ORIGINAL ARTICLE

Preoperative anxiety induces chronic postoperative pain by activating astrocytes in the anterior cingulate cortex region

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SUMMARY

OBJECTIVE: *The study aims to explore the relationship between preoperative anxiety and chronic postoperative pain.*

METHODS: *A total of forty rats were divided into four groups, control, single-prolonged stress alone, Hysterectomy alone, and SPS+ Hysterectomy. The paw withdrawal mechanical thresholds (PWMT) were examined. qRT-PCR and western blotting assay were performed to detect the GFAP expression in astrocytes isolated from the anterior cingulate cortex (ACC) region. In addition, the long-term potentiation (LTP) in ACC was examined.*

RESULTS: *Rats in the SPS group or the Hysterectomy alone group had no significant effect on chronic pain formation, but SPS can significantly induce chronic pain after surgery. Astrocytes were still active, and the LTP was significantly increased three days after modeling in the SPS+Hysterectomy group.*

CONCLUSIONS: *anxiety can induce chronic pain by activating astrocytes in the ACC region.*

KEYWORDS: *Ansiedade. Pain, postoperative. Astrocytes.*

INTRODUCTION

Clinical investigations have demonstrated that patients with serious anxiety have lower pain threshold and higher sensitivity to pain. In particular, patients who experience serious preoperative anxiety may suffer from postoperative chronic pain [1,2]. The mechanism proposed is that anxiety could increase the central sensitivity of pain by regulating corticotropin-releasing and inflammatory factors such as IL-1, IL-6, IL-10, TNF-α, and noradrenaline, which would increase the pain feeling. [3-5] Anxiety can also have a negative impact on the treatment of painful diseases by enhancing the pain feeling. It is important to use psychological intervention to relief patients' pain and improve their quality of life. However, those treatments sometimes may not be effective. Therefore, it is urgent that we find how to mediate pain enhanced by anxiety.

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Neuroactive substances and receptors are abundant on the neurons in the posterior horn of the spinal cord [6,7]. Previous studies have generally believed that only neurons and their associated neurotransmitters played important roles in pain development, while the role of glial cells was ignored. Recently, studies have found that glial cells in the nervous system play various roles. Glial cells are essential in the propagation of pain signaling, especially in the course of neuropathic pain [8]. It was found that the expression of glial fibrillary acidic protein (GFAP) was significantly increased in a rat model of hyperalgesia, suggesting that glial cells may be activated and participate in the process of pain sensitization. Some studies have also found that the activation of glial cells (including astrocytes and microglia) were usually observed during many chronic pain model [9-12].

Astrocytes are particularly sensitive to the changes in the microenvironment around the neurons, and once receiving the signal, activated astrocytes can release massive proinflammatory and neuroactive substances [13]. The anterior cingulate cortex (ACC) region is a cortical area in the brain that contributes to the regulations of pain and emotions and contains abundant astrocytes. [14,15] Some studies using neuroimaging techniques demonstrated increased activity in the ACC during chronic pain formation [16,17]. In addition, it has been reported that astrocytes in the ACC can be activated in the inflammatory pain model [18]. As such, we wondered if preoperative anxiety can activate astrocytes in the ACC, subsequently leading to the formation of postoperative chronic pain.

This study investigated the effects of preoperative anxiety on postoperative chronic pain by stablishing a preoperative anxiety model. By using techniques such as ACC slice, western blot, RT-PCR, immunohistochemistry, immunofluorescence, and electrophysiology, the alterations of astrocyte activation and LTP in the mice model were evaluated. As a result, we found that preoperative anxiety can induce postoperative chronic pain by activating astrocytes in the ACC region.

METHODS

Animals

Adult female Sprague-Dawley rats (210–260 g) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Rats were housed six per cage in a controlled environment, fed a standard rodent food, and allowed distilled water ad libitum. Rats were given at least one week to adapt to the new environment before any manipulation. Forty rats were randomly assigned to four groups (n=10): control group; SPS group (Single-prolonged stress model); Hysterectomy only group; SPS + Hysterectomy group.

Isolation of astrocytes in the rat ACC region.

