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Orexin-A signaling in the paraventricular nucleus promote gastric acid secretion and gastric motility through the activation neuropeptide Y Y_1 receptors and modulated by the hypothalamic lateral area

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ABSTRACT

Objective: Abnormal gastric acid secretion and gastric dyskinesia are common gastroenterological ailments. Our study aims to investigate the effect of orexin-A in the paraventricular nucleus (PVN) gastric motility and gastric acid secretion.

Methods: The source of orexin-A neuronal projections to the PVN were explored by retrograde tracing and fluorescence immunohistochemistry experiments. Neuronal discharge recordings of single cells were taken within the PVN. Gastric motility was recorded using a force transducer implanted into the stomach, and gastric acid secretion measured through a pyloric catheter.

Results: Orexin-A-positive neuronal projections from LHA to PVN were found. Administration of orexin-A to PVN activated the firing of 63.2% NPY-excited/GD-excitatory (GD-E) neurons but suppressed the firing of 55.9% NPY-inhibited/GD-inhibitory (GD-I) neurons, promoted gastric motility and gastric acid secretion in a dose-dependent manner. Responses produced by orexin-A could be partially blocked by Y_1 receptor antagonist GR-231118; Electrical stimulation to the the hypothalamic lateral area (LHA) altered NPY-sensitive/GD neuronal activity in the PVN, stimulated gastric motility and gastric acid secretion. Additionally, these effects induced by LHA electrical stimulation were blocked by administration of the OX1R antagonist SB-334867 to the PVN.

Conclusion: Orexin-A from LHA neurons act on the PVN to enhance gastric motility and gastric acid secretion, with Y_1 receptor signaling playing a critical role.

1. Introduction

The peptide hormones orexin-A and orexin-B regulate many physiological processes including sleep/wakefulness states, energy homeostasis, reward, and autonomic functions (Kodadek and Cai, 2010; Date et al., 1999). Orexin-A and orexin-B are produced from the cleavage of the 130-amino acid peptide pre-orexin in neurons that are primarily located in the LHA and perifornical area (PeF) (Sakurai et al., 1998; Matsuki and Sakurai, 2008). Orexinergic neurons send projections throughout the brain, and notably to the PVN and dorsomedial nucleus of hypothalamic (DMH) (Trivedi et al., 1998; Girault et al., 2012). The OX1R antagonist SB-334867 inhibits orexin-A-mediated appetite stimulation in rats (Dyan and Devanjan, 2013; Kay et al., 2014; Badonnel et al., 2014). In VMH and PVN, orexin-A helps the regulation of the gastric acid secretion and gastric motility (Chaleek et al., 2012; Eliassi et al., 2009). And others in our group indicated that orexin-A changes the GD neurons firing and enhances gastric motility in the LHA, and the possible regulation by the PVN (Hao et al., 2016).

The peptide transmitter neuropeptide Y (NPY), like orexin, promotes feeding and gastric acid secretion, playing an important role in energy balance (Shimizu et al., 2013; Geoghegan and Pappas, 1997; Loh et al., 2015). NPY neurons are distributed primarily in the arcuate nuclei (ARC) and DMH. There, NPY neurons project to the PVN, VMH and LHA (Oh-I et al., 2006). In hypothalamus, Y_1 receptor immunopositive cell were mainly distributed the ARC, PVN and ventromedial nucleus (VMH) (Migita et al., 2001), and expressions of Y_1 receptor were higher than Y_5 receptor in the PVN (Coppola et al., 2004). However, the previous study showed that the supraoptic nucleus (SON) and ARC expressed the higher number of Y_5-immunoreactive cells with fewer immunopositive cells present in the PVN and VMH (Morin and Gehlert, 2006). Previous findings have confirmed that NPY is an important regulator of gastric acid secretion (Matsuda et al., 1991; Geoghegan et al., 1993; Lee et al., 1994).

The PVN is critical for regulating satiety and energy balance

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(Horvath et al., 2001). The PVN receives afferent inputs from many brain regions, including suprachiasmatic nucleus, ARC, subfornical organ and LHA (Morton et al., 2006; Chaleek et al., 2012). In the PVN, information from both orexigenic and anorexigenic signaling systems converge (Broberger et al., 1998; Haskell-Luevano et al., 1999). Further, that PVN is also for the regulation of gastric acid secretion (Shiraishi, 1998), with Y_1, Y_5 receptors, and orexin-A as key factors (Kermani and Eliassi, 2012).

The effects of orexin-A within the LHA-PVN neural pathway on digestion and energy balance have not been fully elucidated. In this study, we explored the effects of orexin-A within a LHA-PVN neural pathway on gastric distention-induced neuronal excitability, gastric acid secretion, and gastric motility. Further, we probed the role for NPY receptor signaling in the regulation of these processes.

2. Materials and methods

2.1. Animals

Male SD rats, weighing 220–280 g, were purchased from Qingdao Institute for Drug Control (Shandong, China), housed in a controlled environment (25 ± 2 °C and 55% relative humidity with a 12 h light/dark cycle, light start at 8:00 am). All rat experimental procedures were approved by the principles of institutional animal care and use in Qingdao University. Animal experiments in vivo were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (United States).

2.2. Retrograde tracing and immunohistochemistry

2.2.1. OrexinA-immunopositive neuronal projections from LHA to PVN

After the rats ($n = 5$) were anesthetized with thiobutabarbitol (0.1 g/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA), 0.2 μL 3% (w/v) fluorescent gold (FG, Sigma-Aldrich, St. Louis, MO, USA) was pressure injected into the PVN (bregma: P: 1.9 mm, L(R) 0.5 mm, H: 7.3 mm) according to Rat Brain Atlas (Paxinos and Charles, 2013). After seven days, rats were anesthetized, perfused with 0.9% saline (200 mL), fixed with 4% paraformaldehyde through the intracardiac system. The brains were removed quickly and post-fixed in 4% paraformaldehyde for 2 h, and subsequently placed on a 30% sucrose solution for 24 h at 4 °C. The brains were cut into 15-μm-thick serial sections in the coronal plane with a freezing microtome (Kryostat 1720, Leica, Germany). The sections for the LHA were selected under a light microscope according to the Rat Brain Atlas (Paxinos and Charles, 2013).

The slices were incubated for 24 h at 4 °C with goat anti-orexin-A antibody (polyclonal, dilution: 1:400, Abcam, UK). Then, they were incubated with Cy3-conjugated donkey anti-goat antibody (dilution: 1:500, Phoenix Pharmaceuticals, Burlingame, CA, USA) at room temperature for 2 h.

Cell counting was done via a BX63F fluorescence microscope and image analysis system (Jeda Science and Technology Company, Nanjing, China). All 30 sections in 5 rats were analyzed for numbers of double-labeled cells in the LHA. Counts of double-labeled cells were taken from six fields randomly spaced throughout the extent of the LHA. The percentage of double-labeled cells (%) = the numbers of double-labeled cells/the numbers of orexin-A positive neurons × 100%.

2.2.2. Expression of OX1R and Y_1 receptors in the PVN

Three rats were used to observe the expressions of OX1R or Y_1 receptor immunopositive neurons. The slices were incubated for 24 h with rabbit anti-Y_1 receptor antibody (dilution: 1:250, Abcam, UK) or rabbit anti-OX1R antibody (dilution: 1:300, Abcam, UK) at 4 °C, then they were incubated with FITC-labeled goat anti-rabbit IgG (dilution: 1: 25, Jackson ImmunoResearch, West Grove, PA, USA) or Cy3-labeled goat anti-rabbit IgG (dilution: 1: 300, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 2 h.

