DIRECT EFFECTS OF GLUCOSE, INSULIN, GLP-1, AND GIP ON BULBOSPINAL NEURONS IN THE ROSTRAL VENTROLATERAL MEDULLA IN NEONATAL WISTAR RATS

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Abstract—Although patients with diabetes mellitus (DM) often exhibit hypertension, the mechanisms responsible for this correlation are not well known. We hypothesized that the bulbospinal neurons in the rostral ventrolateral medulla (RVLM) are affected by the levels of glucose, insulin, or incretins (glucagon like peptide-1 [GLP-1] or glucose-dependent insulinotropic peptide [GIP]) in patients with DM. To investigate whether RVLM neurons are activated by glucose, insulin, GLP-1, or GIP, we examined changes in the membrane potentials of bulbospinal RVLM neurons using whole-cell patch-clamp technique during superfusion with various levels of glucose or these hormones in neonatal Wistar rats. A brainstem–spinal cord preparation was used for the experiments. A low level of glucose stimulated bulbospinal RVLM neurons. During insulin superfusion, almost all the RVLM neurons were depolarized, while during GLP-1 or GIP superfusion, almost all the RVLM neurons were hyperpolarized. Next, histological examinations were performed to examine transporters for glucose and receptors for insulin, GLP-1, and GIP on RVLM neurons. Low-level glucose-depolarized RVLM neurons exhibited the presence of glucose transporter 3 (GLUT3). Meanwhile, insulin-depolarized, GLP-1-hyperpolarized, and GIP-hyperpolarized RVLM neurons showed each of the respective specific receptor. These results indicate that a low level of glucose stimulates bulbospinal RVLM neurons via specific transporters on these neurons, inducing hypertension.

Furthermore, an increase in insulin or a reduction in incretins may also activate the sympathetic nervous system and induce hypertension by activating RVLM neurons via their own receptors. © 2016 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: RVLM neurons, glucose, insulin, GLP-1, GIP, hypertension.

INTRODUCTION

Hypertension is often observed in patients with diabetes mellitus (DM). The detailed mechanisms of hypertension are not known; however, the levels of blood glucose and hormones that regulate the glucose level are considered to be involved in causing high blood pressure (BP). Hypoglycemia causes hypertension (Feldman-Billart et al., 2010), and the rostral ventrolateral medulla (RVLM) is reported to be a key component in hypertension in response to hypoglycemia (Verberne and Sartor, 2010). In contrast, hyperglycemia decreases the BP (Lee et al., 2009). After a meal, the BP tends to decrease (Lipsitz et al., 1993; Imai et al., 1998), and this reduction is thought to be caused by an increase in muscle nerve sympathetic activity after glucose intake has been blunted, even though the splanchnic blood volume is increased (Fagius et al., 1996). However, the details of this mechanism remain unknown.

Insulin stimulates glucose uptake into several organs, which are mainly cardiac muscle, skeletal muscle, liver, and adipose tissue to retain glucose homeostasis (Jia et al., 2016). A decreased response to insulin, or insulin resistance, leads to increased insulin production in the pancreas, resulting in hyperinsulinemia. Hyperinsulinemia, which is often seen in type 2 DM, activates the sympathetic nervous system and the renin–angiotensin–aldosterone system (RAAS), resulting in hypertension (Thorpe and Schlaich, 2015; Jia et al., 2016). Incretins (glucagon-like peptide 1 [GLP-1] and glucose-dependent insulinotropic peptide [GIP]) are released from the gastrointestinal tract and participate in glucose homeostasis (Drucker, 2003). GLP-1 and GIP stimulate glucose-dependent insulin biosynthesis and secretion in pancreatic ß cells after food ingestion to coordinate energy assimilation (Sanusi, 2009). GLP-1 and
GIP are also considered to be associated with a reduction in BP. Fonseca et al. (2010) showed that BP reductions were associated with GIP and GLP-1 levels in clinical trials. Furthermore, a decrease in GIP in obese patients was negatively correlated with the systolic BP (Ceperuelo-Mallafre´ et al., 2014). In patients with type 2 DM, circulating DPP4 is augmented (Röhrborn et al., 2015), and Sanusi (2009) reported reductions in GIP activity and the amount of GLP-1.

The RVLM, which includes presympathetic neurons, plays an important role in the regulation of BP (Madden and Sved, 2003). The RVLM neurons send signals directly to the sympathetic preganglionic neurons (SPNs) in the intermediolateral cell column (IML) and SPNs regulate the function of peripheral sympathetic nerves and BP (Pilowsky and Goodchild, 2002). If glucose, insulin, and incretins affect these neurons, the BP would change. Therefore, in this study, we examined the direct effects of glucose, insulin, GLP-1, and GIP on bulbospinal RVLM neurons using brainstem–spinal cord preparations (Iigaya et al., 2009; Oshima et al., 2013, 2014, 2015).

To the best of our knowledge, the direct effects of glucose, insulin, and incretins on bulbospinal RVLM neurons have not been fully investigated. Previous studies have shown that glucose enters target cells through specific transporters (Mueckler, 1994; Benarroch, 2014), and insulin and incretins activate them via their own receptors (Wideman and Kieffer, 2004; Kanzaki, 2006; Griffioen et al., 2011). Therefore, in this study, we also performed a histological analysis to examine the presence of specific transporters for glucose and specific receptors for insulin and incretins on the RVLM neurons.

