Int J Clin Exp Med 2017;10(2):2353-2359
www.ijcem.com /ISSN:1940-5901/IJCEM0033163

*Original Article*

# Effects of N-methyl-D-aspartate receptor on remifentanil-induced hyperalgesia

Xiaoyun Liao[1], Yan Mou[1], Dayong Xu[2]

[1]*Department of Anesthesiology, Hunan Provincial People's Hospital, Changsha, China;* [2]*Department of General Surgery, The First Hospital of Changsha, Changsha, China*

Received June 2, 2016; Accepted August 8, 2016; Epub February 15, 2017; Published February 28, 2017

**Abstract:** Opioids especially remifentanil have been widely used in clinical operation as inducer for general anesthesia and could cause hyperalgesia. N-methyl and D-aspartate-related receptor (NMDAR) is widely found in animal and plays an important role in central nerve system and hypersensitivity. Our study aimed to investigate the molecular mechanism of N-methyl-D-aspartate receptor on remifentanil-induced hyperalgesia in rat model. A rat model of remifentanil-induced hyperalgesia for intravenous injection was established. Spinal cord of hyperalgesia rat was used to perform PRT-PCR and western blot to detect the expression of NMDAR, PKC and phosphorylate PKC. After intravenous infusion of PKC inhibitor Staurosporine into hyperalgesia rat, algesia time of rat, expression of NMDAR and PKC, as well as activation of PKC were examined to investigate the role of PKC signal pathway on expression of NMDAR and remifentanil-induced hyperalgesia of rat. The expression of NMDAR and PKC in spinal cord of hyperalgesia rat induced by remifentanil was increased. Compared to control group, there was significant difference on the activation of PKC ($P<0.05$). Intravenously injection of PKC inhibitor Staurosporine was obviously inhibit the algesia time induced by remifentanil ($P<0.05$), and the expresssion of NMDAR and PKC were reduced and activation of PKC was inhibited. N-methyl-D-aspartate receptor played an important role in remifentanil-induced hyperalgesia through PKC signal pathway.

**Keywords:** Hyperalgesia, N-methyl-D-aspartate receptor, PKC, remifentanil

## Introduction

Opioids which belong to alkaloid and its ramification [1] could reduce and relieve postoperative pain. However, opioids have some side effects too, especially drug resistance and hyperalgesia [2]. Hyperalgesia often occurs in patients with climacteric syndrome, hypochondrasis, hysteria and neurasthenia who often feel great extent of pain when they were touched on skin slightly, namely algesia sensitivity enhanced [3, 4].

Remifentanil which is one of the opioids and belongs to μ-opioid receptor agonist is widely used in clinical. Remifentanil showed a significant dose effect when it plays analgesic effect in a certain dose. But after repeated administration, hyperalgesia will be present of which molecular mechanism is unknown [5, 6]. Our study will investigate molecular mechanism of remifentanil-induced hyperalgesia.

It was reported that the mechanism of hyperalgesia mainly includes: (1) The concentration of intracellular calcium ($Ca^{2+}$) was increased under general anesthesia leading to activation of protein kinase, especially calcium dependent calmodulin and protein kinase C (PKC) [7]. (2) Previous studies investigated the ERK level associated with hyperalgesia, but later studies confirmed that ERK was not related to hyperalgesia. So we just focused on PKC in this study. (3) The activation of N-methyl-D-aspartate receptor (NMDAR). (4) The release of dynorphin A [10, 11].

N-methyl and D-aspartate-related receptor (NMDAR) is widely found in animal and plays an important role in hypersensitivity of central nerve system [12, 13]. NMDAR, a glutamate receptor subtype, belong to excitatory receptor and play critical role in learning and memory. Some studies found that when hypersensitivity occurs, NMDAR could induce imbalance of

nerve intracellular calcium resulting in excitation of neurotoxicity signal and finally death and dysfunction of neuron [14, 15]. Our study aimed to investigate the molecular mechanism of N-methyl-D-aspartate receptor on remifentanil-induced hyperalgesia in rat model.

