



Selective ablation of TRPV1 by intrathecal injection of resiniferatoxin in rats increases renal sympathoexcitatory responses and salt sensitivity

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Abstract

This study tested the hypothesis that selective ablation of transient receptor potential vanilloid type 1 (TRPV1)-positive nerve fibers by intrathecal injection of resiniferatoxin (RTX) enhances renal sympathoexcitatory responses and salt sensitivity. Intrathecal injection of RTX (1.8 µg/kg) to the levels of lower thoracic and upper lumbar spinal cord (T8–L3) increased mean arterial pressure (MAP) in rats fed a normal (NS, 1% NaCl) or high-sodium (HS, 8% NaCl) diet for 4 weeks compared to vehicle-treated rats (NS: 121 ± 2 vs. 111 ± 2; HS: 154 ± 2 vs. 134 ± 2 mm Hg, both $P < 0.05$), with a greater increase in HS compared to NS rats (9 ± 1% vs. 15 ± 1%, $P < 0.05$). TRPV1 contents were decreased in T8–L3 segments of spinal dorsal horn but not in corresponding dorsal root ganglia and the kidney following RTX treatment ($P < 0.05$). Selective activation of GABA-A receptors with intrathecal T8–L3 segment-injection of muscimol (3 nmol/kg) decreased renal sympathetic nerve activity and increased urinary excretion in both NS and HS rats, with a greater effect in RTX-treated compared to vehicle-treated rats ($P < 0.05$). Chronic activation of GABA-A receptors with muscimol (50 mg/kg/day × 2, p.o.) abolished RTX treatment-induced pressor effects in NS and HS rats. GAD65/67, a GABA synthetase, in the spinal cord was downregulated and tyrosine hydroxylase in the kidney upregulated in NS or HS rats treated with RTX ($P < 0.05$). Thus, selective ablation of TRPV1-positive central terminals of sensory neurons plays a prohypertensive role possibly via inhibition of spinal GABA system especially with HS intake, suggesting that activation of TRPV1 in central terminals of sensory neurons may convey an antihypertensive effect.

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Introduction

Transient receptor potential vanilloid type 1 (TRPV1) is a Ca^{2+} -permeable channel that is activated by temperature above $> 42^\circ\text{C}$ [1, 2]. The TRPV1 channel is expressed by both central terminals in the spinal dorsal horn and peripheral terminals of sensory neurons that innervate peripheral organs such as the skin [3], blood vessels [4, 5], and the kidney [6, 7]. Correspondingly, TRPV1 plays a role in thermal sensation [1, 2, 8, 9], blood pressure regulation [10], and urinary excretion [6, 7].

We have been focusing on defining the role of TRPV1-positive sensory nerves in the regulation of blood pressure and salt sensitivity. Evidence accumulated from our previous studies shows that neonatal degeneration of TRPV1-positive sensory nerves throughout the body by subcutaneous injection of capsaicin leads to increased salt sensitivity of arterial pressure, indicating that TRPV1-positive sensory nerves play a counter-regulatory role against salt-induced increases in blood pressure [11–19].

However, systemic sensory nerve degeneration following subcutaneous capsaicin administration hinders our ability to identify the role of specific organ(s)/tissue(s) in blood pressure regulation. If we can find a method to selectively ablate the sensory nerves located in specific areas, it will be helpful to reveal more deeply the mechanism underlying the counter-regulatory role of TRPV1-positive sensory nerves in salt-induced hypertension. Recently, the approaches of selective ablating of sensory nerves were investigated by two groups: Ulla Kopp group and our group.

The method of dorsal rhizotomy was used by Ulla Kopp group and their results show that arterial pressure was increased in rats fed a high-sodium diet following dorsal rhizotomy [20, 21]. Although these studies are indicative that afferent renal nerves play a role in the regulation of salt sensitivity of arterial pressure, the role of central terminals in blood pressure regulation was unknown.

Our lab used the strategy of intrathecal injection of TRPV1-specific short hairpin RNA (shRNA) to selectively knock down the TRPV1 expressed in dorsal root ganglion (DRG) neurons of T8–L3 segments of the spinal cord and their central and peripheral terminals, and observed that blood pressure was increased and the prohypertensive effects of high-salt intake was enhanced [22, 23]. Although TRPV1 was identified in the study as the key component of afferent nerves that played a role in the regulation of salt sensitivity of arterial pressure, the TRPV1 ablation was not specific enough because TRPV1 in both DRGs and their central and peripheral terminals were affected by intrathecal injection of TRPV1 shRNA. We aimed to investigate the blood pressure-regulatory effect of TRPV1-positive central terminals of DRG.

It is known that resiniferatoxin (RTX), an ultra-potent TRPV1 agonist, can be used as an ablator of TRPV1-containing fiber by causing a sustained and excessive influx of Ca^{2+} , which leads to nerve terminal death [24, 25]. By using intrathecal injection of RTX, we are able to set up a new model to selectively ablate TRPV1-containing central terminals of DRG without affecting the TRPV1 in the soma and peripheral terminals of DRGs. In the present study, we examined the role of TRPV1-positive central terminals in the regulation of arterial pressure and salt sensitivity by selectively ablating them in the spinal dorsal horn using RTX that was intrathecally administered into the lower thoracic and upper lumbar segments (T8–L3) of the spinal cord.

Methods

Animals

Male Wistar rats weighing 201–225 g (Charles River Laboratory, Wilmington, Massachusetts, USA) were

housed on a cycle of 12-h light and 12-h darkness. Food and water were available *ad libitum*. Rats received either a normal-sodium (NS) diet (1% NaCl by weight, Harlan Teklad, Madison, Wisconsin, USA) or a high-sodium (HS) diet (8% NaCl by weight, Harlan Teklad, Madison, Wisconsin, USA). After 1 week, rats received a surgery of intrathecal catheter-implantation and removal of right kidney. Nine days later, rats were injected intrathecally with RTX or vehicle (0.9% NaCl). Therefore, rats were divided into four groups: NS + vehicle, NS + RTX, HS + vehicle, and HS + RTX. Unless mentioned specifically, all experiments were conducted at the 15th day after intrathecal injection of RTX or vehicle. All experimental protocols were approved by the Institutional Animals Care and Use Committee of Michigan State University.

Surgery of intrathecal catheter-implantation and uni-nephrectomy

Catheters were implanted intrathecally in rats according to Yaksh and Rudy [26] with some modifications. Briefly, rats were anesthetized with ketamine/xylazine (85/5 mg/kg, *i.p.*). A small incision was made in the nape and the atlanto-occipital membrane. A PE-10 catheter filled with 0.9% sterile saline was inserted into the subarachnoid space as far as T8 segment of the spinal cord. The catheter was then sutured in place with the skin. The external port of the catheter was sealed with Parafilm film to ensure that the cerebrospinal fluid will not flow out. The right kidney was removed for each rat. Kanamycin (10 mg/kg), penicillin (50,000 unit/kg), and streptomycin (10 mg/kg) were injected subcutaneously daily for 3 days after surgery to prevent infection. The location of the catheter tip was verified at the end of the experiments. The data from rats showing wrong location of the catheter tip were excluded from data analysis.

