Enhanced NMDA receptor NR1 phosphorylation and neuronal activity in the arcuate nucleus of hypothalamus following peripheral inflammation

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Aim: To investigate the role of glutamate and N-methyl-D-aspartate (NMDA) receptors in central sensitization following peripheral inflammation in the arcuate nucleus (ARC) of the mediobasal hypothalamus.

Methods: Mediobasal hypothalamic slices were prepared from rats undergoing peripheral inflammation, which was induced by a unilateral injection of complete Freund’s adjuvant (CFA) into hind paw. Neuronal activation levels in the ARC were monitored by recording extracellular unit discharges. The NMDA receptor NR1 subunit (NR1) was measured using Western blot analysis.

Results: Enhanced NR1 phosphorylation was observed in the ARC of CFA-inflamed rats. Compared with the control rats, the firing rate of spontaneous discharges in ARC neurons of inflamed rats was significantly higher, and it was significantly reduced both by an NMDA receptor antagonist (MK-801, 300 μmol/L) and by a non-NMDA receptor antagonist (CNQX, 30 μmol/L). Application of exogenous glutamate (200 μmol/L) or NMDA (25 μmol/L) resulted in increased neuronal discharges for ARC neurons, which was enhanced to a greater extent in inflamed rats than in control rats.

Conclusion: Glutamate receptor activation in the hypothalamic ARC plays a crucial role in central sensitization associated with peripheral inflammation.

Keywords: arcuate nucleus (ARC); NMDA receptor; NR1 phosphorylation; inflammation; central sensitization; MK-801; CNQX

Original Article

Introduction

Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS). The receptors for glutamate comprise two large families: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs). The iGluRs are classified into the following receptor subtypes: N-methyl-D-aspartate (NMDA), kainate (KA) and alpha-amino 3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). KA and AMPA receptor subtypes are collectively classified as non-NMDA receptors. Molecular studies have indicated that functional NMDA receptors contain a heteromeric combination of NR1 subunits (essential for channel-formation) and one or more NR2A to D subunits[1–3].

NMDA receptors have been shown to play an important role in several physiological processes, such as long-term potentiation, learning and memory, as well as in some pathological conditions, including neurodegenerative diseases, ischemia and persistent nociception. The NMDA receptor has also been shown to be involved in the development of central sensitization, which is believed to underlie hyperalgesia during inflammatory pain and neuropathic pain[2, 3]. The majority of the data describing central sensitization were derived from studies in the dorsal horn of spinal cord, which is the first station to receive and integrate nociceptive information in the CNS[4–9]. However, studies investigating the molecular mechanisms of central sensitization in the supraspinal centers of nociceptive regulation remain elusive[10, 11].

Previous studies have demonstrated that the arcuate nucleus (ARC) of mediobasal hypothalamus, which exhibits large clusters of β-endorphinergic neurons, is one of the critical structures in the modulation of nociception. Electrical
or chemical stimulation of ARC can elicit antinociceptive effects [12–14], while electrolytic or chemical lesioning of ARC attenuates the morphine-induced analgesia, acupuncture-induced analgesia and stress-induced analgesia [12, 15, 16]. Furthermore, peripheral noxious stimulation modulates the spontaneous discharges of neurons in ARC, indicating that peripheral nociceptive information is sent to the hypothalamic nucleus and can result in neuronal activation in ARC [17]. In addition, glutamate and glutamate receptors, such as NMDA receptors, are highly expressed in the nuclei of the medial hypothalamic, including ARC [18–20].

Based on these observations, we hypothesized that hypothalamic ARC, a supraspinal center in pain modulation, might be a locus for central sensitization induced by injuries in peripheral tissues. We investigated the function of the NRI subunit of the NMDA receptor and neuronal activation in ARC in mediobasal hypothalamic slices from rats suffering from peripheral inflammatory injury. We found that peripheral inflammation enhanced NRI phosphorylation and increased neuronal activation in ARC. These findings provide new insights into how noxious signals are centrally processed during persistent nociception, which may be important in the development of novel analgesic strategies.

Materials and methods

Animals

Adult male Wistar rats (180 to 220 g) were housed in a light- (lights on 06:00–18:00 h) and temperature- (22±1 °C) controlled room and were fed rat chow and tap water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the Medical College, Soochow University and were in accordance with the ethical standards of the Helsinki Declaration and the guidelines of the International Association for the study of Pain for pain research in animals.

