The arcuate Src activation-induced phosphorylation of NR2B NMDA subunit contributes to inflammatory pain in rats

Longsheng Xu 1,3, Yanyan Pan 1, Qi Zhu 1, Shan Gong 1, Jin Tao 1, Guang-Yin Xu 1,2*, Xinghong Jiang 1*

1 Key Laboratory of Pain Basic Research & Clinical Therapy, Department of Neurobiology, Medical College of Soochow University, Suzhou 215123, China; 2 Institute of Neuroscience, Soochow University, Suzhou 215123, China; 3 The First Hospital of Jiaxing, Jiaxing 314000, Zhejiang, China

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*Correspondence to:
Xing-Hong Jiang, MD, Ph D
Department of Neurobiology
Medical College of Soochow University
199 Ren-Ai Road
Suzhou 215123, P.R. China
Tel/Fax: +86-512-65880397
E-mail: jiangxinghong@suda.edu.cn

or

Guang-Yin Xu, MD, PhD
Department of Neurobiology
Laboratory of Translation Pain Medicine
Institute of Neuroscience
Soochow University
199 Ren-Ai Road
Suzhou 215123, P.R. China
Tel/Fax: +86-512-65882807
E-mail: guangyinxu@suda.edu.cn

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Abstract

The tyrosine kinases of Src family play an important role in the central sensitization following peripheral inflammation. However, whether Src family in the arcuate nucleus (ARC) of mediobasal hypothalamus is involved in central sensitization remains unknown. The aim of this study was to investigate the role and mechanisms of tyrosine kinases of Src family in NMDA receptor activity in the ARC following peripheral inflammation. Peripheral inflammation was induced by unilateral injection of complete Freund's adjuvant (CFA) into rat hind paw. The neuronal activities of ARC were recorded using electrophysiological field recording from the *in vitro* mediobasal hypothalamic slices from control and CFA rats. Expression of total and phosphorylated Src and NR2B subunit protein was analyzed by western blot and immuoprecipitation. Our results showed that CFA injection resulted in an increase in mechanical and thermal sensitivity, which was partially blocked by neonatal monosodium glutamate treatment. CFA injection also enhanced spontaneous firings of ARC neurons, which were reversed by an NMDA receptor NR2B subunit specific antagonist Ro25-6981 and by PP2, an Src family tyrosine kinase inhibitor. In addition, peripheral inflammation enhanced Src phosphorylation and NMDA receptor NR2B subunit phosphorylation without alteration of total NR2B subunit expression in ARC. Peripheral inflammation also increased the association of NR2B protein with p-Src protein in ARC. Administration of PP2 blocked the upregulation of NR2B phosphorylation induced by CFA injection. Taken together, our present results suggest that the arcuate Src activation-induced tyrosine phosphorylation of NR2B NMDA subunit may contribute to inflammatory pain.
Background

Functional N-methyl-D-aspartate (NMDA) receptors include heteromeric combinations of NR1 subunits and one or more of NR2A through D subunits (Dingledine et al. 1999; Fundytus 2001; Petrenko et al. 2003). The NR1 subunit is essential for pore formation and for the binding of several regulatory ligands, whereas NR2 subunits (NR2A-D) bind glutamate and determine the kinetic characteristics of specific NMDA receptor multimers (Monyer et al. 1994). The NMDA receptor has been found to be involved in the development of the central sensitization during inflammatory pain and neuropathic pain (Fundytus 2001; Li and Neugebauer 2004; Petrenko et al. 2003). A number of signaling proteins including PSD-95/SAP90, P2X receptor and Src tyrosine kinase are involved in the mechanism (Gu and MacDermott 1997; Tao et al. 2001; Yu et al. 1997). Src tyrosine kinase, a non-receptor protein tyrosine kinase, is expressed widely throughout the central nervous system and plays an important role in up-regulating the activity of the NMDA receptors at glutamatergic synapses (Salter and Kalia 2004; Yu et al. 1997). Unlike the NR1 subunit, NR2A and NR2B are tyrosine phosphorylated by Src family kinases in the spinal cord (Lau and Huganir 1995). Protein phosphorylation is a major mechanism for the regulation of receptor function. Phosphorylation of multiple sites of NMDA receptors in the cytoplasmic C termini of the NR1 and NR2 subunits is known to modulate NMDA receptor activity and affect synaptic transmission (Lu et al. 2000; Tingley et al. 1997; Zou et al. 2000). Among the signal transduction pathways for NMDA receptor activation involving protein phosphorylation, tyrosine phosphorylation of the NR2 subunit plays a key role (Lau and Huganir 1995; Moon et al. 1994; Xiong et al. 1999). It has been shown that the major tyrosine phosphorylated protein in the postsynaptic density is the NR2B subunit of the NMDA receptor (Moon et al. 1994). Tyrosine phosphorylation of the NR2 subunits, particularly the NR2B subunit, has been associated with central sensitization (Rosenblum et al. 1996; Rostas et al. 1996) under neuropathological conditions (Dunah et al. 2000; Menegoz et al. 1995).

