

Functional Roles of Bladder P2X3 Receptors in Rat Bladder Overactivity Induced by Low Temperature Exposure or Intravesical Acetic Acid

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To determine whether bladder P2X3 receptors play physiological and pathological roles in bladder overactivity, this study prepared rats with small interfering RNA (siRNA)-transfected bladder P2X3 receptors. The responses to sudden low temperature (LT) exposure or intravesical acetic acid (AA) infusion in the transfected rats were estimated. Female Sprague-Dawley rats were transfected with P2X3 siRNA through bladder wall injections 2 days before cystometric analysis. Control rats received vehicle treatment without siRNA. During cystometry, P2X3 siRNA-transfected rats (transfected rats) were subjected to sudden LT exposure or intravesical infusions of either saline or AA. After cystometry, their bladders were assessed to determine the expression levels of P2X3 mRNA and receptors. The transfected rats exhibited reduced bladder P2X3 mRNA and receptor expression. Before LT exposure or AA infusion, voiding intervals (VI), micturition volumes (MV), and bladder capacities (BC) in transfected rats were comparable to those in control rats. Immediately after LT exposure, VI and BC decreased in both groups. Following AA infusion, MV decreased in control rats, whereas VI, MV, and BC did not change in transfected rats. After 20 min of LT exposure and 90 min of AA infusion, VI, MV, and BC increased in control rats but not in transfected rats. Downregulation of bladder P2X3 receptors could not enhance physiological bladder storage functions and suppress bladder activity induced by LT exposure or AA infusion. The bladder P2X3 receptors might contribute to the recovery modulation of pathological bladder activity. *Shinshu Med J 74 : 157–167, 2026*

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I Introduction

P2X3 receptors are members of the P2X family of ionotropic adenosine triphosphate (ATP) receptors¹⁾²⁾. They primarily function as sensory receptors and are involved in peripheral pain responses³⁾ and nociception signaling⁴⁾. Gefapixant, a P2X3 receptor antagonist, has been used to treat refractory chronic cough resulting from airway dysfunction^{5)–7)}. The clinical mechanisms of action of gefapixant comprise the sup-

pression of afferent sensitization and central activation of sensory neurons involved in the cough reflex⁸⁾⁹⁾. Additionally, P2X3 receptors have been shown to be associated with irritable bowel syndrome¹⁰⁾.

Evidence suggests that P2X3 receptors expressed in the bladder (bladder P2X3 receptors) play a physiological role in sensing bladder filling^{11)–13)}. Studies in P2X3-null mice have shown reduced pain-related behaviors in response to ATP and formalin, along with bladder hyporeflexia characterized by prolonged voiding interval (VI) and enhanced bladder capacity (BC), without altering bladder pressures¹⁴⁾. Furthermore, bladder afferents in P2X3-null mice—particularly those associated with a subset of pelvic afferent fibers—

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did not respond to intravesical administration of P2X agonists such as ATP or α,β -methylene ATP¹⁵. Up-regulation of P2X3 receptors in afferent pathways has been linked to bladder overactivity¹⁶. These findings suggested that bladder P2X3 receptors contribute to both bladder dysfunction and sensory processing during urine storage.

However, since P2X3-null mice might exhibit compensatory neural rearrangements due to the congenital absence of P2X3 receptors, it remains unclear how bladder P2X3 receptors contribute to bladder overactivity. In this study, we employed two rat bladder overactivity models: one induced by sudden exposure to a low environmental temperature¹⁷⁻¹⁹ and the other by intravesical infusion of acetic acid (AA)²⁰. Using small interfering RNA (siRNA) techniques, we prepared rats exhibiting reduced expression of bladder P2X3 receptors. We then evaluated bladder responses to sudden low temperature (LT) exposure or AA infusion to explore the physiological and pathological roles of bladder P2X3 receptors in mediating bladder overactivity.

II Materials and Methods

A Animals

Ten-week-old female Sprague-Dawley rats (n = 36) were obtained from Japan SLC Inc. (Shizuoka, Japan). All animals were housed under standard laboratory conditions with a 12-h light/dark cycle and had *ad libitum* access to food and water. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were carried out in accordance with the regulations for animal experimentation of our institution. The rats were randomly classified into the transfected rats (body weight: 230 ± 22 g, n = 17) and the control rats (body weight: 225 ± 21 g, n = 19).

