British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 3072–3080 3072

RESEARCH PAPER

Brain serotoninergic nervous system is involved in bombesin-induced frequent urination through brain 5-HT$_7$ receptors in rats

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Received 8 March 2017; **Revised** 23 June 2017; **Accepted** 26 June 2017

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BACKGROUND AND PURPOSE

Psychological stress exacerbates symptoms of urinary bladder dysfunction; however, the underlying brain mechanisms are unclear. We have demonstrated that centrally administered bombesin, a stress-related neuropeptide, facilitates the rat micturition reflex. Brain bombesin-like peptides modulate the serotoninergic nervous system activity under stress conditions; therefore, we examined whether brain 5-HT is involved in the bombesin-induced increased frequency of urination in urethane-anaesthetised male Sprague–Dawley rats.

EXPERIMENTAL APPROACH

Evaluation of intercontraction intervals (ICI) and maximal voiding pressure (MVP) during cystometrograms were started 1 h before i.c.v. administration of bombesin or i.c.v. pretreatment with the 5-HT receptor antagonists.

KEY RESULTS

Bombesin (0.03 nmol per animal, i.c.v.) significantly reduced ICI without affecting MVP. The bombesin-induced response was significantly suppressed by acute depletion of brain 5-HT, which was induced by pretreatment with *p*-chlorophenylalanine, a 5-HT synthesis inhibitor. Bombesin at a lower dose (0.01 nmol per animal, i.c.v.) showed no significant effect on ICI, while it significantly reduced ICI in the presence of WAY-100635 (5-HT$_{1A}$ receptor antagonist, 0.1 or 0.3 μg per animal, i.c.v.), which can block the negative feedback control of 5-HT release. Bombesin (0.03 nmol per animal)-induced ICI reduction was significantly attenuated by SB269970 (5-HT$_7$ receptor antagonist, 0.1 or 0.3 μg per animal, i.c.v.) but not by ritanserin (5-HT$_2$ receptor antagonist, 0.3 or 1 μg per animal, i.c.v.).

CONCLUSIONS AND IMPLICATIONS

The brain serotoninergic nervous system is involved in the facilitation of the rat micturition reflex induced by bombesin-like peptides at least in part through brain 5-HT$_7$ receptors.

Abbreviations

DMF, *N*,*N*-dimethylformamide; GRP, gastrin-releasing peptide; IACUC, Institutional Animal Care and Use Committees; ICI, intercontraction intervals; MVP, maximal voiding pressure; PFC, prefrontal cortex; Rv, post-voiding residual urine volume

DOI:10.1111/bph.13941

Introduction

There are previous reports showing a relationship between bladder function and psychological stress not only in experimental animals but also in humans (Lutgendorf *et al.,* 2000; Smith *et al.,* 2011; Merrill *et al.,* 2013; Lai *et al.,* 2015). For example, in rats, psychological stress exposure increases micturition frequency and decreases voiding interval (Smith *et al.,* 2011). In female patients with bladder pain syndrome/interstitial cystitis, exposure to acute mental stress worsens symptoms of bladder pain and urinary urgency, but not in controls (Lutgendorf *et al.,* 2000). These findings suggest that psychological stress plays an important role in the induction of frequent urination and exacerbation of bladder dysfunction including overactive bladder and bladder pain syndrome/interstitial cystitis. Psychological stress-related information is conveyed to the brain, which recruits neuronal and neuroendocrine systems for adaptation to stressful conditions, thereby inducing physical and behavioural responses to psychological stress (stress responses) (Ulrich-Lai and Herman, 2009). However, the brain pathophysiological mechanisms underlying psychological stress-induced effects on bladder function are still unclear.

A brain neuropeptide, **bombesin**, has been reported to regulate stress responses (Merali *et al.,* 2002; Jensen *et al.,* 2008). Bombesin itself is a frog peptide and is not expressed in mammals, while bombesin-like peptides such as neuromedin B and gastrin-releasing peptide (GRP) are expressed in the mammalian brain (Jensen *et al.,* 2008). In fact, in rodent models, acute stress exposure such as immobilization stress increased immunoreactivity and *in vivo* release of these bombesin-like peptides in the brain (Kent *et al.,* 1998; Merali *et al.,* 2008). Recently, we reported that centrally administered bombesin, a non-specific peptide agonist of **bombesin receptors**, reduced intercontraction intervals (ICI) without affecting maximal voiding pressure (MVP) and reduced single-voided volume and bladder capacity without affecting post-voiding residual urine volume (Rv) or voiding efficiency in cystometrogram experiments in rats (Shimizu *et al.,* 2016). These results suggest that brain bombesin-like peptides might facilitate sensory inputs to the micturition centre, thereby inducing frequent urination because the centrally administered bombesin had no significant effect on cystometrogram parameters of bladder efferent activity such as MVP, Rv or voiding efficiency.

