Ketamine Depresses Toll-Like Receptor 3 Signaling in Spinal Microglia in a Rat Model of Neuropathic Pain

Xiao-Peng Mei^{a, b} Yang Zhou^{a, b} Wei Wang^b Jun Tang^a Wen Wang^b
Hui Zhang^a Li-Xian Xu^a Yun-Qing Li^b

^aDepartment of Anesthesiology, School of Stomatology, and ^bDepartment of Anatomy, Histology and Embryology, K.K. Leung Brain Research Centre, Fourth Military Medical University, Xi'an, PR China

Key Words

Glia • Microglia • Mitogen-activated protein kinase • Toll-like receptor • Analgesic

Abstract

Reports suggest that microglia play a key role in spinal nerve ligation (SNL)-induced neuropathic pain, and toll-like receptor 3 (TLR3) has a substantial role in the activation of spinal microglia and the development of tactile allodynia after nerve injury. In addition, ketamine application could suppress microglial activation in vitro, and ketamine could inhibit proinflammatory gene expression possibly by suppressing TLR-mediated signal transduction. Therefore, the present study was designed to disclose whether intrathecal ketamine could suppress SNL-induced spinal microglial activation and exert some antiallodynic effects on neuropathic pain by suppressing TLR3 activation. Behavioral results showed that intrathecal ketamine attenuated SNL-induced mechanical allodynia, as well as spinal microglial activation, in a dose-dependent manner. Furthermore, Western blot analysis displayed that ketamine application downregulated SNL-induced phosphorylated-p38 (p-p38) expression, which was specifically expressed in spinal microglia but not in astrocytes or neurons. Besides, ketamine could reverse TLR3

agonist (polyinosine-polycytidylic acid)-induced mechanical allodynia and spinal microglia activation. It was concluded that intrathecal ketamine depresses TLR3-induced spinal microglial p-p38 mitogen-activated protein kinase pathway activation after SNL, probably contributing to the antiallodynic effect of ketamine on SNL-induced neuropathic pain.

Copyright © 2011 S. Karger AG, Basel

Introduction

Millions of people worldwide suffer from neuropathic pain, which is an intractable clinical problem [1]. It has been reported that ketamine, a noncompetitive blocker for the glutamate N-methyl-D-aspartate (NMDA) receptor, can be applied for treating neuropathic pain both in clinical and lab settings [2–4]. Much research has been carried out to disclose the underlying mechanisms of ketamine analgesia, which are still not fully understood. Studies have suggested that NMDA receptors, the monoaminergic descending inhibitory system, opioid recep-

X.-P.M., Y.Z., and W.W. contributed equally to this work.

tors, nicotinic receptors, voltage-gated ion channels, and the gamma-aminobutyric acid (GABA) receptor are all involved in the analgesia of ketamine [2, 3, 5–9]. Very interestingly, some other studies have suggested that glia are also involved in opioid receptor, NMDA receptor, serotonergic receptor, and GABA receptor function [10–13]. Therefore, the morphology and signaling pathways in glial cells may also be affected by ketamine.

Thanks to decades of investigations, spinal glial cells (microglia and astrocytes) have been implicated as key factors not only in the induction but also in the maintenance of neuropathic pain [14–19]. Spinal microglia have been reported to take part directly in the initiation of peripheral nerve injury-induced neuropathic pain [1, 14, 20]. A previous study confirmed that toll-like receptor 3 (TLR3)-induced spinal dorsal horn microglia p38 mitogen-activated protein kinase (MAPK) activation contributes to the development of spinal nerve ligation (SNL)-induced neuropathic pain [21]. Moreover, spinal microglia could release some proinflammatory factors such as interleukin-1, tumor necrosis factor (TNF), and various chemokines during the development of neuropathic pain [22–25]. Interestingly, recent *in vitro* studies reported that ketamine could inhibit lipopolysaccharide (LPS)-induced microglia activation to exert some anti-inflammatory effects by blocking MAPK pathway activation [26, 27]. In addition, studies confirmed that ketamine could inhibit inflammatory reaction by suppressing the function of TLR [27–30]. Based on the results of those studies, we hypothesized that depressing TLR3-induced spinal microglia MAPK pathway activation may contribute to the antiallodynic effects of ketamine on neuropathic pain.

In the present study, the effects of ketamine on SNL or TLR3 agonist-induced mechanical allodynia were confirmed by behavioral testing. SNL-induced spinal microglia activation was detected by immunofluorescent histochemistry. Subsequently, the variation of microglia activation was investigated after the application of drugs. Finally, the microglia p38 MAPK pathway was investigated by immunofluorescent histochemistry and Western blot after different treatments.

Materials and Methods

Animal Preparation

Male Sprague-Dawley rats (180–200 g) were housed in plastic cages and maintained on a 12:12 h light/dark cycle under conditions of 22–25°C ambient temperature with food and water available. All experimental procedures received prior approval from

the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, China), and the ethical guidelines to investigate experimental pain in conscious animals were followed [31]. Every effort was made to minimize animal suffering and to restrict the number of animals used.

