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Endogenous GLP-1 acts on paraventricular nucleus to suppress feeding: Projection from nucleus tractus solitarius and activation of corticotropin-releasing hormone, nesfatin-1 and oxytocin neurons

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) receptor agonists have been used to treat type 2 diabetic patients and shown to reduce food intake and body weight. The anorexigenic effects of GLP-1 and GLP-1 receptor agonists are thought to be mediated primarily via the hypothalamic paraventricular nucleus (PVN). GLP-1, an intestinal hormone, is also localized in the nucleus tractus solitarius (NTS) of the brain stem. However, the role of endogenous GLP-1, particularly that in the NTS neurons, in feeding regulation remains to be established. The present study examined whether the NTS GLP-1 neurons project to PVN and whether the endogenous GLP-1 acts on PVN to restrict feeding. Intra-PVN injection of GLP-1 receptor antagonist exendin (9–39) increased food intake. Injection of retrograde tracer into PVN combined with immunohistochemistry for GLP-1 in NTS revealed direct projection of NTS GLP-1 neurons to PVN. Moreover, GLP-1 evoked Ca^{2+} signaling in single neurons isolated from PVN. The majority of GLP-1-responsive neurons were immunoreactive predominantly to corticotropin-releasing hormone (CRH) and nesfatin-1, and less frequently to oxytocin. These results indicate that endogenous GLP-1 targets PVN to restrict feeding behavior, in which the projection from NTS GLP-1 neurons and activation of CRH and nesfatin-1 neurons might be implicated. This study reveals a neuronal basis for the anorexigenic effect of endogenous GLP-1 in the brain.

1. Introduction

Glucagon-like peptide-1 (GLP-1), an incretin hormone, is released from the enteroendocrine L-cells in response to meals and enhances glucose-induced insulin secretion from pancreatic islets [1]. GLP-1 is rapidly cleaved by the dipeptidyl peptidase-4 (DPP-4). The DPP-4 inhibitors and DPP-4-resistant GLP-1 receptor agonists have recently been used to treat type 2 diabetes. These medicines have been shown not to increase body weight or even ameliorate obesity [2–4], an action related to suppression of hyperphagia [5–7], providing an advantage over most of other anti-diabetic medicines.

It has been shown that administration of GLP-1 reduces food intake and body weight, and that these effects are mediated largely by its action on the central nervous system [8,9]. Moreover, intracerebroventricular (icv) injection of GLP-1 receptor antagonist exendin (9–39) increases food intake in *ad libitum* fed rats [8,10], suggesting that endogenous GLP-1 in the brain physiologically restricts feeding. GLP-1 is produced by the tissue-specific post-translational processing of proglucagon, which takes place in the nucleus tractus solitarius (NTS) of the brain stem [11], as well as in the intestine. GLP-1-expressing neurons are located in NTS [12], and GLP-1-immunoreactive (IR) nerve terminals are identified in the hypothalamic feeding centers, arcuate nucleus (ARC) and paraventricular nucleus (PVN) [11,13–15], where GLP-1 receptors are expressed [16,17]. Microinjection of GLP-1 into the PVN [18–20], but not ARC [18], suppresses feeding. These reports taken together suggest a possibility that the PVN neurons are projected by GLP-1 neurons of NTS and mediate anorexigenic effect of endogenous GLP-1 in the brain. However this hypothesis remains to be established. Furthermore, the PVN neuron species targeted by GLP-1 remains poorly understood. In the PVN, corticotropin-releasing hormone (CRH) and oxytocin are classically recognized

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anorexigenic neuropeptides [21–23], and nesfatin-1 is a newly discovered neuropeptide implicated in physiological inhibition of feeding [24,25] and in circadian feeding rhythm [26,27].

The present study aimed to clarify whether the endogenous GLP-1 in the PVN regulates food intake, whether the NTS GLP-1 neurons project to the PVN, and whether GLP-1 directly activates PVN neurons. Furthermore, we intended to identify the PVN neuron species targeted by GLP-1. We examined the effects of intra-PVN injection of GLP-1 receptor antagonist on food intake and the projection of the NTS GLP-1 neurons to the PVN by using retrograde tracer and immunohistochemistry. Moreover, we investigated the effect of GLP-1 on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the neurons isolated from PVN, followed by immunostaining for CRH, oxytocin and nesfatin-1.

