P2X3-mediated peripheral sensitization of neuropathic pain in resiniferatoxin-induced neuropathy

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Patients suffering from sensory neuropathy due to skin denervation frequently have paradoxical manifestations of reduced nociception and neuropathic pain. However, there is a lack of satisfactory animal models to investigate these phenomena and underlying mechanisms. We developed a mouse system of neuropathy induced by resiniferatoxin (RTX), a capsaicin analog, and examined the functional significance of P2X3 receptor in neuropathic pain. From day 7 of RTX neuropathy, mice displayed mechanical allodynia (p<0.0001) and thermal hypoalgesia (p=0.0001). After RTX treatment, dorsal root ganglion (DRG) neurons of the periphrine type were depleted (p=0.012), while neurofilament (+) DRG neurons were not affected (p=0.62). In addition, RTX caused a shift in neuronal profiles of DRG: (1) increased in P2X3 receptor (p=0.0002) and ATF3 (p=0.0006) but (2) reduced TRPV1 (p=0.036) and CGRP (p=0.015). The number of P2X3(+)/ATF3(+) neurons was linearly correlated with mechanical thresholds (p=0.0017). The peripheral expression of P2X3 receptor in dermal nerves was accordingly increased (p=0.016), and an intraplantar injection of the P2X3 antagonists, A-317491 and TNP-ATP, relieved mechanical allodynia in a dose-dependent manner. In conclusion, RTX-induced sensory neuropathy with upregulation of P2X3 receptor for peripheral sensitization of mechanical allodynia, which provides a new therapeutic target for neuropathic pain after skin denervation.

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Introduction

Neuropathic pain develops after peripheral nerve degeneration, which presumably reduces nociceptive input. In human peripheral nerve diseases affecting small-diameter nociceptive nerves, some patients could have paradoxical symptoms: neuropathic pain but with reduced sensitivities to noxious stimuli due to skin denervation (Baron et al., 2009; Cheng et al., 2009; Obrosova, 2009; Polydefkis et al., 2004). Similar phenomena are observed after capsaicin-induced skin denervation (Gibbons et al., 2010; Rage et al., 2010). Peripheral sensory nerves are axonal extensions of dorsal root ganglion (DRG) neurons which consist of large-diameter and small-diameter neurons with corresponding sizes of nerve fibers. It is, however, not clear whether pure injury to small neurons could result in such a paradoxical combination of symptoms: reduced nociception and neuropathic pain. There is also a lack of experimental systems to test this hypothesis. We previously established a neuropathy system induced by resiniferatoxin (RTX), a capsaicin analog. Skin denervation with degeneration of unmyelinated nerves is the predominant feature of RTX-induced neuropathy (Hsieh et al., 2008), which can serve as a prototype of pure small-fiber neuropathy and provide a good opportunity to examine underlying molecular mechanisms of clinical presentations: neuropathic pain and reduced nociception.

Nerve injury causes a cascade of responses in neuronal cell bodies, for example, the upregulation of transcription factors, which leads to the generation of effector molecules responsible for maladaptive behaviors of neuropathic pain. Activating transcription factor-3 (ATF3) is a member of the ATF/CREB transcription factor superfamily (Hai and Hartman, 2001) and upregulated in DRG neurons after nerve injury, for example, spinal nerve ligation (Fukuoka et al., 2012). These findings raise the possibility of exploring whether or not ATF3 is upregulated in RTX-induced neuropathy, which only selectively depletes cutaneous nerves (Avelino and Cruz, 2000; Hsieh et al., 2008; Neubert et al., 2003). If so, will the pattern of ATF3 expression in

Abbreviations: ATF3, Activating transcription factor-3; CGRP, Calcitonin gene-related peptide; DRG, Dorsal root ganglion; RTX, Resiniferatoxin; TRPV1, Transient receptor potential vanilloid subtype 1.

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DRG neurons parallel the behavioral manifestations and what are the phenotypes of ATF3(+) neurons?

