


Resveratrol enhances IL-4 receptor-mediated anti-inflammatory effects in spinal cord and attenuates neuropathic pain following sciatic nerve injury

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Abstract

Resveratrol has been showed to relieve neuropathic pain through its anti-inflammatory effects on the peripheral nerve system. However, it is not clear whether resveratrol, especially when administered systemically, is effective in alleviating the peripheral neuropathy-induced imbalance between pro- and anti-inflammatory responses in the central nervous system. To test this, we used a rat neuropathic pain model resulting from chronic constriction injury of the sciatic nerve. Resveratrol (200 mg/kg) or vehicle (dimethylsulfoxide) were administered intraperitoneally once daily for 14 consecutive days after chronic constriction injury. We found that resveratrol attenuated mechanical allodynia and thermal hyperalgesia in rats with chronic constriction injury. After 14 days of resveratrol treatment, expression of several anti-inflammatory cytokine receptors, including IL-1RA and IL-1R2, was increased in the dorsal spinal cord of rats with chronic constriction injury, and IL-4R α was increased in dorsal spinal cord neurons. Knockdown of IL-4R α in a neuronal cell line reversed the resveratrol-induced upregulation of IL-1RA and IL-1R2. These results indicate that resveratrol enhances IL-4 receptor-mediated anti-inflammatory responses in the spinal cord and thus might contribute to the alleviation of central sensitization following peripheral nerve injury.

Keywords

Resveratrol, nerve injury, central sensitization, pro-inflammatory response, anti-inflammatory response

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Introduction

Inflammatory mechanisms in the peripheral nervous system and central nervous system (CNS) play important roles in the development of neuropathic pain. In response to nerve injury, infiltration of inflammatory cells and activation of resident immune cells lead to the production and secretion of various inflammatory mediators.^{1,2} These mediators, including cytokines, can be broadly categorized into two main families based on their effects on inflammation; namely, pro-inflammatory (e.g., tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6) and anti-inflammatory (e.g., IL-4 and IL-10) mediators.^{3,4} After peripheral nerve damage, pro- and anti-inflammatory responses are activated in the injured nerve, the dorsal root ganglion, and in the spinal cord, but the pro-inflammatory

response prevails, thereby contributing to peripheral and central sensitization.^{5–7} Restoring the balance between pro- and anti-inflammatory mediators has been shown to be effective in treating neuropathic pain.

Resveratrol (3,5,4'-trihydroxystilbene, Res) is a natural polyphenolic compound with many beneficial properties, including anti-inflammatory, anti-oxidative

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stress, and anti-tumorigenic activities.^{8–10} Res also has neuroprotective effects and improves the pathological and behavioral outcomes of various types of nerve injury, including stroke,¹¹ traumatic brain injury,¹² spinal cord injury,¹³ and peripheral nerve injury.¹⁴ A number of mechanisms have been proposed to explain Res-induced neuroprotection, many of which invoke its anti-inflammatory effects.^{15,16} For example, Res controls pain by attenuating peripheral sensitization via repression of inflammatory responses in the nerve or local tissue.^{17,18} Res has also been shown to inhibit the expression of pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, and to promote the expression of the anti-inflammatory cytokine IL-10 in the peripheral nerve system of animals suffered from neuropathic pain.^{19,20} The anti-inflammatory effect of Res in the spinal cord also involves inhibition of glial activation.¹⁶ However, it remains unclear whether Res influences the balance between pro- and anti-inflammatory responses in the spinal cord, which is an important contributing factor in central sensitization.

We previously demonstrated that intrathecal administration of Res attenuates neuronal hypersensitivity in the rat spinal cord.²¹ However, drugs are more commonly administered systemically in the clinical setting. Therefore, in this study, we used a rat chronic constriction injury (CCI) model to test the effects of intraperitoneally (i.p.) administered Res on neuropathic pain. We measured the expression of a panel of pro- and anti-inflammatory cytokines and their receptors in the dorsal spinal cord to investigate the ability of Res to regulate the balance between pro- and anti-inflammatory responses to spinal cord injury.

Materials and methods

Animals

Male Sprague Dawley rats (200–250 g) were maintained in a quiet living environment at constant temperature (23°C \pm 2°C) on a 12-h light/dark cycle. Food and water were available ad libitum. All experiments were implemented according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Chinese Council's Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering.

Chronic constriction injury model

CCI was performed according to the protocol of Bennett and Xie.²² Animals were anesthetized with 1% pentobarbital sodium (50 mg/kg, i.p. injection). After blunt separation of the biceps femoris, the left sciatic nerve was exposed. Four ligatures (4–0) were loosely tied

around the nerve at 1-mm intervals from the proximal spinal side to the bifurcation. The ligatures were tightened until they just induced a slight tremor of the operative limb. All nerve ligations were conducted by the same operator. For the sham group, the sciatic nerve was exposed for the same duration without ligation.

Behavioral testing

The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were measured on the plantar surface of a paw on the day before surgery and on days 3, 7, 11, and 14 post-surgery. Von Frey filaments (North Coast Medical, San Jose, CA) were used to examine MWT.²³ Rats were placed on a metal mesh floor, caged with a plastic box, and then left for 30 min to habituate before testing. Von Frey filaments were used to stimulate the plantar skin of the left hind-paw until paw withdrawal was observed. The measurements were repeated three times at 5-min intervals and the average minimum force (g) that initiated a withdrawal response was recorded. A plantar algesimeter (Tes7370, Ugo Basile, Comerio, Italy) was used to measure TWL. Rats were placed on a transparent glass plate in a plastic cage and habituated to the test environment for 30 min. A radiant heat instrument was placed underneath the glass and focused on the plantar skin of the left paw. TWL was defined as the time between the start of heat stimulation and paw withdrawal. The measurements were repeated three times at 5-min intervals and the average duration (s) was recorded.