Brain bombesin-like peptides can modulate activity of the serotoninergic nervous system. Under restraint stress condition, GRP increases serotoninergic neuron activity in the rat hypothalamic paraventricular nucleus (Garrido *et al.,* 2002). In addition, neuromedin B injected into the rat dorsal raphe nucleus, in which 5-HT-containing neurons are widely distributed, promoted *in vivo* release of **5-HT** in the hippocampus (Merali *et al.,* 2006). These findings suggest that the brain serotoninergic nervous system is a downstream pathway of brain bombesin-like peptides. The serotoninergic nervous system in the CNS has been reported to modulate micturition, both inhibitory and excitatory (Sugaya *et al.,* 1998; Ishizuka *et al.,* 2002; Ramage, 2006; Kadekawa *et al.,* 2009; Chiba *et al.,* 2016a). Based on these findings, we hypothesized that brain bombesin-like peptides can induce frequent urination *via* modulation of the central serotoninergic nervous system. Therefore, in this study, we examined the brain mechanisms for the centrally administered bombesin-induced frequent urination in rats focusing on the brain 5-HT and 5-HT receptors. We used ***p*-chlorophenylalanine** (**fenclonine**, an inhibitor of 5-HT biosynthesis enzyme **L-tryptophan hydroxylase**) that has been utilized to investigate the effects of near-total depletion of 5-HT in the CNS, such as the frontal cortex, hypothalamus, brain stem and spinal cord (Steinman *et al.,* 1987; Christianson *et al.,* 2008; Delaville *et al.,* 2012; Yoshimura *et al.,* 2014). In addition, we focused on three 5-HT receptor subtypes, **5-HT$_{1A}$**, 5-HT$_2$ (**5-HT$_{2A}$**, **5-HT$_{2B}$** and **5-HT$_{2C}$**) and **5-HT$_7$**, because several groups have reported that these 5-HT receptor subtypes are involved in the modulation of micturition (Ramage, 2006).

Methods

Animals

All animal experiments were conducted in accordance with the NIH guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committees (IACUC) (#15096571). All efforts were made to minimize the suffering of the animals and the number of animals needed to obtain reliable results. A total of 60 male Sprague–Dawley rats weighing 300–350 g (Harlan Laboratories Inc., Indianapolis, IN, USA) were used in this study, and they were housed with two animals per cage (length, 36.2 cm; width, 24.8 cm; height, 17.8 cm) upon the IACUC recommendation for humane animal care, maintained in an air-conditioned room at 22–24°C under the 12/12 h light–dark cycle with lights on at 0700 h, and given food (LabDiet #5P76, LabDiet, St. Louis, MO, USA) and water *ad libitum*. These rats were divided into 10 groups randomly and were used for the experiments described below. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Surgery

In urethane-anaesthetised (0.9–1.0 g·kg^{-1}, i.p.) male Sprague–Dawley rats, after a laparotomy, a catheter (PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the bladder from the dome in order to perform continuous cystometrogram. Each rat was then placed in the prone position in a stereotaxic apparatus for the brain (SR-6R; Narishige, Tokyo, Japan) until the end of continuous cystometric evaluation, as described previously (Shimizu *et al.,* 2016). The skull was drilled for i.c.v. administration of drugs using a stainless-steel cannula (outer diameter of 0.3 mm). The stereotaxic coordinates of the tip of the cannula were as follows (in mm): AP −1.0, L 1.5, V 4.5 (AP, anterior from the bregma; L, lateral from the midline; V, below the surface of the brain), according to the rat brain atlas (Paxinos and Watson, 2005). Three hours after the surgery, the steel cannula was inserted into the right lateral ventricle and each drug was administered as described below (Figure 1). During the surgery and continuous cystometry, we monitored sufficient levels of anaesthesia by confirming negative reflex responses to toe pinch every 30 min. If the level was insufficient, additional doses of