B Preparation and transfection of P2X3 siRNA

To reduce the expression of bladder P2X3 receptors in the rats, this study used siRNA techniques. To compensate for differences in knockdown efficiency among individual siRNAs and decrease the potential influence of off-target effects, three types of siRNA sequences were designed as follows:

GGAGCAAUUGCUGUUUACUUCUTT and
AGAAGUAAACAGCAAUUGCUCCTT,
CCAGGCAAUAA AUGUCCAATT and
UUGGACAUUUAUUGCCUGGTT, and
CCUCCGAAACUUCAACAUGTT and
CAUGUUGAAGUUUCGGAGGTT,

representing the sense and antisense strands, respectively (KOKEN Co., Ltd., Tokyo, Japan). Each siRNA (25 nmol) was dissolved in RNase-free water to obtain a $0.5 \mu\text{M}$ solution (Qiagen K.K., Tokyo, Japan). Equal volumes ($120 \mu\text{L}$) of the three solutions were then combined to prepare the final siRNA transfection solution using AteloGene[®] Local Use (KOKEN Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. To examine any nonspecific effects of the delivery procedure, a control solution was adequately prepared as a vehicle control (RNase-free water and transfection reagent) in the same manner without siRNA, for which the inclusion of a nontargeting siRNA would not yield additional information. Our siRNA approach employed an atelocollagen-based delivery system, in which the atelocollagen matrix containing the siRNAs solidified at the site of administration. This matrix enabled local retention of the siRNAs, followed by their gradual diffusion throughout the bladder tissue. However, we were unable to determine whether siRNA distribution preferentially reached specific bladder layers, such as the urothelium, smooth muscle, or afferent nerve fibers. Additionally, potential off-target effects of the siRNAs were not examined. Therefore, to show down-regulation of the P2X3 receptor across a broad region of the bladder, real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to assess P2X3 gene expression at the mRNA level, and immunohistochemical analysis was performed to evaluate P2X3 protein expression, as described below.

During the cannulation procedure for cystometric analysis, as described in Section II. C, either the siRNA transfection solution (n = 17, transfected rats) or the control solution (n = 19, control rats) was injected into the bladder walls. Using a 25-G needle attached to a 1-mL syringe (Terumo, Tokyo, Japan), $400 \mu\text{L}$ of the siRNA transfection solution was injected at three to

four locations on the anterior and posterior bladder walls ($n=17$). The control rats ($n=19$) received the control solution in the same manner. Immediately after injection, the P2X3 siRNA transfection or the vehicle control solution was solidified at the regions of administration. If both the solutions leaked into the vesical spaces, we could not confirm the solidified matrix in the bladder walls.

C Transfection and preparation for cystometric analysis

The rats were anesthetized via intraperitoneal injection of a drug mixture containing medetomidine hydrochloride (0.15 mg/kg body weight; Kyoritsu Seiyaku Co., Tokyo, Japan), midazolam (2.0 mg/kg body weight; Sandoz International GmbH, Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg body weight; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Anesthesia was maintained using 3 % sevoflurane (Abbott Japan Co., Ltd., Tokyo, Japan). Then, the bladder was exposed, and a polyethylene catheter (PE50; Becton Dickinson & Company, Sparks, MD, USA) was inserted through the bladder dome. Next, either the siRNA transfection solution ($n=17$) or the control solution ($n=19$) was injected into the bladder walls, as described in a previous section. The external end of the catheter was then tunneled subcutaneously to the back of the neck and secured. Each rat was subsequently housed individually.

Two days after catheter implantation, the treated rats were individually placed in metabolic cages. Each rat's bladder catheter was connected to a pressure transducer (P23 DC; Nihon Kohden, Tokyo, Japan) and a syringe mounted on a syringe pump (TE-351; Terumo, Tokyo, Japan) through a polyethylene tube. A fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments, Tokyo, Japan) was used to measure the micturition volume (MV, mL). Saline was continuously infused into the bladder at a rate of 10 mL/h. Bladder pressure and MV were continuously recorded using a PowerLabs data acquisition system with LabChart software version 8.1.6 (ADInstruments, BRC Bioresearch, Inc., Nagoya, Japan) on a personal computer. From the recorded cystometric traces, VI (min) and

MV (mL) were measured. BC (mL) was calculated by adding MV and residual volume. Residual volume was defined as the difference between the total volume of saline infused and MV.

D Cystometric analysis following sudden exposure to low temperature (LT)

Cystometric analysis with exposure to LT was performed on 19 catheterized rats, comprising the transfected rats ($n=9$) and the control rats ($n=10$). Each rat first underwent baseline measurement during saline infusion for approximately 20 min at room temperature (RT, $27\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$). Subsequently, the rats were gently and rapidly transferred to a large refrigerator (MPR-513; SANYO Tokyo Manufacturing CO., Ltd., Tokyo, Japan) to expose them to an LT of $4\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. Bladder activity was recorded continuously at LT for 40 min. To assess the responses during LT exposure, the recorded measurements were divided into two phases: the measurements recorded in the first 20 min (acute LT phase) and the measurements recorded in the latter 20 min (latter LT phase).

Following cystometric analysis, the rats were anesthetized as described in Section II. C, and their bladders were harvested for real-time RT-PCR and immunohistochemical analyses. After bladder removal, the rats were euthanized with an overdose of pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan).

