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ORIGINAL PAPER

Interleukin-10 of Red Nucleus Plays Anti-Allodynia Effect in Neuropathic Pain Rats with Spared Nerve Injury

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Abstract Our previous studies have shown that pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) in red nucleus (RN) are involved in the development of neuropathic pain and play facilitated roles on the mechanical allodynia induced by peripheral nerve injury. The current study was designed to evaluate the expression and effect of IL-10, an anti-inflammatory cytokine, in the RN of rats with spared nerve injury (SNI). Immunohistochemical staining results demonstrated when 3 weeks after SNI, the expression level of IL-10 in the contralateral RN of SNI rats was apparently higher than those of sham-operated and normal rats. To further study the effect of IL-10 in the development of neuropathic pain, different doses of IL-10 (1.0, 0.5 and 0.1 μg/μl) were microinjected respectively into the RN contralateral to the nerve injury side of SNI rats. Results demonstrated that higher doses of IL-10 (1.0 and 0.5 μg/μl) significantly attenuated the mechanical allodynia of neuropathic rats, while 0.1 μg/μl of IL-10 did not show any analgesic effect. These results suggest that IL-10 of RN participates in the development of neuropathic pain and plays inhibitory roles on the mechanical allodynia induced by SNI.

Keywords Interleukin-10 · Red nucleus · Spared nerve injury · Neuropathic pain

Introduction

Accumulating evidence has shown that peripheral nerve injury can stimulate the central nervous system (CNS) to express both pro-inflammatory and anti-inflammatory cytokines, which play crucial roles in the establishment and maintenance of neuropathic pain [1–3]. Pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α), usually induce or facilitate neuropathic pain [1], while blockade of pro-inflammatory cytokines and/or administration of anti-inflammatory cytokines, such as IL-10, reduce neuropathic pain in animal models [4, 5].

IL-10 is one of the most important regulators in the immune system and secreted by a variety of immune cells including activated type 2 T helper cells (T_H2), B cells and monocytes. Recent studies have shown that IL-10 is also synthesized in the CNS and implicated in the development of neuropathic pain [3, 6–8]. Peripheral nerve injury results in the increased expression of IL-10 in both injured and contralateral noninjured peripheral nerves and their DRG [3, 6, 7]. Some analgesic drugs, such as betamethasone, mirtazapine and adenosine 2A receptor agonist ATL313 can relieve the neuropathic pain through increasing the expression of IL-10, and the analgesic effect can be abolished by administration of neutralizing IL-10 antibodies [9–11]. Moreover, systemic administration or intrathecal

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(i.t.) injection of IL-10 could dose-dependently reduce the dynorphin-induced allodynia, quisqualic acid (QUIS)-induced spontaneous pain-like behaviors, osteoma-induced pain, Leishmania major-induced hyperalgesia and ultraviolet radiation-induced hyperalgesia [12–16]. In rodent experiments, Mahoney, Watkins and co-workers have demonstrated that intrathecal injection of PEGylated IL-10, polymer-based IL-10, viral vectors or non-viral vectors encoding IL-10 could prevent the development of pain or reverse established pain induced by nerve constriction or injection of pain-causing substances into the nerve sheath [17–23]. All of these studies suggest that IL-10 is involved in the development of pain and plays an analgesic effect.

The roles of IL-10 in the peripheral and spinal level of neuropathic pain models seem to be well defined, but in the supraspinal level they remain obscure. Previous studies have demonstrated that TNF-α and IL-1β are up-regulated in the contralateral red nucleus (RN) of spared nerve injury (SNI) rats, and microinjection of their corresponding antibodies could alleviate the mechanical allodynia induced by SNI [24–26], suggesting that TNF-α and IL-β in RN participate in the development of neuropathic pain and play facilitated roles on the mechanical allodynia induced by peripheral nerve injury. In this study, we detected the expression of anti-inflammatory cytokine IL-10 in the RN of SNI rats by immunohistochemistry and an increased IL-10 immunoreactivity was observed in the contralateral RN of SNI rats as compared with those of sham-operated and normal rats. To further explore the effects of IL-10 in RN, different doses of IL-10 were microinjected into the contralateral RN of SNI rats and the results showed that higher doses of IL-10 significantly attenuated the mechanical allodynia of neuropathic rats.

