A Stress-Related Peptide Bombesin Centrally Induces Frequent Urination through Brain Bombesin Receptor Types 1 and 2 in the Rat

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ABSTRACT

Stress exacerbates symptoms of bladder dysfunction including overactive bladder and bladder pain syndrome, but the underlying mechanisms are unknown. Bombesin-like peptides and bombesin receptor types 1 and 2 (BB₁ and BB₂, respectively) in the brain have been implicated in the mediation/integration of stress responses. In this study, we examined effects of centrally administered bombesin on micturition, focusing on their dependence on 1) the sympathoadrenomedullary system (a representative mechanism activated by stress exposure) and 2) brain BB receptors in urethane-anesthetized (1.0–1.2 g/kg, i.p.) male rats. Intracerebroventricularly administered bombesin significantly shortened intercontraction intervals (ICI) at both doses (0.1 and 1 nmol/animal) without affecting maximal voiding pressure. Bombesin at 1 nmol induced significant increments of plasma noradrenaline and adrenaline levels, which were both abolished by acute bilateral adrenalectomy. On the other hand,

adrenalectomy showed no effects on the bombesin-induced shortening of ICI. Much lower doses of bombesin (0.01 and 0.03 nmol/animal, i.c.v.) dose-dependently shortened ICI. Pre-treatment with either a BB₁ receptor antagonist (BIM-23127; D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH₂; 3 nmol/animal, i.c.v.) or a BB₂ receptor antagonist (BEA; H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NH₂; 3 nmol/animal, i.c.v.), respectively, suppressed the BB (0.03 nmol/animal, i.c.v.)-induced shortening of ICI, whereas each antagonist by itself (1 and 3 nmol/animal, i.c.v.) had no significant effects on ICI. Bombesin (0.03 nmol/animal, i.c.v.) significantly reduced voided volume per micturition and bladder capacity without affecting postvoid residual volume or voiding efficiency. These results suggest that brain bombesin and BB receptors are involved in facilitation of the rat micturition reflex to induce bladder overactivity, which is independent of the sympathoadrenomedullary outflow modulation.

Introduction

Several lines of evidence from experiments in rodent models suggest that stress plays a role in exacerbation of bladder dysfunction including overactive bladder and bladder pain syndrome, which share the symptoms of urinary frequency and urgency (Smith et al., 2011; Merrill et al., 2013; Mingin et al., 2014). In addition, patients with bladder pain syndrome reported that stress worsens their symptoms (Lutgendorf

et al., 2000; Rothrock et al., 2001), and Lai et al. (2015) recently reported that there was a positive correlation between stress levels and severity of overactive bladder symptoms. Stress-related information is conveyed to the brain, which recruits neuronal and neuroendocrine systems for adaptation to stressful conditions (Ulrich-Lai and Herman, 2009); therefore, bladder dysfunction characterized by increased urinary frequency and urgency can share complex interactions of neuronal and hormonal factors in the brain. However, the brain pathophysiological mechanisms underlying stress-induced effects on bladder function are still unclear.

Several neural signals, especially neuropeptides, are thought to play an important role in regulation of responses to stress. Bombesin-like peptides are members of a family of the neuropeptides involved in stress responses. Bombesin itself is a tetradecapeptide isolated from the skin of the European frog *Bombina orientalis* (Anastasi et al., 1971) and is not expressed in mammals. On the other hand, the mammalian counterparts of bombesin are neuromedin B (NMB) and gastrin-releasing peptide (GRP), and the receptors for these

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ABBREVIATIONS: ADX, acute bilateral adrenalectomy; BB, bombesin; BC, bladder capacity; BEA, H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NH₂; BIM-23127, D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH₂; CMG, cystometrograms; GRP, gastrin-releasing peptide; ICI, intercontraction intervals; MVP, maximal voiding pressure; NMB, neuromedin B; PAG, periaqueductal gray matter; Rv, postvoiding residual urine volume; VE, voiding efficiency; Vv, single-voided volume.

two peptides are bombesin receptor type 1 (BB₁; NMB-preferring receptor) and type 2 (BB₂; GRP-preferring receptor). Bombesin shows high affinity to both receptor subtypes. These bombesin-like peptides and the receptors are widely distributed in the mammalian brain. A third mammalian bombesin receptor (type 3) is also reported, but its native ligands are unknown to date (see the review by Jensen et al., 2008). Bombesin-like peptides have a wide range of central functions, including learning and memory (Shumyatsky et al., 2002; Presti-Torres et al., 2007; Roesler et al., 2012), thermoregulation (Tsushima et al., 2003), regulation of anxiety and fear response (Merali et al., 2006, 2013; Bédard et al., 2007), and regulation of food intake (Ladenheim and Knipp, 2007), in addition to regulation of stress responses (Merali et al., 2002; Roesler et al., 2014). For instance, in rodent models, exposure to acute stress, such as a restraint and an aversive stimulus, increases immunoreactivity and *in vivo* release of bombesin-like peptides in the brain (Kent et al., 1998; Merali et al., 1998, 2008), and BB receptor antagonists show anxiolytic effects in the elevated plus maze test and attenuating effects on fear-potentiated startle responses (Merali et al., 2006; Bédard et al., 2007). These lines of evidence indicate a possibility that stress exposure can enhance release of bombesin-like peptides in the brain, thereby inducing not only psychologic disorders such as anxiety and depression but also exacerbation in symptoms of bladder dysfunction. However, central effects of bombesin-like peptides on bladder function are not clarified.

Using bombesin as a “nonselective agonist” for BB receptors, we have been examining central regulation mechanisms of stress responses focusing on the sympathoadrenomedullary system, one of the components of the primary systems for maintaining or reinstating homeostasis during stress exposure (Bartolomucci et al., 2003; Ulrich-Lai and Herman, 2009; Fontes et al., 2014). We previously reported that intracerebroventricularly administered bombesin induced secretion of noradrenaline and adrenaline from the rat adrenal medulla through brain BB₂ receptors (Shimizu et al., 2005; Yokotani et al., 2005). In this study, we examined the effects of centrally administered bombesin on micturition, focusing on their dependence on 1) the sympathoadrenomedullary system and 2) brain BB receptors, using anesthetized rats with or without acute bilateral adrenalectomy and BB receptor antagonists such as BIM-23127 (D-Nal-cyclo[Cys-Tyr-D-Trp-Orn-Val-Cys]-Nal-NH₂; a BB₁ receptor antagonist) or BEA (H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NH₂; a BB₂ receptor antagonist).

Materials and Methods

Animals. All animal experiments were conducted in accordance with National Institutes of Health guidelines and were approved by the Kochi University Institutional Animal Care and Use Committee and by the University of Pittsburgh Institutional Animal Care and Use Committee. All efforts were made to minimize the suffering of the animals and the number of animals needed to obtain reliable results. A total of 51 animals (16 male Wistar rats and 35 male Sprague-Dawley rats) were used in this study. All of the Wistar rats were used for continuous cystometrograms (CMG) in combination with measuring of plasma noradrenaline and adrenaline and monitoring of blood pressure, and all of the Sprague-Dawley rats were used for continuous or single CMG, as described below. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) and Sprague-Dawley rats (Harlan Laboratories

Inc., Indianapolis, IN) weighing 200–250 g were housed at two per cage and were maintained in an air-conditioned room at 22–24°C under a constant day/night rhythm for more than 2 weeks; the animals were given food and water *ad libitum*. After their weight reached 300–350 g, the rats were used for experiments.

