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Effects of intravesical instillation of resiniferatoxin on bladder function and nociceptive behavior in freely moving, conscious rats

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Abstract

Purpose—A new animal model that can evaluate bladder function and nociceptive behavior concurrently was developed using freely moving, non-catheterized conscious rats to assess nociceptive behavior responses induced by intravesical instillation of RTx and its relationship with bladder dysfunction.

Materials and Methods—In female SD rats, RTx (0, 0.3 and 3 μ M) was instilled via a catheter temporarily inserted into the bladder through the urethra. Then, after removing the catheter, the incidence of nociceptive behavior (lower abdominal licking and freezing) was scored. Voided urine was collected continuously for the measurement of bladder capacity. In some animals, the pudendal nerves were transected bilaterally (PNT rats) in order to eliminate activation of urethral afferents by RTx.

Results—Intravesical instillation of RTx induced decreased bladder capacity and increased nociceptive behaviors such as licking and freezing, which were blocked by BCTC, a TRPV1 antagonist. In PNT rats, the early phase of RTx-induced licking was decreased without affecting the RTx-induced reduction in bladder capacity and late-phase licking behavior, and RTx-induced late-phase licking in the water-unloaded group was observed to a lesser extent compared with the water-loaded diuresis group.

Conclusions—The intravesical instillation of RTx, which decreases bladder capacity, acts by at least three distinct mechanisms to induce licking behavior; (1) the immediate response mediated by activation of urethral afferents in the pudendal nerve, (2) a late occurring response evoked by direct stimulation of C-fiber afferents in the bladder and (3) gradual facilitation of the response elicited by bladder wall distension induced by rapid bladder filling.

Keywords

rat; nociceptive behavior; urinary frequency; resiniferatoxin

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Introduction

IC/PBS is a chronic inflammatory disease characterized by suprapubic pain related to bladder filling, often coupled with urinary frequency. Although the etiology of IC/PBS is unknown, proposed mechanisms such as altered urothelial barrier function, abnormal urothelial signaling and neurogenic inflammation associated with mast cell activation are likely to contribute to sensitization of bladder afferent pathways to induce the symptoms of IC/PBS.¹ However, it is not well characterized how two major symptoms (i.e., pain and frequency) are correlated following bladder afferent activation.

In rats, intravesical administration of vanilloids such as capsaicin or RTx can induce pain behaviors (intense licking of the lower abdomen or head turning)^{2, 3} as well as increased frequency of voiding.⁴ These agents activate C-fiber bladder afferents by stimulation of TRPV1 receptors, which are nonselective cationic channels that also respond to noxious stimuli such as heat above 43°C and pH less than pH6.⁵ Thus, intravesical instillation of vanilloids elicits responses that mimic some of the symptoms of IC/PBS. However, although it has been shown that afferent signals involved in activation of the spinal NO mechanism are processed differently for capsaicin-induced nociception and micturition in the rat⁶, there have been few studies clarifying a connection between changes in micturition and nociception after intravesical instillation of TRPV1 agonists. Therefore, in the present study, we sought to develop a new animal model that can concurrently evaluate bladder function and nociceptive behavior in freely moving, non-catheterized conscious rats in order to examine the correlation between nociceptive behavioral responses and changes in bladder function induced by intravesical instillation of RTx.

Licking behavior induced by intravesical instillation of capsaicin has previously been studied by Lecci et al. using conscious, freely-moving female rats, indicating that the licking behavior induced by intravesical instillation of capsaicin is mainly produced through the stimulation of urethral afferents traveling in the pudendal nerves rather than activation of bladder afferents in the pelvic nerve.⁷ However, people with IC/PBS often suffer from suprapubic pain that is increased during bladder filling⁸, suggesting the involvement of afferent nerve activation in the bladder. Therefore, we also investigated the effects of PNT and water-loading diuresis, which induces rapid bladder distension, on RTx-induced nociceptive behavior.