Drugs and reagents

In this study L-glutamic acid (glutamate), NMDA, MK-801 (a non-competitive NMDA receptor antagonist), CNQX (a non-NMDA receptor antagonist), rabbit anti-mouse NRI antibody and rabbit anti-serine-897-phosphorylated-NRI (pNR1) antibody were purchased from Sigma Chemical Co. The avidin-biotin complex and the biotinylated goat anti-rabbit IgG secondary antibody were purchased from Vector Laboratories Inc. All other chemical reagents were purchased from Sinopharm Chemical Reagent Co, Ltd.

Inflammatory pain model

Inflammatory pain was induced by injecting complete Freund’s adjuvant (CFA, 50% in saline, with 5 mg/mL heat-killed Mycobacterium tuberculosis, 0.1 mL) into the plantar surface of the left hind paw. Western blotting and electrophysiological experiments were conducted one week after CFA injection, when symptoms of inflammatory pain were evident, such as redness, swelling of the ankle joint, hyperalgesia and impairment in motor activity. Normal rats injected with an equal volume of saline were prepared as a control.

Western blot analysis

One week after CFA injection, rats (including control rats) were deeply anesthetized and decapitated to quickly isolate the ARC region. Three (anterior, middle and posterior) ARC slices (500 μm thick) were isolated from each animal and transferred into the ice-cold artificial cerebrospinal fluid (ACSF). The ARC slices were then homogenized in the presence of protease and phosphatase inhibitors. The homogenate was centrifuged twice at 13000×g for 10 min at 4 °C. The supernatant was used for Western blot analyses. The concentration of protein in the homogenate was measured using a bicinchoninic acid (BCA) kit and was used to calculate volume for equal protein loading in the gel. Proteins were separated and were transferred onto PVDF membranes (Invitrogen) by SDS-PAGE using Criterion XT Precast 6% Bis-Tris gels (Bio-Rad, Hercules, CA) in standard transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 10% v/v methanol, pH 8.3) for 1.5 h at room temperature. After being blocked with 5% milk in Tris-buffered saline and 0.1% Tween 20 (TBS-T) for 1 h, the membranes were incubated in specific antibodies against NRI (1:2000, rabbit affinity-purified polyclonal antibody, Sigma), pNR1-Ser897 (1:500, rabbit affinity-purified polyclonal antibody, Upstate) and β-actin (1:20000, mouse monoclonal, Sigma) overnight at 4 °C. After extensive washing in TBST, the membranes were incubated in goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (1:5000, Jackson Immunoresearch Co) for 1 h at room temperature. After extensive washing, signals were detected by Western Lightning ECL and were quantified relative to a β-actin control by densitometry on Image-Pro Plus 6.0.

Mediobasal hypothalamus slice preparation

Hypothalamic slices (400 μm) were prepared as described previously [25, 26]. In brief, hypothalamic slices containing ARC in a recording chamber were continuously perfused with ACSF (3 mL/min) saturated with 5% CO₂ and 95% O₂ at 33±1 °C. The ACSF media (pH 7.35–7.40) contained the following reagents: NaCl (124 mmol/L), NaHCO₃ (26 mmol/L), KCl (5 mmol/L), CaCl₂ (2.4 mmol/L), MgSO₄ (1.3 mmol/L), NaH₂PO₄ (1.24 mmol/L), and D-glucose (10 mmol/L). After 1 h of ACSF perfusion, extracellular single unit recordings were collected.

Electrophysiological recording

The spontaneous unit discharges from the ARC were recorded extracellularly with glass microelectrodes containing 0.5 mol/L sodium acetate and 2% pontamine sky blue (10–20 MΩ). Using a stereomicroscope and a microelectrode manipulator, the glass microelectrode was lowered into the ARC, which was located anatomically with respect to the third ventricle (3V) and medium eminence (Figure 1). The electrical signals were amplified (microelectrode amplifier, MEZ-8201, Nihon Kohden, Japan) and sent to a dual-beam oscilloscope (VC-10, Nihon Kohden, Japan). Neuronal unit discharges were continuously recorded online. The firing rate and interspike interval of neuronal discharges were analyzed with
Drug application
All the drugs used in our experiments were freshly prepared, dissolved in ACSF saturated with 5% CO\textsubscript{2} and 95% O\textsubscript{2}, and perfused hypothalamic slices via a three-way stopcock near the recording chamber. The baseline activity of neuronal discharges was recorded for 5 to 10 min as control. After establishing a good baseline, glutamate receptor agonists (glutamate 200 μmol/L, NMDA 25 μmol/L) or antagonists (MK-801 300 μmol/L, CNQX 30 μmol/L) were applied. The drug effects were observed continuously for 8 min after application.

Statistical analysis
The ARC neuron responses to glutamate or NMDA were determined according to the critical ratio criterion (CR), using the following formula: CR = (E – S) / (S + E)\textsuperscript{1/2} (where E is discharge frequency after drug application and S is discharge frequency before drug application). The response was considered excitatory, if the ratio exceeded 1.96\textsuperscript{[27]}. All data are presented as mean±SD. Statistical comparisons were performed using one-way ANOVA or student’s t-test. P<0.05 was considered statistically significant.