Puringeric receptors, such as P2X3, play important roles in the development of neuropathic allodynia (Burnstock, 2009; Kaan et al., 2010). Previous studies have documented upregulation of P2X3 receptor in peripheral neuropathic pain, for example, mechanical injury-induced focal neuropathy (Tsuchihara et al., 2009) and chronic visceral pain model (Banerjee et al., 2009; Xu et al., 2008). It is, however, not clear the phenotypes of P2X3(+) neurons in neuropathic pain, i.e., whether these are injured vs. uninjured neurons or large vs. small-diameter DRG neurons. Thus, P2X3 can potentially provide a link between nerve injury and neuropathic pain. However, the role of P2X3 in RTX-induced neuropathy and the contribution of P2X3 to the pathology and underlying molecular mechanisms of peripheral sensitization have not been systematically explored.

To address the above issues, we used RTX-induced neuropathy to investigate (1) neuropathic pain behaviors and their relation to ATF3, (2) the profiles of DRG neurons with induced ATF3 expression, and (3) the pharmacological effects of P2X3 antagonism on RTX-induced neuropathic pain behaviors.

Materials and methods

Systemic RTX treatment

Experiments were performed on 8-week-old male ICR mice (35–40 g). RTX (Sigma, St. Louis, MO) was dissolved in the vehicle (10% Tween 80 and 10% ethanol in normal saline). Mice in the RTX group received a single dose of RTX (50 μg/kg) by an intraperitoneal injection (Hsieh et al., 2008). The other group received an equal volume of the vehicle (the vehicle group) as the control. Mice were housed in plastic cages on a 12-h light/12-h dark cycle and were allowed access water and food ad libitum. All procedures were conducted in accordance with ethical guidelines for laboratory animals (Zimmermann, 1983) and the protocol was approved by the Animal Committee of National Taiwan University College of Medicine, Taipei, Taiwan and Kaohsiung Medical University, Kaohsiung, Taiwan.

Animal behavior evaluation

The behavior evaluation included thermal (hot-plate test) and mechanical (von Frey filament test) responses. Tests were performed before RTX injection (D0) and on day 7 (D7) after RTX treatment, and then weekly until D84, at the end of the experiment.

Hot-plate test

Mice were placed on a 52 °C hot plate (IITC, Woodland Hills, CA), enclosed in a Plexiglas cage. The withdrawal latencies of the hindpaw to thermal stimulations were determined to an accuracy of 0.1 s. Each test session consisted of three trials separated by 30-min 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 the thermal stimulation.

von Frey filament test

Mechanical thresholds of the hindpaw were assessed using the up-and-down method with different calibers of von Frey monofilaments (Somedic Sales AB, Hörry, Sweden). Briefly, a series of monofilaments was applied to the plantar region of the hindpaw. If paw withdrawal occurred, a monofilament of a smaller caliber was applied. In the absence of paw withdrawal, a monofilament of a larger caliber was then used. Four additional stimuli with monofilaments of different calibers based on the preceding responses were applied. The mechanical thresholds were calculated according to a published formula (Chaplan et al., 1994).

Double-labeling immunofluorescent staining of DRG and dermal sheets

In double-label experiments, the well-established tyramide signal amplification (TSA) technique was used for primary antisera raised in the same species (Lin et al., 2008). Sections were sequentially incubated with one primary antisera, a biotinylated-labeled secondary antibody, and streptavidin-horseradish peroxidase (HRP) (1:100, PerkinElmer) for 30 min. Signals were amplified with the fluorescein tyramide reagent (1:50, PerkinElmer) for 3 min. After rinsing in 0.5 M Tris buffer (Tris), sections were incubated with the second primary antisera, followed by a Texas red-conjugated secondary antibody for 1 h (1:100, Jackson Immunoresearch, West Grove, PA). The concentration of the first primary antisera was much lower than that for regular immunostaining (1:6000), which was beyond the detection limit of conventional immunofluorescence. For the two primary antisera raised in different species, conventional immunofluorescent staining was performed, i.e., incubation with Texas red and fluorescein isothiocyanate (FITC)-conjugated secondary antisera (1:100, Jackson Immunoresearch) corresponding to appropriate primary antisera for 1 h. Sections were mounted using Vectashield (Vector, Burlingame, CA). Cryostat sections of 8-μm thickness from the lumbar DRGs were used in this study. For systematic sampling, two ganglia (L4 and L5) per mice were collected for sectioning. Every section of each ganglion with an interval of 80 μm was immunostained and quantified. The primary antisera included ATF3 (rabbit, 1:6000, Santa Cruz Biotechnology, Santa Cruz, CA), peripheral (rabbit, 1:800, Chemicon, Temecula, CA), neuropeptide SM132 (mouse, 1:600, Covance, Emeryville, CA), P2X3 (rabbit, 1:400, Neuromics, Edina, MN), calcitonin gene-related peptide, (CGRP, rabbit, 1:800, Sigma), and transient receptor potential vanilloid subtype 1 (TRPV1, goat, 1:100, Santa Cruz Biotechnology).

