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Concurrent and robust regulation of feeding behaviors and metabolism by orexin neurons

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ABSTRACT

Orexin neurons in the hypothalamus regulate energy homeostasis by coordinating various physiological responses. Past studies have shown the role of the orexin peptide itself; however, orexin neurons contain not only orexin but also other neurotransmitters such as glutamate and dynorphin. In this study, we examined the physiological role of orexin neurons in feeding behavior and metabolism by pharmacogenetic activation and chronic ablation. We generated novel *orexin-Cre* mice and utilized Cre-dependent adeno-associated virus vectors to express Gq-coupled modified GPCR, hM3Dq or diphtheria toxin fragment A in orexin neurons. By intraperitoneal injection of clozapine-N oxide in *orexin-Cre* mice expressing hM3Dq in orexin neurons, we could selectively manipulate the activity of orexin neurons. Pharmacogenetic stimulation of orexin neurons simultaneously increased locomotive activity, food intake, water intake and the respiratory exchange ratio (RER). Elevation of blood glucose levels and RER persisted even after locomotion and feeding behaviors returned to basal levels. Accordantly, 83% ablation of orexin neurons resulted in decreased food and water intake, while 70% ablation had almost no effect on these parameters. Our results indicate that orexin neurons play an integral role in regulation of both feeding behavior and metabolism. This regulation is so robust that greater than 80% of orexin neurons were ablated before significant changes in feeding behavior emerged.

1. Introduction

The neuropeptide orexin (hypocretin) has been known to be involved in feeding behavior since its early identification. In 1998, two groups independently identified this neuropeptide. One group named it "hypocretin" as they had found the peptide in the hypothalamus (De Lecea et al., 1998), and the other group named it "orexin" due to its putative role as a regulator of feeding behavior (Sakurai et al., 1998). Orexin is now known to regulate not only feeding but also various phenomena including arousal, addiction, and metabolism (Sakurai, 2007; Inutsuka and Yamanaka, 2013; De Lecea and Huerta, 2014). Although orexin neurons exist exclusively in the lateral hypothalamic area (LHA), they project their axons throughout the brain including the arcuate nucleus (Arc) where many regulators of feeding behaviors such as proopiomelanocortin and neuropeptide Y (NPY) neurons are present (Peyron et al., 1998; Nambu et al., 1999). Intracerebroventricular (ICV) injection of orexin induces feeding behavior (Sakurai et al., 1998) and water intake (Kunii et al., 1999) in rodents. In addition, injection of orexin-A into the rostral raphe pallidus produces a sustained increase in brown adipose tissue thermogenesis (Tupone et al., 2011).

Although the functions of orexin peptides have already been studied, the contribution of orexin neuronal activity to feeding behavior remains unclear. Narcolepsy is a sleep disorder characterized by primary disorganization of sleep/wakefulness cycles. In narcoleptic patients, the number of orexin neurons is greatly reduced, and orexin peptide levels in the cerebrospinal fluid are reduced to undetectable levels (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). Narcoleptic patients not only exhibit sleep fragmentation but also metabolic changes such as increases in body weight. Interestingly, mice lacking the orexin peptide develop narcolepsy-like symptoms (Chemelli et al., 1999),

Abbreviations: AAV, adeno-associated virus; Arc, arcuate nucleus; CNO, clozapine-N-oxide; DREADD, designer receptors exclusively activated by designer drugs; DTA, diphtheria toxin fragment A; ICV, intracerebroventricular; i.p., intraperitoneal; LHA, lateral hypothalamic area; NPY, neuropeptide Y; RER, respiratory exchange ratio; ZT, zeitgeber time.

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whereas orexin neuron-ablated transgenic mice develop hypophagia and late-onset obesity in addition to narcoleptic symptoms (Hara et al., 2001). Orexin neurons express not only orexins but also dynorphin (Chou et al., 2001) and glutamate (Abrahamson et al., 2001). Therefore, these differences in phenotype might suggest the involvement of other neurotransmitters besides orexin in the regulation of feeding and energy balance. Because it is unlikely that orexin peptides are the only factors released by orexin neurons *in vivo*, it is worth investigating the effects of selective manipulation of orexin neurons.

In this study, we generated a new *orexin-Cre* mouse line and employed a pharmacogenetic technique called designer receptors exclusively activated by designer drugs (DREADD) to selectively manipulate the activity of orexin neurons in free-moving mice. This method utilizes modified GPCRs to achieve selective, rapid and reversible modulation of neuronal activity. In short, muscarinic GPCRs are mutated so that their ability to bind their original ligand, acetylcholine, is lost while the synthetic ligand clozapine-N-oxide (CNO) can activate them (Armbruster et al., 2007). Gq-coupled DREADD, hM3Dq activates neurons through phospholipase C-dependent signal transduction (Alexander et al., 2009). Compared with optogenetic techniques utilizing ion channels, activation of GPCRs has a relatively longer effect on cellular signaling. Therefore, it is reasonable to employ DREADD to modulate the activity of orexin neurons for an extended time in order to examine the effects on feeding behavior and metabolism. We also selectively ablated orexin neurons using diphtheria toxin fragment A (DTA) to investigate the physiological roles of orexin neurons in feeding behaviors and metabolism.

Our results show that selective activation of orexin neurons simultaneously affects feeding behaviors and metabolism and that only 30% of orexin neurons are required to maintain such functions. These findings demonstrate the robust and concurrent regulation of feeding behaviors and wakefulness by orexin neurons.

2. Materials and methods

2.1. Animal usage

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Research Institute of Environmental Medicine, Nagoya University. Mice were maintained under a strict 12 h light: dark cycle (light period: 8:00–20:00; dark period: 20:00–8:00) in a temperature-controlled room (22 °C). Food and water were available *ad libitum* except for in the experiments shown in Fig. 4. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used.