2. Materials and methods

2.1. Animals

Male Wistar rats aged 6 weeks (Nihon SLC, Hamamatsu, Japan) were maintained on a 12 h light/dark cycle (19:30 light off) and given conventional food (CE-2; Clea, Osaka, Japan) and water *ad libitum*. Experimental procedures and care of animals were carried out according to Jichi Medical University Institute of Animal Care and Use Committee.

2.2. Microinjection into the PVN

A stainless steel guide cannula (26-gauge) was stereotaxically inserted into the PVN (1.8 mm caudal to the bregma, 0.3 mm lateral from midline and 7.8 mm below the surface of the skull). The injector needle extended 0.6 mm below the guide cannula. Rats were allowed to recover from the operation for 10 days while they were habituated to handling.

2.3. Effects of GLP-1 receptor blockade in the PVN on food intake

GLP-1 receptor antagonist, exendin (9–39), was injected into unilateral PVN of *ad libitum* fed Wistar rats at 10:30 and cumulative food intake at 1–6 h after injection was measured. Exendin (9–39) (Abgent, San Diego, CA) 10 μg/0.5 μl dissolved in vehicle (saline; NaCl 0.9%) or NaCl 0.9%/0.5 μl (vehicle) was injected.

2.4. Injection of retrograde tracer and immunohistochemistry for GLP-1 neurons

A retrograde tracer, cholera toxin subunit B Alexa Fluor 488 (CTB: Invitrogen, CA), was used to identify the pathway connecting PVN and NTS GLP-1 neurons. CTB, 0.5 μl of 0.5 mg/ml, was injected stereotaxically into unilateral PVN. Four days after injection of CTB, animals were perfused with 4% PFA containing 0.2% picric acid. Brain coronal sections of 40 μm thickness were incubated with mouse anti-glucagon antibody (Sigma–Aldrich, Inc., St. Louis, MO; 1:2000) for overnight at 4 °C, followed by incubation for 40 min with Alexa 594 goat anti-mouse IgG (Molecular Probes, Carlsbad, CA; 1:500) for 30 min. Slices were mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA).

Fluorescence images of proglucagon and CTB labels were acquired with microscope (BX51; Olympus, Tokyo Japan).

2.5. Preparation of single neurons from PVN

Single neurons were prepared from PVN according to procedures reported previously [25,28]. Briefly, the entire PVN from left and right sides was excised and incubated with 20 U/ml papain (Sigma Chemical Co., St. Louis, MO), 0.015 mg/ml deoxyribonuclease, 0.75 mg/ml BSA, and 1 mM cysteine for 15 min at 36 °C, followed by gentle mechanical trituration for 5–10 min. The dispersed cells were plated on coverslips and kept in the humidified chamber for up to 6 h until measurement of $[Ca^{2+}]_i$.

2.6. Measurement of $[Ca^{2+}]_i$ in single PVN neurons and criteria of responses

$[Ca^{2+}]_i$ was measured by ratiometric fura-2 fluorescence imaging, as previously reported [25,28,29]. Briefly, single neurons were superfused at 36 °C with 10 mM HEPES-buffered Krebs–Ringer bicarbonate buffer (HKRB) composed of (in mmol/l) 129 NaCl, 5.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgSO_4$, and 10 HEPES at pH 7.4. Fura-2 ratio (F340/F380) images were produced by Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). Data were obtained from single cells fulfilling the criteria of neurons reported previously [25,28].

When $[Ca^{2+}]_i$ changed within 5 min after administration of agents with amplitudes larger than 0.25 ratio unit, they were considered the responses. The viability of cells was checked by responses to 30 mM KCl at the end of measurements.