**EXPERIMENTAL PROCEDURES**

**General preparations**

We performed experiments using brainstem–spinal cord preparations collected from 1- to 5-day-old Wistar rats of either sex, as previously described (Iigaya et al., 2009; Oshima et al., 2013, 2014, 2015). In the electrophysiological study, we used a single value from individual rats and 249 neurons were used from 249 rats (one neuron/rat). In only histological study we used nine rats. In total, 258 rats were used. The experiment protocols were approved by the Institutional Review Board of the National Defense Medical College and were in accordance with the National Guidelines for the Conduct of Animal Experiments. Briefly, we placed the animals under deep ether anesthesia, and isolated the brainstem–spinal cord at the Th2 level. Then we sectioned the brainstem between the roots of cranial nerve VI and the lower border of the trapezoid body (Fig. 1A). We continuously superfused the preparations with a solution containing (in mmol/L) 124 NaCl, 5.0 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgCl2, 26 NaHCO3, and 30 glucose and maintained at 25–26 °C (artificial cerebrospinal fluid [aCSF]). For Experimental Protocol (1) and (3), we used 4.4 mmol/L-glucose-aCSF as a standard solution (osmotic pressure was adjusted to 330 mOsm/L by adding sucrose). We main-

![Fig. 1. Brainstem–spinal cord preparation and an antidromic AP. (A) Brainstem–spinal cord preparation of a neonatal Wistar rat. IX, X, XI, and XII are the numbers of cranial nerves. Electrical stimulation was applied to the IML at the Th2 level via an electrode. (B) Antidromic AP in an RVLM neuron that was evoked by electrical stimulation (†) to the IML at the Th2 level.](image)
Experimental protocols

**Protocol (1):** To examine the effect of glucose on the bulbospinal RVLM neurons, the glucose level of aCSF was changed from 4.4 mmol/L (80 mg/dL) to 1.1, 11, or 30 mmol/L (20, 200, or 540 mg/dL). The osmotic pressure of each solution was adjusted to 330 mOsm/L by adding sucrose. In protocol (1), 4.4 mmol/L-glucose-aCSF (4.4 mmol/L is the physiological glucose level of cerebrospinal fluid) was used as the standard solution. During the recording of the MPs of the bulbospinal RVLM neurons, we superfused the preparations with the 1.1, 11, or 30 mmol/L-glucose solution. We determined the changes in the MPs 2–5 min after the start of superfusion using each solution. We superfused the bulbospinal RVLM neurons with a tetrodotoxin (TTX, 0.5 mmol/L, Sigma) solution for 10 min to block synaptic transmissions from other neurons to the recorded bulbospinal...
RVLM neurons. Thereafter, we superfused the neurons with the 1.1, 11, or 30 mmol/L-glucose solution dissolved in a TTX solution and we recorded the MPs.

**Protocol (2):** The preparations were superfused with solutions of insulin (200–1000 pmol/L, Sigma), GLP-1 (100–500 pmol/L, Sigma), or GIP (100–500 pmol/L, Sigma) dissolved in aCSF. The duration of superfusion with each drug was 2–10 min. The changes in the MPs were determined 2–5 min after the start of superfusion with each drug. We superfused the bulbospinal RVLM neurons with a TTX solution for 10 min. Thereafter, we superfused the neurons with each hormone (insulin, 2000 pmol/L; GLP-1, 200 pmol/L; and GIP, 200 pmol/L) dissolved in a TTX solution and we recorded the MPs.

To examine whether the effects of these hormones on RVLM neurons were caused by the action on insulin receptors (IRs), GLP-1 receptors (GLP-1Rs), or GIP receptors (GIPRs), the bulbospinal RVLM neurons were superfused with each receptor antagonist (S961 [IR antagonist, 5 nmol/L; Funakoshi, Tokyo, Japan], exendin(9–39) [GLP-1R antagonist, 200 nmol/L; Wako, Osaka, Japan], or (Pro3)GIP [GIPR antagonist, 500 nmol/L; Sigma]).

Fig. 3. Bulbospinal RVLM neurons showing depolarization during insulin superfusion. (A) The neuron was depolarized by insulin. During insulin superfusion, the RVLM neurons (n = 8) were depolarized (B) and the FAP in the RVLM neurons tended to increase (C). White bar, before insulin superfusion; gray bar, during insulin superfusion; values are mean ± SEM. **P < 0.01 vs. before insulin superfusion using Paired t-test. (D) The neuron was hyperpolarized by S961. During S961 superfusion, the RVLM neurons were hyperpolarized (E) and the FAP in the RVLM neurons tended to decrease (F). White bar, before S961 superfusion; gray bar, during S961 superfusion; values are mean ± SEM. *P ≤ 0.05 vs. before S961 superfusion using Wilcoxon’s signed-rank test (E). (G) The hyperpolarizing effect of S961 was suppressed by insulin. During insulin superfusion, the addition of S961 decreased the MPs of the RVLM neuron slightly, but the change in the MPs was not significant (H), and no significant changes in the FAP in the RVLM neurons were observed (I). Gray bar, before S961 superfusion; white bar, during S961 superfusion; values are mean ± SEM. (J) The neuron was depolarized during superfusion with insulin dissolved in a TTX solution.
Protocol (3): To examine whether the changes in RVLM neuron activities caused by low-level or high-level glucose superfusion were transmitted to the IML neurons, the MPs of the IML neurons at the Th2 level were recorded and the RVLM areas were microsuperfused (30–100 µL) with the 1.1, 11, or 30 mmol/L-glucose solution (Fig. 6A). In protocol (3), the whole preparation was superfused with a solution of 4.4 mmol/L-glucose.

Protocol (4): We recorded the MPs of the IML neurons and microsuperfused (10–30 µL) the RVLM areas with each hormone solution (insulin, 1000 pmol/L; GLP-1, 200 pmol/L; GIP, 200 pmol/L).

Immunofluorescence staining
To determine the presence of specific transporters for these toxins histologically, we performed immunofluorescence staining. After the aforementioned experiments, we fixed the preparations for 1 h at 4 °C in 4% paraformaldehyde in 0.1 M PBS, immersed them in 18% sucrose–PBS overnight, embedded them in optimal cutting temperature (OCT) compound (Sakura Finetek).
Japan Co., Ltd., Tokyo, Japan), froze them on dry ice, and cut them into 20-μm-thick transverse sections. Then we stained them using immunofluorescence method. We obtained the images using a conventional fluorescence microscope (LSM510; Carl Zeiss Co., Oberkochen, Germany).