Materials and Methods

Animals

Male Sprague-Dawly rats weighing 200–230 g were used for the study, all of which were purchased from the Experimental Animal Center of Shaanxi Province, China. All animals were housed with *ad libitum* access to food and water and maintained on a 12/12 light/dark cycle. All experiments were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University in accordance with the ethical guidelines of the International Association for the Study of Pain [27].

Spared Nerve Injury

The neuropathic pain was induced by tightly ligating the tibial and common peroneal nerve with leaving the sural nerve intact as reported previously [28]. Briefly, after rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), the sciatic nerve and its three terminal branches were exposed by directly incising through the biceps femoris muscle of the right hind limb. The tibial and common peroneal branches were tightly ligated by 5–0 silk sutures and sectioned distal to the ligation, removing 2–4 mm of the distal nerve stump. Great care was taken to avoid contacting or stretching the intact sural nerve. Muscle and skin were closed in two layers. In sham-operated group, rats were treated in the same way but the nerve was neither ligated nor sectioned. After surgery, rats were allowed to recover from anaesthesia in an observation chamber with a warming light. In normal control group, rats were free of any treatment. Rats of SNI group were used for further experiments only when the withdrawal threshold of right hind paw was less than 4.0 g in response to von Frey filaments stimulation.

Immunohistochemistry

Three weeks after surgery, 24 rats including SNI group ($n = 8$), sham-operated group ($n = 8$) and normal control group ($n = 8$) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Each rat was fixed by perfusion through the aortic arch with 250 ml ice-cold heparinized normal saline, followed by 420 ml Bouin's fluid (300 ml saturate nitroxanthic acid solution, 100 ml 40 % formaldehyde and 20 ml glacial acetic acid). The brain region containing red nucleus was harvested, postfixed in Bouin's fluid for 2 days and then dehydrated by 30 % sucrose.

All brain tissues were embedded in OCT and sectioned coronally into 20 μm thick sections using a LEICACM 1850 ultramicrotome. One slice from 100 μm was picked and three slides were used for analyzing one animal. After routine treatments of acetone and 3 % hydrogen peroxide, slides were blocked with 5 % goat serum in PBS for 1 h and then incubated overnight with rabbit anti-rat IL-10 polyclonal antibody (working dilution 1:100, Boster Bio-Engineering Limited Co., Wuhan, China) at 4 °C. Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG for 30 min and DAB was used for staining. As a control, the primary antibody or secondary antibody was omitted and isotypic antibody (normal rabbit IgG, Boster Bio-Engineering Limited Co., Wuhan, China) was used to confirm the immunospecificity of the IL-10 reaction.

Histological sections were viewed with Olympus DP70 microscope and the images were captured with Olympus BX-51 camera. The area ratio of IL-10 positive cells (area of positive signal/area of interesting) and the integrated optical density (IOD) were analyzed using Image pro-plus (IPP) and Motic Med (version 6.0) software. Each slide

was analyzed with the same size of arbitrary areas (600 μm × 800 μm) and total 24 blinded slides (3 arbitrary slides/rat) for each experimental group were calculated. All data were analyzed by an assistant who was unaware of the treatment groups.

Catheterization and Drug Administration

Two weeks after SNI, total 32 rats with SNI were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) for surgery. The rat skull was exposed and a stainless steel guide cannula (0.8 mm in diameter) was stereotaxically implanted at a position 2.0 mm dorsal to the left RN, where an increased IL-10 immunoreactivity was observed after SNI according to the results of immunohistochemistry, at the following coordinates: 5.2–6.7 mm to bregma, 0.6–1.4 mm lateral, 4.4–5.4 mm below cortical surface [29]. The guide cannula was fixed on the skull with four microscrews and dental cement. A stainless steel plug was inserted into the guide cannula and kept in place until the intracerebral injection. After surgery, each rat was injected intraperitoneally with penicillin 0.2 million units per day for consecutive 4 days. During this period, rats were housed individually in cage with free access to water and food.