Surgery. In urethane-anesthetized (1.0–1.2 g/kg, *i.p.*) Wistar rats, three catheters were inserted into the femoral artery and vein as well as the bladder from the bladder dome to collect blood samples, infuse saline (1.2 ml/h), and perform continuous CMG, respectively. The arterial catheter filled with heparinized saline (100 U/ml). In some experiments, acute bilateral adrenalectomy (ADX) [plus hydrocortisone (5 mg/kg per animal, *i.m.*)] was performed just before the cannulation by an abdominal midline incision (Shimizu et al., 2006; Nakamura et al., 2014). Subsequently, each rat was placed in a stereotaxic apparatus for the brain (SR-6R; Narishige, Tokyo, Japan) until the end of each experiment, as described previously by our laboratory (Shimizu et al., 2004). The skull was drilled for intracerebroventricular 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: anterior from the bregma, –0.8 mm; lateral from the midline, 1.5 mm; and below the surface of the brain, 4.5 mm, according to the rat brain atlas (Paxinos and Watson, 2005).

When Sprague-Dawley rats were used, a catheter was inserted into the bladder from the bladder dome under urethane anesthesia (1.0–1.2 g/kg, *i.p.*) to perform continuous or single CMG. After the cannulation, each rat was treated for intracerebroventricular administration of drugs as described above.

Three hours after the surgery, the steel cannula was injected into the right lateral ventricle and each drug was administered as described below.

Drugs. Bombesin was purchased from R&D Systems Inc. (Minneapolis, MN) and BIM-23127 and BEA were purchased from Bachem AG (Bubendorf, Switzerland).

Drug Administration. Bombesin dissolved in sterile saline was slowly administered into the right lateral ventricle using a cannula connected to a 10- μ l Hamilton syringe at a rate of 10 μ l/min, and the cannula was retained until the end of the experiment. In Wistar rats, 1 and 9 μ l bombesin solution (0.1 nmol/ μ l) containing the doses of 0.1 and 1 nmol in total, respectively, were intracerebroventricularly administered serially at an interval of 60 minutes. In Sprague-Dawley rats, 3.3 and 6.7 μ l bombesin solution (0.003 nmol/ μ l) containing the doses of 0.01 and 0.03 nmol in total, respectively, were intracerebroventricularly administered serially at an interval of 60 minutes. The exact location of the cannula inserted in the brain was confirmed at the end of each experiment by verifying that Cresyl Violet, injected through the cannula, had spread throughout the entire ventricular system.

For the pretreatment with BB receptor antagonists in Sprague-Dawley rats, each antagonist was dissolved in 5 μ l sterile saline and intracerebroventricularly administered using a cannula connected to a 10- μ l Hamilton syringe at a rate of 10 μ l/min. The cannula was retained in the ventricle for 15 minutes to avoid the leakage of each antagonist and then removed from the ventricle. Subsequently, bombesin (0.03 nmol/10 μ l) was intracerebroventricularly administered 30 minutes after pretreatment.

Continuous CMG. Cystometric studies were performed according to previously reported methods (Inoue et al., 2012) with slight modification. After the surgery described above, the bladder catheter was connected to a pressure transducer (DX-100; Nihon Koden, Tokyo, Japan) for measurement of intravesical pressure and to an infusion pump (5200; TOP, Tokyo, Japan) for continuous infusion of sterile saline at a rate of 12 ml/h. Intravesical pressure was recorded by a personal computer (Macintosh G4; Apple Computer, Cupertino, CA) through a bridge amplifier (CASE 7903; San-ei Instruments, Tokyo, Japan) and a multipoint controller (PowerLab/8sp; AD Instruments, Castle Hill, Australia). Saline infusion and measurements of intercontraction intervals (ICI) and maximal voiding pressure (MVP) were started 1 hour before the first intracerebroventricular administration.

Single CMG. In some Sprague-Dawley rats, CMG studies were performed according to previously reported methods (Yoshiyama and de Groat, 2005) with slight modification. After the surgery described above, the bladder catheter was connected to a pressure transducer (DX-100; Nihon Koden) and to an infusion pump (5200; TOP) as described above. Sterile saline was infused into the bladder at a rate of 12 ml/h through the bladder catheter until the peak of a voiding bladder contraction; then the infusion was stopped and the saline voided from the bladder was collected and measured [single-voided volume (Vv)]. The bladder was then emptied to measure residual volume [postvoiding residual urine volume (Rv)]. This procedure was performed three times before bombesin administration (0.03 nmol/10 μ l per rat, i.c.v.) and from 20 to 60 minutes after the bombesin injection. After the experiments, by using the parameters of Vv and Rv, bladder capacity (BC = Vv + Rv) and voiding efficiency (VE = Vv/BC) were calculated.

Measurement of Plasma Noradrenaline and Adrenaline. In Wistar rats, blood samples (250 μ l) were collected through the arterial catheter at just before the initial intracerebroventricular administration of bombesin and at 30 minutes after each bombesin injection. The samples were preserved on ice during experiments. Plasma was prepared immediately after the final sampling. Noradrenaline and adrenaline (catecholamines) in the plasma were extracted by previously reported methods (Anton and Sayre, 1962) with slight modification and were assayed electrochemically with high-performance liquid chromatography (Shimizu et al., 2004). Briefly, after centrifugation (1500 $\times$ g for 10 minutes, at 4°C), the plasma (100 μ l) was transferred to a centrifuge tube containing 30 mg activated alumina, 2 ml water deionized in a MilliQ water purification system (Millipore, Billerica, MA.), 1 ml 1.5 M Tris buffer (pH 8.6) containing 0.1 M disodium EDTA, and 1 ng 3,4-dihydroxybenzylamine as an internal standard. The tube was shaken for 10 minutes and the alumina was washed three times with 4 ml ice-cold deionized water. Then, catecholamines adsorbed onto the alumina were eluted with 300 μ l 4% acetic acid containing 0.1 mM disodium EDTA. A pump (EP-300; Eicom, Kyoto, Japan), a sample injector (Model-231XL; Gilson, Villiers-le-Bel, France), and an electrochemical detector (ECD-300; Eicom) equipped with a graphite electrode were used with high-performance liquid chromatography. Analytical conditions were as follows: detector, +450 mV potential against an Ag/AgCl reference electrode; column, Eicompact CA-50DS, 2.1 $\times$ 150 mm (Eicom); mobile phase, 0.1 M NaH₂PO₄-Na₂HPO₄ buffer (pH 6.0) containing 50 mg/l disodium EDTA, 0.75 g/l sodium 1-octanesulfonate and 15% methanol at a flow rate of 0.18 ml/min; and injection volume, 40 μ l. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine. In this assay, coefficients of variation for the intra- and interassay were 3.0% and 3.7%, respectively, and 0.5 pg catecholamines was accurately determined.

