Dexmedetomidine relieved neuropathic pain and inflammation response induced by CCI through HMGB1/TLR4/NF-κB signal pathway

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Abstract

Neuropathic pain is one of the most intractable diseases. The lack of effective therapy measures remains a critical problem due to the poor understanding of the cause of neuropathic pain. The aim of this study was to investigate the effect of dexmedetomidine (Dex) in trigeminal neuropathic pain and the underlying molecular mechanism in order to identify possible therapeutic targets. We used a chronic constriction injury (CCI) model of mice to investigate whether Dex prevents neuropathic pain and the inflammation response. The α 2-adrenoceptors (α2AR) inhibitor BRL44408 and adenovirus for knocking down High mobility group box 1 (HMGB1) was administrated to confirm whether Dex exert its effect through targeting α2AR and HMGB1. The results indicated that Dex significantly inhibited CCI induced neuropathic pain through targeting α2AR and HMGB1. Dex inhibited the inflammatory response through decreasing the release and the mRNA expression of IL-1β, IL-6, and TNF-α while increasing that of IL-10. Moreover, Dex participates in the regulation of HMGB1, Toll-like receptor 4 (TLR4), NFκb (p-65) expression and the phosphorylation of IκB-α. In conclusion, Dex could relieve neuropathic pain through α2AR and HMGB1 and attenuate inflammation response.

Key words
Dexmedetomidine, neuropathic pain, High-mobility group protein B1, nuclear transcription factor-κ B
Introduction

Neuropathic pain (NP) which is characterized by spontaneous pain, allodynia, and hyperalgesia ranks one of the most intractable diseases\textsuperscript{[1,2]}. NP was caused by the dysfunction or primary injury of the somatosensory nervous system\textsuperscript{[3,4]}. Neuropathic pain impaired the quality of life and caused depression. Furthermore, the lack of satisfactory treatment make neuropathic pain one of the most serious health problems as the understanding of the molecular mechanisms underlying the neuropathic pain remain limited. Thus, it is of great importance to extend our understanding of the progression of neuropathic pain.

Dexmedetomidine (Dex) is an alpha-2 adrenergic receptor agonist to suppress nociceptive neurotransmission through spinal cord as well as the locus coeruleus. The α2 adrenergic receptor binding properties in inflammatory and neuropathic endow it with analgesic effect\textsuperscript{[5,6]}. As one of the primary narcotic and adjuvant drugs, Dex has also been found to possess various biological functions such as anxiolytic, anticonvulsion and anti-epileptic effects\textsuperscript{[7,8]}. Numerous studies have demonstrated that Dex exerts an analgesic roles through multiple mechanisms.

HMGB1 is an important chromatin protein interacts with nucleosomes, transcription factors, and histones to regulate the transcription of target genes. HMGB1 contributed to the development of pain hypersensitivity. TLR4 is expressed mostly on the surface of astrocytes resulting in NF-κB activation followed by inflammatory response\textsuperscript{[9]}. There have been lot of studies focus on the effect of Dex in neuropathic pain. Dex exerts an anti-nociceptive effect in monoarthritis and vincristine induced neuropathic pain via suppression of the TLR4/NF-κB p65 pathway\textsuperscript{[10-11]}, Dex was also capable of alleviating neuropathic pain in chronic constriction injury through inhibiting the activation of NR2B and inducible NOS (iNOS) as well as the expression level of Purinergic Receptor P2X 7 (P2X7R) and ERK in Rats\textsuperscript{[12,13]}. However, no reports have published to explore the underlying of HMGB1/TLR4/NFκB pathway through which Dex exerts its anti-nociceptive role.

In the present study, we evaluated the expression and effect of Dex in chronic constriction injury (CCI) induced neuropathic pain and the underlying mechanism involving in the regulation of HMGB1/TLR4/NFκB pathway.
Materials and methods

Animals

C57BL/6 mice (18–20 g) were obtained from Beijing Vital River Laboratory Animal Technology Co. and housed at 23°C with a 12 h light and 12 h dark cycle under 40-50% humidity. Animals had free access to food and water *ad libitum*. The animal study was approved by the Zhejiang University committee on Animal Research and was performed following the guidelines for the care and use of laboratory animals.

