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Original Article

Paroxetine alleviates rat limb post-ischemia induced allodynia through GRK2 upregulation in superior cervical ganglia

Jun Tang[1], Jing Dong[2], Li Yang[1], Lingqi Gao[2], Jijian Zheng[2]

[1]Department of Anesthesiology, Jinshan Hospital, Fudan University, Shanghai, China; [2]Department of Anesthesiology, Shanghai First People's Hospital, Shanghai Jiaotong University Affiliated Shanghai First People's Hospital, 650 Xin Songjiang Road, Shanghai 201620, China

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Abstract: Long-lasting neuroplastic changes induced by transient decrease in G protein-coupled receptor kinase 2 (GRK2) in nociceptors enhances and prolongs inflammatory hyperalgesia. Here, we investigated the effects of paroxetine (a selective serotonin reuptake inhibitor and GRK2 inhibitor) on GRK2 expression in superior cervical ganglion (SCG) in a rat model of complex regional pain syndrome type I (CRPS-I). After ischemia-reperfusion (I/R) injury, the ipsilateral 50% paw withdrawal thresholds (PWTs) to mechanical stimuli and the expression levels of GRK2 protein and mRNA in the ipsilateral SCGs all decreased significantly; the ipsilateral cold allodynia scores increased significantly. No significant differences were found in the contralateral side except GRK2 mRNA reduced significantly at day 2-day 9 after I/R injury, but still higher than those in ipsilateral SCGs. After paroxetine administration, the ipsilateral 50% PWTs at day 2, 7, 14, and 21 were significantly higher than those in control group; The GRK2 protein and mRNA levels in ipsilateral SCGs were also significantly up-regulated after day1; The ipsilateral cold allodynia scores were significantly reduced after day7. No significant differences were found in the contralateral 50% PWTs, cold allodynia scores, and GRK2 protein level except GRK2 mRNA levels increased significantly at day1-day7 after paroxetine administration. Therefore, a transient decrease of GRK2 expression in SCG neurons might be involved in the development and maintenance of allodynia in CRPS-I and paroxetine might alleviate this allodynia through GRK2 protein upregulation in SCGs.

Keywords: G protein-coupled receptor kinase 2, complex regional pain syndrome type I, mechanical and cold allodynia, superior cervical ganglion neurons, paroxetine, selective serotonin reuptake inhibitor

Introduction

Complex regional pain syndrome (CRPS) is a devastating pain condition characterized by chronicity and relapses that can cause significant disability [1, 2]. Different from other neuropathic pain syndromes, CRPS is seemingly disproportionate in time or degree to the usual course of any known trauma or lesions, and usually characterized by regional sensory abnormality (allodynia or hyperalgesia), motor dysfunction (paresis, tremor, dystonia), and autonomic changes (edema, alterations in sudomotor, skin color or temperature, etc.) [3-5]. In spite of many years of intensive studies, the underlying mechanisms of CRPS and its pathophysiology are still not clear. Therefore, there is no effective treatment for CRPS so far, and polypharmacy is often used to address different CRPS symptoms [6].

Anti-depressants have long been used for managements of neuropathic pain and several other intractable pain conditions, such as trigeminal neuralgia, low back pain, fibromyalgia, etc. [7-9]. Tri-cyclic antidepressants are superior to newer antidepressants including selective serotonin reuptake inhibitors (SSRIs) in treating patients with diabetic neuropathy and postherpetic neuralgia, but SSRIs (fluoxetine, paroxetine, citalopram) are better tolerated [10-12]. Due to the difference in the development and/or maintenance of neuropathic pain, the efficacy of SSRIs on different types of pain varies dramatically. Paroxetine is more effective than fluoxetine and citalopram in reducing pain in patients with diabetic neuropathy and postherpetic neuralgia, but no analgesic effect has been reported in low back pain, rheumatoid arthritis, osteoarthritis [11, 13, 14]. The analgesic effect on neuropathic pain induced by

chronic constriction injury of the sciatic nerve in rats is also inconsistent [9, 15]. Whether paroxetine can be used to manage CRPS is unknown.

Our recent study showed that a transient decrease in G protein-coupled receptor kinase 2 (GRK2), a member of the GRK family that primarily regulates the desensitization of G protein-coupled receptors (GPCRs) in SCG neurons might be involved in the development and maintenance of allodynia in a rat model of CRPS-I. Our findings are in line with other studies that a transient GRK2 decrease was found in inflammatory hyperalgesia and epinephrine-induced hyperalgesia [16-18]. It is known that paroxetine has diverse mechanisms in controlling the anti-allodynic pain independent of its antidepressant effect [9, 19, 20]. Whether paroxetine can alleviate the allodynic pain of CRPS through modulating GRK2 expression in SCG is unknown.

The present study aimed to examine the effects of paroxetine on GRK2 expression in SCGs using a rat limb ischemia-reperfusion injury model to explore the mechanism of sympathetic nerve-mediated allodynia associated with CRPS-I.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 210-270 g were purchased from Experimental Animal Center, Shanghai First People's Hospital, Shanghai, China. They were housed in groups of 3-4 under a 12-h light/dark cycle, with food and water available *ad libitum*. All experimental procedures were approved by the Animal Care Committee at Shanghai First People's Hospital affiliated to Shanghai Jiaotong University and were conducted according to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised in 1996) and the ethical guidelines of the International Association for the Study of Pain (Zimmerman, 1983).