Finally, the slices were mounted in citifluor (citifluor, London, UK). Fluorophores were visualized under a BX63F fluorescence microscope, and images were obtained with a DP80 digital camera (Olympus, Tokyo, Japan).

2.3. Electrophysiological recording

After fasting overnight, rats ($n = 26$) were anesthetized with thiobutabarbitol (0.1 g / kg, i.p.), and then fixed on the brain stereotaxic apparatus; Brain surgery and balloon implantation in the stomach were performed as previously described (Hao et al., 2016). A glass microelectrode with five-barrels (tip diameter approximately 10–15 μm, electrode impedance: 5–10 MΩ) was stereotaxically inserted into the PVN (position described as above) according to the the Rat Brain Atlas (Paxinos and Charles, 2013) for the electrophysiological recording of cell firing and drug microinjection. In the 5-barreled microelectrode, one is the recording electrode, filled with 2% pontamine sky blue and 0.5 M sodium acetate for recording the firing. The other four tubes were sequentially contained with normal saline (NS), 15 nM orexin-A (Sigma-Aldrich, St. Louis, MO, USA), 125 nM NPY (Sigma-Aldrich, St. Louis, MO, USA), 25 nM SB334867 (Sigma-Aldrich, St. Louis, MO, USA) or 150 nM GR-23118 (Sigma-Aldrich, St. Louis, MO, USA). The drugs were given on the surface of neurons by short pulse gas pressure from the pressure injector (PM2000B; Micro Data Instrument Inc., NJ, USA), and the injection volumes of drugs were < 1 nL (Gao et al., 2017). In a pre-experiment, a series of orexin-A concentrations (1.0 nM, 5.0 nM, 10.0 nM, 15.0 nM, 25 nM, 50.0 nM, 100.0 nM) were used. Since 15 nM orexin-A produced a half maximal response (pEC50), this dose was selected for experiments. Different concentrations of SB-334867 (5 nM, 10 nM, 15 nM, 20 nM, 25 nM, 35 nM and 40 nM) and GR-231118(60 nM, 90 nM, 120 nM, 150 nM, 180 nM, 210 nM and 240 nM) were tested with 25 nM SB334867 or 150 nM GR-231118 selected as it was the minimum dose that completely or partly block the effects of orexin-A on neuronal firing.

Once the glass microelectrode was positioned in the PVN, the extracellular action potential of single neurons was recorded. The firing signals were amplified through MEZ8201 amplifier (Nihon Kohden, Tokyo, Japan), viewed on an oscilloscope (VC-11; Nihon Kohden). The amplified electrical signals were passed through low (5000 Hz) - and high (1 Hz)-pass filters into a bioelectricity signal analyzer and computer. Spike parameters were pre-processed online and further analyzed offline using Micro 1401 and Spike 2 software (Cam-bridge Electronic Design Limited, Cambridge, UK) for spike data analysis. When the firing pattern became stable for at least 120 s, 3–5 mL warm saline was injected into the stomach soft balloon at a rate of 0.5 mL/s and maintained at a stable volume for 30 s to determine whether the PVN neuron was response to stomach mechanical distention. The gastric distention (GD) responsive neuron was identified depending on the change in the firing rate ≥ 20%. The GD responsive neurons were called as GD-excitatory (GD-E) neurons or GD-inhibitory (GD-I) neurons according to whether firing activity was excited or suppressed by GD, respectively.

2.4. Electrical stimulation

Rats were anesthetized and subsequently mounted in a stereotaxic frame. The stimulation electrode (David Kopf Instruments, Tujunga, CA, USA) was implanted into the LHA (bregma: P: 1.3–2.3 mm, L (R): 1.5–2.5 mm, H: 8.0–9.0 mm). After one-week recovery, the electrode was used to stimulate nucleus with a radio-frequency output of square-wave current impulses. Electrical stimulation LHA parameters: 0.5 ms in duration and 20 μA in intensity, sustained for 10 s at 50 Hz. The control group was given sham stimulation, that is, only the buried stimulation electrode was not stimulation.

2.5. Implantation of cannula and drug injection

For direct PVN injections, a cannula was implanted. Anesthetized rats (thiobutabarbitol, 0.1 g/kg. i.p.) were fixed in the stereotaxic frame. After craniotomy surgery, a 24-gauge stainless steel cannula (diameter: 1.5 mm) was inserted into the PVN (position described as above) according to the Paxinos-Watson (Paxinos and Charles, 2013). The 29-gauge injection needle was jointed to a syringe using the polyethylene tubing drugs injection.

The action of PVN injected different concentrations of orexin-A (1.0 μg orexin-A; 5.0 μg orexin-A; 10.0 μg orexin-A) on gastric motility and gastric acid secretion was observed. The mixture of 20.0 μg SB-334867 and 5.0 μg orexin-A, or mixture of 5.0 μg GR-231118 and 5.0 μg orexin-A was microinjected into the PVN through the ventricular catheter, evaluating the action of OX1R antagonist SB-334867 and Y_1 receptor antagonist GR-231118 on orexin A-induced gastric motility and acid secretion. In the control group, 0.5 μL saline were injected into the PVN. A series of SB-334867 concentrations (5 μg, 10 μg, 15 μg, 20 μg, 25 μg, 30 μg) and GR-231118 concentrations (0.5 μg, 1.0 μg, 3.0 μg, 5.0 μg, 7.0 μg, 8.5 μg) were used in the pre-experiment. Since 20 μg SB334867 or 5.0 μg of GR-231118 is the minimum dose that completely or partially blocks the effect of orexin-A on gastric motility and gastric acid secretion, this dose was selected for experiments. Orexin A, GR-231118 administered in 0.9% saline. SB-334867 was dissolved in three drops of DMSO and diluted in 0.9% saline (final the DMSO concentration 0.1%, Łupina et al., 2018).

2.6. Gastric motility recording

To explore the action of orexin-A injected to the PVN on gastric motility in conscious rats, 48 rats with implanted cannulas were divided into 8 groups randomly ($n = 6$): (1) Normal saline (NS); (2) 1.0 μg/0.5 μL orexin-A; (3) 5.0 μg/0.5 μL orexin-A; (4) 10.0 μg/0.5 μL orexin-A; (5) 20.0 μg/ 0.5 μL SB-334867; (6) 20.0 μg/0.25 μL SB-334867 + 5.0 μg/0.25 μL orexin-A; (7) 5.0 μg/0.5 μL GR-231118; (8) 5.0 μg/0.25 μL GR-231118 + 5.0 μg/0.25 μL orexin-A. To observe the actions of electrical stimulation to the LHA on gastric motility in conscious 36 rats, implanted with electrodes, were randomly divided into 6 groups (n = 6): sham stimulation (SS); electrical stimulation (ES); NS + SS group; NS + ES; SB-334867 + SS; SB-334867 + ES.

The process of implanting a force transducer (Beijing Xinhang Xingye Electronics Co., Ltd.) was conducted as previously described (Sun et al., 2017). The rats recovered for 72 h prior to the gastric motility recordings. Rats were placed in the experimental area to adapt to the new environment 30 min before gastric motility recordings experiment. Rats were fasted during the gastric motility recording, but allowed drinking water freely. The polygraph (3066–23; Chengdu Precision Instruments, Sichuan, China) was used to record gastric contraction activity. First basal gastric motility were observed and recorded around 30 min, then gastric contraction were recorded after drugs was administrated through the cranial cannula respectively or electrical stimulation to the LHA. For each rat, gastric motility recording was performed for at least 2 h on 2 different days.

