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ORIGINAL ARTICLE

Promotion of thermal analgesia and neuropeptidergic skin reinnervation by 4-methylcatechol in resiniferatoxin-induced neuropathy

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Abstract To investigate whether 4-methylcatechol (4MC) could decrease the duration of the thermosensation disorder and promote the innervation of peptidergic intraepidermal nerve fibers (IENFs), we developed a resiniferatoxin (RTX)-induced neuropathic mouse model with thermal analgesia and skin denervation that was followed by daily 4MC treatment. On day 7 after RTX administration (RTXd7), the substance P (SP)(+) IENFs were completely depleted compared with the vehicle group ($p < 0.0001$), whereas the calcitonin gene-related peptide (CGRP)(+) IENFs were dramatically, but not completely, depleted ($p < 0.0001$). While SP(+) IENFs remained depleted ($p = 0.0043$), CGRP(+) IENFs were recovered by RTXd84 ($p = 0.78$). 4MC had no effect on the reinnervation of SP(+) IENFs, but markedly promoted the reinnervation of CGRP(+) IENFs on RTXd35 ($p = 0.035$). On RTXd56, CGRP(+) IENFs were comparable with the vehicle group ($p = 0.39$). In addition, 4MC normalized thermal analgesia on RTXd35 compared with RTX group ($p = 0.007$). In the current study, the significant promotion of reinnervation of CGRP(+) IENFs and

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thermal latencies on RTXd35 by 4MC indicated that CGRP(+) IENFs were responsible for the thermal transmission in chronic phase of RTX-induced neuropathy.

Introduction

Calcitonin gene-related peptide (CGRP) and substance P (SP) are neurotransmitters that are used by peptidergic small-diameter dorsal root ganglia (DRGs) neurons and are presumably responsible for transmitting nociceptive stimuli from intraepidermal nerve fibers (IENFs) in the skin. These neurons also express transient receptor potential vanilloid subtype 1 (TRPV1) [1,2], which can be depleted by an ultrapotent capsaicin agonist, resiniferatoxin (RTX) [2—4]. RTX is widely used in pain studies to deplete the IENFs, which leads to thermal analgesia [5—8]. Previous studies focused on the depletion of TRPV1(+) neurons and their cutaneous terminal, which corresponds to the thermal transmission [3,4]. Together with the development of thermal analgesia by RTX, those observations raise the following issues: what is the effect of RTX in the depletion of SP(+) and CGRP(+) IENFs and are they in the same degree? If there is a differential effect by RTX, which phenotypic IENFs are susceptible and which profiles of phenotypic IENFs parallel the recovery of thermal sensation in the chronic phase of RTX-induced neuropathy?

4-Methylcatechol (4MC) has been considered as a potent stimulator of endogenous nerve growth factor (NGF) [9—11], which enhanced skin reinnervation [8,11]. In clinics, patients with thermal analgesia are accompanied by skin denervation. These observations implied that the IENFs reinnervation could relieve thermosensation disorders. Our previous study demonstrated that 4MC promoted the synthesis of NGF [11] and skin reinnervation, particularly by CGRP IENFs [8]. Taken together, these results suggest the potential for a therapeutic strategy to improve the skin reinnervation and to reverse the thermal analgesia. The following issues were raised: (1) whether 4MC has the differential effect in peptidergic IENFs, and (2) whether 4MC could promote specific phenotypic IENFs reinnervation that reversed thermal analgesia?

To address these issues, we generated a RTX-induced thermal analgesic mouse model and investigated the depletion of SP(+) and CGRP(+) IENFs with footpad skin sections. After the administration of 4MC, there were significant increases in the densities of CGRP(+) IENFs, while there were reductions in the duration of thermal analgesia, suggesting that CGRP(+) IENFs are responsible for the transmission of thermal nociception.