Previous studies have demonstrated that arcuate nucleus (ARC) of mediobasal hypothalamus contains clusters of β-endorphinergic neurons and is thought to be one of the critical structures in the modulation of nociception. Electrical or chemical stimulation of ARC can elicit antinociceptive effects (Wang et al. 1990a; Wang et al. 1990b; Zhang and Yin 1988), while electrolytic or chemical lesion of ARC attenuates the morphine-, acupuncture-,
stress-induced analgesia (Guo et al. 1983; Wang et al. 1990a; Zhu et al. 1987). These data indicate that ARC is an important area for suppression of nociceptive information. In contrast, peripheral noxious stimulation can change the spontaneous discharges of neurons in ARC, indicating that the peripheral nociceptive information can access this hypothalamic nucleus and change neuronal function (Yu and Yin 1984). Src kinase is also highly expressed in ARC and regulates NMDA receptor activities (Gotoh et al. 2008). However, whether NR2B are tyrosine phosphorylated by Src family kinases in the ARC under peripheral inflammation remains unknown.

In the present study, we showed that peripheral inflammation increased Src and NR2B phosphorylation in ARC in association with enhanced spontaneous firing in ARC neurons. Src family tyrosine kinase inhibitor PP2 reversed the increased NR2B tyrosine phosphorylation and attenuated the spontaneous firing frequency of ARC neurons. These findings suggest new insights into how noxious signals are centrally processed by Src and NR2B subunit of NMDA receptor in ARC during persistent pain.

Materials and methods

Animals

Adult male Sprague-Dawley rats (200±20 g, n=296) were housed in a temperature-controlled room (22 ± 1 °C) and fed with 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 IASP's guidelines for pain research in animals.

Drugs and Reagents

In this study, PP2 (a selective inhibitor for Src-family kinases), MK-801 (a non-competitive NMDA receptor antagonist), Ro25-6981 (NR2B subunit of NMDA receptor antagonist) and monosodium glutamate were purchased from Sigma Chemical Co. The rabbit anti-mouse Src (pTyr^{418}) antibody, rabbit anti-NR2B (pTyr1472) antibody, and rabbit anti-NR2B antibody were purchased from Merck & Co., Inc. The biotinylated goat anti-rabbit and goat anti-mouse secondary antibody IgG were purchased from Vector Laboratories Inc, and the other chemical reagents were all purchased from Sinopharm Chemical Reagent Co., Ltd.

Induction of Inflammatory Pain Model

For inducing inflammatory pain, complete Freund’s adjuvant (CFA, 50 % in saline, with 5 mg/ml heat-killed mycobacterium tuberculosis, 0.1 ml) was injected 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 such as redness, swelling of
the ankle joint, hyperalgesia and impairment in motor activity were evident. Normal rats
injected with the same volume of saline were used as control.

Implantation of Intracerebral Guide Cannula and Drug Administration

The rats were anesthetized with chloral hydrate (4%). Sterilized stainless-steel guide
cannulae (20 gauge) were positioned 2.8 mm dorsally to the ARC [AP: −4.0, L: 0.5, V: 9.8
mm; AP, anterior (+) or posterior (−) to Bregma; L, lateral to midline; V, ventral to the
surface of skull], according to Paxinos and Watson (Watson and Paxinos 1986) and fixed to
the skull by dental acrylic. After surgery, when the animals had regained their preoperative
weight, PP2/PP3 (5 nmol 0.5 µl) dissolved in DMSO were injected in a 1-µl volume via an
acute injection needle (26 gauge) inserted into the guide cannula. The injection needle, which
extended 2.8 mm (for ARC) beyond the tip of the guide cannula, was connected by a catheter
to a microsyringe. The injection needle was left in place for 30 sec after each injection before
removal.

Histological Verification of the Injection Sites

At the end of the experiments, blue ink was injected in a 1 µl volume via an acute
injection needle (26 gauge) inserted into the guide cannula. Rats were euthanized, and the
brain was removed and stored in 10% formalin for a week or more. On a cryostat, frozen
sections (150 µm) were cut and the point of injection verified. Because the animals were
always killed immediately after the experiment, the trace of the injection cannula was easy to
determine, even though the injection tip was determined according to the atlas of Paxinos and
Watson (Watson and Paxinos 1986). Results from animals with injections within the ARC
were included for statistical analysis.

Monosodium Glutamate (MSG) Treatment

Twenty-eight (n=28) male rat pups from our breeding colony were injected
intraperitoneally with MSG (2 mg/kg body weight, 28.8%, pH 7.4, Sigma-Aldrich, St. Louis,
MO) or equivalent volumes of normal saline (NS, 10%) on day 5 to 10 after birth as
previously described (GONG et al. 1994; Leitner and Bartness 2009). This dose and injection
schedule was chosen based on the successful production of the MSG-destruction of ARC
neurons (GONG et al. 1994). To minimize pup variation between groups, half of the rats
within a litter were injected with MSG and the other half normal saline. When they were
11-12 week old, half of the rats from each group were injected with CFA and the other half
normal saline as described above.
Behavioral Studies

All behavioral studies were carried out in a quiet room from 9:00 am to 11:00 am. Rats were allowed to habituate to the environment for 30 min before the experiments were begun. Mechanical withdrawal thresholds (MWT) were evaluated using the Analgesy-Meter (Ugo Basile, Italy) as described previously (Hayes et al. 1987). Briefly, rats were gently held and incremental pressure (maximum 25 g) was applied onto the dorsal surface of the left hind paw. The pressure required to elicit paw withdrawal, the MWT (in grams), was determined. The cut-off point was set at 25 g to prevent tissue damage. In that case, 25 g was determined as the MWT. The pressure-bearing spot of dorsal surface was marked to make sure the repeated-measured MWT was from the same point, and the averages were taken as MWT (g).