Monitoring of Blood Pressure. In Wistar rats, after the surgery described above, the arterial catheter was connected to a pressure transducer (DX-100; Nihon Koden) that was connected to a carrier amplifier (AP-601G; Nihon Koden). The signals provided by the transducer were monitored by a personal computer (Macintosh G4; Apple Computer) through a multipoint controller (PowerLab/8sp; AD Instruments). The measurement was started after placement in the stereotaxic apparatus. The pressure transducers were calibrated daily using a mercury manometer.

Data Analysis and Statistics. All values are expressed as means $\pm$ S.E.M. Relative values of ICI and MVP were calculated as the ratio of averaged ICI and MVP measured for each 10 minutes after bombesin administration to those measured for 10 minutes just before the initial bombesin administration. Relative values of Vv, Rv, BC, and VE were calculated as the ratio of averages of these four parameters measured for each 10 minutes after bombesin administration to those measured three times just before bombesin administration. Statistical differences were determined using one-way analysis of variance, followed by post hoc analysis with the Bonferroni

method. When only two means were compared, an unpaired *t* test was used. *P* values less than 0.05 were taken to indicate statistical significance.

Results

Centrally Administered Bombesin Shortened ICI without Affecting MVP. Results of continuous CMG studies in Wistar rats are shown in Figs. 1 and 2. There were no significant differences in baseline values of ICI or MVP prior to bombesin administration between ADX plus BB and non-ADX (vehicle and BB rats) groups (Table 1), indicating that ADX by itself had no significant effects on baseline bladder activity. Centrally administered bombesin at a dose of 0.1 nmol/animal (i.c.v.) rapidly shortened ICI compared with the vehicle-treated group (ICI changes at 0–10 minutes: 37.2% $\pm$ 9.0% and 106.7% $\pm$ 1.0% in bombesin- and vehicle-treated groups, respectively), and the bombesin-induced response was sustained at least 60 minutes after the administration (ICI changes at 50–60 minutes: 54.8% $\pm$ 15.4% and 97.2% $\pm$ 2.5% in bombesin- and vehicle-treated groups, respectively) (Fig. 2). After that, serially administered bombesin at a higher dose (1 nmol/animal, i.c.v.) further shortened ICI compared with the vehicle-treated group (ICI changes at 0–10 minutes: 26.9% $\pm$ 6.8% and 101.4% $\pm$ 2.5% in bombesin- and vehicle-treated groups, respectively), and the shortening was sustained at least 60 minutes after the second administration (ICI changes at 50–60 minutes: 19.8% $\pm$ 7.9% and 108.4% $\pm$ 6.4% in bombesin- and vehicle-treated groups, respectively) (Fig. 2). The bombesin-induced shortening effect was also observed in the ADX group (Fig. 2). On the other hand, centrally administered bombesin at both doses (0.1 and 1 nmol/animal) had no significant effects on MVP (Fig. 2).

Centrally Administered Bombesin Induced Elevation of Plasma Noradrenaline and Adrenaline. In our previous studies, centrally administered bombesin (0.1 and 1 nmol/animal, i.c.v.) dose-dependently elevated plasma noradrenaline and adrenaline (adrenaline > noradrenaline) and the levels of both catecholamines peaked at 30 minutes after the administration in Wistar rats (Tanaka et al., 2014). In the present experiments with Wistar rats, centrally administered bombesin at a lower dose (0.1 nmol/animal, i.c.v.) significantly elevated plasma adrenaline but not noradrenaline at 30 minutes after the administration (increments of noradrenaline: -23 ± 7 and -18 ± 36 pg/ml in bombesin- and vehicle-treated groups, respectively; increments of adrenaline: 348 ± 152 and -13 ± 20 pg/ml in bombesin- and vehicle-treated

Fig. 1. Representative in vivo continuous CMG traces in a Wistar rat. BB was administered serially at an interval of 60 minutes (0.1 and 1 nmol/animal, i.c.v.). Arrows indicate the timing of BB injection.

Fig. 2. Effects of centrally administered BB on ICI and MVP. Vehicle indicates Wistar rats treated with vehicle (1 and 9 μ l saline/animal, i.c.v.) serially at an interval of 60 minutes. BB indicates Wistar rats treated with BB (0.1 and 1 nmol/animal, i.c.v.) serially at an interval of 60 minutes. ADX plus BB indicates adrenalectomized Wistar rats treated with BB (0.1 and 1 nmol/animal, i.c.v.) serially at an interval of 60 minutes. Data were calculated as the ratio to the pretreatment (–10 to 0 minutes) values presented as means $\pm$ S.E.M. * $P < 0.05$ (compared with the Bonferroni method to the vehicle group). The number of animals per group is indicated in parentheses.

groups, respectively) (Fig. 3). After that, serially administered bombesin at a higher dose (1 nmol/animal, i.c.v.) significantly elevated plasma levels of both noradrenaline and adrenaline at 30 minutes after the second administration (increments of noradrenaline: 364 ± 9 and -56 ± 21 pg/ml in bombesin- and vehicle-treated groups, respectively; increments of adrenaline: 578 ± 98 and -3 ± 27 pg/ml in bombesin- and vehicle-treated groups, respectively) (Fig. 3).

The bombesin-induced elevation of plasma noradrenaline and adrenaline was almost abolished in the ADX group (increments of noradrenaline and adrenaline at 1 nmol bombesin: 88 ± 47 and -7 ± 9 pg/ml, respectively) (Fig. 3), in agreement with our previous results (Yokotani et al., 2005). There were no significant differences among three groups in baseline values of plasma noradrenaline and ADX by itself had no significant effects on noradrenaline, whereas baseline values

TABLE 1

Baseline values of ICI, MVP, plasma noradrenaline and adrenaline, and blood pressure prior to bombesin administration in each group of Wistar rats

Values are presented as means $\pm$ S.E.M.

Evaluation parameters	Vehicle (n = 5)	BB (n = 5)	ADX Plus BB (n = 6)	Non-ADX (n = 10)
ICI (s)	233 $\pm$ 66	172 $\pm$ 11	169 $\pm$ 25	207 $\pm$ 38
MVP (cmH ₂ O)	33.0 $\pm$ 3.4	35.3 $\pm$ 5.1	41.3 $\pm$ 2.5	34.2 $\pm$ 2.9
Noradrenaline (pg/ml)	258 $\pm$ 22	220 $\pm$ 32	314 $\pm$ 50	239 $\pm$ 19
Adrenaline (pg/ml)	107 $\pm$ 37	178 $\pm$ 42	31 $\pm$ 19*	142 $\pm$ 29 [†]
Systolic blood pressure (mmHg)	154.1 $\pm$ 9.6	153.9 $\pm$ 9.6	140.2 $\pm$ 6.2	154.0 $\pm$ 6.3
Mean blood pressure (mmHg)	101.4 $\pm$ 9.7	105.0 $\pm$ 8.0	93.7 $\pm$ 6.4	103.2 $\pm$ 5.8
Diastolic blood pressure (mmHg)	75.1 $\pm$ 9.7	80.5 $\pm$ 8.3	70.5 $\pm$ 6.9	77.8 $\pm$ 6.0

Baseline values of ICI and MVP were averaged ICI and MVP measured for 10 minutes just before the initial BB injection (0.1 nmol/animal, i.c.v.). Baseline values of plasma noradrenaline and adrenaline were measured just before the initial BB injection. Baseline values of systolic, mean, and diastolic blood pressure were measured for 10 minutes just before the initial BB injection. Vehicle indicates Wistar rats treated with vehicle. BB indicates Wistar rats treated with BB. ADX plus BB indicates adrenalectomized Wistar rats treated with BB. Non-ADX indicates Wistar rats without adrenalectomy (i.e., a group combining vehicle and BB rats). * $P < 0.05$ (compared with the Bonferroni method to the BB group); [†] $P < 0.05$ (compared with the *t* test to the ADX plus BB group).