Dermal nerves of different phenotypes

We also investigated the changes in dermal nerves of different phenotypes. Briefly, the plantar skin of the hindpaw was removed and incubated in an EDTA solution at 37 °C for 30 min. The dermis was separated from the epidermis and subcutaneous tissues were trimmed (Tschaechler et al., 2004). Dermal sheets were immersed in 4% paraformaldehyde overnight and then incubated with different primary antisera following the same immunofluorescence procedures described above.

Quantitation of DRG neurons and dermal nerves of different phenotypes

For DRG neuron quantitation, each DRG section was photographed at 200× under a fluorescence microscope (Axioptih microscope, Carl Zeiss, Heidelberg, Germany) in a systematic fashion to produce a montage of the entire DRG section following established procedures (Hsieh et al., 2008). To avoid density bias, only the area containing neurons was measured and only neurons with a clear nuclear profile were counted. The diameter of each P2X3(+) DRG neuron was measured with Image J version 1.44d software (National Institutes of Health, Bethesda, MD). The density was expressed as neurons/mm2 and a histogram of the diameter of P2X3(+) DRG neurons was plotted. The quantitation of dermal nerve fibers followed our established protocols (Lin et al., 2008). For each dermal sheet, photographs were taken at 200× under an Axioptih fluorescence microscope (Carl Zeiss) from 10 randomly selected areas, covering 30–40% of the entire dermal sheet. The intensities of the immunofluorescence signals were subtracted from the background, and each pixel (intensity signal) was equivalent to 0.1445 μm2. The areas of each dermal nerve of different phenotypes were divided by the selected area of the dermis. The dermal nerve density was expressed as μm2/mm2.

Pharmacological intervention with P2X3 by an intraplantar injection

Pharmacological experiments were performed with single dose of P2X3 antagonism on D7 after RTX treatment. Two antagonists were used: (Attal et al., 2010) A-317491 (Sigma), a P2X3-specific antagonist, at concentrations of 2, 20, and 200 μg/paw and (Avelino and Cruz, 2000) TNP–ATP (Sigma), a P2X1-4 antagonist, at concentrations of 16, 160, and 240 μg/paw (Oliveira et al., 2009). Drugs were dissolved in normal saline and administrated by an intraplantar injection at one hindpaw (10 μl/paw). The other group of mice received normal saline as the control for comparison (the saline group). Changes in mechanical thresholds were assessed at 0.5, 1, 2, 4, and 6 h after the intraplantar injection.

Experimental design and statistical analysis

In the set-up of RTX-induced neuropathy, there were two groups: the RTX group and the vehicle group. The pharmacological intervention study of P2X3 antagonist, A-317491, consisted of the experimental group and the saline group. The coding information was masked during the behavioral tests and the quantification procedures. There were 180 mice in total with 5–8 mice in each group at different time points. All data are expressed as the mean ± standard derivation of the mean and t test was performed for data with a Gaussian distribution. For data which did not follow a Gaussian distribution, a nonparametric Mann–Whitney test was conducted. pb 0.05 was considered statistically significant.