Intrathecal injection

Ten microliters of RTX (1.8 $\mu\text{g/kg}$), capsaicin (1 $\mu\text{g/kg}$), or muscimol (3 nmol/kg) were injected intrathecally via the catheter. The catheter was flushed immediately every time with 10 μl of saline to ensure complete release of the drugs into the subarachnoid space.

Measurement of systolic blood pressure

Systolic blood pressure of conscious rats was measured using the indirect tail cuff method with a sphygmomanometer (Hatteras Instruments SC1000 Blood Pressure Analysis System, Cary, North Carolina, USA). Rats were acclimatized to the testing environment for 3 days prior to the testing day to decrease the effect of stress on blood

pressure. The blood pressure value was the average of nine separate measurements.

Measurement of mean arterial pressure

Rats were anesthetized with ketamine/xylazine (85/5 mg/kg, i.p.). A PE-50 catheter was implanted into the right carotid artery for monitoring of mean arterial pressure (MAP) with a Statham 231D pressure transducer coupled to a Gould 2400s recorder (Gould Instruments, Cleveland, Ohio, USA). Baseline MAP was obtained 3 h after surgery on fully awake and unstrained rats.

Response of mean arterial pressure to intrathecal injection of capsaicin

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). A PE-50 catheter was implanted into the right carotid artery for monitoring of MAP with a Statham 231D pressure transducer coupled to a Gould 2400s recorder (Gould Instruments, Cleveland, Ohio, USA). The response of MAP to intrathecal injection of capsaicin (1 µg/kg) was recorded in anesthetized rats.

Activation of GABA-A receptor by pretreatment with muscimol

Muscimol, a GABA-A receptor agonist, was administered into the rat stomach via gastric gavage at a dose of 50 µg/ml/kg twice a day during the period from 3 days before RTX treatment to 15 days after RTX treatment.

Recording of renal sympathetic nerve activity (RSNA)

Recording of RSNA was performed as described previously [27]. Briefly, anesthesia on the rat was induced by urethane (1.5 g/kg, i.p.). The renal nerve branch was dissected free from fat and connective tissue for a length of approximately 10 mm. The nerve was sectioned, and its proximal part was placed on one pair of stainless steel electrodes for recording of sympathetic nerve activity. The nerve was fixed to the electrodes and was prevented from drying by applying silicone elastomer (Kwik-CastTM, World Precision Instruments, Sarasota, Florida, USA). The signals were amplified 20,000 folds, filtered with a high-frequency cutoff at 1000 Hz and a low-frequency cutoff at 100 Hz by an AC pre-amplifier (G Model P511, Grass Technologies, West Warwick, Rhode Island, USA) and recorded by Gould 2400s recorder (Gould Instruments, Cleveland, Ohio, USA). Renal nerve activity was integrated over 1-s intervals by the P3 Plus software and was expressed in percentage change compared to its basal value. The

background activity was determined by recording the activity when crushing the renal nerve bundle central to the recording electrodes and was subtracted from all values of renal nerve activity.

Measurement of urine excretion

A PE-50 catheter was inserted into the ureter via midline incision for urine collection. Urine samples were collected for seven 10-min periods, i.e., 10–1 min before intrathecal injection of muscimol (3 nmol/kg), 1–10, 11–20, 21–30, 31–40, 41–50, and 51–60 min after intrathecal injection of muscimol. Urine flow rate was expressed per minute per gram of kidney weight (µl/min/g) and was expressed in change compared to its basal value.