Five rats in each group were euthanized around three weeks after modeling for astrocytes isolation by placing rats in a CO2 enriched tank. The ACC region in the brain was dissected, and the meninges were removed. The rodent brain was kept in Hank's Balanced Salt Solution (HBSS) containing 0.05% trypsin and 0.005% DNase at 4 °C during the dissection process. The tissue was then triturated for around 5 min by pipetting up and down using a Pasteur pipette. Triturated tissue was then centrifuged at 400 g for 5 min at 22 °C. After removing the supernatant, the pellet was resuspended into the HBSS containing 34 U papain/ml, 0.02% cysteine and 0.005% DNase and triturated for further 5 min. Followed by another centrifugation at 400 g for 5 min at 22 °C, the cells were resuspended in HBSS containing 0.005% DNase and put on ice for 30 min. The supernatant was collected and centrifuged again for another 10 min at 400 g.

Single-prolonged stress (SPS) modeling

When establishing the SPS model, rats were first restrained for 2 hr by placing them in plastic bags individually and immobilizing tightly. Several holes were made on the bag to make sure rats could breathe freely. After restraint, the rats were placed in a swimming pool (24 cm in diameter, 50 cm in height) at 24°C and forced to swim for 20 min. After 15-min rest, the rats in all groups were anesthetized with isoflurane. Thereafter, the rats were placed in a ventilated place until they waked up naturally and returned to the cage.

Hysterectomy

24 hr after the SPS modeling, the rats in the Hysterectomy and SPS+Hysterectomy groups underwent a hysterectomy. The rats were anesthetized with isoflurane and then fixed onto a warm pad. The fur over the surgery area was shaved, and the skin was sterilized with ethanol. The skin was cut with approximately 1.5cm length at 0.5 cm above the

midline of the pubic symphysis. The abdominal muscles and peritoneum were bluntly separated. Thereafter, the uterus was exposed by pushing intestine upwards. The lower edge of the cervix was severed, and the cut was sealed with wire. Then, cellulite was removed, and pink or yellow-red ovary was exposed. The fallopian tube, peri-uterine, and fascia were ligatured, and the uterus was removed. At last, the surgical wound was double sutured. A sham operation was performed in the control and SPS groups, and the wounds were sutured only at 0.5 cm above the midline of the pubic symphysis

Animal behavioral test

The mechanical paw withdrawal threshold (PWMT) was performed by Von Frey cilia at 0d (before modeling) every three days after the operation. It was examined by Chaplan's "Up-and-Down" method. Briefly, the rats were placed on a metal frame with a clear plexiglass box cover. The bottom of the box was an empty metal grid. The Electronic von Frey monofilament was used to stimulate the pain at the inner and outer sides of the rat's paw with a series of upward forces. We gradually increased the force until the appearance of a sharp retraction of the hind paw. The number on the electronic display was recorded as a mechanical pain threshold. The threshold was measured three times for each rat with a 5-10 min break interval. The average of three measurements was taken for statistical analysis

qRT-PCR

One-Step SYBR ® PrimeScript ™ (Takara, Japan) qPCR Kit and 7300 real-time fluorescence quantitative PCR instrument were used to detect GFAP, per kit instructions. The reaction conditions were as follows: 95°C for 30 s; 95°C for 5 s; 60°C for 30 s, 40 cycles. The relative mRNA expression level of GFAP was calculated by the $2^{-\Delta\Delta Ct}$ method. β-actin was used as an internal reference, and the primer sequences were:

GFAP forward-5'-GTACCAGGACCTGCTCAAT-3', reverse-5'-CAACTATCCTGCTTCTGCTC-3';

Actin forward-5'-AGAGCTACGAGCTGCCT-GAC-3', reverse-5'-AGCACTGTGTTGGCGTACAG-3'.