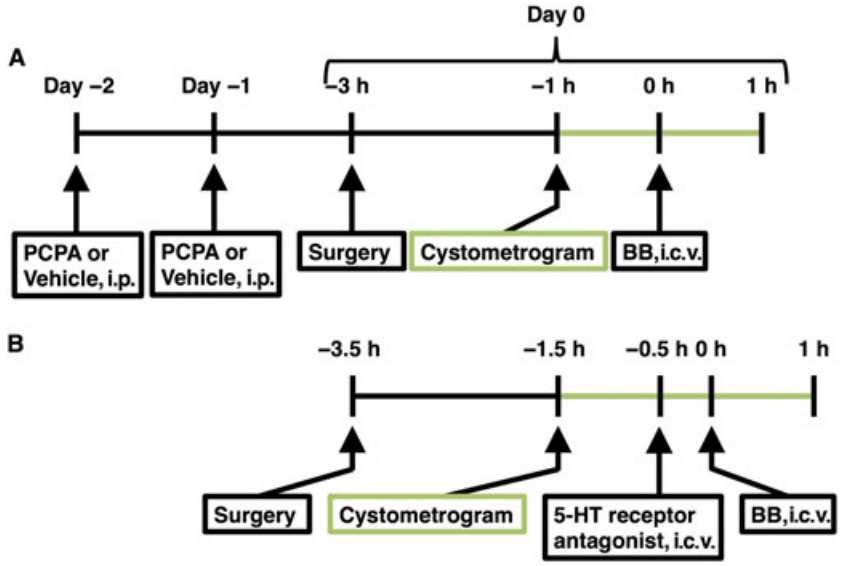

Figure 1

Experiment outline of this study. (A) In some rats, *p*-chlorophenylalanine (PCPA) (200 mg·kg^{-1}, i.p.), a 5-HT synthesis inhibitor, or vehicle (saline containing 1% tween 80, 10 mL·kg^{-1}, i.p.) was administered once a day for 2 days. At 1 day after the second administration, bombesin (BB) was i.c.v. administered 3 h after the surgery for continuous cystometrogram and i.c.v. administration. *In vivo* continuous cystometrograms were started 1 h before the i.c.v. administration and continued for 1 h after the BB administration. (B) In other rats without PCPA or vehicle treatment, 3 h after the surgery, each 5-HT receptor antagonist was i.c.v. pretreated 30 min before the BB administration. *In vivo* continuous cystometrograms were started 1 h before the first i.c.v. administration and continued for 1 h after the BB administration

urethane (0.05 g·kg^{-1} per injection, i.p.) were administered. In some experiments, *p*-chlorophenylalanine or vehicle (saline with 1% Tween 80) was administered (200 mg·kg^{-1}, i.p.) in a volume of 10 mL·kg^{-1} once a day for 2 days (Figure 1A). It has been reported that the administration of *p*-chlorophenylalanine at this dose resulted in approximately 80–95% depletion of 5-HT in the rat brain (Christianson *et al.,* 2008; Yoshimura *et al.,* 2014). At 1 day after the second administration, *p*-chlorophenylalanine-treated rats, which exhibited no obvious behavioural abnormality, underwent the surgery described above (Figure 1A).

Drug administration

Bombesin dissolved in sterile saline (0.001 or 0.003 nmol·μL^{-1}) was slowly administered into the right lateral ventricle in a volume of 10 μL using a cannula connected to a 10 μL Hamilton syringe at a rate of 10 μL·min^{-1}, and the cannula was retained until the end of the experiment. For pretreatment with 5-HT receptor antagonists, **WAY-100635** dissolved in 5 μL of sterile saline, or **ritanserin** or **SB269970** dissolved in 3 μL of *N*, *N*-dimethylformamide (DMF) was i.c.v. administered using a cannula connected to a 10 μL Hamilton syringe at a rate of 10 μL·min^{-1}. Sterile saline in a volume of 5 μL was administered as a vehicle of WAY-100635, and DMF in a volume of 3 μL was administered as a vehicle of ritanserin and SB269970. The cannula was retained in the ventricle for 15 min to avoid the leakage of each antagonist and then removed from the ventricle. Subsequently, bombesin (0.01 or 0.03 nmol·10 μL^{-1}) was i.c.v. administered 30 min after each pretreatment (Figure 1B). One hour after the bombesin administration, Cresyl Violet solution was injected through the cannula. Thereafter, the rats were decapitated under anaesthesia, and the brains were removed in order to confirm the exact location of the cannula inserted in the brain and to verify whether the solution had spread throughout the entire ventricular space. Due to cannula misplacement, six rats were excluded from 60 rats used; therefore, the data were obtained from 54 rats.

Continuous cystometrogram

Cystometrogram studies were performed according to the methods previously reported (Shimizu *et al.,* 2016). Briefly, after the surgery described above, the bladder catheter was connected to a pressure transducer for measurements of intravesical pressure and to a syringe pump for continuous infusion of sterile saline into the bladder at a rate of 12 mL·h^{-1}. Intravesical pressure was recorded using a PowerLab System (AD Instruments, Bella Vista, Australia). Continuous infusion of saline and measurements of ICI, which was the interval of two voiding bladder contractions, and MVP, which was the maximum pressure during a micturition cycle, were started 1 h before the first i.c.v. administration, and the infusion was continued for 1 h after bombesin administration (Figure 1).