## Materials and methods

### *Experimental reagents and devices*

Hydrochloric remifentanil for injection was bought from Yichang Human well Pharmaceutical Co., LTD. NMDAR and internal reference antibody actin, anti-PKC and activated phosphorylated PKC antibody (p-PKC) was bought from Sigma corporation of America. RNA isolation kit was bought from Tiangen Biotech (Beijing) Co., LTD. Reverse transcription RT-PCR kit was bought from Beijing Dingguo Changsheng Biotechnology Co., LTD. Other reagents were bought from Beiyotime Biotechnology Co., LTD. Rat algesimeter was bought from Shanghai Yuyan Instruments Co., LTD.

### *The establishment of animal model of hyperalgesia*

10 weeks old male Sprague-Dawley (SD) rats were bought from Beijing HFK bioscience Co., LTD. As methods described in previous studies [16, 17], rat models of hyperalgesia with hydrochloric remifentanil for injection were established. The detailed methods are showed as follow: 16 10-week-old male SD rats were randomly divided into hyperalgesia group and control group through random number method. Three hours before operation, thermal paw withdrawal latency of each rat was measured. After the rats were anesthetized with an intraperitoneal injection of 2% chloral hydrate, normal saline (control group) or remifentanil were infused at a rate of 1.6 μg/kg/min for 45 minutes through caudal vein. After infusion, thermal paw withdrawal latency of each rat was measured again, namely algesia time. After models were successfully established, subsequent experiments were carried out. In the experiment of PKC signal pathway, 2 hours before establishment of model, intraperitoneal injection of PKC inhibitor Staurosporine of 5 mg/kg were performed.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Hunan Provincial People's Hospital.

### *RT-PCR*

After spinal cords of rats in hyperalgesia group and control group were separated, tissue RNA was extracted using RNA isolation kit and performed reverse transcription according to instruction of RT-PCR reagent kit. Primer sequence of NMDAR and internal reference actin were 5'TAAGTCCAGAATGTAAGTAACAGAACATA3' and 5'CAACAGAATAAGTAAAGATGTTGATCACA3' 5'ACCACCACACCTTACAGTGTCTAATGCCA3' and 5'CCTACAGTGACTCTACTCCAACAATGCCA3', respectively. The PCR system is showed following: cDNA solution: 1 μl, 10×PCR Buffer 2 μl, dNTP Mixture (1 mM) 1 μl, primer 1 (10 μM) 2 μl, primer 2 (10 μM) 2 μl, Taq DNA Polymerase 1 μl, $MgCl_2$ (25 mM) 1 μl, $H_2O$ 16 μl.

The reaction program of PCR is showed as follow: 95°C, 4 min, 95°C, 60 S, 58°C, 30 S, 30 Cycles, 72°C, 60 S, 72°C, 4 min, 4°C keep warm.

Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference actin.

### *Western blot*

To prepare for western blot sample (containing buffer solution above) of spinal cord tissue of rats, spinal cord tissue of rats in hyperalgesia group and control group were homogenized in ice-cold lysis buffer for 30 minutes.

Protein samples were performed polyacrylamide gel electrophoresis on SDS-PAGE with 10 μg sample in every pore for 30 min under 60 V and then 120 V for 90 min. After electrophoresis samples were transferred onto membrane with 300 mA for 180 min. At the end of membrane transferred, NC membrane with protein were closed with milk/PBST under room temperature for 60 min. NMDAR antibodies (1:2000) and p-PKC antibodies (1:1000) and internal reference antibody actin (1:2000) were diluted and incubated under room temperature for 2 hours. After washing with PBST for 3 times, secondary antibodies (diluted 1:2000) of goat anti-mouse were incubated for 2 hours and wash-

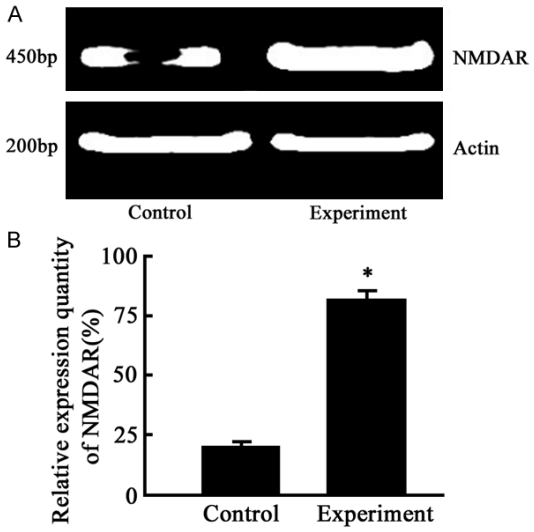


**Figure 1.** Level of mRNA of NMDAR in spinal cord of rat model of hyperalgesia was increased. Note: *compare with control group ($P$<0.05). A. One result of the three times of electrophoresis. B. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference Actin.