Cell culture

The rat neuronal cell line PC12 (Cellbank, Shanghai, China) was maintained at 37°C in a 5% CO₂ incubator in complete medium consisting of RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA), 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), and 1% penicillin and streptomycin (Thermo Fisher Scientific). The medium was changed every 2 days. To determine the optimal Res dose, cells were treated with 10, 50, or 100 μ M Res (Sigma-Aldrich, Santa Clara, CA) for 24 h,²⁴ and IL-4R α messenger RNA (mRNA) levels were measured. Substantial upregulation was seen in the presence of 50 μ M Res, which was taken as the optimal dose for further experiments. The same volume of 0.1% dimethylsulfoxide (DMSO) in saline solution was used as vehicle. Expression of IL-4R α was silenced by transfection of cells with siRNA against rat IL-4R α (siRNA-1479 sense GCCUUCAGAAAGUGGGCAATT and antisense UU GCCCACUUUCUGAAGGCTT) or with a non-targeting negative control (NC) siRNA (sense UUCUU CGAACGUGUCACGUTT and antisense ACGUGA CACGUUCGGCGGAGAATT).

Eight groups of cells were tested in this study: blank control (no treatment, addition of medium alone), mock control (cells treated with transfection reagent without siRNA), si-NC, si-IL-4R α , Res, Res + si-IL-4R α , Res + si-NC, and Res + mock. Cells were transfected with 50 nM siRNA, based on the manufacturer's recommendation, using riboFect CP reagent (RiboBio, Guangzhou, China) for 24 h. For the Res + mock, Res + si-IL-4R α , and Res + si-NC groups, Res (50 μ m) was added to the cells before transfection. Cells were cultured for 24 h, and total RNA and proteins were extracted for analysis.

Drug administration

Res (Sigma-Aldrich) was dissolved in 2% DMSO and saline solution and injected (i.p. 200 mg/kg) into rats once daily for 14 days starting on the day of surgery. This dose was selected based on a literature review,²⁵ as well as previous studies which examined the effect of i.p. treatment of resveratrol on spinal pathology.^{13,26} The control groups were administered the same volume of 2% DMSO in saline.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed to quantify expression of inflammatory cytokines and receptors. On the 14th day post-surgery, rats were placed under deep anesthesia and euthanized. The ipsilateral dorsal part of the lumbar spinal cord was harvested and stored at -80°C until use for RNA or protein extraction. Total RNA was extracted using E.Z.N.A. Total RNA Kit II (Omega, Stamford, CT), and cDNA was synthesized using a reverse transcription kit (Takara Bio Inc., Otsu, Japan) and amplified using corresponding primers (Table 1). RT-qPCR was conducted with an ABI Prism, 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following thermal cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 29 s, and 72°C for 24s. β -Actin was probed as an endogenous control. Relative expression of the target genes compared with β -actin was calculated using the comparative threshold cycle ($\Delta\Delta\text{Ct}$) method.⁶

Immunofluorescence microscopy

On the 14th day post-surgery, rats were placed under deep anesthesia and infused with phosphate-buffered saline (PBS, 1 M, pH 7.4) and 4% paraformaldehyde. The lumbar spinal cord was harvested, frozen, and cut into 10- μ m transverse sections, and the sections were placed on glass slides. Slides were washed with PBS three times, each for 10 min, and blocked with 5%

Table 1. Primers for real-time quantitative polymerase chain reaction.

Gene	Oligonucleotides	(5' to 3')
β -actin	Forward	TCGTGCGTGACATTAAGAG
	Reverse	ATTGCCGATAGTGACCT
TNF- α	Forward	CCCAGACCCCTCACACTCAGAT
	Reverse	TTGTCCCTTGAAGAGAACCTG
TNFR1	Forward	CTTTCTAAGCGGAAATGAG
	Reverse	TCGGCACAGTAGACTGA
TNFR2	Forward	GATGTTAGGACTGGCGA
	Reverse	CGCTGTGACTCTTGCT
IL-1 β	Forward	CACCTTCTTTTCCTTCATCTTTG
	Reverse	GTCGTTGCTTGTCTCTCCTTGTA
IL-1RA	Forward	CTTATTGCCTCTGCCCTCTG
	Reverse	TGATTGGTCTGGACTGTGGA
IL-1RI	Forward	AGGGACAGACCTGTGATTA
	Reverse	TTCCAGTAGACAAGGTCCG
IL-1R2	Forward	CAGCCAAGAACTGCAAGGT
	Reverse	CGGGAGACTCAGAAACCCTT
IL-4	Forward	GGTCACAGAAAAAGGGACTCCAT
	Reverse	GCTCGTTCTCCGTGGTGTC
IL-4R α	Forward	GCAGACACATACTGGCTGGA
	Reverse	CATTGGTGTGGAGTGTGAGG
IL-10	Forward	TAAGGGTTACTTGGGTTGC
	Reverse	TATCCAGAGGGTCTTCAGC
IL-10R	Forward	CTGGTCACCCTGCCATTGAT
	Reverse	AGGCATGGCTAAAATCAAAAGAAAC

donkey serum in PBS for 1 h. The slides were then incubated with primary antibodies: rabbit anti-IL-4R α (1:200; Santa Cruz Biotechnology, Dallas, TX), mouse anti-NeuN (1:400; Cell Signaling Technology, Danvers, MA), goat anti-Iba-1 (1:400; Abcam, Cambridge, UK), and mouse anti-glial fibrillary acidic protein (GFAP) (1:400; Novus Biologicals, Littleton, CO) for 12 h at 4°C . The slides were washed with PBS and incubated with Dylight 488 donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA), Cy3 donkey anti-goat IgG (1:400; Abcam), or Dylight 549 donkey anti-mouse IgG (1:400; Jackson ImmunoResearch Laboratories) for 2 h at 4°C . The slides were washed, sealed with a coverslip using 4',6-diamino-2-phenylindole Fluoromount-G (AmyJet Scientific, Wuhan, China), and visualized with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired with a digital camera (Carl Zeiss) under the same light intensity for each stain.