Data analysis and statistics

All values are expressed as means ± SEM. Relative values of ICI and MVP were calculated as the ratio of averaged ICI and MVP measured for each 10 min after bombesin administration to those measured for 10 min prior to the bombesin administration. During each 10 min evaluation period, five to eight micturition cycles were recorded and used to calculate the average value of each cystometric parameter. These data

analyses were performed by an investigator (Y.H.) blinded to experimental conditions. The sample size in each experimental group was determined based on the expected difference in a desired endpoint measurement between the test and control groups and the mean of the standard deviations for the two groups reported in our previous report (Shimizu *et al.,* 2016). In experiments in two groups of rats pretreated with *p*-chlorophenylalanine or vehicle, five rats per group were used at first to examine the tendency of *p*-chlorophenylalanine to produce an inhibitory effect. However, two more rats were added to the *p*-chlorophenylalanine-pretreated group because of a high dispersion of values in this group. In experiments in eight groups of rats treated with 5-HT receptor antagonists or corresponding vehicle of each antagonist, six rats per group were used. However, in six out of eight groups, one rat per group was excluded due to cannula misplacement as described above. Statistical differences were determined using one-way ANOVA, followed by *post hoc* analysis with the Bonferroni method when relative data values were compared at the same time range. When only two means were compared at the same time range, Student's unpaired *t*-test was used. When values were compared with those measured for 10 min prior to the bombesin administration, raw data values were compared using one-way ANOVA, followed by *post hoc* analysis with the Bonferroni method. *P* values less than 0.05 were taken to indicate statistical significance. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Materials

The following drugs were used: bombesin (catalogue #, 1149) from R&D Systems Inc. (Minneapolis, MN, USA); *p*-chlorophenylalanine (fenclonine) (catalogue #, C6506) from Sigma Aldrich (St. Louis, MO, USA); WAY-100635 maleate (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate) (catalogue #, 4380) and ritanserin (6-[2-[4-[bis(4-fluorophenyl)methylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-a]pyrimidin-5-one) (catalogue #, 1955) from Tocris Bioscience (Bristol, UK); SB269970 hydrochloride (3-[[(2*R*)-2-[2-(4-methyl-1-piperidinyl)ethyl]-1-pyrrolidinyl]sulfonyl]-phenol, monohydrochloride) (catalogue #, 17 081) from Cayman Chemical (Ann Arbor, MI, USA). All other reagents of the highest grade available were obtained from Sigma Aldrich. The dosage of drugs was determined according to previous studies (Read *et al.,* 2003; Yoshiyama *et al.,* 2003; Christianson *et al.,* 2008; Yoshimura *et al.,* 2014; Shimizu *et al.,* 2016) and our preliminary experiments.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.,* 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

Acute depletion of brain 5-HT by p-chlorophenylalanine attenuated the centrally administered bombesin-induced ICI reduction

Results of continuous cystometrograms in rats with or without *p*-chlorophenylalanine pretreatment are shown in Figures 2 and 3. The baseline values of ICI (s) and MVP (cmH_2O) during the −10 to 0 min period were 98 ± 17 and 44.5 ± 0.9 in the vehicle (saline containing 1% tween 80, 10 $mL \cdot kg^{-1}$)-pretreated group (n = 5), and 90 ± 18 and 45.3 ± 2.6 in the *p*-chlorophenylalanine (200 $mg \cdot kg^{-1}$)-pretreated group (n = 7), respectively, and there were no significant differences in baseline values of ICI or MVP prior to bombesin administration between *p*-chlorophenylalanine-treated and vehicle-treated groups. In vehicle-pretreated rats, centrally administered bombesin at a dose of 0.03 nmol per animal (i.c.v.) significantly reduced ICI without affecting MVP compared with the values before the bombesin

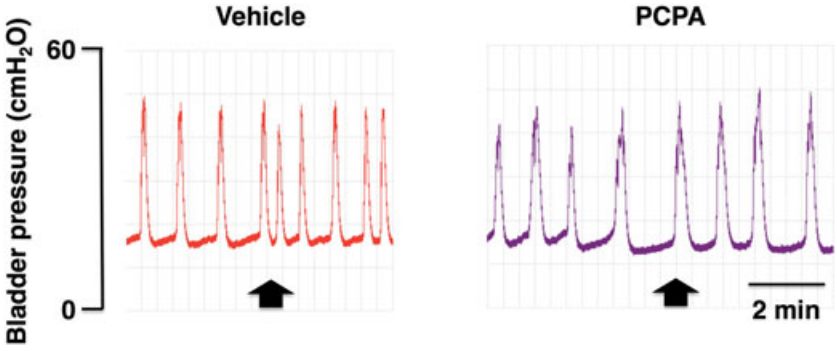

Figure 2

Representative *in vivo* continuous cystometrogram traces in Sprague–Dawley rats. Para-chlorophenylalanine (PCPA), a 5-HT synthesis inhibitor, was administered (200 $mg \cdot kg^{-1}$, i.p.) once a day for 2 days. At 1 day after the second administration, bombesin was administered (0.03 nmol per animal, i.c.v.). Arrows indicate the timing of bombesin injection. Left and right panels show traces in a vehicle- or PCPA-pretreated rat respectively. Note that acute depletion of brain 5-HT attenuated the centrally administered bombesin-induced changes.