**Immunofluorescence staining protocols**

The Lucifer Yellow-stained RVLM neurons that responded to the change in glucose level were examined for the type of glucose transporter. Also, the Lucifer Yellow-stained RVLM neurons that responded to insulin, GLP-1, and GIP were examined for the specific receptors of each hormone. The following primary antibodies (1:400 dilution) were used for immunofluorescence: goat anti-glucose transporter 1 (GLUT1) antibody (Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-GLUT2 antibody, rabbit anti-GLUT3 antibody (Sigma), mouse anti-GLUT4 antibody (Santa Cruz Biotechnology), rabbit anti-GLP-1 antibody (Santa Cruz Biotechnology), rabbit anti-GLP-1 receptor antibody (Sigma), rabbit anti-GIP antibody (Santa Cruz Biotechnology), and rabbit anti-GIP receptor antibody (Sigma).

RVLM neurons include C1 catecholaminergic neurons (Madden and Sved, 2003; Oshima et al.)

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**Fig. 5.** Bulbospinal RVLM neurons showing hyperpolarization during GIP superfusion. (A) The neuron was hyperpolarized by GIP. During GIP superfusion, the RVLM neurons (n = 7) were hyperpolarized (B) and the FAP in the RVLM neurons decreased (C). White bar, before GIP superfusion; gray bar, during GIP superfusion; values are mean ± SEM. *P < 0.05 vs. before GIP superfusion using Paired t-test. (D) The neuron was depolarized by (Pro3)GIP. During (Pro3)GIP superfusion, the RVLM neurons were depolarized (E) and the FAP in the RVLM neurons increased (F). White bar, before (Pro3)GIP superfusion; gray bar, during (Pro3)GIP superfusion; values are mean ± SEM. *P < 0.05, **P < 0.01 vs. before GLP-1 superfusion using Paired t-test. (G) The depolarizing effect of (Pro3)GIP was suppressed by GIP. In this figure, the addition of (Pro3)GIP caused a slight increase in the MPs and EPSPs of the recorded neurons. During GIP superfusion, added (Pro3) GIP increased the MPs of the RVLM neurons, but the change in the MPs was not significant (H), and no significant changes in the FAP in the RVLM neurons were observed (I). Gray box, before (Pro3)GIP superfusion; white box, during (Pro3)GIP superfusion; within each box plot, the center line shows the median, the box shows the IQR, and whiskers represent the minimum and maximum values. (J) The neuron was hyperpolarized during superfusion with GIP dissolved in a TTX solution.
To confirm that the examined area was a C1 area, we also examined the existence of tyrosine hydroxylase (TH)-positive neurons in the RVLM using mouse anti-TH antibody (Sigma).

The secondary antibodies for fluorescence staining (1:1000 dilution) were Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes/Invitrogen, Eugene, OR, USA), Alexa Fluor 546 goat anti-rabbit IgG, and Alexa Fluor 633 goat anti-mouse IgG (Molecular Probes/Invitrogen).

Statistics
In each neuron, we compared the activities of the neuron before and during (or after) superfusion with the drug. We used a single value from individual rats (one neuron/rat) and we used non-independent data in our analyses. Comparisons of the MPs or FAP recorded before and during (or after) superfusion with the drugs were performed using Paired t-test or Wilcoxon’s signed-rank tests for paired observations (n = 249). We checked all the data to find normal distributions and equal variances. When the variables had them, we used Paired t-test. When the variables did not have them, we used Wilcoxon’s signed-rank tests. The results were expressed as means ± SEM when Paired t-test was applied and the results were expressed as the median (interquartile range [IQR]) when Wilcoxon’s signed-rank tests were applied. The statistical significance was set at P < 0.05. Data were analyzed using JMP Pro 12 statistical software (SAS Inc, Cary, NC, USA).

RESULTS

Effects of glucose level on bulbospinal RVLM neurons
To examine the effects of glucose on the bulbospinal RVLM neurons, we changed the glucose level of aCSF from 4.4 to 1.1, 11, or 30 mmol/L.

Change in the level of glucose from 4.4 to 1.1 mmol/L
To examine the effect of a low level of glucose on RVLM neurons, RVLM neurons were superfused with a 1.1 mmol/L-glucose solution. Of the 16 recorded bulbospinal RVLM neurons, 14 showed depolarization and an increased frequency of action potential (FAP) during 1.1 mmol/L-glucose superfusion {MP before: –45.4 ± 0.9 mV, MP during: –43.0 ± 1.2 mV; t(15)}

Fig. 6. Micro-superfusion with a low-level or high-level glucose solution over the RVLM area either depolarized or hyperpolarized the IML neurons. (A) Brainstem–spinal cord preparation. Micro-superfusion with a low-level or high-level glucose solution was performed over the RVLM area during whole-cell recordings of the IML neuron at the Th2 level. In this experiment, the whole preparation was superfused with a solution of 4.4 mmol/L-glucose. (B) The IML neuron at the Th2 level was depolarized after 1.1 mmol/L-glucose micro-superfusion over the RVLM area. (C) The IML neuron at the Th2 level was depolarized after 11 mmol/L-glucose micro-superfusion over the RVLM area. (D) The IML neuron at the Th2 level was hyperpolarized after 30 mmol/L-glucose micro-superfusion over the RVLM area.
activities of the RVLM neurons directly.

suggest that a high level of glucose suppressed the activities of the RVLM neurons.