E Cystometric analysis with intravesical 0.1 % acetic acid (AA) infusion

Cystometric analysis with intravesical infusion of saline and 0.1 % AA was conducted on 17 catheterized rats, comprising the transfected rats ($n=8$) and the control rats ($n=9$), under RT. Baseline measurements were first recorded during saline infusion for approximately 20 min (first saline infusion). Then, measurements were recorded during an infusion of 0.1 % AA for approximately 20 min (AA infusion). After this phase, the cystometric recordings were paused, and the rats were removed from the recording setup for a 90-min interval. Subsequently, cystometric measurements were resumed during a second saline infusion lasting approximately 20 min. Cystometric parameters were evaluated as previously described. To assess bladder responses to the 0.1 %

AA infusion, the parameters were analyzed separately for each infusion phase.

Following cystometric analysis, rat bladders were harvested for real-time RT-PCR and immunohistochemical analyses. The rats were then euthanized, as described in Section II. D.

F Real-time RT-PCR analysis

Following each cystometric analysis, bladder samples were preserved in RNAlater (Thermo Fisher Scientific K.K., Foster City, CA, USA). Total RNA was extracted from the bladder tissues using the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan), as per the manufacturer's instructions. cDNA was synthesized from 0.1 μ g of total RNA using the SuperScript VILO Master Mix (Thermo Fisher Scientific K.K.). The resulting cDNA was combined with TaqMan Universal PCR Master Mix (Thermo Fisher Scientific K.K.) and gene-specific assay probes targeting either *P2X3* (*P2rx3*, Rn00579301_m1) or the internal control gene *β -actin* (*Actb*, Rn00667869_m1), both obtained from Thermo Fisher Scientific K.K. The RT-PCR protocol was as follows: initial incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min, followed by 40 amplification cycles of incubation at 95 °C for 15 s and 60 °C for 1 min. Relative gene expression was quantified using the comparative threshold cycle ($\Delta\Delta$ Ct) method, normalized to *β -actin* expression.

G Immunohistochemical analysis

Following each cystometric analysis, the harvested bladder tissues were fixed in 4 % paraformaldehyde (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan) and embedded in paraffin. Serial sections (5- μ m-thick) were deparaffinized and subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0) for 5 min. The sections were then incubated with a blocking solution, containing 1.5 % normal donkey serum (Chemicon International, Inc., Temecula, CA, USA) and 1.5 % non-fat milk dissolved in 0.01 M phosphate-buffered saline (PBS), for 1 h at 4 °C. Next, the sections were incubated at 4 °C for 12 h with primary antibodies. These antibodies included anti-P2X purinoceptor 3 (anti-P2X3; EPR26568-3, 1 : 2000, rabbit monoclonal, Abcam, Cambridge, UK), uroplakin III (UPIII; SFI-1, 1 : 20, mouse monoclonal,

Abcam), smooth muscle actin (SMA; 61001, 1 : 100, mouse monoclonal, Progen Biotechnik GmbH, Heidelberg, Germany), or calcitonin gene-related peptide (CGRP; 16013, 1 : 800, guinea pig polyclonal, Progen Biotechnik GmbH). After the primary antibody incubation, the sections were washed thrice with PBS for 5 min per wash. The sections were then incubated at 4 °C for 1 h with appropriate secondary antibodies. The secondary antibodies included Alexa Fluor 488-conjugated donkey anti-rabbit IgG (green, 1 : 250, Thermo Fisher Scientific K.K.) and Alexa Fluor 594-conjugated donkey anti-mouse or anti-guinea pig IgG (red, 1 : 250, Thermo Fisher Scientific K.K.), based on the corresponding primary antibody. After washing the sections three times with PBS for 5 min per wash, the nuclei were counterstained with 5 μ g/mL of 4',6-diamidino-2-phenylindole dihydrochloride (Thermo Fisher Scientific K.K.). Each section was then mounted with fluorescent mounting medium (Dako Cytomation, Carpinteria, CA, USA). After drying, stained sections were examined from the apex to the trigone of the bladder, and images were captured using a fluorescence microscope (Keyence, Osaka, Japan).

H Statistical analysis

All values are presented as mean \pm standard error of the mean. Statistical analyses were conducted using the Excel[®] Statistics program, File ystat2006.xls (Igakutosho Shuppan, Tokyo, Japan). Intragroup comparisons were performed using two-way repeated measures analysis of variance followed by the Student-Newman-Keuls test. Unpaired *t*-tests were used for intergroup comparisons of micturition parameters. A *P*-value of <0.05 was considered statistically significant.