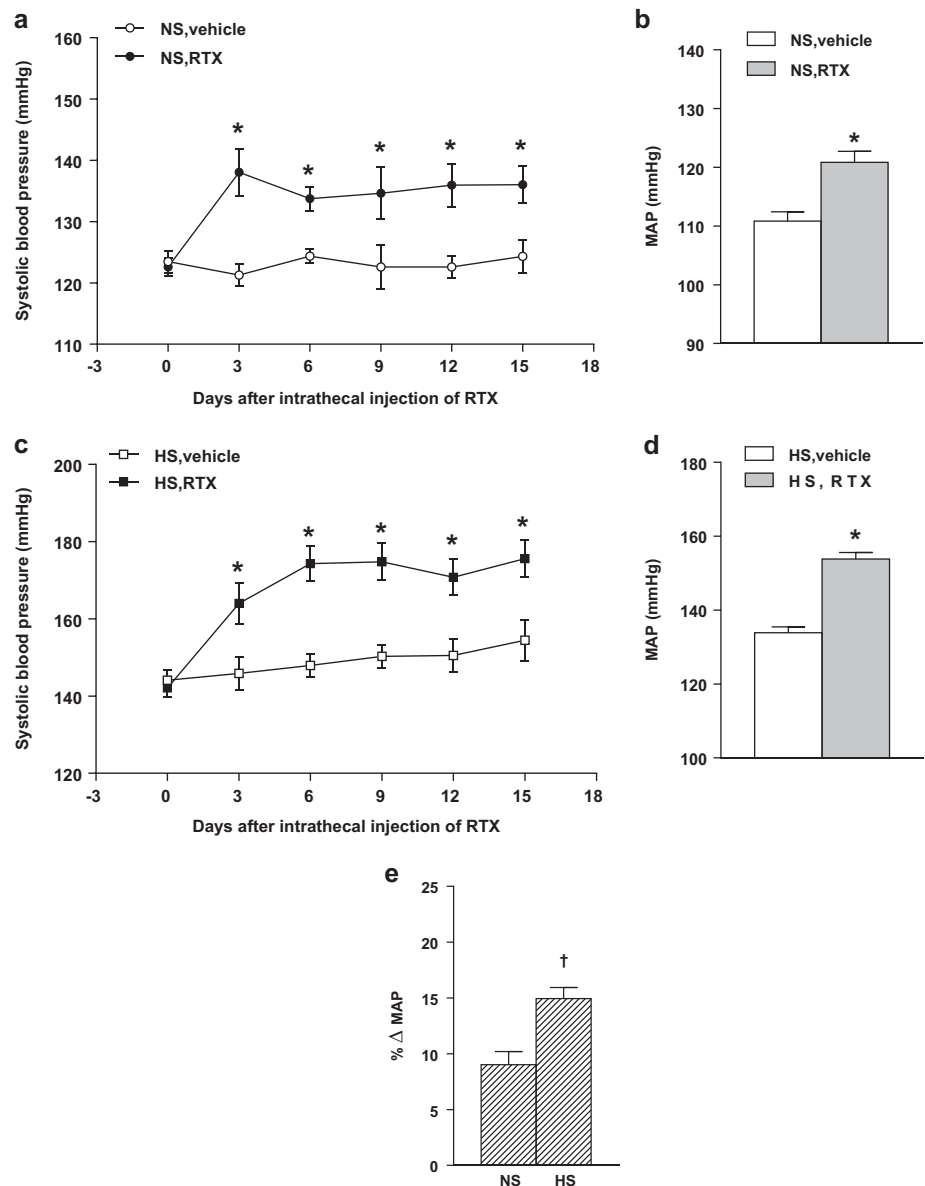
Immunohistochemistry

Rats were anesthetized by ketamine/xylazine (85/5 mg/kg, i.p.) and perfused transcardially by a fixative (4% paraformaldehyde in PBS). The samples of spinal cord of T8–L3 segments were harvested and cut into sections of 20 µm by a cryostat (Leica CM 1850). The sections were incubated with rabbit anti-TRPV1 polyclonal antibody (1:1000, RA10110, Neuromics, Edina, Minnesota, USA) or mouse anti-GAD65/67 monoclonal antibody (1:200, MSA-225, Assay Designs, Ann Arbor, Michigan, USA) for 1 h at room temperature, then incubated with Rhodamine Red (TM)-X donkey anti-rabbit IgG (1:100, 711-295-152, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) or FITC anti-mouse IgG (1:100, 715-095-150, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 1 h at room temperature, mounted with Vectashield mounting medium (H-1400, Vector Laboratories, Burlingame, California, USA). Pictures were taken with a fluorescent microscope (Olympus BX41 model, Olympus Optical Co. Ltd, Tokyo, Japan; Olympus MicroSuiteTM-Basic software, Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Western blot analysis

Western blot analysis was performed as described previously [14] with some modifications. Briefly, each sample was homogenized and sonicated in 2 ml of 10 mmol/L Tris buffer (pH 7.6) containing 0.5 mmol/L MgCl₂, 50 mmol/L NaCl, 10 mg/ml aprotinin, 18 mg/ml iodoacetamide, 10 mg/ml leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 500 × g for 5 min at 4 °C. The supernatant was added with 10 µl of 0.5 mol/L EDTA and 100 µl of 10% Triton X-100, incubated on ice for 45 min, and centrifuged at 22,000 × g for 30 min at 4 °C. The supernatant was saved. The protein concentration

Fig. 1 Intrathecal injection of RTX increased blood pressure in rats fed a NS or HS diet. Effect of intrathecal injection of resiniferatoxin (RTX) on systolic blood pressure (**a, b**) and mean arterial pressure (MAP) (**c, d**) in rats fed a normal-sodium (NS) or high-sodium (HS) diet. MAP was measured at 15th day after intrathecal injection of RTX or vehicle. **e** The percentage change of MAP of RTX group from that of corresponding vehicle group at 15th day after vehicle or RTX treatment. Values are mean \pm SE ($n = 7$ to 8). * $P < 0.05$ compared with the corresponding vehicle-treated group; $^{\dagger}P < 0.05$ compared with the corresponding NS diet group



was determined with a BCA assay kit (QBCA-1KT, Sigma-Aldrich, St. Louis, Missouri, USA). Equal amount of proteins was electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane (162-0180, Bio-Rad Laboratories, Hercules, California, USA). Membranes were incubated with rabbit anti-TRPV1 (1:1000, RA10110, Neuromics, Edina, Minnesota, USA), mouse anti-GAD65/67 monoclonal antibody (1:200, MSA-225, Assay Designs, Ann Arbor, Michigan, USA) or mouse anti-tyrosine hydroxylase (1:1000, 657010, Calbiochem, EMD Chemicals Inc. Gibbstown, New Jersey, USA) at 4 °C overnight, followed by incubation with HRP-donkey anti-rabbit IgG (1:10,000, 711-035-152, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) or HRP-donkey anti-mouse IgG (1:10,000, 711-035-151, Jackson

ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 2 h at room temperature. The immunoreactive bands were visualized by ECL reagents (RPN 2106, Amersham, GE Healthcare, Piscataway, New Jersey, USA) and a film (Kodak® X-Omat LS film, Eastman Kodak company, Rochester, New York, USA). The intensity of the bands was determined by using ImageJ (NIH, Bethesda, Maryland, USA). The protein loading was normalized by β -actin (mouse anti- β -actin antibody, 1:2000, sc-69879, Santa Cruz Biotechnology, Santa Cruz, California, USA).

Drugs

RTX (R8756, Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in saline including 1% ethanol. Capsaicin

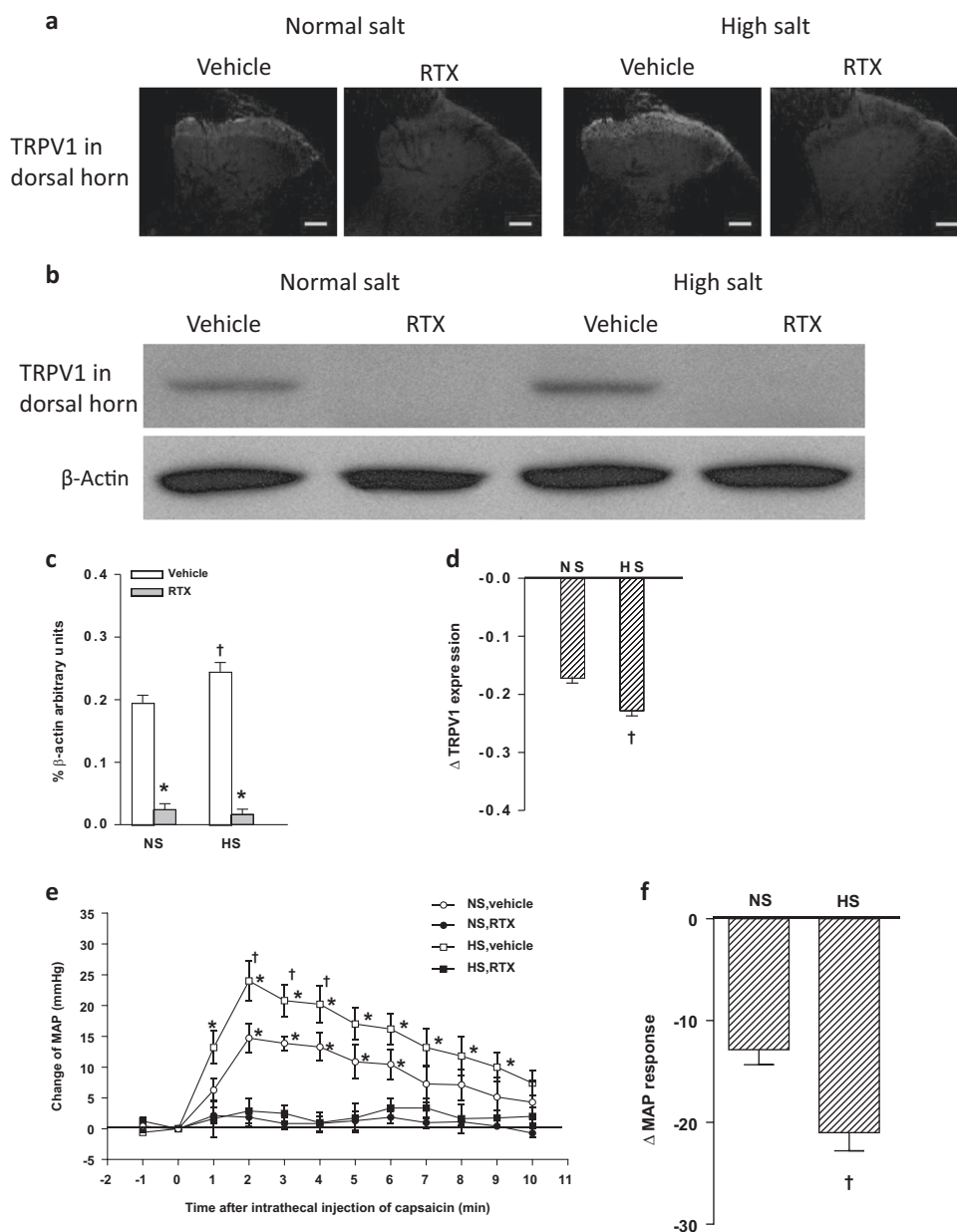
Fig. 2 TRPV1 expression and function were reduced in spinal cord after RTX treatment.