Materials and methods

RTX-induced neuropathy and 4MC treatment

Experiments were performed on 8-week-old adult male imprinting control region mice (35—40 g). RTX (Sigma, St. Louis, MO, USA) was dissolved in a vehicle (10% Tween-80 and 10% ethanol in normal saline) [2,8]. The animals received a single dose of RTX by intraperitoneal injection (50 μg/kg, defined as the RTX group). One group received an equal volume of vehicle to serve as the controls (the vehicle group). 4MC (10 μg/kg; Wako, Osaka, Japan) was dissolved in phosphate-buffered saline and was intraperitoneally injected to mice on day 7 of RTX-induced neuropathy (RTXd7). The group that received the 4MC injections was defined as the 4MC group [11]. After treatment, mice were housed in plastic cages on 12-hour light/12-hour dark cycle and had access to water and food *ad libitum*. All procedures were conducted in accordance with the ethical guidelines for laboratory animals [12] and the protocol (Permit No. 100055) was approved by Kaohsiung Medical University, Kaohsiung, Taiwan. All experimental procedures were performed using 4% chloral hydrate (dose: 1 mL/100 g), and all efforts were made to minimize suffering.

Evaluation of hot plate withdrawal latencies

Mice were placed on a 52 °C hot plate (IITC, Woodland Hills, CA, USA) enclosed in a Plexiglass cage. The withdrawal latencies of the hind paw to thermal stimulations were determined to an accuracy of 0.1 seconds. Each test session consisted of three trials separated by 30-minute intervals. The criteria of withdrawal included shaking, licking, or jumping on the hot plate. The mean latency was expressed as the threshold of an individual animal to thermal stimulation.

Immunohistochemistry of footpad

Mice were killed by intracardiac perfusion with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde (4P) in 0.1 M PB (pH = 7.4) [13]. Footpad tissues were removed after perfusion and postfixed in 4P overnight, followed by cryoprotection with 30% sucrose. Tissues sections, 30-μm thick, were cut on a sliding microtome. Briefly, the sections were quenched with 1% H_2O_2 in methanol and blocked with 0.5% nonfat dry milk and 0.1% Triton X-100 in 0.5 M Tris buffer (Tris). The tissue sections were incubated with primary anti-SP (1:1000; DiaSorin, Stillwater, MN, USA) and CGRP (1:1000; Sigma) antibodies overnight at 4 °C, followed by incubation with biotinylated secondary antibody for another 1 hour. Then the avidin—biotin complex (Vector Labs, Burlingame, CA, USA) method was used and the activity of the reaction product was demonstrated with 3,3′-diaminobenzidine (Sigma). The sections were mounted on gelatin-coated slides for quantification.

Quantification of SP(+) and CGRP(+) IENFs

SP(+) and CGRP(+) IENFs were counted under 400× magnification (Axiophot microscope; Zeiss, Oberkochen,

Germany). The protocol for counting followed established criteria in a coded fashion [14]. Fibers with branching points within the epidermis were counted as a single IENF. Fibers with branching points in the dermis were counted as each single IENF. The length along the lower margin of the stratum corneum was defined as epidermal length and determined using Image J software (version 1.44d; National Institutes of Health, Bethesda, MD, USA). The density of IENF was expressed as the counted epidermal fibers divided by the epidermal length (fibers/cm).

Statistic analysis

All data were expressed as mean ± standard derivation of the mean. The means among the vehicle, RTX, and 4MC group were analyzed by nonparametric Mann–Whitney test. A p value < 0.05 was taken to be significant.

Results

Depletion of SP(+) and CGRP(+) IENFs in RTX-induced neuropathy

In the vehicle group, typical SP(+) and CGRP(+) IENFs emerged from the subepidermal plexuses with varicose appearances. The number of CGRP(+) IENFs were more abundant than SP(+) IENFs (Fig. 1A and B). On RTXd7, SP(+) IENFs were completely depleted (Fig. 1C) and CGRP(+) IENFs were significantly reduced (Fig. 1D). The observations of reduced SP(+) and CGRP(+) IENFs were verified by quantitative comparison (Fig. 1E).