In neonatal monosodium glutamate treated rats, von Frey filaments (VFF) were used to measure the mechanical hyperalgesia, as described previously (Cao et al. 2009; Chaplan et al. 1994). A series of calibrated VFF (ranging from 0.4 to 25.0 g) were applied perpendicularly to the plantar surface of the hind paw with sufficient force to bend filaments for 60 s or until it withdrew. In the presence of a response, filament of next greater force was applied. In the absence of a response, filament of next lower force was applied. To avoid injury during tests, cutoff strength of von Frey filament was 25.0 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined by means of the "up-down" calculating method, as described previously (Cao et al. 2009; Chaplan et al. 1994). Each trial was repeated 2-3 times at approximately 2 min intervals, and the mean value was used as the force to produce withdrawal response.

As for thermal withdrawal latency (TWL), the tail flick unit (Ugo Basile, Italy) was used (Julien et al. 2005). Rats were gently held and the left hind paw pad was put over the flush mounted window containing the heat source set at 50 °C. The paw withdrawal latencies were defined as the time taken by the rat to remove its hind paw from the heat window. The cut-off point was set at 10 s to prevent tissue damage. All pain thresholds were measured three times at intervals of 1-2 min, and the averages were taken as TWLs.

Western Blot Analysis and Co-immunoprecipitation

One week after CFA injection, rats (including NS rats) were deeply anesthetized, decapitated and the ARC region was cut out quickly. Three ARC slices (500 μm thick) were obtained from each animal and moved into ice-cold artificial CSF. The ARC slices were then
homogenized with the inhibitors of phosphatases and proteases. The homogenate was centrifuged 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. Proteins were separated by SDS-PAGE using Criterion XT Precast 6 % Bis-Tris gels (Bio-Rad, Hercules, CA) and electrotransferred onto PVDF membranes (Invitrogen) in standard transfer buffer (25 mM Tris, 192 mM glycine, 10% vol/vol methanol, pH 8.3) for 1.5 h at room temperature. After the membranes were blocked with 5% non-fat milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T) for 1 h, bound proteins were exposed to specific antibodies against NR2B (1:500, rabbit affinity-purified polyclonal antibody, Merck), NR2B-pTyr1472 (1:1000, rabbit affinity-purified polyclonal antibody, Merck), Src-pTyr418 (1:1000, mouse affinity-purified monoclonal antibody, Merck) and β-actin (1:20000, rabbit monoclonal, Sigma) overnight at 4°C. After extensive washing in TBST, a 1:5000 dilution of goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Jackson Immunoresearch Co) was used as appropriate and incubated for 1 h at room temperature. After extensive washing, signals were detected with Western Lightning ECL and quantified relative to β-actin control by densitometry on Image-Pro Plus 6.0.

For co-immunoprecipitate, after protein titration using a BCA protein assay the equivalent of 500 μg of total protein was added to 5 μg of rabbit anti-NR2B antibody (1:500, rabbit affinity-purified polyclonal antibody, Merck) and gently shaken overnight at 4°C. Protein-A sepharose beads were added to the samples and gently shaken for 4 h at 4°C. Beads were then rinsed and removed in lysis buffer and boiled in loading buffer made of 2% sodium dodecyl sulfate (SDS), 100 mM DTT, 10% glycerol, 0.25% Bromophenol Blue for 5min. A set of samples was boiled for 5 min before immunoprecipitation to separate the pSrc and to verify the NR2B of antibodies. The protein-rich supernatants were used for western blot analysis for p-Src.

**Mediobasal Hypothalamus Slice Preparation and Electrophysiological Recordings**

Hypothalamic slices (400 μm) were prepared as described previously (Zhang and Yin 1988). In brief, hypothalamic slices containing ARC in a recording chamber were continuously superfused with artificial cerebrospinal fluid (ACSF, 3 ml/min) saturated with 5 % CO₂ and 95 % O₂ at 33 ± 1 °C. The ACSF contains (in mM): NaCl 124, NaHCO₃ 26, KCl 5, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.24, and D-glucose 10, pH 7.35 ~ 7.40. After 1 h ACSF superfusion, extracellular single unit recordings were performed.

The spontaneous unit discharges of ARC were recorded extracellularly with glass microelectrodes filled with 0.5 M sodium acetate and 2 % pontamine sky blue (10 ~ 20 MΩ). With assistance of a stereomicroscope and a microelectrode manipulator the glass
microelectrode was lowered into ARC, which was located according to the anatomical
relation of the third ventricle (3V) and medium eminence. The electrical signals were
amplified (microelectrode amplyfier, MEZ-8201, Nihon Kohden, Japan), and fed to a
dual-beam oscilloscope (VC-10, Nihon Kohden, Japan). Neuronal unit discharges were
continuously recorded on-line. The firing rate and interspike interval of neuronal discharges
were analyzed with Powerlab/4SP (AD Instruments, Australia).

**Drug Application**

All drugs used in our experiments were freshly prepared in ACSF saturated with 5 % CO₂
and 95 % O₂, and perfused to hypothalamic slices via three-way stopcock near the recording
chamber. The baseline activity of neuronal discharges was recorded for 5-10 min as control.
Then, the glutamate receptor antagonists (MK-801 300 μM and Ro25-6981 20 μM) or Src
inhibitor PP2 (300 μM) were applied. The drug effects were observed continuously for 8 min
after application. For behavioral study, PP2 or PP3 was administered by microinjection via
the implanted cannula as described above.