Western blot analysis

The ipsilateral dorsal part of the lumbar spinal cords was collected from animals on the 14th day after CCI. Spinal tissue and PC12 cells were lysed in radioimmuno-precipitation assay buffer and centrifuged at $14,000 \times g$ for 30 min at 4°C . Proteins were electrophoresed in 10%

polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA). The membranes were blocked with 5% bovine serum albumin for 1 h and then incubated with rabbit polyclonal anti-IL-4R α (1:200; Santa Cruz Biotechnology) or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000; Cell Signaling Technology) for 24 h at 4°C. The membrane was washed and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:4000; ConWin Biotech, Beijing, China) for 2 h at room temperature. Immunoreactive bands were visualized using super ECL detection reagent (Merck Millipore). ImageJ software (National Institutes of Health, Bethesda, MD) was used to analyze band densities.

Statistical analysis

Data are presented as the mean \pm standard error of the mean. Two-way analysis of variance with repeated measures followed by Dunnett's test was used to compare

behavioral responses. All other data were analyzed using the Kruskal–Wallis test followed by Dunn's test for comparison of multiple groups, and the Mann–Whitney *U* test for comparison of two groups. $p < 0.05$ was defined as statistically significant.

Results

CCI induces neuropathic pain and upregulates pro- and anti-inflammatory signaling in the dorsal spinal cord

CCI induces obvious neuropathic pain in rats, manifesting as reductions of both MWT and TWL from 3 days to 14 days after surgery (compared with sham-operated rats, MWT $p < 0.001$ on days of 3, 7, 11, and 14 post-surgery, TWL $p < 0.05$ on days of 3 and $p < 0.001$ on days of 7, 11, and 14 post-surgery (Figure 1(a) and (b)).

We performed RT-qPCR analysis to examine mRNA levels of various cytokines and receptors in the ipsilateral dorsal spinal cord on day 14 after CCI or sham surgery.