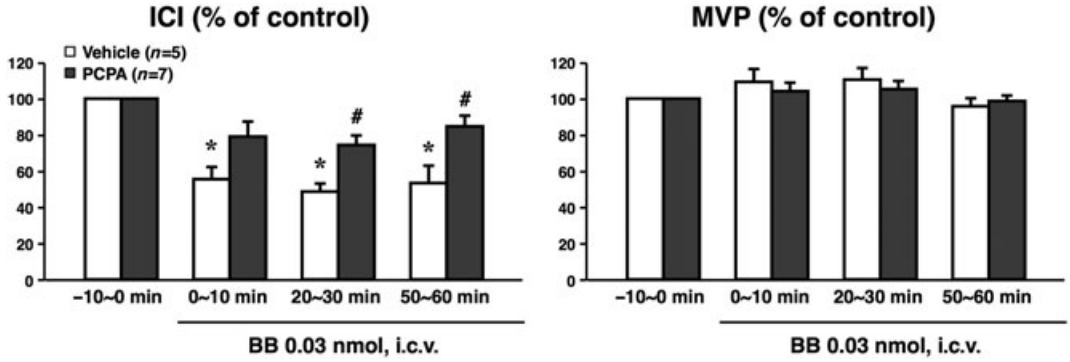

Figure 3

Effects of centrally administered bombesin (BB) on ICI and MVP. Sprague–Dawley rats were pretreated with vehicle (saline containing 1% tween 80, 10 mL·kg^{-1}, i.p.) once a day for 2 days (Vehicle, $n = 5$) or with *p*-chlorophenylalanine (200 mg·kg^{-1}, i.p.) once a day for 2 days (PCPA, $n = 7$). At 1 day after the second PCPA administration, BB was administered (0.03 nmol per animal, i.c.v.). Data calculated as the ratio to the values during the −10 to 0 min period prior to BB administration (−10 to 0 min) present means ± SEM. *$P < 0.05$, when raw data values were compared with the Bonferroni method to the values prior to BB (−10 to 0 min). $^{\#}P < 0.05$, when relative data values were compared with Student's *t*-test to the Vehicle group. The number of animals per group is indicated in parentheses. Note that acute depletion of brain 5-HT by PCPA attenuated the centrally administered BB-induced reduction in ICI.

administration (−10 to 0 min) (Figure 3). These data are in agreement with our previous results (Shimizu *et al.,* 2016). However, in *p*-chlorophenylalanine-pretreated rats, ICI values were not significantly decreased after bombesin administration, indicating that the bombesin-induced reduction in ICI was significantly suppressed after 5-HT depletion (Figure 3).

Centrally administered bombesin-induced ICI reduction was potentiated by WAY-100635, a 5-HT$_{1A}$ receptor antagonist

The baseline values of ICI during the −10 to 0 min period were 92 ± 14 s in the vehicle (5 μL saline per animal, i.c.v.)-pretreated group ($n = 6$), 74 ± 7 s in the WAY-100635 (0.1 μg per animal, i.c.v.)-pretreated group ($n = 5$) and 100 ± 20 s in the WAY-100635 (0.3 μg per animal, i.c.v.)-pretreated group ($n = 5$), and there were no significant differences among three groups. Centrally administered bombesin at a lower dose of 0.01 nmol per animal (i.c.v.) showed no significant effect on ICI compared with the values prior to bombesin administration (−10 to 0 min) (Figure 4). Pretreatment with WAY-100635 at a lower dose of 0.1 μg per animal (i.c.v.) had no significant effect on the bombesin (0.01 nmol per animal, i.c.v.)-induced response, while the bombesin significantly reduced ICI in the presence of a higher dose of WAY-100635 (0.3 μg per animal, i.c.v.) (Figure 4). There were no significant effects of the treatment with WAY-100635 alone (0.1 and 0.3 μg per animal, i.c.v.) on ICI or MVP (data not shown).