Fig. 3. Effects of centrally administered BB on plasma noradrenaline and adrenaline. Δ Noradrenaline and Δ Adrenaline indicate the increments of noradrenaline and adrenaline, respectively, at 30 minutes after each BB administration compared with noradrenaline and adrenaline measured just before the initial BB administration. Data were obtained in the same rats as in Fig. 2. Values are presented as means $\pm$ S.E.M. * $P < 0.05$; † $P < 0.05$ (compared with the Bonferroni method to the vehicle group or BB group, respectively). The number of animals per group is indicated in parentheses.

of plasma adrenaline were significantly reduced by ADX (Table 1).

Effects of Centrally Administered Bombesin on Blood Pressure. In the present experiments with Wistar rats, centrally administered bombesin at both doses (0.1 and 1 nmol/animal, i.c.v.) had no significant effects on changes of systolic, mean, and diastolic blood pressure compared with the vehicle-treated group (Table 2). In the ADX group, however, changes of these blood pressure parameters were higher 20 minutes after administration of bombesin at a lower dose (0.1 nmol/animal) and 0 minutes after administration of bombesin at a higher dose (1 nmol/animal) compared with each non-ADX group (Table 2). There were no significant differences among three groups in baseline values of systolic, mean, or diastolic blood pressure, and ADX by itself had no significant effects on these parameters (Table 1).

Centrally Administered Bombesin at Lower Doses Shortened ICI in a Dose-Dependent Manner. Results of continuous CMG studies in Sprague-Dawley rats are shown in

Figs. 4 and 5. The baseline values of ICI at -10 to 0 minutes prior to bombesin administration were 95 ± 19 and 145 ± 17 seconds in vehicle-treated ($n = 4$) and bombesin-treated groups ($n = 5$), respectively, and there was no significant difference between these two values. Centrally administered bombesin at a lower dose (0.01 nmol/animal, i.c.v.) showed a tendency to shorten ICI compared with the vehicle-treated group, and significant shortening was observed at 50–60 minutes (ICI changes: $81.5\% \pm 5.8\%$ and $112.9\% \pm 10.7\%$ in bombesin- and vehicle-treated groups, respectively) (Fig. 5). After that, serially administered bombesin at a higher dose (0.03 nmol/animal, i.c.v.) rapidly and significantly shortened ICI compared with the vehicle-treated group (ICI changes at 0–10 minutes: $61.7\% \pm 2.3\%$ and $103.9\% \pm 8.7\%$ in bombesin- and vehicle-treated groups, respectively), and the response was sustained at least 60 minutes after the second administration (ICI changes at 50–60 minutes: $60.0\% \pm 9.6\%$ and $99.2\% \pm 6.8\%$ in bombesin- and vehicle-treated groups, respectively) (Fig. 5). On the other hand, centrally

TABLE 2

Effects of centrally administered BB on blood pressure
Values are presented as means $\pm$ S.E.M.

Timing of evaluation	0.1 nmol BB			1 nmol BB		
	Vehicle (n = 5)	BB (n = 5)	ADX Plus BB (n = 6)	Vehicle (n = 5)	BB (n = 5)	ADX Plus BB (n = 6)
Δ SBP (mmHg)						
0–10 min	-4.5 ± 3.1	-0.9 ± 8.4	-2.3 ± 3.1	-1.4 ± 3.4	6.7 ± 2.7	$23.2 \pm 5.0^*$
20–30 min	0.7 ± 2.9	-6.0 ± 1.9	$18.6 \pm 3.8^{*,\dagger}$	-0.3 ± 3.0	24.1 ± 12.9	26.2 ± 3.9
50–60 min	-0.3 ± 1.2	4.4 ± 1.3	17.5 ± 7.0	-3.2 ± 2.1	18.8 ± 9.1	22.4 ± 2.4
Δ MBP (mmHg)						
0–10 min	-7.6 ± 2.2	-6.7 ± 4.3	-2.5 ± 2.9	3.8 ± 1.9	2.1 ± 2.1	$13.2 \pm 0.4^{*,\dagger}$
20–30 min	1.7 ± 3.2	3.4 ± 3.4	$16.1 \pm 2.8^{*,\dagger}$	0.0 ± 1.0	9.7 ± 5.9	20.6 ± 2.4
50–60 min	0.1 ± 3.5	3.2 ± 3.3	10.3 ± 4.0	-5.9 ± 1.5	7.1 ± 5.4	$17.6 \pm 1.4^*$
Δ DBP (mmHg)						
0–10 min	-7.3 ± 2.6	-11.8 ± 1.5	-2.6 ± 3.3	4.8 ± 1.9	-2.1 ± 2.5	12.3 ± 3.5
20–30 min	3.7 ± 3.6	2.6 ± 1.1	$14.9 \pm 2.5^{*,\dagger}$	-0.4 ± 1.1	4.6 ± 4.5	$17.8 \pm 3.1^*$
50–60 min	0.7 ± 4.5	-1.7 ± 0.6	6.6 ± 3.6	-7.3 ± 3.3	3.9 ± 4.2	$15.3 \pm 2.4^*$

Both doses of BB (0.1 and 1 nmol/animal, i.c.v.) were serially injected at an interval of 60 minutes. Δ SBP, Δ MBP, and Δ DBP indicate increments of systolic, mean, and diastolic blood pressure, respectively, for each 10 minutes after each BB injection compared with each blood pressure measured for 10 minutes just before the initial BB injection. Vehicle indicates Wistar rats treated with vehicle (1 and 9 μ l saline/animal, i.c.v.) serially at an interval of 60 minutes. BB indicates Wistar rats treated with BB (0.1 and 1 nmol/animal, i.c.v.) serially at an interval of 60 minutes. ADX plus BB indicates adrenalectomized Wistar rats treated with BB (0.1 and 1 nmol/animal, i.c.v.) serially at an interval of 60 minutes. * $P < 0.05$; † $P < 0.05$ (compared with the Bonferroni method to the vehicle-treated group or the BB-treated group, respectively).