2.7. Gastric acid secretion measurement

To investigate the effects of orexin-A injected into on gastric acid secretion and the actions of electrical stimulation to the LHA on gastric acid secretion in anesthetized rats, the grouping is the same as the Materials and Methods 2.6.

In this experiment, we used the pylorus-ligation experimental technology test for gastric acid secretion as presented (Gao et al., 2017). Following rats are anesthetized thiobutabarbitol (0.1 g/kg, i.p.), a small incision was performed in the middle of the abdomen in order to expose the stomach and duodenum. After incision at the abdominal midline, the esophagus was ligated at the gastroesophageal junction (to preserve the vagal trunks carefully). A double-lumen catheter was inserted into the stomach via the ligated pylorus. After administration of the drug in the PVN or electrical stimulation to the LHA 1 h, gastric fluid was gathered every 15 min for 4 times. The gastric acid secretion was determined by titration with 0.01 N NaOH to a pH of 7.0 using the automatic titrator (COM-2500; Hiranuma Sangyo Co Ltd., Japan, Gao et al., 2017).

2.8. Histological verification

After electrophysiological recording, the site for the recording electrode was marked by pontamine sky blue using the direct current (10 μA, 20 min) passed through the electrode; At the end of the experiment, 0.5 μL pontamine sky blue was microinjected through the cannula to check the target of the drug administration. The rat was perfused and fixed with saline and 4% paraformaldehyde (Described in Materials and methods 2.2.1), brains were cut into 50 μm thickness, stained with neutral red and then observed under light microscope. If the injection site or the recorded cell was not in the PVN, the experimental data was eliminated from the statistical analysis.

2.9. Statistical analysis

Data were processed with Prism 5 (GraphPad Software Inc., San Diego, CA, USA) and are displayed as mean ± SD. For electrophysiological experiments, paired *t*-test was used to compare the firing rates before (mean firing frequency of 60s before administration) and after (mean firing frequency of 120 s after administration) a treatment in the same neuron; unpaired *t*-test was used to compare data of two unrelated groups. For gastric motility experiments and gastric acid secretion, one-way ANOVA and posthoc Bonferroni's tests were used to compare data of multiple groups. $P < .05$ was considered statistically significant.

3. Results

3.1. Anatomical evidence for a LHA-PVN orexin-A pathway

We first sought to define the chemical phenotype of LHA neurons that send projections to the PVN by performing retrograde tracing and immunofluorescence experiments. After microinjection of the retrograde tracer FG into the PVN, FG-labeled cells were observed in the LHA (Fig. 1B). Immunostaining for orexin-A revealed the presence of orexin-A within the LHA (Fig. 1A), and that 24.3% orexin-A-immunopositive neurons were also FG-positive (Fig. 1C). The above results suggested that a portion of the LHA orexinergic neurons project to the PVN.

We further observed that not only the expression of OX1R (Fig. 1D) but also the expression of Y_1 receptor (Fig. 1E) in the PVN of rats, indicating that the PVN neurons contain receptors for orexin-A and NPY.

3.2. Electrophysiological recordings

3.2.1. Identification of the PVN NPY- sensitive/GD neurons

To assess the relevance of this LHA-PVN orexin-A pathway to brain homeostatic systems, recordings were taken from GD-sensitive neurons within the PVN. To accomplish this, spontaneous firing of 163 PVN neurons were recorded from 28 rats. After experimental distension of the stomach, the PVN neurons where experimental distension of the stomach altered firing frequency were categorized as GD- sensitive neurons.

Among the 163 recorded neurons, 106 (65.0%) neurons were GD-sensitive. After stomach distension, 34.9% (57/163) GD- sensitive neurons significantly increased their firing frequency and were defined as GD-E neurons, whereas 30.1% (49/163) neurons significantly decreased in firing frequency, and were defined as GD-I neurons. The

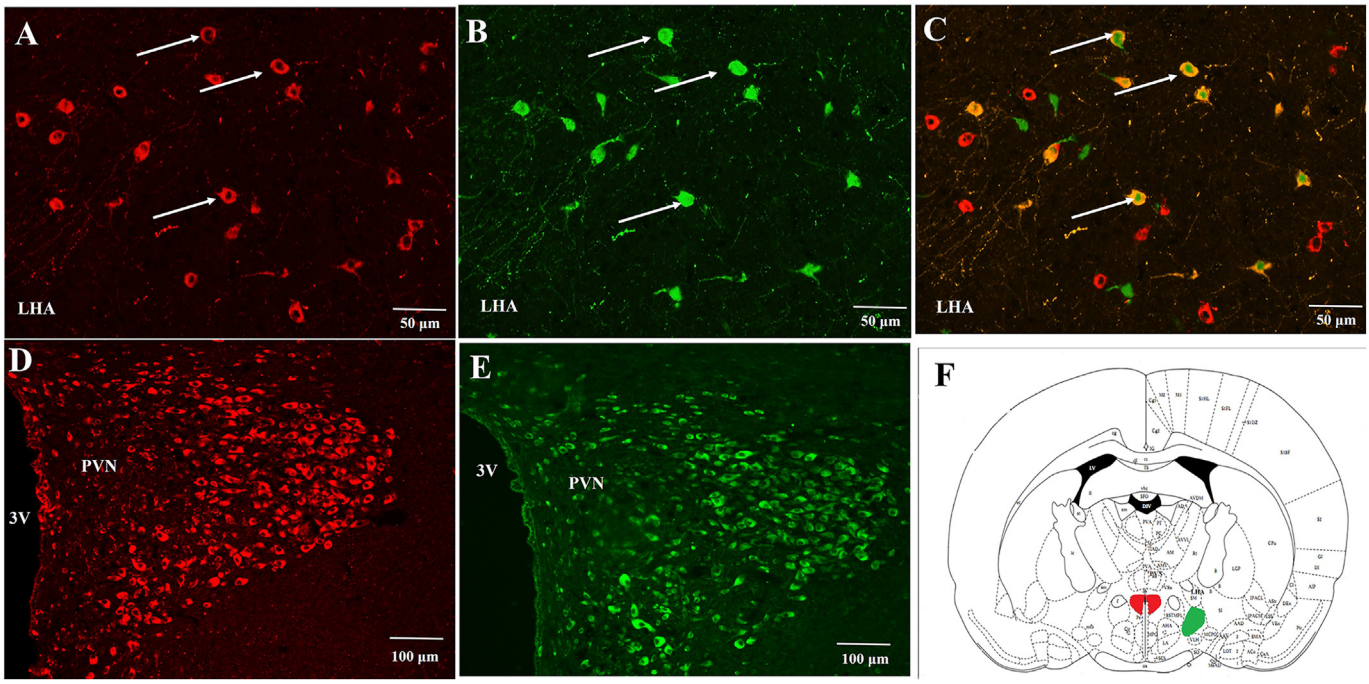

Fig. 1. Retrograde tracing and immunohistochemical staining. Orexin-A-immunopositive neurons (A) and FG-labeled neurons (B) were observed in the same LHA section. (C) Double visualization of FG-labeled and orexin-A-immunopositive neurons. OX1R (D) and Y_1 receptor (E) expression were observed in the PVN. (F) Diagram for the sites of the LHA (green area) and PVN (red area) according to the Paxinos and Waston. FG: Fluorescent gold; LHA: Hypothalamic lateral area; PVN:Hypothalamic paraventricular nucleus; 3 V: Third ventricle; Fluorescent immunohistochemical staining; Scale bars: 100 μm and 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