One week after catheterization, a 1.0 μl microsyringe (0.4 mm in diameter) with its tip extending 2.0 mm beyond the end of the guide cannula was inserted into RN through the guide cannula after the plug was removed. Drugs dissolved in normal saline (0.5 μl) were then slowly infused into RN within 60 s and the microsyringe was left in place for an additional 30 s to minimize the drug solution flowing back into the injector track. Drugs used in this study included different doses of recombinant rat IL-10 (1.0, 0.5 and 0.1 μg/μl; PeproTech Inc., USA). The same volume of 0.9 % normal saline was injected in control rats. At the end of experiments, pontamine sky blue was injected into RN and the microinjection sites were histologically verified.

Behavioral Measures

One week after catheterization (i.e., 3 weeks after SNI), the mechanical withdrawal threshold of SNI rats were measured blindly by an experimenter before and 10, 20, 30, 40, 50, 60, 70 min after drug administration by using the up-down method [30]. The rat was placed in transparent plastic box (280 × 250 × 210 mm) with a metal wire mesh floor that allowed full access to the paws from underneath. Ten von Frey filaments (Stoelting Company, Wood Dale, IL, USA) ranged from 0.4 to 15.0 g were used to measure the mechanical allodynia. Starting with filament 4.31 (0.2 g) which is one of the middle of the series of filaments, von Frey filaments were applied from underneath perpendicularly to the right hind paw with sufficient force to cause slight bending and held for 6–8 s. The pattern of positive and negative responses was converted into a 50 % withdrawal threshold using the formula given by Dixon [31]: 50 % withdrawal threshold $= 10^{(X+kd)}/10^4$, where X is the value of the final von Frey hair used (in log units), k is the tabular value for the pattern of positive/negative responses modified from Dixon [30], and d is the mean difference between stimuli in log units (0.17). In the cases where continuous positive or negative responses were observed all the way out to the end of the stimulus spectrum, values of 0.25 or 15.0 g were assigned, respectively.

Data Analysis

Statistical analyses were performed by using SigmaStat 2.03 and all data were presented as mean ± standard deviation. Linear regression was used to assess the correlation between the effects and doses of IL-10. Differences in the area ratio of IL-10 positive cells and IOD were tested statistically by one-way analysis of variance (one-way ANOVA). Differences in drug effect among groups were tested statistically by two-way repeated measures of analysis of variance (two-way RM ANOVA) with a multiple comparison for analysis of the differences in entire observation time or at each time point among different groups. $P < 0.05$ was considered to be statistically significant.

Results

General

Three weeks after SNI, the mechanical withdrawal threshold of hind paw ipsilateral to the nerve injury (1.35 ± 0.64 g, $n = 8$) was significantly decreased ($P < 0.001$) as compared with those from the sham-operated group (12.21 ± 2.47 g, $n = 8$) and normal control group (11.95 ± 4.1 g, $n = 8$), while no difference of withdrawal threshold was observed in the contralateral hind paw. These results suggest that the neuropathic pain model with monolateral mechanical allodynia has been created successfully and is consistent with previous report [32].