Establishment of an ischemia-reperfusion injury model of CRPS-I in the rat forelimb

Rat ischemia-reperfusion injury model of CRPS-I was developed according to the protocol reported by Coderre et al. [21]. Briefly, after anesthesia with intraperitoneal injection of 10% chloral hydrate, a Nitrile 70 Durometer rubber O-ring (Internal diameter 2.5-4.0 mm, Wuxi Wo'erding Trade Co., Ltd, Wuxi, China) was placed around rat's left forelimb instead of the hindlimb, just proximal to the ankle joint and left it on the forelimb for 3 h. Adjusting the tight fit of O-ring to make sure a complete blockade of rat forelimb arterial blood flow. Anesthesia was controlled to ensure that the rats recovered from anesthesia within an hour after reperfusion.

Assessments of mechanical and cold allodynia

To more precisely assess the mechanical and cold allodynia, all of the studied rats were placed in a cage (21 × 16 × 27 cm^3) with a wire grid bottom for adaption at least 30 min until cage exploration and major grooming activities stopped. we first assessed mechanical allodynia, then cold allodynia (5-10 min later) to reduce the effects of cold stimulation on the mechanical threshold [22]. Mechanical and cold allodynia were measured before ischemia; at 24 h and 48 h after reperfusion; and on days 1, 2, 7, 14, and 21 after paroxetine or normal saline administration. Paroxetine was administered daily for a maximum of 7 days after 48 h of reperfusion.

Mechanical allodynia was assessed by von Frey filament (Stoelting) stimulation [23]. A filament with bending force of 8 g in our study was first used, then the filaments were subsequently changed according to the up-and-down method first described by Dixon [24] since our preliminary experimental results and other study results showed that 8 g von Frey filament bending force did not elicit a response in normal rats. The filament was vertically applied to the plantar surface of forepaws until buckling against for approximately 6-8 s. The stimulation intervals were 30 s for a positive response and 15 s for a negative response. Rapid withdrawal of the paw or flinching on removal of the filaments was considered as a positive response to mechanical allodynia. Ambulation was considered an ambiguous response, and the stimulus was repeated. The 50% response threshold was calculated using the following formula: 50% threshold = $(10^{[Xf + K\delta]})/10{,}000$, where X_f is the log value of the last used von Frey filament, K is the tabular value for the pat-

tern of positive/negative responses, and δ is the log value of mean differences between stimuli (0.224) [23]. Animals with a >30% mechanical threshold decrease after I/R were included in the study [25].

Cold allodynia was assessed using the method reported by Flatters and Bennett [26]. Briefly, 50 μL of acetone was dropped onto the plantar surface of the rats' forepaws without touching the skin, and assessed within 20 s. Normal rats do not respond or occasionally respond to acetone with a very small and brief withdrawal. Behavioral responses to the acetone drops after I/R were rated by a 4-point scale: 0, no response; 1, quick withdrawal or flicking or stamping of the paw; 2, prolonged withdrawal or repeated flicking of the paw; and 3, repeated flicking of the paw with persistent licking directed at the ventral side of the paw. The acetone was applied three times to each forelimb at 5-min intervals. The sum of three individual scores was the final score of cold allodynia threshold. Therefore, the minimum score was 0 and the maximum possible score was 9.

Drug administration

Sixty rats with limb allodynia induced by I/R were randomly allocated to control group and paroxetine group (n = 30 in each group). Each group was further divided into 5 subgroups (n = 6, per subgroup) according to the time points of treatment after reperfusion (day 1, day 2, day 7, day 14, and day 21). 10 mg/kg paroxetine (Sigma-Aldrich, St. Louis, MO, USA) was daily given to the rats in paroxetine group through rat's tail vein under sevoflurane anesthesia for consecutive 7 days except the rats in day1 subgroup received only one day of paroxetine injection and the rats in day 2 subgroup received two days of paroxetine injection. The control group rats received same volumes of 0.9% normal saline.

GRK2 immunohistochemistry

GRK2 immunohistochemistry was performed on both sides of SCGs before ischemia; at 48 h after reperfusion, and on days 1, 2, 7, 14, and 21 after paroxetine or saline injection. All fixed SCGs with 4% paraformaldehyde were sectioned into 4-6-μm-thick tissue slides. Endogenous peroxidase was inactivated by 3% hydrogen peroxide and epitope retrieval was done with EDTA in a microwave oven, Affinity-purified rabbit polyclonal anti-GRK2 antibody (#3982, dilution:1/100, Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase-conjugated goat anti-rabbit antibody (PV-9001, ZSGB-BIO, Beijing, China) were used to detect GRK2 immunoreactivity. A horseradish peroxidase-based chromogens-substrate system, including DAB (brown) chromogen (ZLI-9017, ZSGB-BIO, Beijing, China), combined with hematoxylin (blue) counterstain of the nuclei was used to facilitate the visualization of GRK2 immunoreaction. The integrated optical density (IOD) of GRK2 protein expression was analyzed using Image-Pro Plus 6.0 (Media Cybemetics Company, USA).

GRK2 mRNA in situ hybridization

5 μm thick SCG tissue sections mounted onto poly-D-lysine-coated glass slides were used to do GRK2 mRNA in situ hybridization. The slides were first incubated at 65°C for a day, then deparaffinized with xylene and rehydrated. These sections were further prepared by 20 μg/ml proteinase K (Roche Diagnostics, Basel, Switzerland) for 30 minutes at 37°C, and finally hybridized with GRK2 gene fluorescence probe with a 33-base oligonucleotide (agcaaccct-cagcaccctcttgcccctccatg) (labeled Spectrum Green) for 16 h at 37°C after probe denaturation in 70% formamide for 5 min at 70°C. Fluorescence microscope was used to detect GRK2 mRNA expression, and Image-Pro Plus 6.0 (Media Cybemetics Company, USA) was used to analyze mean optical density (IOD/Area) of GRK2 mRNA expression.