Immunohistochemistry staining (a) and western blot analysis (b, c, d) showing TRPV1 expression in the dorsal horn of spinal cord (T8–L3) of rats fed a NS or HS diet at 15th day after vehicle or RTX treatment.

b, c, and d indicate representative western blots, quantification results (% β -actin arbitrary units), and the change of TRPV1 expression of RTX group from that of corresponding vehicle group, respectively. Values are mean \pm SE ($n = 5$). * $P < 0.05$ compared with the corresponding vehicle-treated group; † $P < 0.05$ compared with the corresponding NS diet group.

Scale bars, 100 μ m. Time course responses of mean arterial pressure (MAP) to bolus intrathecal injection of capsaicin (1 μ g/kg) in pentobarbital-anesthetized NS and HS rats at 15th day after vehicle or RTX treatment (e). f The change of MAP response at 2 min after intrathecal injection of capsaicin of RTX group from that of corresponding vehicle. Values are mean \pm SE ($n = 7$ to 8).

* $P < 0.05$ comparison between vehicle-treated group and corresponding RTX-treated group receiving the same dose of capsaicin; † $P < 0.05$ compared with the corresponding NS diet group



(M2028, Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in saline including 0.1% ethanol. Muscimol (M1523, Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in saline.

Statistical analysis

All data were expressed as mean \pm SE. Unpaired Student's t -test was used to analyze the differences between two groups. One-way ANOVA followed by a Bonferroni adjustment for multiple comparisons was used to analyze the differences among groups. Differences at $P < 0.05$ were considered statistically significant.

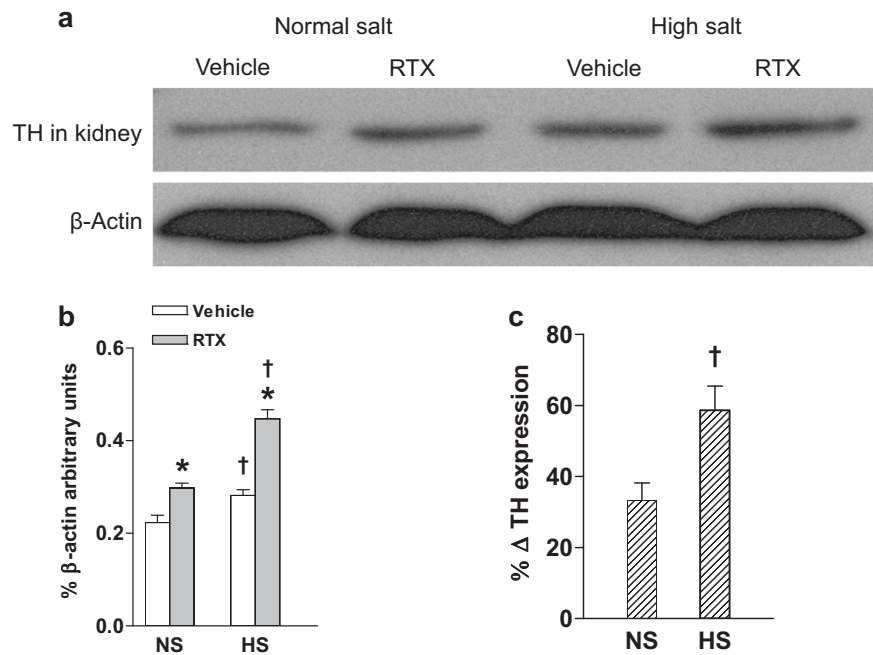
Results

Body weight in all groups increased with age (data not shown). There was no difference in body weight between NS rats and HS rats given vehicle or RTX at any time point, indicating that general condition of rats was not affected by HS feeding.

Intrathecal injection of RTX increased blood pressure in rats fed a NS or HS diet

Intrathecal injection of RTX was conducted at the time when rats had already received 2-week salt treatment.

Fig. 3 Tyrosine hydroxylase (TH) was upregulated in the kidney after RTX treatment. Western blot analysis showing TH expression in the kidney of rats fed a NS or HS diet at 15th day after vehicle or RTX treatment. **a**, **b**, and **c** indicate representative western blots, quantification results (% β -actin arbitrary units), and the change of TH expression of RTX group from that of corresponding vehicle group, respectively. Values are mean \pm SE ($n = 5$). * $P < 0.05$ compared with the corresponding vehicle-treated group; $^\dagger P < 0.05$ compared with the corresponding NS diet group. Scale bars, 100 μ m



Systolic blood pressure was elevated after intrathecal injection of RTX in both NS rats and HS rats and the elevation lasted more than 15 days (Fig. 1a, b), which was confirmed by direct measurement of MAP (Fig. 1c, d) (in NS rats, vehicle: 111 ± 2 vs. RTX: 121 ± 2 mm Hg, $P < 0.05$; in HS rats, vehicle: 134 ± 2 vs. RTX: 154 ± 2 mm Hg, $P < 0.05$). Moreover, the increases in MAP were greater in HS rats given RTX compared with NS rats given RTX (Fig. 1e) (NS: $9 \pm 1\%$ vs. HS: $15 \pm 1\%$, $P < 0.05$).

