Enhancement of Cutaneous Nerve Regeneration by 4-Methylcatechol in Resiniferatoxin-Induced Neuropathy

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Abstract
To generate an experimental neuropathy model in which small-diameter sensory nerves are specifically affected and to test a potential treatment, adult mice were given a single injection (50 µg/kg, i.p.) of the capsaicin analog resiniferatoxin (RTX). On Day 7 after RTX treatment, there was a 53% reduction in unmyelinated nerve density in the medial plantar nerve (p = 0.0067) and a 66% reduction in epidermal nerve density of hind paw skin (p = 0.0004) compared with vehicle-treated controls. Substance P–immunoreactive dorsal root ganglion neurons were also markedly depleted (p = 0.0001). These effects were associated with the functional deficit of prolonged withdrawal latencies to heat stimuli (p = 0.0007) on a hot plate test. The potential therapeutic effects of 4-methylcatechol (4MC) on this neuropathy were then tested by daily injections of 4MC (10 µg/kg, i.p.) from Days 7 to 35 after neuropathy induction. On Day 35, 4MC-treated mice had an increase in unmyelinated (p = 0.014) and epidermal nerve (p = 0.0013) densities and a reduction in thermal withdrawal latency (p = 0.0091) compared with RTX-only controls. These results indicate that 4MC promoted regeneration of unmyelinated nerves in experimental RTX-induced neuropathy and enhanced function.

Key Words: 4-Methylcatechol, Calcitonin gene–related peptide, Capsaicin, Nerve regeneration, Neuropathy, Resiniferatoxin, Skin denervation, Substance P.

INTRODUCTION
Neuropathies affecting small-diameter sensory nerves (small-fiber sensory neuropathy) are manifested by skin denervation and are common in various diseases, including diabetes mellitus and chemotherapeutic agent–associated neurotoxicities (1–4). Cutaneous nerve degeneration occurs in several experimental models, including cisplatin-induced neuropathy and nerve injury caused by compression or transection (5, 6). In most of those models, however, large-diameter myelinated nerves are affected, and it is difficult to evaluate the functional deficits of small-diameter sensory nerves independent of large-diameter nerves. For example, examination of thermosensory loss by withdrawal latencies depends on intact motor nerve fibers (7).

Resiniferatoxin (RTX) is an ultrapotent capsaicin analog that acts on transient receptor potential vanilloid receptor 1 by increasing calcium permeability and causing activation of the transient receptor potential vanilloid receptor 1 nonselective cation channel (8), which mediates nociceptive processing (9–11). Resiniferatoxin has recently been used in the treatment of neurogenic bladder hyperreflexia (12) and arthritic pain (13). An intraganglionic injection of RTX induces irreversible loss of dorsal root ganglion (DRG) neurons (11). The investigation of skin innervation has traditionally depended on immunohistochemical demonstration of nerve terminals in the skin by protein gene product 9.5 (PGP 9.5) (1, 14, 15). Some PGP 9.5–immunoreactive nerves in the epidermis are capsaicin-sensitive and responsible for transmitting thermal stimuli (16, 17). Taken together, these data suggest that RTX induces cutaneous nerve degeneration, and that it may be used to generate and assess the functional consequences of an experimental model of neuropathy that predominantly affects small-diameter sensory nerves.

Sensory nerve terminals in the skin are the peripheral processes of small-diameter DRG neurons. The development of these neurons depends on various neurotrophins (18–20). The maintenance and regeneration of these sensory neurons and their processes presumably also require neurotrophins, but there is limited literature exploring this hypothesis. The treatment of neuropathies affecting small-diameter sensory nerves is also clinically challenging. To date, there are no satisfactory therapeutic strategies for repairing and improving the functions of these nociceptive nerves (21). 4-Methylcatechol (4MC), an inducer of nerve growth factor synthesis, has been used to increase the synthesis of neurotrophins (22–24). We therefore hypothesized that the administration of 4MC might also promote cutaneous nerve regeneration in an experimental model of small-fiber neuropathy.

To address the issues mentioned in the preceding paragraph, we developed a model of RTX-induced small-fiber sensory neuropathy in mice and investigated 1) whether RTX induces degeneration of cutaneous nerves, 2) whether DRG neurons are depleted by RTX, and 3) whether...
4MC can promote their regeneration and improve clinical function.

