Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Orexin-A signaling in the paraventricular nucleus modulates spontaneous firing of glucose-sensitive neurons and promotes food intake via the NPY pathway in rats

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ARTICLE INFO

Article history:
Received 10 September 2018
Accepted 14 September 2018
Available online xxx

Keywords:
Hypothalamus paraventricular nucleus
Glucose-sensitive neurons
Food intake
Orexin-A
NPY-1 receptor

ABSTRACT

Understanding the mechanisms regulating feeding is crucial to unraveling the pathogenesis of obesity. The study primary explored the effects of orexin-A and neuropeptide Y (NPY) signaling in the hypothalamic paraventricular nucleus (PVN) on feeding and glucose-sensitive (GS) neuron activity in rats. Microinjection of orexin-A into the PVN promoted feeding and modulated the spontaneous firing of GS neurons. Those effects were eliminated by pre-injection of the orexin-A receptor-1 (OX1R) antagonist SB-334867 and weaken by the NPY-1 receptor (NPY-1R) antagonist BMS-193885. After orexin-A administration into the PVN, the number of c-fos cells in the arcuate nucleus (ARC) was significantly higher than that in the group receiving normal saline. Furthermore, most cells exhibited co-expression of NPY and c-fos, indicating activation of NPY neurons in the ARC by PVN-administered orexin-A, which might be involved in feeding regulation. These findings indicate that orexin-A and NPY signaling in the PVN are essential to regulating GS neuronal excitability and feeding in rats.

1. Introduction

In developing countries, obesity is a growing health problem affecting more than 100 million people worldwide [1]. Orexins are orexigenic peptides that were first identified in the brains of rats in 1998. Orexin neurons are primarily located in the lateral hypothalamus area (LHA) [2,3] and their fibers widely project to the other brain area, including the PVN [4] and the dorsomedial nucleus of hypothalamus (DMH) and so on [5]. Orexin has been found to regulate many physiological processes related to obesity including sleep/wakefulness states [6,7], energy homeostasis [8], reward [9] and autonomic functions [10,11]. Studies have shown that intracerebroventricular (icv) injection of orexin-A induces feeding behavior in a dose-dependent manner.

NPY, a peptide hormone of 36 amino acids, was first reported in 1982 [12]. NPY is an endogenous orexigenic factor with substantial regulatory effects on feeding, body weight and energy balance [13–17]. Administration of NPY into the rat brain directly increases food intake, mainly through the NPY-1R and NPY-5R [16]. Icv injection of the NPY-1R antagonist BMS-193885 decreases food intake [18,19].

The PVN has been speculated to play a key role in the brain network that mediates satiety and energy balance [20]. Information on both orexigenic peptidergic systems and anorexigenic systems appears to converge and integrate within the PVN [21,22]. However, the mechanism through which orexin-A regulates PVN glucose-sensitive (GS) neuronal excitability and feeding remains unclear. Therefore, in this study, we investigated the regulatory effects of orexin-A on feeding and the excitability of PVN GS neurons in rats, and explored the functional relationship between orexin-A and NPY-1R signaling as it relates to orexin-A's regulatory effects.

2. Material and methods

2.1. Animals

Sprague-Dawley rats weighing 200–250 g (male) were provided by the Qing Dao Institute for Drug Control and were housed under controlled light conditions (12 h:12 h light-dark cycles starting at 8 a.m.) and temperature (25 ± 2 °C). All animal protocols were approved by the Qingdao University Animal Care and Use Committee.

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https://doi.org/10.1016/j.bbrc.2018.09.091
0006-291X/

2.2. Implantation of brain cannula and drug administration

The rats were anesthetized with thiobutabarbitol (100 mg/kg, i.p.) and were subsequently placed in a stereotaxic apparatus (Narashige SN-3, Tokyo, Japan). A cannula (26 gauge, Plastics One, VA, USA) was implanted into the PVN (bregma: P: 1.6–1.8 mm, L (R): 0.1–0.7 mm, H: 7.7–8.4 mm) for drug administration. A stainless steel inner tube was inserted into the cannula and connected to the syringe via a 10-cm-long polyethylene tube. The drugs were injected into the PVN. After surgery, the rats were allowed to recover for at least 7 days before the experiments.

2.3. Immunohistochemistry

2.3.1. Expression of OX1R and NPY-1R in the PVN

Briefly, four normal rats were intraperitoneally (i.p.) anesthetized with thiobutabarbital (100 mg/kg; Sigma-Aldrich Chemical, USA). Subsequently, they were perfused with 0.9% saline (200 mL) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) through the intracardiac system. The brains were removed and post-fixed in 4% paraformaldehyde for 2 h, and they were subsequently placed in a 30% sucrose solution for 24 h at 4 °C. The rat brains were cut into 15-μm-thick serial sections along the coronal plane.

The 15 μm slices were incubated for 24 h at 4 °C with rabbit anti-NPY-1R antibody (1:250, Abcam, UK) or rabbit anti-OX1R antibody (1:300, Sigma, St. Louis, MO, USA). The sections were incubated with goat anti-rabbit FITC (1: 25, Jackson ImmunoResearch, West Grove, PA, USA) or goat anti-rabbit cy3 (1: 300, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 2 h.