TRPV1 expression was reduced in spinal cord after RTX treatment

Immunohistochemistry studies revealed that TRPV1 labeling in the dorsal horn of spinal cord (T8–L3 segments) was eliminated in RTX-treated rats fed a NS or HS diet compared with corresponding vehicle-treated rats (Fig. 2a). Negative controls in which TRPV1 antibody was omitted showed no staining (data not shown). Consistently, western blot analysis revealed that TRPV1 expression was reduced markedly by RTX treatment in NS rats and HS rats in the spinal dorsal horn (T8–L3 segments) (Fig. 2b–d) (in NS rats, vehicle: 0.19 ± 0.01 vs. RTX: 0.02 ± 0.01 , $P < 0.05$; in HS rats, vehicle: 0.24 ± 0.01 vs. RTX: 0.02 ± 0.01 , $P < 0.05$). However, there were no changes in TRPV1 levels in DRG (Supplementary Fig. S1, in NS rats, vehicle: 0.32 ± 0.02 vs. RTX: 0.31 ± 0.02 , $P > 0.05$; in HS rats, vehicle: 0.38 ± 0.01 vs. RTX: 0.36 ± 0.02 , $P > 0.05$), kidney (in NS rats, vehicle: 0.24 ± 0.02 vs. RTX: 0.22 ± 0.03 , $P > 0.05$; in HS rats, vehicle: 0.33 ± 0.01 vs. RTX: 0.31 ± 0.03 , $P > 0.05$), and cervical spinal cord (in NS rats, vehicle: 0.20 ± 0.01 vs. RTX: 0.18 ± 0.02 , $P > 0.05$; in HS

rats, vehicle: 0.25 ± 0.01 vs. RTX: 0.22 ± 0.01 , $P > 0.05$) in NS rats or HS rats after RTX treatment. Moreover, TRPV1 expression in the dorsal horn, DRG, and kidney was higher in HS rats given vehicle compared with NS rats given vehicle (in dorsal horn, NS: 0.19 ± 0.01 vs. HS: 0.24 ± 0.01 , $P < 0.05$; in DRG, NS: 0.32 ± 0.02 vs. HS: 0.38 ± 0.01 , $P < 0.05$; in kidney, NS: 0.24 ± 0.02 vs. HS: 0.33 ± 0.01 , $P < 0.05$) and the decrease of TRPV1 by RTX in spinal cord was more in HS rats than in NS rats (NS: -0.17 ± 0.01 vs. HS: -0.23 ± 0.01 , $P < 0.05$).

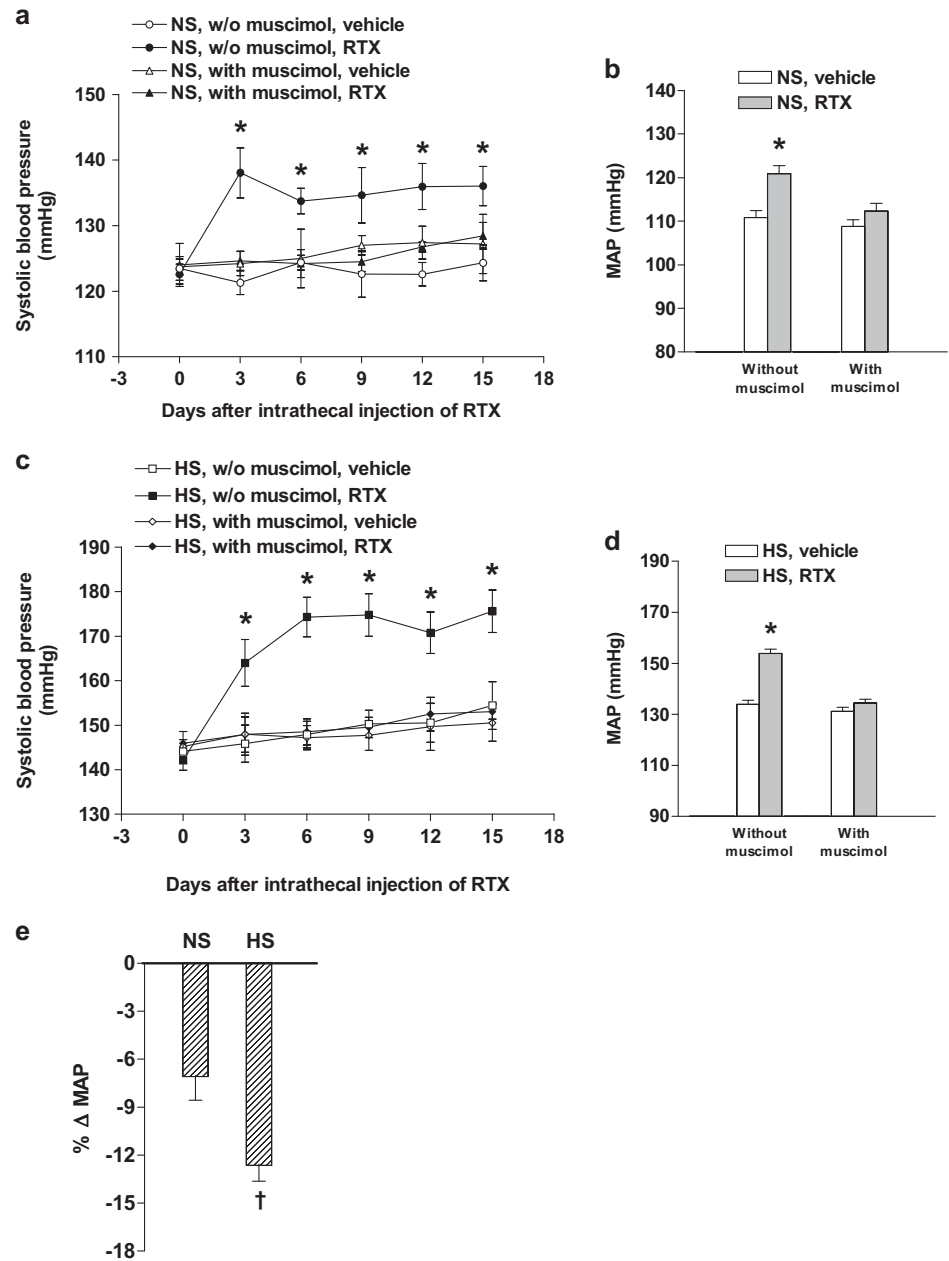
MAP response to capsaicin was weaker after RTX treatment

To further examine the elimination of RTX on TRPV1, a functional experiment was conducted. Bolus intrathecal injection of a TRPV1 agonist, capsaicin (1 μ g/kg), caused transient elevation of MAP in vehicle rats under pentobarbital anesthesia and the elevation was greater in HS rats than that in NS rats (Fig. 2e). The pressor responses to capsaicin disappeared in both NS rats and HS rats after RTX treatment; and the decreased pressor response was more remarkable in HS rats than in NS rats (NS: 14 ± 2 vs. HS: 21 ± 2 mm Hg, $P < 0.05$) (Fig. 2f). These data on alteration of TRPV1 function was consistent with corresponding data on alteration of TRPV1 expression (Fig. 2c).