MATERIALS AND METHODS

Systemic RTX and 4MC Treatment

Experiments were performed on 8-week-old adult male ICR (Harlan, Oxfordshire, UK) mice weighing 35 to 40 g. Resiniferatoxin (Sigma, St. Louis, MO) was dissolved in a vehicle (10% Tween-80 and 10% ethanol in isotonic sodium chloride solution) (25). A single dose of RTX (50 μg/kg, i.p.) was administered to a group of mice via injection (RTX group). Control mice received an equal volume of vehicle (vehicle group). After the intraperitoneal injections, the mice were housed in plastic cages on a 12-hour-light/12-hour-dark cycle and were allowed ad libitum access to food and water.

**FIGURE 1.** Skin denervation in resiniferatoxin (RTX)-induced neuropathy. The footpad skin in the vehicle (A, C, E) and RTX groups (B, D, F) was immunostained for protein gene product (PGP) 9.5 (A, B), calcitonin gene-related peptide (CGRP) (C, D), and substance P (SP) (E, F) on Day 7 after RTX treatment. (A, B) In the vehicle group, PGP 9.5(+) epidermal nerves arise from the subepidermal nerve plexus with a typical varicose appearance. In the RTX group, PGP 9.5(+) epidermal nerves are markedly reduced. (C, D) The number of CGRP(+) epidermal nerves is relatively smaller compared with that of PGP 9.5(+) epidermal nerves in the vehicle group (C, compared with A). In the RTX group, CGRP(+) epidermal nerves are markedly reduced compared with the vehicle group. (E, F) There are only sparse SP (+) epidermal nerves in the vehicle group, and SP (+) epidermal nerves are almost completely depleted. Bar = 50 μm.

**FIGURE 2.** Changes in epidermal nerve density (END) in resiniferatoxin (RTX)-induced neuropathy. Epidermal nerve densities were calculated as described in the Materials and Methods section for epidermal nerves of different phenotypes: protein gene product (PGP) 9.5 (A), calcitonin gene-related peptide (CGRP) (B), and substance P (SP) (C). *Statistically significant.

For treatment of the neuropathy, 4MC (10 μg/kg; Wako, Osaka, Japan) dissolved in PBS was given to RTX-induced neuropathic mice via daily intraperitoneal injections from Day 7 (D7) to D35 after RTX treatment. This group was designated the RTX + 4MC group.

All procedures were conducted in accordance with ethical guidelines set up by National Research Council’s Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Animal Committee of National Taiwan University College of Medicine, Taipei, Taiwan.

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Immunohistochemistry of Footpad Skin

Mice were killed by intracardiac perfusion with 0.1 mol/L phosphate buffer (PB), followed by 4% paraformaldehyde in 0.1 mol/L PB (pH 7.4) (7). Footpads were removed after perfusion and postfixed in 4% paraformaldehyde for another 6 hours. Tissues were then cryoprotected with 30% sucrose in PB overnight. Sections were cut at 30-μm thickness on an HM440R sliding microtome (Microm, Walldorf, Germany), labeled sequentially, and stored at −20°C. To ensure adequate sampling, every third section of each footpad was immunostained. Briefly, sections were quenched in 1% H2O2 in methanol and blocked with 0.5% nonfat dry milk and 0.1% Triton X-100 in 0.5 mol/L Tris buffer. Footpad sections were incubated with antisera against PGP 9.5 (1:1000; UltraClone, Isle of Wight, UK), substance P (SP; 1:1000; DiaSorin, Stillwater, MN), and calcitonin gene–related peptide (CGRP; 1:1000; Amersham, Buckinghamshire, UK) overnight at 4°C. Sections were then incubated with a biotinylated secondary antibody and the avidin-biotin complex (Vector, Burlingame, CA). The reaction product was demonstrated with 3,3'-diaminobenzidine (Sigma).