Data analysis

All data were presented in figures as means ± standard errors of the mean (SEM) and analyzed with Origin 8.0 (OriginLab Corp., Northampton, USA). Unpaired or paired Student's t-tests were used to compare GRK2 protein and mRNA levels in rat SCGs between groups, while one-way ANOVA was used for comparison within groups. For comparison of nonparametric data between groups including mechanical and cold allodynia scores, the Mann-Whitney U test or paired-sample Wilcoxon signed rank test was used. Kruskal-Wallis ANOVA was used for data analysis within each group. A *P*-value of <0.05 was denoted statistically significant.

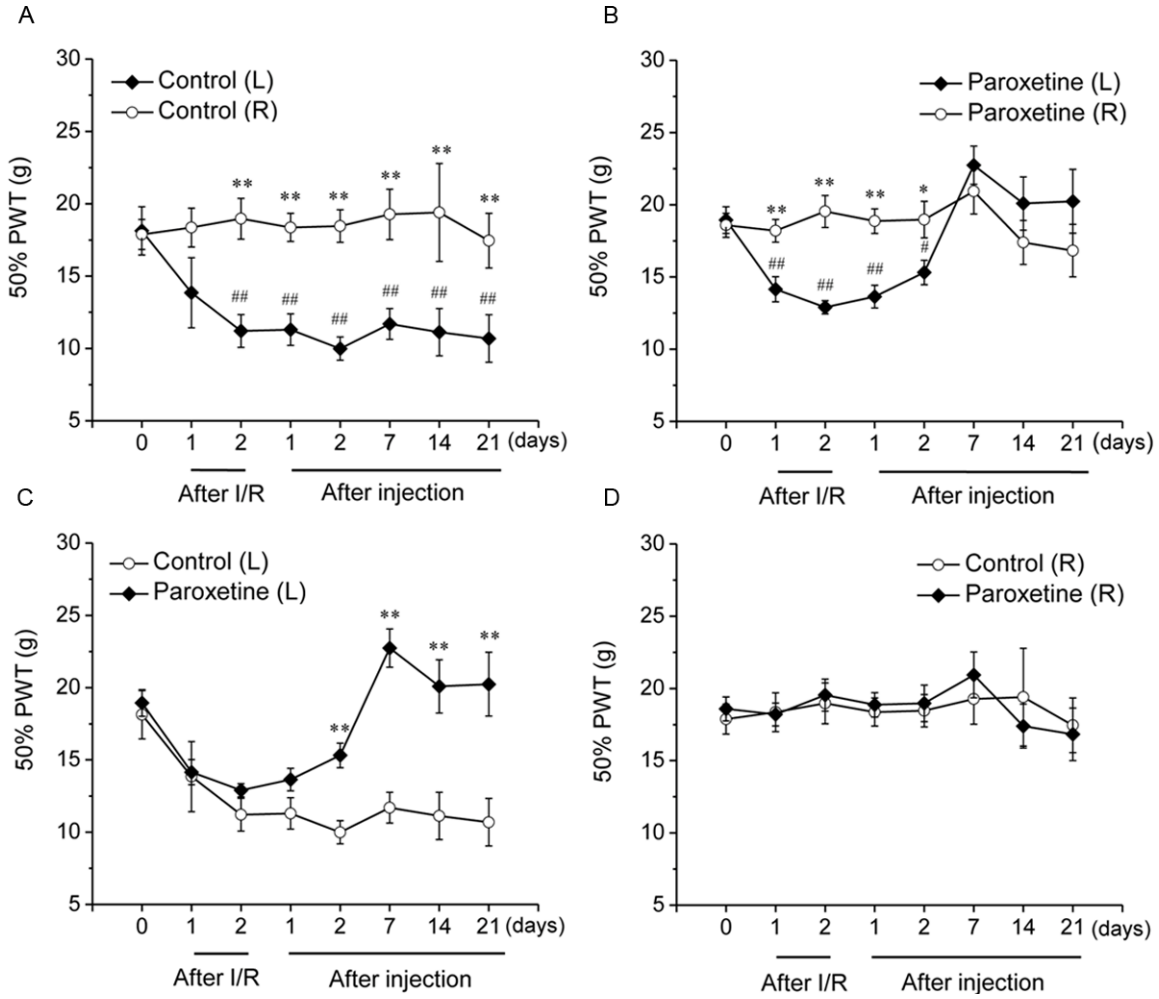

Figure 1. Effects of paroxetine on PWTs to mechanical stimuli applied to the plantar surface of forepaws by von Frey filaments in rat ischemia-reperfusion model of CRPS-I. Mechanical allodynia was induced in rat left forelimb by I/R. The 50% PWT was determined before ischemia, 24 h and 48 h after reperfusion, and on days 1, 2, 7, 14, and 21 after drug or normal saline administration. All the results are expressed as means ± standard errors of mean (n = 6 per subgroup at each time point). A: Time course of 50% PWTs changes in the ipsilateral (L) and contralateral (R) forelimbs after rat left forelimb I/R injury. B: Effects of paroxetine on the ipsilateral (L) and contralateral (R) forelimbs 50% PWTs. C: Effects of paroxetine on the ipsilateral (L) forelimb 50% PWTs. D: Effects of paroxetine on the contralateral (R) forelimb 50% PWTs. PWTs, paw withdrawal thresholds; I/R, ischemia-reperfusion; chronic regional pain syndrome type I (CRPS-I). The Mann-Whitney U test or paired-sample Wilcoxon signed rank test was used to compare data between Control (L) and (R), Paroxetine (L) and (R), Control (L) and Paroxetine (L), Control (R) and Paroxetine (R) at each time point. *P<0.05, **P<0.01. The Kruskal-Wallis ANOVA test was used to determine statistical significance within Control (L), Control (R), Paroxetine (L), Paroxetine (R) at each time point. #P<0.05, ##P<0.01 compared with baseline values (before ischemia).