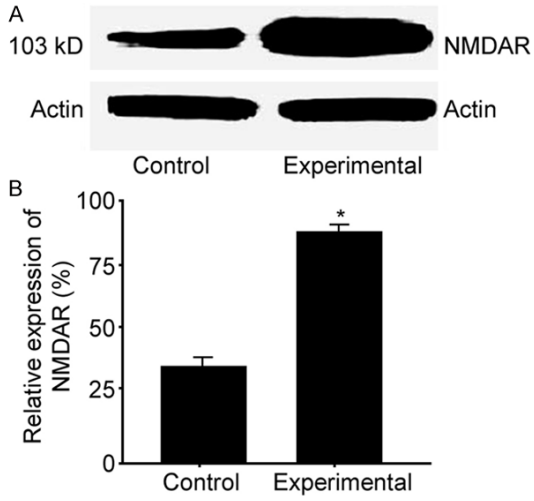


**Figure 2.** Level of protein of NMDAR in spinal cord of rat model with hyperalgesia was increased. Note: *compare with control group ($P$<0.05). A. One result of the three times of electrophoresis. B. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference Actin.

ed with PBST for 3 times then developed and fixing [19]. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target

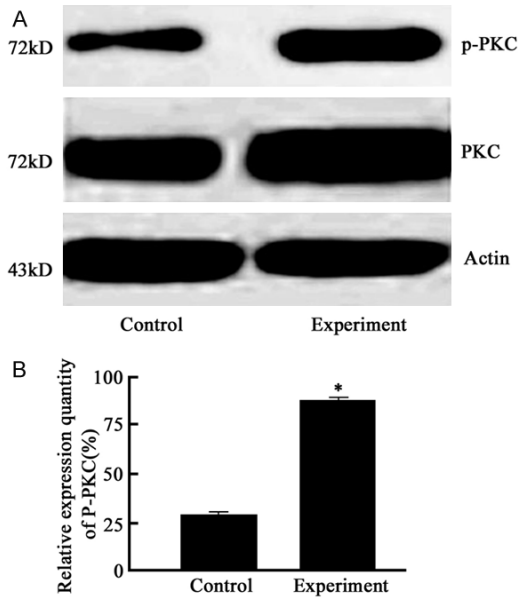


**Figure 3.** Level of protein of activated PKC in spinal cord of rat model with hyperalgesia was increased. Note: *compare with control group ($P$<0.05). A. One result of the three times of electrophoresis. B. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference Actin.

gene divides gray value of internal reference actin.

*Measurement of algesia time*

We used routine method to measure algesia time of rats [13] with rat algesimeter. The detailed steps are following: cold-hot plate algesimeter was used to measure reaction time of rats to pain in order to screen anesthesia analgesics rapidly and precisely. For rats in a group, before medication, average time of paw lick of rats on hot plate corresponding to reaction time of rats to heat stimulation was measured. After medication, average time of paw lick of rats on hot plate corresponding to reaction time of rats to heat stimulation after medication was measured. Comparison with time of the two groups was to assess the effect of analgesia or drugs to rats.

*Statistical analysis*

Statistical analysis was performed with SPSS 13.0. Values were expressed as mean ± SEM. The comparison of NMDAR level of spinal cord, PKC level and activity of PKC protein kinase