**Statistical Analysis**

The responses of ARC neurons to PP2 or MK-801 and Ro25-6981 were determined
according to the critical ratio criterion (CR), using the formula: \( CR = (E-S)/(S+E)^{1/2} \) (where E
is the discharge frequency after drug application and S is discharge frequency before drug
application). The response was considered as inhibition if the CR was lower than -1.96. All
data were presented as mean ± SEM. Statistical comparisons were performed using two-way
repeated measures ANOVA or student’s \( t \)-test. P<0.05 was considered statistically significant.

**Results**

**Peripheral inflammation enhanced spontaneous discharge frequency of ARC neurons**

Rats were randomly assigned to 2 groups: saline injection as NS group (n=7) and
complete Freund’s adjuvant injection as CFA group (n=7). Sub-plantar injection of Freund’s
adjuvant produced a pronounced local edema, redness, swelling of the ankle joint after a few
hours. In agreement with previous reports (Xu et al. 2008), both thermal withdrawal latency
(TWL, Fig. 1A) and mechanical withdrawal threshold (MWT, Fig. 1B) were significantly
reduced 1 day after CFA injection and persisted for 9 days, within our time period of
observation. To determine the responsiveness of ARC neurons following CFA injection, we
first examined the frequency of spontaneous discharge of ARC neurons from inflammatory
rats. The spontaneous firing frequencies varied between neurons and ranged from 0.72 Hz to
3.5 Hz (average: 1.87±0.10 Hz, n=60) in inflamed rats and from 0.65 Hz to 3.8 Hz (average:
1.54±0.08 Hz, n=60) in NS-treated rats. Our results showed that the mean firing rate was significantly increased as compared with that in NS group (**P < 0.01, Fig. 1C & D).

Furthermore, these spontaneous firing neurons were divided into three groups, i.e. regular (NS: n=11, 18.3%; CFA: n=8, 13.3%), irregular (NS: n=35, 58.3%; CFA: n=37, 61.7%) and burst (NS: n=14, 23.3%; CFA: n=15, 25.0%) firing (Fig. 1C &D). In CFA-treated rats, spontaneous irregular and burst firing frequency were significantly higher in CFA-treated rats than in NS-treated rats while the regular firing frequency was not altered.

**Monosodium glutamate (MSG) treatment attenuated the inflammatory hyperalgesia**

To further determine whether ARC nucleus is involved in inflammatory hyperalgesia, MSG was administered into neonatal rats (post natal day 5–10). In line with previous studies, neonatal MSG treatment produced a selective ARC lesion (GONG et al. 1994; Sanchis-Segura and Aragon 2002). As shown in figure 2, neonatal MSG injection did not produce any changes in baselines of thermal threshold in rats between NS-NS and MGS-NS group (Fig. 2A, n=7) and mechanical threshold (Fig. 2B, n=7) in rats between NS-NS and MSG-NS group. However, neonatal MSG treatment partially blocked the CFA-induced thermal (Fig. 2A, n=7) and mechanical hyperalgesia (Fig. 2B, n=7) in adult rats compared with neonatal normal saline (NS) treatment, indicating a pro-nociceptive role of ARC under inflammatory conditions.

**Blockade of NMDA NR2B decreased the spontaneous discharge of ARC neurons in inflamed rats**

To examine the role of NMDA receptors and their NR2B subunits in the neuronal activities of ARC during peripheral inflammation, we tested the effects of MK-801 (NMDA receptors antagonist) and Ro25-6981 (NMDA receptor NR2B subunit antagonist) on the increased spontaneous discharges of ARC neurons of inflamed rats. After MK-801 (300 μM) application, the discharge frequency reduced from 2.32 ±0.29 Hz to 1.23±0.17 Hz (n = 11, P < 0.01, Fig. 3A & B) in slices from inflammatory rats. In contrast, MK-801 (300 μM) did not induce significant reduction in discharge frequency of ARC neurons from NS rats (Fig. 3B). These results suggested that the increase in spontaneous discharges of ARC neurons in inflamed rats was mediated by NMDA receptors. Furthermore, application of Ro25-6981 (20 μM) produced a significant reduction in discharge frequency of ARC neurons in inflammatory rats. After Ro25-6981 application, the discharge frequency reduced from 2.10±0.19 Hz to 1.42±0.10 Hz (n=10, P<0.01, Fig. 3C & D) in inflammatory rats. In NS group, however, Ro25-6981 did not induce significant reduction in discharge frequency of ARC neurons (Fig.
These results suggested that the increase in spontaneous discharges of ARC neurons in inflamed rats was mediated by NMDA receptor NR2B subunits.

**Inflammation increased phosphorylation of the NR2B subunit in ARC**

NMDA receptors are composed of NR1 subunits and one of NR2A, NR2B, NR2C or NR2D. Among them the NR2B is an auxiliary functional subunit. In the present study, we measured expression of NR2B and tyr1472 phosphorylated NR2B (p-NR2B) protein in ARC from NS and inflammatory animals by western blotting analysis. The relative densitometry of pNR2B in CFA-treated rats was significantly increased as compared with those of NS rats (n = 6, P < 0.001, Fig. 4A & B). Meanwhile the relative densitometry of NR2B in inflamed rats had no statistical difference as compared with those of NS rats (n = 6, P>0.05, Fig. 4C & D). These data suggested that NMDA receptor NR2B phosphorylation was enhanced in ARC after peripheral inflammation.