Intrathecal Implantation and Drug Administration

Intrathecal implantation was performed by inserting polyethylene (PE) tubing to inject the drug directly into the subarachnoid space of the lumbar enlargement. Briefly, a midline incision (3 cm) was made at the back of the rat at the level of the thoracic vertebrae under pentobarbital anesthesia (45 mg/kg, *i.p.*). A pre-measured length of PE-10 tubing (inner diameter 0.28 mm and outer diameter 0.61 mm) was passed caudally from the T8 level to the L3 level of the spinal cord; 2 cm of it was left exposed in the upper thoracic region. The rats were allowed to recover for 3–5 days before further use. Only the animals judged as neurologically normal and showing complete paralysis of both hind legs and tail after administration of 2% lidocaine (10 μ l) through the intrathecal catheter were used for the subsequent experiments.

S-(+)-ketamine hydrochloride (Sigma, St. Louis, Mo., USA) was dissolved and diluted with preservative-free normal saline solution for administration. Normal saline (0.9%) was used as the negative control. Animals were divided into the following 4 groups for administration: sham-saline, SNL-saline, SNL-ketamine, and sham-ketamine. Drugs and normal saline were injected intrathecally over 30 s, followed by a 10- μ l flush of normal saline.

Polyinosine-polycytidylic acid (poly I:C; Sigma) was injected intrathecally (100 μ g, 10 μ l) to the normal rats, followed by ketamine (300 μ g/kg, 10 μ l) or normal saline (10 μ l). The dosages of ketamine and poly I:C used in the present study were in accordance with previous research [2, 21, 32] and our pilot experiment.

Spinal Nerve Ligation

To create the SNL model, the left L6 transverse process was first removed to expose the L4 and L5 spinal nerves under pentobarbital anesthesia (45 mg/kg, *i.p.*). The L5 spinal nerve was then carefully isolated and tightly ligated with 6-0 silk thread [33]. The surgical procedure for the sham group was identical to that of the SNL group, except that the spinal nerve was not ligated.

Nociceptive Behavioral Tests

The animals were acclimatized to the testing environment for 3 days before baseline testing; they were then placed under inverted plastic boxes (30 $\times$ 30 $\times$ 50 cm³) on an elevated mesh floor and allowed habituation for 30 min before threshold testing. Room temperature (22–25°C) and humidity remained stable for all experiments. An electronic von Frey anesthesiometer (model 2390-5, blunt polypropylene tip, diameter 0.5 mm; IITC Life Science, Woodland Hills, Calif., USA) was used to determine the mechanical threshold. Behavioral testing was performed blindly with respect to drug administration. A paw flick response was elicited by applying increasing force (measured in g) using a plastic filament focused on the middle of the plantar surface of the ipsilateral hind paw. The force applied was initially below the detection threshold; it was increased from 1 to 50 g in 0.1-gram steps over 20 s and then maintained at 50 g for a further 10 s. The rate of force increase was 2.5 g/s. The threshold was taken as the force applied to elicit a reflex removal of the hind paw. The cutoff value was 50 g. This was defined as the mean of 3 measurements at 1-min intervals.

Immunofluorescence

After deep anesthesia produced with pentobarbital (60 mg/kg, i.p.), the rats were perfused through the ascending aorta with 100 ml 0.9% saline followed by 500 ml 0.1 M phosphate buffer (pH 7.3) that contained 4% paraformaldehyde and 2% picric acid. After perfusion, the L5 spinal segment was removed and postfixed in the same fixative for 2–4 h and then cryoprotected for 24 h at 4°C in 0.1 M phosphate buffer that contained 30% sucrose. Transverse frozen spinal sections (30 μm thick) were cut in a cryostat (CM1800; Leica, Heidelberg, Germany) and collected serially in 3 dishes. Each dish contained a complete set of serial sections that were processed for immunofluorescent staining. One of the dishes was selected randomly. The sections in that dish were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.3) 3 times (10 min each), blocked with 2% goat serum in 0.01 M PBS that contained 0.3% Triton X-100 for 1 h at room temperature, and then used for immunofluorescent staining.

The sections were incubated with primary antibodies, i.e. mouse anti-OX42 (1:500; Abcam, Cambridge, UK) and rabbit anti-phosphorylated p38 (p-p38) antibody (1:500; Cell Signaling Technology, Beverly, Mass., USA) overnight at 4°C. Other primary antibodies used in this study were monoclonal antibodies, i.e. mouse anti-neuronal-specific nuclear protein (NeuN) (1:3,000; Chemicon, Temecula, Calif., USA) and mouse anti-glial fibrillary acidic protein (GFAP) (1:5,000; Chemicon). For double immunofluorescence, sections were incubated with a mixture of 2 primary antibodies followed by a mixture of the 2 respective secondary antibodies (Alexa 488 donkey anti-rabbit IgG and Alexa 594 donkey anti-mouse IgG, 1:500; Invitrogen, Carlsbad, Calif., USA). The specificity of the staining was tested on the sections in another dish by omission of the primary specific antibodies. No immunoreactive products were found on the sections (data not shown). Confocal images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan; 1- μm -thick optical section), and digital images were captured with a Fluoview 1000 (Olympus).