Fig. 4. Representative in vivo continuous CMG traces in a Sprague-Dawley rat. BB was administered serially at an interval of 60 minutes (0.01 and 0.03 nmol/animal, i.c.v.). Arrows indicate the timing of BB injection.

administered bombesin at both doses (0.01 and 0.03 nmol/animal) had no significant effect on MVP (data not shown).

Centrally Administered Bombesin-Induced Shortening Effect on ICI Was Suppressed by Each Antagonist for BB₁ or BB₂ Receptors. The baseline values of ICI at -10 to 0 minutes prior to bombesin administration were 105 ± 13 , 104 ± 14 , and 86 ± 13 seconds in vehicle-pretreated ($n = 5$), BIM-23127-pretreated ($n = 5$), and BEA-pretreated groups ($n = 5$), respectively, and there were no significant differences among three groups. The bombesin (0.03 nmol/animal, i.c.v.)-induced shortening of ICI in Sprague-Dawley rats was significantly attenuated by pretreatment with each BB receptor antagonist, BIM-23127 (3 nmol/animal, i.c.v.) or BEA (3 nmol/animal, i.c.v.). ICI changes at 20–30 minutes were $49.4\% \pm 8.1\%$, $77.3\% \pm 3.3\%$, and $94.2\% \pm 5.2\%$ in vehicle-, BIM-23127-, and BEA-treated groups, respectively; ICI changes at 50–60 minutes were $64.3\% \pm 8.6\%$, $96.5\% \pm 1.6\%$, and $95.4\% \pm 5.8\%$ in vehicle-, BIM-23127-, and BEA-treated groups, respectively (Fig. 6). On the other hand, there were no significant effects of the treatment with either BB receptor antagonist, BIM-23127 or BEA (1 and 3 nmol/animal, i.c.v.), on ICI or MVP in Sprague-Dawley rats (data not shown).

Centrally Administered Bombesin Reduced Vv and BC without Affecting Rv or VE. The actual values of Vv, Rv, BC, and VE before central administration of bombesin (0.03 nmol/animal, i.c.v.) were 0.39 ± 0.05 ml, 0.14 ± 0.04 ml, 0.53 ± 0.07 ml, and $74.38\% \pm 6.71\%$, respectively ($n = 7$). The

bombesin administration significantly reduced Vv and BC but showed no significant changes of Rv or VE (Table 3).

Discussion

In this study, we demonstrated that intracerebroventricularly administered bombesin dose-dependently shortened ICI without affecting MVP. The ICI shortening was induced at lower doses compared with those inducing elevation of plasma noradrenaline and adrenaline. ADX, which almost abolished the bombesin-induced elevation of both catecholamines, had no effect on the bombesin-induced shortening of ICI. Pretreatment with BIM-23127 (a BB₁ receptor antagonist) or BEA (a BB₂ receptor antagonist) reduced the bombesin-induced shortening of ICI, respectively, whereas each antagonist by itself had no significant effects on ICI. Furthermore, intracerebroventricularly administered bombesin reduced Vv and BC but not Rv or VE in the single CMG study. Taken together, these results suggest that brain bombesin-like peptides and BB receptors are involved in facilitation of the rat micturition reflex independent of the sympathoadrenomedullary outflow modulation. In addition, although we used Wistar and Sprague-Dawley rats in the first and second series of experiments, respectively, centrally administered bombesin induced similar effects on bladder activity by shortening ICIs, indicating that there is no species difference in bombesin-mediated responses in the rat brain.

Bombesin-like peptides have been reported to stimulate smooth muscle contraction peripherally in the gastrointestinal tract (Milusheva et al., 1998; Degen et al., 2001) and also to mediate central effects on the gastrointestinal tract (Martinez and Taché, 2000). In the lower urinary tract, bombesin-like peptides and BB receptors are expressed in the bladder and urethra (Ghatei et al., 1985; Kilgore et al., 1993; Radziszewski et al., 1996), and exogenously administered bombesin-like peptides produce smooth muscle contraction in these tissues (Watts and Cohen, 1991; Maggi et al., 1992; Radziszewski et al., 2011; Kullmann et al., 2013). Furthermore, Kullmann et al. (2014) reported that systemically administered bombesin-like peptides facilitated micturition in the rat. On the other hand, unlike the gastrointestinal tract, central effects of bombesin-like peptides on the lower urinary tract have not been investigated previously. Since we previously

Fig. 5. Effect of centrally administered BB at lower doses on ICI. Vehicle indicates Sprague-Dawley rats treated with vehicle (3.3 and 6.7 μ l saline/animal, i.c.v.) serially at an interval of 60 minutes. BB indicates Sprague-Dawley rats treated with BB (0.01 and 0.03 nmol/animal, i.c.v.) serially at an interval of 60 minutes. Data were calculated as the ratio to the pretreatment (-10 to 0 minutes) values presented as means $\pm$ S.E.M. * $P < 0.05$ (compared with an unpaired t test to the vehicle group). The number of animals per group is indicated in parentheses.

Fig. 6. Effects of BB receptor antagonists on the centrally administered BB-induced shortening of ICI. BIM-23127 or BEA (a BB₁ or BB₂ antagonist, respectively) (3 nmol/animal, i.c.v.) or vehicle (5 μ l saline/animal, i.c.v.) was administered 30 minutes before the administration of BB (0.03 nmol/animal, i.c.v.) in Sprague-Dawley rats. Data were calculated as the ratio to the pretreatment (–10 to 0 minutes) values presented as means $\pm$ S.E.M. * P < 0.05 (compared with the Bonferroni method to the vehicle- and BB-treated group). The number of animals per group is indicated in parentheses.

reported that centrally administered bombesin induced central activation of sympathoadrenomedullary outflow (Shimizu et al., 2005; Yokotani et al., 2005; Tanaka et al., 2014), we examined whether central effects of bombesin on micturition involve activation of this outflow system. In this study, intracerebroventricularly administered bombesin (0.1 and 1 nmol/animal) dose-dependently induced elevation of plasma noradrenaline and adrenaline and the elevation of catecholamines was almost abolished by ADX, in agreement with our previous results (Yokotani et al., 2005; Tanaka et al., 2014). These results suggest that brain bombesin-like peptides can activate the adrenomedullary system. However, centrally administered bombesin significantly shortened ICI even at a lower dose (0.1 nmol/animal) without affecting MVP, and the bombesin-induced ICI shortening was not affected by ADX. Thus, it is assumed that brain bombesin-like peptides can facilitate micturition independent of the sympathoadrenomedullary outflow modulation.