Results

Distinct neuropathic pain behaviors in RTX-induced neuropathy

To explore the behavioral patterns of neuropathic pain in RTX-induced neuropathy, we examined thermal latencies and mechanical thresholds. Mice with RTX-induced neuropathy exhibited thermal hypoalgesia and mechanical allodynia. On D7, the thermal latencies had markedly increased (10.3 ± 2.3 vs. 22.7 ± 2.9 s, pb 0.0001). Those thermal latencies were gradually decreased on D14 (22.9 ± 2.2 s, pb 0.0001) and were normalized from D56 (12.1 ± 2.8 s, p = 0.21) to D84 (10.8 ± 2.9 s, p = 0.85) (Fig. 1A). In contrast, the mechanical threshold had reached a nadir on D7 (513.7 ± 97.2 vs. 255.2 ± 84.6 mg, pb 0.0001). The degree of reduced mechanical thresholds gradually lessened had normalized by D56 (423.2 ± 120.1 mg, p = 0.103) (Fig. 1B). In summary, changes in the thermal latencies and mechanical thresholds followed similar temporal courses with a reversed pattern (r = −0.62, p < 0.0001), but this pattern was not observed in the vehicle group (r = 0.024, p = 0.82) (Fig. 1C vs. D).

Effects of RTX on DRG neurons of different phenotypes

To evaluate the effects of RTX on DRG neurons of different phenotypes, we first examined ATF3(+) DRG neurons. RTX had depleted peripherin(+) DRG neurons on D7 and D84 (Figs. 2A–C), but did not influence SMI32(+) DRG neurons (Figs. 2D–F). There were no ATF3(+) DRG neurons in the vehicle group (Figs. 2A,D). ATF3(+)}
neurons had markedly increased by D7 (Figs. 2B,E) and had returned to the baseline level in the vehicle group by D84 (Figs. 2C,F). Most ATF3(+) neurons were co-expressed with peripherin, but only rarely with SMI32 on D7 (Figs. 2B,E). Quantitatively, the density of peripherin(+) neurons was lower on D7 (367.7±51.3 neurons/mm²; \( p = 0.012 \)) and D84 (369.6±103.3 neurons/mm²; \( p = 0.02 \)) in the RTX group compared to those in the vehicle group. ATF3(+) neurons were increased on D7 in the RTX group and co-localized with peripherin(+) neurons (arrow in B). (D–F) There were no changes in SMI32(+) neurons in the vehicle (D) or RTX groups on D7 (E) and D84 (F). ATF3(+) neurons were mainly in SMI32(−) neurons (arrow in E), and rare in SMI32(+) neurons (arrowhead in E). (G–J) The graphs show neuronal densities of peripherin(+) (G) and SMI32(+) (H), and their co-localized expression with ATF3 (I, J) based on Figs. A–F. Peripherin(+) neurons were reduced but co-expressed with ATF3 in the RTX group. * Statistically significant at \( p < 0.05 \). Bar, 50 μm.

Fig. 2. Phenotypical changes in dorsal root ganglion (DRG) neurons after resiniferatoxin (RTX)-induced neuropathy. Double-labeling immunofluorescent staining was performed with anti-activating transcription factor-3 (ATF3; A–F, in green) and peripherin (A–C, in red) or neurofilament (SMI32, D–F, in red) in the vehicle group (A, D) and on day 7 (D7; B, E) and D84 (C, F) after the administration of RTX. (A–C) Peripherin(+) neurons were reduced on D7 (B) and on D84 (C) compared to those in the vehicle group. ATF3(+) neurons were increased on D7 in the RTX group and co-localized with peripherin(+) neurons (arrow in B). (D–F) There were no changes in SMI32(+) neurons in the vehicle (D) or RTX groups on D7 (E) and D84 (F). ATF3(+) neurons were mainly in SMI32(−) neurons (arrow in E), and rare in SMI32(+) neurons (arrowhead in E). (G–J) The graphs show neuronal densities of peripherin(+) (G) and SMI32(+) (H), and their co-localized expression with ATF3 (I, J) based on Figs. A–F. Peripherin(+) neurons were reduced but co-expressed with ATF3 in the RTX group. * Statistically significant at \( p < 0.05 \). Bar, 50 μm.