Materials and methods

Animals

Female Sprague-Dawley rats weighing 235–280 g from Hilltop Animal Care (Pittsburgh, PA) were used. Care and handling of animals were in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Pudendal nerve transection (PNT)

In some rats, pudendal nerves were transected bilaterally in order to eliminate afferent inputs from the urethra through these nerves.⁷ Rats were anesthetized with 2% halothane (Halocarbon Laboratories, River Edge, NJ). Pudendal nerves were exposed bilaterally through a midline abdominal incision and transected near internal iliac vessels using microscissors. Then, the abdominal wall and the skin were closed with sutures. Experiments were performed two weeks after PNT.

Simultaneous recordings of bladder activity and nociceptive behavior

After an acclimation for at least two hours in a transparent metabolic cage (Nalgene, Rochester, NY), rats were placed in a Ballman-type restraining device (KN-326, Natsume, Tokyo, Japan).

A polyethylene tube (PE-50, Clay Adams, NJ) was inserted into the bladder through the external urethral orifice, and residual urine was withdrawn. RTx (0.3 or 3 μ M) or vehicle was then instilled into the bladder via the catheter in a volume of 0.3ml and kept for one minute. Thereafter, the transurethral catheter was removed and rats were placed back in the metabolic cage. In a previous study, intravesical instillation of RTx induced nine different behaviors, in which abdominal licking and head-turning were characterized as RTx-induced nociceptive responses.² Therefore, in the present study, the incidence of lower abdominal licking was scored as “licking behavior”. We also scored head-turning as “freezing behavior”, during which animals stopped moving and pointed their nose toward the lower abdomen without licking, but sometimes exhibited hunching posture or eye-closing. The observation time was divided into 5 sec intervals. If licking or freezing behavior occurred during a 5 sec interval, it was scored as one positive event. The number of positive events of respective behavior was then counted in each rat for 15 min after intravesical instillation of RTx. At the same time, voided urine was collected continuously using a cup placed underneath the metabolic cage and specially fitted onto a force displacement transducer (Fort 100, World Precision Instruments, FL) connected to an amplifier (Transbridge TBM4M, World Precision Instruments, FL) in order to measure voided volume. The data were collected using a data acquisition system equipped with an analog-to-digital converter (Power Lab, AD Instruments, CO) for 75 minutes after intravesical instillation of RTx. Bladder capacity was determined by dividing the total voided volume by the number of micturitions. In these animals, in which the effects of bladder distension on pain behavior were investigated, distilled water (30mL/kg, po) was administered 15 minutes before the RTx treatment to increase urine production (water-loaded diuresis group). Water was not given before RTx treatment to a second group (water-unloaded group).

Measurement of plasma protein extravasation in the bladder

Bladder tissue damage were quantified by an increase in bladder vascular permeability as measured by an EB dye leakage technique.⁹ After physiological measurements, rats were anesthetized with pentobarbital (50mg/kg, ip), and EB (50mg/kg) was injected into the left femoral vein 15 minutes before sacrifice. The bladders were then dissected and dried at 50 C for 24 hours. The dried bladder was weighed, and immersed in tubes containing 1 mL formamide for 72 hours. The content of dye was determined in duplicate using a Microplate Reader (ELx800, Bio-Tek instrument, VT) set at 620 nm wavelength, and the results are expressed as micrograms of EB per dry weight.

Statistics

The values of bladder capacity and EB concentration are expressed as mean $\pm$ SEM. Behavioral scores are expressed as median and interquartile range because they are nonparametric data. Statistical significance was determined with Student's t-test or ANOVA followed by Dunnett's multiple test for parametric data, and with Wilcoxon rank sum test or Steel's multiple test for nonparametric data. P-values less than 0.05 were considered to be significant. All data analyses were performed using the SAS statistical software (SAS Institute, Cary, NC).

Drugs

RTx and EB were purchased from Sigma (St. Louis, MO). BCTC was purchased from BIOMOL (Butler Pike, PA). RTx was dissolved in a small amount of ethanol and diluted to desired concentrations in 10% ethanol, 10% Tween 80 and 80% saline (vehicle). BCTC was dissolved in DMSO and diluted to a concentration of 2mg/mL in 10% DMSO and 90% saline. BCTC was given orally at a volume of 5mL/kg 15 minutes before the instillation of RTx. EB was dissolved in saline, and given intravenously at a volume of 1mL/kg.