III Results

A Expression levels of bladder P2X3 receptors in the transfected rats

The expression level of bladder P2X3 mRNA in the transfected rats (0.46 ± 0.28 , $n = 17$) was significantly lower than that in the control rats (2.06 ± 2.54 , $P < 0.05$, $n = 19$; **Fig. 1A**). Immunohistochemical analysis showed that P2X3 receptors were localized in the UPIII-

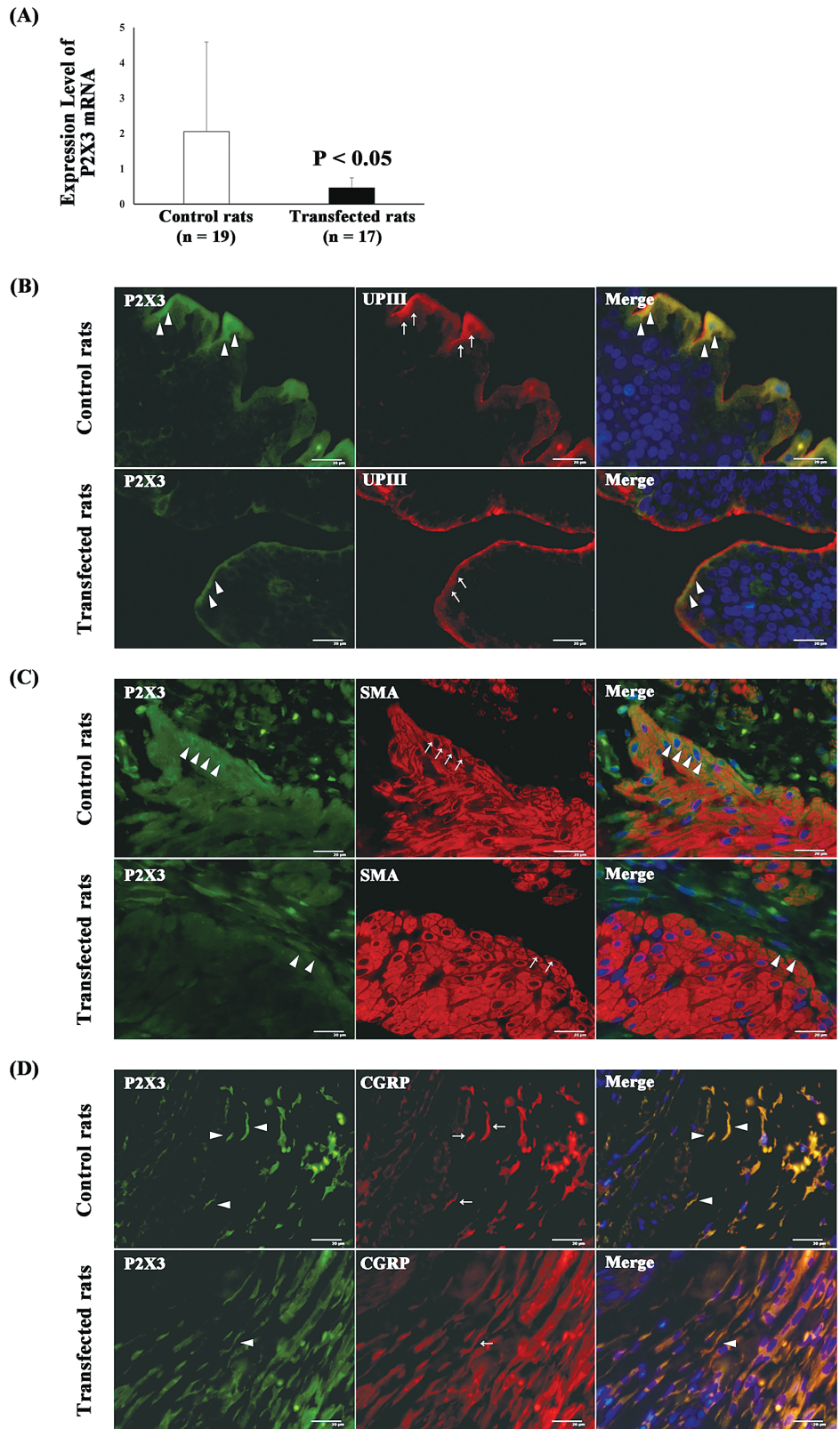


Fig. 1 Expression level of P2X3 mRNA and receptors in the bladders of the transfected rats (A) Expression level of P2X3 mRNA in the bladders of the transfected rats (n = 17) was lower than that of the control rats (n = 19). (B-D) Immunohistochemical images showing P2X3 expression (green, arrowheads) in (B) UPIII-positive urothelium (red, arrows), (C) SMA-positive smooth muscle cells (red, arrows), and (D) CGRP-positive afferent nerve fibers (red, arrows). P2X3 receptor expression in the bladders of the transfected rats (bottom panels) was lower than that of the control rats (upper panels). Blue indicates nuclei.

positive urothelium (**Fig. 1B**), SMA-positive smooth muscle cells (**Fig. 1C**), and CGRP-positive afferent nerve fibers (**Fig. 1D**). However, the expression of these bladder P2X3 receptors in the transfected rats tended to be lower than that in the control rats across all regions.

B Effects of P2X3 receptors on bladder overactivity induced by sudden LT exposure

Prior to LT exposure, micturition of the transfected rats was similar to that of the control rats (**Fig. 2**). In the acute LT phase, the control rats exhibited bladder overactivity with an increased micturition frequency (**Fig. 2A**). In the latter LT phase, the bladder activity of the control rats was mitigated (**Fig. 2A**). Similarly, the transfected rats also showed bladder overactivity during the acute LT phase (**Fig. 2B**). However, in contrast to the control rats, the bladder activity of transfected rats did not mitigate during the latter LT phase (**Fig. 2B**).