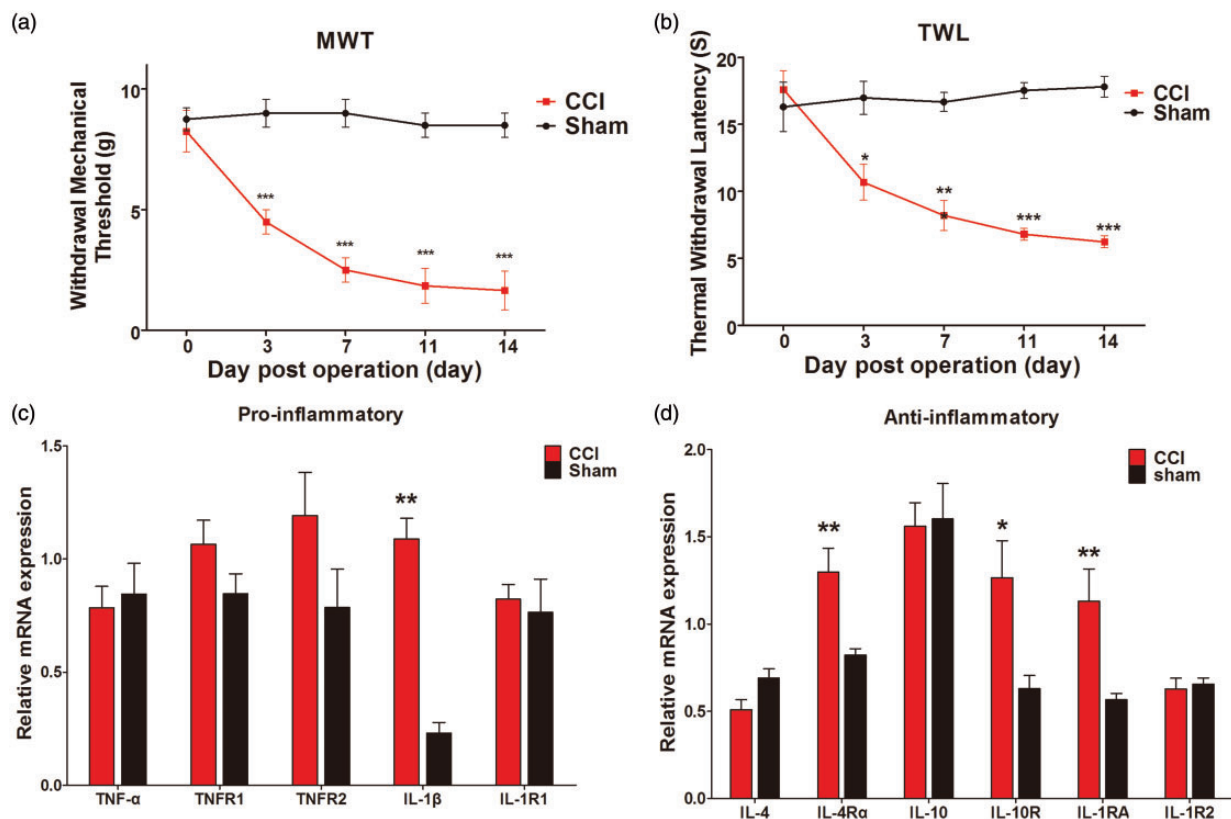


Figure 1. Evaluation of neuropathic pain and expression of pro- and anti-inflammatory cytokines and receptors in dorsal spinal cord after chronic constriction injury or sham surgery. (a) and (b) Mechanical withdrawal threshold and thermal withdrawal latency in the ipsilateral hindpaw were measured 1 day before and on days 3, 7, 11, and 14 after chronic constriction injury or sham surgery. (c) and (d) RT-qPCR analysis of mRNA expression of pro-inflammatory (TNF- α , TNFR1, TNFR2, IL-1 β , and IL-1R1) and anti-inflammatory (IL-4, IL-4R α , IL-10, IL-10R, IL-1RA, and IL-1R2) cytokines and receptors in the rat dorsal spinal cord after chronic constriction injury or sham surgery. Data are represented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for chronic constriction injury + Vehicle group versus Sham + Vehicle group ($n = 8$).

The pro-inflammatory genes examined were TNF- α and its receptors TNFR1 and TNFR2, and IL-1 β and its receptor IL-1R1, and the anti-inflammatory genes were IL-4 and its receptor IL-4R α , IL-10 and its receptor IL-10R, the IL-1R antagonist (IL-1RA), and the IL-1 decoy receptor IL-1R2. We found that CCI dramatically increased expression of the pro-inflammatory cytokine IL-1 β ($p < 0.01$) (Figure 1(c)), consistent with our previous work.⁶ Alternatively, several anti-inflammatory factors were also enhanced; namely, IL-4R α ($p < 0.01$), IL-1RA ($p < 0.01$), and IL-10R ($p < 0.05$), where especially the latter was highly amplified (Figure 1(d)). These results suggest that sciatic nerve injury activates both pro- and anti-inflammatory signaling in the spinal cord.

Res attenuates the duration of neuropathic pain and further enhances the anti-inflammatory signaling in the spinal cord

After CCI or sham surgery, rats were treated by i.p. injection with Res (200 mg/kg) or 2% DMSO (vehicle) in saline once daily for 14 days. As shown in Figure 2(a) and (b), the CCI + vehicle rat group showed significantly greater sensitivity to mechanical and thermal stimulation than did the sham + vehicle group ($p < 0.001$). Compared with vehicle treatment, i.p. injection of Res significantly increased tolerance in the MWT test on days 11 and 14 post-surgery ($p < 0.001$) (Figure 2(a)) and in the TWL test on day 14 post-surgery ($p < 0.001$) (Figure 2(b)). These results indicate that treatment with Res attenuated the duration of CCI-induced neuropathic pain.

Treatment of CCI rats with Res further upregulated IL-4R α , IL-1RA, and IL-1R2 mRNA levels compared with vehicle treatment ($p < 0.05$) (Figure 2(d)), whereas IL-1 β mRNA levels were unaffected by Res (Figure 2(c)). Since IL-1RA and IL-1R2 mRNA levels can be regulated by IL-4R signaling, we also examined the expression of IL-4R α protein in spinal cord by western blotting and immunofluorescence staining. Consistent with the mRNA results, IL-4R α protein levels were increased after CCI ($p < 0.05$), and the expression was further elevated by Res treatment ($p < 0.01$) (Figure 2(e) and (f)). Immunofluorescence staining revealed that IL-4R α expression was mainly increased in the superficial dorsal horn, indicating a potential relationship between IL-4R α and nociceptive signaling (Figure 2(g) and (h)). To identify the cell type(s) expressing IL-4R α after the treatment of Res, we performed double immunofluorescence staining for IL-4R α and NeuN (neuronal marker), GFAP (astrocyte marker), or Iba-1 (microglial marker) (Figure 3(g) to (i)).

IL-4R α knockdown reverses Res-activated transcription of anti-inflammatory genes

To investigate the relationship between IL-4R α , IL-1RA, and IL-1R2 expression further, we examined the effects of small interfering RNA (siRNA)-mediated silencing of IL-4R α expression in PC12 cells, a rat neuronal cell line. In preliminary experiments, we performed a dose-response analysis of Res effects on IL-4R α expression and found marked upregulation of IL-4R α mRNA in cells treated with 50 μ M Res compared with vehicle ($p < 0.001$) (Figure 4(a)). Therefore, we compared the effects of 50 μ M Res in PC12 cells on the mRNA expression of IL-4R α , IL-1RA, IL-1R2 as well as STAT6, a transcription factor which is required for gene transcription responding to IL-4 signaling.^{27,28} Notably, all these genes were significantly upregulated by treatment with Res compared with vehicle ($p < 0.01$ for STAT6, $p < 0.05$ for IL-1RA, $p < 0.001$ for IL-4R α and IL-1R2) (Figure 4(b)). Similarly, IL-4R α protein expression was upregulated by Res treatment ($p < 0.01$) (Figure 4(c)).

Next, cells were transfected with IL-4R α -targeting or non-targeting NC siRNAs. We verified that IL-4R α expression was specifically knocked down with high efficiency. The IL-4R α mRNA and protein levels were reduced over 70% and 50%, respectively, compared with si-NC-transfected cells ($p < 0.01$) (Figure 4(d) and (e)). Importantly, mRNA levels of STAT6 as well as IL-1RA and IL-1R2 in Res-treated cells were decreased by transfection with si-IL-4R α compared with si-NC ($p < 0.05$) (Figure 4(f)). These results indicated that knockdown of IL-4R α inhibits Res-activated transcription of anti-inflammatory genes.

Discussion

In this study, we demonstrated that Res treatment significantly alleviates neuropathic pain in rats subjected to CCI, a model of sciatic nerve injury. Res treatment also enhanced IL-4R α receptor-mediated signaling in dorsal spinal cord neurons, leading to increased expression of IL-1RA and IL-1R2 mRNA. Thus, Res-induced upregulation of these anti-inflammatory molecules might contribute to the ability of Res to reduce central sensitization following peripheral nerve injury.