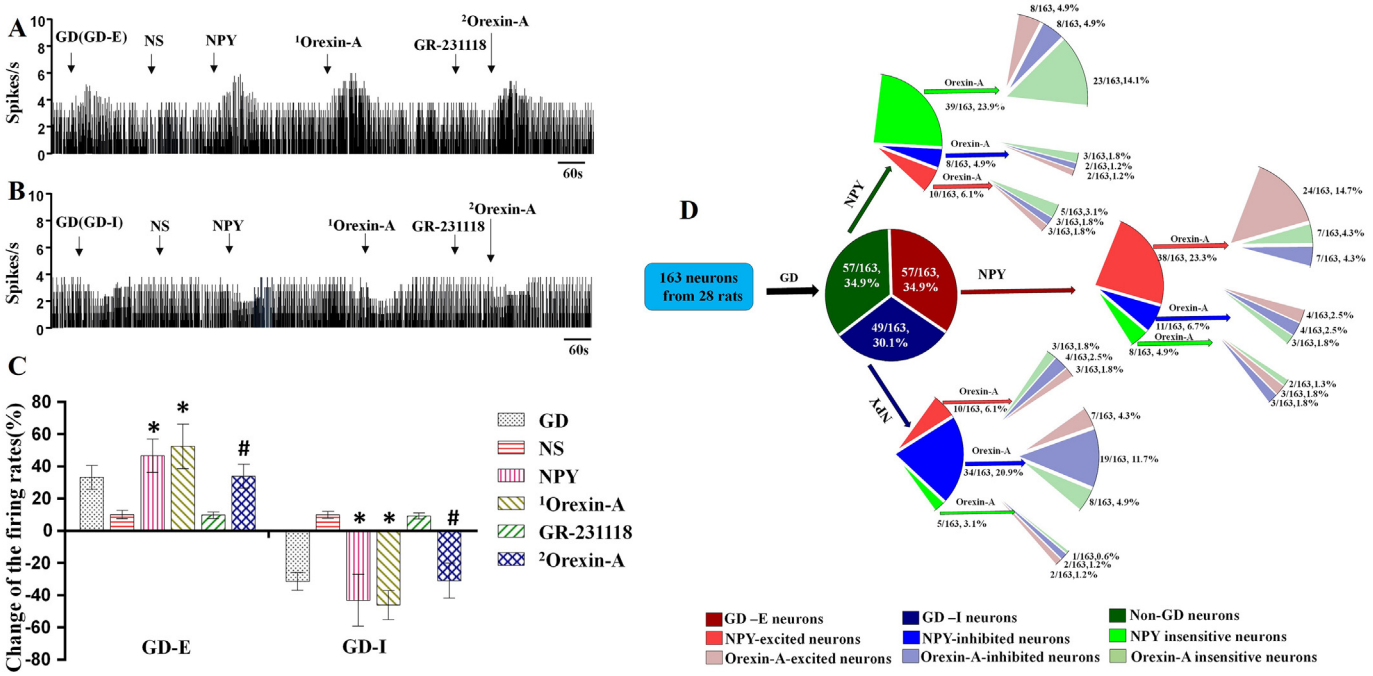

Fig. 2. Effects of orexin-A on the firing frequency of NPY-sensitive/GD neurons in the PVN. Orexin-A induced a significant increase in neuronal firing frequency of NPY- excited/GD-E neurons (A) and a marked decrease in NPY-inhibited/GD-I neurons (B). Pre-administration of GR-334867 blocked the discharge-promoting effect of orexin-A. The percent changes in the firing rates for A-B are shown in C. (D) indicates final the percentage of an individual group against whole the population of the neurons. Scale bar, 60 s. $^{*}P < .05$ vs NS; $^{\#}P < .05$ vs ^{1}orexinA. ^{1}orexin-A:First injection of orexin-A into the PVN. GD: gastric distension; GD-E: gastric distension excited; GD-I: gastric distension inhibited; Non-GD: non- gastric distension. The change in firing rate of the GD neurons was calculated by 100 × (firing rate of GD neurons after treatment-firing rate of GD neurons before treatment)/(firing rate of GD neurons before treatment). Bars represent 60 s of recording. Data are presented as mean ± S.D.

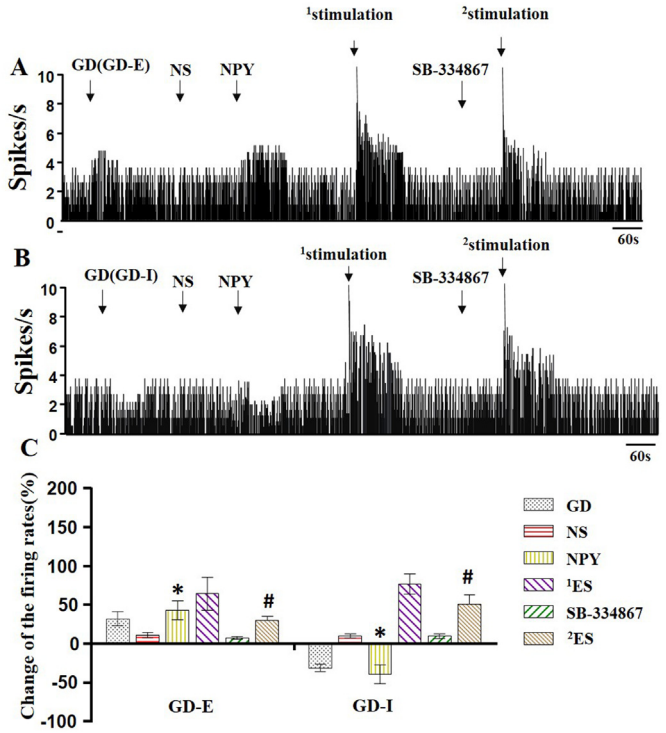

Fig. 3. Effect of electrical stimulation to the LHA on the firing rate of NPY-sensitive/GD neurons in the PVN. The NPY- excited/GD-E neurons (A) and NPY-inhibited/GD-I neurons (B) in the PVN were mainly activated by electrical stimulation of LHA. (C) Shows the change in firing rate (%) of NPY- sensitive/GD neurons in the PVN caused by electrical stimulation of LHA. Pre-injection of SB-334867 in the PVN weakened the effects of electrical stimulation of LHA. $^{*}P < .05$, vs. NS group; $^{\#}P < .05$, vs. ^{1}electrical stimulation group. The change in firing rate of the GD neurons was calculated by 100 × (firing rate of GD neurons after treatment-firing rate of GD neurons before treatment)/(firing rate of GD neurons before treatment). Bars represent 60 s of recording. Data are presented as mean ± S.D.

remaining are non-GD neurons (57/163, 34.9%,).

To assess, the effects of NPY on GD neurons, NPY was superfused on the surface of PVN neuron. 38 of 57 (66.7%) GD-E neurons were excited by NPY, leading to a significant increase in the discharge activity comparing with normal saline (NS) injection. Conversely, 11 (11/57, 19.3%) were suppressed and 8 (8/57, 14.0%) had no significant change in firing frequency following the PVN injection of NPY.

After NPY, 34 of 49 (69.4%) PVN GD-I neurons were inhibited. 10 (20.4%) GD-I neurons were excited and 5 (10.2%) GD-I neurons had no change after administration of NPY. The lack of response after NS treatment confirmed that the responses were specific to NPY.