Western Blot

Isolated astrocytes from the rats were spin down, and 200 μl of protein lysis buffer was added into each tube and mixed well. After 30 min of lysis, the cell lysates were transferred into another EP tube and centrifuged at 12000 r/min for 10 min. The protein concentration was measured by BCA assay. The SDS buffer was added into cell lysates followed by 10 min protein denature. SDS-PAGE gel electrophoresis was performed, and the wet transfer method was applied to transfer the protein gel onto a PVDF membrane. After 5% skimmed milk powder block at room temperature for 2h, samples were incubated with primary antibody anti-GFAP at 4°C overnight (ab7260, Abcam, USA, dilution ratio of 1: 10000). On the next day, samples were incubated with corresponding secondary antibodies at room temperature for 1h (Abcam, USA) Membranes were washed three times by TBST containing ECL chemiluminescence solution (Promega). Gel imaging equipment was used for band observation. The results were presented as the ratio of the optical density of the target strip to internal GAPDH.

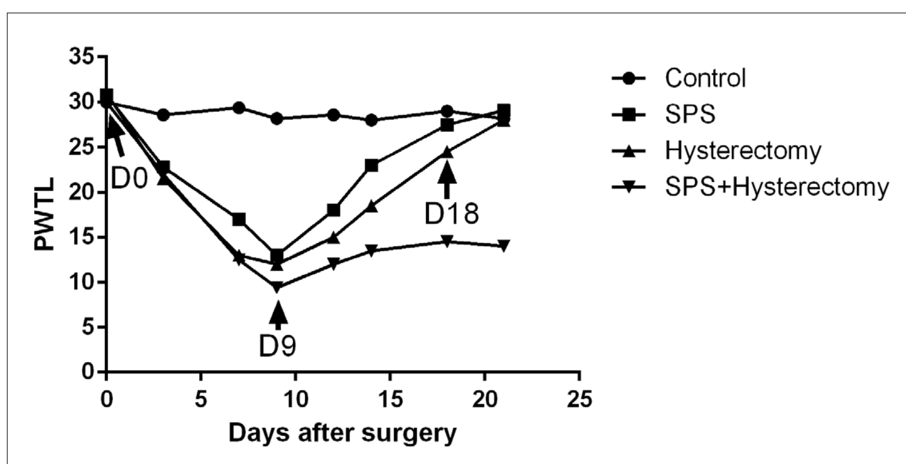


FIGURE1. POSTOPERATIVE CHRONIC PAIN FORMATION INDUCED BY SPS+HYSTERECTOMY.

The PWMT of rats in each group were examined before modeling and every three days after it for three weeks. n=10

LTP measurement

Experiments were performed in a recording chamber on the stage of an Axioskop 2FS microscope with infrared DIC optics for visualizing whole-cell patch-clamp recordings. Excitatory postsynaptic currents (EPSCs) were recorded from layer II-III neurons using an Axon 200B amplifier (Axon Instruments, CA) and stimulations were delivered using a bipolar tungsten stimulating electrode placed in layer V of the ACC. The EPSCs were induced by repetitive stimulations at 0.02 Hz, and the neurons were voltage-clamped at -70 mV. LTP was induced within 10 min after obtaining stable EPSCs to prevent the washout effect. The protocol involved paired presynaptic 80 pulses at 2 Hz with postsynaptic depolarization at +30 mV (referred to as pairing training). The neurons were then voltage-clamped at -30 mV and EPSCs were evoked at 0.05 Hz. The access resistance was 15-30 MΩ and was monitored throughout the experiment. Data were discarded if access resistance changed by more than 15% during an experiment.