Results
Expression of NMDA receptor in ARC
NMDA receptors are composed of NR1 subunits, which are essential, and one of the NR2 subunits (NR2A, NR2B, NR2C, or NR2D). In the present study, we used Western blotting to measure the relative amounts of NR1 and pNR1 in ARC isolated from control and inflamed animals. Figure 2 illustrates that the relative amount of pNR1 in CFA-induced inflamed rats was significantly increased as compared with control rats (n=6, P<0.01, Figure 2A and 2B). Meanwhile the relative amounts of NR1 in inflamed and control rats were not statistically different (n=6, P>0.05, Figure 2A, 2C). These data suggest that peripheral inflammation increased NR1 phosphorylation in ARC, but it had no effect on NR1 subunit upregulation.

The discharge patterns of ARC neurons
We also measured the neuronal discharge activity in ARC. We found that the spontaneous discharges of ARC neurons in the slices could be divided into three firing patterns: regular, irregular and burst firing (Figure 3). Irregular discharges were observed in the majority of recorded neurons. As indicated in Table 1, the number of neurons exhibiting regular, irregular and burst firing was similar across control and inflamed rats (Table 1).

Discharge frequency in ARC neurons
The frequency of spontaneous discharge in ARC neurons was calculated for each discharge pattern, as well as for the mean of the three discharge patterns. The mean firing rate and the
firing rate of each discharge pattern in ARC neurons from inflamed rats were significantly elevated as compared with the normal group ($P<0.05$, Table 1). This significant difference was also observed for the inter-spike interval histograms. As shown in Figure 3, as the firing rate increased, the inter-spike intervals shifted to the left in the inflamed animals.

**Effect of glutamate on the electrophysiological activities of ARC neurons**

We investigated the response of ARC neurons to application of exogenous glutamate (200 μmol/L) to monitor their electric activities. In control animals, neuronal discharges increased significantly following glutamate application with a latency of $28.5\pm5.9$ s ($n=26$). The firing rate increased by $44.5\%\pm9.9\%$, and this increase persisted for up to $(152.5\pm15.3)$ s. In inflamed rats, the glutamate-induced excitatory response was more rapid, with a latency of $(19.8\pm2.8)$ s ($n=23$), and was more long lasting $(188.5\pm20.1)$ s. The firing rate increased by $84.0\%\pm32.7\%$. These results indicate that the glutamate-induced excitatory response in ARC neurons from inflamed rats exhibited a shorter latency ($P<0.05$), longer duration ($P<0.05$) and an enhanced firing rate ($P<0.01$) as compared with control rats (Figure 4).

**Effect of NMDA on the electric activities of ARC neurons**

Similar to application of exogenous glutamate, application of an NMDA receptor agonist, NMDA (25 μmol/L), also induced an excitatory response in ARC neurons in both the control and inflamed rats. In inflamed rats ($n=24$), the excitatory response exhibited a shorter latency, larger increment and longer duration, all of which were significantly different ($P<0.05$) from the control group.

**Figure 3.** The discharge patterns of arcuate neurons. Examples of electrical signals (left) and their inter-spike interval histograms (right) recorded from arcuate neurons in control and inflamed rats are shown in A and B, respectively. a, b, and c show examples of regular, irregular and burst firing, respectively.

**Figure 4.** Effects of exogenous glutamate and NMDA on neuronal discharges in the ARC. Relative increases in neuronal discharge frequency following glutamate (200 μmol/L, $n=26$ in control rats, $n=23$ in inflamed rats) or NMDA (25 μmol/L, $n=29$ in control rats, $n=24$ in inflamed rats) application are shown in A. The baseline discharge frequency before glutamate application in control and inflamed rats were $1.26\pm0.31$ Hz and $1.58\pm0.22$ Hz, respectively; before NMDA application they were $1.27\pm0.22$ Hz and $1.69\pm0.34$ Hz, respectively. The latency and duration of glutamate or NMDA-induced increases in neuronal discharges are shown in B and C, respectively. $abP<0.05$, $bcP<0.01$ as compared with control rats.
ARC neurons in control animals ($n=29$, Figure 4). These results indicate that both NMDA and glutamate could induce a stronger excitatory response in inflamed rats than in control rats.