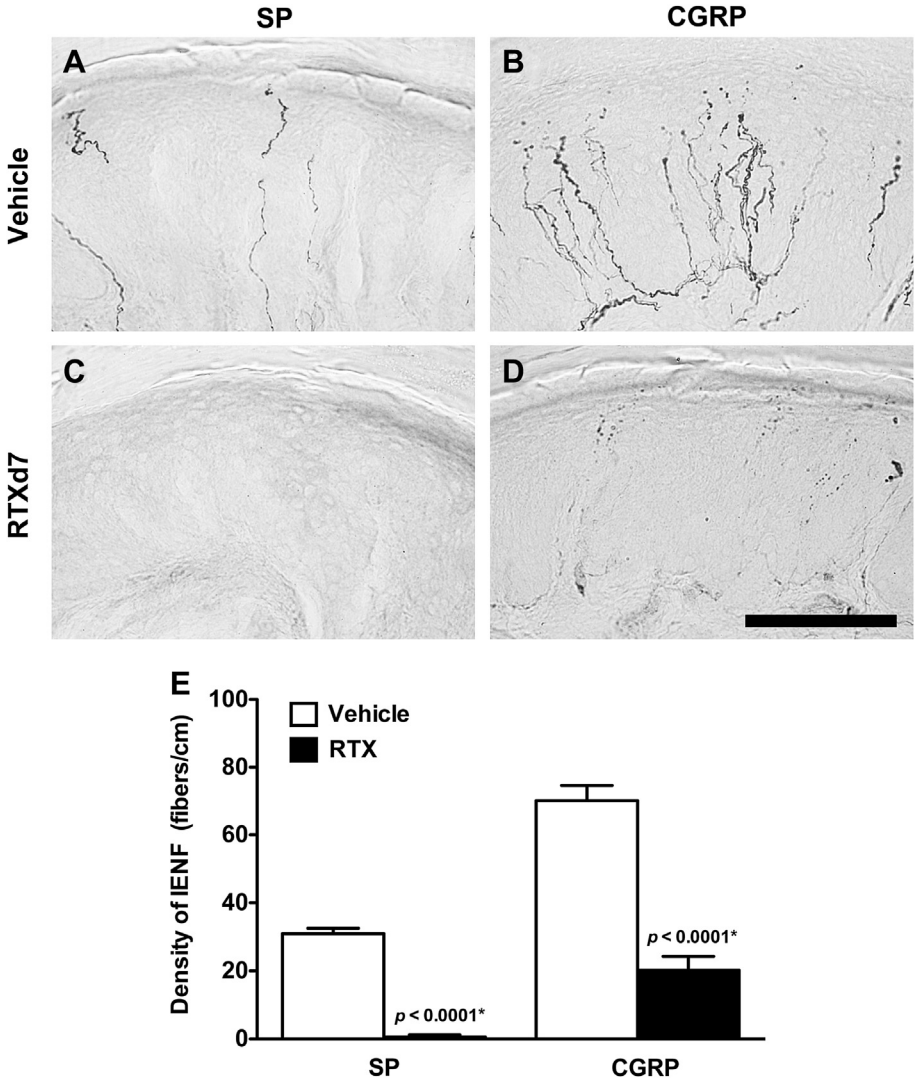

Figure 1. Differential denervation of peptidergic intraepidermal nerve fibers (IENFs) after resiniferatoxin (RTX)-induced neuropathy. Different phenotypes of peptidergic IENFs were demonstrated by (A and C) anti-substance P (SP) and (B and D) calcitonin gene-related peptide (CGRP) antiserum in (A and B) vehicle and (C and D) on day 7 (RTXd7) of RTX-induced neuropathy. In the vehicle group, (A) SP(+) and (B) CGRP(+) IENFs penetrated into epidermis from subepidermal plexus. CGRP(+) IENFs were more abundant than SP(+) IENFs. On RTXd7, (C) SP(+) IENFs were completely depleted and (D) there was partial denervation by CGRP(+) IENFs. (E) The graph shows the quantitation of IENF according to Fig. 1A–D. Bar = 50 μm; *statistically significant at $p < 0.05$.