CCI model establishment

After intraperitoneal injection of sodium pentobarbital anesthesia (1%, 40mg/kg), an incision was made in the skin on the lateral surface of the thigh of mice. Light the left common sciatic nerve with a 5.0 silk suture tightly threads at 4 sites around the nerve with a 1.0-1.5 mm interval between them. Muscle and skin were enclosed. The same procedures were carried out in the mice of sham group with the sciatic nerve untied.

Drug administration

Dex and BRL44408 were dissolved in sterile saline. In the function investigation of Dex, 60 mice were randomly divided into 6 groups that are sham group, CCI group, CCI+vehicle, CCI+Dex (1μg/kg/day), CCI+Dex (5μg/kg/day), CCI+Dex (10μg/kg/day). In the investigation of the relation between Dex and α2AR receptor, 40 mice were divided into 4 gorup that are sham, CCI+vehicle, CCI+Dex and CCD+Dex+BRL44408 groups. The Dex was administrated intrathecally to the mice in CCI+Dex (1μg/kg/day), CCI+Dex (5μg/kg/day), CCI+Dex (10μg/kg/day) groups. The dosage of BRL44408 was 500μg/kg/day. Intrathecal injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the fifth or sixth lumbar vertebra. A 25-μL Hamilton syringe (Hamilton Co, NV, USA) attached to a 27-G stainless steel needle was used to perform the injection. Animals in sham group were treated with equal volume of normal saline. Dex and BRL44408 was administered once a day for a period of continuous 7 days following the CCI model establishment.
Construction of adenovirus

The adenoviruses used in the present study was prepared by Raybio company. The CDS sequence of HMGB1 was subcloned into the pAdTrack-ChAT-CMV/GFP vector. For the control adenovirus, a scramble sequence was subcloned into the pAdTrack-ChAT-CMV/GFP vector. The pAdEasy-1 vector (Santa Clara, CA, USA) was used to generate stable homologous recombinants with pAdTrack-ChAT-hGARSwt-CMV/GFP, pAdTrack-ChAT-hGARSL129P-CMV/GFP, and pAdTrack-ChAT-hGARSL129P-CMV/GFP. The adenoviruses were generated with an Adenovirus Expression Vector kit (Takara, Dalian, China) following the protocols. 1 × 10^8 pfu recombinant adenoviruses were injected using a microinjection syringe, which was connected to the intrathecal catheter each time at 4 days before as well as 0 day and 7 day after CCI operation.

Mechanical allodynia test

Mechanical allodynia was detected through measuring the paw withdrawal threshold (PWT) in response to a series of Von Frey hairs stimulation (Stoelting, Wood Dale, IL, USA) and using a Dixon’s up-down method. A series of von Frey filaments started with the filament 0.5g were applied to the dorsal surface of the hindpaw with a sufficient force. PWT was defined as the pressure (g) at which the mice withdrew its paw. The foot withdrawal threshold was recorded while the paw was withdrawn. Each trial was repeated six times at approximately 3 min intervals.

Thermal hyperalgesia test

Thermal hyperalgesia was detected through measuring paw withdrawal thermal latency (PWTL). Briefly, an infrared light beam made by a modified Hargreaves machine (UARDG of UCSD, USA) was irradiated on the plantar surface of the hind paw. The withdrawal latency (s) will be immediately recorded once the mice retract their paws.