**NR2B interacted with p-Src in rat ARC following peripheral inflammation**

Previous reports had shown that Src phosphorylation promoted the association of the proteins to NR2B in the spinal dorsal horn (Yu et al. 1997). To assess the molecular basis of NR2B-pSrc interaction underlying inflammation-induced NR2B tyr-P, we further determined whether the NR2B subunit of the NMDAR is linked to Src tyrosine kinase in ARC. Total proteins were extracted from ARC tissues. When anti-NR2B antibodies were used to immunoprecipitate NR2B proteins, p-Src was detected in immunoprecipitates in ARC from CFA-treated rats (Fig. 5A left). In contrast, we did not detect or barely see any proteins in immunoprecipitates in ARC from normal rats (NS, Fig. 5 left) in this study. To further confirm the association of these two proteins, tissue extracts from normal and CFA-treated animals were boiled before immunoprecipitation. This interrupted the coimmunoprecipitation of associated proteins, leaving blank in the immunoprecipitates in ARC from CFA-treated animals (Fig. 5A right). These results indicate that CFA-induced inflammation increased NR2B interaction with p-Src in rat ARC.

**Inflammation increased tyr1472 phosphorylation of NR2B subunit via Src kinase**

To determine the involvement of Src protein tyrosine kinase in NR2B tyrosine phosphorylation, PP2, a selective Src protein tyrosine kinase inhibitor, was microinjected into ARC 10 min before injection of CFA. ARC tissues were collected 30 min after CFA injection. PP2 (5 nmol in 0.5 µl, n=6) abolished inflammation-induced NR2B tyrosine phosphorylation (p<0.05 vs PP3, Fig. 5B & C). PP3 (5 nmol in 0.5 µl, n=6, Fig. 5B & C) was used as a negative control for PP2. PP3 administration did not alter the expression of NR2B. These data indicate that an increase in tyr1472 phosphorylation of the NR2B subunit is mediated by...
Src-family kinases in ARC following peripheral inflammation.

**Inflammation induced an increase in tyr418 phosphorylation of Src in ARC**

We next measured the relative densitometry of immunoblots of tyr418 phosphorylated Src (p-Src) protein in ARC from rats 1 week following NS or CFA injection. Using western blot, we showed that the state of phosphorylation of non-receptor Src-family tyrosine kinases in ARC was increased after CFA-induced inflammation (n = 6, \( P < 0.001 \), Fig 6A & B). These data suggested that peripheral inflammation led to a significant increase in tyr418 phosphorylation of Src tyrosine kinase in ARC.

**Blockade of Src kinase decreased the spontaneous discharge of ARC neurons in inflamed rats**

To further examine the role of Src in the neuronal activities of ARC during peripheral inflammation, we tested the effects of PP2 on the spontaneous discharges of ARC neurons from normal and inflamed rats. Application of PP2 (300 \( \mu \)M) produced a significant reduction in discharge frequency of ARC neurons in inflammatory rats. The discharge frequency was reduced from 2.19 Hz to 1.55 Hz after application of PP2 (n =11, \( P < 0.01 \), Fig. 6C & D). In NS group, PP2 did not induce significant reduction in discharge frequency of ARC neurons (Fig. 6D). These results suggested that the involvement of the Src tyrosine kinase in the increase of spontaneous discharges of ARC neurons in inflammatory rats.

**Src kinase inhibitor attenuated inflammatory hyperalgesia**

We next determined whether Src kinase inhibitor reduced the inflammatory pain. When PP2 (5 nmol in 0.5 \( \mu \)l; n=7) was injected into ARC area 24 hr after CFA injection, there was an increase in mechanical threshold from 6.63 g to 7.29 g and an increase in thermal withdrawal latency from 2.91 s to 3.94 s at 5 min after PP2 (\( *P<0.05 \) vs pre-drug; \( ^*P<0.05 \) vs PP3, Fig. 7A & B). The effect of PP2 post-treatment on mechanical threshold and thermal withdrawal lasted for more than 15 min. However, microinjection of PP3 (5 nmol 0.5 \( \mu \)l; n=7) into the ARC did not inhibit hyperalgesia after hindpaw inflammation (\( P > 0.05 \) vs pre-drug). These results indicate that the Src family tyrosine kinase in ARC is involved in the development of hyperalgesia after hindpaw inflammation.
**Discussion**

We have previously demonstrated that peripheral inflammation enhanced the neuronal activities of ARC neurons *in vivo* following peripheral inflammation induced by CFA injection into rat hind paw (Peng et al. 2011). The enhanced ARC neuronal activity was blocked *in vivo* by NMDA receptor antagonist microinjected into ARC. In the present study, we provide new evidence to demonstrate that NMDA receptors in ARC play an important role in the development of hyperalgesia and that NMDA activities are regulated by Src family following peripheral inflammation. Firstly, CFA-induced inflammation significantly enhanced the spontaneous discharges of ARC neurons *in vitro*. Secondly, Peripheral inflammation increased the phosphorylation of NMDA receptor NR2B subunit and the interaction of pNR2B with pSrc tyrosine kinase in ARC. Thirdly, both MK-801 and So25-6981 inhibited the spontaneous firings of ARC neurons from CFA-injected rats, but not in control healthy rats. Fourthly, PP2 microinjection into the ARC in inflamed rats reversed the upregulation of tyrosine-phosphorylated NR2B protein. Finally, Src antagonist greatly lowered frequency of neuronal discharges of ARC neurons and increased pain threshold in CFA-injected rats.