2.7. Post-$[Ca^{2+}]_i$ immunocytochemistry for CRH, oxytocin or nesfatin-1

Post-$[Ca^{2+}]_i$ immunostaining was performed as previously reported [12,13]. In brief, after $[Ca^{2+}]_i$ measurements, the cells were incubated with primary rabbit antiserum against CRH (Peninsula Laboratories Inc., San Carlos, CA) diluted 1:2000, against oxytocin (Chemicon International Inc., Temecula, CA; 1:5000) or against nesfatin-1 (Chemicon; 1:2000). Then, they were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:500, and color was developed with 3,3′-diaminobenzidine. Immunocytochemical results were correlated with $[Ca^{2+}]_i$ data in each cell with the aid of pictures taken at the end of $[Ca^{2+}]_i$ measurements [30,31].

2.8. Data presentation and statistical analysis

The data were presented as the mean ± SEM. Two-way ANOVA followed by Tukey's test were used to compare multiple test groups. Incidence of responses or fraction of the neurons was analyzed by χ^2 test. Values of $p < 0.05$ were considered significant.

3. Results

In order to assess a possible effect of endogenous GLP-1 in the PVN, a GLP-1 receptor antagonist exendin (9–39) (Abgent, San Diego, CA) was injected unilaterally into the PVN. The intra-PVN injection of 10 μg exendin (9–39) significantly increased cumulative food intake at 2 h (Fig. 1), suggesting that endogenous GLP-1 acts on PVN to decrease food intake.

In the brain, GLP-1 is known to be produced in the neurons of the NTS [11,12]. Hence, we examined whether the NTS GLP-1 neurons project to the PVN. We injected the retrograde fluorescence tracer CTB into unilateral PVN (Fig. 2A) and examined the appearance of the fluorescence in NTS neurons and its localization in GLP-1 neurons by immunohistochemical staining. We used an antibody recognizing proglucagon that is the precursor of GLP-1 and present in GLP-1 neurons in the NTS. The CTB fluorescence was detected in the NTS at 3 days after injection (Fig. 2B). The CTB-labeled neurons and proglucagon-immunoreactive (IR) neurons highly coincided in the NTS, showing that GLP-1 neurons in the NTS substantially project to the PVN (Fig. 2C–H).

The direct effect of GLP-1 (7–36) amide (Peptide Institute Inc., Osaka, Japan) on single PVN neurons was determined by $[Ca^{2+}]_i$

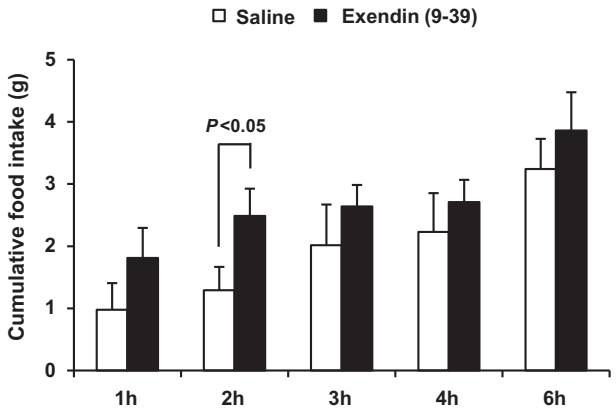

Fig. 1. Cumulative food intake for 1–6 h after focal injection of vehicle or exendin (9–39) into unilateral PVN. $n = 7$ for vehicle and 8 for exendin (9–39). Bars represent mean ± SEM. $p < 0.05$ indicates the significant difference by two-way ANOVA followed by Tukey's test.

imaging. GLP-1 concentration-dependently increased $[Ca^{2+}]_i$ in PVN neurons; at 10^{-16} M in none of 36 neurons (0%), at 10^{-14} M in 3 of 99 neurons (3%), at 10^{-12} M in 10 of 110 neurons (9%), at 10^{-10} M in 8 of 95 neurons (8%) and at 10^{-8} M in 24 of 247 neurons (10%) (Fig. 3A). We next examined whether GLP-1 increases $[Ca^{2+}]_i$ in the specific subpopulation of PVN neurons expressing anorexigenic neuropeptides CRH, oxytocin or nesfatin-1. For this, $[Ca^{2+}]_i$ measurements were followed by immunocytochemical staining with specific antiserum against CRH, oxytocin and nesfatin-1. As shown in Fig. 3B, administration of GLP-1 at 10^{-14} and 10^{-8} M increased $[Ca^{2+}]_i$ in a PVN neuron (left panel) that was subsequently proved to be IR to CRH (right panel). Administration of GLP-1 at 10^{-10} and 10^{-8} M induced small and sound increases in $[Ca^{2+}]_i$, respectively, in a PVN neuron IR to oxytocin (Fig. 3C). Administration of GLP-1 at 10^{-12} M increased $[Ca^{2+}]_i$ in a PVN neuron IR to nesfatin-1 (Fig. 3D).