Quantitation of Epidermal Innervation

Epidermal nerves were counted under 400× magnification (Axioskop microscopy; Zeiss, Oberkochen, Germany), and the quantitation protocol followed established criteria in a coded fashion (6). Epidermal nerves with branching points within the epidermis were counted as a single epidermal nerve, whereas those with a branching point in the dermis were each counted as single epidermal nerves. The length along the lower margin

**FIGURE 3.** Ultrastructural changes in unmyelinated nerves after resiniferatoxin (RTX)-induced neuropathy. The medial plantar nerves of the vehicle (A, B) and RTX groups (C, D) were examined by electron microscopy on Day 7 after RTX administration. (A) In the vehicle group, several unmyelinated nerves are enclosed by a Schwann cell. Together, they form a Remak bundle. (B) Each unmyelinated fiber shows intact organelles and axonal membrane in the vehicle group. (C) The number of unmyelinated nerves enclosed by a Schwann cell is reduced in the RTX group. (D) In the RTX group, the organelles of some unmyelinated nerves seem swollen, and the contents have become amorphous or condensed, indicating an early stage of degeneration. Scale bars = (A, C) 0.5 μm; (B, D) 0.25 μm.

**FIGURE 4.** Effects of resiniferatoxin (RTX) on the ultrastructural morphometry of unmyelinated nerves. The quantitative analysis was based on electron microscopy examinations as illustrated in Figure 3. (A, B) Compared with the vehicle group, the spectrum of unmyelinated nerves/Remak bundle is shifted to the left in the RTX group, indicating a loss of unmyelinated nerves after RTX treatment. (C) The unmyelinated nerve density of the RTX group is lower than that of the vehicle group. *p = 0.0067*, Statistically significant.
of the stratum corneum was defined as the epidermal length and determined with National Institutes of Health (NIH; Bethesda, MD) image v1.6.3 for Macintosh. The epidermal nerve density (END) was defined as the number of epidermal nerves divided by the epidermal length.

Quantitation of the DRG Neurons by Double Labeling Immunofluorescence

After perfusion, the fifth lumbar dorsal root ganglia were removed and postfixed for another 2 hours. Tissues were then cryoprotected with 30% sucrose in PB overnight, and sections were cut to an 8-μm thickness on a Leica CM3050S cryostat (Leica, Wetzlar, Germany) and stored at −80°C. To ensure adequate sampling, each section at 80-μm intervals was immunostained. For double labeling immunofluorescence, sections were incubated with a mixture of antisera containing mouse anti-neurofilament (SMI-32; 1:200; Sternberger-Meyer, Baltimore, MD) and either rabbit anti-SP (1:200; Diasorin, Stillwater, MN) or anti-CGRP (1:200; Amersham) overnight at 4°C. Sections were then incubated with a combination of secondary antisera conjugated fluorescein isothiocyanate and Texas Red (1:100; Jackson ImmunoResearch, West Grove, PA). These sections were observed under fluorescence microscopy (Axioskop Microscope; Zeiss) with appropriate filters. Photographs of immunofluorescence images were taken in a systematic fashion with a magnification of 200×. A montage photograph of the entire DRG sections was created. Immunoreactive DRG neurons with an obvious nucleus were counted, and the area of DRG was measured with NIH image v1.6.3 for Macintosh with the neuron density expressed as neurons/square millimeter.

Ultrastructural Morphometric Studies of Unmyelinated Nerves

Medial plantar nerves were dissected after intracardiac perfusion with 5% glutaraldehyde following established protocols (26). After rinsing in PB, tissues were postfixed in 2% osmium tetroxide for 2 hours, dehydrated through a graded ethanol series, and embedded in Epon 812 resin (Polyscience, Philadelphia, PA). Thin sections were cut on an ultramicrotome (Reichert Ultracut E; Leica). Sections were stained with uranyl acetate and lead citrate, observed under electron microscopy (EM; Hitachi H-7100, Tokyo, Japan), and photographed.

Ultrastructural morphometry was performed according to a previously published protocol with some modification (27). Electron photomicrographs were taken in a systematic fashion with a magnification of 8,000×. A montage was created from all photographs, and the areas of each montage covered 60% to 75% of the entire cross section of a medial plantar nerve. Numbers of unmyelinated nerves enclosed within the basal lamina of a Schwann cell (Remak bundle) were counted. The unmyelinated nerve density was defined as the number of unmyelinated nerves divided by the area of the endoneurium (nerves/square millimeter).