Finally, after unbound secondary antibodies were washed away with PBS, the sections were mounted in Citifluor (Citifluor, London, UK). Fluorophores were visualized under a BX63F fluorescence microscope, and images were acquired with a DP80 digital camera (Olympus, Tokyo, Japan).

2.3.2. Effects of PVN injection of orexin-A on c-fos expression in NPY-containing neurons in the ARC

Rats were randomly divided into orexin-A and NS groups (n = 4 per group). After orexin-A (0.5 nmol) or NS was injected into the PVN for 2 h, the rats were perfusion-fixed with 0.9% saline and 4% paraformaldehyde. The rat brains were cut into 15-μm-thick serial sections in the coronal plane. A series of ARC sections was selected under a light microscope according to the Rat Brain Atlas [25]. The slices were incubated overnight at 4 °C with mouse anti-*c*-fos antibody (1:100, Santa Cruz Biotechnology, USA) and rabbit anti-NPY antibody (1:200, Santa Cruz Biotechnology, USA). The sections were incubated for 2 h with Cy3-conjugated goat anti-mouse rabbit antibody (1:100, Jackson ImmunoResearch, West Grove, PA, USA) and the fluorochrome-labeled secondary antibody FITC-conjugated goat anti-rabbit (1:25, Jackson ImmunoResearch, USA) at room temperature.

2.3.3. Quantification

To quantify immunopositive cell numbers, six sections from the middle of the ARC (3.10–3.40 mm posterior to bregma) were selected. The number of immunopositive neurons in ARC with clear profiles from each section was counted with a Macintosh-based image analysis system (Image, NIH). The results are expressed as the number of cells per 0.1 mm^2. Only clear cytoplasmic or nuclear staining of the cell body was recorded as a positive neuron.

2.4. Electrophysiological experiment

Rats (26 rats) were anesthetized with thiobutabarbitol (100 mg/kg, i.p.) and then were fixed in a stereotaxic apparatus (Narashige SN-3, Tokyo, Japan). After craniotomy, a five-barrel glass microelectrode (rotal tip diameter: 10–20 μm, resistance: 5–15 MΩ) was stereotaxically guided into the PVN on the basis of known coordinates, with a multi-channel pressure injector, which was used for electrophysiological recording. The recording electrode was filled with 0.5 M sodium acetate and 2% pontamine sky blue to record neuronal discharge, and the other four barrels were filled with 5 mM glucose solution [23], orexin-A (15 nM, Phoenix Pharmaceuticals CA, USA), SB-334867 (25 nM, Sigma-Aldrich Chemical, USA) or BMS-193885 (150 nM, NPY-1R antagonist, Sigma-Aldrich Chemical, USA). Less than 1 nL of the compounds [24] was superfused on the surfaces of firing neurons through a short pulse of pressurized gas (1500 ms, 5.0–15.0 psi). After the microelectrode was advanced into the PVN, the unit firing activity was recorded with the glass microelectrode. Extracellular action potentials were sent via a MEZ8201 amplifier (Nihon Kohden, Tokyo, Japan) inorder to expand the exhibit on the oscilloscope (VC-II, Nihon Kohden, Tokyo, Japan) and were import in a SUMP-PC biological signal processing system. All data were stored for later analysis.

The GS neurons were identified through microinjection of glucose into the PVN. If the average firing increased or decreased to at least 20% of basal firing after glucose administration, the neurons were identified as GS neurons. Neurons exhibiting decreased firing frequency with an increase in local glucose level were termed glucose-inhibitory (GI) neurons, and neurons exhibiting increased discharge with an increase in local glucose level were defined as glucose-excitatory (GE) neurons.

2.5. Food intake measurement

In this study, 30 normal rats were selected. Each group was further randomly divided into the following groups (n = 5 per group): NS, BMS-193885 (5 nmol), SB-334867 (1.0 nmol), orexin-A (0.5 nmol), orexin-A (0.5 nmol) + BMS-193885 (5 nmol), and orexin-A (0.5 nmol) + SB-334867 (1.0 nmol). After the injection of the drugs into the PVN, the rats were placed in their cages with food that had been weighed. The remaining and spilled food was collected and weighed after 2 h and 4 h, respectively.

2.6. Histological verification

At the end of the experiment, to verify the recording position of the glass microelectrode, a direct current (10 μA, 20 min) was run through the electrode, and an iron deposit of pontamine sky blue formed. To verify the target of the implanted cannula for drug administration in the PVN or icv, 0.5 μl pontamine sky blue was microinjected at the end of each experiment. The rat brains were perfused and fixed with saline followed by paraformaldehyde, and frozen coronal sections of 50 μm thickness were cut. Light microscopy was used to verify all of the recording and microinjection sites. If the center of the pontamine sky blue staining in the sections was not within the PVN or icv, the corresponding experimental data of the animal were removed from analysis.

2.7. Statistical analysis

Data were processed with Prism 5 statistical software and are displayed as mean ± SD. Comparisons between two groups were analyzed with Student's t-test or one-way analysis of variance, which was followed by Bonferroni's post hoc test to compare means. The difference in firing rates before and after treatment was assessed with a paired *t*-test. $P < 0.05$ was considered statistically significant.

Please cite this article in press as: C. Wang, et al., Orexin-A signaling in the paraventricular nucleus modulates spontaneous firing of glucose-sensitive neurons and promotes food intake via the NPY pathway in rats, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.091