Increased Expression of IL-10 Protein in Red Nucleus

Three weeks after SNI, a stronger immunoreactivity of IL-10 was observed in the contralateral red nucleus of SNI rats (Fig. 1a, d, g) and a weaker immunoreactivity of IL-10 was observed in the ipsilateral RN of SNI rats and sham-operated rats (Fig. 1b, e, h). In normal rats, no obvious immunoreactivity of IL-10 was found in the both sides of

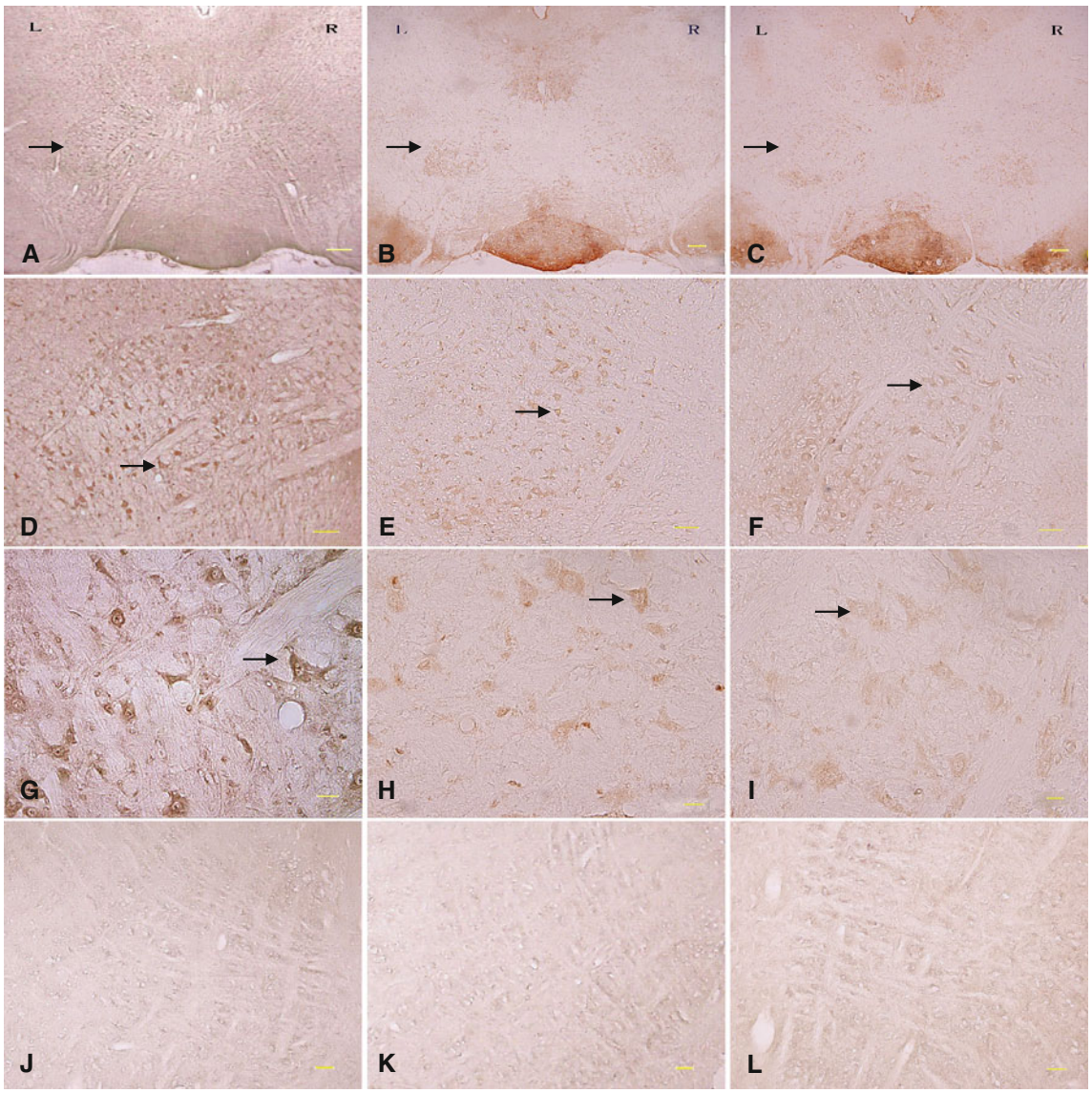

Fig. 1 Immunohistochemical staining of IL-10 in the red nucleus (RN) at 3 weeks following spared nerve injury (SNI). Three weeks after SNI, a stronger immunoreactivity of IL-10 was observed in the contralateral red nucleus of SNI rats (**a**, **d** and **g**) and a weaker immunoreactivity of IL-10 was observed in the ipsilateral RN of SNI rats and sham-operated rats (**b**, **e** and **h**). In normal rats, no obvious immunoreactivity of IL-10 was found in the both sides of RN (**c**, **f** and **i**). The control experiments, in which the primary antibody or secondary antibody was omitted and normal rabbit IgG was used, did not find any positive staining, suggesting that the immunoreactivity of IL-10 in red nucleus was specific (**j**, **k** and **l**). *L* left, *R* right. *Scale bars* **a**, **b** and **c**: 200 μm; **d**, **e** and **f**: 100 μm; **g**, **h** and **i**: 10 μm; **j**, **k** and **l**: 50 μm

RN (Fig. 1c, f, i). The control experiments, in which the primary antibody or secondary antibody was omitted and normal rabbit IgG was used, did not find any positive staining, suggesting that the immunoreactivity of IL-10 in red nucleus was specific (Fig. 1j, k, l). The area ratio of IL-10 positive cells and IOD values of sham and SNI groups above those of control group were analyzed. In SNI group, the area ratio of IL-10 positive cells and the IOD values in the contralateral RN were 0.08 ± 0.02 and 10.98 ± 3.46, respectively. While in the sham-operated group, the area ratio of IL-10 positive cells and the IOD values in the contralateral RN were 0.08 ± 0.02 and 2.65 ± 1.23, respectively (Fig. 2). After statistic analysis, no significant difference ($P > 0.05$) was found in the area ratio of IL-10 positive cells between SNI and sham-operated groups (Fig. 2a), but a significantly increased IOD level ($P < 0.01$) was observed in the contralateral RN of SNI group (Fig. 2b). These results indicated that SNI rats and sham-operated rats had the same amount of IL-10 positive cells in their contralateral RN, while the protein expression