Change in the level of glucose from 4.4 to 1.1 mmol/L in a TTX solution

To examine that a low level of glucose activates RVLM neurons directly, the neurons were superfused with a 1.1 mmol/L-glucose solution dissolved in a TTX solution. All six recorded bulbospinal RVLM neurons showed depolarization during superfusion with 1.1 mmol/L-glucose dissolved in a TTX solution (MP before: 45.6 ± 1.4 mV, MP during: 42.6 ± 1.2 mV, t(9) = 8.620, P < 0.01) (Fig. 2D). These results suggest that a low level of glucose increased the activities of the RVLM neurons directly.

Change in the level of glucose from 4.4 to 11 mmol/L

To examine the effect of a high level of glucose on RVLM neurons, RVLM neurons were superfused with a 11 mmol/L-glucose solution. Of the 10 recorded bulbospinal RVLM neurons, RVLM neurons were superfused with an 11 mmol/L-glucose solution dissolved in a TTX solution. All seven recorded bulbospinal RVLM neurons showed hyperpolarization during superfusion with 11 mmol/L-glucose superfusion {MP before: −40.5 ± 0.9 mV, MP during: −42.9 ± 1.6 mV, t(9) = −3.073, P < 0.05; FAP before: 0.4 ± 0.1 Hz, FAP during: 0.1 ± 0.1 Hz, t(9) = −5.284, P < 0.01} (Fig. 2E –G). These results suggest that a high level of glucose suppressed the activities of the RVLM neurons directly.

Change in the level of glucose from 4.4 to 11 mmol/L in a TTX solution

To examine that a high level of glucose activates RVLM neurons directly, the neurons were superfused with a 11 mmol/L-glucose solution dissolved in a TTX solution. All six recorded bulbospinal RVLM neurons showed hyperpolarization during superfusion with 11 mmol/L-glucose dissolved in a TTX solution (MP before: 45.5 ± 1.0 mV, MP during: 44.2 ± 1.3 mV, t(17) = 5.642, P < 0.01; FAP before: 0.6 ± 0.1 Hz, FAP during: 0.3 ± 0.1 Hz, t(17) = −3.141, P < 0.01) (Fig. 2L). These results suggest that a high level of glucose suppressed the activities of the RVLM neurons.

Change in the level of glucose from 4.4 to 30 mmol/L

To confirm that a high level of glucose suppressed the activities of RVLM neurons, RVLM neurons were superfused with a 30 mmol/L-glucose solution. All 18 recorded bulbospinal RVLM neurons showed hyperpolarization and a decreased FAP during 30 mmol/L-glucose superfusion {MP before: −41.6 ± 0.8 mV, MP during: −45.2 ± 1.3 mV, t(17) = −5.642, P < 0.01; FAP before: 0.6 ± 0.1 Hz, FAP during: 0.3 ± 0.1 Hz, t(17) = −3.141, P < 0.01} (Fig. 2I–K). These results suggest that a high level of glucose suppressed the activities of the RVLM neurons.

Change in the level of glucose from 4.4 to 30 mmol/L in a TTX solution

To confirm that a high level of glucose suppressed RVLM neurons directly, the neurons were superfused with a 30 mmol/L-glucose solution dissolved in a TTX solution. All 6 recorded bulbospinal RVLM neurons showed hyperpolarization during superfusion with 30 mmol/L-glucose dissolved in a TTX solution (MP before: −44.2 ± 2.1 mV, MP during: −46.9 ± 2.4 mV, t(5) = −6.507, P < 0.01} (Fig. 2L). These results suggest that a high level of glucose suppressed the activities of the RVLM neurons directly.

Effects of insulin, GLP-1 and GIP on RVLM neurons

Insulin superfusion. To examine the effect of insulin on RVLM neurons, RVLM neurons were superfused with solution of insulin. Of the 22 recorded bulbospinal RVLM neurons, 20 showed depolarization (Fig. 3A –C, Table 1). The results suggest that insulin increased the RVLM neurons activities.

S961 superfusion. To examine the effect of S961 (an antagonist of IRs) on RVLM neurons, RVLM neurons were superfused with solution of S961. All seven bulbospinal RVLM neurons were hyperpolarized {MP before: −42.7 ± 1.3 mV, MP during: −45.6 ± 1.6 mV, t(6) = −7.823, P < 0.01; FAP before: 0.3 Hz (IQR 0.2–0.4 Hz), during: 0.2 Hz (IQR 0–0.3 Hz), z (7, 7) = −13.500, P ≤ 0.05} (Fig. 3D –F). These results

| Concentration (pmol/L) | Depolarized/examined | MP (mV) | FAP (Hz) |
|------------------------|-----------------------|---------|----------|
| 200 (n = 7)            | 6 (no change, 1)      |         |          |
|                        | Before during         | −44.9 ± 0.7 | 0.1 ± 0.1 |
|                        |                       | −41.7 ± 0.8” | 0.4 ± 0.2 |
| 600 (n = 7)            | 6 (no change, 1)      |         |          |
|                        | Before during         | −44.1 ± 0.8 | 0.3 ± 0.2 |
|                        |                       | −41.1 ± 1.1” | 0.8 ± 0.3 |
| 1000 (n = 8)           | 8                     |         |          |
|                        | Before during         | −44.3 ± 1.0 | 0.3 ± 0.1 |
|                        |                       | −40.1 ± 1.1” | 0.7 ± 0.2” |

MP: membrane potential; FAP: frequency of action potential.
Values are mean ± SEM, *P < 0.05, **P < 0.01 vs. before insulin superfusion using Paired t-test.
For MP and FAP, data were obtained from all the examined neurons.

Table 1. Depolarization of bulbospinal RVLM neurons during insulin superfusion

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suggest that S961 suppressed the activities of the bulbospinal RVLM neurons by inhibiting IRs.