Bombesin reportedly has pressor effects when administered intracerebroventricularly in the conscious rat (Fisher et al., 1985). The pressor effect was abolished by ADX or systemic pretreatment with an α -adrenoceptor antagonist phentolamine (Fisher et al., 1985), indicating that the adrenal medulla-derived noradrenaline and adrenaline are involved

in the bombesin-induced elevation of blood pressure. In our study, however, intracerebroventricularly administered bombesin showed no significant pressor effects, and the bombesin-induced significant elevation of blood pressure was seen in ADX rats. Because we performed blood pressure monitoring in combination with blood sampling for measurements of plasma catecholamines in this study, the bombesin-induced pressor effect shown by Fisher et al. might be influenced by blood sampling. In fact, intracerebroventricularly administered bombesin normalized blood pressure in hemorrhaged rabbits (Maryanovich et al., 1994). In our ADX rats, centrally administered bombesin induced pressor responses independent of plasma catecholamines derived from the adrenal medulla. In addition, some reports show that centrally administered bombesin induced bradycardia (Fisher et al., 1985; Carver-Moore et al., 1991) and the response was not altered by ADX (Fisher et al., 1985). These findings suggest a possibility that the bombesin-induced pressor effects in our ADX rats might be a compensatory response to the bombesin-induced bradycardia through the adrenal medulla-independent mechanisms. The reasons underlying the contradictory results between Fisher et al. and our group are not clear; however, they could be related in part to differences in the animal conditions such as conscious and free-moving ADX rats without supplementation of glucocorticoid in the study by Fisher et al. versus anesthetized ADX rats supplied with glucocorticoid in our study.

Because intracerebroventricularly administered bombesin shortened ICI even at a dose (0.1 nmol/animal), which only induced weak activation of the sympathoadrenomedullary outflow, we examined a dose dependence of the bombesin effects on ICI without blood sampling. In this study, centrally administered bombesin at low doses (0.01 and 0.03 nmol/animal) dose-dependently shortened ICI without affecting MVP, indicating that the dose range of bombesin that induces the ICI shortening effect and the activation of the sympathoadrenomedullary outflow is different. Furthermore, we characterized brain BB receptor subtypes involving the bombesin-induced shortening of ICI using BIM-23127 and BEA, peptidergic antagonists for BB₁ and BB₂ receptors, respectively. BIM-23127 selectively reversed NMB-induced feeding suppression but had no effect on the suppression induced by GRP in the rat (Ladenheim et al., 1997). BEA showed high affinity for BB₂ receptors and >1000-fold selectivity for BB₂ over BB₁ receptors from results of the binding assay using radioactive substrates, ¹²⁵I-[Tyr⁴]bombesin and ¹²⁵I-labeled Bolton-Hunter-NMB, in the rat esophageal tissue sections (von Schrenck et al., 1990). In our study, pretreatment with either BIM-23127 or BEA significantly reduced the

TABLE 3

Effects of centrally administered BB on cystometric parameters of single CMG

Data were calculated as the ratio to the pretreatment (before i.c.v.) control values presented as means $\pm$ S.E.M.

Timing of evaluation	Vv	Rv	BC	VE
	% of Control			
0.03 nmol BB ($n = 7$)				
Before i.c.v. administration	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
After i.c.v. administration				
20–30 min	56.70 $\pm$ 3.94*	89.51 $\pm$ 20.80	64.94 $\pm$ 6.19*	92.93 $\pm$ 7.02
30–40 min	65.34 $\pm$ 8.65*	77.44 $\pm$ 3.53	67.66 $\pm$ 1.98*	96.16 $\pm$ 2.01
40–50 min	62.76 $\pm$ 5.08*	92.40 $\pm$ 19.25	79.76 $\pm$ 8.55*	94.61 $\pm$ 9.88
50–60 min	104.40 $\pm$ 15.81	86.74 $\pm$ 17.55	88.58 $\pm$ 3.85	104.85 $\pm$ 8.93

* P < 0.05 (compared with the respective parameters before administration by the Bonferroni method).

bombesin-induced shortening of ICI, respectively. In the last series of experiments, we performed single CMG because ICI shortening can be induced not only by frequent urination but also by an increase in residual urine volume. We have found that centrally administered bombesin showed reductions in Vv and BC without affecting Rv or VE. Taken together, it seems likely that brain bombesin-like peptides centrally induce bladder overactivity without changing voiding function through brain BB₁ and BB₂ receptors. Considering that intracerebroventricularly administered BIM-23127 or BEA by itself had no effect on ICI or MVP, endogenous bombesin-like peptides in the brain do not seem to affect bladder function at least in normal conditions. Therefore, it seems reasonable to assume that enhancement of brain bombesin-like peptides release in response to stress might be involved in stress-induced exacerbation in bladder dysfunction characterized by increased urinary frequency and urgency shown in patients with overactive bladder or bladder pain syndrome (Smith et al., 2011; Merrill et al., 2013; Mingin et al., 2014), and that BB receptor antagonists could be useful candidates for alleviation of stress-induced exacerbation in bladder dysfunction.

The underlying mechanism inducing bladder overactivity by activation of the central bombesin-like peptide system is not clear; however, it is possible that BB receptor activation in the brain might facilitate sensory inputs to the pontine micturition center (also called the Barrington's nucleus) (Blok, 2002; Valentino et al., 2011), thereby inducing frequent urination, because bombesin had no significant effects on the CMG parameters of bladder efferent activity such as MVP, Rv, or VE. The Barrington's nucleus plays a key role in communication between the brain and the bladder (see the review by Valentino et al., 2011). The nucleus neurons that project to the preganglionic parasympathetic column of the lumbosacral spinal cord also project to the locus coeruleus (Valentino et al., 1996; Samuels and Szabadi, 2008), whose neurons have massive collateralized projections that innervate the entire neuraxis, particularly in the forebrain (Swanson and Hartman, 1975). The locus coeruleus initiates and maintains arousal in response to stimuli and modulates the attention process (Berridge and Waterhouse, 2003; Bouret and Sara, 2005). This complex circuit can coordinate activity of forebrain regions that underlies voiding behavior. In addition, the Barrington's nucleus is mainly controlled by the midbrain periaqueductal gray matter (PAG) (Holstege, 2010), where inputs from the prefrontal cortex, amygdala, and hypothalamus converge (Rizvi et al., 1991; Keay and Bandler, 2001). In the rat prefrontal cortex and amygdala, exposure to acute stress such as a restraint and an aversive stimulus evokes release of bombesin-like peptides (Merali et al., 1998, 2008) and GRP-containing cell bodies are found in the rat hypothalamus (Moody and Merali, 2004). Considering that the PAG contains bombesin-like immunoreactive fibers (Marcos et al., 1994) and BB receptors (Pert et al., 1980), bombesin-like peptides released by stress exposure might influence the bladder function due to abnormal activation of a projection from the PAG to the Barrington's nucleus, thereby exacerbating symptoms of bladder dysfunction characterized by increased urinary frequency and urgency. However, because our study used intracerebroventricular administration of bombesin and its receptor antagonists, it is not possible to clarify the brain circuits involved in bombesin-induced stimulation of

the micturition reflex. Further studies are therefore needed to examine the specific regions in the brain including the PAG that contribute to bombesin-mediated bladder dysfunction using animal models of stress exposure.

In summary, our results suggest that brain bombesin-like peptides and both BB₁ and BB₂ receptors are involved in facilitation of the rat micturition reflex to induce bladder overactivity, which is independent of the sympathoadrenomedullary outflow modulation.

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Authorship Contributions

Participated in research design: T. Shimizu, S. Shimizu, Yoshimura, Saito.