Pro-inflammatory response in the spinal cord contributes to central sensitization after CCI

Consistent with our previous work,⁶ we found that the pro-inflammatory cytokine IL-1 β is increased in the dorsal spinal cord of rats on day 14 after CCI. Considerable evidence suggests that IL-1 β expression

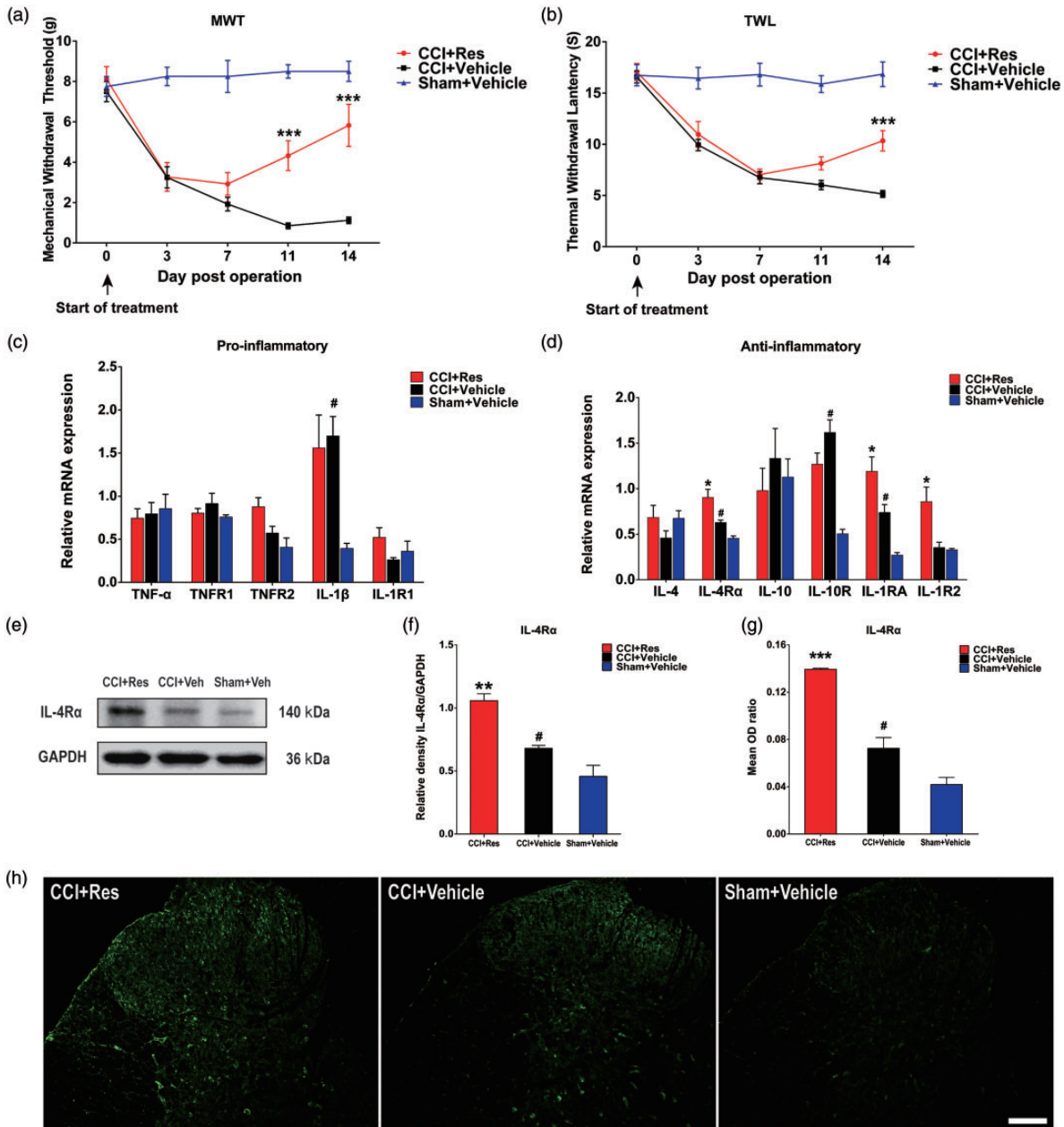


Figure 2. Effects of Res treatment on neuropathic pain and expression of pro- and anti-inflammatory cytokines and receptors in dorsal spinal cord of chronic constriction injury rats. Res (200 mg/kg) or DMSO (vehicle) in saline solution were intraperitoneally injected once daily for 14 days starting on the day of chronic constriction injury or sham surgery. (a) and (b) Mechanical withdrawal threshold and thermal withdrawal latency in the ipsilateral hindpaw were measured 1 day before and on days 3, 7, 11, and 14 after surgery ($n = 12$). (c) and (d) RT-qPCR analysis of mRNA expression of pro-inflammatory (TNF- α , TNFR1, TNFR2, IL-1 β , and IL-1R1) and anti-inflammatory (IL-4, IL-4R α , IL-10, IL-10R, IL-1RA, and IL-1R2) cytokines and receptors in rats subjected to chronic constriction injury or sham surgery and treated with Res or vehicle ($n = 8$). (e) and (f) Western blot (e) and densitometric quantification (f) of IL-4R α protein expression ($n = 8$). GAPDH was probed as a loading control. (g) and (h) Quantified immunofluorescence intensity (g) and images (h) of IL-4R α staining in the ipsilateral dorsal spinal cord ($n = 4$). Data are represented as mean \pm SEM. # $p < 0.05$ for CCI + Vehicle group versus Sham + Vehicle group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for CCI + Res group versus CCI + Vehicle group. Scale bar = 100 μ m.