In the RT phase, the transfected rats showed a tendency toward a decrease in VI, MV, and BC (3.21 ± 2.27 min, 0.49 ± 0.36 mL, and 0.54 ± 0.39 mL, respectively; $n=9$) compared with the control rats (4.55 ± 2.67 min, 0.72 ± 0.41 mL, and 0.76 ± 0.44 mL, respectively; $n=10$). However, these differences did not reach statistical significance (all $P>0.05$). In the control rats, VI (1.89 ± 1.08 min, $P<0.01$), MV (0.35 ± 0.21 mL, $P<0.05$), and BC (0.36 ± 0.21 mL, $P<0.01$) significantly decreased during the acute LT phase (**Fig. 2C-E**). During the latter LT phase, VI (3.28 ± 1.42 min, $P<0.01$) and BC (0.56 ± 0.24 mL, $P<0.05$) increased, while MV (0.47 ± 0.23 mL, $P>0.05$) remained unchanged (**Fig. 2C-E**). In the transfected rats, VI (1.87 ± 1.30 min, $P<0.05$) and BC (0.36 ± 0.21 mL, $P<0.05$) decreased significantly during the acute LT phase (**Fig. 2C-E**). However, neither VI nor BC increased during the latter LT phase (1.84 ± 1.09 min and 0.31 ± 0.18 mL, respectively; **Fig. 2C-E**). MV of the transfected rats remained unchanged in both phases (acute LT phase: 0.35 ± 0.22 mL, $P>0.05$; latter LT phase: 0.26 ± 0.16 mL, $P>0.05$; **Fig. 2C-E**). During the latter LT phase, VI, MV, and BC of the transfected rats were significantly lower than those of the control rats (all $P<0.05$, **Fig. 2C-E**).

C Effects of P2X3 receptors on bladder overactivity induced by intravesical AA infusion

In the first saline infusion, micturition of the transfected rats was also similar to the control rats (**Fig. 3**). Following the switch from saline to AA infusion, the control rats exhibited bladder overactivity with an increased micturition frequency (**Fig. 3A**). After a 90-min interval, during the second saline infusion, bladder overactivity was attenuated in the control rats (**Fig. 3A**). Similarly, the transfected rats exhibited bladder overactivity during the AA infusion (**Fig. 3B**). However, unlike the control rats, the transfected rats did not show mitigation of bladder overactivity during the second saline infusion (**Fig. 3B**).

In the first saline infusion, the VI, MV, and BC of the transfected rats (2.99 ± 1.11 min, 0.53 ± 0.20 mL, and 0.55 ± 0.20 mL, respectively; $n=8$) tended to decrease compared with those of the control rats (4.14 ± 1.34 min, 0.69 ± 0.16 mL, and 0.74 ± 0.20 mL, respectively; $n=9$). However, these values did not achieve statistical significance (all $P>0.05$). In the control rats, MV significantly decreased during AA infusion (0.53 ± 0.20 mL; $P<0.05$), while VI and BC remained unchanged (3.69 ± 1.75 min, $P>0.05$ and 0.62 ± 0.29 mL, respectively; both $P>0.05$; **Fig. 3C-E**). During the second saline infusion phase, VI (5.45 ± 1.33 min, $P<0.01$), MV (0.83 ± 0.22 mL, $P<0.01$), and BC (0.91 ± 0.22 mL, $P<0.01$) significantly increased in the control rats (**Fig. 3C-E**). Conversely, the transfected rats showed no significant changes in VI (2.75 ± 0.94 min), MV (0.40 ± 0.13 mL), and BC (0.47 ± 0.16 mL) between the saline and AA infusion phases (all $P>0.05$, **Fig. 3C-E**). These parameters also remained unchanged during the second saline infusion (VI: 2.65 ± 0.99 min, $P>0.05$, MV: 0.40 ± 0.13 mL, $P>0.05$, and BC: 0.46 ± 0.15 mL; all $P>0.05$; **Fig. 3C-E**). During the second saline infusion, the VI, MV, and BC values of the transfected rats were significantly lower than those of the control rats (all $P<0.05$, **Fig. 3C-E**).

IV Discussion

To explore the role of bladder P2X3 receptors, we downregulated their expressions using siRNA techniques, in which the siRNAs were gradually diffused