Statistical analysis

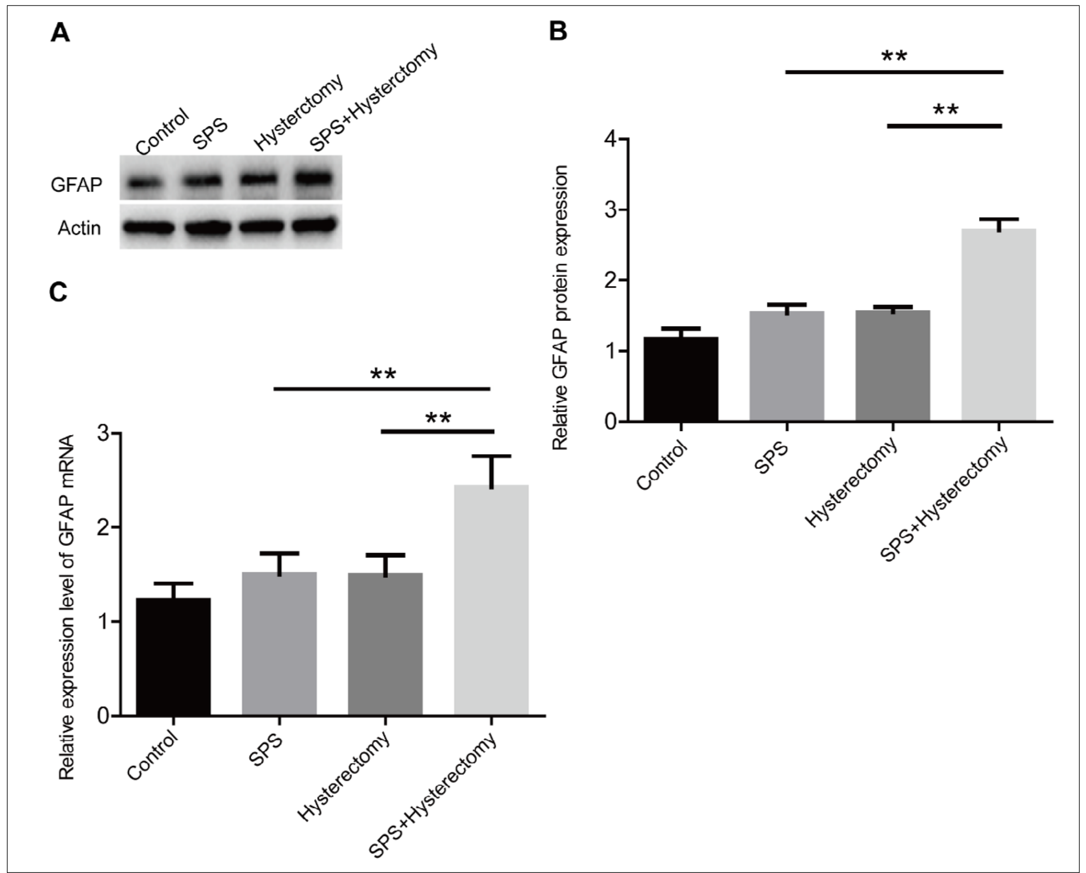
All the statistical analysis was performed using SPSS 17.0(SPSS, USA) and Prism 6 software. Statistical analysis for all the raw data was then performed using student t-test.

RESULTS

PWMT test after modeling

The mechanical PWMT in each group were tested before modeling and every three days after it. As shown in figure 1, these two thresholds in the SPS, Hysterectomy, and SPS+Hysterectomy groups were decreased significantly within the first week compared to day0 or the control group. Notably, there was no obvious variation of PWMT between different model groups for the first week, suggesting the modeling did not have an impact on the acute pain. However, around one week later, the thresholds of the SPS and Hysterectomy alone groups started to increase and recovered back to the control level at

FIGURE2. ASTROCYTES WERE STILL ACTIVE 3 WEEKS AFTER MODELING IN THE SPS+HYSTERECTOMY GROUP.



Astrocytes were isolated from the ACC region, and the expression of GFAP was detected by Westernport. B. The protein expression level was normalized to actin. C. The expression of GFAP was further confirmed by RT-PCR. n=5. *P < 0.05; **P < 0.01. Data are presented as mean ± SEM.

around day18. However, rats in the SPS+Hysterectomy did not present any improvement on pain thresholds at all after one week and showed a noticeable difference compared to the SPS or Hysterectomy groups after day10. This indicates chronic pain was formed in the SPS+Hysterectomy group but not in the SPS or Hysterectomy groups.

GFAP expression in astrocytes isolated from the ACC region

To investigate the reason why chronic pain was formed in the SPS+Hysterectomy group but not in the others, the astrocytes from the ACC region were isolated from the rats in each group three weeks after the modeling. Since GFAP is the activation marker of astrocytes, its expression level was detected in these cells. The western blot results presented that GAFP in the SPS+Hysterectomy group had higher expression than in the other groups. The GAFP expressions in the SPS and Hysterectomy alone groups did not show a significant difference compared to the control, suggesting astrocytes were only activated in the SPS+Hysterectomy group three weeks after the modeling. This observation was further confirmed by RT-PCR(Figure 2).