Morphometric Studies of Myelinated Nerves

Sural nerves were dissected and processed as described in the preceding paragraph for the ultrastructural morphometry of medial plantar nerve. Semithin sections were stained with toluidine blue. Photographs were taken at a magnification of 400× under an Axioskop microscope (Zeiss). The numbers of myelinated nerves were counted, and myelinated
The criteria of withdrawal included shaking, licking, or jumping while on the hot plate. The cutoff limit was 25 seconds to avoid potential tissue damage. The mean latency was expressed as the threshold of each individual mouse to the noxious thermal stimulation. Mice were tested before the RTX injection (D0) and weekly until D35.

**Neurophysiologic Studies**

To evaluate the effects of RTX on large myelinated motor and sensory nerves, amplitudes of the compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) were recorded with a Nicolet VikingQuest System (Nicolet Biomedical, Madison, WI) on D7 after RTX treatment (28). For recording CMAPs, monopolar stimulating needle electrodes were placed at the sciatic notch, and 2 surface recording electrodes with an interval of 0.5 cm were placed on the plantar muscle. A monopolar electrode was inserted into the tail as the ground electrode. The parameters of stimulation included a stimulating intensity of 100 V, duration of 0.1 milliseconds, and nonrepeated (manual) stimuli. Filters were set at 2 to 10,000 Hz. For recording SNAPs, orthodromic stimulations were used on the sural nerves. The stimulating electrodes were placed on the lateral side of the ankle, and recording electrodes were placed on the sural nerve. The parameters of stimuli included a stimulus intensity of 10 mA, duration of 0.1 milliseconds, and a repeated stimulus rate of 0.7 Hz. Filters were set at 20 to 3,000 Hz. Amplitudes of CMAPs and SNAPs were calculated by baseline-to-peak values of the recorded waveforms.

**Experimental Design and Statistical Analysis**

The study was divided into 2 parts: 1) RTX-induced neuropathy and 2) therapeutic effects of 4MC. In the first part, determining the neurotoxic effects of RTX on D7 after RTX injection, there were 2 groups (RTX and vehicle) with 5 animals in each group. In the second part, investigating the effects of 4MC on the RTX-induced neuropathy, there were 2 groups (RTX and RTX + 4MC groups) with 5 animals in each group.
examined on D35 of RTX treatment. All data are expressed as mean ± SD. The means between group parameters were analyzed with the t-test. p < 0.05 was considered statistically significant.

RESULTS

Patterns of Skin Denervation in RTX-Induced Neuropathy

To understand the effects of RTX on skin innervation, we immunostained the footpad skin and quantitated ENDS. In the vehicle group, typical PGP 9.5(+) epidermal nerves emerged from the subepidermal plexus through the epidermis and had a varicose appearance (Fig. 1A). On D7, there was a significant reduction in PGP 9.5(+) epidermal nerves (Fig. 1B). This observation was verified by quantitative comparison of skin innervation with a 66% reduction of ENDS in the RTX group compared with the vehicle group (38.2 ± 15.2 vs 109.6 ± 18.6 fibers/centimeter; p = 0.0004; Fig. 2A).

To determine if epidermal nerves of different phenotypes were susceptible to RTX to the same degree, we examined CGRP(+) and SP(+) epidermal nerves in hind paw footpad skin sections. In the vehicle group, the appearance of CGRP(+) and SP(+) epidermal nerves was similar to that of PGP 9.5(+) epidermal nerves, but there were fewer CGRP(+) and SP(+) epidermal nerves than PGP 9.5(+) epidermal nerves (Fig. 1C, E). In the RTX group, there were fewer CGRP(+) epidermal nerves than in the vehicle group (Fig. 1D), and SP(+) epidermal nerves were nearly completely depleted (Fig. 1F). These observations were confirmed by a quantitative comparison of ENDS. On D7, the END of CGRP in the RTX group was lower than that in the vehicle group (19.6 ± 10.3 vs 70.5 ± 11.5 fibers/centimeter; p = 0.0006; Fig. 2B). Similarly, the END of SP was markedly reduced in the RTX group compared with that of the vehicle group (0.9 ± 1.9 vs 31.7 ± 3.6 fibers/centimeter; p < 0.0001; Fig. 2C). Taken together, RTX caused skin denervation, and the degree of epidermal nerve depletion differed among epidermal nerves of different phenotypes.