in the CNS induces hyperalgesia in animal models.^{29–31} However, we also found increased mRNA expression of the anti-inflammatory receptors IL-1RA, IL-4R α , and IL-10R in the dorsal spinal cord following CCI. These

results indicate that both pro- and anti-inflammatory cytokine signaling are activated in the spinal cord after CCI, but the pro-inflammatory response may dominate and contribute to central sensitization.³²

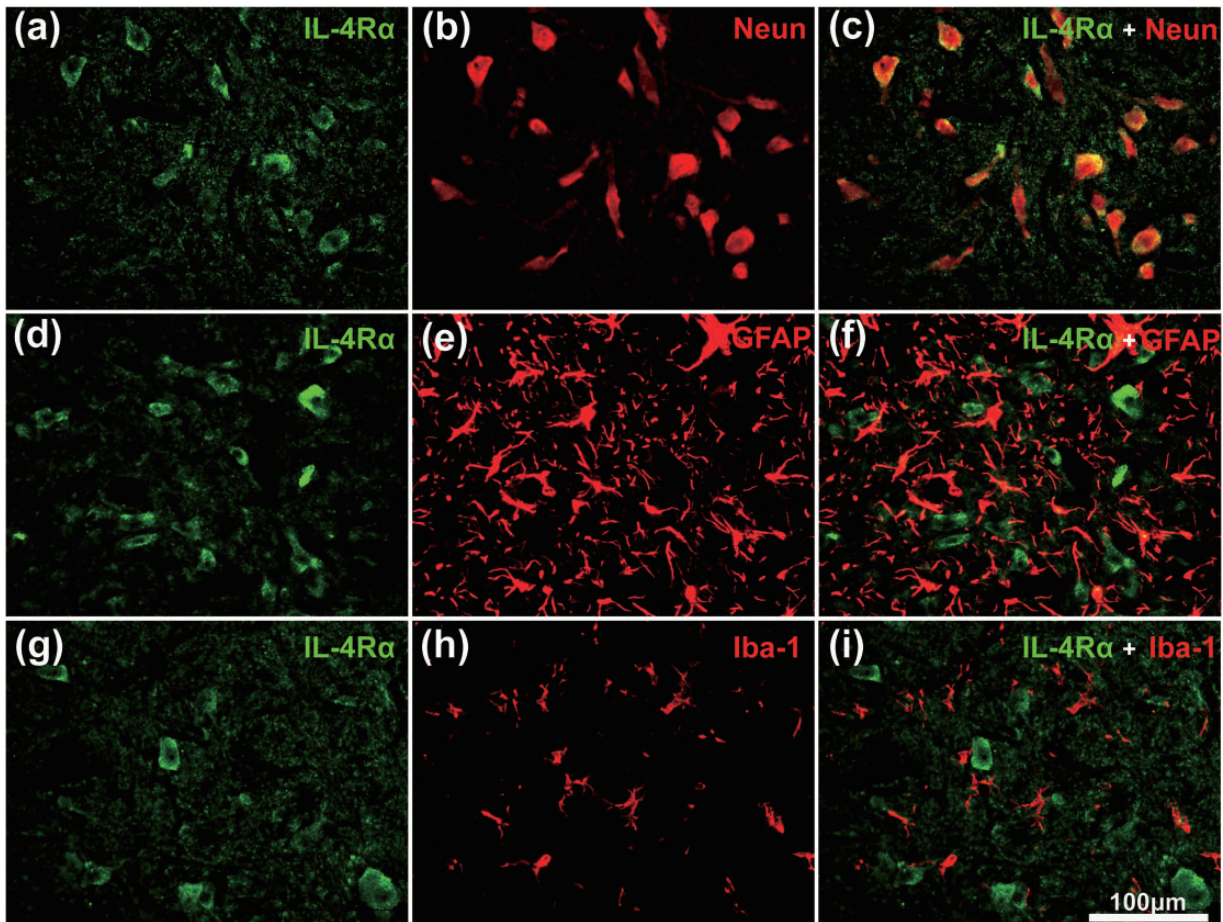


Figure 3. Double immunofluorescence staining for IL-4R α with NeuN, GFAP, or Iba-1 in the dorsal spinal cord of Res-treated chronic constriction injury rats. Sections were stained with antibodies to IL-4R α (green) alone (a), (d) and (g), or together with a neuronal marker (b, NeuN), an astrocyte marker (e, GFAP), or a microglial marker (h, Iba-1) (red). The merged images (c), (f), and (i) show that IL-4R α is mostly localized in neurons (c). Scale bar = 100 μ m.

Res may inhibit IL-1 β -mediated inflammation by enhancing the expression of IL-1RA and IL-1R2

Daily injections of CCI rats with Res alleviated pain-related behavior on days 11 and 14 after surgery, indicating that systemic Res treatment can attenuate the duration of neuropathic pain. Although Res has previously been shown to have anti-inflammatory effects in the peripheral nervous system,¹⁹ our study shows that Res also restores the balance between pro- and anti-inflammatory responses in the CNS following peripheral nerve injury. Although Res cannot suppress upregulation of IL-1 β in the dorsal spinal cord, it can block its effect.^{33,34} Current data suggest that all known actions of IL-1 are mediated via a single cell surface receptor, IL-1R1.^{35,36} We demonstrated that treatment with Res increased IL-1RA and IL-1R2 mRNA levels in the rat dorsal spinal cord. IL-1RA binds more efficiently to IL-1R1 than does IL-1 β and is thus able to block IL-1R1–IL-1 β interactions.³⁷ IL-1R2 is structurally

similar to IL-1R1 and acts as a decoy receptor to block IL-1 signaling.^{38,39} By enhancing the expression of IL-1RA and IL-1R2, Res may inhibit IL-1 β -mediated inflammation^{40,41} in the spinal cord and thus attenuate central sensitization following peripheral nerve injury.⁴²