The fraction of GLP-1 responsive neurons among the specific neuropeptide-IR neurons is shown in Fig. 4A. One of 7 CRH-IR neurons (14%) responded to 10^{-14} M GLP-1, 1 of 6 (17%) to 10^{-12} M GLP-1, and 4 of 16 (25%) responded to 10^{-8} M GLP-1. One of 26 (4%) and 1 of 13 (8%) oxytocin-IR neurons responded to 10^{-10} and 10^{-8} M GLP-1, respectively, while none of 18 responded to 10^{-12} M GLP-1. Two of 10 (20%) and 5 of 44 (11%) nesfatin-1-IR neurons responded to 10^{-12} and 10^{-8} M GLP-1, respectively, while none of 21 responded to 10^{-14} M GLP-1. The fraction of the specific neuropeptide-IR neurons among the neurons that responded to GLP-1 is shown in Fig. 4B. Four of 8 GLP-1 responsive neurons (50%) were IR to CRH, 2 of 16 (13%) to oxytocin, and 7 of 16 (44%) to nesfatin-1. These results show that CRH and nesfatin-1 neurons are the major, while oxytocin neurons are the minor, targets of GLP-1 in the PVN.

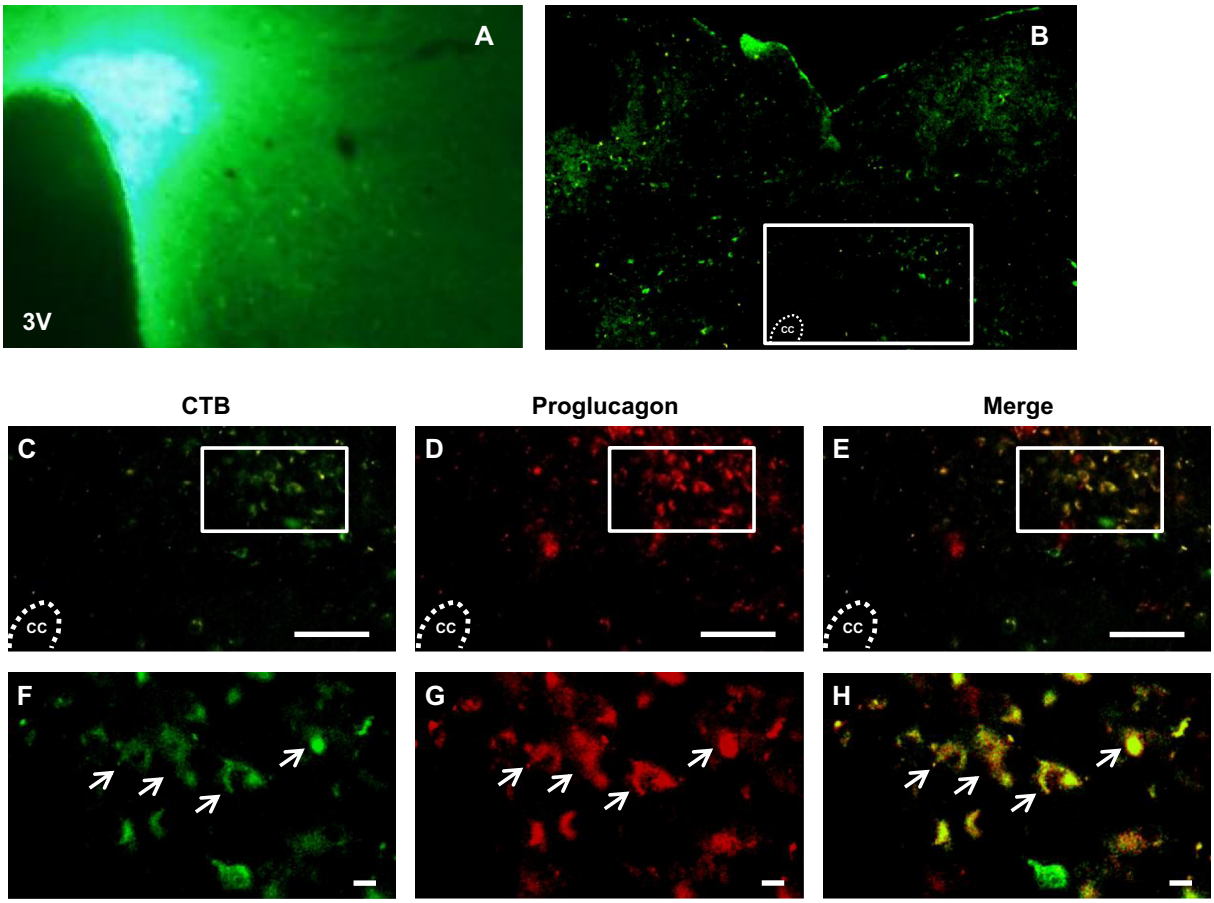

Fig. 2. NTS GLP-1 neurons project directly to PVN. (A) A retrograde tracer, cholera toxin subunit B Alexa Fluor 488 (CTB), was injected into unilateral PVN. (B) CTB-labeled neurons was identified in the NTS. (C–H) Fluorescence (C–E) and confocal (F–H) images of the NTS stained for CTB with Alexa 488 (green) (C and F), for proglucagon with Alexa 594 (red) (D and G), and for both by merged images (E and H). Arrows indicate the neurons labeled for CTB and immunoreactive to proglucagon. 3V, third ventricle; CC, central canal. Scale bars represent 100 μm in (C–E) and 10 μm in (F–H).