Results

Effects of paroxetine on mechanical allodynia induced by I/R injury in the rat forelimb

In present study, the 50% PWTs on the ipsilateral forelimb of CRPS-I model rats decreased significantly, but no significant threshold difference was found in the contralateral forepaw among the different time points (**Figure 1A**); Daily injection of 10 mg/kg of paroxetine for a maximum of 7 days significantly increased the 50% PWTs in the ipsilateral forelimb, and the 50% PWTs gradually returned to baseline after 7 days of paroxetine administration, no significant effects were found (**Figure 1B**, **1C**). Compared to control group, the mechanical withdrawal thresholds on the ipsilateral forelimb significantly increased on days 1, 2, 7, 14, and 21 after paroxetine injection (**Figure 1C**);

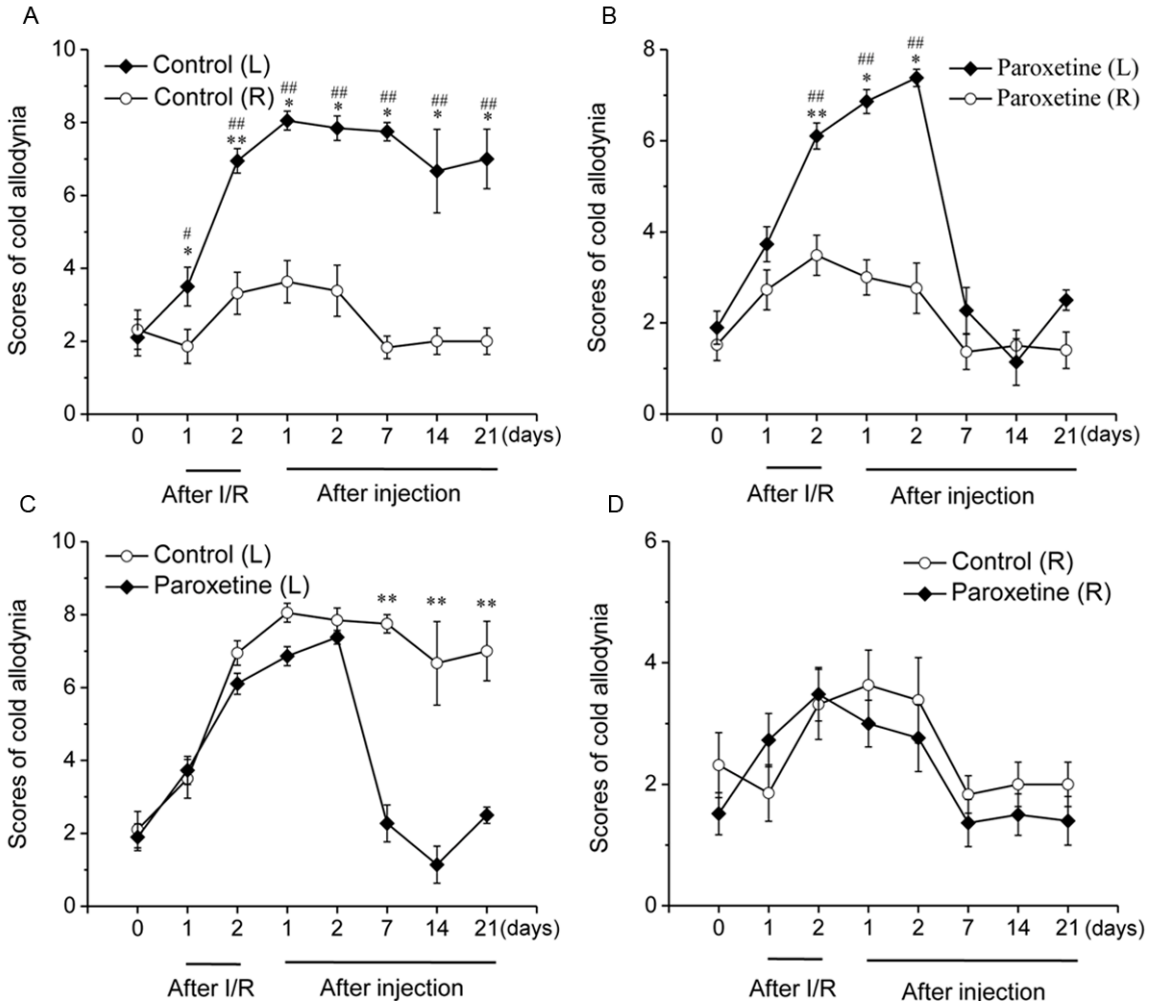

Figure 2. Effects of paroxetine on cold allodynia score induced by dropping acetone on the plantar surface of forepaws in rat ischemia-reperfusion model of CRPS-I. Scores were assessed before ischemia, at 24 h and 48 h after reperfusion, and on days 1, 2, 7, 14, and 21 after drug or normal saline injection. All the results are expressed as means ± standard errors of the mean (n = 6 per group at each time point). A: Time course of cold allodynia score changes in the ipsilateral (L) and contralateral (R) forelimbs after rat left forelimb ischemia-reperfusion injury. B: Effects of paroxetine on the ipsilateral (L) and contralateral (R) forelimbs cold allodynia scores. C: Effects of paroxetine on the ipsilateral (L) forelimb cold allodynia scores. D: Effects of paroxetine on the contralateral (R) forelimb cold allodynia scores. I/R, ischemia-reperfusion; CRPS-I, chronic regional pain syndrome type I. The Mann-Whitney U test or paired-sample Wilcoxon signed rank test was used to compare data between groups, *P<0.05, **P<0.01; The Kruskal-Wallis ANOVA test was used to determine the statistical significance within each group at each time point, #P<0.05, ##P<0.01 compared with baseline values (before ischemia).