Quantitative real-time PCR (qPCR)

The total RNA was isolated with a TRIzol kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. Thereafter, RNA was reversed transcribed into cDNA which was further used as the template in PCR reaction using SYBR Premix Ex Taq (Takara, Dalian, China) on a Light Cycler®480 System (Roche). mRNA levels were normalized to GAPDH. The relative
expression level was calculated using $2^{-\Delta\Delta Ct}$ method. The sequence of primers are as follows:

- **IL-10** forward: 5’GCTGGACAACATACTGCTAACC3’, reverse:
- 5’ATTTCCGATAAGGCTTGGCA3’
- **TNF-α** forward:
- 5’CACAGAAAGCATCCCGAGCAGT3’, reverse:
- 5’CGGCAGAGGAGGTTGACTTTCG3’
- **IL-6** forward:
- 5’CCAAGAGGTGAGTGCTTC3’, reverse: 5’CTGTTGGCTAGGATC3’
- **IL-1β** forward:
- 5’CCAGCTTCAAACTCTCACAGC3’
- reverse:
- 5’CTTCTTTGGGTATTGCTTGGGAT3’
- **GAPDH** forward:
- 5’AGGTCGGTGTGAACGGA3’, reverse: 5’TGTAGACCATGTAGGGTCA3’.

**Western blot**

The L4–L6 spinal cord segments of each mouse were removed and treated with lysis buffer (Sigma, CA, USA). 40 μg proteins extracted from spinal cord were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by transferring onto a polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blockage with QuickBlock<sup>™</sup> Blocking buffer for 2 h, the blots were probed with the primary antibodies against HMGB1, TLR4, P65, IκB-α (1:1000, Abcam, England) at 4°C overnight. Then, the HRP labeled secondary antibody (1:2000) was added into the incubation solution for another 2 h. An enhanced chemiluminescence kit (ECL kit, Solarbio, Beijing, China) visualized using Immunofluorescence

The spinal cord tissues were removed from the mice followed by fixing in 4% paraformaldehyde. After dehydration and embedding, the tissues were cut into 4μm sections. The sections were incubated with 0.25% Triton X-100 for 20 minutes. After blockage, the slice were treated with primary antibodies at 4°C overnight, then incubated with the secondary antibody for another 2 h. Followed by DAPI staining, the sections were examined under fluorescence microscope (CLSM 800, Zeiss, Germany).

**Enzyme linked immunosorbent assay (ELISA)**

All spinal cord samples were removed and cut into small pieces. The tissues were homogenized
with PBS in a LAWSON-24 tissue grinder (LARSON, China). After centrifugation at 5000 g and
4°C for 12 min, the supernatants were collected for the further use. ELISA assays to assess the
amount of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interleukin (IL)-10 and interleukin
(IL)-1β were performed according to the instructions (Jiancheng, Nanjing, China).

Statistical analysis
All experiments were performed independently triplicates. Data were presented as the mean ±
standard deviation (SD) and analyzed using SPSS 17.0. Differences between two groups were
determined by the Student’s t-test. The comparison of multiple groups was performed by Analysis
of Variance (ANOVA) followed by Turkey’s test. It was considered statistically significant when p
value < 0.05.

Results
Effects of Dex on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced
by CCI
As figure 1 showed, CCI decreased the threshold of paw withdrawal and the paw withdrawal
latency, meantime elevated the number of paw lifts after CCI surgery in comparison to sham
group. The low dose of Dex treatment was not revealed to have influence on these three indicators.
However, the medium and high dose of Dex administration significantly reversed the effect of
CCI on the threshold paw withdrawal, paw withdrawal latency and the number of paw lifts.

Dex inhibited the release and expression of IL-1β, IL-6, and TNF-α but increased that of IL-10
induced by CCI
We evaluate the amount and expression of the inflammatory factors including IL-1B, IL-6, IL-10
and TNF-α in the spinal cord tissue using ELISA and qPCR. The results revealed that CCI
significantly increased the release and expression of IL-1β, IL-6 and TNF-α, meanwhile, reduced
that of IL-10 in the spinal cord tissue. Furthermore, medium and high dose of Dex decreased the
elevated release and mRNA expression of IL-1β, IL-6 and TNF-α, meantime, increased the
inhibited release (Figure 2 A-D) and mRNA expression of IL-10 remarkably in comparison to the
The α2AR inhibitor BRL44408 reversed the effect of Dex on the CCI induced neuropathic pain. As Dex is a selective agonist of α2AR, in order to detect whether Dex exert its anti-nociceptive role through binding to the α2AR, we applied α2AR inhibitor BRL44408 administration. The results indicated that BRL44408 notably reversed the effect of high dose of Dex (10μg/kg) on the threshold of paw withdrawal, the paw withdrawal latency as well as the number of paw lifts which indicated that BRL44408 was capable of reversing the effect of Dex on the CCI induced neuropathic pain (Figure 3 A-C).