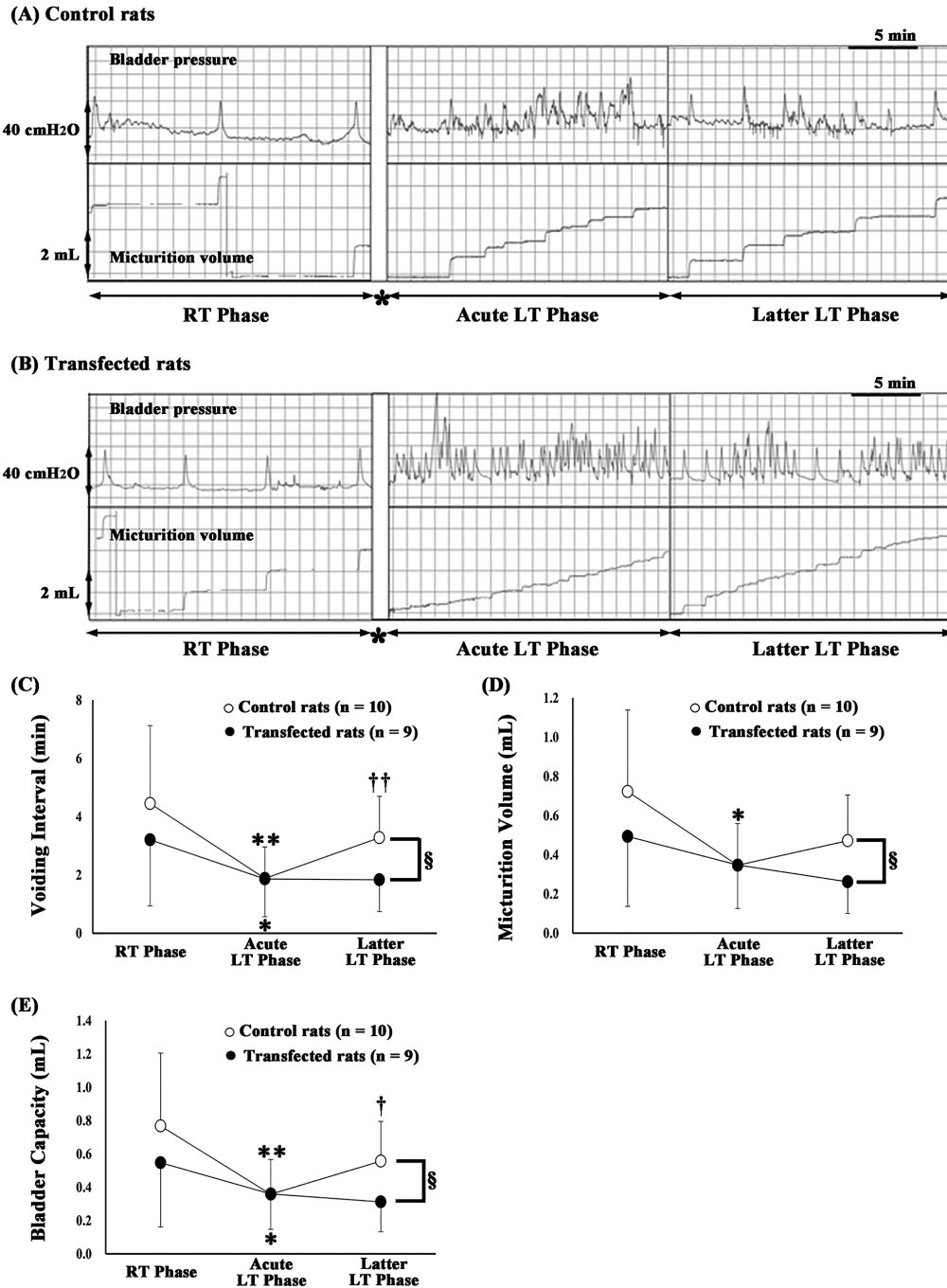


Fig. 2 Bladder overactivity induced by sudden exposure to low temperature (LT)

(A) The control rats (n=10) showed bladder overactivity, as evidenced by increased micturition frequency during the acute LT phase. This overactivity was recovered during the latter LT phase. Asterisks indicate approximately 30 s required to transition from RT to LT. (B) The transfected rats (n=9) also showed bladder overactivity during the acute LT phase; however, this overactivity persisted and was not attenuated during the latter LT phase. Asterisks indicate approximately 30 s required to transition from room temperature (RT) to LT. (C-E) Micturition parameters—VI (C), MV (D), and BC (E)—tended to be lower in the transfected rats (n=9, black dots) compared to the control rats (n=10, white dots) during the RT phase. In the control rats, VI, MV, and BC significantly decreased during the acute LT phase. Subsequently, VI and BC increased during the latter LT phase, while MV remained unchanged. In the transfected rats, VI and BC decreased during the acute LT phase but remained unchanged during the latter LT phase. MV remained unchanged across all phases. During the latter LT phase, VI, MV, and BC of the transfected rats were lower than those of the control rats. *P<0.05 and **P<0.01 compared with the RT phase for each parameter, †P<0.05 and ††P<0.01 compared with the acute LT phase for each parameter, §P<0.05 compared with the control rats. VI: voiding interval, MV: micturition volume, BC: bladder capacity.