Results

Effects of intravesical instillation of RTx on bladder function and nociceptive behavior in normal rats

Intravesical instillation of RTx (0.3 and 3 μ M) decreased bladder capacity in a concentration-dependent manner (fig. 1A). At the same time, RTx (0.3 and 3 μ M) increased both licking and freezing behaviors, which were evaluated for 0–5, 5–10 and 10–15 min after RTx treatment (fig. 2). After RTx instillation, licking behavior was immediately increased, and a significant increase of this behavior was observed in the immediate phase (0–5 min) at 0.3 μ M and in the immediate to later phases (0–5 and 5–10 min) at 3 μ M. Freezing behavior, which was less frequently observed than licking behavior, was increased for 10–15 min and 0–10 min after RTx instillation (0.3 and 3 μ M, respectively). However, RTx (0.3 or 3 μ M) had no effect on EB levels in the bladder (fig. 1B). These RTx-induced changes in bladder function and pain behavior were completely prevented by BCTC (10 mg/kg, po), a TRPV1 antagonist, applied before RTx treatment (table 1). BCTC (10 mg/kg, po) itself had no effect on bladder capacity, licking/freezing behavior or EB levels in the bladder in normal rats.

Effects of intravesical instillation of RTx on bladder function and nociceptive behavior in PNT rats

Bladder capacity was reduced by intravesical RTx application in PNT rats, and the changes in bladder capacity were comparable to those in normal rats, indicating that RTx-induced reduction in bladder capacity was unaffected by pudendal nerve transection (fig. 3). In contrast, the immediate phase (0–5 min) of RTx (3 μ M)-induced licking behavior was decreased in PNT rats although the late-phase (5–15 min) licking behavior was still observed in these rats (fig. 4A, table 2). In addition, the late-phase licking was attenuated in PNT rats without water loading before RTx treatment (the water-unloaded group) compared with the water-loaded diuresis group although licking behavior was still significantly enhanced after RTx treatment in the water-unloaded group, when compared with RTx-untreated, water-loaded PNT rats (table 2). Similar results were obtained in RTx-induced freezing behavior (fig. 4B, table 2). These results indicate that the early phase, but not late phase, nociceptive behavior is reduced in PNT rats and that the late-phase nociceptive behavior is enhanced by increased bladder distension.

Correlation between the RTx-induced reduction in bladder capacity and nociceptive behavior in normal and PNT rats

Reduced bladder capacity and nociceptive behavior induced by RTx were individually plotted in order to clarify their relationship. In normal rats, the correlation was not clearly shown ($r=-0.48$, $p>0.05$, $n=15$) (fig. 5A). However, in PNT rats, there was an apparent negative correlation between changes in bladder capacity and licking behavior following RTx treatment ($r=-0.85$, $p<0.01$, $n=11$) (fig. 5B). Similar results were obtained in the correlation between the RTx-induced reduction in bladder capacity and the freezing behavior in normal rats ($r=-0.20$, $p>0.05$, $n=15$) and PNT ($r=-0.78$, $p<0.01$, $n=11$) (data not shown).

Discussion

In the present study, we developed an animal model that can evaluate bladder function and animal pain behavior simultaneously using freely moving, non-catheterized conscious rats in order to study the correlation between pain and decreased bladder capacity induced by intravesical instillation of RTx. Our non-catheterization method seems to have an advantage over the intravesical catheter implantation methods used in previous studies^{2,3,6,7} because implanted catheters reportedly induce edema and an increase in prostaglandin E2 production in the bladder¹⁰, which potentially interfere urodynamic and behavioral parameters.

We have demonstrated that intravesical instillation of RTx decreases bladder capacity and at the same time induces licking and freezing behaviors and that RTx-induced licking behavior is more frequently observed than freezing behavior. The latter finding is in accordance with the earlier report in which licking behavior was observed most frequently among nine different behaviors induced by RTx.² In addition, because we did not find changes in plasma extravasation (i.e., no increase in EB concentration) after RTx treatment, RTx-induced tissue injury in the bladder seems minimal using the short-period of application.