Centrally administered bombesin-induced ICI reduction was suppressed by SB269970, a 5-HT$_7$ receptor antagonist, but not by ritanserin, a non-selective 5-HT$_2$ receptor antagonist

The same vehicle-treated rats were used for statistical comparison of SB269970 and ritanserin experiments in Figure 5A, B respectively. The baseline values of ICI during the −10 to 0 min period were 135 ± 21 s in the vehicle (3 μL DMF per animal, i.c.v.)-pretreated group ($n = 5$), 140 ± 29 s in the ritanserin (0.3 μg per animal, i.c.v.)-pretreated group ($n = 5$),

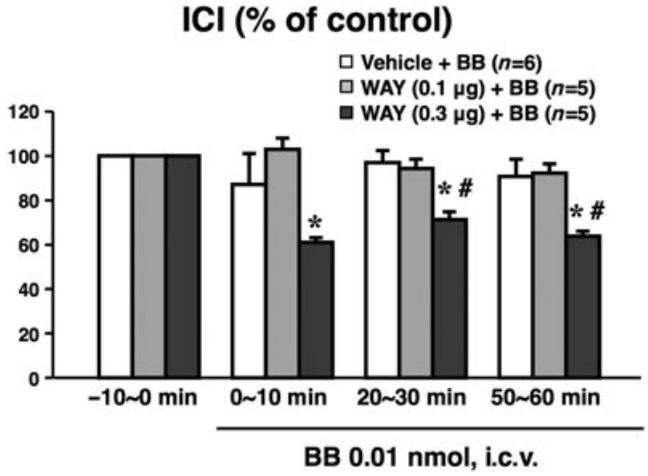

Figure 4

Effect of pretreatment with WAY-100635 (WAY), a 5-HT$_{1A}$ receptor antagonist, on the centrally administered bombesin (BB)-induced reduction in ICI. WAY (0.1 or 0.3 μg per animal, $n = 5$ each) or vehicle (5 μL saline per animal, $n = 6$) was i.c.v. administered 30 min before the administration of BB (0.01 nmol per animal, i.c.v.). Data calculated as the ratio to the values during the −10 to 0 min period prior to BB administration (−10 to 0 min) present means ± SEM. *$P < 0.05$, when raw data values were compared with the Bonferroni method to the values prior to BB (−10 to 0 min). $^{\#}P < 0.05$, when relative data values were compared with the Bonferroni method to the Vehicle + BB group. The number of animals per group is indicated in parentheses. Note that the centrally administered BB-induced ICI reduction was potentiated by WAY.

137 ± 3 s in the ritanserin (1 μg per animal, i.c.v.)-pretreated group ($n = 5$), 96 ± 18 s in the SB269970 (0.1 μg per animal, i.c.v.)-pretreated group ($n = 5$) and 76 ± 9 s in the SB269970 (0.3 μg per animal, i.c.v.)-pretreated group ($n = 6$). There were no significant differences in these baseline values among the three groups in Figure 5A or the three groups in Figure 5B.

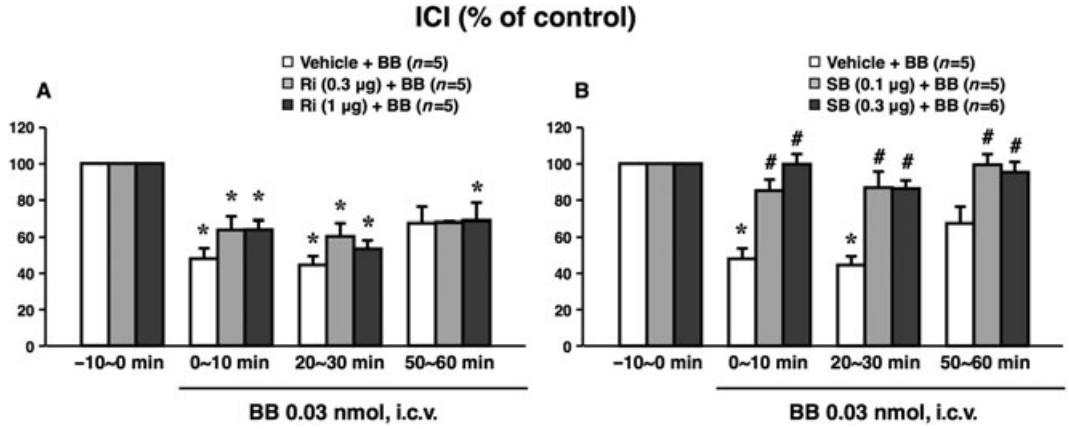

Figure 5

Effects of pretreatment with ritanserin (Ri), a 5-HT_2 receptor antagonist, or SB269970 (SB), a 5-HT_7 receptor antagonist, on the centrally administered bombesin (BB)-induced reduction in ICI. Ri (0.3 or 1 μg per animal, $n = 5$ each, A), SB (0.1 or 0.3 μg per animal, $n = 5$ or 6, respectively, B) or vehicle (3 μL DMF per animal, $n = 5$) was i.c.v. administered 30 min before the administration of BB (0.03 nmol per animal, i.c.v.). Data calculated as the ratio to the values during the −10 to 0 min period prior to BB administration (−10 to 0 min) present means ± SEM. The same vehicle-treated rats were used for statistical comparison in (A) and (B) respectively. $^*P < 0.05$, when raw data values were compared with the Bonferroni method to the values prior to BB (−10 to 0 min). $^{\#}P < 0.05$, when relative data values were compared with the Bonferroni method to the Vehicle + BB group. The number of animals per group is indicated in parentheses. Note that the centrally administered BB-induced ICI reduction was suppressed by SB, but not by Ri.