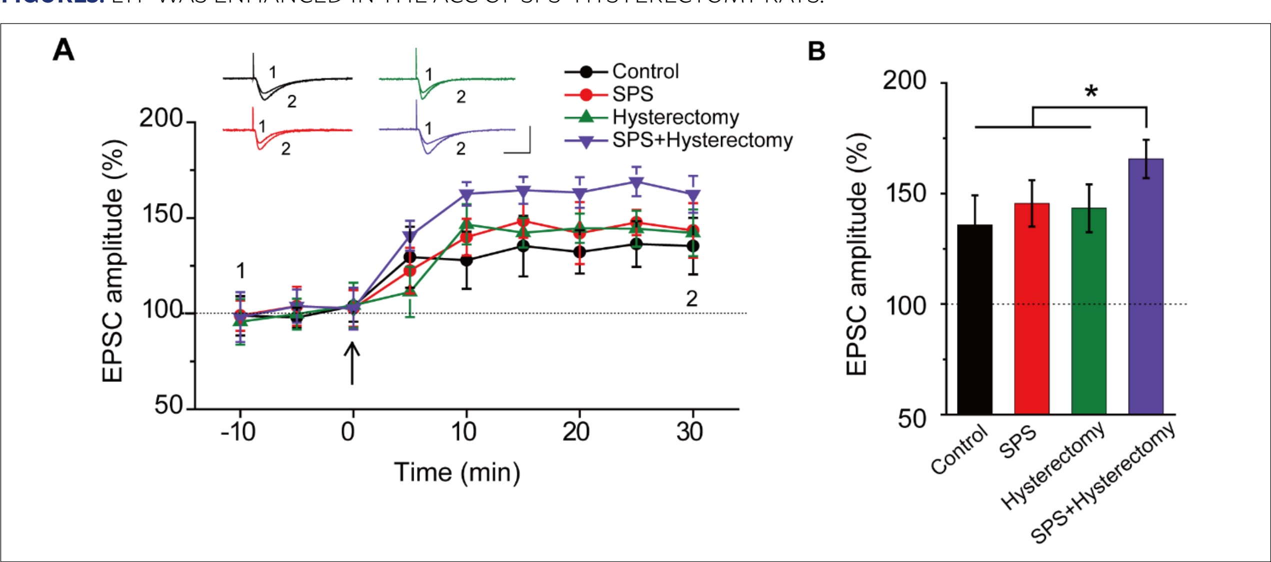
LTP change after modeling

To determine the LTP in different rat models, the ACC region were isolated and sliced for electrophysiological test three weeks after the modeling. As a result, the EPSC amplitude in the ACC region of the SPS+Hysterectomy group was significantly enhanced after induction compared to the control (Figure 3), SPS, and Hysterectomy groups. This suggests the LTP in the SPS+Hysterectomy group was enhanced, and chronic pain occurred after the modeling. Though a small enhancement of LTP in the SPS and Hysterectomy rats was recorded, the difference is not significant compared to the control group. We did not observe any noticeable change of LTP in the ACC region between the SPS and Hysterectomy groups. This indicates that SPS+Hysterectomy affects synaptic potentiation in the ACC.

DISCUSSION

Negative emotions, including anxiety, fear, and depression, are common among patients before operation. Preoperative anxiety, in particular, is very common and apparent. This kind of anxiety can reduce postoperative pain tolerance, diminish treatment efficiency, and damage patients' physical and mental health. It has been reported that patients who undergo operations tend to stimulate pain after the surgery, especially those receiving local anesthesia such as peripheral nerve block anesthesia, epidural anesthesia, and spinal anesthesia. The body damage caused by the surgery and postoperative pain they experience are usually overestimated, resulting in unnecessary anxi-

FIGURE3. LTP WAS ENHANCED IN THE ACC OF SPS+HYSTERECTOMY RATS.