In this study, BCTC (10mg/kg) that was sufficient to block TRPV1¹¹ had no effect on bladder capacity, licking/freezing behavior or EB levels in RTx-untreated normal rats. It has also been reported that systemic capsaicin pretreatment had no effect on cystometric parameters in conscious, freely-moving rats.¹² These findings suggest that TRPV1 receptors are not involved in normal micturition under conscious conditions in rats. In addition, BCTC completely abolished the RTx-induced decrease in bladder capacity and increase in nociceptive behavior, indicating that these RTx-induced responses were mediated by activation of TRPV1 receptors.

It has been documented that crushing the pudendal nerves in female rats induces mild urinary incontinence, which however recovered in two weeks following the injury.¹³ The present study demonstrated that bladder capacity in PNT rats was comparable to that in normal rats. Furthermore, the RTx-induced reduction in bladder capacity was similar in normal and PNT rats. These results suggest that urethral afferents carried through the pudendal nerve are minimally involved in normal voiding or RTx-induced urinary frequency in freely moving, conscious rats.

However, PNT significantly decreased the immediate phase of RTx-induced licking behavior, indicating that the early-phase licking behavior is dependent on activation of pudendal urethral afferents. These findings are in accordance with a previous report by Lecci et al., in which capsaicin-induced licking behavior was reduced after the transection of the pudendal nerves.⁷ In contrast, PNT did not affect the RTx-induced late-phase (5–15min) licking behavior, suggesting that the RTx-induced late-phase licking behavior is triggered by activation of bladder afferent pathways. This assumption is further supported by the findings in the individual plotting analysis (fig. 5). Since bladder capacity and the incidence of licking behavior after RTx treatment were negatively correlated in PNT rats, but not in normal rats, it is assumed that, after PNT, decreased bladder capacity and the remaining licking behavior in the late phase induced by RTx are elicited by the same mechanism (i.e., activation of bladder afferent nerves) although the possibility of an additional involvement of urethral afferents carried through pelvic or hypogastric nerves can not be excluded. In addition, in normal rats, licking behavior in an early phase that is additionally induced via activation of pudendal nerve afferents seems to mask the correlation seen in PNT rats.

We also found that RTx-induced late phase licking behavior in PNT rats was attenuated in the water-unloaded condition when compared with PNT rats with water-loaded diuresis, suggesting that bladder distension due to increased urine output (approximately 8 times increase in volume after 30mL/kg water loading) can enhance bladder afferent nerve activity leading to increased licking behavior in the late phase. In addition, the late-phase licking behavior was still enhanced after RTx stimulation in water-unloaded PNT rats when compared with RTx-untreated, water-loaded PNT rats, indicating that RTx can also directly stimulate bladder afferent pathways to induce pain behavior in the absence of increased rates of bladder distension. Therefore, RTx-induced late-phase licking behavior is very likely to be mediated by at least two mechanisms; (1) the response evoked by direct stimulation to C-fiber afferents in the bladder and (2) facilitation of the afferent response due to gradual bladder wall distension induced by bladder filling. Because similar results were obtained in RTx-induced freezing

behavior, two different types of nociceptive behaviors seem to be elicited through the similar sensory mechanism.

In the present study, the late-phase pain behavior (licking) was enhanced by bladder filling and correlated with decreased bladder capacity following RTx treatment. Interestingly, these findings well represent clinical symptoms of patients with IC/PBS, such as suprapubic pain increased during bladder filling and concomitant urinary frequency. Therefore, our animal model that can evaluate bladder function and nociceptive behavior simultaneously under uncatheterized, conscious conditions would be useful for the study of mechanisms inducing bladder pain and the evaluation of new treatments of bladder hypersensitive disorders such as IC/PBS.

Conclusions

Intravesical instillation of RTx decreases bladder capacity associated with increased nociceptive behavior in freely moving, conscious rats. At least three components of RTx-induced licking has been identified; (1) an immediate response mediated by urethral afferents in the pudendal nerves, (2) a late response evoked by direct stimulation to C-fiber afferents in the bladder and (3) a gradual facilitation of the response due to activation of afferents in the bladder wall during bladder filling. Simultaneous recordings of bladder activity and nociceptive behavior during bladder irritation could be a useful method for the study of mechanisms inducing bladder dysfunction and pain.