There were no significant differences in the mechanical withdrawal thresholds on the contralateral forelimb between the control and paroxetine groups (**Figure 1D**).

Effects of paroxetine on cold allodynia induced by I/R in the rat forelimb

Cold allodynia scores in the ipsilateral side of CRPS - I model rats increased significantly starting from 24 h after reperfusion compared with preischemia scores and those in the contralateral side at each time point (**Figure 2A**), no significant changes of cold allodynia scores in the contralateral forepaw were found even though the absolute values of cold allodynia scores increased at 48 h, 72 h, 96 h after reperfusion. Daily injection of 10 mg/kg paroxetine for a maximum of 7 days significantly reduced the cold allodynia scores in the ipsilateral side on days 7, 14, 21, and the cold allodynia scores nearly returned to the baseline values (**Figure 2B**). There were no significant effects of paroxetine injection on the cold allo-

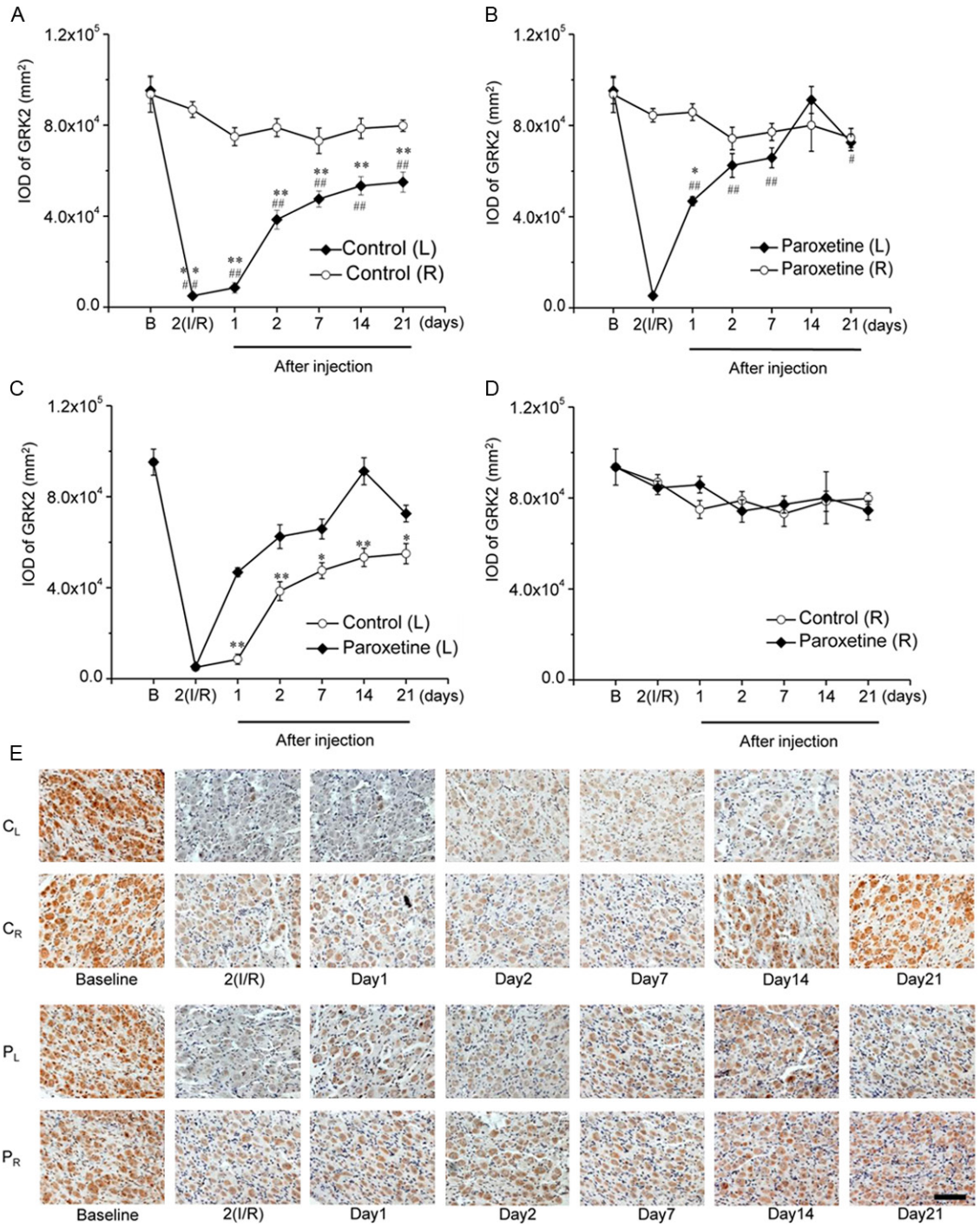

Figure 3. Effects of paroxetine on GRK2 protein expression in SCGs in rat ischemia-reperfusion model of CRPS-I. GRK2 protein expression in SCGs was determined by measuring the integrated optical density using Image-pro plus 6.0 software before ischemia, 48 h after reperfusion, and on days 1, 2, 7, 14, and 21 after the daily injection of paroxetine for a maximum of 7 days. All the results are expressed as means ± standard errors of the mean (n = 6 per group at each time point). A: Time course of GRK2 protein expression changes in ipsilateral (L) and contralateral (R) SCGs after rat left forelimb I/R