Effects of S961 on insulin-depolarized RVLM neurons. To confirm whether the effects of insulin on RVLM neurons were caused by the action on IRs, S961 was added to an insulin solution. In 5 of the 6 cases, the addition of S961 did not hyperpolarize the insulin-depolarized RVLM neurons (before: $-41.8 \pm 1.4$ mV, during: $-42.5 \pm 1.3$ mV, $t(5) = -2.519$; before: $0.4 \pm 0.1$ Hz, during: $0.2 \pm 0.1$ Hz, $t(5) = -2.324$) (Fig. 3 G–I). The hyperpolarizing effect of S961 was suppressed in the presence of an insulin solution. These results suggest that insulin increased the activities of the bulbospinal RVLM via IRs.

Superfusion with insulin dissolved in a TTX solution. To examine that insulin activated RVLM neurons directly, the neurons were superfused with an insulin solution dissolved in a TTX solution. Of the six recorded bulbospinal RVLM neurons, five showed depolarization during superfusion with insulin dissolved in a TTX solution (before: $-44.9 \pm 0.6$ mV, during: $-42.0 \pm 0.6$ mV, $t(5) = 7.885$, $P < 0.01$) (Fig. 3J). These results suggest that insulin activated the RVLM neurons directly via IRs.

GLP-1 superfusion. To examine the effect of GLP-1 on RVLM neurons, RVLM neurons were superfused with a GLP-1 solution. Of the 22 recorded bulbospinal RVLM neurons, 20 showed hyperpolarization during GLP-1 superfusion (Fig. 4A –C, Table 2). The results suggest that GLP-1 suppressed the RVLM neurons' activities via GLP-1Rs.

Exendin(9–39) superfusion. To examine the effect of exendin(9–39) (an antagonist of GLP-1R) on RVLM neurons, RVLM neurons were superfused with solution of exendin(9–39). Of the 20 recorded bulbospinal RVLM neurons, 19 showed depolarization (MP before: $-43.2 \pm 0.7$ mV, MP during: $-40.5 \pm 0.8$ mV, $t(19) = 5.330$, $P < 0.01$; FAP before: $0.1 \pm 0.1$ Hz, FAP during: $0.5 \pm 0.1$ Hz, $t(19) = -3.642$, $P < 0.01$) (Fig. 4D–F). These results suggest that exendin(9–39) increased the activities of the bulbospinal RVLM neurons by inhibiting GLP-1Rs.

Effects of exendin(9–39) on GLP-1-hyperpolarized RVLM neurons. To confirm whether the effects of GLP-1 on RVLM neurons were caused by the action on GLP-1Rs, exendin(9–39) was added to a GLP-1 solution. In all 4 cases, the addition of exendin(9–39) did not depolarize the GLP-1-hyperpolarized RVLM neurons (before: $-42.7$ mV (IQR $-44.4$ to $-39.6$ mV), during: $-42.3$ mV (IQR $-42.7$ to $-38.7$ mV), z (4, 4) = 5.00; before: $0.1$ Hz (IQR 0.1–0.3 Hz), during: $0.2$ Hz (IQR 0.1–0.4 Hz), z (4, 4) = 3.500) (Fig. 4G–I). The depolarizing effect of exendin(9–39) was suppressed in the presence of a GLP-1 solution. These results suggest that GLP-1 decreased the RVLM neurons' activities via GLP-1Rs.

Superfusion with GLP-1 in a TTX solution. To examine that GLP-1 suppressed RVLM neurons' activities directly, the neurons were superfused with a GLP-1 solution dissolved in a TTX solution. All six recorded bulbospinal RVLM neurons showed hyperpolarization during superfusion with GLP-1 dissolved in a TTX solution (before: $-45.6 \pm 1.1$ mV, during: $-48.0 \pm 1.2$ mV, $t(5) = -11.068$, $P < 0.01$) (Fig. 4J). These results suggest that GLP-1 suppressed the RVLM neurons' activities directly via GLP-1Rs.

GIP superfusion. To examine the effect of GIP on RVLM neurons, RVLM neurons were superfused with a GIP solution. Of the 21 recorded RVLM neurons, 18 showed hyperpolarization during GIP superfusion (Fig. 5A–C, Table 3). The results suggest that GIP suppressed RVLM neurons' activities.

(Pro3)GIP superfusion. To examine the effect of (Pro3)GIP (an antagonist of GIPR) on RVLM neurons, RVLM neurons were superfused with solution of (Pro3)GIP (9–39). All 6 bulbospinal RVLM neurons were depolarized (before: $-44.6 \pm 2.9$ mV, during: $-40.4 \pm 2.7$ mV, $t(5) = 4.602$, $P < 0.01$; before: $0.1 \pm 0.1$ Hz, during: $0.5 \pm 0.1$ Hz, $t(5) = 3.503$, $P < 0.05$) (Fig. 5D–F). These results suggest that (Pro3)GIP increased the activities of the bulbospinal RVLM neurons by inhibiting GIPRs.

Effects of (Pro3)GIP on GIP-hyperpolarized RVLM neurons. To confirm whether the effects of GIP on RVLM neurons were caused by the action on GIP-1Rs, (Pro3) GIP was added to a GIP solution. In all four cases, the addition of (Pro3)GIP did not depolarize the GIP-hyperpolarized RVLM neurons (before: $-42.2$ mV (IQR $-43.8$ to $-41.2$ mV), during: $-41.8$ mV (IQR $-43.0$ to $-40.8$ mV), z (4, 4) = 4.00; before: $0.1$ Hz (IQR 0.1–0.3 Hz), during: $0.2$ Hz (IQR 0.1–0.3 Hz), z (4, 4) = 1.500) (Fig. 5G–I). The depolarizing effect of (Pro3)GIP was suppressed in the presence of a GIP solution. These results suggest that GIP suppressed the RVLM neurons' activities via GIPRs.