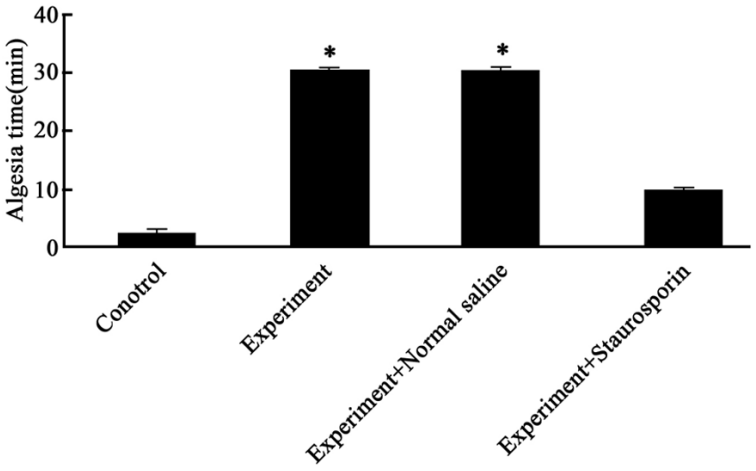


**Figure 4.** Inhibitor of PKC Staurosporine reduced algesia time of rat model with hyperalgesia. Note: *Compare with control group ($P$<0.05).

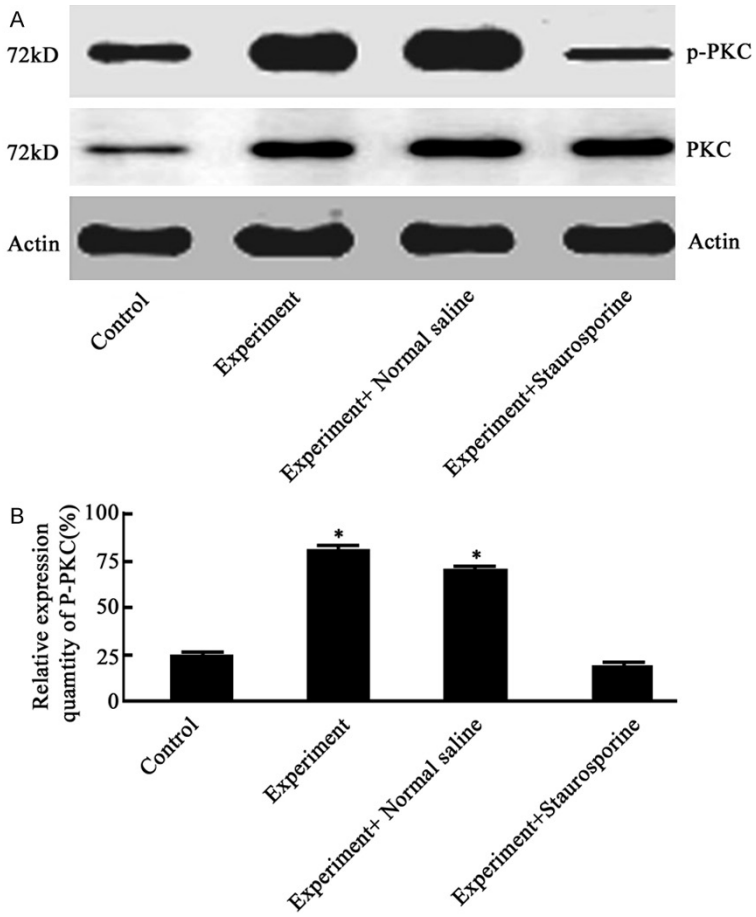


**Figure 5.** Inhibitor of PKC Staurosporine reduced activation of PKC in spinal cord of rat model with hyperalgesia. Note: *compare with control group ($P$<0.05). A. One result of the three times of electrophoresis. B. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference Actin.

were analyzed by one-way ANOVA. $P$<0.05 was considered statistically significant.

## Results

### *Spinal cord NMDAR mRNA increased in hyperalgesia rat model*

As the results of RT-PCR showed in **Figure 1**, compared with control group, level of mRNA of NMDAR in spinal cord of rat model with hyperalgesia was increased. There was significant difference in level of mRNA of NMDAR in spinal cord between two groups ($P$<0.05).

### *Spinal cord NMDAR protein increased in hyperalgesia rat model*

As the results of western blot showed in **Figure 2**, compare with control group, level of Protein of NMDAR in spinal cord of rat model with hyperalgesia was increased. There was significant difference in level of protein of NMDAR in spinal cord between two groups ($P$<0.05).

### *Spinal cord activated PCK protein increased in hyperalgesia rat model*

As the results of western blot showed in **Figure 3**, compare with control group, level of protein of activated PKC in spinal cord of rat model with hyperalgesia was increased. There was significant difference in level of protein of activated PKC in spinal cord between two groups ($P$<0.05).