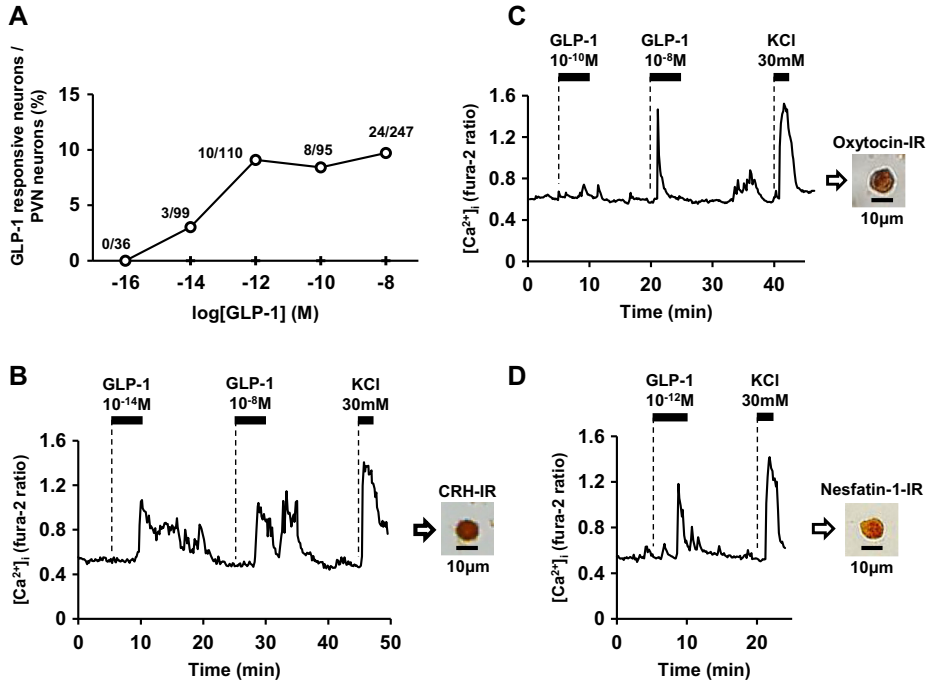

Fig. 3. GLP-1 concentration-dependently increases $[Ca^{2+}]_i$ in CRH, oxytocin and nesfatin-1 neurons of PVN. (A) Incidence of $[Ca^{2+}]_i$ response is shown by the number of neurons that responded to GLP-1 with $[Ca^{2+}]_i$ increases over that examined, as expressed by percentage. The numbers above the circles indicate the number of PVN neurons that responded over that examined. (B) GLP-1 at 10^{-14} and 10^{-8} M increased $[Ca^{2+}]_i$ in a single neuron isolated from PVN (left panel), and this neuron was subsequently shown to be immunoreactive (IR) to CRH (right panel). This neuron also responded to 30 mM KCl with an increase in $[Ca^{2+}]_i$. (C) GLP-1 at 10^{-8}, but not 10^{-10} M, increased $[Ca^{2+}]_i$ in a single PVN neuron that was subsequently shown to be IR to oxytocin. (D) GLP-1 at 10^{-12} M increased $[Ca^{2+}]_i$ in a single PVN neuron that was subsequently shown to be IR to nesfatin-1. Superfusate contained 1 mM glucose and the bars above the tracings indicate the periods of administration of agents in (B–D).

4. Discussion

We have shown that intra-PVN injection of GLP-1 receptor antagonist exendin (9–39) increases food intake under fed states. Furthermore, NTS GLP-1 neurons directly project to the PVN. GLP-1 activates PVN CRH and nesfatin-1 neurons as its major targets and also oxytocin neurons. This study indicates that endogenous GLP-1 acts on PVN to restrict feeding behavior, in which the projection of NTS GLP-1 neurons to PVN and activation of CRH and nesfatin-1 neurons could be implicated.

In the present study, intra-PVN injection of GLP-1 receptor antagonist exendin (9–39) increased food intake by two fold or greater in rats fed *ad libitum* at the light phase. This result is in agreement with previous report that intracerebroventricular (icv) injection of exendin (9–39) doubled food intake in *ad libitum* fed, but not 24-h fasted, rats [8,10]. These results by us and others support that endogenous GLP-1 in the brain mainly acts on the PVN and physiologically restricts feeding.