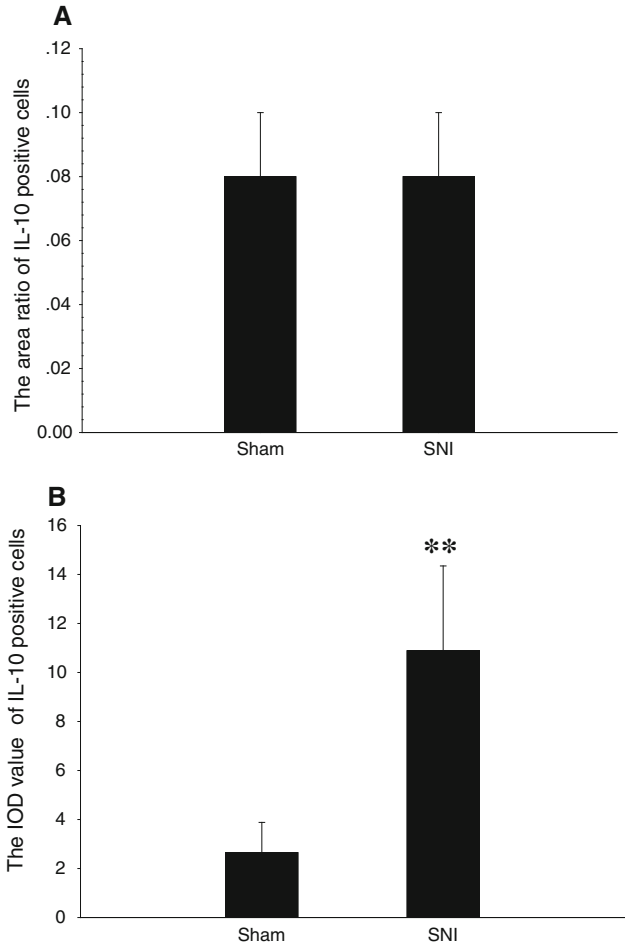

Fig. 2 Quantitative analysis of IL-10 immunoreactive intensity in the contralateral red nucleus (RN) of spared nerve injury (SNI) and sham-operated rats. The area ratio of IL-10 positive cells (**a**) in the RN did not show any significant difference between SNI and sham-operated groups ($P > 0.05$), while the integrated optical density (IOD) level (**b**) in the RN of SNI rats was significantly higher than that of sham-operated rats ($P < 0.01$), suggesting that SNI rats and sham-operated rats have the same amount of IL-10 positive cells in their contralateral RN, while the protein expression of IL-10 in positive cells was up-regulated significantly in the SNI rats as compared with that of sham-operated rats. ** $P < 0.01$, compared with sham-operated group (one-way ANOVA)

of IL-10 in positive cells was up-regulated significantly in the SNI rats as compared with that of sham-operated rats.

Effect of IL-10 on the Allodynia Induced by SNI

Three weeks after SNI, total 32 rats with mechanical allodynia (the withdrawal thresholds before and after SNI were 11.73 ± 2.59 g and 0.89 ± 0.10 g, respectively) was divided into four groups randomly. After microinjection of different doses of IL-10 (1.0, 0.5, 0.1 μg/μl) and normal saline into the RN contralateral to the nerve injury paw, the mechanical allodynia induced by SNI was depressed in a dose-dependent manner ($r = 0.999$, $P < 0.001$). The mean withdrawal thresholds during the 60 min (10–70 min) observation period were 2.31 ± 1.26 g ($n = 8$) for 1.0 μg/μl of IL-10, 1.61 ± 0.58 g ($n = 8$) for 0.5 μg/μl of IL-10, 1.19 ± 0.30 g ($n = 8$) for 0.1 μg/μl of IL-10 and 1.06 ± 0.09 g ($n = 8$) for normal saline, respectively. As shown in Fig. 3, the time course curves (i.e., normal saline treated group and three different doses of IL-10 treated groups) were significantly different among treatments ($F(3,126) = 5.046$, $P = 0.009$), across times ($F(6,126) = 47.864$, $P < 0.001$) and for their interaction ($F(18,126) = 8.807$, $P < 0.001$). Further analyses indicated that 1.0 μg/μl of IL-10 microinjected into RN significantly increased the withdrawal threshold of SNI rats ($t = 3.54$, $P = 0.012$), the peak analgesic effect occurred at 50 min and thereafter gradually reduced to the baseline at 70 min. IL-10 at dose of 0.5 μg/μl also increased the withdrawal threshold of SNI rats and displayed obvious analgesic effect at 50 min ($t = 3.678$, $P = 0.004$). However, 0.1 μg/μl of IL-10 did not show any effect on the mechanical allodynia ($P > 0.05$) as compared with the normal saline treated group. The detailed comparisons at each time point among groups and the microinjection sites of IL-10 and normal saline in RN region are shown in Fig. 3.

Discussion

Red nucleus is an important nucleus of extracorticospinal tract, and comprises an important subcortical relay station of a massive descending motor tract (rubrospinal tract). Previous studies have suggested that RN is involved in regulating muscle tension, motor learning, triggering conditioned motor responses, postural corrections, modification of jaw movements and the recovery of movement after spinal injury [33–38]. Most neurons in the RN in the intact and decerebrate cat exhibit phasic discharge preferentially in the swing phase of locomotion, during which they influence the activity of flexor muscles [34]. Unilateral lesions of the RN in rats give rise to a characteristic asymmetry in which abnormal braking and propulsive forces are produced during locomotion [35]. Apart from its well established roles in motor system, recent studies suggest that RN is involved in pain processing and aversive events. The studies have shown that stimulation of peripheral nerve or limbs could cause the changes of electrical activities of neuron in RN and chemical or electrical stimulation of RN increases the pain threshold and produces analgesic effect assessed during nociceptive pain experiments [39–41]. Furthermore, RN receives the fibers from the sensorimotor cortex and it has been suggested that cortex employs the rubrospinal tract to suppress the nociceptive transmission from the spine.