injury. B: Effects of paroxetine on GRK2 protein expression in ipsilateral (L) and contralateral (R) SCGs. C: Effects of paroxetine on GRK2 expression in ipsilateral (L) SCGs. D: Effects of paroxetine on GRK2 protein expression in contralateral (R) SCGs. E: Representative photomicrographs of GRK2 protein expression (brown color) in ipsilateral (L) and contralateral SCGs. EC_L: GRK2 protein expression in ipsilateral (L) SCGs in control group. EC_R: GRK2 protein expression in contralateral (R) SCGs in control group. EP_L: GRK2 protein expression in ipsilateral (L) SCGs in paroxetine group. EP_R: GRK2 protein expression in contralateral (R) SCGs in paroxetine group.

I/R, ischemia/reperfusion; SCGs, superior cervical sympathetic ganglions; CRPS-I, chronic regional pain syndrome type I; GRK2, G protein-coupled receptor kinase 2. GRK2 protein expression in rat SCGs was compared between groups by paired or unpaired Student's t-test (*P<0.05, **P<0.01) and one-way ANOVA (#P<0.05, ##P<0.01) within groups. Scale bar, 50 μm.

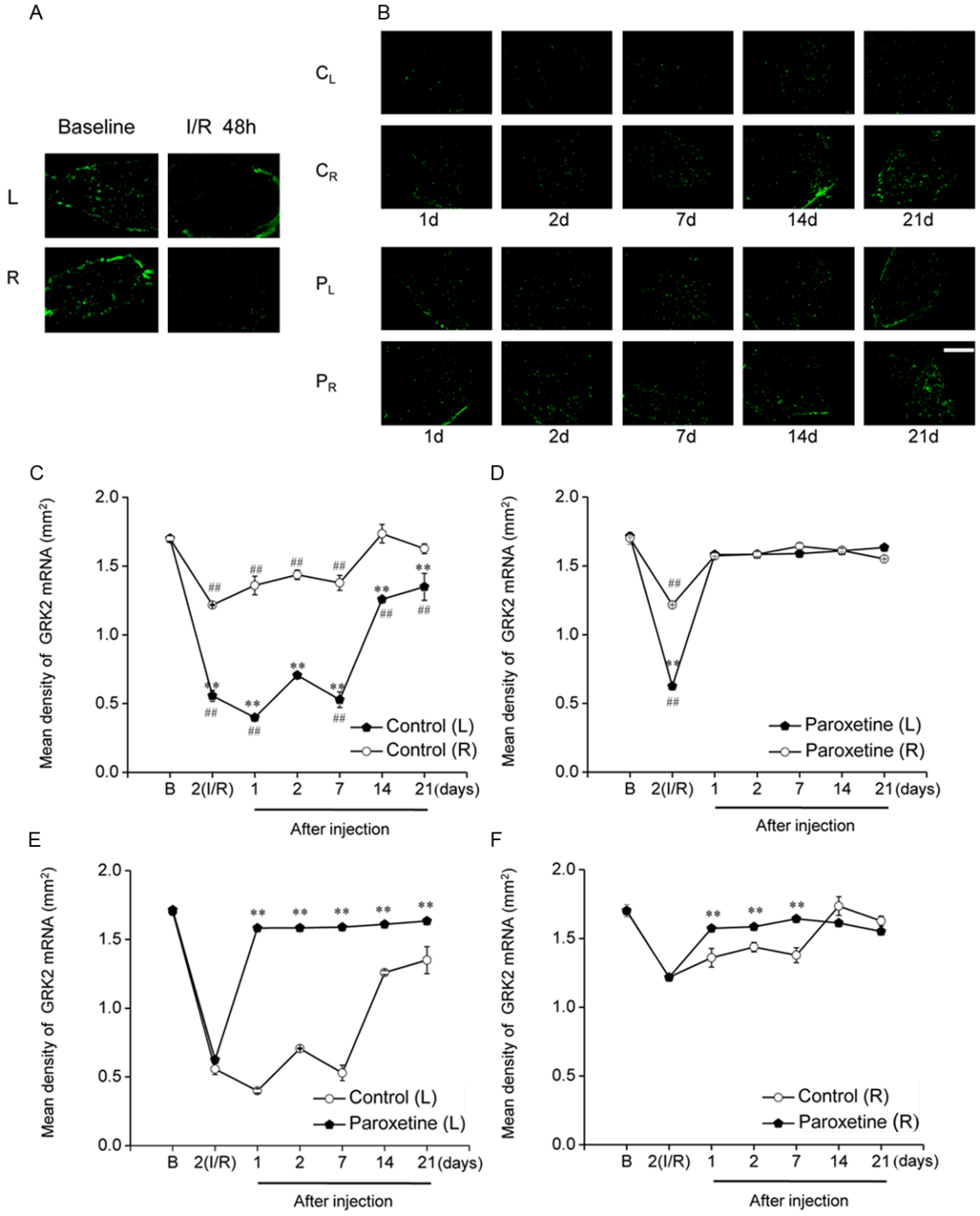

Figure 4. Effects of paroxetine on GRK2 mRNA expression in in SCGs in rat ischemia-reperfusion model of CRPS-I. GRK2 mRNA expression in SCGs was determined by measuring the mean optical density using Image-pro plus 6.0 software before ischemia, 48 h after reperfusion, and on days 1, 2, 7, 14, and 21 after the daily injection of paroxetine for a maximum of 7 days. All the results are expressed as means ± standard errors of the mean (n = 6 per group at each time point). A: Representative photomicrographs of GRK2 mRNA expression (green color) in ipsilateral

