in the ferret. *Eur J Neurosci* 25: 2773–2782.
- Tanasescu R, Constantinescu CS (2010) Cannabinoids and the immune system: an overview. *Immunobiology* 215: 588–597.
- Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83: 393–411
- Van Sickle MD, Oland LD, Ho W, Hillard CJ, Mackie K, Davison JS, Sharkey KA (2001) Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. *Gastroenterology* 121: 767–774.
- Van Sickle MD, Oland LD, Mackie K, Davison JS, Sharkey KA (2003) Delta9-tetrahydrocannabinol selectively acts on CB1 receptors in specific regions of dorsal vagal complex to inhibit emesis in ferrets. *Am J Physiol Gastrointest Liver Physiol* 285: 566–576.
- Vaseghi G, Rabbani M, Hajhashemi V (2012) The CB(1) receptor antagonist, AM281, improves recognition loss induced by naloxone in morphine withdrawal mice. *Basic Clin Pharmacol Toxicol* 111: 161–165.
- Viganò D, Grazia Cascio M, Rubino T, Fezza F, Vaccani A, Di Marzo V, Parolaro D (2003) Chronic morphine modulates the contents of the endocannabinoid, 2-arachidonoyl glycerol, in rat brain. *Neuropsychopharmacology* 28: 1160–1167.
- Wang J, Charboneau R, Balasubramanian S, Barke RA, Loh HH, Roy S (2002) The immunosuppressive effects of chronic morphine treatment are partially dependent on corticosterone and mediated by the mu-opioid receptor. *J Leukoc Biol* 71: 782–790.
- Wang XM, Zhou Y, Spangler R, Ho A, Han JS, Kreek MJ (1999) Acute intermittent morphine increases prepro-dynorphin and kappa opioid receptor mRNA levels in the rat brain. *Mol Brain Res* 66: 184–187.
- Wilson RI, Nicoll RA (2002) Neuroscience-Endocannabinoid signaling in the brain. *Science* 296: 678–682.
- Zelek-Molik A, Taracha E, Nawrat D, Bielawski A, Lehner M, Plaznik A, Nalepa I (2010) Effects of morphine and methadone treatment on mRNA expression of G alpha(i) subunits in rat brains. *Pharmacol Rep* 62: 1197–1203.