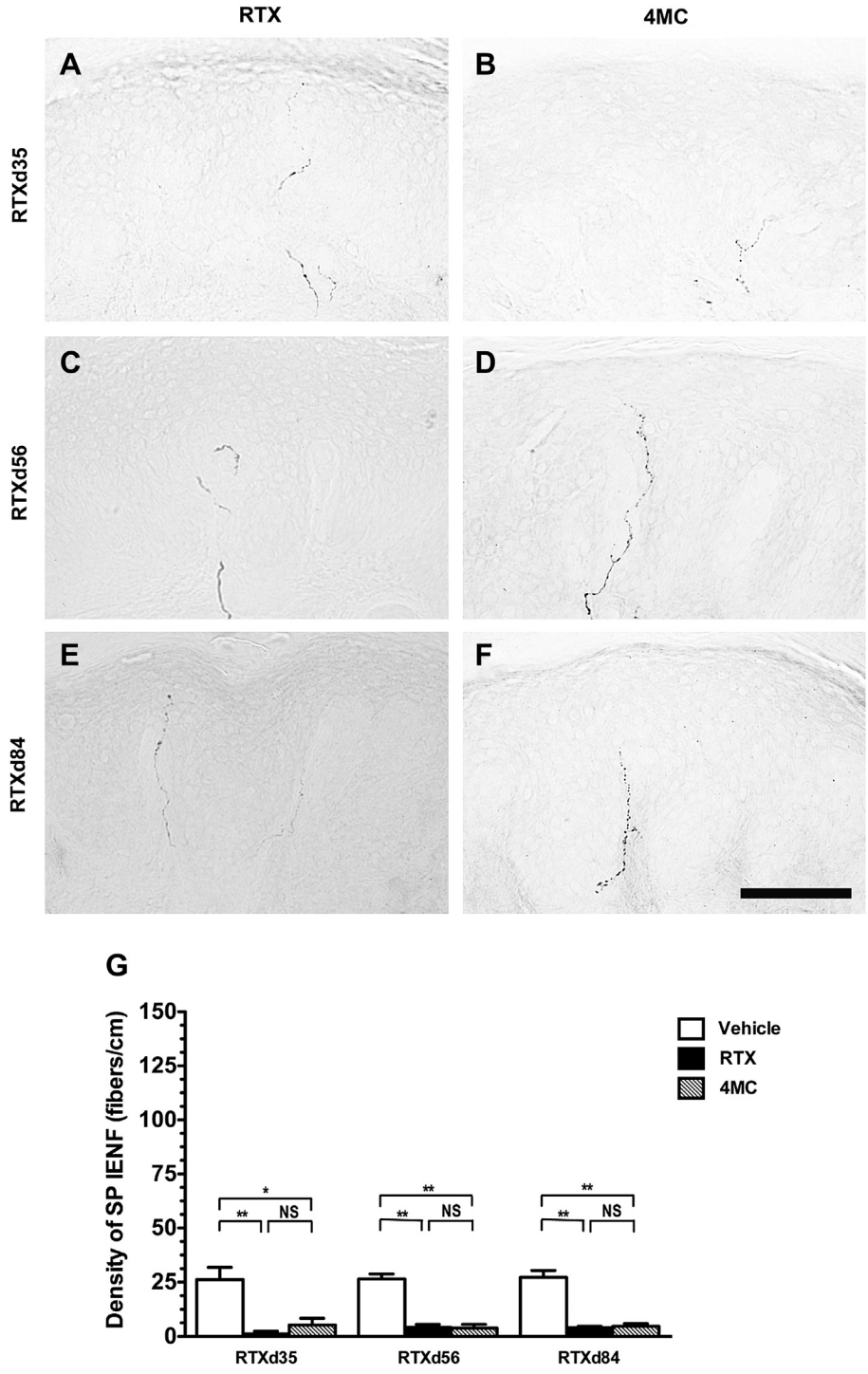

Figure 2. Effect of 4-methylcatechol (4MC) on the reinnervation of substance P (SP)(+) intraepidermal nerve fibers (IENFs) in resiniferatoxin (RTX)-induced neuropathy. SP(+) IENFs were demonstrated by anti-SP antiserum in RTX (A, C, E) and 4MC (B, D, F) groups on (A and B) day 35 (RTXd35), (C and D) RTXd56, and (E and F) RTXd84 after RTX-induced neuropathy. There was no significant reinnervation of SP(+) IENF in the RTX and 4MC groups. (G) The graph shows the quantitation of SP(+) IENFs according to Fig. 2A–F. Bar = 50 μm; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS = no significant difference.