Superfusion with GIP in a TTX solution. To examine that GIP suppressed RVLM neurons' activities directly, the neurons were superfused with a GIP solution dissolved in a TTX solution. All six recorded bulbospinal RVLM neurons recorded showed hyperpolarization during superfusion with GIP dissolved in a TTX solution (before: $-43.5 \pm 1.2$ mV, during: $-45.8 \pm 1.4$ mV, $t(5) = -9.136$, $P < 0.01$) (Fig. 5J). These results suggest that GIP suppressed the RVLM neurons' activities directly via GIPRs.

Micro-superfusion with each solution over the RVLM area. To examine whether the changes in the RVLM neurons' activities induced by glucose were transmitted to the IML neurons, the RVLM areas were micro-superfused with a low-level or high-level glucose solution and the changes in the MPs of the IML neurons were observed (Fig. 6A). In all six cases, micro-
superfusion over the RVLM with a 1.1 mmol/L-glucose solution caused depolarization of the IML neurons at the TH2 level (before: −44.2 ± 1.6 mV, after: −41.5 ± 1.6 mV, t(5) = 5.743, P < 0.01; before: 0.1 ± 0.1 Hz, after: 0.7 ± 0.1 Hz, t(5) = 7.319, P < 0.01). The latency, which is the period from the end of micro-superfusion over the RVLM areas to the beginning of the change in the MPs of the IML neurons, was 74.7 ± 4.8 s (Fig. 6B). In all six cases, micro-superfusion over the RVLM with an 11 mmol/L-glucose solution caused hyperpolarization of the IML neurons (before: 41.6 ± 1.2 mV, after: 43.9 ± 0.9 mV, t(5) = −5.021, P < 0.01; before: 0.3 ± 0.1 Hz, after: 0.1 ± 0.1 Hz, latency, t(5) = −2.390: 74.5 ± 19.2 s) (Fig. 6C). In all nine cases, micro-superfusion over the RVLM with a 330 mmol/L-glucose solution caused hyperpolarization of the IML neurons (before: −43.2 ± 1.1 mV, after: −46.6 ± 1.3 mV, t(8) = −8.551, P < 0.01; before: 0.3 ± 0.1 Hz, after: 0.1 ± 0.1 Hz, t(8) = −8.575, P < 0.01; latency: 75.8 ± 9.2 s) (Fig. 6D).

The RVLM areas were micro-superfused with insulin, GLP-1, or GIP solution and the changes in the MPs of the IML neurons were observed. In 12 of the 14 cases, micro-superfusion over the RVLM with an insulin solution caused depolarization of the IML neurons (before: −42.7 ± 1.1 mV; after: −40.1 ± 1.2 mV, t(13) = 10.048, P < 0.0001; before: 0.1 ± 0.1 Hz, after: 0.5 ± 0.1 Hz, t(13) = 4.534, P < 0.01; latency: 28.2 ± 2.9 s) (Fig. 7A). In 12 of the 13 cases, micro-superfusion over the RVLM with a GLP-1 solution hyperpolarized the IML neurons (before: −41.9 ± 0.5 mV, after: −45.0 ± 0.9 mV, t(12) = −3.086, P < 0.05; before: 0.4 Hz (IQR 0.2–1.3 Hz), during: 0.1 Hz (IQR 0.01–0.3 Hz), z(13, 13) = −7.500, P < 0.05; latency: 25.6 ± 2.3 s) (Fig. 7B). In eight of the nine cases, micro-superfusion over the RVLM with a GIP solution hyperpolarized the IML neurons (before: −40.7 ± 1.8 mV, after: −42.6 ± 1.7 mV, t(8) = −3.745, P < 0.05; before: 0.6 ± 0.1 Hz, after: 0.1 ± 0.1 Hz, t(8) = −3.931, P < 0.01; latency: 31.3 ± 5.7 s) (Fig. 7C).

Localization of transporters and receptors. To examine the presence of specific transporters for glucose histologically, immunofluorescence staining was performed. Lucifer Yellow staining was performed after the recordings of the MPs of the RVLM neurons were completed (Fig. 8A3–D3). Five RVLM neurons that were depolarized during 1.1 mmol/L-glucose superfusion were examined for GLUT3 immunoreactivity, and we found that all five neurons were located in the RVLM area and showed GLUT3 immunoreactivity (Fig. 8A1). Three of these 5 neurons also demonstrated TH immunoreactivity (Fig. 8A4). GLUT1 immunoreactivity was observed in the RVLM and almost overlapped with CD34 immunoreactivity (examined in three rats) (Fig. 9A3). These results suggest that GLUT1 is present on vascular endothelial cells. Immunoreactivity for GLUT2 (examined in three rats) or GLUT4 (examined in three rats) was not observed in the RVLM neurons.

Five RVLM neurons that were depolarized during insulin superfusion were examined for IRs, and all five insulin-depolarized neurons that were recorded

### Table 2. Hyperpolarization in bulbospinal RVLM neurons during GLP-1 superfusion

| Concentration (pmol/L) | Depolarized/examined | MP (mV) | FAP (Hz) |
|------------------------|----------------------|---------|----------|
| 100 (n = 6)            | 6                    | Before during | −40.6 ± 1.4, 0.5 ± 0.3 |
|                        |                      | After     | −43.1 ± 1.4 * , 0.2 ± 0.2 |
| 200 (n = 9)            | 8 (depolarization, 1) | Before during | −45.3 ± 0.9, 0.5 ± 0.2 |
|                        |                      | After     | −47.7 ± 1.1 * , 0.2 ± 0.1 |
| 500 (n = 7)            | 6 (no change, 1)     | Before during | −41.3 ± 1.4, 0.3 ± 0.1 |
|                        |                      | After     | −43.5 ± 1.3 * , 0.1 ± 0.1 * |

Total 20/22

MP: membrane potential; FAP: frequency of action potential.