Western Blot

All animals were rapidly sacrificed and the L5 dorsal horns were promptly removed and frozen on dry ice. The spinal dorsal horn was dissected using the open-book method [34]. The selected region was homogenized with a hand-held pestle in SDS sample buffer (10 ml/mg tissue) which contained a cocktail of proteinase and phosphatase inhibitors. The electrophoresis samples were heated at 100°C for 5 min and loaded onto 10% SDS-polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, Hercules, Calif., USA). The proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, Mass., USA). The membranes were placed for 1 h in a blocking solution which contained Tris-buffered saline with 0.02% Tween (TBS-T) and 5% nonfat dry milk, and they were incubated for 2 nights at 4°C under gentle agitation with primary antibodies, i.e. rabbit anti-p-p38 antibody (1:1,000 in 5% BSA; Cell Signaling Technology) and mouse anti- β -actin (1:1,000; Sigma), respectively. Bound primary antibodies were detected with the anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech, Inc., Piscataway, N.J., USA). After each step, the immunoblots were rinsed with TBS-T. All reactions were detected using the enhanced chemiluminescence (ECL) detection method (Am-

ersham). The densities of protein blots were analyzed using Labworks software (Ultra-Violet Products, UK). The densities of p-p38 and β -actin immunoreactive bands were quantified with background subtraction. A square of the same size was drawn around each band to measure the density, and the background near that band was subtracted. Since β -actin levels did not change significantly after inflammation and nerve injury [35], we used β -actin levels as a loading control and the p-p38 level was normalized against β -actin levels.

Quantification and Statistical Analysis

Data from immunofluorescence were calculated as detailed in our previous report [36]. For the quantification of OX42 immunopositive cell profiles in the spinal cord, 5 nonadjacent sections (30 μm) from the L5 segments were selected randomly from each animal. In each group, 6 rats were used for statistical analysis. An image (450 $\times$ 338 μm^2) of the medial two thirds of the superficial dorsal horn (laminae I to III) was captured under a 20 $\times$ objective [19]. All of the positively stained cells in the area were evaluated by a computer-assisted image analysis program (MetaMorph 6.1) which set the low and high thresholds for the immunofluorescent intensity which was determined to be a signal. The same configuration was used to measure cell areas in all experimental groups. The measured areas were transferred to Excel automatically for the following statistical analysis. MetaMorph 6.1 is calibrated to provide standardization of area measurements. A standardized field area was sampled arbitrarily from regions within randomly selected dorsal horn sections [19]. Data from immunofluorescence were expressed as a fold change against that of the sham-saline (or saline-saline) group. ANOVA followed by the least significant difference test was used for statistical analysis.

Double-labeled cells were counted on 6 sections randomly selected from 6 rats (1 section per animal) after superposition of the 2 images from the same field but with different markers.

All data are presented as means $\pm$ SD and were collected by researchers blinded to the surgery and reagents used. Data from the Western blot were tested using one-way ANOVA followed by the least significant difference test. Data from the von Frey test were analyzed by repeated measures ANOVAs followed by Fisher's protected least significant difference post hoc comparisons where appropriate. All statistical analyses were performed using SPSS® version 16.0 software (SPSS, Inc., Chicago, Ill., USA). $p < 0.05$ was considered statistically significant.

Results

Ketamine Relieved SNL-Induced Mechanical Allodynia in a Dose-Dependent Manner

In order to detect the effects of intrathecal ketamine on SNL-induced mechanical allodynia, ketamine was injected once a day at 3 different dosages (30, 100, and 300 $\mu\text{g/kg}$), and changes in the paw withdrawal threshold (PWT) were observed 30 min after injection and from postoperative day (POD) 0 to POD 3.

SNL could induce rapid and significant mechanical allodynia as shown in the SNL-saline group (fig. 1). In-

Fig. 1. Effect of intrathecal ketamine (KTM) on SNL-induced neuropathic mechanical allodynia. SNL could induce significant mechanical allodynia shown by the pain behavioral tests. Ketamine application (100 and 300 µg/kg) showed an effective and reliable antiallodynia effect in a dose-dependent manner on SNL-induced mechanical allodynia, whereas intrathecal ketamine 30 µg/kg did not affect the pain threshold after SNL. Additionally, intrathecal ketamine 300 µg/kg had no effect on the normal pain threshold of the sham-operated group. Drugs were given intrathecally once a day from POD 0 to POD 3. * $p < 0.05$ vs. SNL-saline. # $p < 0.05$ vs. SNL-ketamine 100 µg/kg. There were 12 rats in each group.

trathecal ketamine (30 µg/kg) did not obviously influence the PWT. However, intrathecal ketamine (100 µg/kg) elevated the PWT significantly after administration ($p < 0.05$ vs. SNL-saline). Moreover, a higher dose of ketamine (300 µg/kg) relieved the allodynia ($p < 0.05$ vs. SNL-ketamine 100 µg/kg). In addition, neither a high dose (300 µg/kg) nor a lower dose (30 and 100 µg/kg, data not shown) of ketamine affected the basal threshold in the sham-operated group. All of these results suggest that intrathecal ketamine (30, 100, and 300 µg/kg) exerted an effective and reliable effect on SNL-induced mechanical allodynia in a dose-dependent manner.