It was not entirely clear whether NTS GLP-1 neurons project directly to the PVN. In the present study, we injected retrograde tracer CTB into PVN and immunostained proglucagon/GLP-1 in NTS, and has revealed a direct projection of NTS GLP-1 neurons to PVN. This result is in accordance with previous studies reporting GLP-1-IR nerve terminals or fibers and GLP-1 receptor mRNA expression in the PVN [11,13–17], and confirms the previous immunohistochemical study with CTB and GLP-2 immunostaining [32].

We have found that the CRH and oxytocin neurons were, respectively, the major and minor populations of the neurons that responded to GLP-1 with $[Ca^{2+}]_i$ increases. This result is in agreement with previous immunohistochemical and pharmacologic studies. Icv injection of GLP-1 induces c-Fos expression in 80% of CRH-IR and 38% of oxytocin-IR neurons in the PVN [33]. Abundant GLP-1-IR terminal contacted CRH-IR neurons while fewer contacted oxytocin-IR neurons in the PVN [14,15]. GLP-1-induced suppression of feeding in rats is blocked by pretreatment with a CRH receptor antagonist α-helical CRH [34] but not with an oxytocin receptor antagonist [35]. These results by us and others indicate that CRH neuron is the primary target of GLP-1 in PVN. In addition, we found that the PVN neurons IR to nesfatin-1, a newly discovered satiety peptide [24], were also activated by GLP-1. Close to half of GLP-1-responsive neurons in PVN were nesfatin-1–IR neurons. The current study is the first to demonstrate that GLP-1 directly activates the nesfatin-1 neuron as its substantial target in the PVN. Nesfatin-1 located in the PVN was shown to play a role in suppressing light phase feeding [25,26] and producing circadian feeding rhythm [27]. Oxytocin in the PVN is also implicated in inhibition of feeding [25]. Hence, it is suggested that CRH, nesfatin-1 and oxytocin neurons serve as the targets and mediators of the anorexigenic effect of GLP-1.

Summation of the frequencies of CRH-IR, nesfatin-1-IR and oxytocin-IR neurons among GLP-1-responsive neurons apparently exceeded 100%. This result could be due to that nesfatin-1 neurons overlap with CRH and oxytocin neurons by 13% and 24%, respectively, in the PVN [25,36].