3.2.2. Effects of PVN injection of orexin-A on the firing of NPY-sensitive/GD neurons

To explore whether NPY- sensitive/GD neurons are responsive to the orexin-A, we assessed the effects of orexin-A on the firing of NPY-sensitive/GD neurons. Compared with the NS group, 24 out of 38 (63.2%) NPY- excited/GD-E neurons were further activated by administration of orexin-A in the PVN with the firing frequency significantly increasing from 3.84 ± 0.09 Hz to 5.85 ± 0.87 Hz ($P < .01$, Fig. 2A, C); 7 neurons (7/38, 18.4%) were inhibited and 7 (7/38, 18.4%) showed no significantly change. Among the 34 NPY-inhibited/GD-I neurons, 19 (19/34, 55.9%) were inhibited by orexin-A, where firing frequency decrease from 3.8 1 ± 0.14 Hz to 2.05 ± 0.05 Hz ($P < .01$, Fig. 2B, C); 7 (7/34, 20.6%) were excited and 8 (8/34, 23.5%) showed no change. The final % of an individual group against whole the population of the recording neurons is shown in Fig. 2D, For example, the net % of GD-excited/ NPY-excited/Orexin-A-excited neurons is 0.349 × 0.667 × 0.632 = 14.7%.

To determine whether the NPY receptor pathway was involved in the effect of orexin-A on NPY- sensitive/GD neurons, the Y_1 receptor antagonist GR-23118 was administered into the PVN previous to orexin-A administration. The excitatory effects of orexin-A on NPY- excited/GD-E neurons or NPY-inhibited/GD-I neurons were blunted by pre-administration of GR-23118 ($P < .05$, Fig. 2A, B, C). It could be argued that desensitization of the neuron, and not the Y_1 receptor antagonist, reduced firing rate. To address this possibility, we administered orexin-A (1 nL, Gao et al., 2017) without the antagonist as a control in the pre-experiment. There was no significant difference in firing rate throughout the relevant time course (interval 3 min, 4 min or 5 min, $P > .05$, data not shown). Further, there was no change in firing rate when GR-23118 was administered alone ($P > .05$, Fig. 2A, B, C). These results, and the lack of response in the NS treatment group, confirmed that the responses were specific to NPY and orexin-A. Further, our results demonstrate that orexin-A regulates the electrophysiological properties of NPY- sensitive/GD neurons in the PVN, and involve NPY receptor signaling pathway.

3.2.3. Effects of electrical stimulation to LHA on the discharge of NPY-sensitive/GD neurons in the PVN

To confirm whether the activity of NPY- sensitive/GD neurons in the PVN is mediated by the LHA-PVN neural pathway, electrical stimulation of LHA neurons was performed. Out of 38 NPY-excited/GD-E neurons in the PVN, 16 (16/38, 43.5%) were activated by electrical stimulation of the LHA, an increase in firing rate of 64.9 ± 21.3% ($P < .01$, Fig. 3 A, C). Among these NPY-inhibited/ GD-I neurons, 15 of 34 (44.1%) neurons were activated by LHA stimulation (an increase in firing frequency from 3.82 ± 1.03 Hz to 6.87 ± 1.36 Hz, $P < .01$, Fig. 3B, C). These LHA-induced excitatory responses could be altered by pre-injection of SB334867 into the PVN ($P < .05$, Fig. 3), strongly suggesting that orexin-A neurons from the LHA mediate the changes to NPY-sensitive/GD neuronal excitability in the PVN.

3.3. Gastric motility recordings

3.3.1. The effects of PVN orexin-A on gastric motility involve NPY receptor signaling

The effects of orexin-A on gastric motility was assessed in awake, behaving rats. Different concentrations orexin-A (1.0 μg, 5.0 μg, and 10.0 μg) were injected into the PVN and gastric contraction amplitude and frequency were significantly increased compared to control and in a dose-dependent manner ($P < .01$–0.05, Fig. 4A, B, C). The increase in gastric contraction amplitude and frequency started 5 min post-injection and reached a peak roughly 10–15 min post-injection (Fig. 4A, B, C). The addition of 20.0 μg SB-334867 blocked the effect of 5.0 μg orexin-A on gastric motility ($P < .01$, Fig. 4A, B, C), showing that these effects were OX1R receptor dependent. Additionally, 5.0 μg of the Y_1 receptor antagonist GR-231118, applied to the PVN, also weakened the effect of 5.0 μg orexin-A on gastric motility ($P < .05$, Fig. 4A, B, C). Importantly, GR23118, SB-334867, nor saline altered the the frequency and amplitude of gastric contraction, alone ($P > .05$, Fig. 4A, B, C). These results suggests that orexin-A signaling in the PVN plays an important role in the regulation of gastric motility, mainly through the OX1R receptors, though Y_1 receptor signaling may also play a role in this process.

We also assessed whether a Y_5 receptor antagonist CGP-71583 would have an inhibitory effect on orexin-A-induced gastric motility. We found that the inhibitory effect of Y_1 receptor on gastric motility was more pronounced than that of Y_5 receptor(MI% 10 min: 33.2 ± 5.7% vs. 43.3 ± 6.2%; 15 min: 20.2 ± 4.3% vs. 33.5 ± 5.8%; Values of the %MI for a 5-min period were calculated by 100 x (area under the manometric trace for each 5-min period after the durgs)/(area under the manometric trace for the 5-min period