Values are the mean ± SEM; *P < 0.05; **P < 0.01 vs. before GLP-1 superfusion using Paired t-test.

For MP and FAP, data were obtained from all examined neurons.

### Table 3. Hyperpolarization in bulbospinal RVLM neurons during GIP superfusion

| Concentration (pmol/L) | Depolarized/examined | MP (mV) | FAP (Hz) |
|------------------------|----------------------|---------|----------|
| 100 (n = 6)            | 5 (no change, 1)     | Before during | −43.7 ± 1.7, 0.3 ± 0.1 |
|                        |                      | After     | −46.4 ± 1.9 * , 0.1 ± 0.1 |
| 200 (n = 7)            | 5 (no change, 2)     | Before during | −43.2 ± 1.4, 0.5 ± 0.1 |
|                        |                      | After     | −45.4 ± 1.2 * , 0.3 ± 0.1 |
| 500 (n = 8)            | 8                    | Before during | −42.7 ± 2.1, 0.4 ± 0.1 |
|                        |                      | After     | −45.6 ± 2.1 * , 0.1 ± 0.1 * |

Total 18/21

MP: membrane potential; FAP: frequency of action potential.

Values are the mean ± SEM; *P < 0.05; **P < 0.01 vs. before GIP superfusion using Paired t-test.

For MP and FAP, data were obtained from all the examined neurons.

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demonstrated IRs’ immunoreactivity (Fig. 8B1). Three of the four IR-immunoreactive neurons also demonstrated immunoreactivity for TH (Fig. 8C4). Four RVLM neurons that were hyperpolarized during GIP superfusion were examined for GIPR immunoreactivity, and all four neurons demonstrated immunoreactivity for GIPRs (Fig. 8D1). Two of these four neurons also demonstrated immunoreactivity for TH (Fig. 8D4).

To determine the presence of insulin, GLP-1, or GIP histologically, the immunoreactivity for insulin, GLP-1 was examined, or GIP. In the RVLM area, most GLP-1R-immunoreactive neurons also demonstrated GLP-1R immunoreactivity (examined in three rats) (Fig. 9C3). However, immunoreactivity for insulin (examined in three rats) or GIP (examined in three rats) was not observed in the neurons.

**DISCUSSION**

In this study, we examined whether glucose, insulin, or incretins influenced the activities of bulbospinal RVLM neurons. The results showed that low-level glucose and insulin depolarized bulbospinal RVLM neurons, while incretins hyperpolarized the neurons (Figs. 2–5, Tables 1–3).

**Effects of glucose on bulbospinal RVLM neurons**

Since Verbeek et al. (2016) showed that the level of glucose in CSF fluctuates with the level of glucose in the blood, we examined the effects of low-level and high-level glucose on RVLM neurons. In this study, the level of glucose in aCSF was changed from 4.4 (within the physiological range for the glucose level in CSF) to 1.1, 11, or 30 mmol/L. As a result, the activities of the RVLM neurons were increased by lowering the glucose level and suppressed by raising it (Fig. 2A–L). Furthermore, since the RVLM neurons showed similar responses to the changes in the glucose level when in the presence of TTX (Fig. 2D, H, L), glucose was thought to affect the bulbospinal RVLM neurons themselves directly. To examine whether these increased or decreased activities of the neurons in the RVLM were transmitted to the IML neurons, micro-superfusion with each level of glucose was performed over the RVLM area during recordings of MPs of an IML neuron at the Th2 level. IML neurons, which receive direct signals sent from bulbospinal RVLM neurons, regulate the functions of peripheral sympathetic nerves and BP (Oshima et al., 2015). Consequently, stimulated IML neurons increase peripheral sympathetic nerve activity (SNA). These results suggest that the glucose level affects the bulbospinal RVLM neurons directly and may change BP through the peripheral SNA. In vivo study, elucidation whether or not bulbospinal RVLM neurons themselves sense the level of glucose is expected.

In this study, the IML neurons were activated about 75 s after micro-superfusion with 1.1 mmol/L-glucose and were suppressed about 75 s after micro-superfusion with 11 or 30 mmol/L-glucose over the RVLM area (Fig. 6B–D). The latency of 75 s was much longer than the latency seen in cases in which uremic toxins were micro-superfused over the RVLM area (2–3 s; Oshima et al., 2016).
et al., 2015). Since the signal transmission time from RVLM to IML (Th2 level) is approximately 45 ms (Fig. 1B), the “75 s” is considered to represent the period that was spent from the time when the glucose level was sensed through its own transporter on RVLM neurons to the time when the change in their MPs occurred. The detailed mechanism by which glucose affects the MPs of RVLM neurons is unknown. However, Chen et al. (2012) showed that lowering the glucose level enhances the activity of P/Q-type Ca^{2+} channels and elevates the intracellular Ca^{2+} level in hypothalamic arcuate nucleus neurons. A similar mechanism might exist in RVLM neurons. Further studies are needed.

In most mammalian cells, the transportation of glucose is mediated by a family of facilitative glucose transporters (GLUT1–GLUT4) (Mueckler, 1994). In this study, GLUT1 was observed in endothelial cells (Fig. 9A3) and GLUT3 was observed in the recorded neurons (Fig. 9A4) within the RVLM area. Since all the examined RVLM neurons that were depolarized during superfusion with low-level glucose showed immunoreactivity for GLUT3 (Fig. 9A4), low-level glucose was suggested to activate RVLM neurons through GLUT3 on those neurons. GLUT4 is known to appear on the cell surface of adipocytes and skeletal muscle cells in response to insulin (Kanazaki, 2006). Although we examined the IRs in the bulbospinal RVLM neurons, the presence of GLUT4 was not confirmed (Fig. 9B2).