Activation of IL-4R signaling contributes to Res-mediated upregulation of IL-1RA and IL-1R2

Res-mediated upregulation of IL-4R α , IL-1RA and IL-1R2 may be due to the activation of intracellular signal transduction pathways. In the cellular level, Res works as a nutrient signaling molecular that activates a number of stimulus-responsive transcription factors such as STAT6, CREB, AP-1, Egr-1, Elk-1, and Nrf2.^{43,44} Gene transcription of IL-4R α , IL-1RA, and IL-1R2 has been shown to be resulted from these activations.^{27,45} Besides, the upregulation of IL-1RA and IL-1R2 requires IL-4R signaling. Although we did not detect a change in IL-4 mRNA levels on day 14 of Res

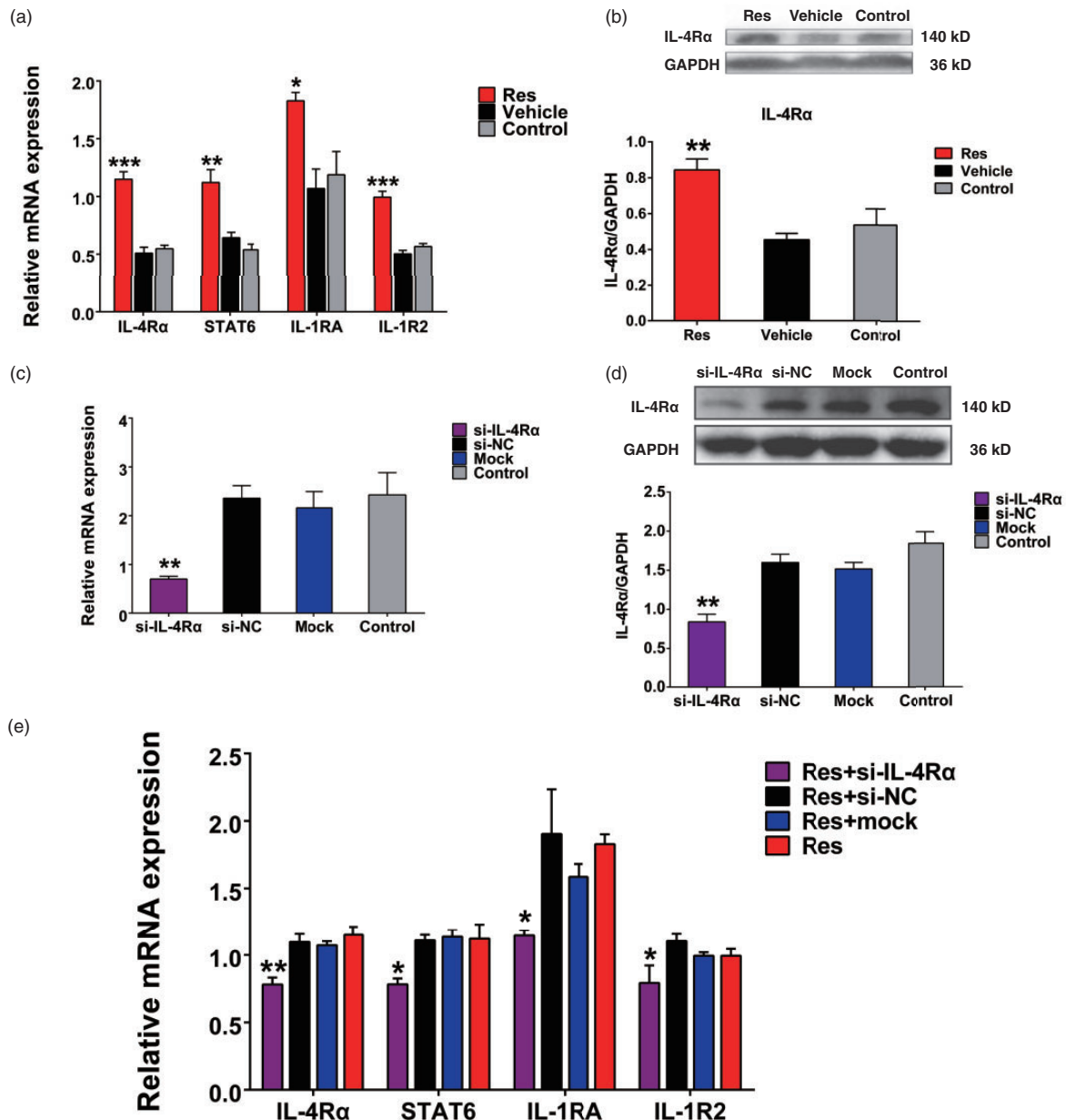


Figure 4. Effect of IL-4R α knockdown in Res-treated PC12 cells. (a) Dose-response analysis on the mRNA expression of IL-4R α in PC12 cells treated 10 μ M, 50 μ M, and 100 μ M Res, medium (control), or DMSO (vehicle). *** p < 0.001 for 50 μ M Res group versus vehicle group (n = 3) (b) RT-qPCR analysis of IL-4R α , IL-1RA, and IL-1R2 mRNA levels in PC12 cells treated 50 μ M Res. * p < 0.05, *** p < 0.001 for Res group versus vehicle group (n = 3). (c) Western blot analysis (upper) and densitometric quantification (lower) of IL-4R α protein expression in cells treated 50 μ M Res. GAPDH was probed as a loading control ** p < 0.01 for Res group versus vehicle group (n = 4). (d) and (e) Cells were untreated, mock-transfected, or transfected with negative control or IL-4R α -specific siRNA for 24 h and then tested for mRNA (d) and protein (e) expression of IL-4R α . Data are represented as mean \pm SEM. ** p < 0.01 for si-IL-4R α group versus si-NC group (n = 3). (f) RT-qPCR analysis of IL-4R α , IL-1RA, and IL-1R2 mRNA levels in PC12 cells 24 h after treatment as in (d) and (e) with 50 μ M Res added before transfection. Data are represented as mean \pm SEM. * p < 0.05 and ** p < 0.01 for Res + si-IL-4R α group versus Res + si-NC group (n = 3).