### *PKC inhibition reduced algesia time in hyperalgesia rat model*

As the results of western blot showed in **Figure 4**, compare

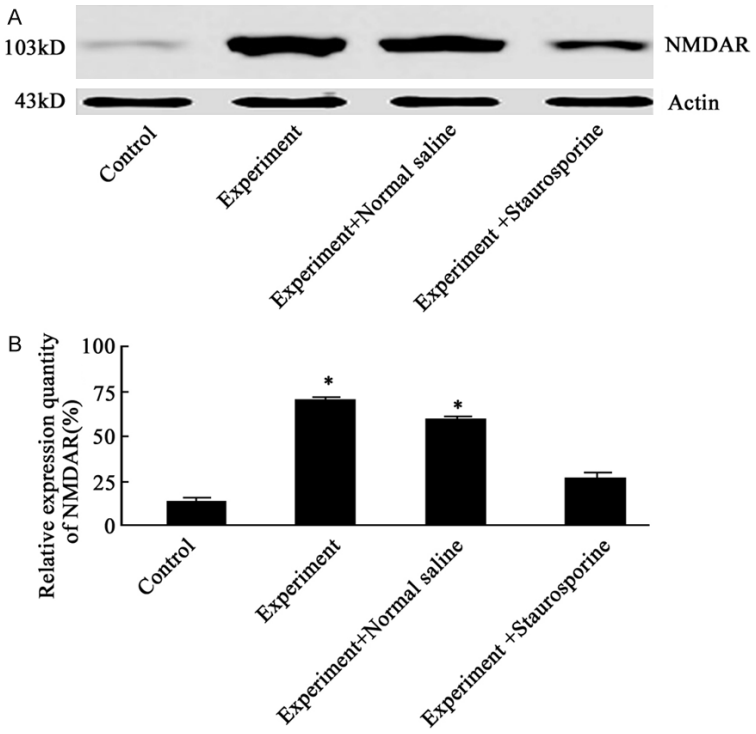


**Figure 6.** Inhibitor of PKC Staurosporine reduced protein level of NMDAR in spinal cord of rat model with hyperalgesia. Note: *compare with control group ($P$<0.05). A. One result of the three times of electrophoresis. B. Gray value of electrophoretic band was anlyzed using Image J sofrware. Expression quantives of target gene is gray value of target gene divides gray value of internal reference Actin.

with control group, after infusion of Staurosporine in model group, the algesia time of rats with hyperalgesia was significantly reduced ($P$<0.05).

*PKC inhibition reduced PKC activation in spinal cord in hyperalgesia rat model*

As the results of western blot showed in **Figure 5**, compare with control group, after infusion of Staurosporine in model group, level of PKC was not changed. Activation of PKC in spinal cord of rat model with hyperalgesia was significantly inhibited ($P$<0.05).

*PKC inhibition reduced NMDAR protein expression in spinal cord in hyperalgesia rat model*

As the results of western blot showed in **Figure 6**, compare with control group, after infusion of Staurosporine in model group, NMDAR in spinal cord of rat model with hyperalgesia was significantly reduced ($P$<0.05).

## Discussion

In clinical practice, the phenomenon of anesthetic-induced hyperalgesia has been in existence for a long time. But it is not able to demonstrate the molecular mechanism of hyperalgesia [1]. There are both theoretic meaning and practice meaning for medication development in clinical practice to investigate the molecular mechanism of remifentanil-induced hyperalgesia [6]. Therefore, our study aimed to investigate the possible effect of NMDAR on remifentanil-induced hyperalgesia.

There were three main findings: (1) The expression level of NMDAR and PKC of rats' spinal cord with remifentanil-induced hyperalgesia were increased. (2) PKC of rats' spinal cord with remifentanil-induced hyperalgesia was activated. (3) Intravenous infusion of PKC inhibitor Staurosporine significantly reduced remifentanil-induced hyperalgesia. All these results indicated that NMDAR play a critical role in remifentanil-induced hyperalgesia, possibly through PKC signal pathway. Base on general anesthesia, controlling level of NMDAR and activation of protein kinase may be one of the methods to relieve remifentanil-induced hyperalgesia.