Induction of ATF3 in DRG neurons

To investigate the nociceptor phenotypes of ATF3(+) neurons and their functional significance, we first analyzed the co-expression of
P2X3 and CGRP with ATF3 (Fig. 3). On D7, RTX had induced P2X3 expression (234.2 ± 36.4 vs. 125.5 ± 13.4 neurons/mm²; p = 0.0002), and the expression in the RTX group had become comparable to the vehicle group by D84 (134.6 ± 29.3 neurons/mm²; p = 0.27) (Figs. 3A-C and G). In contrast, the expression of CGRP was lower on D7 (206.7 ± 19.6 vs. 165.8 ± 32.8 neurons/mm²; p = 0.015) and had returned to the baseline by D84 (206.4 ± 32.6 neurons/mm²; p = 0.98) (Figs. 3D-F and H). ATF3(+) neurons had increased by D7 (157.4 ± 54.3 vs. 4.1 ± 1.3 neurons/mm²; p = 0.0006), but the density had returned to the same level as the vehicle group by D84 (5.2 ± 1.7 neurons/mm²; p = 0.21) (Fig. 3I). The ratio of P2X3(+) / ATF3(+) neurons was higher than that of CGRP(+) / ATF3(+) neurons (36.5% ± 10.3% vs. 19.4% ± 6.2%; p = 0.0002) (Fig. 3J). The morphometric analyses indicated that P2X3(+) DRG neurons were similar in size between the vehicle and

Fig. 3. Changes in the expression of different nociceptors after resiniferatoxin (RTX)-induced neuropathy. Double-labeling immunofluorescent staining was performed with antisera against activating transcription factor-3 (ATF3; A–F, in green) and P2X3 (A–C, in red) or calcitonin gene-related peptide (CGRP; D–F, in red) in the vehicle group (A, D), and on day 7 (D7; B, E) and D84 (C, F) after RTX administration. (A–C) There were more P2X3(+) neurons on D7 in the RTX group than in the vehicle group. On D84, P2X3(+) neurons were comparable to those of the vehicle group. ATF3(+) neurons were higher on D7, and some were co-expressed with P2X3 (arrow in B). (D–F) CGRP(+) neurons were lower on D7 of RTX-induced neuropathy compared to the vehicle group. On D84, the number of CGRP(+) neurons was similar to that in the vehicle group. A small proportion of ATF3(+) neurons also expressed CGRP (arrowhead in E). (G–J) The graphs show neuronal densities of P2X3(+) (G), CGRP(+) (H) and ATF3(+) (I) according to the Figs. 3A–F, and (J) a comparison of co-expression ratio with ATF3(+) neurons. (K, L) The graphs show the morphometry analyses of P2X3(+) neurons including the diameter histogram (K) and mean neuronal diameter (L). * Statistically significant at p < 0.05. Bar, 50 μm.

the RTX group in terms of the diameter histograms (p = 0.51) and mean neuronal diameters (22.8 ± 0.8 vs. 21.9 ± 2.5 μm, p = 1.00) (Figs. 3K and L).

We further investigated the relationship between mechanical thresholds and nociceptor expression by performing a linear regression analysis (Fig. 4). On D7 of RTX-treated mice, the ratios of P2X3 (+)/ATF3(+) were correlated with the mechanical thresholds (r = -0.73, p = 0.0017, Fig. 4A). However, such a pattern was not observed for CGRP(+/)ATF3(+) neurons (r = -0.39, p = 0.16, Fig. 4B).