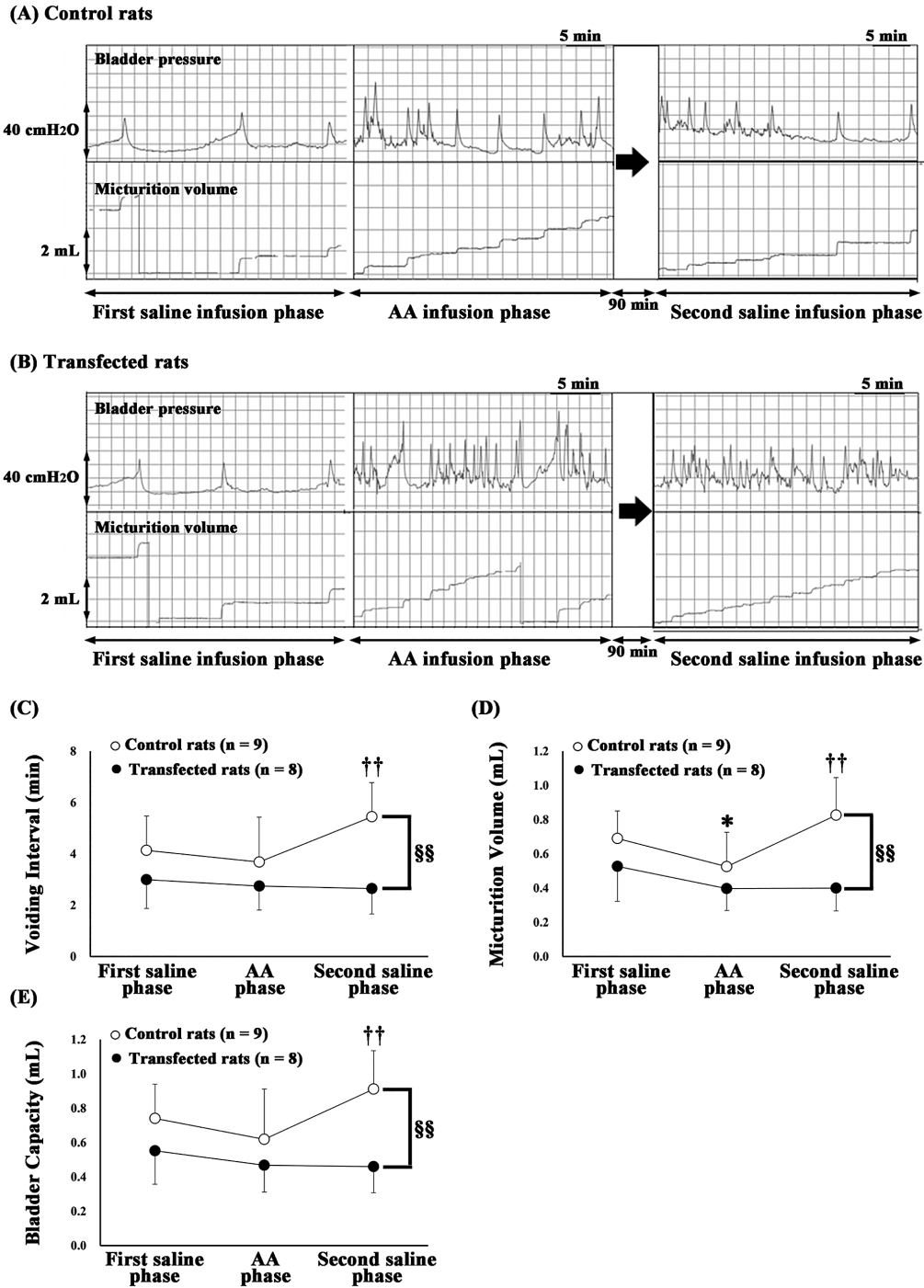


Fig. 3 Bladder overactivity induced by intravesical infusion of acetic acid (AA)

(A) The control rats (n=9) exhibited bladder overactivity, characterized by increased micturition frequency during the AA infusion phase. Following a 90-min interval, during the second saline infusion phase, the induced bladder overactivity was recovered. (B) The transfected rats (n=8) showed bladder overactivity during the AA infusion phase; however, this overactivity was not recovered during the second saline infusion phase. (C-E) Micturition parameters—VI (C), MV (D), and BC (E)—tended to be lower in the transfected rats (n=8, black dots) than in the control rats (n=9, white dots). In the control rats, MV significantly decreased during AA infusion, while VI and BC remained unchanged. After the 90-min interval, VI, MV, and BC of the control rats increased during the second saline infusion phase. In the transfected rats, VI, MV, and BC remained unchanged throughout the cystometric analysis. During the second saline infusion phase, VI, MV, and BC were significantly lower in the transfected rats than in the control rats. *P<0.05 compared with the saline phase for each parameter; ††P<0.01 compared with the AA phase for each parameter; §P<0.05 and §§P<0.01 compared with the control rats.

throughout the bladder tissue. We were not able to examine whether the P2X3 siRNA reached specific layers. Additionally, three distinct siRNA sequences were employed to enhance the efficiency and reliability of target gene downregulation and to minimize the potential influence of off-target effects. Thus, to show P2X3 receptor downregulation across a wide area of the bladder, real-time RT-PCR was used to analyze the mRNA expression levels, and immunohistochemical analysis was performed to examine the receptor expressions. In the bladders of the transfected rats, the expression levels of P2X3 mRNA and P2X3 receptors—localized to the urothelium and smooth muscle layers, and CGRP-positive afferent nerve fibers—were partially reduced compared to the control rats.