Reinnervation of SP(+) and CGRP(+) IENFs after RTX treatment

To explore the long-term effects of RTX, we quantified the skin innervation for SP(+) and CGRP(+) IENFs on RTXd35, RTXd56 and RTXd84, respectively. There were negligible SP(+) IENFs on RTXd35 (1.2 ± 2.6 vs. 26.3 ± 12.6 fibers/cm, $p = 0.0024$), RTXd56 (4.2 ± 4.0 vs. 26.5 ± 5.2 fibers/cm, $p = 0.0016$), and RTXd84 (4.0 ± 1.8 vs. 27.3 ± 7.1 fibers/cm, $p = 0.0043$) (Fig. 2A, C, E and G). 4MC had no effect on

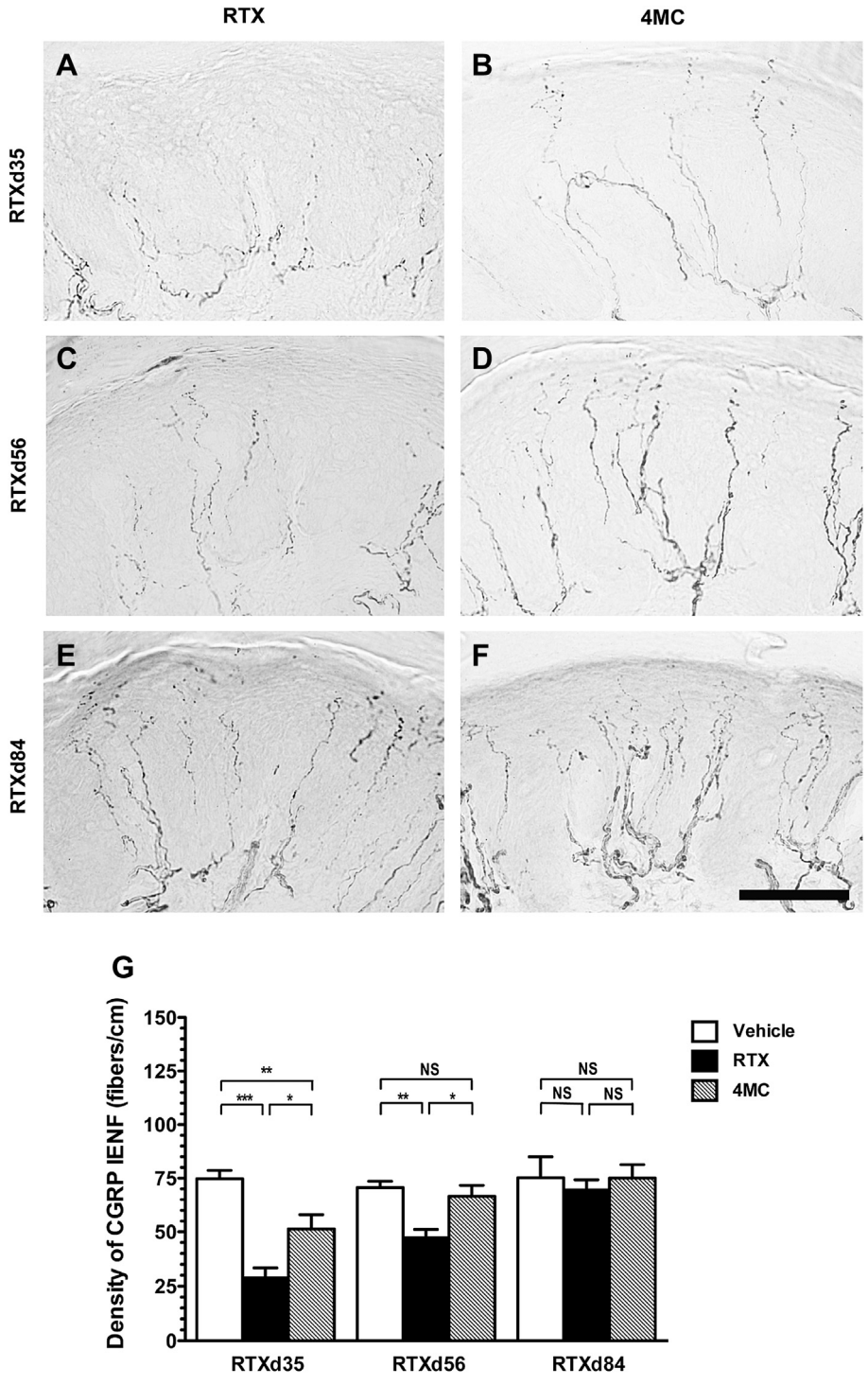

Figure 3. Effect of 4-methylcatechol (4MC) on the reinnervation of calcitonin gene-related peptide (CGRP)(+) intraepidermal nerve fibers (IENFs) in resiniferatoxin (RTX)-induced neuropathy. CGRP(+) IENFs were demonstrated by anti-CGRP antiserum in (A, C, E) RTX and (B, D, F) 4MC groups on (A and B) day 35 (RTXd35), (C and D) RTXd56, and (E and F) RTXd84 after RTX-induced neuropathy. (A, C, E) There was gradual reinnervation of CGRP(+) IENFs from RTXd35 to RTXd84 of the RTX group. (B, D, F) There was marked reinnervation of CGRP(+) IENFs on (B) RTXd35, (D) RTXd56 and (F) RTXd84 upon 4MC treatment. (G) The graph shows the quantitation of CGRP(+) IENFs according to Fig. 3A–F. Bar = 50 μm; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. NS = no significant difference.