Ketamine Attenuated SNL-Induced Microglia Activation in the Ipsilateral Spinal Dorsal Horn

SNL induced marked microglia activation in the ipsilateral spinal dorsal horn (fig. 2a), while only a few positive cells could be detected in the contralateral spinal dorsal horn 3 days after SNL (fig. 2b). The expression of spinal microglial OX42 was very low in the sham-saline

Fig. 2. Effect of intrathecal ketamine (KTM) on SNL-induced spinal microglia activation. SNL induced remarkable spinal microglia activation indicated by OX42 upexpression in the ipsilateral (ipsi) dorsal horn (a) vs. the contralateral (contra) side (b). The spinal microglia activation level is low in the ipsilateral dorsal horn in the sham-operated rat (c), and intrathecal ketamine 300 µg/kg did not have any obvious effect on microglia activation (d). SNL-induced microglia activation is confirmed by the higher expression of OX42 (e). Intrathecal administration of ketamine (30, 100, and 300 µg/kg) indicates suppressive effects on SNL-induced microglia activation in a dose-dependent manner (f–h). i Statistical analysis of spinal microglia activation after different treatments with the density of OX42 expression. *, #, and \$ each indicate a statistically significant difference with $p < 0.05$ between groups. There were 6 rats in each group. Scale bar = 100 µm.

group (fig. 2c) and in the group which received sham-ketamine 300 µg/kg (fig. 2d). Compared with the sham-saline group, SNL induced a much higher expression of OX42 in the spinal dorsal horn (SNL-saline group; fig. 2e) as indicated by the increased density of immunoreactive staining (fig. 2i).

Ketamine (30, 100, and 300 µg/kg) inhibited SNL-induced spinal microglia activation in a dose-dependent

manner ($p < 0.05$ compared with SNL-saline; fig. 2i). Intrathecal ketamine (30 $\mu\text{g/kg}$) did not have any obvious effect on SNL-induced spinal microglia activation (fig. 2f), but higher doses (100 and 300 $\mu\text{g/kg}$) significantly attenuated SNL-induced spinal microglia activation after administration (fig. 2g, h).

SNL Induced Significant Upregulation of p-p38 in the Ipsilateral Spinal Dorsal Microglia

SNL induced remarkable p-p38 upregulation in the ipsilateral spinal dorsal horn (fig. 3a) compared with that in the contralateral spinal dorsal horn (fig. 3b) 3 days after SNL.

In order to detect the cellular localization of p-p38 expression, double immunofluorescent staining with antibodies against p-p38 and neuronal marker NeuN, microglial specific marker OX42, and astrocytic specific marker GFAP was performed. The double immunolabeled cells were counted (table 1). No colocalization could be observed either between p-p38 and NeuN (fig. 3c–e) or between p-p38 and GFAP (fig. 3f–h), which suggests that neither neurons nor astrocytes expressed p-p38 in the spinal dorsal horn 3 days after SNL. However, almost all p-p38-positive cells were OX42-positive microglia (fig. 3i–k). p-p38-positive staining was localized in the cell body, which was surrounded by the OX42-positive staining and localized in the cell membrane or processes (fig. 3l–n).

Intrathecal Ketamine Downregulated SNL-Induced p-p38 Expression

We then tested whether intrathecal ketamine had an effect on the spinal microglia p38 signal pathway. The results from the Western blot showed that SNL induced an upregulation of p-p38 in the spinal dorsal horn in the SNL-saline group vs. the sham-saline group ($p < 0.05$; fig. 4). Intrathecal ketamine 30 $\mu\text{g/kg}$ did not affect p-p38 expression compared with that in SNL-saline group (fig. 4). However, intrathecal ketamine 100 $\mu\text{g/kg}$ significantly attenuated the expression of p-p38 ($p < 0.05$ vs. the SNL-saline group; fig. 4). Intrathecal injection of ketamine 300 $\mu\text{g/kg}$ apparently downregulated p-p38 expression ($p < 0.05$ vs. the SNL-saline group; fig. 4), which indicates that ketamine (300 $\mu\text{g/kg}$) showed stronger inhibition effects on p-p38 expression compared with SNL-ketamine 100 $\mu\text{g/kg}$ ($p < 0.05$; fig. 4). However, ketamine (300 $\mu\text{g/kg}$) did not show any effect on p-p38 expression in sham-operated rats compared with the sham-saline group (fig. 4). These results showed that intrathecal ketamine has a dose-dependent effect on the suppression of SNL-induced p-p38 upregulation in spinal dorsal horn.

Fig. 3. SNL-induced p-p38 expression and cellular localization in the spinal dorsal horn. SNL induced remarkable p-p38 upregulation in the ipsilateral spinal dorsal horn (a) vs. the contralateral spinal dorsal horn (b). High-magnification images demonstrating double immunofluorescent staining between NeuN and p-p38 (c–e), glial fibrillary acidic protein (GFAP), and p-p38 (f–h), or between OX42 and p-p38 (i–k). l–n Images were individually captured from i–k showing the colocalization of OX42 and p-p38. Scale bars = 100 μm (a, b), 50 μm (c–k), and 10 μm (l–n).