Conducted experiments: T. Shimizu, Nakamura.

Performed data analysis: T. Shimizu, Nakamura.

Wrote or contributed to the writing of the manuscript: T. Shimizu, S. Shimizu, Higashi, Yoshimura, Saito.

References

- Anastasi A, Erspamer V, and Bucci M (1971) Isolation and structure of bombesin and alkytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. *Experientia* **27**:166–167.
- Anton AH and Sayre DF (1962) A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. *J Pharmacol Exp Ther* **138**:360–375.
- Bartolomucci A, Palanza P, Costoli T, Savani E, Laviola G, Parmigiani S, and Sgoifo A (2003) Chronic psychosocial stress persistently alters autonomic function and physical activity in mice. *Physiol Behav* **80**:57–67.
- Bédard T, Mountney C, Kent P, Anisman H, and Merali Z (2007) Role of gastrin-releasing peptide and neuromedin B in anxiety and fear-related behavior. *Behav Brain Res* **179**:133–140.
- Berridge CW and Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev* **42**:33–84.
- Blok BF (2002) Central pathways controlling micturition and urinary continence. *Urology* **59** (Suppl 1):13–17.
- Bouret S and Sara SJ (2005) Network reset: a simplified overarching theory of locus coeruleus noradrenaline function. *Trends Neurosci* **28**:574–582.
- Carver-Moore K, Gray TS, and Brown MR (1991) Central nervous system site of action of bombesin to elevate plasma concentrations of catecholamines. *Brain Res* **541**:225–231.
- Degen LP, Peng F, Collet A, Rossi L, Ketterer S, Serrano Y, Larsen F, Beglinger C, and Hildebrand P (2001) Blockade of GRP receptors inhibits gastric emptying and gallbladder contraction but accelerates small intestinal transit. *Gastroenterology* **120**:361–368.
- Fisher LA, Cave CR, and Brown MR (1985) Central nervous system cardiovascular effects of bombesin in conscious rats. *Am J Physiol* **248**:H425–H431.
- Fontes MA, Xavier CH, Marins FR, Limbório-Filho M, Vaz GC, Müller-Ribeiro FC, and Nalivaiko E (2014) Emotional stress and sympathetic activity: contribution of dorsomedial hypothalamus to cardiac arrhythmias. *Brain Res* **1554**:49–58.
- Ghatei MA, Gu J, Allen JM, Polak JM, and Bloom SR (1985) Bombesin-like immunoreactivity in female rat genito-urinary tract. *Neurosci Lett* **54**:13–19.
- Holstege G (2010) The emotional motor system and micturition control. *NeuroUrol Urodyn* **29**:42–48.
- Inoue S, Saito M, and Takenaka A (2012) Hydroxyfasudil ameliorates bladder dysfunction in male spontaneously hypertensive rats. *Urology* **79**:1186.e9–1186.e14.
- Jensen RT, Battey JF, Spindel ER, and Benya RV (2008) International Union of Pharmacology. LXVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. *Pharmacol Rev* **60**:1–42.
- Keay KA and Bandler R (2001) Parallel circuits mediating distinct emotional coping reactions to different types of stress. *Neurosci Biobehav Rev* **25**:669–678.
- Kent P, Anisman H, and Merali Z (1998) Are bombesin-like peptides involved in the mediation of stress response? *Life Sci* **62**:103–114.
- Kilgore WR, Mantyh PW, Mantyh CR, McVey DC, and Vigna SR (1993) Bombesin/GRP-preferring and neuromedin B-preferring receptors in the rat urogenital system. *Neuropeptides* **24**:43–52.
- Kullmann FA, McKenna D, Wells GI, and Thor KB (2013) Functional bombesin receptors in urinary tract of rats and human but not of pigs and mice, an *in vitro* study. *Neuropeptides* **47**:305–313.
- Kullmann FA, Wells GI, McKenna D, and Thor KB (2014) Excitatory effects of bombesin receptors in urinary tract of normal and diabetic rats *in vivo*. *Life Sci* **100**:35–44.
- Ladenheim EE and Knipp S (2007) Capsaicin treatment differentially affects feeding suppression by bombesin-like peptides. *Physiol Behav* **91**:36–41.
- Ladenheim EE, Taylor JE, Coy DH, Carrigan TS, Wahn A, and Moran TH (1997) Caudal hindbrain neuromedin B-preferring receptors participate in the control of food intake. *Am J Physiol* **272**:R433–R437.