A growing body of evidence has demonstrated that spinal NMDA receptors are involved in the initiation and maintenance of central sensitization, a persistent increase in the excitability of nociceptive neurons (Fundytus 2001; Petrenko et al. 2003). NMDA receptor antagonists, such as ketamine, dextromethorphan and MK-801, block wind-up in the spinal cord and produce analgesic effects in animal models of chronic pain (Chaplan et al. 1997; Mao et al. 1993; Ren and Dubner 1993), but their clinical use is limited by numerous intolerable side effects due to the essential functional contribution of NMDA receptors throughout the central nervous system (Backonja et al. 1994; Jorum et al. 2003; McQuay et al. 1994). However, subtype-specific drugs, such as those, which selectively target NR2B subunit-containing receptors, may show great promise as analgesics for neuropathic pain states since they have significantly less side effects (Boyce et al. 1999; Qu et al. 2009). We showed here that Ro25-6981 significantly inhibited the spontaneous discharge frequency of ARC neurons in CFA-injected rats, indicating the involvement of NR2B subunit receptors in inflammatory pain. To exclude the non-specific effects, we administrated these two antagonists in normal animals. Neither MK-801 nor Ro25-6981 produced any effect on the spontaneous firings of ARC neurons in normal animals, suggesting that these two antagonists play a specific effect under inflammatory conditions. Ro25-6981 was used in this experiment since this antagonist reduced C-fiber-evoked neuronal responses, blocked spinal LTP in SNL rats (Qu et al. 2009) and inhibited pain-related sensitization of amygdala neurons (Ji et al. 2009). The reason why Ro25-6981 did not completely abolish the spontaneous firings of ARC neurons is likely because of the low dose of Ro25-6981 or the intrinsic...
properties of spontaneous firings. The residential spontaneous firing may be mediated by other signaling molecules, such as NR1 (Peng et al. 2011) or non-NMDA receptors and galanin-like peptide receptors (Saito et al. 2003). In our previous study, we showed that NMDA NR1 subunits were phosphorylated following peripheral inflammation (Peng et al. 2011), suggesting a role for NR1 subunit in ARC under peripheral inflammation. Since the spontaneous firing frequency after R025-6981 application is markedly reduced, it is therefore tempting to hypothesize that Ro25-6981 may be a potential therapeutic drug for treatment for inflammatory pain. Further experiments are warranted to identify the origin of spontaneous firings in these rats and to obtain the best doses.

The most interesting finding in the present study is that peripheral inflammation enhanced the interaction of pNR2B and pSrc in ARC since more proteins were immunoprecipitated in ARC from CFA-treated rats than those from NS-treated rats. Possible mechanisms for potentiation of the interaction include an upregulation of pScr expression and/or an increase in physical association induced by peripheral inflammation. Although an increase in physical association in ARC of inflamed rats has yet to be elucidated, an increase in pSrc expression is most likely. Studies in recent years have focused on trafficking of NMDA receptors from storage site, endoplasmic reticulum, to synaptic plasma membrane and a hyperfunction of NR2B subunit of the receptor in nociceptive transmission (Gogas 2006). The hyperfunction of NMDA receptor could be due to receptor phosphorylation (Wu et al. 2005; Zou et al. 2000) by neighboring activated receptors including TrkB, mGluRs and the protein kinases (Guo et al. 2004; Mantyh and Hunt 2004; Woolf and Salter 2000) both in the hippocampus and in the spinal cord (Guo et al. 2002; Rosenblum et al. 1996). However, the different roles of the multiple NMDA receptor subunits are still controversial, and it is also not clear whether the same mechanism occurs in different nucleus in the central nervous system. There are several lines of evidence to show that inflammation-induced increase in NR2B tyrosine phosphorylation was abolished by genistein, a tyrosine kinase inhibitor, and by PP2, a Src family protein tyrosine kinases inhibitor in the spinal cord (Ali and Salter 2001; Chen and Leonard 1996). Post-treatment of PP2 also blocked NR2B tyrosine phosphorylation in the spinal dorsal horn and inflammatory hyperalgesia, suggesting that Src phosphorylation plays a role in maintaining central hyperexcitability (Guo et al. 2002). Our present study is in agreement with previous studies and provides an extension that Src phosphorylation enhanced NR2B activities in ARC following peripheral inflammation.

Another important finding is that arcuate Src plays a role in NR2B subunit receptor activation in ARC thus contributing to inflammatory hyperalgesia. pSrc and NMDA receptors NR2B co-immunoprecipitated in ARC preparation, indicating that they are associated in a complex. After PP2 microinjection into the ARC in inflamed rats, the protein level of tyrosine-phosphorylated NR2B was greatly decreased, spontaneous firing rate in
ARC neurons was markedly inhibited, and the pain threshold was significantly increased. In contrast, PP3, a negative control for PP2, did not produce any effect in this model. Together with previous report that intrathecal administration of PP2 before CFA injection delayed the onset of mechanical hyperalgesia and allodynia (Lu et al. 2000), we therefore conclude that PP2 may play a specific effect on inflammatory pain. Although we cannot exclude the possibility that Src activation may regulate activities of other receptors or that NR2B may be regulated by other kinases, the present findings support our hypothesis that NR2B tyrosine phosphorylation by Src is involved in the development of ARC neuronal plasticity and hyperalgesia following peripheral inflammation. There are several lines of evidence showing that pituitary and hypothalamic glycoprotein hormone (van Zeijl et al. 2011) and interleukin-1beta (DeBoer et al. 2009) might be up-stream regulators for phosphorylation of Src in the ARC. However, the detailed mechanisms underlying phosphorylation of Src in the ARC needs to be further investigated.