Degeneration of Unmyelinated Nerves After RTX Treatment

To determine whether the degeneration of nerve terminals was responsible for the RTX-induced skin denervation, we performed an ultrastructural analysis on medial plantar nerves on D7 in RTX-treated and control mice. In the vehicle group, each single Schwann cell enclosed several unmyelinated nerves as a Remak bundle (Fig. 3A) with distinct organelles and membranous structures in each axon (Fig. 3B). In the RTX group, the numbers of unmyelinated nerves enclosed by each Schwann cell were reduced compared with the vehicle group (Fig. 3C). In addition, the axoplasm in some unmyelinated nerves was amorphous, and some axonal organelles had become swollen, indicating degeneration (Fig. 3D). The numbers of unmyelinated nerves in each Remak bundle were lower in the RTX group than in the vehicle group (2.7 ± 0.8 vs 5.3 ± 0.6 nerves/Remak bundle; p = 0.011) (Fig. 4A vs B). In addition, there was a 53% reduction in the unmyelinated nerve density in the RTX group compared with the vehicle group (10,505.8 ± 705.3 vs

FIGURE 8. Effect of resiniferatoxin (RTX) on neurophysiologic examinations of myelinated motor and sensory nerves. The neurophysiology of myelinated motor and sensory nerves in the vehicle (A, D) and RTX groups (B, E) was assessed with motor (A, B) and sensory (D, E) nerve conduction studies on Day 7 of RTX treatment. The analyses were based on amplitudes of the compound muscle action potential (CMAP) and sensory nerve action potential (SNAP) between both groups (C, F).
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that RTX induced a significant loss of peptidergic DRG neurons to different degrees but spared large-diameter DRG neurons.

Functional Deficit in RTX-Induced Neuropathy

Because skin innervation is responsible for transmitting thermal stimuli, we then explored whether thermal responses were impaired after RTX treatment. Before RTX treatment, withdrawal latencies to the thermal stimuli were similar between the 2 groups (10.5 ± 1.6 vs 11.4 ± 1.5 second; \( p = 0.186 \)). On D7 after RTX treatment, withdrawal latencies were increased in the RTX group compared with those in the vehicle group (22.2 ± 3.8 vs 9.5 ± 2.8 s; \( p = 0.0007 \)) (Fig. 7). These findings provided functional evidence of RTX-induced skin denervation.

Effects of RTX on Myelinated Motor and Sensory Nerves

To understand whether RTX caused neurophysiologic functional deficits of myelinated motor and sensory nerves, we compared the nerve conduction (CMAPs and SNAPs) and morphometric studies between the RTX and vehicle groups on D7 after RTX treatment. Compound muscle action potential waveforms were biphasic in the vehicle group (Fig. 8A). The patterns and amplitudes did not change after RTX treatment (9.5 ± 1.5 vs 9.9 ± 2.9 mV; \( p = 0.84 \); Fig. 8B, C). Similarly, the waveforms and amplitudes of SNAPs were the same in both groups (554.8 ± 64.5 vs 567.7 ± 42.8 \( \mu \)V; \( p = 0.55 \); Fig. 8D–F).

To understand the effects of RTX on myelinated nerves, we performed morphometric studies on sural nerves. In the vehicle group, myelinated nerves were distributed throughout the entire cross sections of the sural nerves (Fig. 9A), and there were no significant differences in the integrity or distribution of myelinated nerve fibers between RTX and vehicle groups (Fig. 9B). Myelinated nerve densities were similar between these 2 groups (28,029 ± 4,475 vs 29,550 ± 5,203 nerves/square millimeter; \( p = 0.72 \)) (Fig. 9C). These findings suggest that RTX did not induce physiologic or pathologic alterations of myelinated nerves.