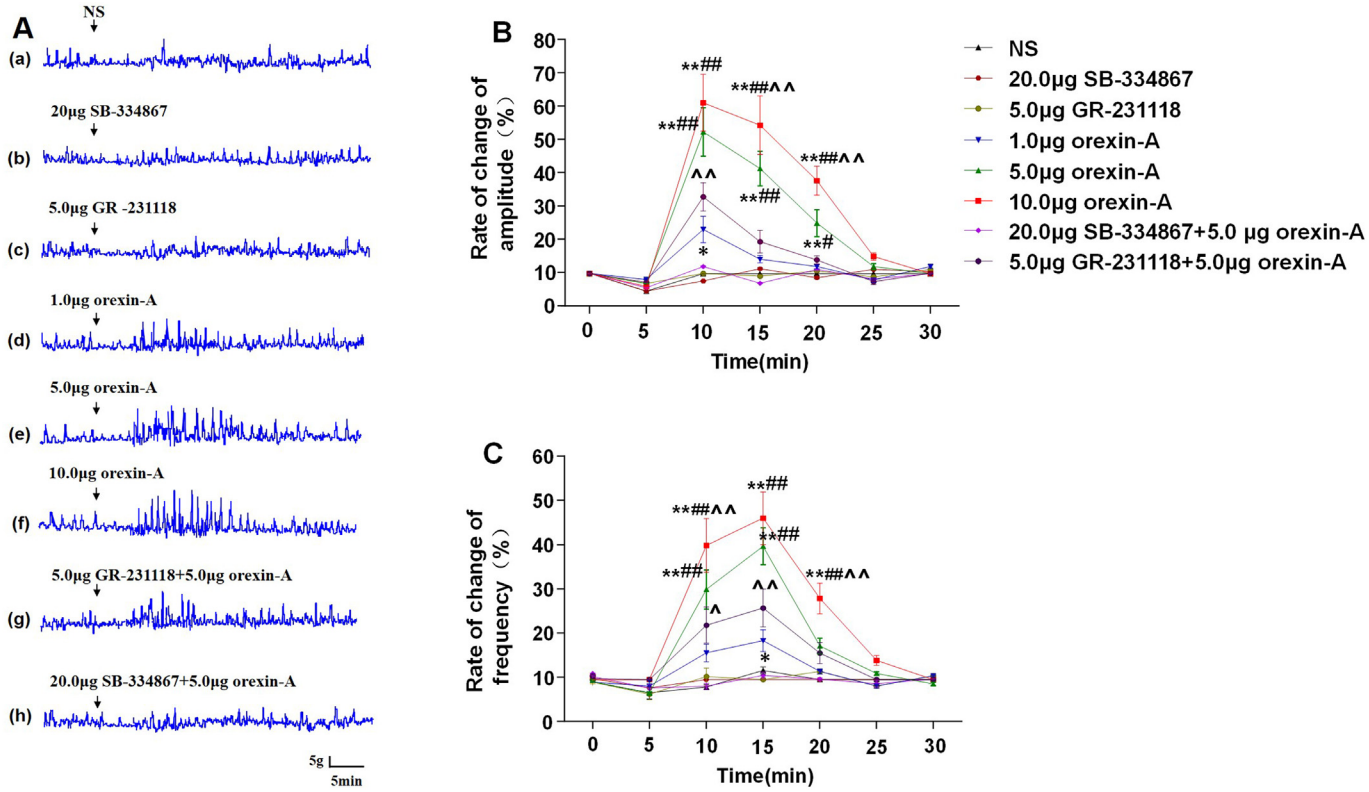

Fig. 4. Effects of orexin-A on gastric motility in the PVN. (A) Recording curves of gastric motility: Gastric motility was dose-dependently increased by administration of orexin-A into the PVN at doses of 1.0 μg, 5.0 μg, and 10.0 μg (d–f). The excitatory effects induced by orexin-A were completely eliminate by 20.0 μg SB-334867 (h) and weakened by 5.0 μg GR −231,118 (g). Injection of neither NS, SB-334867 nor GR-231118, alone, had an effect on gastric motility (a-c). After injection of orexin-A in the PVN, the amplitude (B) and frequency (C) of gastric contraction increased significantly comparing with rats receiving NS injection, and showed a dose-dependent relationship. *P < .05, **P < .01, vs. NS group; #P < .05, ##P < .01, vs. 1.0 μg orexin-A group; ^P < .05, ^^P < .01, vs. 0.5 μg orexin-A group. Per group $n = 6$. Data are presented as mean ± S.D. Percentages of frequency and amplitude changes were derived from the equation: frequency or amplitude change = (frequency or amplitude after microinjection-frequency or amplitude before microinjection)/frequency or amplitude before microinjection × 100%.

immediately before the drugs). This result suggests that Y_1, not Y_5 receptors, seem to be more central to PVN orexin-A-dependent changes in gastric motility.

3.3.2. Effects of LHA electrical stimulation on gastric motility

Next, we explored the effect of LHA electrical stimulation on gastric motility in awake, behaving rats. The gastric contraction amplitude and frequency started 3 min post-stimulation, and peaked 13 min after LHA electrical stimulation, significantly increasing gastric motility ($P < .01$, Fig. 5A, B). This effect was inhibited by pre-injection of SB-334867 in the PVN ($P < .05$, Fig. 5A, B), demonstrating that endogenous orexin-A released from LHA projections in PVN is important to this effect. Importantly, injection of SB-334867 or saline in the PVN, alone, had no significant effect on gastric motility.

3.4. Gastric acid secretion

3.4.1. The effects of PVN orexin-A on gastric acid secretion involve NPY receptor signaling

Next, we assessed the role of PVN orexin-A on gastric acid secretion in anesthetized rats. Orexin-A administration to the PVN caused an increase in gastric secretion compared to rats receiving NS injection. Gastric acid secretion increased following 5.0 μg and 10.0 μg orexin-A, but not 1.0 μg, showing a dose-dependent response (P < .05–0.01, Fig. 6). The OX1R antagonist SB-334867 fully blocked orexin-A-induced gastric acid secretion ($P < .01$, Fig. 6). Further, administration of 5.0 μg GR-23118 in the PVN partially blocked orexin-A-induced gastric acid secretion (P < .05, Fig. 6). These results demonstrate that orexin-A signaling in the PVN induces gastric acid secretion through the OX1R receptors, while NPY receptor signaling also plays a role in this process. GR-231118 in the PVN, alone, slightly, though significantly, reduced gastric acid secretion. However, saline and SB -334,867 did not significantly change gastric acid secretion (Fig. 6). In the pre-experiment, showing that a Y_5 receptors antagonist CGP-71683 injected into the PVN was more effective at blocking orexin-A-dependent gastric acid secretion. The effect of orexin-A was 16.8 ± 3.2% when the Y_1 receptors antagonist GR-231118 was applied and 52.9 ± 11.3% with a Y_5 receptors antagonist CGP-71683. This is consistent with previous research (Kermani and Eliassi, 2012).

3.4.2. Effects of LHA electrical stimulation on gastric acid secretion

Next, we assessed the effect of LHA electrical stimulation on gastric acid secretion in anesthetized rats. Electrical stimulation to the LHA significantly increased gastric acid secretion in the rats comparing with SS group (P < .01, Fig. 7). This effect was reduced by pre-injection of the OX1R antagonist SB-334867 to the PVN (SB-334867 + ES vs. NS + ES, P < .01, Fig. 7). Importantly, NS or SB-334867, alone, had no significant effect on gastric acid secretion.

4. Discussion

In this study, we illustrate the central regulatory mechanisms of an LHA to PVN orexinergic circuit on gastric motility and gastric acid secretion. We show that GD alters the activity of PVN neurons, which are also sensitive to NPY. Orexin-A activated the majority of NPY-excited/GD-E PVN neurons and inhibited the majority of NPY-inhibited/ GD-I PVN neurons, while also promoting gastric contractions and gastric acid secretion. Furthermore, electrical stimulation of the LHA influenced