Tyrosine hydroxylase (TH) was upregulated in the kidney after RTX treatment

Quantitative measurement by western blot analysis revealed that TH levels in the kidney were markedly enhanced in NS

Fig. 4 Activation of GABA-A receptor prevented RTX-induced elevation of blood pressure. Effect of pretreatment with muscimol (p.o.) on RTX-induced elevation of systolic blood pressure (**a**, **b**) and mean arterial pressure (MAP) (**c**, **d**) in rats fed a NS or HS diet. MAP was measured at 15th day after intrathecal injection of RTX or vehicle. **e** The percentage change of MAP of RTX-with muscimol group from that of corresponding RTX-without muscimol group at 15th day after vehicle or RTX treatment. Values are mean \pm SE ($n = 6$). * $P < 0.05$ compared with the corresponding vehicle-treated group; † $P < 0.05$ compared with the corresponding NS diet group

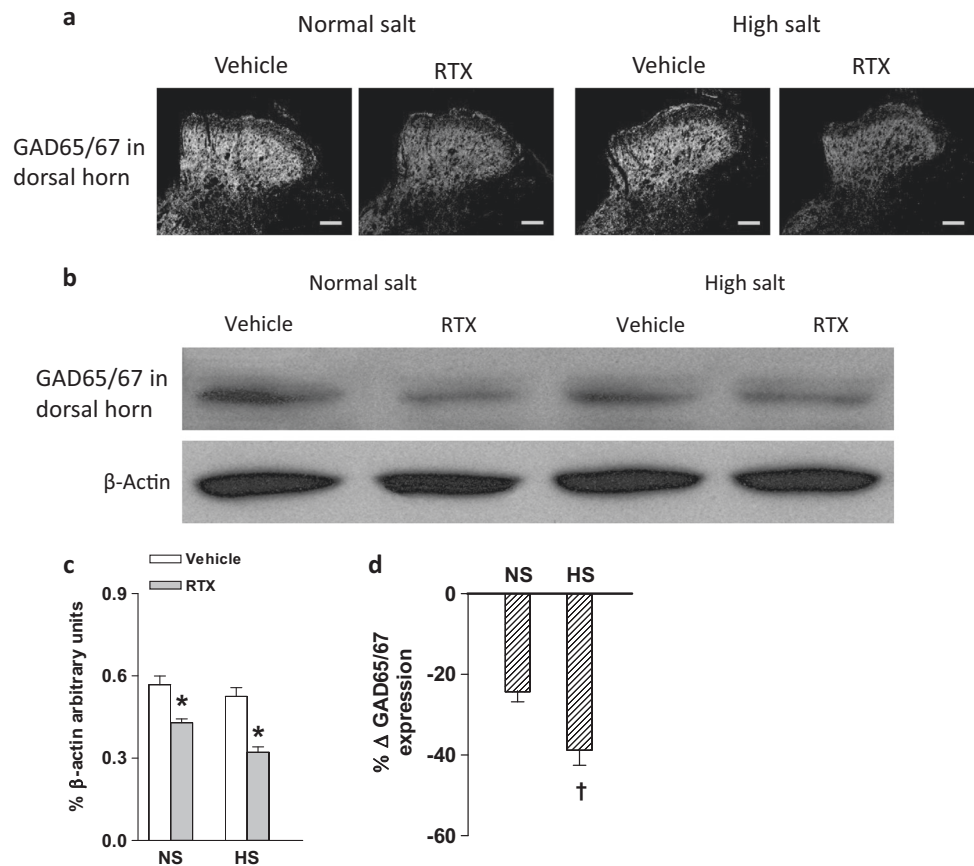


rats and HS rats given RTX compared with corresponding vehicle treatment (Fig. 3) (in NS rats, vehicle: 0.22 ± 0.02 vs. RTX: 0.30 ± 0.01 , $P < 0.05$; in HS rats, vehicle: 0.28 ± 0.01 vs. RTX: 0.45 ± 0.02 , $P < 0.05$). Moreover, in rats treated with vehicle, HS intake increased TH expression in the kidney in comparison with NS intake (NS: 0.22 ± 0.02 vs. HS: 0.28 ± 0.01 , $P < 0.05$). Interestingly, RTX treatment induced greater enhancement of TH in HS rats than in NS rats (NS: $33 \pm 5\%$ vs. HS: $59 \pm 7\%$, $P < 0.05$). HS diet increased the urine excretion, water intake, and urine excretion/water intake, whereas RTX treatment had no effects on these parameters (Supplementary Fig. S2).

Activation of GABA-A receptor prevented RTX-induced elevation of blood pressure

Activation of GABA-A receptor by muscimol pretreatment prevented RTX-induced elevation of systolic blood pressure in NS and HS rats (Fig. 4a, b), which was confirmed by direct measurement of MAP (Fig. 4c, d) (in NS rats, vehicle: 109 ± 2 vs. RTX: 114 ± 2 mm Hg, $P > 0.05$; in HS rats, vehicle: 132 ± 2 vs. RTX: 134 ± 2 mm Hg, $P > 0.05$). Muscimol played a greater depressor role in HS rats given RTX compared with NS rats given RTX (Fig. 4e) (NS: $-6 \pm 2\%$ vs. HS: $-13 \pm 1\%$, $P < 0.05$).

Fig. 5 GAD65/67 expression was attenuated in spinal cord after RTX treatment. Immunohistochemistry staining (a) and western blot analysis (b, c, d) showing GAD65/67 expression in the dorsal horn of spinal cord (T8–L3) of rats fed a NS or HS diet at 15th day after vehicle or RTX treatment. b, c, and d indicate representative western blots, quantification results (% β -actin arbitrary units), and the change of GAD65/67 expression of RTX group from that of corresponding vehicle group, respectively. Values are mean \pm SE ($n = 5$). * $P < 0.05$ compared with the corresponding vehicle-treated group; † $P < 0.05$ compared with the corresponding NS diet group. Scale bars, 100 μ m



GAD65/67 expression was attenuated in spinal cord after RTX treatment

Immunohistochemistry studies suggested that the expression of GABA synthetases, glutamate decarboxylases of 65 kDa and 67 kDa (GAD65/67), in the dorsal horn of spinal cord (T8–L3 segments) was attenuated in RTX-treated rats fed a NS or HS diet compared with corresponding vehicle-treated rats (Fig. 5a). Negative controls in which GAD65/67 antibody was omitted showed no staining (data not shown). Consistently, western blot analysis revealed that GAD65/67 levels were reduced by RTX treatment in NS rats and HS rats in the spinal dorsal horn (T8–L3 segments) (in NS rats, vehicle: 0.57 ± 0.03 vs. RTX: 0.43 ± 0.01 , $P < 0.05$; in HS rats, vehicle: 0.53 ± 0.03 vs. RTX: 0.32 ± 0.02 , $P < 0.05$) and the decrease of GAD65/67 was more in HS rats than in NS rats (NS: -24 ± 2 vs. HS: -39 ± 4 , $P < 0.05$) (Fig. 5b, d).

RTX treatment enhanced muscimol-induced increase in urine flow and decrease in RSNA

Bolus intrathecal injection of muscimol (3 nmol/kg) caused a decrease in RSNA in rats under pentobarbital anesthesia and the decrease was greater in RTX-treated rats than that

in vehicle-treated rats (30 min after muscimol in NS rats, vehicle: $-19 \pm 4\%$ vs. RTX: $-38 \pm 5\%$, $P < 0.05$; 30 min after muscimol in HS rats, vehicle: $-42 \pm 6\%$ vs. RTX: $-66 \pm 5\%$, $P < 0.05$) (Fig. 6a–c). Moreover, the decrease in RSNA was greater in HS rats given RTX compared with NS rats given RTX (30 min after muscimol, NS: $-38 \pm 5\%$ vs. HS: $-66 \pm 5\%$, $P < 0.05$).