Pretreatment with ritanserin had no significant effect at either dose (0.3 or 1 μg per animal, i.c.v.) on the bombesin (0.03 nmol per animal, i.c.v.)-induced reduction in ICI (Figure 5A). In contrast, SB269970 at both doses (0.1 and 0.3 μg per animal, i.c.v.) significantly suppressed the bombesin-induced ICI reduction (Figure 5B). There were no significant effects of the treatment with SB269970 alone (0.1 and 0.3 μg per animal, i.c.v.) on ICI or MVP (data not shown).

Discussion and conclusions

In the present study, we demonstrated that i.c.v. administered bombesin-induced ICI reduction was attenuated in *p*-chlorophenylalanine-pretreated rats, in which brain 5-HT was depleted. In the presence of WAY-100635, i.c.v. administered bombesin induced ICI reduction even at a dose that had no effect in the absence of WAY-100635. Central pretreatment with SB269970, but not ritanserin, significantly inhibited the reduction of ICI induced by i.c.v. administered bombesin. These results suggest that the brain serotoninergic nervous system is involved in bombesin-induced frequent urination in rats at least through brain 5-HT_7 receptors, whereas WAY-100635 that can inhibit 5-HT_{1A} receptors-mediated negative feedback control of serotoninergic neuron firing and 5-HT release at serotoninergic nerve terminals (Mundey *et al.,* 1996; Adell and Artigas, 1998) is likely to strengthen the 5-HT-mediated enhancement of the micturition reflex induced by centrally administered bombesin.

The serotoninergic pathways in the CNS have been shown to modulate micturition. Electric stimulation of the raphe nucleus, a major 5-HT-containing area, causes inhibition of micturition in decerebrated rats (Sugaya *et al.,* 1998), and intrathecally administered 5-HT inhibits bladder contractions in rats (Kadekawa *et al.,* 2009). These lines of evidence suggest that 5-HT-containing pathways play an inhibitory role in the control of micturition through the descending pathway from the raphe nucleus to the spinal cord. In addition, 5-HT is reported to be involved in suppression of micturition in the rat prefrontal cortex (PFC) (Chiba *et al.,* 2016a), which is known to perform executive functions to suppress voiding until a socially appropriate timing (Funahashi and Andreau, 2013). On the other hand, i.c.v. administered 5-HT and some agonists for 5-HT receptor subtypes such as 5-HT_{1A}, 5-HT_2 and 5-HT_4 enhance the micturition reflex in rats (Ishizuka *et al.,* 2002), indicating that 5-HT-containing pathways might be excitatory in the control of micturition through the ascending pathway. Considering that 5-HT in the CNS can play both inhibitory and excitatory roles in regulation of micturition, the findings that the pretreatment with *p*-chlorophenylalanine alone showed no effect on micturition, in current and previous studies (Yoshiyama *et al.,* 1994), are not unexpected. However, in this study, in *p*-chlorophenylalanine-pretreated rats, centrally administered bombesin-induced frequent urination was attenuated compared with control rats. Even though *p*-chlorophenylalanine pretreatment can suppress the inhibitory regulation of 5-HT-containing descending pathways in the micturition control, the pretreatment suppressed the bombesin-induced stimulation of micturition. Therefore, brain bombesin-like peptides seem to induce frequent urination at least through 5-HT-containing ascending pathways, which are excitatory, in the brain.

Subsequently, we used a potent 5-HT_{1A} receptor antagonist, WAY-100635, which displays 100-fold selectivity for 5-HT_{1A} over other 5-HT receptor subtypes (Forster *et al.,* 1995). 5-HT_{1A} receptors are well known as 5-HT autoreceptors, which can inhibit 5-HT neuron firing and 5-HT release from the presynaptic nerve terminals (Mundey *et al.,* 1996; Adell and Artigas, 1998). In this study, in rats pretreated with

WAY-100635, centrally administered bombesin induced frequent urination, even at a dose which alone had no effect on normal micturition. On the other hand, centrally administered WAY-100635 by itself had no effect on micturition. It has previously been reported that systemically administered WAY-100635 alone had no effect on *in vivo* release of 5-HT in the median raphe nucleus in rats, while the WAY-100635 inhibited 5-HT release reduction induced by systemically administered 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist (Adell and Artigas, 1998). These findings suggest that the 5-HT_{1A} receptors-mediated negative feedback control of 5-HT neuron firing and 5-HT release is put into effect when 5-HT release is enhanced or 5-HT_{1A} receptors are stimulated. Therefore, it might be reasonable to assume that WAY-100635 can inhibit activation of the 5-HT_{1A} receptors-mediated negative feedback control after administration of bombesin, which induces enhancement of 5-HT release, to potentiate the bombesin-induced frequent urination. Taken together, our results indicate that brain bombesin-like peptides are involved in facilitation of the rat micturition reflex through the brain serotoninergic nervous system.