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Abbreviations and Acronyms

IC/PBS	interstitial cystitis/painful bladder syndrome
RTx	Resiniferatoxin
TRPV1	Transient receptor potential vanilloid receptor 1
BCTC	N-(4-t-Butylphenyl)-4-(3-Chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide
PNT	Pudendal nerve transection
EB	Evans blue

References

1. Chancellor MB, Yoshimura N. Treatment of interstitial cystitis. *Urology* 2004;63:85. [PubMed: 15013658]
2. Craft RM, Carlisi VJ, Mattia A, Herman RM, Porreca F. Behavioral characterization of the excitatory and desensitizing effects of intravesical capsaicin and resiniferatoxin in the rat. *Pain* 1993;55:205. [PubMed: 7508591]

3. Craft RM, Henley SR, Haaseth RC, Hruby VJ, Porreca F. Opioid antinociception in a rat model of visceral pain: systemic versus local drug administration. *J Pharmacol Exp Ther* 1995;275:1535. [PubMed: 8531126]
4. Ishizuka O, Mattiasson A, Andersson KE. Urodynamic effects of intravesical resiniferatoxin and capsaicin in conscious rats with and without outflow obstruction. *J Urol* 1995;154:611. [PubMed: 7609147]
5. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816. [PubMed: 9349813]
6. Pandita RK, Persson K, Andersson KE. Capsaicin-induced bladder overactivity and nociceptive behaviour in conscious rats: involvement of spinal nitric oxide. *J Auton Nerv Syst* 1997;67:184. [PubMed: 9479670]
7. Lecci A, Giuliani S, Lazzeri M, Benaim G, Turini D, Maggi CA. The behavioral response induced by intravesical instillation of capsaicin rats is mediated by pudendal urethral sensory fibers. *Life Sci* 1994;55:429. [PubMed: 8035660]
8. Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A, Wein A. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Neurourol Urodyn* 2002;21:167. [PubMed: 11857671]
9. Jasmin L, Janni G, Manz HJ, Rabkin SD. Activation of CNS circuits producing a neurogenic cystitis: evidence for centrally induced peripheral inflammation. *J Neurosci* 1998;18:10016. [PubMed: 9822756]
10. Morikawa K, Ichihashi M, Kakiuchi M, Yamauchi T, Kato H, Ito Y, Gomi Y. Effects of various drugs on bladder function in conscious rats. *Japan J Pharmacol* 1989;50:369. [PubMed: 2779004]
11. Valenzano KJ, Grant ER, Wu G, Hachicha M, Schmid L, Tafesse L, Sun Q, Rotshteyn Y, Francis J, Limberis J, Malik S, Whittemore ER, Hodges D. N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties. I. in vitro characterization and pharmacokinetic properties. *J Pharmacol Exp Ther* 2003;306:377. [PubMed: 12721338]
12. Maggi CA, Conte B. Effect of urethane anesthesia on the micturition reflex in capsaicin-treated rats. *J Auton Nerv Syst* 1990;30:247. [PubMed: 2229891]
13. Sakamoto K, Smith GM, Storer PD, Jones KJ, Damaser MS. Neuroregeneration and voiding behavior patterns after pudendal nerve crush in female rats. *Neurourol Urodyn* 2000;19:311. [PubMed: 10797587]

FIG. 1. Effects of intravesical instillation of RTx on bladder capacity (A) and Evans blue (EB) concentration in the bladder (B) in normal rats. Each bar represents the mean $\pm$ SEM of 5 rats.

* $P < 0.05$: compared to the vehicle group (Dunnett's multiple comparison test).

FIG. 2.

Effects of intravesical instillation of RTx on licking behavior (A) and freezing behavior (B) in normal rats. In upper panels, the number of licking (A) or freezing behavior (B), which was scored as one positive event when it was present during a 5-second observation period, was counted for every 1 min in each rat, then expressed as median in 5 rats and plotted against the time (min). In lower panels, the number of licking (A) or freezing behavior (B) was summed up for each 5 min period (0–5, 5–10 or 10–15 min) following RTx treatment in each rat, and the median value and interquartile range of 5 rats were represented in bar graphs.