Co-localization of TRPV1 with P2X3(+) and CGRP(+) neurons

To understand the potential mechanisms for different expression patterns of P2X3 and CGRP after RTX treatment, we performed double-labeling experiments with TRPV1 (Fig. 5). TRPV1 was rarely colocalized with P2X3 (Figs. 5A,B) compared to CGRP (Figs. 5C,D), which was confirmed by quantifying the ratio of TRPV1(+)/P2X3 (+) relative to that of TRPV1(+)/CGRP(+) neurons (2.9% ± 1.5% vs. 19.0% ± 4.0%, p = 0.008, Fig. 5G). TRPV1(+) neurons were completely depleted on D7 (87.8 ± 13.9 vs. 1.6 ± 1.4 neurons/mm², p = 0.036), and on D84 (2.3 ± 2.5 neurons/mm², p = 0.036) (Figs. 5E, F, and H). Taken together, this observation provides an explanation for the reduced expression of CGRP in RTX-induced neuropathy: the depletion of CGRP(+) neurons was related to their co-localization with TRPV1 (+) neurons.

Changes in phenotypes of dermal nerves after RTX-induced neuropathy

To explore whether the peripheral process of DRG neurons had similar changes as the cell body, we assessed the phenotypes of dermal nerves in the skin. In the vehicle group, peripherin(+) dermal nerves exhibited an interlacing network pattern and the abundance of peripherin(+) dermal nerves was decreased on D7 and on D84 (Figs. 6A–C). TRPV1(+), CGRP(+) and P2X3(+) dermal nerves showed punctate profiles (Figs. 6D–L). The patterns of dermal nerves were similar to those of DRG neurons, i.e. disappearance of TRPV1 (+) dermal nerves on D7 and D84 (Figs. 6D–F). CGRP(+) dermal nerves were reduced on D7 and reappeared on D84 (Figs. 6G–I). In contrast, the density of P2X3(+) dermal nerves was increased on D7 (Figs. 6J vs. K) and became normalized on D84 (Fig. 6L). Quantitatively, the changes in dermal nerves of different phenotypes paralleled those of their DRG neurons (Fig. 6M–P).

Effects of P2X3 antagonism on mechanical allodynia

To determine the functional significance of increased P2X3 expression in the skin, we conducted pharmacological interventions by an intraplantar injection of P2X3 antagonists: A-317491 and TNP–ATP (Fig. 7). Before the injection, both hindpaws showed mechanical allodynia (p < 0.0001), and there was no difference in the mechanical thresholds between the two paws (p = 0.16) on D7 of RTX treatment. At the dose of 200 μg/paw for A-317491, mechanical allodynia had become normalized by 0.5 h (597.7 ± 167.0 vs. 248.8 ± 66.9 mg, p = 0.016) which lasted for 4 h (461.0 ± 158.8 vs. 241.3 ± 30.2 mg, p = 0.008). Mechanical allodynia reappeared at 6 h with similar mechanical thresholds compared to those of the control group (294.5 ± 115.5 vs. 284.6 ± 95.4 mg, p = 0.45) and to the contralateral paws (p = 0.5). The antagonistic effect was dose-dependent (Fig. 7A): the relief of mechanical allodynia by 20 μg/paw of A-317491 only lasted for 0.5 h (531.6 ± 195.2 mg, p = 0.029), and mechanical allodynia had re-developed by 1 h later. There was no effect on the mechanical thresholds with an injection of 2 μg/paw (p = 0.31).

There were similar dose-dependent effects of TNP–ATP on the changes in mechanical thresholds after RTX-induced neuropathy (Fig. 7B). Both hindpaws on D7 of mice after RTX-treatment had similar levels of mechanical allodynia (p = 0.23). At a dose of 240 μg/paw, mechanical thresholds were higher at 0.5 h (706.3 ± 230.9 vs. 253.3 ± 38.7 mg, p = 0.029), and the effect lasted for 2 h (363.1 ± 89.5 vs. 276.5 ± 62.6 mg, p = 0.029). Mechanical allodynia had reappeared by 4 h post-injection (p = 0.11). With 160 μg/paw administration, mechanical allodynia was attenuated at 0.5 h (727.5 ± 162.3 mg, p = 0.018) and 1 h (425.1 ± 95.0 mg, p = 0.036). With an injection of 16 μg/paw, the antagonistic effect was only observed at 0.5 h (451.4 ± 75.1 mg, p = 0.029).