Prior to LT exposure and AA infusion, VI, MV, and BC of the transfected rats did not reduce compared to the control rats. Other studies have implicated P2X3 receptors—members of the ATP-activated receptor family—in physiological and pathological mechanisms affecting bladder storage sensitivity and dysfunctions^{14,21}. It is expected that the downregulation of the bladder P2X3 receptors decreased the afferent activity. These theories are obtained by using P2X3-null mice or systemic administration of antagonists. In addition, compensatory neuroplasticity or pathway alterations may exist in congenitally P2X3-null mice. This study used rats having bladders with downregulated P2X3 expression. However, the rats retained a few P2X3 receptors in the bladder and a normal expression of P2X3 receptors in the spinal cord and other regions of the central nervous system. In relation to these, the downregulated bladder P2X3 receptors did not alter VI, MV, and BC under normal conditions, representing the physiological functions of bladder storage. Therefore, this study focused on functional responses to sudden LT exposure and intravesical AA infusion in rats with downregulated bladder P2X3 receptors.

We first analyzed bladder overactivity induced by sudden LT exposure, which involves systemic stimulation. Our previous research showed that LT exposure triggers bladder overactivity in rats via the activation of unmyelinated afferent C fibers¹⁸, cutaneously expressed transient receptor potential melastatin 8

channels^{22,23}, and sympathetic nerves²⁴. Furthermore, we showed that bladder overactivity induced by LT exposure resolved in a time-dependent manner with acclimation to LT. Immediately after LT exposure, VI and BC decreased in both control and transfected rats. However, after 20 min of continued LT exposure (the latter LT phase), VI, MV, and BC significantly increased in the control rats but remained unchanged in the transfected rats. Also, those parameters of the transfected rats were lower than those of the control rats. Next, we assessed bladder overactivity induced by intravesical AA infusion, which was mediated by local stimulation. The AA infusion-induced bladder overactivity disappeared after stopping the infusion. Thus, we especially focused on the bladder function 90 min after AA infusion. The control rats showed higher VI, MV, and BC in this phase compared to the AA infusion phase. However, these parameters in the transfected rats were remained unchanged, and lower compared to the control rats. Together, these results suggested that bladder P2X3 receptors might contribute to the recovery modulation of pathological bladder activity in response to systemic and local stimulation.

In general, stimulation of P2X3 receptors, which are expressed in the urothelium and afferent nerves (unmyelinated afferent C fibers), develops bladder overactivity¹⁶. ATP released from nerve terminals in the bladder functions as a nonadrenergic, noncholinergic neurotransmitter. This ATP induces smooth muscle contractions through P2X receptors, including P2X3 receptors. Activated P2X3 receptors are implicated in urinary frequency or bladder pain. Furthermore, we previously showed that reductions in P2X3 receptor protein levels within unmyelinated afferent C fibers, achieved using an extract of Ba-Wei-Die-Huang-Wan (Hachimi-jio-gan), mitigated bladder overactivity induced by ATP infusion in the spontaneously hypertensive rats²⁵. However, the mechanisms underlying the recovery modulation of the developed bladder activity remain poorly understood. King et al. reported that P2X3 receptors expressed in inhibitory nerves might facilitate the relaxation of gastrointestinal smooth muscle and regulate intestinal motility²⁶.

Thus, we considered that bladder P2X3 receptors might mediate the recovery modulation of LT exposure- and AA infusion-induced bladder overactivity.

As mentioned in the previous paragraph, since the transfected rats retained a normal P2X3 receptor expression, the precise role of spinal cord or central nervous system P2X3 receptors in the micturition reflex pathways remains unclear. The bladder overactivity observed in control rats, including normal rats, gradually resolved in a time-dependent manner; however, bladder overactivity persisted in the transfected rats. Therefore, while the bladder P2X3 receptors are promising clinical targets to treat lower urinary tract symptoms, such as urinary frequency, bladder overactivity, or bladder pain, our findings might provide valuable insights for the development of novel drugs for bladder P2X3 receptor-targeted treatments for patients with lower urinary tract symptoms.

V Conclusion

The transfected rats showed decreased expression of bladder P2X3 mRNA and receptors. Immediately following the transition from RT to LT and switching from saline to AA infusion, the control and transfected rats exhibited bladder overactivity with an increased micturition frequency. At 20 min post-LT exposure and 90 min post-AA infusion, bladder overactivity of the control rats had recovered; however, it persisted in the transfected rats. This result was supported by increased VI and BC in the control rats, and lower VI, MV and BC in the transfected rats compared to the control rats. Based on these findings, bladder P2X3 receptors might contribute to the recovery modulation in the bladder overactivity induced by LT exposure and AA infusion.

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