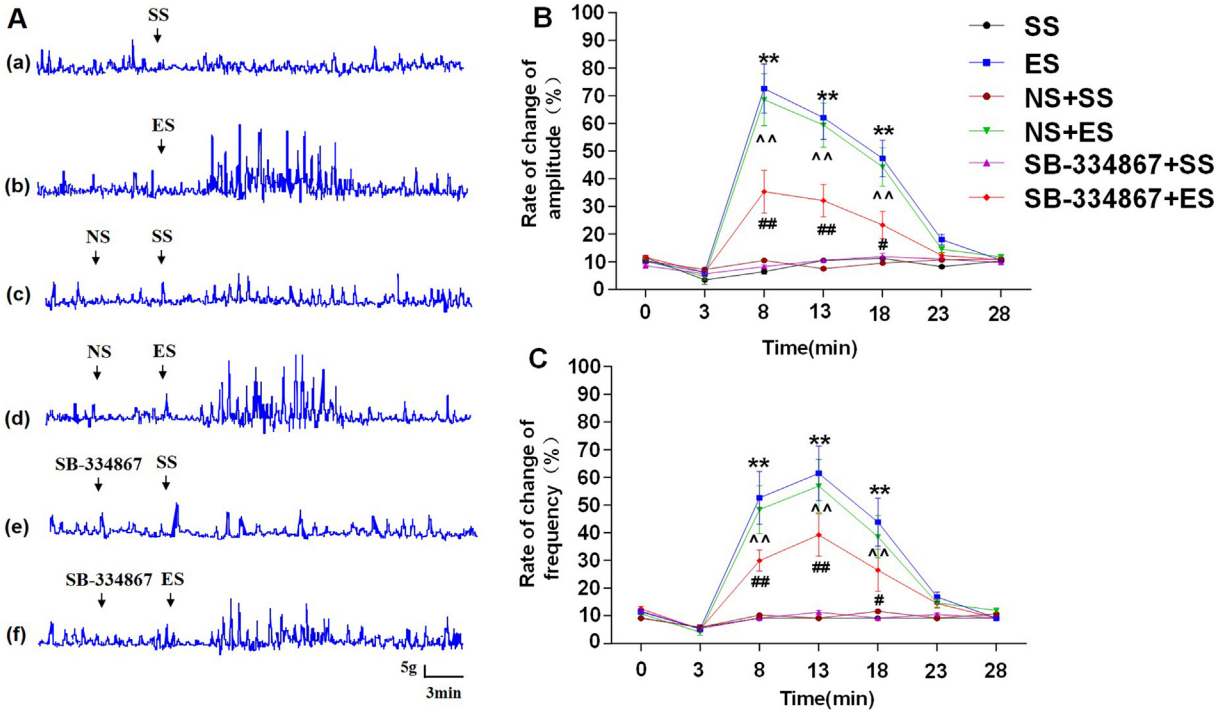

Fig. 5. Effect of LHA electrical stimulation (ES) on gastric motility. (A) Recording curves of gastric motility: ES enhanced gastric motility (b and d). The excitatory effect of ES was partially blocked by pre-administration of 20.0 μg SB-334967 in the PVN (f). After ES, the amplitude (B) and frequency (C) of the gastric contraction were increased compared to the SS group. **P < .01, vs. SS group; ^^P < .01, vs. NS + SS group; #P < .05, ##P < .01, vs. SB-334867 + SS group. Per group n = 6. Data are presented as mean ± S.D. SS: Sham stimulation; ES: Electrical stimulation. NS: Normal saline. The percentage of the frequency and amplitude changes were derived from the equation: frequency or amplitude change = (frequency or amplitude after microinjection–frequency or amplitude before microinjection)/frequency or amplitude before electrical stimulation × 100%.

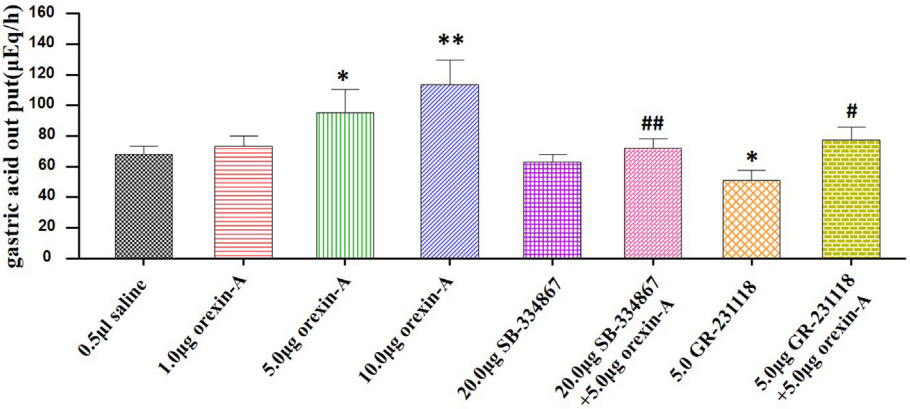

Fig. 6. Effects of PVN administration of orexin-A on gastric acid secretion. After administration of orexin-A in the PVN, gastric acid secretion increased significantly in a dose-dependent manner. However, the excitatory effect of orexin-A was eliminated by 20.0 μg SB-334867, and partially blocked by 5.0 μg GR-231118. Administration of NS, SB-334867 did not change gastric acid secretion. Per group n = 6. *P < .05, **P < .01, vs. 5.0 μL saline group; #P < .05, ##P < .01, vs. 5.0 μg orexin-A; Data are presented as mean ± S.D.

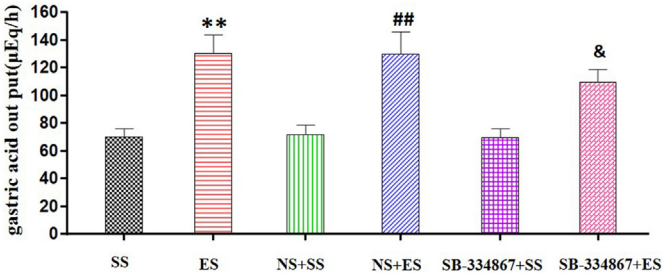

Fig. 7. The effect of LHA electrical stimulation on gastric acid secretion. LHA electrical stimulation significantly increased gastric acid output in rats. The excitatory effect induced by the electrical stimulation was partially blocked by pre-administration of 20.0 μg SB-334967 to the PVN (per group n = 6). **P < .01, vs. SS group; ##P < .01, vs. NS + SS group; &P < .05, vs. SB-334867 + SS group. Data are presented as mean ± S.D.

NPY- sensitive/GD neurons firing in the PVN, and promoted the gastric contractions and gastric acid secretion through action at OX1R receptors. Retrograde tracing and immunohistochemical labeling confirmed this LHA-PVN orexinergic pathways. Orexin-A's effects in the PVN were partially blocked by the Y_1 receptor antagonist GR-231118. Together, these findings suggest that orexin-A, originating from LHA, acts on the PVN to modulate gastric contractions and gastric acid secretion mainly through OX1R receptors, while Y_1 receptor signaling also participates in this modulatory process.

GD imitates feeding and induces negative regulatory feedback of appetite. Gastric acid secretion and gastric motility are also necessary for feeding, and, like GD are regulated by the hypothalamus (Takahashi et al., 1999; Sun et al., 2016). It has been previously reported that LHA orexin-A neurons, specifically, are sensitive to GD (Luan et al., 2017). We show here that these LHA orexin-A neurons regulate firing of GD-sensitive neurons in the PVN. These modulatory effects may be