A. Synaptic potentiation of EPSCs in the ACC of SPS+Hysterectomy rats was compared with that in the control, SPS, and Hysterectomy rats. B. Summarized data of EPSC amplitude 30 min after LTP induction in the ACC slices of rats. n=5. *P < 0.05. Data are presented as mean ± SEM.

ety in patients [19]. Clinical approaches, such as non-steroidal anti-inflammatory drugs and non-medical intervention, have been applied to relieve postoperative pain [20] while the treatment outcomes are not ideal in many cases. Despite an increased focus and development of new standards for pain management, many patients still experience intense pain after surgery. Additional efforts are required to improve patients' postoperative pain relief [21].

The ACC region is important to control the nociceptive emotion. Some studies suggest that astrocytes in this region may be activated and participate in the process of pain sensitization. Therefore, we wondered if the activation of astrocytes could be the link between preoperative anxiety and postoperative pain. In this project, we used a SPS model to investigate the relationship between preoperative anxiety and postoperative pain. SPS is a frequently used rat model of posttraumatic stress disorder (PTSD) that involves exposure to several successive stressors. [22] Hereby, we report a novel animal model which combines SPS and hysterectomy to study the formation of postoperative pain induced by preoperative anxiety. In addition, we found that chronic pain was formed in the SPS+Hysterectomy group but not in the SPS or Hysterectomy groups, indicating preoperative anxiety did enhance the postoperative pain. Moreover, astrocytes were found to be still activated, and increased LTP was also observed in the ACC region of rats in the SPS+Hysterectomy group three weeks after modeling, suggesting the formation of chronic pain.

CONCLUSION

Anxiety can induce chronic pain by activating astrocytes in the ACC region.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Author Contributions

Conceptualization, H.C. .and T.L.M..; methodology, D.Y.L..; software D.Y.L..; validation, S.H.X..; formal analysis, D.Y.L.; investigation, T.P.; resources, H.C.; data curation, Y.B.Y.; writing—original draft preparation, D.Y.L.; writing—review and editing, T.L.M..; visualization, T.L.M..; supervision, T.L.M..; project administration, H.C.; funding acquisition, T.L.M.

RESUMO

OBJETIVO: *O objetivo deste estudo é explorar a relação entre a ansiedade no pré-operatório e a dor crônica no pós-operatório.*

MÉTODOS: *Um total de 40 ratos foram divididos em quatro grupos: controle, estresse prolongado (SPS), histerectomia e SPS + histerectomia. Os limiares de retirada da pata em resposta a estímulo mecânico (PWMT) foram examinados. Ensaios qRT-PCR e imunoenzimáticos (western blotting) foram realizados para detectar a expressão de GFAP em astrócitos isolados da região do córtex cingulado anterior (CCA). Além disso, a potenciação de longa duração (LTP) no CCA também foi examinada.*

RESULTADOS: *Os ratos no grupo de estresse prolongado e no grupo de histerectomia não apresentaram nenhum efeito significativo na formação de dor crônica. Porém, o estresse prolongado foi capaz de induzir dor crônica significativamente após a cirurgia. Três dias após o modelo, o grupo de SPS + histerectomia ainda apresentava astrócitos ativos e LTP significativamente maior.*

CONCLUSÃO: *A ansiedade pode provocar dor crônica através da ativação de astrócitos na região do CCA.*

PALAVRAS-CHAVE: *Ansiedade. Pós-operatória, dor. Astrócitos.*

REFERENCES

1. Tanasale B, Kits J, Kluin PM, Trip A, Kluin-Nelemans HC. Pain and anxiety during bone marrow biopsy. Pain Manag Nurs. 2013;14(4):310-7.
2. Johanes C, Monoarfa RA, Ismail RI, Umbas R. Anxiety level of early- and late-stage prostate cancer patients. Prostate Int. 2013;1(4):177-82.
3. Phyomaung PP, Dubowitz J, Cicuttini FM, Fernando S, Wluka AE, Raaijmaakers P, et al. Are depression, anxiety and poor mental health risk factors for knee pain? A systematic review. BMC Musculoskelet Disord. 2014;15:10.
4. Merritt C. Fear and loathing in the ER: managing procedural pain and anxiety in the Pediatric Emergency Department. R I Med J (2013). 2014;97(1):31-4.