Discussion

This study demonstrates a mouse system of neuropathy with mechanical allodynia and thermal hypoalgesia, activation of ATF3 in DRG neurons with concomitant upregulation of P2X3 receptor and downregulation of CGRP, and structural evidence of peripheral sensitization in the development of neuropathic pain behaviors after cutaneous nerve degeneration.

Manifestations of neuropathic pain due to small-diameter sensory nerve injury

The major theme of the report is the establishment of a mouse system with reduced nociception but with mechanical hypersensitivity. In RTX neuropathy, mice showed thermal hyperalgesia and mechanical allodynia due to selective depletion of small-diameter nociceptive nerves. Previous studies attributed injury-induced mechanical hypersensitivity to degeneration or dysfunctions of large DRG neurons and their nerves (Baron 2009). For example, a report indicated an abnormal pathology of myelinated fibers in RTX-induced mechanical allodynia (Pan et al., 2003). The role of small DRG neurons in this issue has received little attention. The current report demonstrates the parallel appearance of mechanical allodynia with corresponding upregulation of the purinergic P2X3 receptor in DRG neurons with increased P2X3(+) nerve fibers in the skin, which was responsible for the development of mechanical allodynia.
In contrast, large DRG neurons and their nerve fibers in RTX-induced neuropathy remained intact according to normal myelinated nerve morphometry and nerve conduction studies (Hsieh et al., 2008). RTX is an ultrapotent capsaicin analog (Brown et al., 2005; Iadarola and Mannes, 2011; Kissin and Szallasi, 2011). The current study documents that thermal hypoalgesia and mechanical allodynia were inversely correlated. What is the possible mechanisms underlying these two distinct neuropathic behaviors? One putative mechanism is the influence of RTX on different populations of ATF3(+) small-diameter neurons independent of TRPV1 phenotype (Braz and Basbaum, 2010). In the present report, mechanical thresholds were linearly correlated with ratio of P2X3(+)/ATF3(+) but not with CGRP(+)/ATF3(+) neurons, suggesting that abnormal mechanical sensations could be initiated by injuries to small-diameter sensory neurons and mediated by enhanced purinergic signaling.

In human small-fiber neuropathy due to skin denervation, most patients noticed a loss of thermal nociception. A proportion of patients experienced additional neuropathic pain (Bouhassira et al., 1999). The former symptom of reduced sensitivity is straightforward as the loss of cutaneous nerve terminals lead to impaired transmission of nociceptive stimuli. However, the pathophysiology and mechanisms of concomitant pain on a background of skin denervation is not fully understood. Furthermore there is no correlation between the degree of pain and the degree of neuropathy (Kalliomaki et al., 2011; Wildgaard et al., 2012). This report provides possible scenario for the combination of both symptoms: the expression of ATF3 was increased in injured small-diameter neurons and the preferential expression of P2X3 led to mechanical allodynia.

Activation of ATF3 and its consequences in RTX-induced neuropathy

The phenotypic profiles of ATF3(+) DRG neurons after RTX treatment provide molecular explanations for the behavioral manifestations. Recently, a comprehensive study demonstrated increased expression of ATF3 in DRG neurons after a variety of nerve injuries and noxious stimuli (Braz and Basbaum, 2010). Those observations raise intriguing issues: what are the phenotypic features of ATF3(+) DRG neurons and how do such changes contribute to neuropathic pain behaviors? The current study demonstrates increased ATF3 expression with RTX-induced neuropathy and its link to denervation of cutaneous nerves. The higher percentage of CGRP/TRPV1 neurons compared to P2X3/TRPV1 neurons explains the difference in the degree of cutaneous nerve denervation after RTX treatment.

In addition to being an injury marker, roles of ATF3 in previous studies mainly focused on promoting nerve regeneration (Seijffers et al., 2007). That report indicated the association of ATF3 with the expression of nociceptive molecules in DRG neurons. In the present study, ATF3 was induced after RTX treatment, mainly on peripherin (+) DRG neurons, and the pattern of ATF3 expression had become peptidergic fibers on the skin. Our previous study indicated the thermal latencies was linearly correlated with CGRP(+) cutaneous innervation (Hsieh et al., 2008). In the current report, the temporal expression of CGRP on neuronal soma (DRG) and their peripheral processes (dermal nerves) were correlated with withdrawal latencies to thermal stimuli. Further studies are required to unravel the role of peptidergic fibers in the absence of TRPV1.