treatment, IL-4R α expression was significantly increased in the dorsal spinal cord neurons, suggesting that there is indeed an increased response to IL-4.⁴⁶ Moreover, IL-4 signaling is known to increase both IL-1RA and IL-1R2

mRNA expression.^{47–49} We found not only that IL-4R α , IL-1RA, and IL-1R2 are all elevated in the spinal cord of Res-treated rats, but also that IL-4R α knockdown in PC12 neurons reversed the Res-induced upregulation of

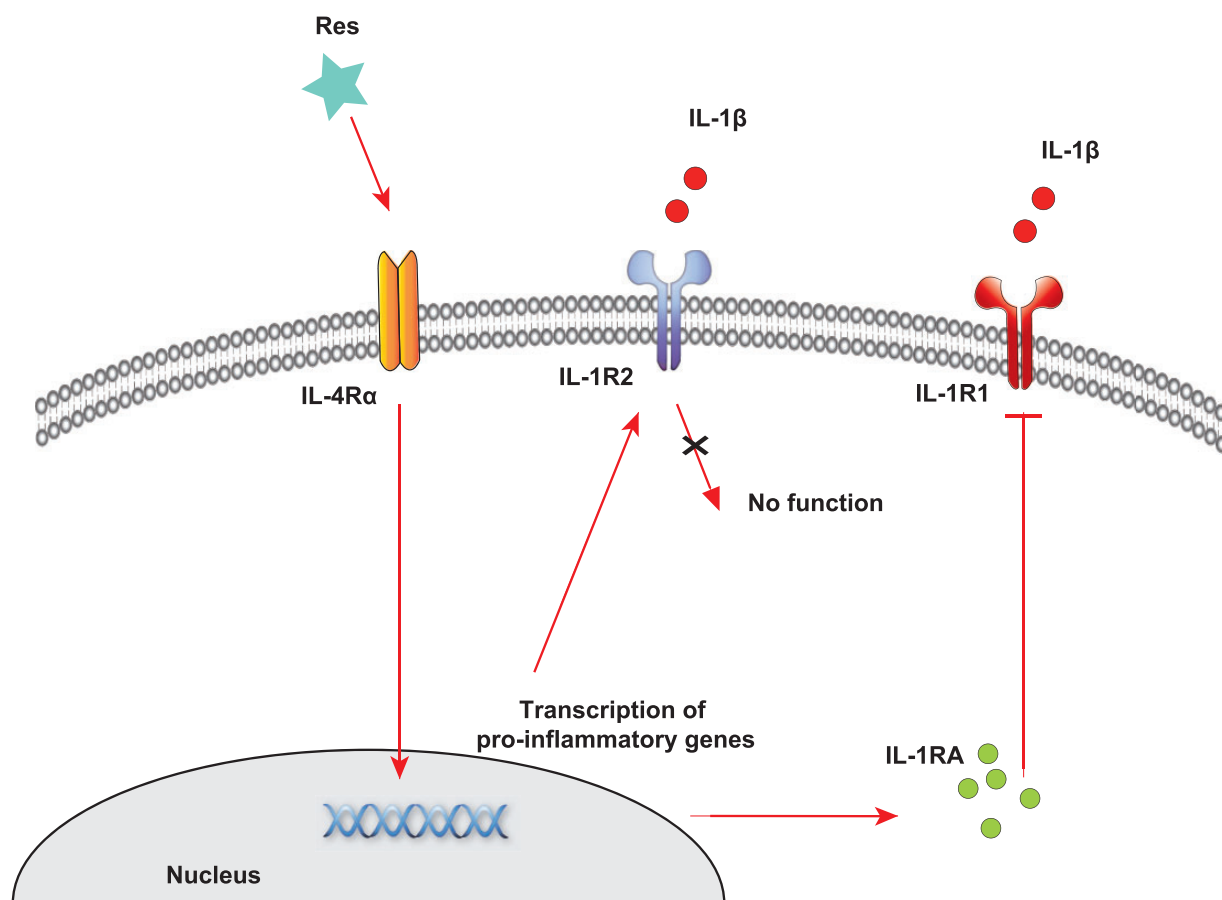


Figure 5. Schematic representation of Res-mediated anti-inflammatory effects in neurons. Res enhances the transcription of IL-1RA and IL-1R2 via IL-4R α signaling. IL-1RA prevents IL-1 β from interacting with IL-1R1, and IL-1R2 acts as a decoy target for IL-1 signaling.

IL-1RA and IL-1R2. These results suggest that Res might act on the IL-4R α to facilitate its anti-inflammatory effects (Figure 5).

Conclusion

Systemic administration of Res attenuated neuropathic pain in the CCI rat model. Res may alleviate central sensitization by enhancing IL-4R-mediated anti-inflammatory signaling in the dorsal spinal cord.

Author Contributions

CH conceived and designed the experiments. MX and ZC performed the experiments. ZD analyzed the data. CH, YW, and QG wrote the paper.

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Declaration of Conflicting Interests

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