The results of the study indicated that expression level of NMDAR in spinal cord of rats with hyperalgesia was increased by remifentanil, which is consistent with the results of previous studies on NMDAR and hyperalgesia [17, 18]. What was different to previous studies was that the results of this study indicated that PKC signal pathway was associated with NMDAR involved in hyperalgesia. In contrast, previous studies found that PKC signal pathway was not associated with NMDAR involved in hyperalgesia [16]. There may be two reasons for the difference of the results, firstly, the difference of experiment condition espe-

cially animal model of hyperalgesia which was rat model of remifentanil-induced hyperalgesia, whereas was model of surgery-cut induced hyperalgesia. The other reason was that the concentration of calcium and PKC activation is a transient process, with the time goes by, the activation of PKC signal pathway will be reduced and even disappeared. We had examined the calcium concentration, but for various reasons, we didn't observe the change of calcium concentration. The reason may be that in one hand our lab had not mastered the technology of detecting calcium concentration, in the other hand the change of calcium concentration was too fast to detect because it took a long time to separate animal spinal cord. It was reported that when sensitivity reaction happened, NMDAR could cause imbalance of calcium concentration of nerve cells resulting in activation of neurotoxicity signal and finally apoptosis and dysfunction of neuron. Our results were consistent with these study ideas.

There are some limitations about this study. (1) Though we investigate the relationship between remifentanil-induced hyperalgesia and NMDAR through rat model with hyperalgesia, results of clinical specimen of patients with hyperalgesia was lacking. (2) The number of animal model in this study is limited. The future study should expand the samples to further confirm the role of NMDAR and PKC on remifentanil-induced hyperalgesia [18]. (3) The study didn't change the NMDAR level with siRNA and overexpression technology in the animal level to investigate the role of NMDAR and PKC on remifentanil-induced hyperalgesia. Previous study [20] investigated the ERK level in hyperalgesia. But afterwards ERK was confirmed to be not associated with hyperalgesia. So PKC was the only one investigated in our study. (4) The study didn't perform correlation analysis between NMDAR and remifentanil-induced hyperalgesia through using different doses of remifentanil to investigate whether different doses of remifentanil were dose-dependently associated with the change of NMDAR.

In conclusion, N-methyl-D-aspartate receptor played a critical in remifentanil-induced hyperalgesia, possibly through PKC signal pathway. Base on general anesthesia, controlling level of NMDAR and activation of protein kinase may be one of the methods to relieve remifentanil-induced hyperalgesia and represent one of the new strategies of medication development of controlling hyperalgesia.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Dayong Xu, Department of General Surgery, The First Hospital of Changsha, 311 Yinpan Road, Changsha 410008, China. Tel: +86-731-84667533; Fax: +86-731-84667533; E-mail: dayongxu221@sina.com

## References

[1] Wei H, Viisanen H, You HJ, Pertovaara A. Spinal histamine in attenuation of mechanical hypersensitivity in the spinal nerve ligation-induced model of experimental neuropathy. Eur J Pharmacol 2016; 772: 1-10.

[2] Lefèvre Y, Amadio A, Vincent P, Descheemaeker A, Oliet SH, Dallel R, Voisin DL. Neuropathic pain depends upon D-serine co-activation of spinal NMDA receptors in rats. Neurosci Lett 2015; 603: 42-7.

[3] Hama A, Sagen J. Combinations of intrathecal gamma-amino-butyrate receptor agonists and N-methyl-d-aspartate receptor antagonists in rats with neuropathic spinal cord injury pain. Eur J Pharmacol 2012; 683: 101-8.

[4] Benson C, Paylor JW, Tenorio G, Winship I, Baker G, Kerr BJ. Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE). Exp Neurol 2015; 271: 279-90.

[5] Pagadala P, Park CK, Bang S, Xu ZZ, Xie RG, Liu T, Han BX, Tracey WD Jr, Wang F, Ji RR. Loss of NR1 subunit of NMDARs in primary sensory neurons leads to hyperexcitability and pain - hypersensitivity: involvement of Ca(2+)-activated small conductance potassium channels. J Neurosci 2013; 33: 13425-30.

[6] Sagalajev B, Bourbia N, Beloushko E, Wei H, Pertovaara A. Bidirectional amygdaloid control of neuropathic hypersensitivity mediated by descending serotonergic pathways acting on spinal 5-HT3 and 5-HT1A receptors. Behav Brain Res 2015; 282: 14-24.