Taken together, the inverse correlation between thermal withdrawal latencies and mechanical thresholds suggests that two distinct small-diameter nociceptors underlie distinct behaviors: CGRP(+) for thermal hypoalgesia and P2X3(+) for mechanical allodynia.
nearly normalized by D84, consistent with the normalization of neuropathic pain behaviors. P2X3 purinoceptor was associated with ATF3(+) DRG neurons after skin denervation. A potential link between these molecular signatures might be mas-related G protein-coupled receptors D (mrgprD)(+) neurons, which are sensitive to mechanical stimuli and ATP (Cavanaugh et al., 2009; Dussor et al., 2008; Zylka et al., 2005). In summary, the concomitant changes in the co-localization of P2X3 (+)/ATF3(+) could act as an indicator for the neuropathic pain behaviors. How these signaling pathways contributed to RTX-mediated mechanical allodynia will require further investigations.

Structural evidence of peripheral sensitization and its clinical implications

This study documented the increased expression of P2X3 in dermal nerves as morphological evidences of peripheral sensitization in

Fig. 6. Expression of different phenotypic dermal nerves in resiniferatoxin (RTX)-induced neuropathy. The expression of different phenotypic dermal nerves was assessed by immunofluorescent staining (A–L) and quantification accordingly (M–P) with the staining included peripherin (A–C in red), transient receptor potential vanilloid subtype 1 (TRPV1; D–F in green), calcitonin gene-related peptide (CGRP; G–I in red), and P2X3 (J–L in green) in the vehicle group (A, D, G, J) and on day 7 (D7; B, E, H, K) and D84 (C, F, I, L) after RTX administration. (A–C) Peripherin(+) dermal nerves showed interlacing network patterns (A) and mildly depleted on D7 (B) and D84 (C) after RTX. (G–I) CGRP(+) dermal nerves exhibited punctate appearances (G), which were decreased on D7 (H) but reappeared on D84 (I) after RTX. (D–F) TRPV1 showed punctate pattern in dermal nerves (D) and those patterns disappeared on D7 (E) and D84 (F) after RTX. (J–L) P2X3 was expressed on dermal nerves with a punctate pattern (J). The punctate profiles of P2X3 were increased on D7 (K) after RTX treatment but became normalized by D84 (L). (M–P) The graphs showed the quantification of dermal nerves immunoreactive for peripherin(+) (M), TRPV1(+) (N), CGRP(+) (O) and P2X3(+) (P) according to Figs. 6A–L. * Statistically significant at p < 0.05. Bar, 50 μm.
RTX-induced neuropathic pain. Previously, most studies mainly depended on a pharmacological intervention of peripheral tissues to document peripheral sensitization, for example, demonstrating changes in neuropathic pain behaviors by an intraplantar injection. A gap exists between structural data and pharmacological results, i.e., a lack of evidence showing that P2X3 expression increased in cutaneous nerve terminal regions. The current report shows structural evidence that increased P2X3 expression at dermal nerve terminals is responsible for injury-induced mechanical allodynia. Such observations were supported by an intraplantar injection of P2X3 antagonists to normalize mechanical allodynia. Currently, most treatment guidelines for neuropathic pain recommend the use of antidepressants and anticonvulsants, for example, the blockade of the α2δ subunit of the calcium channel (Attal et al., 2010; O’Connor and Dworkin, 2009) and treatment responses are not satisfactory (Attal et al., 2010; Finnerup et al., 2010). Taken together with a recent report on cancer pain (Kaan et al., 2010), this work was supported by grants from National Health Research Institute (NHRI-EX99-9736NI), National Science Council (NSC97-2320-B-002-042-MY3, NSC100-2320-B-037–018), Kaohsiung Medical University Research Foundation (KMU-Q090005, KMU-M100004), and the Excellent Translational Medicine Research Projects of NTUMC and NTUH (98C101-201).

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References