the reinnervation of SP(+) IENFs (Fig. 2B, D, F and G). In contrast to the absence of SP(+) IENFs, CGRP(+) IENFs were gradually reinnervated from RTXd35 to RTXd84 (Fig. 3A, C, E and G). Interestingly, there was significant reinnervation of CGRP(+) IENFs on RTXd35 by 4MC compared with CGRP(+) IENFs in the RTX group (51.3 ± 20.0 vs. 28.8 ± 14.4 fibers/cm, $p < 0.012$; Fig. 3A vs. 3B); such reinnervation was complete as compared with the vehicle group on RTXd56 (66.6 ± 12.4 vs. 70.6 ± 7.2 fibers/cm, $p = 0.39$; Fig. 1B vs. D) and RTXd84 (75.1 ± 15.3 vs. 75.2 ± 16.9 fibers/cm, $p = 0.71$) (Fig. 3F). Taken together, 4MC could accelerate the reinnervation of CGRP(+) IENFs but it had no effect on SP(+) IENFs.

4MC normalized the thermal analgesia in RTX-induced neuropathy corresponding with the skin innervation

The typical thermal analgesia was induced on RTXd7 (22.8 ± 1.9 vs. 10.5 ± 1.6 seconds, $p < 0.0001$) and persisted through RTXd42 (18.3 ± 2.3 vs. 10.3 ± 0.7 seconds, $p = 0.016$). On RTXd49, thermal latencies became normalized (14.2 ± 5.2 vs. 9.4 ± 2.2 seconds, $p = 0.40$), until the end of the experimental period on RTXd84 (11.3 ± 3.5 vs. 10.8 ± 1.9 seconds, $p = 0.85$). In the 4MC group, the thermal latency was reduced on RTXd35 as compared with the RTX group (12.9 ± 1.7 vs. 20.1 ± 4.0 seconds; $p = 0.007$) (Fig. 4).

In summary, the patterns of thermal latencies in the 4MC group correlated to the patterns of CGRP(+) IENFs' innervation but not to those of SP(+) IENFs, indicating that CGRP(+) IENFs were responsible for the transmission of thermal nociception.