Effects of MK-801 and CNQX on the spontaneous discharge of ARC neurons

It is well known that the increased excitability of nociceptive neurons in the spinal cord during persistent inflammation is mediated by glutamate receptors, including NMDA and non-NMDA receptor subtypes. To examine the role of glutamate receptors in the neuronal activation of ARC neurons during peripheral inflammation, we tested the effects of MK-801 (NMDA receptor antagonist) and CNQX (non-NMDA receptor antagonist) on the increased spontaneous discharges of ARC neurons from inflamed rats. Application of either MK-801 (300 µmol/L) or CNQX (30 µmol/L) resulted in a significant reduction in the discharge frequency of ARC neurons from inflamed rats. After MK-801 application, the discharge frequency was reduced from (2.32±0.29) Hz to (1.25±0.17) Hz ($n=11$, $P<0.01$, Figure 5A). After CNQX application, the discharge frequency decreased from (2.17±0.25) Hz to (1.32±0.15) Hz ($n=11$, $P<0.01$, Figure 5B). In control rats neither antagonist induced a significant reduction in the discharge frequency of ARC neurons (Figure 5A, 5B). These results suggest that the increase in spontaneous discharges of ARC neurons from inflamed rats was mediated by NMDA and non-NMDA receptors.

Discussion

The results of this study were threefold: 1) CFA-inflamed rats exhibited elevated levels of phosphorylated NR1 but not unphosphorylated NR1; 2) ARC neurons from CFA-inflamed rats exhibited a higher frequency of neuronal discharges, which was both NMDA and non-NMDA receptor dependent; and 3) ARC neurons from CFA-inflamed rats exhibited enhanced responses to exogenous glutamate and NMDA.

NMDA receptors in spinal dorsal horn have been shown to play a critical role in nociceptive transmission[2, 3]. Peripheral tissue injury dramatically enhances the function of spinal NMDA receptor that is involved in the initiation and maintenance of central sensitization, a persistent increase in the excitability of nociceptive neurons[2, 3, 28, 29]. The hyperfunction of the NMDA receptor could result from phosphorylation, upregulation, or a combination of both[30, 31]. Our western blot analysis indicated that the NMDA receptor NR1 subunit was phosphorylated but not upregulated in the ARC, and this effect was enhanced in inflamed rats. These results are consistent with previous studies. Yang et al found that CFA treatment in mice did not affect total NR1 levels, but it did result in a marked increase in NR1 phosphorylation[32]. Bird et al demonstrated that mono-arthritis induced by an injection of a kaolin suspension with carrageenan into the knee joint in rats resulted in increased NR1 phosphorylation rather than receptor upregulation in the amygdala[33]. Similarly, Maneepak reported that dural stimulation by topical application of an inflammatory soup enhanced phosphorylation but not expression of NR1 in the trigeminal nucleus caudalis of the spinal cord in rats[34].

Phosphorylation of NR1 has been shown to induce NMDA receptor trafficking from storage sites in the endoplasmic reticulum to the synaptic plasma membrane, leading to a hyperactivation state of NMDA receptor in nociceptive transmission[35, 36]. Furthermore, NR1 phosphorylation has been correlated with hyperalgesia and allodynia, which are characteristic behavioral manifestations of central sensitization[31, 37]. Previous investigations have demonstrated that the blockage of NR1 phosphorylation could reverse allodynia[37]. These data indicate that NR1 phosphorylation plays an important role in the initiation and maintenance of central sensitization. In the present study, NR1 phosphorylation was enhanced in ARC neurons from inflamed rats, thereby suggesting that central sensitization during peripheral inflammation might also occur in this supraspinal center.

Many studies have demonstrated that increased excitability of nociceptive neurons in the spinal dorsal horn is an important manifestation of central sensitization[39]. In addition, central sensitization has been shown to result from glutamate-induced activation of multiple signaling pathways in dorsal horn neurons, which involve both ionotropic (NMDA and non-NMDA) receptors and metabotropic receptors[38, 39]. In the present study we also observed increased excitability of ARC neurons from CFA-inflamed rats, as evidenced by the increased frequency of spontaneous discharges that was
NMDA and non-NMDA receptor dependent. The enhanced responsiveness of ARC neurons from inflamed rats by glutamate and NMDA application indicates that the excitability of ARC neurons increased following peripheral inflammation.

In the present study, hypothalamic ARC neurons from CFA-inflamed rats exhibited enhanced NR1 phosphorylation, increased excitability and increased responsiveness. These data suggest that the hypothalamic ARC may be important in the development of central sensitization associated with inflammatory injuries.

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Author contribution

Xing-hong JIANG, Xian-min YU, and Gen-cheng WU designed the experiments; Long-sheng XU, Jian-ming PENG, Qi ZHU, and Shan GONG performed all experiments; Shi-yu GUO and Jin TAO analyzed data; Xing-hong JIANG and Jin TAO wrote the manuscript.

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