5. Dogan MC, Yazicioglu I, Antmen B. Anxiety and pain during dental treatment among children with haemophilia. Eur J Paediatr Dent. 2013;14(4):284-8.

6. González S, Labombarda F, Gonzalez Deniselle MC, Saravia FE, Roig P, De Nicola AF. Glucocorticoid effects on Fos immunoreactivity and NADPH-diaphorase histochemical staining following spinal cord injury. Brain Res. 2001;912(2):144-53.

7. Fürst S. Transmitters involved in antinociception in the spinal cord. Brain Res Bull. 1999;48(2):129-41.

8. Tsuda M, Inoue K, Salter MW. Neuropathic pain and spinal microglia: a big problem from molecules in „small" glia. Trends Neurosci. 2005;28(2):101-7.

9. Hashizume H, DeLeo JA, Colburn RW, Weinstein JN. Spinal glial activation and cytokine expression after lumbar root injury in the rat. Spine (Phila Pa 1976). 2000;25(10):1206-17.

10. Scheggi S, Mangiavacchi S, Masi F, Gambarana C, Tagliamonte A, De Montis MG. Dizocilpine infusion has a different effect in the development of morphine and cocaine sensitization: behavioral and neurochemical aspects. Neuroscience. 2002;109(2):267-74.

11. Mika J, Wawrzczak-Bargiela A, Osikowicz M, Makuch W, Przewlocka B. Attenuation of morphine tolerance by minocycline and pentoxifylline in naive and neuropathic mice. Brain Behav Immun. 2009;23(1):75-84.

12. Maolood N, Meister B. Protein components of the blood-brain barrier (BBB) in the brainstem area postrema-nucleus tractus solitarius region. J Chem Neuroanat. 2009;37(3):182-95.

13. Allen NJ, Barres BA. Signaling between glia and neurons: focus on synaptic plasticity. Curr Opin Neurobiol. 2005;15(5):542-8.

14. Vogt BA. Pain and emotion interactions in subregions of the cingulate gyrus. Nat Rev Neurosci. 2005;6(7):533-44.

15. Xie YF, Huo FQ, Tang JS. Cerebral cortex modulation of pain. Acta Pharmacol Sin. 2009;30(1):31-41.

16. Hsieh JC, Belfrage M, Stone-Elander S, Hansson P, Ingvar M. Central representation of chronic ongoing neuropathic pain studied by positron emission tomography. Pain. 1995;63(2):225-36.

17. Tseng MT, Chiang MC, Chao CC, Tseng WY, Hsieh ST. fMRI evidence of degeneration-induced neuropathic pain in diabetes: enhanced limbic and striatal activations. Hum Brain Mapp. 2013;34(10):2733-46.

18. Chen FL, Dong YL, Zhang ZJ, Cao DL, Xu J, Hui J, et al. Activation of astrocytes in the anterior cingulate cortex contributes to the affective component of pain in an inflammatory pain model. Brain Res Bull. 2012;87(1):60-6.

19. Gerbershagen HJ, Aduckathil S, van Wijck AJ, Peelen LM, Kalkman CJ, Meissner W. Pain intensity on the first day after surgery: a prospective cohort study comparing 179 surgical procedures. Anesthesiology. 2013;118(4):934-44.

20. Møiniche S, Kehlet H, Dahl JB. A qualitative and quantitative systematic review of preemptive analgesia for postoperative pain relief: the role of timing of analgesia. Anesthesiology. 2002;96(3):725-41.

21. Apfelbaum JL, Chen C, Mehta SS, Gan TJ. Postoperative pain experience: results from a national survey suggest postoperative pain continues to be undermanaged. Anesth Analg. 2003;97(2):534-40.

22. Souza RR, Noble LJ, McIntyre CK. Using the single prolonged stress model to examine the pathophysiology of PTSD. Front Pharmacol. 2017;8:615.