Therapeutic Effect of 4MC on Skin Reinnervation of PGP 9.5(+) and CGRP(+) Epidermal Nerves

To determine whether 4MC can improve skin reinnervation, we administrated 4MC daily beginning from D7. At the end of the experimental period on D35, the epidermis in the RTX group remained denervated of PGP 9.5(+) nerve fibers, similar to that on D7 (Fig. 10A vs 1B). In contrast, the epidermis was significantly reinnervated with PGP 9.5(+) nerves in the RTX + 4MC group (Fig. 10B). The quantitative analysis indicated that the END was higher in the RTX + 4MC group compared with that of the RTX group (86.4 ± 21.8

Depletion of SP(+) DRG Neurons and Mild Reduction of CGRP(+) DRG Neurons after RTX Treatment

To understand the neurotoxicity of RTX on neuronal cell bodies, we performed double labeling immunofluorescence on DRG sections and quantified changes in different subpopulations of DRG neurons. Representing the large-diameter DRG neurons, SMI-32(+) DRG neurons remained similar between the RTX and vehicle groups (222.4 ± 19.0 vs 229.0 ± 24.7 neurons/square millimeter; Figs. 5, 6A). In contrast, the numbers of small-diameter peptidergic DRG neurons were significantly reduced, with an 88% reduction in SP(+) DRG neurons (6.3 ± 5.4 vs 50.2 ± 2.4 neurons/square millimeter; Figs. 5A, B and 6B) and a 32% reduction in CGRP(+) DRG neurons (214.4 ± 33.4 vs 319.9 ± 42.2 neurons/square millimeter; Figs. 5C, D and 6C). Taken together, these data indicated that RTX induced a significant loss of peptidergic DRG neurons to different degrees but spared large-diameter DRG neurons.

FIGURE 9. Effect of resi
erferoxin (RTX) on myelinated nerve fibers of sural nerves. (A, B) These cross sections show myelinated nerve fibers of the sural nerves in the vehicle (A) and RTX groups (B) on Day 7 of RTX treatment. (C) The myelinated nerve densities are similar between the 2 groups. Bar = (A, B) 10 \( \mu \)m.

22,812.2 ± 5,001.6 nerves/square millimeter; \( p = 0.0067 \) (Fig. 4C). Taken together, the quantitative analysis provided direct evidence that unmyelinated nerve degeneration after RTX treatment was a mechanism of skin denervation.

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vs 36.1 ± 12.0 fibers/centimeter; p = 0.0013) (Fig. 11A), reaching 75% of the value in the vehicle group on D7. After 4MC treatment, CGRP innervation was significantly increased compared with that in the RTX group (Fig. 10C, D), with an END of 49.0 ± 17.5 fibers/centimeter in the RTX + 4MC group compared with 22.0 ± 3.7 fibers/centimeter in the RTX group (p = 0.0048; Fig. 11B). The END of CGRP in the RTX + 4MC group on D35 reached 70% of the value of the vehicle group on D7. In contrast, the epidermis remained depleted of SP(+) nerves in both the RTX + 4MC and RTX groups (1.2 ± 2.6 vs 3.1 ± 2.2 fibers/centimeter; p = 0.13) (Figs. 10E, F and 11C). Taken together, the results indicate that 4MC treatment significantly improved skin reinnervation by PGP 9.5(+) and CGRP(+) epidermal nerves, but not SP(+) epidermal nerves.

Enhancement of Unmyelinated Nerve Regeneration by 4MC

To explore whether nerve regeneration was responsible for the reinnervation of the skin by 4MC treatment, we analyzed the ultrastructural morphometry of unmyelinated nerves in medial plantar nerves on D35. Each Remak bundle in the RTX + 4MC group enclosed more unmyelinated nerves than that in the RTX group, and the number of unmyelinated nerves/unit area also increased in the RTX + 4MC group (Fig. 12). The numbers of unmyelinated nerves within each Remak bundle were significantly higher in the RTX + 4MC group than in the RTX group on D35 (4.5 ± 0.5 vs 2.4 ± 0.1 nerves/Remak bundle; p = 0.002) (Fig. 13A, B). The value for the RTX + 4MC group was similar to that of the vehicle group on D7 (4.5 ± 0.5 vs 5.3 ± 0.6 nerves/Remak bundle;

![RTX and RTX + 4MC images](image_url)