The α2AR inhibitor BRL44408 reversed the effect of Dex on the CCI induced inflammatory response. Next, we investigated whether α2AR inhibitor BRL44408 could reverse the effect of Dex on the inflammatory response to further explore the mechanism underlying the effect of Dex. Interestingly, the results indicated that BRL44408 increased the release and expression level of IL-1β, IL-6 as well as TNF-α compare to the CCI+Dex group. Moreover, BRL44408 reduced the release and mRNA expression of IL-10 which was increased by Dex treatment (Figure 4 A-H). These results confirmed that Dex regulated inflammatory response induced by CCI through regulating α2AR receptor.

Dex regulated the protein expression of HMGB1/TLR4/NFκB signal pathway. To further elucidate the underlying mechanism of Dex on the regulation of CCI induced neuropathic pain, we carried out western blot assay to evaluate the neuropathic pain related protein expression. We found that CCI significantly increased the expression of HMGB1, TLR4, p65 as well as the phosphorylation of IκB-α. As expected, Dex treatment reversed this effect of CCI on the expression of HMGB1, TLR4, p65 and the phosphorylation of IκB-α. Moreover, the α2AR inhibitor BRL44408 reversed the effect of Dex notably indicating that Dex exert its role through targeting to the α 2-adrenoceptors (Figure 5 A-B). Immunofluorescence was performed to further confirm the expression alteration of HMGB1 and TLR4. Double staining of HMGB1 and TLR4 results revealed the similar results as the western blot assay, Dex decreased the elevated...
expression of HMGB1, TLR4 induced by CCI in the spinal cord, however, the α2AR inhibitor BRL44408 significantly increased the level of both HMGB1, TLR4 compare to the CCI+Dex group (Figure 5 C).

Overexpression of HMGB1 reversed the effect of Dex on the CCI induced inflammatory response. We observed that Dex inhibited the expression level of HMGB1 and TLR4. However, we can not figure out whether HMGB1 regulate the expression of TLR4 or vice versa. Moreover, we want to investigate whether HMGB1 overexpression was capable of directly influencing the effect of Dex. As the results revealed, HMGB1 overexpression elevated the level of TLR4, p-IκBα and p65 which was reduced by Dex treatment (Figure 6A, B). As expected, like α2AR inhibitor BRL44408, HMGB1 overexpression increased the release of IL-1β, IL-6 as well as TNF-α, Meantime, reduced that of IL-10 compare to CCI+Dex group (Figure 6C). Finally, HMGB1 overexpression notably reversed the effect of high dose of Dex on the threshold of paw withdrawal, the paw withdrawal latency as well as the number of paw lifts which indicated that HMGB1 is directly involved in the effect of Dex on the CCI induced neuropathic pain (Figure 6D-F).

Discussion

Here, we explored the anti-nociceptive effect of Dex on the inflammatory response in CCI induced neuropathic pain. The results revealed that Dex administration remarkably inhibited the mechanical and thermal hyperalgesia. Further study indicated an inhibitory effect of Dex on the expression of HMGB1, TLR4 and NFκB which lead to the increased inflammatory response.