Bolus intrathecal injection of muscimol (3 nmol/kg) caused an increase in urine flow in rats under pentobarbital anesthesia and the increase was greater in RTX-treated rats than that in vehicle-treated rats (20–30 min after muscimol in NS rats, vehicle: 0.90 ± 0.17 vs. RTX: 1.39 ± 0.17 μ l, $P < 0.05$; 20–30 min after muscimol in HS rats, vehicle: 1.15 ± 0.23 vs. RTX: 2.20 ± 0.22 μ l, $P < 0.05$) (Fig. 6d–f). Moreover, the increase in urine flow was greater in HS rats given RTX compared with HS rats given RTX (20–30 min after muscimol, NS: 1.64 ± 0.12 vs. HS: 2.39 ± 0.23 μ l, $P < 0.05$). The representative RSNA recordings are shown in Fig. 6g.

Discussion

The main findings of the present study are as follows: (1) intrathecal injection of RTX increased blood pressure in rats fed a NS or HS diet with a greater magnitude of the

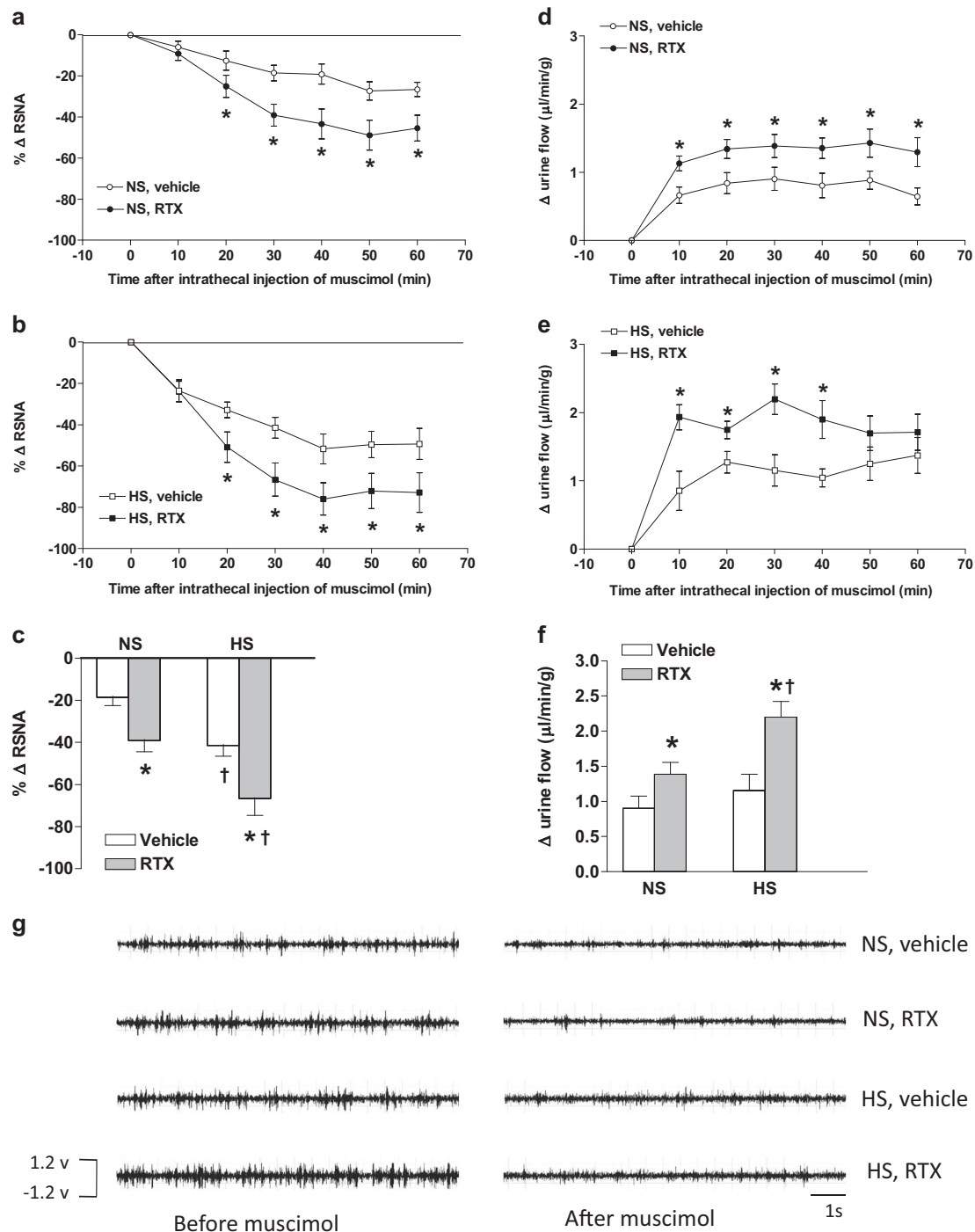


Fig. 6 RSNA was enhanced after RTX treatment. Effect of intrathecal injection of muscimol on renal sympathetic nerve activity (RSNA) (**a**, **b**, **c**, **g**) and urine flow (**d**, **e**, **f**) in rats fed a NS or HS diet at 15th day after vehicle or RTX treatment. **a**, **d** The time course percent change of RSNA. **b**, **e** The time course change of urine flow. **c** The

percent change of RSNA at 30 min after intrathecal injection of muscimol. **f** The change of urine flow at 20–30 min after intrathecal injection of muscimol. **g** Original tracings. Values are mean \pm SE ($n = 6$). * $P < 0.05$ compared with the corresponding vehicle-treated group; † $P < 0.05$ compared with the corresponding NS diet group

increase in HS treated rats; (2) HS intake increased TRPV1 expression in the spinal cord that was eliminated by RTX treatment in HS- as well as NS-treated rats; (3) RTX treatment enhanced muscimol-induced increase in urine flow and decrease in RSNA.

Accumulating evidence showed that TRPV1 plays a role in the regulation of blood pressure and cardiovascular function [10, 28]. We have previously shown that neonatal degeneration of TRPV1-positive sensory nerves leads to salt-induced hypertension in adult rats [11–19]. Recent

studies have investigated the role of TRPV1-positive renal sensory nerves in the regulation of salt sensitivity of arterial pressure by using dorsal rhizotomy or intrathecal injection of TRPV1-specific shRNA [20–23]. Here in the present study, we further affected TRPV1 by intrathecal injection of RTX to selectively ablate the TRPV1-positive central terminals of DRG neurons (T8–L3) in an attempt to answer the question as to whether degenerating TRPV1-positive central terminals of sensory neurons is sufficient in altering blood pressure in the presence or absence of salt loading.