**FIGURE 10.** Effect of 4-methylcatechol (4MC) on skin innervation after resiniferatoxin (RTX)-induced neuropathy. To investigate the therapeutic effect of 4MC on RTX-induced neuropathy (the RTX group), 4MC was given daily to another group of mice with RTX-induced neuropathy beginning from Day 7 of RTX treatment (the RTX + 4MC group). On Day 35 after RTX treatment, footpad skin sections of hind paws in the RTX (A, C, E) and RTX + 4MC groups (B, D, F) were stained by immunohistochemistry with antibodies against protein gene product (PGP) 9.5 (A, B), calcitonin gene-related peptide (CGRP) (C, D), and substance P (SP) (E, F). (A, B) In the RTX group, PGP 9.5(+) epidermal nerves remain reduced. In the RTX + 4MC group, the epidermis has become reinnervated by PGP 9.5(+) epidermal nerves. (C, D) There are more CGRP(+) epidermal nerves in the RTX + 4MC group compared with the RTX group. (E, F) Only limited SP(+) epidermal nerves can be seen in the RTX and RTX + 4MC groups. Bar = 50 μm.
p = 0.14) (Fig. 4A vs 13B). Moreover, the unmyelinated nerve density was higher in the RTX + 4MC group than in the RTX group (23,077.5 ± 6,316.7 vs 9,852.9 ± 2,138.1 nerves/square millimeter; p = 0.014) (Fig. 13C), and this value was comparable to that in the vehicle group on D7 (22,812.2 ± 5,001.6 nerves/square millimeter; p = 0.48). Taken together, these findings indicated that 4MC promoted the regeneration of unmyelinated nerves, leading to skin reinnervation.

**Functional Consequences of 4MC-Promoted Skin Reinnervation**

To determine whether skin reinnervation by 4MC can reverse functional deficits induced by RTX, we measured thermal withdrawal latencies with the hot plate test. On D35, withdrawal latencies in the RTX + 4MC group were significantly decreased compared with those in the RTX group (14.1 ± 3.6 vs 20.2 ± 4.0 seconds; p = 0.0091) (Fig. 14A); these values, however, were still higher than those in the vehicle group on D7 (9.5 ± 2.8 seconds; p = 0.02).

To explore the effect of epidermal innervation on thermal responses further, we analyzed the relationship between withdrawal latencies and ENDs of PGP 9.5, CGRP, and SP on D35. The END of PGP 9.5 was linearly correlated with the withdrawal latency (r = 0.82; p = 0.004; Fig. 14B); a similar relationship was obtained for the correlation between the END of CGRP and the withdrawal latency (r = 0.86; p = 0.0014; Fig. 14C). The END of SP, however, was not correlated with the withdrawal latency (r = 0.40; p = 0.25; Fig. 14D). These findings indicated that the skin reinnervation by PGP 9.5(+) and CGRP(+) epidermal nerves contributed to the recovery of withdrawal latencies to thermal stimuli.

**DISCUSSION**

The present study provides pathologic and functional evidence for a new model of a small-diameter sensory neuropathy in mice induced with a single intraperitoneal dose of RTX. The major features of the model include unmyelinated nerve degeneration with subsequent skin denervation and a loss of thermal sensation and marked depletion of SP(+) DRG neurons with a mild reduction of CGRP(+) DRG neurons. Moreover, 4MC promoted regeneration of unmyelinated nerves, skin reinnervation, and a consequent reversal of thermal responses.
RTX-Induced Nerve Degeneration and Skin Denervation

For the present study, we created a model of neuropathy that specifically affects small-diameter sensory nerves, particularly with differential involvement of peptidergic nerve terminals in the skin. Previous models of neuropathy usually affected both large- and small-diameter sensory nerves, for example, cisplatin- and taxol-induced neuropathies (5, 30, 31). Thus, the effects of motor weakness in behavioral tests of these animals could not be completely excluded. In the current experimental system, small-diameter nerves were specifically affected, whereas large-diameter nerves remained intact. In particular, RTX induced an irreversible loss of SP(+) nerves innervating the skin, whereas CGRP(+) epidermal nerves can reinnervate the skin after 4MC treatment.