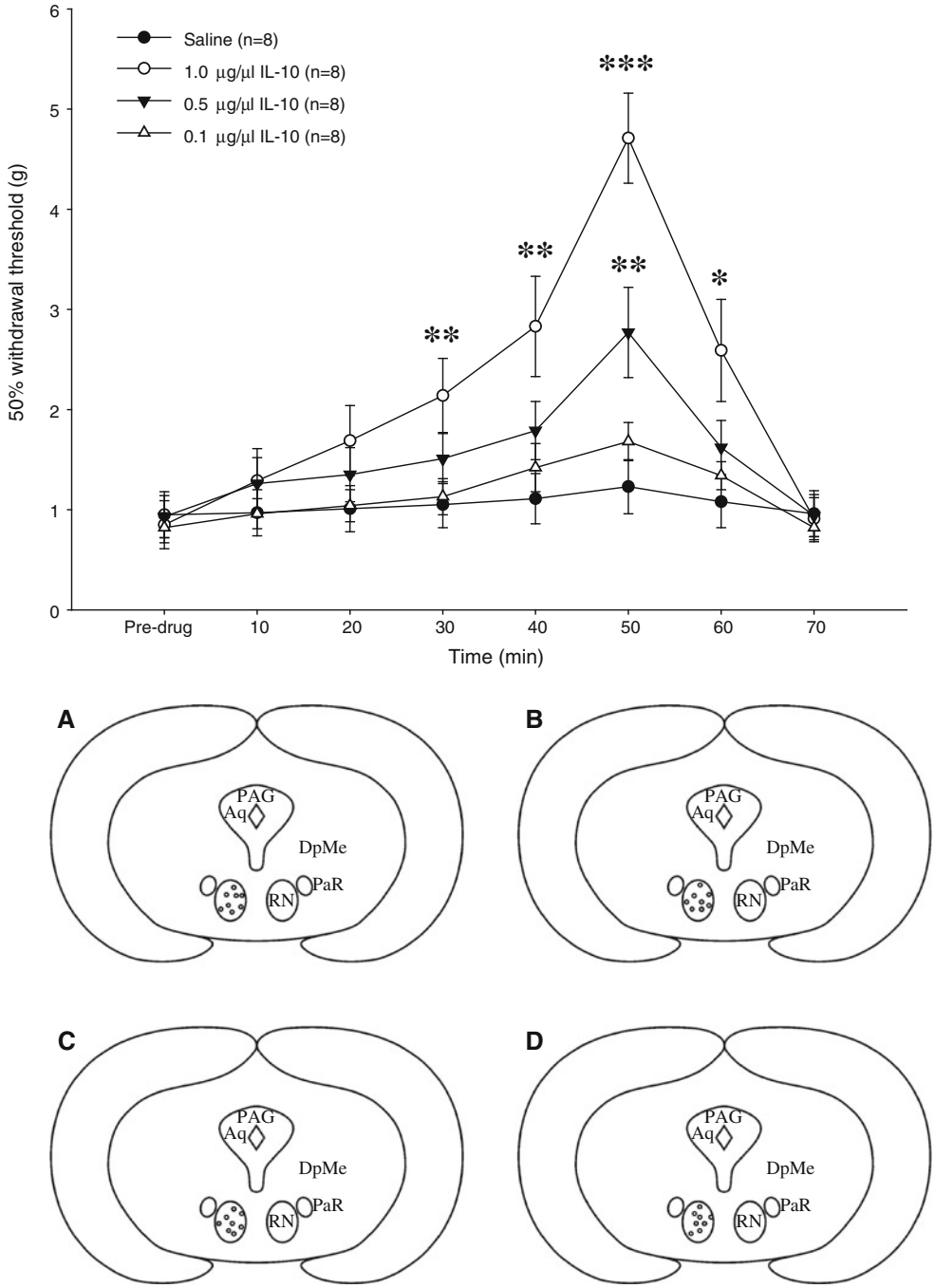

Fig. 3 Time course curve graph showing the anti-allodynia effect of different doses (1.0, 0.5 and 0.1 μg/μl) of IL-10 microinjected into the contralateral red nucleus (RN) of spared nerve injury (SNI) rats. During 60 min (10–70 min) observation period, 1.0 μg/μl of IL-10 microinjected into RN significantly increased the withdrawal threshold ($P = 0.012$) of SNI rats. 0.5 μg/μl of IL-10 also increased the withdrawal threshold of SNI rats and displayed obvious analgesic effect at 50 min ($P = 0.004$). However, 0.1 μg/μl of IL-10 did not show any effect on the mechanical allodynia ($P > 0.05$) as compared with normal saline treated group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with normal saline group at those time points (two-way RM ANOVA). **a** The locations of 1.0 μg/μl of IL-10 microinjection sites in RN region. **b** The locations of 0.5 μg/μl of IL-10 microinjection sites in RN region. **c** The locations of 0.1 μg/μl of IL-10 microinjection sites in RN region. **d** The locations of normal saline microinjection sites in RN region. *RN* red nucleus, *PAG* periaqueductal gray, *Aq* aqueduct, *PaR* pararubral nucleus, *DpMe* deep mesencephalic nucleus

Our previous studies have demonstrated that the expression of TNF-α, IL-1β and nerve growth factor (NGF) are up-regulated in the contralateral RN of SNI rats, and microinjection of their corresponding antibodies could alleviate the mechanical allodynia induced by SNI [24, 25, 42]. These results suggest that TNF-α, IL-β and NGF in RN are involved in the development of neuropathic pain and play facilitated roles on the mechanical allodynia induced by SNI. In the current study, we observed an increased expression of IL-10 in the contralateral RN of rats at 3 weeks following SNI, suggesting that not only the pro-inflammatory cytokines but also anti-inflammatory cytokine IL-10 may be involved in the pain modulation mediated by RN. This is consistent with previous studies that IL-10 is up-regulated in CNS during inflammatory or neuropathic pain [3, 6, 7].