Comparison between the results in the previous study and those in this study

In contrast to the previous study in vivo (Verberne et al., 2010), our results showed that almost all bulbospinal RVLM neurons (in 14 of 16 neurons) were activated by a low level of glucose. We don’t know the reasons clearly. The RVLM neurons in neonatal rats might be more sensitive to a low level of glucose than those in adult rats, or some of hypoglycemia-sensitive neurons in the RVLM might receive suppressive signals from other hypoglycemia-sensitive neurons in vivo (for example, neurons in the hypothalamus). In this study, the brainstem–spinal cord preparations were used and inputs from neurons in the hypothalamus to RVLM were blocked.

Furthermore, compared to the study by Verberne et al. (2010), the magnitude of the changes in the MPs and the FAP of the RVLM neurons induced by a low level of glucose were very small in this study. RVLM neurons receive many inputs from other neurons, such as neurons in the paraventricular nucleus or neurons in the supraoptic nucleus (Guyenet et al., 2013). In this study, the inputs from neurons in those nuclei to RVLM were blocked. Moreover, the temperature of aCSF in this study was low (25–26 °C). These factors may lead to the decreased magnitude of the changes in the MPs and the FAP of the RVLM neurons induced by glucose reduction.

However, considering these differences between the results in the previous study (Verberne et al., 2010) and those in this study, the functions of RVLM neurons in...
Effects of insulin on bulbospinal RVLM neurons

In this study, insulin depolarized the bulbospinal RVLM neurons themselves (Fig. 3A, J, Table 1). S961 hyperpolarized the RVLM neurons, and this hyperpolarizing effect was suppressed in the presence of an insulin solution (Fig. 3D, G). All the insulin-depolarized RVLM neurons showed immunoreactivity for IRs (Fig. 8B4). These results show that insulin activates bulbospinal RVLM neurons via IRs on these neurons. Furthermore, since the IML neurons were activated after micro-superfusion with insulin over the RVLM area (Fig. 7A), the administration of insulin to RVLM neurons was thought to increase the peripheral SNA and raise the BP.

Insulin is reported to cross the blood–brain barrier (BBB) (Banks, 2004), and the serum and CSF insulin concentrations are correlated (Sartorius et al., 2015). Consequently, a high level of insulin in the CSF, which is seen during hyperinsulinemia and insulin resistance, may stimulate RVLM neurons via IRs expressed on them, thereby causing a high BP. Lakhi et al. (2013) show that insulin depolarized subfornical organ neurons by activating Ih channels (known as hyperpolarization-activated cation channels). A similar mechanism might exist for RVLM neurons.

Effects of GLP-1 on bulbospinal RVLM neurons

Circulating GLP-1 can pass across the BBB, and GLP-1 regulates food intake, thermoregulation, BP, and glucose homeostasis via GLP-1Rs in the neurons of the hippocampus, hypothalamus, and NTS (Trapp and Cork, 2015). However, the effect of GLP-1 on RVLM neurons is not known.

In this study, GLP-1 hyperpolarized the bulbospinal RVLM neurons themselves (Fig. 4A, J, Table 2), and IML neurons were suppressed after micro-superfusion with GLP-1 over the RVLM area (Fig. 7B). These results suggest that the administration of GLP-1 to RVLM neurons may decrease the peripheral SNA and reduce BP. In the present study, all the GLP-1-hyperpolarized RVLM neurons showed immunoreactivity for GLP-1Rs (Fig. 8C4), and the presence of GLP-1 itself was found in RVLM neurons (Fig. 9C). These results suggest that GLP-1, which may be produced in RVLM neurons, suppresses bulbospinal RVLM neurons through GRP-1Rs on these neurons. The mechanism by which GLP-1 hyperpolarized the RVLM neurons is not known. A previous study showed that GLP-1 contributes to neuroprotection against oxidative stress using cultured cortical neurons in rats (Nakajima et al., 2016). Since some studies show that oxidative stress increases neuronal activities (Nani et al., 2010; Oshima et al., 2015), GLP-1 might decrease the activities of RVLM neurons by suppressing oxidative stress. Further studies are needed.

Effects of GIP on bulbospinal RVLM neurons

GIP hyperpolarized the bulbospinal RVLM neurons themselves (Fig. 5A, J, Table 3) and the hyperpolarized effect was transmitted from RVLM neurons to IML neurons (Fig. 7C). Incretin-based drugs, which increase GLP-1 and GIP, could potentially prevent cardiovascular events by suppressing SNA.
In brain regions, the expression of GIP has been reported in the olfactory bulb, hippocampus, cerebral cortex, hypothalamus, and brainstem (Nyberg et al., 2007). GIPRs are expressed in the olfactory bulb, hippocampus and cerebral cortex (Nyberg et al., 2007). The roles of GIP in the brainstem are not clear, but a previous report showed that a GIP analog, which is beneficial for treating neurodegenerative conditions such as Alzheimer’s disease, reduces oxidative stress in the brain (Duffy and Hölscher, 2013). GIP may also reduce oxidative stress in RVLM neurons, leading to a reduction in the activities of RVLM neurons.

CONCLUSION
Low-level glucose increased the activities of bulbospinal RVLM neurons and high-level glucose decreased them. Insulin increased the activities of bulbospinal RVLM neurons, and incretins (GRP-1 and GIP) decreased them. The presence of a transporter of GLUT3 and receptors of insulin, GRP-1, and GIP in the bulbospinal RVLM neurons was demonstrated histologically. Based on these results, hypoglycemia may induce high BP by stimulating bulbospinal RVLM neurons via specific transporters on these neurons. Furthermore, hyperinsulinemia and a reduction in GLP-1 or GIP may also cause a high BP by increasing the activity of RVLM neurons via their own receptors. Incretins may reduce cardiovascular events via the suppression of SNA.

CONFLICT OF INTEREST
None

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