Previous studies have reported that Dex attenuated monoarthritis induced neuropathic pain in rat model via of TLR4/NFκB p65 pathway\[14\]. However, we discovered the effect of Dex in a CCI induced neuropathic pain model of mice. Moreover, we evaluate the expression level of HMGB1 and the phosphorylation IκB-α which has not been studied before. High-mobility group protein B1 (HMGB1) is a crucial chromatin protein interacts with nucleosomes, transcription factors, and histones to involve in regulating target gene transcription \[15\]. It was reported that HMGB1 was capable of inducing nerve injury which can be reversed by treatment of anti-HMGB1 antibody \[16\]. Once cells subjected to damage stimulation, HMGB1
located in nucleus would be transferred to the cytoplasm and even released out of the neurons to mediate the initiation of NP [17,18]. Moreover, HMGB1 contributed to the development of pain hypersensitivity [19].

HMGB1 receptors in neuropathic pain include TLR4 and advanced glycation end products [20]. It is also demonstrated that HMGB1 can interact with CXCL12 and CXCR4 to form a complex [21]. In addition, HMGB1 may interact with other molecules such as LPS, IL-1, bacterial DNA, and viral RNA to promote inflammatory responses [22,23].

Toll-like receptors 4 (TLR4) is expressed mostly on the surface of astrocytes resulting in NF-κB activation followed by inflammatory response. TLR4 was reported to participate in the activation of astrocytes and microglia in the progression of chronic pain [24]. Numerous reports have demonstrated the interaction of HMGB1 and TLR4. However, the function and mechanism of HMGB1/TLR4 signal axis in NP remain still limited.

In the present research work, Dex was found to be capable of inhibiting the expression of HMGB1, TLR4 as well as the phosphorylation of NFκB (p65). Moreover, we firstly indicated that HMGB1 influenced the expression of TLR4 and other downstream proteins such as IκB-α and p65. The previous studies merely realized that phosphorylation of NFκB (p65) altered the synthesis and release of inflammatory factors including IL-1β, IL-6, IL-10 and TNF-α [25-27]. We found that Dex is capable of inhibiting the phosphorylation of IκB-α and afterwards release the p65 subunit of NFκB. The released p65 transfer to nucleus and regulate the downstream protein expression transcriptionally such as IL-1β, IL-6, IL-10 and TNF-α. These findings extended the understanding of the effect of Dex on neuropathic pain. It may provide novel therapy methods for neuropathic pain. However, much more work are remaining to be performed to realize this dream.

However, how Dex activated α2 adrenergic receptors influence the expression of HMGB1 has not been elucidated here. Previous studies reported that HMGB1 is a targeted gene of let-7a-1/2-3p, while Dex inhibited the expression of let-7a-1/2-3p [28]. MiRs are a group of non-coding RNAs that negatively regulate the expression of their target genes. We speculate the Dex activated α2 adrenergic receptors may regulate the expression of HMGB1 through miRNAs.

In addition, DEX was indicated to down-regulat SNHG14 to inhibit the stability of HMGB1 mRNA [29]. These investigation will be carried out in our future research.
Astrocytes and microglia both play critical roles in the progression of neuropathic pain. Activation of microglia was shown to be mostly related to development, maintenance, and potentiation of neuropathic pain. In addition, fluorocitrate administration, which is known to inhibit the activation of astrocytes, can alleviate neuropathic pain. The expression of HMBG1 and TLR4 in the astrocytes or microglia may be related to the molecular mechanism of Dex. Thus, there is a limitation here, we did not perform the double-staining using biomarkers for astrocytes or microglia, such as GFAP and Iba-1 in the immunofluorescence experiment. As a result, we cannot confirm which kind of cell accurately express HMBG1 or TLR4. We will further perform these investigations in our further research work.

**Conclusion**

In general, the present study elucidate the effect of Dex in the CCI induced neuropathic pain through regulating HMGB1/TLR4/NF-κB signal pathway. These findings enriched the knowledge about the mechanism of Dex in the neuropathic pain.

**Conflict of Interest**

The authors declare no conflict of interest.
References


Synergistic Interaction Between Dexmedetomidine and Ulinastatin Against Vincristine-Induced Neuropathic Pain in Rats. J Pain. 18, 1354-1364.