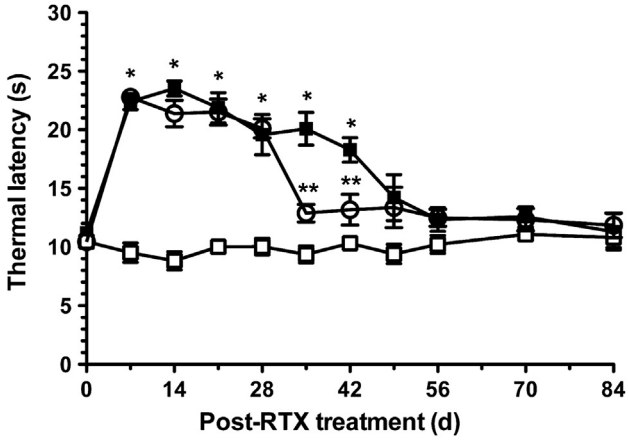

Figure 4. 4-Methylcatechol (4MC) reduced the duration of thermal analgesia in resiniferatoxin (RTX)-induced neuropathy. The thermal latencies were evaluated by hot plate test before and weekly after RTX-induced neuropathy. There was no change of thermal latencies in the vehicle group (open squares). The thermal analgesia was induced on day 7 of RTX-induced neuropathy (RTXd7) and up to RTXd42 (filled squares). After the administration of 4MC, the duration of thermal analgesia was significantly reduced (open circles). *Statistically significant at $p < 0.05$ compared with the vehicle group. **Statistically significant at $p < 0.05$ compared with the RTX group.

Discussion

The main findings of this report include the following: (1) differential depletion of SP(+) and CGRP(+) IENF resulted in reversible reinnervation of CGRP(+) IENFs but not in SP(+) IENFs after RTX treatment. (2) 4MC could promote the reinnervation of CGRP(+) IENFs. (3) Finally, these patterns of CGRP(+) IENFs were parallel to the course of thermal analgesia.

RTX induced discrepancy in denervation and reinnervation of SP(+) and CGRP(+) IENFs

RTX induced the depletion of TRPV1(+) neurons [2] and skin denervation [8]. In addition, RTX depleted the small-diameter nerves in genitourinary system with a different effect [15]. However, there was no report addressing the reinnervation of IENFs in terms of different phenotypic small-diameter nerves in late-stage RTX-induced neuropathy. In this report, we have demonstrated that RTX induces the denervation of SP(+) and CGRP(+) IENFs to varying degrees. These discrepancies in SP(+) and CGRP(+) IENFs corresponded to the differential depletion of SP(+) and CGRP(+) neurons in lumbar DRG whose peripheral terminal innervated the hind paw skin [2]. This discrepancy in the depletion of SP(+) and CGRP(+) DRG neurons was due to different ratios of co-localization with TRPV1(+) DRG neurons, which were depleted by RTX. CGRP(+) neurons account for the relative resistance of CGRP(+) neurons to RTX treatment as compared with SP(+) neurons [16] and they provide the possibility of reinnervation by CGRP(+) IENFs after RTX-induced neuropathy [17], that is, the reinnervation in the late phase may result from the survival of CGRP(+) neurons that contributed to the reinnervation of CGRP(+) IENFs in the late phase. In this report, reversible CGRP(+) IENFs in the late phase provided a possible pathway for thermal nociceptive transmissions.

4MC attenuated the duration of thermal analgesia and promoted the reinnervation of CGRP(+) IENFs

This report demonstrated that 4MC attenuated the duration of thermal analgesia and promoted the reinnervation of CGRP(+) IENFs in the late phase of RTX-induced neuropathy. The densities of CGRP(+) IENFs reached a nadir on RTXd7 and then gradually increased from RTXd35 to RTXd56, corresponding to the course of thermal analgesia. This report suggests that CGRP(+) IENFs contributes to the thermal nociceptive transmission. In addition, we further demonstrated the effect of 4MC on both attenuating the duration of thermal analgesia and promoting the reinnervation of CGRP(+) IENFs. 4MC is a stimulator of several neurotrophins and promotes skin reinnervation [11]. In this report, we further addressed the effect of 4MC on the reinnervation of CGRP(+) IENFs. The effect of 4MC for the promotion of reinnervation of CGRP(+) IENFs may be due to peptidergic DRG neurons and is neurotrophin dependent.

The cascade pathways after 4MC treatment are not clear, however, and these merit further investigation.

In summary, this report addressed the effect of 4MC on the promotion of CGRP(+) IENFs' innervation and the recovery of thermosensation disorders. The findings raise the potential to improve peripheral regeneration and peripheral neuropathy by increasing neurotrophins synthesis [11,18].

Acknowledgments

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