Fig. 4. Effect of different treatments on ipsilateral spinal dorsal horn p-p38 expressions. There was no obvious difference in p-p38 expression after intrathecal 300 μ g/kg ketamine (KTM) in sham-operated rats vs. the sham-saline group. p-p38 was apparently upregulated in the SNL-saline group. Ketamine administration effectively decreased p-p38 expression in a dose-dependent manner. *, #, and \$ each indicate a statistically significant difference with $p < 0.05$ between groups. There were 6 rats in each group.

Fig. 5. Effect of intrathecal ketamine (KTM) on poly I:C injection-induced neuropathic mechanical allodynia. Intrathecal poly I:C (100 μ g) induced marked mechanical allodynia shown by the pain behavioral tests. Ketamine administration (300 μ g/kg) did not affect the pain threshold of normal rats but reversed poly I:C-induced mechanical allodynia. Drugs were given intrathecally only once and the behavioral test was performed during the following 3 days. * $p < 0.05$ vs. the saline-saline group. # $p < 0.05$ vs. the poly I:C-saline group. There were 6 rats in each group.

Table 1. Quantitation of double immunofluorescent staining between p-p38 and NeuN, GFAP, or OX42

	p-p38-positive cells, n	Double-labeled cells, n
NeuN group	47 $\pm$ 7	0 (p-p38/NeuN)
GFAP group	45 $\pm$ 7	0 (p-p38/GFAP)
OX42 group	51 $\pm$ 5	51 $\pm$ 5 (p-p38/OX42)

Values are presented as means $\pm$ SD. There were 6 rats in each group.

Ketamine Reversed Poly I:C Injection-Induced Mechanical Allodynia and Spinal Dorsal Microglia Activation

In order to verify whether TLR3 was involved in the inhibiting effects of ketamine on neuropathic pain, we intrathecally injected a kind of TLR3 agonist poly I:C

(100 μ g, 10 μ l) together with ketamine (300 μ g/kg, 10 μ l) into the normal rats and observed the changes in PWT during the following 3 days. Subsequently, microglia activation was detected.

Poly I:C induced significant mechanical allodynia shown in the poly I:C-saline group 1 day after injection ($p < 0.05$ vs. the saline-saline group; fig. 5). Intrathecal ketamine (300 μ g/kg) obviously reversed poly I:C-induced mechanical allodynia ($p < 0.05$ vs. poly I:C-saline). Moreover, poly I:C induced significant microglia activation in the spinal dorsal horn 3 days after injection (fig. 6c). There was no marked microglia activation in the saline-saline group (fig. 6a) or in the group receiving saline-ketamine 300 μ g/kg (fig. 6b). Ketamine (300 μ g/kg) notably suppressed poly I:C-induced spinal microglia activation (fig. 6d).

All of these results suggest that inhibiting TLR3 activation could be an underlying mechanism of intrathecal ketamine in SNL-induced neuropathic pain.

Color version available online

Fig. 6. Effect of intrathecal ketamine (KTM) on poly I:C injection-induced spinal microglia activation. Poly I:C (100 μ g) injection induced a distinguished spinal microglia activation confirmed by the increased OX42 expression (c). Intrathecal ketamine (300 μ g/kg) suppressed poly I:C-induced microglia activation (d). **e** Statistical analysis of spinal microglia activation after different treatments with the density of OX42 expression. * and # indicate a statistical difference with $p < 0.05$ between groups. There were 6 rats in each group. Scale bar = 100 μ m.

Discussion

The present study suggests that intrathecal injection of ketamine could relieve SNL-induced mechanical allodynia, probably by suppressing TLR3-induced spinal microglia activation during the initiation of SNL-induced neuropathic pain. Moreover, it may be one of the underlying mechanisms of the analgesic effect of intrathecal ketamine on peripheral nerve injury-induced neuropathic pain.

Both clinical and animal studies have shown that ketamine can be applied for treating many kinds of chronic pain, such as neuropathic pain, cancer pain, and complex regional pain syndrome [4, 5, 37]. The present results show that a low dose of ketamine (30 μ g/kg) administration did not affect the pain threshold of the neuropathic rats, while 100 μ g/kg ketamine showed obvious effects on mechanical allodynia. However, high-dose ketamine (300 μ g/kg) administration raised the SNL-induced mechanical pain threshold without any impairment on motor function. These results confirm that ketamine is effective in treating peripheral nerve injury-induced mechanical allodynia, which is accordant with previous reports [5, 38]. Although the analgesic effect of ketamine is undoubted, the underlying mechanisms are not yet fully understood.