RTX is an ultra-potent TRPV1 agonist and has been used as an ablator of TRPV1-positive nerve terminals by causing sustained Ca^{2+} influx into the nerve terminals [24, 25]. Sensory nerves that innervate the kidney project mainly to T8–L3 segments of the spinal cord [29]. In the present study, RTX was administered to the lower thoracic and upper lumbar segments of the spinal cord (T8–L3), which caused a localized, selective ablation of TRPV1-positive central terminals of sensory neurons innervating the abdominal organs including the kidney. Indeed, western blot analysis showed that TRPV1 was knocked down within the targeted segments of the spinal cord (T8–L3), i.e., the TRPV1-positive central terminals of sensory nerves (Fig. 2), with other segments such as the cervical spinal cord unaffected.

After being administrated to the region of the lower thoracic and upper lumbar spinal cord, RTX activated the TRPV1 located in the central terminals of the afferent nerves in the dorsal horn of spinal cord and caused a sustained and excessive influx of Ca^{2+} , leading to nerve terminal death. RTX's toxic effect was confined only in the central projections in the dorsal horn, without reaching the DRG soma and its peripheral terminals in the kidney (Fig. 2 and Supplementary Fig. S1). The loss of TRPV1 terminals in the dorsal horn was further confirmed by the functional study in which MAP responses to intrathecal injection of capsaicin, a selective TRPV1 agonist, were attenuated after RTX treatment (Fig. 2).

Correlated to the loss of TRPV1 expression in the spinal cord (T8–L3), blood pressure in rats fed NS or HS diets increased after intrathecal injection of RTX (Fig. 1). These results support the notion that ablating the TRPV1-positive central terminals of the sensory nerves innervating the kidney conveys a prohypertensive effect. HS diet-fed rats showed a greater increase in blood pressure compared to HS rats, a result consistent with our previous reports showing that neonatal systemic degeneration of TRPV1-positive sensory nerves increased salt sensitivity of arterial pressure [11–19].

Since the TRPV1 expression in DRG and kidney was intact after RTX treatment, it suggested that the efferent

functions of sensory nerves such as TRPV1-mediated CGRP release at the peripheral nerve terminals [30, 31] will not be affected. As a result, CGRP-mediated vasodilatation in the kidney may not play a key role in RTX-induced prohypertensive effect. Given that increased RSNA has been shown to contribute to elevated blood pressure induced by afferent renal nerve denervation by dorsal rhizotomy [21], we focused on the role of the sympathetic nervous system in RTX-induced prohypertensive effect in the present study.

Renal sensory nerves interact with sympathetic nerves to regulate the blood pressure [32–34]. Previous study showed that renal sympathetic nerve activity can modulate the renal afferent nerve activity [35]. Our previous study demonstrated that renal sensory nerves regulate blood pressure through influencing sympathetic nerve activity [23]. However, Foss et al. [36] showed that degeneration of renal sensory nerves by capsaicin painting did not affect the increase in blood pressure in Dahl salt-sensitive rats, suggesting that central and peripheral terminals of renal sensory nerves have different effects in the regulation of salt sensitivity.

Expression of TH, a sympathetic nerve marker [37], in the kidney was markedly upregulated by RTX treatment (Fig. 3). Furthermore, RSNA was suppressed to a greater extent in RTX-treated rats than vehicle-treated rats after intrathecal injection of muscimol, a GABA-A receptor agonist (Fig. 6). These results suggest that elimination of TRPV1 in central terminals of sensory nerves innervating the kidney might increase blood pressure through regulating sympathetic nerve system.

To explore the detailed process in the above-mentioned reflex, it's necessary to identify the link between TRPV1-positive nerve terminals in the dorsal horn and the sympathetic nervous system. Considering a great number of interneurons located between the dorsal horn and the lateral horn were GABAergic neurons, we focused on assessing the role of spinal GABA system in the link between TRPV1-positive central terminals located in the dorsal horn and sympathetic preganglionic neurons located in the lateral horn. It's reported that TRPV1 activation on primary afferent fibers excites inhibitory neurons in the spinal cord, leading to an increased release of GABA [38]. It's also reported that dorsal rhizotomy decreased the number of GAD67 mRNA-expressing neurons in the superficial layers of the deafferented dorsal horn [39]. Then, a question need to be answered that whether TRPV1 suppression (like TRPV1 deletion by RTX treatment in the present study) on primary afferent fibers inhibits GABAergic inhibitory neurons in the spinal cord. Pretreatment with muscimol, a GABA-A receptor agonist, abolished RTX-induced prohypertensive effects (Fig. 4), suggesting the possible

involvement of GABA system in RTX-induced hypertension. Furthermore, the expression of GABA synthetases, GAD65/67, in the spinal cord was downregulated by RTX treatment (Fig. 5). These data suggest that ablation of TRPV1 nerve terminals in the dorsal horn suppresses the GABAergic interneurons in the spinal cord. It's well documented that sympathetic preganglionic neurons are innervated by GABAergic interneurons in the spinal cord [40–42]. Therefore, the RTX-induced ablation of TRPV1-containing nerve terminals in the dorsal horn has a suppressive effect on the GABAergic interneurons, which may attenuate the inhibition of GABAergic interneurons on the sympathetic preganglionic neurons, leading to an increase in the activities of renal sympathetic nerves.

Our data provide evidence that how an increase in RSNAs may produce an elevation of blood pressure. In spite of the over 20 mm Hg increase in MAP in the RTX-treated rats compared with vehicle-treated rats, 24 h urine excretion and the ratio of urine to water intake remained unchanged, suggesting that the pressure-natriuresis relationship at the kidney was attenuated by RTX, which was probably mediated by the renal sympathetic nerves.

After analyzing the mechanism underlying RTX-induced hypertension, we want to know why the RTX-induced pressor effect was greater in HS rats than in NS rats. HS intake enhanced the levels of TH in the kidney and TRPV1 in the dorsal horn of the spinal cord (Figs. 2 and 3). RTX treatment produced a greater TRPV1 ablation in the dorsal horn, a bigger decrease in GAD65/67 expression in the spinal cord and a higher level of TH in the kidney in HS rats in comparison with NS rats (Figs. 2, 3, and 5). A possible relation that link the changes in TRPV1, GAD65/67 and TH is that RTX treatment caused a bigger ablation of TRPV1 nerve terminals in the dorsal horn, which caused a greater decrease in the production of GABA, producing a weaker inhibition of GABAergic interneurons on the sympathetic preganglionic neurons, resulting in an increase of activities of sympathetic nerve, inducing more retention of sodium and water and finally leading to a more pronounced pressor effect in HS rats than in NS rats.

Conclusion

In conclusion, the present study shows that selective ablation of TRPV1-positive central terminals of sensory nerves innervating the abdominal organs including the kidney by intrathecal injection of RTX increases blood pressure in both NS rats and HS rats with a greater effect in the latter. The pressor effect of RTX may be mediated by, at least in

part, the inhibition of spinal GABA system and the enhancement of the renal sympathoexcitatory response.

Study limitations

TRPV1 colocalizes with other components (CGRP, SP, glutamate, and other TRP channels) in the same central terminals of TRPV1-positive sensory neurons [43–45]. These components may also be affected by RTX, which contributes to altered blood pressure. More selectively inhibition or activation of each of these components is needed to dissect the role of these molecules in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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