Resiniferatoxin is an ultrapotent capsaicin analog, and its affinity with the transient receptor potential vanilloid receptor 1 can cause a loss of peptidergic DRG neurons in culture (32). Previous studies on RTX and capsaicin mainly focused on the morphologic or functional loss of DRG neuronal cell bodies (33–35). The current report indicates that a proportion of DRG neurons, particularly SP(+) neurons, are depleted by systemic administration of RTX. Taken together, these data suggest that in addition to neuronal loss, RTX also contributes to the degeneration of unmyelinated peripheral processes of DRG neurons, which leads to skin denervation and impairment of nociception.

Skin innervation has become a novel approach to investigating neuropathies affecting small-diameter nociceptive nerves both clinically and experimentally (36–39). The reduction of PGP 9.5(+) epidermal nerves in skin biopsies can be attributed to the degeneration of unmyelinated nerve terminals (2, 6) or the downregulation of neuronal proteins (7). The application of skin biopsies in diagnosing neuropathies is usually based on the assumption that nerve degeneration underlies skin denervation (40). Only limited studies have provided direct evidence of unmyelinated nerve degeneration (2). By providing ultrastructural evidence of a significant reduction in unmyelinated nerve densities in mediatal plantar nerves, we demonstrate that degeneration of unmyelinated nerves can be responsible for skin denervation. The degrees of nerve fiber loss seem different between unmyelinated nerve density of mediatal plantar nerve (~53%) and skin innervation (~66%), suggesting that RTX also produces distally accentuated nerve degeneration.

The mechanisms that mediate RTX-induced unmyelinated nerve degeneration are presently uncertain. Resiniferatoxin and capsaicin can evoke calcium influx (33, 41–43), and those influxes can elicit downstream responses, including degradation of the axonal cytoskeleton (44, 45). Moreover, a recent study suggested that RTX and capsaicin act as mitochondrial inhibitors (35). Because axons are mainly dependent on the cell body for energy, such a demand might render peripheral nerve terminals susceptible to RTX as well.

Promotion of Unmyelinated Nerve Regeneration by 4MC

This report also documents the therapeutic potential of 4MC in promoting skin reinnervation, particularly of CGRP(+) nerve terminals. Previous studies mainly focused on the induction of endogenous synthesis of nerve growth factor by 4MC (22) and implied potential therapeutic effects
FIGURE 14. Effect of 4-methylcatechol (4MC) on the thermal response and correlations with skin innervation after resiniferatoxin (RTX)-induced neuropathy. The thermal response was assessed as withdrawal latencies on a hot plate test in the RTX and RTX + 4MC groups on Day 35 of RTX treatment (A). The relation between the withdrawal latency and epidermal nerve density (END) of different phenotypes was analyzed with a linear regression model for protein gene product (PGP) 9.5 (B), calcitonin gene-related peptide (CGRP) (C), and substance P (SP) (D). (A) The withdrawal latency in the RTX + 4MC group was significantly reduced compared with that of the RTX group. (B–D) The withdrawal latencies were linearly correlated with the ENDs of PGP 9.5 and CGRP, but not with that of SP. *, Statistically significant.

of 4MC on certain types of toxic neuropathies such as amino-glycoside and pyridoxin-induced neuropathies (23, 46). Both types of neuropathies affect large-diameter DRG neurons that mainly depend on neurotrophin 3 (47).

Various neurotrophins have been implicated in the development and survival of small-diameter DRG neurons. Except for the central process of DRG neurons (48), the beneficial effect on the regeneration of small-diameter DRG neurons with their peripheral processes has rarely been documented. Such studies were performed on cultured DRG neurons (49) or in developing animals (18). There is a lack of studies exploring the potential for regenerating unmyelinated nerves in adult animals.

Because the unmyelinated nerve density of the medial plantar nerve was significantly increased after 4MC treatment to a level comparable to controls, the current study provides direct evidence that 4MC promotes regeneration of unmyelinated nerves and subsequent reinnervation of the skin. Cutaneous nerve terminals are the peripheral processes of small-diameter DRG neurons, presumably depending on trk activation (47). 4-Methylcatechol may restore the downregulation of trk in DRG neurons, as suggested by a recent study that demonstrated the stimulating effect of 4MC on trk phosphorylation (50). Further studies are required to test this hypothesis. Nevertheless, this report documents an experimental neuropathy with selective involvement of small-diameter sensory nerves and possible therapeutic effects by 4MC.

REFERENCES


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