Numerous reports have confirmed that spinal glial cells (microglia and astrocytes) are essential and sufficient for the development of peripheral nerve injury-induced neuropathic pain [1, 14, 17], and an interesting in vitro study demonstrated that ketamine can inhibit LPS-induced microglial activation via downregulation of the inflammatory factor TNF- α [27]. Our present study demonstrates that intrathecal ketamine could inhibit microglial activation with OX42 downregulation after SNL. Peripheral nerve injury induced spinal microglia activation as early as 1 day after the lesion [16]. Activated spinal microglia could release many inflammatory cytokines and chemokines which seriously facilitate the lesion [15]. It has been confirmed that peripheral nerve injury-induced spinal microglial activation is one of the key factors in the initiation of neuropathic pain development [34]. Therefore, the present result of inhibiting SNL-induced spinal microglial activation might contribute to the analgesic effect of intrathecal ketamine on mechanical allodynia. But, how does ketamine act on microglia to exert the inhibiting effect?

TLRs play an essential role in innate immune responses and in the initiation of adaptive immune responses [21]. TLRs, especially TLR2–4, have been reported as im-

portant for the development of neuropathic pain [21, 39, 40]. Previous studies have shown that SNL induces neuropathic pain with a significant upregulation of TLR2–4 [39, 40]. TLR-deficient mice display significantly attenuated behavioral hypersensitivity and decreased expression of spinal glial activation and proinflammatory cytokines [41]. Microglia constitutively express a wide range of TLR2–4 at high levels [42]. A previous study [21] observed that intrathecal administration of TLR3 antisense oligodeoxynucleotide suppressed SNL-induced mechanical allodynia and decreased the phosphorylation of p38 MAPK, but not extracellular signal-regulated protein kinases 1/2, in spinal microglia. These results suggest that TLRs have a substantial role in the activation of spinal glial cells and the development of mechanical allodynia after peripheral nerve injury. Interestingly, studies have demonstrated that ketamine could inhibit proinflammatory gene expression, such as TNF- α , IL-1 β , and IL-6, among others, possibly by suppressing TLR-mediated signal transduction [27–30]. Moreover, other *in vitro* studies have suggested that ketamine could inhibit microglia activation and block MAPK pathway activation, which are induced by LPS, a ligand for TLR4 [26, 27]. The present study observed that ketamine could suppress TLR3 agonist-induced mechanical allodynia and spinal dorsal horn microglia activation, which was accordant with these previous reports in this respect. Since TLR3 contributes greatly to SNL-induced spinal microglia p38 MAPK activation and the development of mechanical allodynia, ketamine might act on TLR3 located on microglia and thus inhibit microglia p38 activation after SNL.

Ketamine is known to be a nonselective NMDA receptor antagonist, and a recent study reported that the NMDA receptor subunit NR1 is phosphorylated after peripheral nerve injury [43]. Moreover, a study indicated that NMDA injection leads to increased p-p38 MAPK immunoreactivity predominantly in spinal microglia [44]. This report suggested that the microglial NMDA-p-p38 pathway is involved in spinal nociceptive processing. Our present study demonstrated that ketamine application could effectively depress the spinal microglial p-p38 level and relieve mechanical allodynia induced by SNL. Therefore, ketamine could possibly directly act on the microglial NMDA-p-p38 pathway to suppress spinal microglial activation.

In addition to this possibility, ketamine could suppress SNL-induced spinal microglial activation through the NMDA receptor indirectly. The NMDA receptor mainly distributes in the superficial dorsal horn, which has a much closer relationship with neuropathic pain

[45]. Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in rats [46]. Meanwhile, it has been reported that NMDA receptor activation could facilitate neuronal nitric oxide (NO) release, which contributes to the progression of pain transmission after nerve injury [47]. It has also been reported that inhibition of spinal neuronal NO (nNO) synthesis by the selective inhibitor of neuronal NO synthesis (nNOS) could suppress spinal microglia activation by attenuating p38 MAPK pathway activation [48]. This suggests that suppressing nNO signals to microglia leads to the downregulation of microglial p38 MAPK pathway activation, which is accordant with the findings of the present study that intrathecal NMDA antagonist ketamine could inhibit SNL-induced spinal microglia activation and downregulate microglial p38 MAPK pathway activation. Such p38 MAPK activation in microglia is consistent with a potential role in the development of neuropathic pain, which was verified by a previous report [16].

It could be concluded that the NMDA-nNO-microglia-p38 pathway plays an important role in the development of peripheral nerve injury-induced neuropathic pain. Therefore, blocking NMDA-nNO-microglia-p38 pathway activation, to some extent, could be the indirect action of intrathecal ketamine on inhibiting SNL-induced microglia activation.

In conclusion, our present study indicates that inhibiting TLR3-induced spinal microglia p38 MAPK pathway activation after SNL could be one of the antiallodynic mechanisms of intrathecal ketamine at the spinal level. The present work could be a basis for ketamine application when treating neuropathic pain in the clinical setting.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30901400, 30772073, 30771133, and 30971123), the Eleventh Five-Year Project of Science and Technology (06G093), the National Program of Basic Research of China (G2006CB500808), and the Program for New Century Excellent Talents in University (NCET-06-0931). Our thanks also go to Dr. Jerome Staal from the Menzies Research Institute Tasmania in Australia for his critical scientific opinions and help in English.