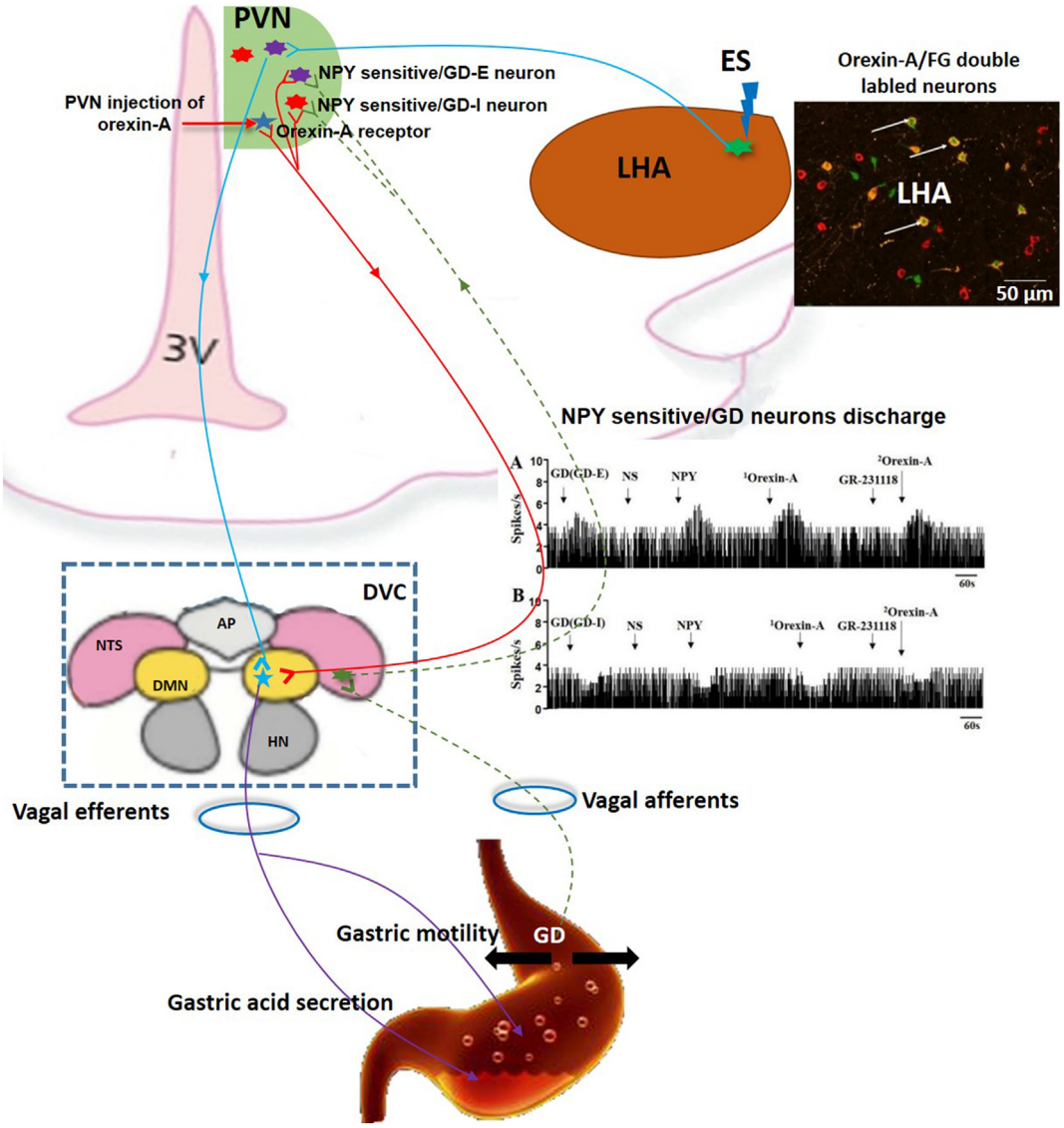

Fig. 8. Summary of the orexinergic pathway from LHA to PVN and its regulation of gastric distension (GD) and gastric function. When GD occurs, a signal is transmitted to the NTS through the afferent nerve, and subsequently transmitted directly or indirectly to the PVN, causing a change in the activity of GD neurons (the green dotted line). Administration of orexin-A to the PVN, binding to the orexin-A receptor, and then the orexin-A signaling in the PVN is directly or indirectly transmitted through the DMN and gastric motility and gastric secretion are increased (the red solid lines). Orexin-A/FG double-labeled neurons were observed in the LHA which indicated that orexin-A immunoreactive neurons send their fibers into the PVN. Endogenous orexin-A which was induced by electrical stimulating the LHA could regulate gastric function (the blue solid lines). The purple solid line indicates that pathway from the DMN to the stomach. PVN: paraventricular nucleus; GD: Stomach distension; GD-E: gastric distension excited; GD-I: gastric distension inhibited; ES: Electrical stimulation; LHA: the lateral hypothalamus area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contributed to GD-induced signals that are important for hypothalamic regulation of satiety. The above results suggest that orexin-A can enhance the excitability of gastric-associated afferent neurons in the PVN, which might be contribute to further regulate gastrointestinal function.

In the hypothalamus, and PVN specifically, many neuronal subtypes are involved in the regulation of gastric functions (Matsuki and Sakurai, 2008). It is likely that these subtypes are incorporated into a regulatory network (Sohn, 2015). Excitatory neurons (perhaps GD-inhibited/NPY-inhibited/orexin-A-excited) and inhibitory neurons (perhaps GD-inhibited/NPY-inhibited/orexin-A-inhibited) may interact with other neurons (Luccioli et al., 2018), sending out a comprehensive signal to regulate gastric motility or gastric acid secretion regulation. One possible target is the vagus nerve complex (DVC), which contains the dorsal motor nucleus of the vagus (DMN) (Willett et al., 1987). The activity of this PVN network and its downstream effectors, like the DMN, should be the subject of future research.

The vagus nerve complex (DVC) is an important structure regulating the gastrointestinal tract, including area postrema (AP), the dorsal motor nucleus of the vagus (DMN), and solitary tract (NTS) (Wang et al., 2014). In this experiment, when the gastric distension is performed, the mechanoreceptors of the muscular layer in the stomach wall are excited, and the satiety signal is transmitted to the NTS through the afferent nerve (Zhang et al., 2002). Subsequent the information is directly or indirectly transmitted to the hypothalamic feeding center, such as PVN. And the GD-excitatory or GD-inhibiting neurons were observed in the PVN. Similarly, And while the neurons in the CNS, such as PVN, were activated, the nerve impulse may be transmitted to the DMN (Willett et al., 1987) through direct or multiple synapse neural pathways, followed by to the stomach by the efferent nerve. In our study, the orexin-A signaling in PVN may participate in the regulation of gastric motility and gastric acid secretion through the above regulatory pathway (Fig. 8), and its specific mechanism pathway

needs further study.

Interestingly, previous studies have shown that bilateral LHA electrolytic lesion leads to attenuated gastric acid secretion and eating disorders in rats (Anand and Brobeck, 1951; Sun et al., 2016). Conversely, LHA electrical stimulation has been shown to cause a significant increase in food intake in cats (Hoebel and Teitelbaum, 1962). Our results showed that LHA electrical stimulation influenced the neuronal activity of the NPY-sensitive/GD neurons in the PVN, and promoted gastric contraction and gastric acid secretion, through OX1R receptors. Previous to this study, little was known about the molecular or neurotransmitter mechanisms by which electrical stimulation of LHA neurons increases gastric acid production and gastric contraction activity. Our results argue that orexin-A released from the terminal nerve endings of LHA neurons in the PVN may stimulate gastric acid secretion and gastric motility. Another possible mechanism is that electrical stimulation of LHA activates orexin-A neurons as well as other neurons associated with gastrointestinal function. Further research is needed to identify other neurotransmitter and molecular systems in the LHA that may be involved in these effects.

A study by Horvath et al. (1999) found a direct synaptic connection between orexin positive neuronal fibers and NPY neurons. The sites of action of NPY and orexin are primarily in the hypothalamus (Dube et al., 1999). NPY acts through six receptor subtype, though only Y_1 and Y_5 receptors appear to be involved in the regulation of food intake (Coppola et al., 2004). Importantly, the locations of Y_1 receptors are similar to the locations of orexin receptor mRNA expression in the hypothalamus, particularly in the PVN (Gehlert, 2004). Further, orexin and NPY neuronal pathway functionally interact (Horvath et al., 1999; Dube et al., 1999; Jain et al., 2000). Because of this close morphological and functional relationship between orexin-A and NPY neurons, these two orexinergic signals may also cooperate in the regulation of gastric motility and gastric acid secretion. In our study, orexin-A in the PVN stimulated gastric motility and gastric acid secretion, which was partially blocked by the Y_1 receptor antagonist GR-231118. This result indicate that orexin-A at least partially relies on the Y_1 receptor signaling to mediate the gastric functions.

In conclusion, orexin-A from LHA neurons act on PVN to enhance gastric motility and gastric acid secretion mainly through OX1R receptors, with Y_1 receptor signaling playing a critical role. This research provides deeper insight into the actions of the LHA-PVN projection and the PVN orexin-A signaling in regulation gastric motility and gastric acid secretion and may provide a novel treatment strategy for gastric acid secretion abnormalities and gastric dyskinesia.

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Conflict of interest

There is no conflict of interest in this study.

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