The ARC is thought to be the site of origin of neurons that produce endorphins, that project to the spinal cord and other areas to modulate pain pathways. However, our molecular and electrophysiological data here are correlated with the behavioral readout, which suggest that this activity in the ARC is actually contributing to the pain state. Previous reports suggest that ARC, presumably endorphin releasing cells, are activated in inflammatory pain and that reducing this activity can alleviate the sensation of pain i.e. behaviorally. Although the detailed mechanism has yet to be investigated, it is tempting to speculate that activation of these neurons is leading to the release of more endorphins and this is pro-nociceptive and capable of completely by-passing the spinothalmic-cortical pathways. Studies showing a reduction of spontaneous and evoked firing frequencies after icv MK801 administration in CFA treated animals are in line with this hypothesis (Peng et al. 2011). Unfortunately, we don’t know if these cells really are the endorphin producing cells. To better correlate the behavior with the molecular and electrophysiology, further studies are warranted to measure endorphin release to determine whether persistent peripheral inflammation maybe recruit, via hypothalamic circuits, endorphin cells or other systems (Balthasar et al. 2004; Plum et al. 2006), for example orexin (Ma et al. 2007), from lateral hypothalamus projecting to spinal cord.

It is noteworthy that in ARC the spontaneous firing rate was increased after CFA treatment. Under control conditions, Arc neurons are firing at ~1.5 Hz from in vitro brain slices, which is similar to those reported previously for identified POMC cells (Ma et al. 2007). There is no effect
in the behavior when they are lesioned, indicating that they do not normally contribute to endogenous tone. However, under inflammatory conditions, these neurons increase firing by ~22% at ~2 Hz. It is noteworthy that the small increase in spontaneous firing makes significant contribution to the behavior since lesion of ARC mitigates the enhanced pain behavior. The detailed mechanism for this activity translating remains unknown. It is likely that increase in numbers of action potential enhances neurotransmitter release thus causing pain sensitization. Further experiments are needed to determine the powerful and remarkable activity translating under inflammatory conditions.

In summary, the present study showed for the first time that Src tyrosine kinase activation in the arcuate nucleus contributes to inflammatory pain, which is at least in a large part mediated by NMDA receptor NR2B. In agreement with previous studies in the spinal cord and the amygdala (CeA) (Ji et al. 2009), our present studies provide further evidence to support the hypothesis that Src family plays an important role in pain-related neuroplasticity in ARC under peripheral inflammation.

**Competing interests**

The authors declare that they have no competing interests.

**Author contribution**

Xing-Hong JIANG designed research; Long-Sheng XU, Yanyan PAN, Qi ZHU and Shan GONG performed experiments; Long-Sheng XU and Jin TAO analyzed data; Xing-Hong JIANG, Guang-Yin XU, and Jin TAO wrote the manuscript.

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**Figure legends**

**Fig. 1.** CFA produced inflammatory hyperalgesia in association with increased spontaneous firings in ARC neurons. The thermal withdrawal latency (TWL, A) and mechanical withdrawal threshold (MWT, B) were significantly decreased in CFA-treated rats compared to NS-treated rats. Data are expressed as mean ± SEM (n=7 for each group), *p < 0.05, **p < 0.01 versus NS-treated rats. CFA-induced inflammation increased neuronal discharges in ARC (C and D). C, Three types of spontaneous firings were observed in NS and CFA rats. D, The mean discharge frequency in NS and inflamed rats were 1.54±0.08Hz (n=60) and 1.87±0.10 Hz (n=60), respectively. *p <0.05, **p < 0.01 versus NS-treated rats.

**Fig. 2.** Neonatal monosodium glutamate (MSG) injection attenuated the CFA-induced hyperalgesia. MSG or saline was injected intraperitoneally at postnatal day 5 and consecutively for another 5 days. At the age of 11-12 weeks, these rats were injected with CFA to induce inflammation or NS as controls. Neonatal MSG injection attenuated the CFA-induced thermal (A) and mechanical hyperalgesia (B). *p<0.05, **p<0.01, compared with NS-CFA.

**Fig. 3.** MK-801 or Ro25-6981 inhibited the spontaneous discharges of ARC neurons only in inflammatory rats. A and B, Significant decrease in neuronal discharge frequency following MK-801 application (300 µM) in inflamed rats (n = 11; P < 0.01, paired t test), but not in NS rats (n = 12). C and D, Significant decrease in neuronal discharge frequency following Ro25-6981 application (20 µM) in inflamed rats (n = 10; P < 0.01, paired t test), but not in NS rats (n = 10). ** p<0.01 versus predrug.