21. Venereau, E., Schiraldi, M., Uguccioni, M., Bianchi, M. E. (2013) HMGB1 and leukocyte...
migration during trauma and sterile inflammation. Mol Immunol. 55, 76-82.


Figure 1 Effects of Dexmedetomidine (Dex) on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by CCI. The mice were randomly divided into 6 groups that are sham, CCI, CCI+vehicle, CCI+Dex (1 μg/kg/day), CCI+Dex (5 μg/kg/day), CCI+Dex (10 μg/kg/day). The Dex was administrated intrathecally to the mice in CCI+Dex (1 μg/kg/day), CCI+Dex (5 μg/kg/day), CCI+Dex (10 μg/kg/day) groups once a day for a period of continuous 7 days following the CCI model establishment. (A) Threshold of paw withdrawal was detected to evaluate the mechanical allodynia.(B) Number of paw lifts was detected to evaluate the thermal allodynia. (C) Paw withdrawal latency was detected to evaluate the thermal hyperalgesia. (n=6, *p<0.05 verses sham group, †p<0.05 verses CCI+vehicle group)
Figure 2 Dex inhibited the release and mRNA expression of IL-1β, IL-6 and TNF-α while promoted that of IL-10. (A-D) ELISA assay was conducted to evaluate the amount of inflammatory factors including IL-1β, IL-6, IL-10 and TNF-α in the spinal cord. (E-H) qPCR was carried out to evaluate the mRNA expression of IL-1β, IL-6, IL-10 and TNF-α. (n=6, *p<0.05 verses sham group).
Figure 3 The α2-adrenoceptors (α2AR) inhibitor BRL44408 reversed the effect of Dex on the CCI induced neuropathic pain. Dex (10μg/kg/day) and BRL44408 (500 μg/kg/day) was administered once a day through intrathecal injection for a period of continuous 7 days following the CCI model establishment. (A) Threshold of paw withdrawal was detected to evaluate the mechanical allodynia. (B) Number of paw lifts was detected to evaluate the thermal allodynia. (C) Paw withdrawal latency was detected to evaluate the thermal hyperalgesia. (n=6, *p<0.05 versus sham group).
Figure 4 BRL44408 reversed the effect of Dex on the CCI induced inflammatory response. (A-D) ELISA assay was conducted to evaluate the amount of inflammatory factors including IL-1β, IL-6, IL-10 and TNF-α in the spinal cord of each group. (E-H) qPCR was carried out to evaluate the mRNA expression of IL-1β, IL-6, IL-10 and TNF-α in the spinal cord of each group. (n=6, *p<0.05 verses sham group).
Figure 5 Dex regulated the protein expression of HMGB1/TLR4/NFkB signal pathway. (A, B) Western blot assay was conducted to measure the protein expression level of HMGB1, TLR4, NFkB (p-65) and the phosphorylation of IxB-a. (C) Immunofluorescence staining was carried out to evaluate the protein expression of HMGB1 and TLR4 in the spinal cord tissue. (n=6, *p<0.05 verses sham group. #p<0.05 verses CCI+vehicle group. &p<0.05 verses CCI+Dex group).
Figure 6 HMGB1 overexpression reversed the Dex function. (A, B) Western blot assay was conducted to measure the protein expression of HMGB1, TLR4, NFκB (p-65) and the phosphorylation of IκB-α. (C) ELISA assay was conducted to evaluate the amount of inflammatory factors including IL-1β, IL-6, IL-10 and TNF-α in the spinal cord of each group. (D) Threshold of paw withdrawal was detected to evaluate the mechanical allodynia. (E) Number of paw lifts was detected to evaluate the thermal allodynia. (F) Paw withdrawal latency was detected to evaluate the thermal hyperalgesia. (n=6, *p<0.05 verses sham group. #p<0.05 verses CCI group. &p<0.05 verses CCI+Dex group).