To further study the roles of IL-10 in the development of neuropathic pain, different doses of IL-10 were microinjected into the RN contralateral to the nerve injury side of SNI rats and the changes of mechanical withdrawal threshold were measured dynamically. Results indicate that IL-10 could dose-dependently decrease the mechanical allodynia induced by SNI, suggesting that IL-10 of RN is involved in the development of neuropathic allodynia and plays inhibitory roles in SNI rats. This is consistent with studies that systemic or intrathecal administration of IL-10 protein or IL-10 gene therapy can attenuate the pain-related behaviors [12–23]. Combining with our previous studies [24, 25, 42], we conclude that RN is involved in the modulation of neuropathic pain and plays both facilitated roles through pro-inflammatory cytokines and inhibitory roles through anti-inflammatory cytokine IL-10.

Previous studies have identified that IL-10 receptors express on the membrane surface of only astrocytes and microglia, but not spinal cord neurons [8, 43]. That is, IL-10 can not play the analgesic effect by directly acting on neurons, but by acting on activated astrocytes and microglia and then indirectly affects the activation of neurons. Further studies indicate that IL-10 plays the analgesic effect mainly by suppressing the production of some chemokines and cell adhesion molecules, and further inhibiting the recruitment and activation of immune cells [4]; reducing the production of pro-inflammatory cytokines (e.g., IL-1, IL-6 and TNF-α) at multiple levels, including transcription, translation and release [4, 15, 44–46]; interrupting the pro-inflammatory cytokines signaling by down-regulating the expression of pro-inflammatory cytokine receptors [44, 45]. In addition, IL-10 can inhibit the production of reactive oxygen and nitrogen intermediates [46]. Although various ways are involved in IL-10 mediated analgesic effect, which ways are involved in the analgesic effect of red nucleus IL-10 on earth still need further studies to verify.

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