[7] Choi SR, Roh DH, Yoon SY, Kang SY, Moon JY, Kwon SG, Choi HS, Han HJ, Beitz AJ, Oh SB, Lee JH. Spinal sigma-1 receptors activate NADPH oxidase 2 leading to the induction of pain hypersensitivity in mice and mechanical allodynia in neuropathic rats. Pharmacol Res 2013; 74: 56-67.

[8] Chen YW, Lin MF, Chen YC, Hung CH, Tzeng JI, Wang JJ. Exercise training attenuates postop-

erative pain and expression of cytokines and N-methyl-D-aspartate receptor subunit 1 in rats. Reg Anesth Pain Med 2013; 38: 282-8.
[9] Verne GN, Price DD, Callam CS, Zhang B, Peck J, Zhou Q. Viscerosomatic facilitation in a subset of IBS patients, an effect mediated by N-methyl-D-aspartate receptors. J Pain 2012; 13: 901-9.
[10] Chen SR, Zhou HY, Byun HS, Chen H, Pan HL. Casein kinase II regulates N-methyl-D-aspartate receptor activity in spinal cords and pain hypersensitivity induced by nerve injury. J Pharmacol Exp Ther 2014; 350: 301-12.
[11] Chen Y, Chen AQ, Luo XQ, Guo LX, Tang Y, Bao CJ, Lin L, Lin C. Hippocampal NR2B-containing NMDA receptors enhance long-term potentiation in rats with chronic visceral pain. Brain Res 2014; 1570: 43-53.
[12] D'Mello R, Marchand F, Pezet S, McMahon SB, Dickenson AH. Perturbing PSD-95 interactions with NR2B-subtype receptors attenuates spinal nociceptive plasticity and neuropathic pain. Mol Ther 2011; 19: 1780-92.
[13] Hu YM, Chen SR, Chen H, Pan HL. Casein kinase II inhibition reverses pain hypersensitivity and potentiated spinal N-methyl-D-aspartate receptor activity caused by calcineurin inhibitor. J Pharmacol Exp Ther 2014; 349: 239-47.
[14] Ciftci T, Erbatur S, Ak M. Comparison of the effects of dexmedetomidine and remifentanil on potential extreme haemodynamic and respiratory response following mask ventilation and laryngoscopy in patients with mandibular fractures. Eur Rev Med Pharmacol Sci 2015; 19: 4427-33.
[15] Zhou L, Huang J, Gao J, Zhang G, Jiang J. NMDA and AMPA receptors in the anterior cingulate cortex mediates visceral pain in visceral hypersensitivity rats. Cell Immunol 2014; 287: 86-90.
[16] Li S, Cao J, Yang X, Suo ZW, Shi L, Liu YN, Yang HB, Hu XD. NR2B phosphorylation at tyrosine 1472 in spinal dorsal horn contributed to N-methyl-D-aspartate-induced pain hypersensitivity in mice. J Neurosci Res 2011; 89: 1869-76.
[17] Zhang L, Shu R, Wang C, Wang H, Li N, Wang G. Hydrogen-rich saline controls remifentanil-induced hypernociception and NMDA receptor NR1 subunit membrane trafficking through GSK-3β in the DRG in rats. Brain Res Bull 2014; 106: 47-55.
[18] Eisenach JC, Tong C, Curry RS. Failure of intrathecal ketorolac to reduce remifentanil-induced postinfusion hyperalgesia in humans. Pain 2015; 156: 81-7.
[19] Romero A, González-Cuello A, Laorden ML, Campillo A, Vasconcelos N, Romero-Alejo E, Puig MM. Effects of surgery and/or remifentanil administration on the expression of pERK1/2, c-Fos and dynorphin in the dorsal root ganglia in mice. Naunyn Schmiedebergs Arch Pharmacol 2012; 385: 397-409.
[20] Yang F, Sun W, Yang Y, Wang Y, Li CL, Fu H, Wang XL, Yang F, He T, Chen J. SDF1-CXCR4 signaling contributes to persistent pain and hypersensitivity via regulating excitability of primary nociceptive neurons: involvement of ERK-dependent Nav1.8 up-regulation. J Neuroinflammation 2015; 12: 219.