References

- 1 Tsuda M, Inoue K, Salter MW: Neuropathic pain and spinal microglia: a big problem from molecules in 'small' glia. *Trends Neurosci* 2005;28:101–107.
- 2 Nadeson R, Tucker A, Bajunaki E, Goodchild CS: Potentiation by ketamine of fentanyl antinociception. 1. An experimental study in rats showing that ketamine administered by non-spinal routes targets spinal cord antinociceptive systems. *Br J Anaesth* 2002;88:685–691.
- 3 Christoph T, Schiene K, Englberger W, Parsons CG, Chizh BA: The antiallodynic effect of NMDA antagonists in neuropathic pain outlasts the duration of the in vivo NMDA antagonism. *Neuropharmacology* 2006;51:12–17.
- 4 Kiefer RT, Rohr P, Ploppa A, Dieterich HJ, Grothusen J, Koffler S, Altemeyer KH, Unertl K, Schwartzman RJ: Efficacy of ketamine in anesthetic dosage for the treatment of refractory complex regional pain syndrome: an open-label phase II study. *Pain Med* 2008;9:1173–1201.
- 5 Hota D, Bansal V, Pattanaik S: Evaluation of ketamine, nimodipine, gabapentin and imipramine in partial sciatic nerve transection model of neuropathic pain in rat: an experimental study. *Methods Find Exp Clin Pharmacol* 2007;29:443–446.
- 6 Schnobel R, Wolff M, Peters SC, Brau ME, Scholz A, Hempelmann G, Olschewski H, Olschewski A: Ketamine impairs excitability in superficial dorsal horn neurones by blocking sodium and voltage-gated potassium currents. *Br J Pharmacol* 2005;146:826–833.
- 7 Koizuka S, Obata H, Sasaki M, Saito S, Goto F: Systemic ketamine inhibits hypersensitivity after surgery via descending inhibitory pathways in rats. *Can J Anaesth* 2005;52:498–505.
- 8 Abelson KS, Goldkuhl RR, Nylund A, Høglund AU: The effect of ketamine on intraspinal acetylcholine release: involvement of spinal nicotinic receptors. *Eur J Pharmacol* 2006;534:122–128.
- 9 Hevers W, Hadley SH, Luddens H, Amin J: Ketamine, but not phencyclidine, selectively modulates cerebellar GABA(A) receptors containing alpha6 and delta subunits. *J Neurosci* 2008;28:5383–5393.
- 10 Rahman W, Suzuki R, Webber M, Hunt SP, Dickenson AH: Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. *Pain* 2006;123:264–274.
- 11 Aicher SA, Sharma S, Cheng PY, Pickel VM: The N-methyl-D-aspartate (NMDA) receptor is postsynaptic to substance P-containing axon terminals in the rat superficial dorsal horn. *Brain Res* 1997;772:71–81.
- 12 Muller T, Fritschy JM, Grosche J, Pratt GD, Mohler H, Kettenmann H: Developmental regulation of voltage-gated K⁺ channel and GABAA receptor expression in Bergmann glial cells. *J Neurosci* 1994;14:2503–2514.
- 13 Holdridge SV, Armstrong SA, Taylor AM, Cahill CM: Behavioural and morphological evidence for the involvement of glial cell activation in delta opioid receptor function: implications for the development of opioid tolerance. *Mol Pain* 2007;3:7.
- 14 Watkins LR, Milligan ED, Maier SF: Spinal cord glia: new players in pain. *Pain* 2001;93:201–205.
- 15 Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y: BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 2005;438:1017–1021.
- 16 Jin SX, Zhuang ZY, Woolf CJ, Ji RR: p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 2003;23:4017–4022.
- 17 Scholz J, Woolf CJ: The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 2007;10:1361–1368.
- 18 Watkins LR, Milligan ED, Maier SF: Glial activation: a driving force for pathological pain. *Trends Neurosci* 2001;24:450–455.
- 19 Zhuang ZY, Wen YR, Zhang DR, Borsello T, Bonny C, Strichartz GR, Decosterd I, Ji RR: A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *J Neurosci* 2006;26:3551–3560.
- 20 Terayama R, Omura S, Fujisawa N, Yamaai T, Ichikawa H, Sugimoto T: Activation of microglia and p38 mitogen-activated protein kinase in the dorsal column nucleus contributes to tactile allodynia following peripheral nerve injury. *Neuroscience* 2008;153:1245–1255.
- 21 Obata K, Katsura H, Miyoshi K, Kondo T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Akira S, Noguchi K: Toll-like receptor 3 contributes to spinal glial activation and tactile allodynia after nerve injury. *J Neurochem* 2008;105:2249–2259.
- 22 Detloff MR, Fisher LC, McGaughy V, Longbrake EE, Popovich PG, Basso DM: Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp Neurol* 2008;212:337–347.
- 23 Inoue K: The function of microglia through purinergic receptors: neuropathic pain and cytokine release. *Pharmacol Ther* 2006;109:210–226.
- 24 Kawasaki Y, Zhang L, Cheng JK, Ji RR: Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci* 2008;28:5189–5194.
- 25 Ohtori S, Takahashi K, Moriya H, Myers RR: TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: studies in murine DRG and spinal cord. *Spine* 2004;29:1082–1088.
- 26 Chang Y, Lee JJ, Hsieh CY, Hsiao G, Chou DS, Sheu JR: Inhibitory effects of ketamine on lipopolysaccharide-induced microglial activation. *Mediators Inflamm* 2009;2009:705379.
- 27 Shibakawa YS, Sasaki Y, Goshima Y, Echigo N, Kamiya Y, Kurahashi K, Yamada Y, Andoh T: Effects of ketamine and propofol on inflammatory responses of primary glial cell cultures stimulated with lipopolysaccharide. *Br J Anaesth* 2005;95:803–810.
- 28 Chang HC, Lin KH, Tai YT, Chen JT, Chen RM: Lipoteichoic acid-induced TNF-alpha and IL-6 gene expressions and oxidative stress production in macrophages are suppressed by ketamine through downregulating Toll-like receptor 2-mediated activation of ERK1/2 and NF-kappaB. *Shock* 2010;33:485–492.
- 29 Yu M, Shao D, Yang J, Feng S, Xu J: Ketamine suppresses intestinal TLR4 expression and NF-kappaB activity in lipopolysaccharide-treated rats. *Croat Med J* 2006;47:825–831.
- 30 Chen TL, Chang CC, Lin YL, Ueng YF, Chen RM: Signal-transducing mechanisms of ketamine-caused inhibition of interleukin-1 beta gene expression in lipopolysaccharide-stimulated murine macrophage-like Raw 264.7 cells. *Toxicol Appl Pharmacol* 2009;240:15–25.
- 31 Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109–110.
- 32 Mei X, Wang W, Wang W, Li Y, Zhang H, Wu S, Li Y, Xu L: Inhibiting astrocytic activation: a novel analgesic mechanism of ketamine at the spinal level? *J Neurochem* 2009;109:1691–1700.
- 33 Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992;50:355–363.
- 34 Zhuang ZY, Gerner P, Woolf CJ, Ji RR: ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 2005;114:149–159.