(L) and contralateral (R) SCGs at the baseline and I/R 48 h. B: Representative photomicrographs of GRK2 mRNA expression (green color) in ipsilateral (L) and contralateral (R) SCGs after paroxetine or normal saline injection. BC_L: GRK2 mRNA expression in ipsilateral (L) SCGs in control group. BC_R: GRK2 mRNA expression in contralateral (R) SCGs in control group. BP_L: GRK2 mRNA expression in ipsilateral (L) SCGs in paroxetine group. BP_R: GRK2 mRNA expression in contralateral (R) SCGs in paroxetine group. C: Time course of GRK2 mRNA expression changes in ipsilateral (L) and contralateral (R) SCGs after rat left forelimb I/R injury. D: Effects of paroxetine on GRK2 mRNA expression in ipsilateral (L) and contralateral (R) SCGs. E: Effects of paroxetine on GRK2 mRNA expression in ipsilateral side (L) SCGs. F: Effects of paroxetine on GRK2 mRNA expression in contralateral (R) SCGs. I/R, ischemia/reperfusion; SCGs, superior cervical sympathetic ganglions; CRPS-I, chronic regional pain syndrome type I; GRK2, G protein-coupled receptor kinase 2. GRK2 mRNA expression in rat SCGs was compared between groups by paired or unpaired Student's t-test (*P< 0.05, **P<0.01) and one-way ANOVA within groups (#P<0.05, ##P<0.01). Scale bar, 50 μm.

dynia scores in the contralateral side (**Figure 2B**). Compared to control group, the cold allodynia scores in the ipsilateral side in the paroxetine group were significantly lower on days 7, 14, and 21 (**Figure 2C**). No significant differences of cold allodynia scores in the contralateral side were found between the control and paroxetine groups on days 1, 2, 7, 14, and 21 after paroxetine or saline injection (**Figure 2D**).

Effects of paroxetine on the expression of GRK2 protein in rat SCGs

Rat forelimb I/R injury induced a transient and significant decrease of GRK2 expression in the ipsilateral SCGs, which decreased to the lowest level at 48 h after reperfusion, and then it gradually recovered. However, it did not return to the baseline level even at 23 days after I/R injury (**Figure 3A** and **3EC$_L$**). Rat forelimb I/R injury did not induce any significant changes of GRK2 expression in the contralateral SCGs (**Figure 3A** and **3EC$_R$**). Daily injection of 10 mg/kg paroxetine for a maximum of 7 days significantly increased the expression of GRK2 in the ipsilateral SCGs starting from day 1 after paroxetine injection, and gradually returned to the baseline value at day 14 (**Figure 3B** and **3EP$_L$**). The IODs of GRK2 in the ipsilateral SCGs in paroxetine group were significantly higher than those in control group at each time point (**Figure 3C**, **3EC$_L$** and **3EP$_L$**). No significant differences of GRK2 expression in the contralateral side were found between the paroxetine and control groups (**Figure 3D**, **3EC$_R$** and **3EP$_R$**).

Effects of paroxetine on the expression of GRK2 mRNA in rat SCGs

In addition to the effects of paroxetine on GRK2 protein expression in CRPS-I model rat SCGs, we further explored the effects of paroxetine on the expression of GRK2 mRNA in rat SCGs through in situ hybridization experiments. Rat forelimb I/R injury also induced a transient and significant decrease of GRK2 mRNA expression in the ipsilateral SCGs, and lasted for over 3 weeks; GRK2 mRNA levels in the contralateral SCGs also decreased at day2 - day9 after I/R injury, but still much higher than those in the ipsilateral SCGs (**Figure 4A**, **4BC$_L$**, **4BC$_R$** and **4C**). Daily injection of 10 mg/kg paroxetine for a maximum of 7 days significantly increased the expression of GRK2 in the ipsilateral SCGs starting from day1 after paroxetine injection and nearly returned to baseline levels (**Figure 4BP$_L$**, **4D** and **4E**); In the contralateral SCGs, GRK2 mRNA levels returned to baseline levels at day1 after paroxetine injection (**Figure 4BP$_R$**, **4D** and **4F**).

Discussion

Complex regional pain syndrome type I characterized not only the features of inflammation pain, but also the features of sympathetic disturbance. The sympathetic disturbance is not just the symptoms of CRPS-I, but it also contributes to the pathophysiology of CRPS-I through interaction with inflammation [3, 27, 28]. Recent studies show that transient decrease of GRK2 expression in peripheral nociceptors, spinal cord microglia/macrophages, peripheral blood mononuclear cells, etc. can regulate the severity and duration of inflammatory hyperalgesia and neuropathic pain [17, 29-32]. In current study, rat forelimb ischemia-reperfusion induced a long time and significant decrease of GRK2 expression in ipsilateral SCGs lasting longer than 23 days after reperfusion, which corresponded with the time course of changes in ipsilateral limb mechanical and cold allodynia. Paroxetine, a selective serotonin reuptake inhibitor alleviated the mechanical and cold allodynia and normalized the decreased GRK2 expression in the ipsilateral sympathetic gan-

glia induced by I/R injury in rat forelimb, indicating that GRK2 upregulation in he ipsilateral SCGs might be involved in the anti-allodynic effects of paroxetine in rat ischemia-reperfusion model of CRPS-I.