The activation of PVN CRH and nesfatin-1 neurons by GLP-1 could also be related to stress responses. Icv injection of GLP-1 reportedly stimulates ACTH and corticosterone release [34,37], and the stress-induced secretion of these hormones are blocked by a GLP-1 receptor antagonist [37]. Nesfatin-1 was shown to directly interact with the PVN CRH neurons and activate hypothalamo–pituitary–adrenocortical (HPA) axis [26,38]. The activation of CRH and nesfatin-1 neurons by GLP-1, demonstrated in this study, could be relayed to the release of ACTH and corticosterone, thus evoking the HPA axis. It is also known that GLP-1 induces transient increases in heart rate and blood pressure possibly through the

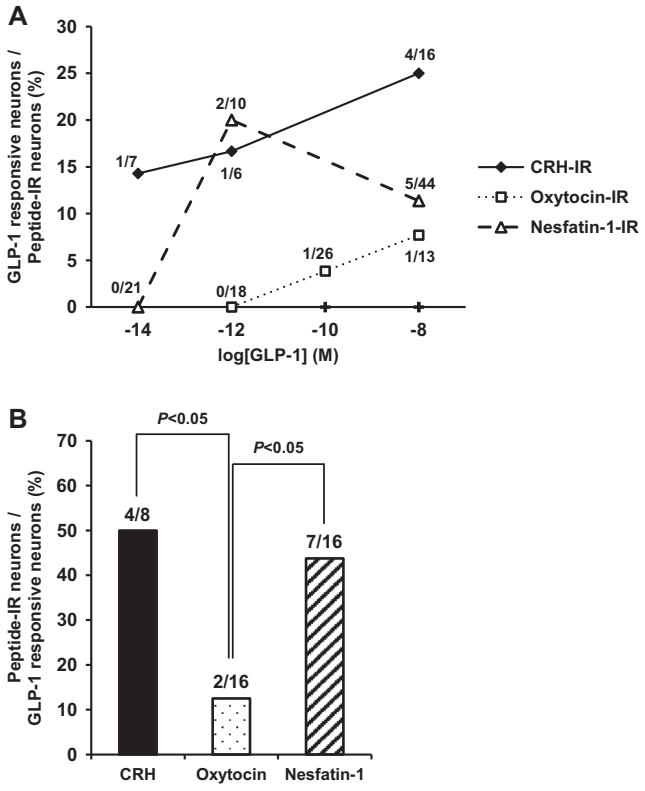

Fig. 4. Relationship between GLP-1-responder and neuropeptide identity in PVN neurons. (A) Incidences of $[Ca^{2+}]_i$ responses to 10^{-14}–10^{-8} M GLP-1 in CRH, oxytocin and nesfatin-1 neurons, expressed by percentage. The numbers beside each point indicate the number of neurons that responded over that examined. (B) Fraction of the neurons that express each peptide over those responded to GLP-1 in the PVN. The numbers above the bars indicate the number of neurons IR to each peptide over that responded to 10^{-14}–10^{-8} M GLP-1. $p < 0.05$ indicate significant differences by χ^2 test.

sympathetic and parasympathetic nervous systems [39]. Icv injection of nesfatin-1 stimulates sympathetic nerve activity and increases mean arterial pressure in conscious rats [40,41]. Hence, the GLP-1-induced activation of nesfatin-1 neurons in the PVN could be involved in the effect of GLP-1 on the cardiovascular functions.

In conclusion, we have demonstrated that the endogenous GLP-1 acts on PVN neurons to restrict food intake in fed states, that the NTS GLP-1 neurons project to PVN, and that GLP-1 directly targets CRH, nesfatin-1 and oxytocin neurons in the PVN. In the past several years of clinical experience, GLP-1 receptor agonists and DPP-4 inhibitors have been shown to exhibit pleiotropic effects beyond glycemic control, which include the effects on feeding, body weight and cardiovascular system. This study suggest that the central GLP-1 activation of CRH, nesfatin-1 and oxytocin neurons in the PVN underlies the anorexigenic and other pleiotropic effects of endogenous GLP-1 and GLP-1-based medicines.

Acknowledgments

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