- 35 Guo W, Wang H, Watanabe M, Shimizu K, Zou S, LaGraize SC, Wei F, Dubner R, Ren K: Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. *J Neurosci* 2007;27:6006–6018.
- 36 Wang W, Wang W, Mei X, Huang J, Wei Y, Wang Y, Wu S, Li Y: Crosstalk between spinal astrocytes and neurons in nerve injury-induced neuropathic pain. *PLoS One* 2009;4:e6973.
- 37 Lauretti GR, Lima IC, Reis MP, Prado WA, Pereira NL: Oral ketamine and transdermal nitroglycerin as analgesic adjuvants to oral morphine therapy for cancer pain management. *Anesthesiology* 1999;90:1528–1533.
- 38 Gammaitoni A, Gallagher RM, Welz-Bosna M: Topical ketamine gel: possible role in treating neuropathic pain. *Pain Med* 2000;1:97–100.
- 39 Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Peri F, Costa B: Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* 2008;56:1312–1319.
- 40 Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB, Lee SJ: A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem* 2007;282:14975–14983.
- 41 Cao L, Tanga FY, Deleo JA: The contributing role of CD14 in toll-like receptor 4 dependent neuropathic pain. *Neuroscience* 2009;158:896–903.
- 42 Bsibsi M, Ravid R, Gveric D, van Noort JM: Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol* 2002;61:1013–1021.
- 43 Daulhac L, Maffre V, Mallet C, Etienne M, Privat AM, Kowalski-Chauvel A, Seva C, Fialip J, Eschaliere A: Phosphorylation of spinal N-methyl-D-aspartate receptor NR1 subunits by extracellular signal-regulated kinase in dorsal horn neurons and microglia contributes to diabetes-induced painful neuropathy. *Eur J Pain* 2011;15:169.e1–169.e12.
- 44 Svensson CI, Hua XY, Protter AA, Powell HC, Yaksh TL: Spinal p38 MAP kinase is necessary for NMDA-induced spinal PGE(2) release and thermal hyperalgesia. *Neuroreport* 2003;14:1153–1157.
- 45 Chizh BA, Headley PM, Tzschentke TM: NMDA receptor antagonists as analgesics: focus on the NR2B subtype. *Trends Pharmacol Sci* 2001;22:636–642.
- 46 Ultenius C, Linderöth B, Meyerson BA, Wallin J: Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in the rat. *Neurosci Lett* 2006;399:85–90.
- 47 Yonehara N, Kudo C, Kamisaki Y: Involvement of NMDA-nitric oxide pathways in the development of tactile hypersensitivity evoked by the loose-ligation of inferior alveolar nerves in rats. *Brain Res* 2003;963:232–243.
- 48 Liu W, Wang CH, Cui Y, Mo LQ, Zhi JL, Sun SN, Wang YL, Yu HM, Zhao CM, Feng JQ, Chen PX: Inhibition of neuronal nitric oxide synthase antagonizes morphine antinociceptive tolerance by decreasing activation of p38 MAPK in the spinal microglia. *Neurosci Lett* 2006;410:174–177.