In addition to anti-depression, paroxetine is often used to manage neuropathic pain and some other intractable pain conditions. However, its analgesic mechanism is SSRI independent [9, 33]. Recent studies show that antidepressant treatments including SSRIs can normalize the down-regulated GRK2 expression in blood cells and up-regulated GRK2 expression in brain in major depression, indicating that SSRIs can bidirectionally modulate GRK2 expression [34, 35]. In our study, paroxetine reversed the induced low GRK2 mRNA expression in both sides of SCGs on day1 after paroxetine injection, the induced low GRK2 protein expression in the ipsilateral SCGs returned to normal level on day 14 even though the ipsilateral GRK2 protein expression was significantly increased starting from day1, no significant changes of GRK2 protein expression in contralateral side, and finally the ipsilateral mechanical allodynia became much better starting from day 2 after paroxetine injection and the ipsilateral cold allodynia became much better starting from day 7. Although the recovery timings of GRK2 mRNA and protein expression in the ipsilateral SCGs and the mechanical and cold allodynia caused by paroxetine were different, the pain behaviors were alleviated with the recovery of GRK2 mRNA and protein expression in the ipsilateral SCGs.

Heat and mechanical hyperalgesia have been shown to be mediated by different afferent fibers and different adrenergic responses after neuropathic injury [36-38]. C-fibers mediate heat hyperalgesia rather than mechanical allodynia because capsaicin can semi-selectively spare small myelinated A_{δ}-afferent fibers and destroy C-fibers [36, 37, 39]. In a postherpetic pain model, mechanical dynamic allodynia was associated with injury to mouse sensory C-fiber neurons and little damage to A-fiber neurons [38]. In addition, sympathectomy prior to neuropathic injury was found to eliminate or delay the onset of heat hyperalgesia, but not mechanical allodynia [40, 41]. Therefore, the different effects of paroxetine on mechanical and cold allodynia in the rat CRPS-I models in our study might be due to the different mechanisms of mechanical and cold allodynia.

GRK2 was usually thought as the kinase that mediates G protein-coupled receptor (GPCR) desensitization and internalization through phosphorylate serine/threonine residues in the intracellular loops and the carboxyl-termini of GPCRs, and finally blocks the detrimental effects of persistent stimulation [42]. Therefore, inhibition of GRK2 activity might inhibit GPCR desensitization and internalization and enhance the detrimental effects of persistent stimulation. Different from other SSRIs, paroxetine was recently shown to be an effective inhibitor of GPCR phosphorylation and desensitization via direct binding to GRK2 [43]. Therefore, we first speculated that paroxetine might not produce analgesic effects or worsen pain behaviors if transient GRK2 decrease was involved in the development of allodynia. In fact, patoxetine produced significant analgesic effects and simultaneously increased the expression of GRK2 mRNA and protein in the ipsilateral SCGs. Recent studies showed that the desensitization of mGluR1, 5, and $GABA_B$ receptor is independent of GRK2 catalytic activity since overexpress of phosphorylating-deficient GRK2-K220R mutant can facilitate GPCR desensitization, indicating the possibility of GRK2 kinase activity independent pathways of GPCR desensitization and internalization [44-47]. Therefore, it is possible for paroxetine to produce antiallodynic effect through phosphorylation-independent pathway even if GRK2 catalytic activity is inhibited by paroxetine. Whether this phosphorylation-independent mechanism was involved in analgesic effects of paroxetine is needed for further research, other signaling pathways such as β-arrestins signaling, or mechanisms such as inhibition on P2X4 receptors, increase of noradrenaline (NA) in spinal cord, allopregnanolone-mediated, etc., are also not excluded [9, 33, 34].

There are several limits in our studies. In this study, we only focused on the characteristics of mechanical and cold allodynia of CRPS, we did not explore the changes of edema, skin temperature and motor function, which are also the important characteristics of CRPS due to our lab limitation. The roles of GRK2 in other sites such as spinal cord, brain, peripheral blood cells, etc. are not simultaneously investigated. Due to the lack of targeted knockout techniques and SCG focal administration in our laboratory, whether the transient decrease in GRK2 expression in SCGs is an adaptive

change induced by forelimb I/R injury or a mediator of mechanical and cold allodynia associated with CRPS-I or the transition from acute to chronic pain remained unclear. Therefore, the precise role of GRK2 in the sympathetic nervous system of rat CRPS-I models and the possible sympathetic mechanism of paroxetine in alleviating mechanical and cold allodynia is still required to investigate.

In conclusion, a transient decrease in GRK2 expression in sympathetic ganglionic neurons might be involved in the development and maintenance of allodynia associated with CRPS-I. Paroxetine, a selective serotonin reuptake inhibitors and direct GRK2 inhibitor might alleviate allodynia associated with CRPS-I through GRK2 protein upregulation in sympathetic postganglia.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jijian Zheng, Department of Anesthesiology, Shanghai First People's Hospital, Shanghai Jiaotong University Affiliated Shanghai First People's Hospital, 650 Xin Songjiang Road, Shanghai 201620, China. Tel: (86) 021-37798758; Fax: (86) 021-63241377; E-mail: zhengjijian626@sina.com

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