**Fig. 4.** Enhanced phospho-NR2B, but not total NR2B, expression in the ARC from CFA-treated rats. A, Immunoblots of phosphorylated NR2B (pNR2B) in ARC from rats one week following NS and CFA injection. β-actin was used as loading control. B, Averaged data of immunoblot densitometry showed that the relative level of pNR2B protein was significantly increased in ARC from CFA-treated rats (n= 6) compared to NS rats (n= 6; *p<0.001, unpaired t test). C, Immunoblots of NR2B in ARC of NS and CFA-inflamed rats. D, There was no significant difference of NR2B protein expression between NS (n= 6) and CFA-treated rats (n= 6).

**Fig. 5.** Effect of PP2 on tyrosine phosphorylation of NR2B. A, Proteins were immunoprecipitated (IP) with NR2B antibodies and probed with antibodies against pSrc. There was obviously a pSrc band pulled down by NR2B antibodies in CFA-treated rats but not in NS-treated rats (top left). Note that boiling of the samples before IP eliminated pSrc precipitation, leaving only blank (top right). B, PP2 (5 nmol in 0.5µl) was microinjected into
ARC 10 min before CFA injection. The ARC region of inflamed rats 30 min after injection of CFA into the hindpaw were dissected and the proteins were extracted. Representative immunoblots against anti-PY1472-NR2B (*top*) and β-actin (*bottom*) antibodies. PP3 (5 nmol in 0.5µl) was used as a negative control for PP2. PP2 application significantly suppressed the pNR2B expression. C, Bar graph showed the mean relative levels of tyrosine-phosphorylated NR2B proteins from four individual experiments. *p < 0.05 versus PP3 rats.

**Fig. 6. Involvement of Src protein tyrosine kinase.** A, Immunoblots of phosphorylated Src (pSrc) in ARC from rats one week following NS and CFA treatment. B, Bar graph showed that the relative level of pSrc (or Tyr418) protein was markedly increased in ARC of CFA-treated rats (n= 6) compared to NS rats (n= 6; P < 0.001, unpaired t test). C and D, PP2 application (300 µM) significantly decreased neuronal discharge frequency in inflamed rats (n = 10; P < 0.05, paired t test), but not in NS rats (n = 12).

**Fig. 7. Inhibition of PP2 post-treatment on inflammatory pain.** Twenty-four hours (24 h) after CFA injection, PP2 (5 nmol in 0.5 µl; n=7) or PP3 (5 nmol in 0.5 µl; n =7) were microinjected into ARC. A, The latency of thermal withdrawal response started to increase 5 min after PP2 injection and lasted for about 15 mins. PP3, a negative control for PP2, did not produce any effect. B, The threshold of mechanical started to increase 5 min after PP2 injection and lasted for about 15 mins. (*P<0.05, **P<0.01 vs. pre-drug  #P<0.05, ##P<0.01 vs. PP3; Two-Way ANOVA).
Fig 1

A

Time post-CFA (day)

pre- 1 2 3 4 5 6 7 8 9

TWL (s)

0 1 2 3 4 5 6 7

CFA

NS

CFA

NS

** *** ** ** ** **

CFA

5 s

B

Time post-CFA (day)

pre- 1 2 3 4 5 6 7 8 9

MWT (g)

0 2 4 6 8 10 12

CFA

NS

CFA

NS

** *** ** ** ** **

CFA

5 s

C

Regular

Irregular

Burst

D

Discharge frequency (Hz)

0.0 0.5 1.0 1.5 2.0 2.5

NS

CFA

Regu‌lar

Irregular

Burst

5 s
Fig 2

A

![TWL (s) graph](image)

B

![MWT (g) graph](image)

- **TWL (s)**
  - NS-CFA
  - MSG-CFA
  - NS-NS
  - MSG-NS

- **MWT (g)**
  - NS-CFA
  - MSG-CFA
  - NS-NS
  - MSG-NS

- **Time after CFA injection**
Fig 3

A

B

C

D

**Discharge frequency (Hz)**

**Counts/bin**
Fig 4

A

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<td>β-actin</td>
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**Relative density of pNR2B**

\[ \text{Relative density of pNR2B} \]

B

![Bar chart showing relative density of pNR2B](chart)

C

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**Relative density of NR2B**

\[ \text{Relative density of NR2B} \]
Fig 5

A

NR2B

pSrc

IgG

~59kD

~50kD

NS CFA NS CFA

IP Boil + IP

B

NS CFA

pNR2B

β-actin

~180kD

~42kD

PP2 PP3

C

Relative density of pNR2B

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* indicates a statistically significant difference compared to NS.
**Fig 6**

A. Western blot analysis showing the relative density of pSrc (Tyr418) for NS and CFA conditions. The pSrc band is approximately 59kD, and the β-actin band is approximately 42kD.

B. Bar graph showing the relative density of pSrc for NS and CFA conditions. The CFA condition shows a statistically significant increase (*P<0.05) compared to the NS condition.

C. Discharge frequency histogram showing the counts/bin for PP2 treatment over time (s).

D. Discharge frequency (Hz) comparison between predrug, PP2, and PP2 in CFA conditions. The PP2 condition shows a statistically significant decrease in discharge frequency (**P<0.01) compared to the predrug condition.
Fig 7

A

Time post-drugs (min)

pre- 0 5 10 15 25 50 120

TWL (s)

CFA  PP2/PP3

PP3  PP2

B

Time post-drugs (min)

pre- 0 5 10 15 25 50 120

MWT (g)

CFA  PP2/PP3

PP3  PP2