We further investigated the effects of ritanserin and SB269970, potent antagonists of 5-HT_2 (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) and 5-HT_7 receptors respectively (Watanabe *et al.,* 1992; Lovell *et al.,* 2000). In this study, central pretreatment with SB269970 almost completely suppressed the centrally administered bombesin-induced frequent urination in rats. On the other hand, ritanserin had no effects on the bombesin-induced response. These results indicate that brain bombesin-like peptides facilitate the micturition reflex through brain 5-HT_7 receptors, but not 5-HT_2 receptors. It has previously been reported that i.c.v. administered SB269970 alone at a higher dose compared with those used in this study abolished the micturition reflex in rats, but not when given intrathecally (Read *et al.,* 2003; Ramage, 2006). These findings suggest that 5-HT_7 receptors play an excitatory role in the control of bladder function at a supraspinal level and can support our present data. On the other hand, in the PFC, 5-HT_7 receptors are reported to mediate the inhibitory control of micturition (Chiba *et al.,* 2016b), suggesting that roles of brain 5-HT_7 receptors on micturition might be either excitatory or inhibitory depending on different brain regions where 5-HT_7 receptors are expressed and activated. We used i.c.v. administration of bombesin and each 5-HT receptor antagonist; therefore, a limitation of this study is that it is unclear which 5-HT-containing pathway in the brain is involved in the bombesin-induced frequent urination. Further studies are therefore needed to examine the specific regions in the brain that contribute to bombesin and 5-HT-mediated frequent urination. In addition, another limitation of this study was to use anaesthetised rats because urethane anaesthesia can affect brain activity. Therefore, future studies under the conscious condition are necessary in order to clarify the physiological mechanisms of stress underlying lower urinary tract dysfunction.

Chronic reductions of monoamines, 5-HT and noradrenaline in the CNS reportedly lead to frequent urination and bladder overactivity in rats, which were reversed by fluoxetine, a selective 5-HT reuptake inhibitor (Lee *et al.,* 2003). Imipramine, a tricyclic antidepressant, and duloxetine, a 5-HT and noradrenaline reuptake inhibitor, ameliorated frequent urination in a mouse model of chemically induced cystitis (Redaelli *et al.,* 2015). In addition, duloxetine improved symptoms of overactive bladder in human patients (Steers *et al.,* 2007). Thus, chronic alterations in central serotoninergic and/or noradrenergic nervous systems can induce bladder dysfunction. The present study has demonstrated that the brain serotoninergic nervous system is involved in frequent urination induced by bombesin, a stress-related neuropeptide, in rats. Although it might be difficult to directly apply the findings in our study using acute experimental protocols to the chronic disease condition, the central serotoninergic nervous system could be a useful target for alleviation of psychological stress-induced exacerbation of bladder dysfunction.

In summary, our results suggest that the brain bombesin-like peptide system is involved in facilitation of the rat micturition reflex through the brain serotoninergic nervous system and that frequent urination is mediated by activation of brain 5-HT_7 receptors. These findings would be useful for understanding the underlying mechanisms of stress-related exacerbation of lower urinary tract symptoms in overactive bladder and bladder pain syndrome/interstitial cystitis, for which brain 5-HT_7 receptors could be a new therapeutic target.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (no. JP26460909 and JP17K09303 to T.S.) from Japan Society for the Promotion of Science, a grant from Narishige Neuroscience Research Foundation in Japan, a grant from Smoking Research Foundation in Japan, a grant from The Japan Health Foundation, a grant from the National Institutes of Health National Institute of Diabetes and Digestive and Kidney, USA (R01-DK088836 to N.Y.) and a Grant from the Department of Defence, USA (W81XWH-12-1-0565 to N.Y.).

Author contributions

T.S., S.S., M.S. and N.Y. designed this research. T.S., N.W., S.T, N.S., K.K. and T.M. performed the experiments. T.S. and Y.H. analysed the data. T.S., S.S., M.S. and N.Y. interpreted the results of experiments. T.S. and N.Y. drafted the manuscript. T.S., S.S., N.W., S.T., N.S., Y.H., K.K., T.M., M.S. and N.Y. approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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