- Lai H, Gardner V, Vetter J, and Andriole GL (2015) Correlation between psychological stress levels and the severity of overactive bladder symptoms. *BMC Urol* **15**:14.
- Lutgendorf SK, Kreder KJ, Rothrock NE, Ratliff TL, and Zimmerman B (2000) Stress and symptomatology in patients with interstitial cystitis: a laboratory stress model. *J Urol* **164**:1265–1269.
- Maggi CA, Coy DH, and Giuliani S (1992) Effect of [D-Phe⁶] bombesin (6-13) methyl ester, a bombesin receptor antagonist, towards bombesin-induced contractions in the guinea-pig and rat isolated urinary bladder. *J Auton Pharmacol* **12**:215–222.
- Marcos P, Coveñas R, Narváez JA, Tramu G, Aguirre JA, and González-Barón S (1994) Distribution of gastrin-releasing peptide/bombesin-like immunoreactive cell bodies and fibres in the brainstem of the cat. *Neuropeptides* **26**:93–101.
- Martínez V and Taché Y (2000) Bombesin and the brain-gut axis. *Peptides* **21**:1617–1625.
- Maryanovich AT, Aprelkov PA, Shipilov YI, Tchurkina SI, and Kostjuchenko AL (1994) Intracerebroventricular injection of bombesin (6-14) restores blood pressure in hemorrhaged rabbits. *Resuscitation* **27**:73–76.
- Merali Z, Anisman H, James JS, Kent P, and Schulkin J (2008) Effects of corticosterone on corticotrophin-releasing hormone and gastrin-releasing peptide release in response to an aversive stimulus in two regions of the forebrain (central nucleus of the amygdala and prefrontal cortex). *Eur J Neurosci* **28**:165–172.
- Merali Z, Bédard T, Andrews N, Davis B, McKnight AT, Gonzalez MI, Pritchard M, Kent P, and Anisman H (2006) Bombesin receptors as a novel anti-anxiety therapeutic target: BB₁ receptor actions on anxiety through alterations of serotonin activity. *J Neurosci* **26**:10387–10396.
- Merali Z, Kent P, and Anisman H (2002) Role of bombesin-related peptides in the mediation or integration of the stress response. *Cell Mol Life Sci* **59**:272–287.
- Merali Z, McIntosh J, Kent P, Michaud D, and Anisman H (1998) Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *J Neurosci* **18**:4758–4766.
- Merali Z, Mountney C, Kent P, and Anisman H (2013) Activation of gastrin-releasing peptide receptors at the infralimbic cortex elicits gastrin-releasing peptide release at the basolateral amygdala: implications for conditioned fear. *Neuroscience* **243**:97–103.
- Merrill L, Malley S, and Vizzard MA (2013) Repeated variate stress in male rats induces increased voiding frequency, somatic sensitivity, and urinary bladder nerve growth factor expression. *Am J Physiol Regul Integr Comp Physiol* **305**:R147–R156.
- Milushcheva EA, Kortezova NI, Mizhorkova ZN, Papasova M, Coy DH, Bálint A, Vizi ES, and Varga G (1998) Role of different bombesin receptor subtypes mediating contractile activity in cat upper gastrointestinal tract. *Peptides* **19**:549–556.
- Mingim GC, Peterson A, Erickson CS, Nelson MT, and Vizzard MA (2014) Social stress induces changes in urinary bladder function, bladder NGF content, and generalized bladder inflammation in mice. *Am J Physiol Regul Integr Comp Physiol* **307**:R893–R900.
- Moody TW and Merali Z (2004) Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. *Peptides* **25**:511–520.
- Nakamura K, Shimizu T, Yanagita T, Nemoto T, Taniuchi K, Shimizu S, Dimitriadis F, Yawata T, Higashi Y, and Ueba T, et al. (2014) Angiotensin II acting on brain AT₁ receptors induces adrenaline secretion and pressor responses in the rat. *Sci Rep* **4**:7248.
- Paxinos G and Watson C, editors (2005) *The Rat Brain in Stereotaxic Coordinates*, Elsevier Academic Press, Burlington, MA.
- Pert A, Moody TW, Pert CB, Dewald LA, and Rivier J (1980) Bombesin: receptor distribution in brain and effects on nociception and locomotor activity. *Brain Res* **193**:209–220.
- Presti-Torres J, de Lima MN, Scalco FS, Caldana F, Garcia VA, Guimarães MR, Schwartzmann G, Roesler R, and Schröder N (2007) Impairments of social behavior and memory after neonatal gastrin-releasing peptide receptor blockade in rats: implications for an animal model of neurodevelopmental disorders. *Neuropharmacology* **52**:724–732.
- Radziszewski P, Crayton R, Persson K, Andersson KE, and Mattiasson A (2011) Exogenously administered bombesin and gastrin releasing peptide contract the female rat urethra in vivo and in vitro. *Neurourol Urodyn* **30**:1681–1685.
- Radziszewski P, Ekblad E, Sundler F, and Mattiasson A (1996) Distribution of neuropeptide-, tyrosine hydroxylase- and nitric oxide synthase containing nerve fibers in the external urethral sphincter of the rat. *Scand J Urol Nephrol Suppl* **179**:81–85.
- Rizvi TA, Ennis M, Behbehani MM, and Shipley MT (1991) Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. *J Comp Neurol* **303**:121–131.
- Roesler R, Kent P, Luft T, Schwartzmann G, and Merali Z (2014) Gastrin-releasing peptide receptor signaling in the integration of stress and memory. *Neurobiol Learn Mem* **112**:44–52.
- Roesler R, Kent P, Schroder N, Schwartzmann G, and Merali Z (2012) Bombesin receptor regulation of emotional memory. *Rev Neurosci* **23**:571–586.
- Rothrock NE, Lutgendorf SK, Kreder KJ, Ratliff T, and Zimmerman B (2001) Stress and symptoms in patients with interstitial cystitis: a life stress model. *Urology* **57**:422–427.
- Samuels ER and Szabadi E (2008) Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation. *Curr Neuropharmacol* **6**:235–253.
- Shimizu T, Okada S, Yamaguchi N, Arai J, Wakiguchi H, and Yokotani K (2005) Brain phospholipase C/diacylglycerol lipase are involved in bombesin BB₂ receptor-mediated activation of sympatho-adrenomedullary outflow in rats. *Eur J Pharmacol* **514**:151–158.
- Shimizu T, Okada S, Yamaguchi N, Sasaki T, Lu L, and Yokotani K (2006) Centrally administered histamine evokes the adrenal secretion of noradrenaline and adrenaline by brain cyclooxygenase-1- and thromboxane A₂-mediated mechanisms in rats. *Eur J Pharmacol* **541**:152–157.
- Shimizu T, Okada S, Yamaguchi-Shima N, and Yokotani K (2004) Brain phospholipase C-diacylglycerol lipase pathway is involved in vasopressin-induced release of noradrenaline and adrenaline from adrenal medulla in rats. *Eur J Pharmacol* **499**:99–105.
- Shumyatsky GP, Tsvetkov E, Malleret G, Vronskaya S, Hatton M, Hampton L, Batten JF, Dulac C, Kandel ER, and Bolshakov VY (2002) Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. *Cell* **111**:905–918.
- Smith AL, Leung J, Kun S, Zhang R, Karagiannides I, Raz S, Lee U, Glovatska V, Pothoulakis C, and Bradesi S, et al. (2011) The effects of acute and chronic psychological stress on bladder function in a rodent model. *Urology* **78**:967.e1–967.e7.
- Swanson LW and Hartman BK (1975) The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. *J Comp Neurol* **163**:467–505.
- Tanaka K, Shimizu T, Yanagita T, Nemoto T, Nakamura K, Taniuchi K, Dimitriadis F, Yokotani K, and Saito M (2014) Brain RVD-haemopressin, a haemoglobin-derived peptide, inhibits bombesin-induced central activation of adrenomedullary outflow in the rat. *Br J Pharmacol* **171**:202–213.
- Tsushima H, Mori M, Fujiwara N, and Moriama A (2003) Pharmacological characteristics of bombesin receptor mediating hypothermia in the central nervous system of rats. *Brain Res* **969**:88–94.
- Ulrich-Lai YM and Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci* **10**:397–409.
- Valentino RJ, Chen S, Zhu Y, and Aston-Jones G (1996) Evidence for divergent projections to the brain noradrenergic system and the spinal parasympathetic system from Barrington's nucleus. *Brain Res* **732**:1–15.
- Valentino RJ, Wood SK, Wein AJ, and Zderic SA (2011) The bladder-brain connection: putative role of corticotropin-releasing factor. *Nat Rev Urol* **8**:19–28.
- von Schrenck T, Wang LH, Coy DH, Villanueva ML, Mantey S, and Jensen RT (1990) Potent bombesin receptor antagonists distinguish receptor subtypes. *Am J Physiol* **259**:G468–G473.
- Watts SW and Cohen ML (1991) Effect of bombesin, bradykinin, substance P and CGRP in prostate, bladder body and neck. *Peptides* **12**:1057–1062.
- Yokotani K, Okada S, Nakamura K, Yamaguchi-Shima N, Shimizu T, Arai J, Wakiguchi H, and Yokotani K (2005) Brain prostanoid TP receptor-mediated adrenal noradrenaline secretion and EP₃ receptor-mediated sympathetic noradrenaline release in rats. *Eur J Pharmacol* **512**:29–35.
- Yoshiyama M and de Groat WC (2005) Supraspinal and spinal alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and N-methyl-D-aspartate glutamatergic control of the micturition reflex in the urethane-anesthetized rat. *Neuroscience* **132**:1017–1026.

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