* $P < 0.05$: compared to the vehicle group (Steel's multiple comparison test).

FIG. 3.

Effects of RTx on bladder capacity in normal and PNT rats.

Each bar represents the mean $\pm$ SEM of 5–6 rats.

** $P < 0.01$: compared to the vehicle group (Student's t-test).

FIG. 4.

Time course of nociceptive response to RTX in normal rats (A) and PNT rats (B). The number of licking (A) or freezing behavior (B), which was scored as one positive event when it was present during a 5-second observation period, was counted for every 1 min in each rat, then expressed as median in 5–6 rats/group and plotted against the time (min).

FIG. 5.

Relationship between the RTx-induced reduction in bladder capacity and licking behavior in normal rats (A) ($n=5/\text{group}$) and PNT rats (B) ($n=5-6/\text{group}$). The total number (Score) of licking (A) or freezing behavior (B) counted during the 15 min period after vehicle (RTx (0 μ M)) or RTx treatment (0.3 or 3 μ M) was plotted against averaged bladder capacity in each rat.

Table 1
Effects of BCTC on RTx-induced decrease in bladder capacity and increase in nociceptive behavior in normal rats

Treatment	n	Bladder function		Behavior		
		Bladder capacity (mL)		Licking (Score)	Freezing (Score)	
Vehicle	5	0.85±0.09		13 [13–14]	0 [0–5]	
RTx	5	0.47±0.07 ^{**}		52 [52–64] ^{\$}	30 [18–36] ^{\$}	
BCTC + Vehicle	5	0.96±0.15		7 [6–10]	0 [0–0]	
BCTC + RTx	5	0.84±0.11 [#]		2 [0–4] ^{&}	0 [0–0] ^{&&}	

Each data for bladder function represents mean ± SEM from 5 rats. Each data for nociceptive behavior represents the median value and interquartile range from 5 rats.

^{**} P<0.01; compared to the vehicle group.
[#] P<0.05; significant difference to the RTx group. (Student's t-test)
^{\$} P<0.05; compared to the vehicle group.
[&] P<0.05,
^{&&} P<0.01; compared to the RTx group. (Wilcoxon rank sum test)

Table 2

Effects of RTx on behavior in normal and PNT rats

Treatment	n	Licking behavior (Score)			Freezing behavior (Score)		
		0-5	6-10	11-15 (Min)	0-5	6-10	11-15(Min)
Normal rats							
Vehicle	5	6 [6-8]	1 [0-5]	2 [1-2]	0 [0-0]	0 [0-0]	0 [0-1]
RTx 3μM	5	33 [33-34] [*]	15 [14-20] [*]	5 [4-5]	8 [4-10] [*]	13 [12-14] ^{**}	10 [3-12]
PNT rats							
Vehicle	6	4 [2-4]	2 [1-2]	2 [1-4]	0 [0-0]	0 [0-0]	0 [0-0]
RTx 3μM	5	12 [10-12] ^{##}	17 [16-24] ^{**}	13 [10-15] ^{**}	0 [0-2] [#]	10 [4-12] [*]	9 [4-27] ^{**}
PNT rats - Water unloaded							
RTx 3μM	5	6 [5-8]	10 [10-12] ^{\$&}	8 [3-9] ^{\$}	0 [0-1]	3 [3-6] ^{&}	4 [2-4] ^{& &}

The number (Score) of licking (A) or freezing behavior (B) was counted for each 5 min period (0-5, 5-10 or 10-15 min) after vehicle or RTx treatment in each rat. Each data represents the median value and interquartile range (in parentheses) obtained from 5-6 rats.

^{*} P<0.05,

^{**} P<0.01: compared to the vehicle group.

[#] P<0.05,

^{##} P<0.01: compared to the RTx 3μM group in normal rats.

^{\$} P<0.05: compared to the RTx 3μM group in PNT rats.

[&] P<0.05,

[&] P<0.01: compared to the vehicle group in PNT rats. (Wilcoxon rank sum test)