2.2. Generation of orexin-Cre mice

The transgene was constructed with a 3.2-kb fragment of the 5′-upstream region of the human prepro-orexin gene as a promoter, Cre recombinase cDNA fused to EGFP with the 2A peptide, and the murine protamine-1 gene intron and poly(A) fragment (Fig. 1A). The transgene was excised and microinjected into pronuclei of fertilized mouse eggs (C57BL/6J mice) to generate transgenic founders. Founders were bred with C57BL/6J mice to produce stable *orexin-Cre* transgenic lines. Five transgene-positive founders were obtained from the *orexin-Cre* transgenic mice. Analysis of the N1 generation demonstrated that only two lines showed sufficient expression of Cre recombinase. The *orexin-Cre* transgenic mouse line that showed the highest Cre expression in orexin neurons was used for subsequent experiments.

2.3. Adeno-associated virus (AAV) production and purification

All AAV vectors were produced using the AAV Helper-Free System (Agilent Technologies, Inc., Santa Clara, CA, USA), and purified according to published methods (Lazarus et al., 2011). Briefly, HEK293 cells were transfected with a pAAV vector plasmid that included a gene of interest, pHelper, and pAAV-RC (serotype 10; provided by Penn Vector Core) using a standard calcium phosphate method. Three days later, the transfected cells were collected and suspended in artificial CSF (124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1.25 mM KH_2PO_4, 10 mM D-Glucose). After 4 freeze–thaw cycles, the cell lysate was treated with benzonase nuclease (Merck, Darmstadt, Germany) at 45 °C for 15 min, and centrifuged 2 times at 16,000 *g* for 10 min. The supernatant was used as the virus-containing solution. To measure the titer of purified virus dissolved in artificial CSF, quantitative PCR was performed; the virus was stored at −80 °C in aliquots before use. The pAAV-hSyn-FLEX-hM3Dq-mCherry plasmid was purchased from Addgene (ID: 44361).

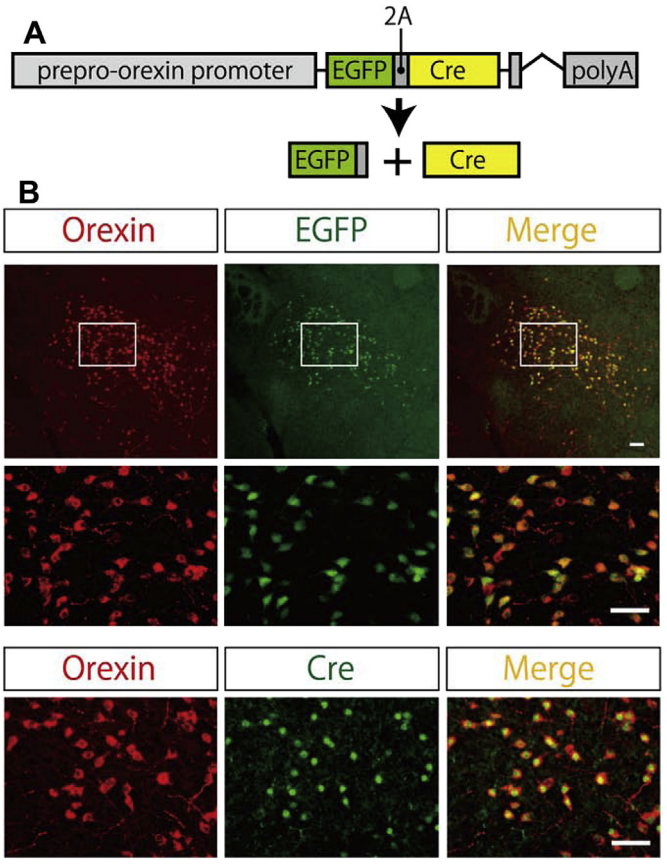

Fig. 1. Generation of *orexin-Cre* mice that express EGFP and Cre recombinase exclusively in orexin neurons. A: Schematic representation of the *orexin-Cre* transgene. To achieve orexin neuron-specific expression of Cre recombinase, we used a 3.2 kb 5′-upstream region of human prepro-orexin gene as a promoter. The transgene consists of the orexin promoter, EGFP-2A-Cre cDNA, and mouse protamine intron and poly(A) signal. Viral 2A peptide is cleaved just after translation, and EGFP and Cre recombinase localize independently. B: Specific expression of EGFP and Cre recombinase in orexin neurons. Immunohistochemistry showed specific expression of EGFP or Cre recombinase in orexin neurons in the LHA. Quantitative analysis showed 91.2 ± 0.9% ($n = 8$) colocalization of Cre recombinase with orexin. Scale bars, 100 μm (upper row) and 50 μm (middle and lower row).

2.4. Stereotaxic AAV injection

Surgeries for AAV injections were conducted under pentobarbital anesthesia (50 mg/kg, i.p.) using a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Eight-week-old mice were injected stereotaxically into the LHA with recombinant AAV-hSyn-FLEX-hM3Dq-mCherry (600 nl/injection, 3×10^{12} copies/ml) or AAV-hSyn-FLEX-diphtheria toxin A fragment (DTA) (600 nl/injection, 3×10^{12} copies/ml) with a glass micropipette and an air pressure injector system (Pneumatic PicoPump; World Precision Instruments, Inc., Sarasota, FL, USA). Injection sites were as follows: bregma 1.5 mm, lateral ±0.7 mm, ventral −5.0 mm, for AAV-hSyn-FLEX-hM3Dq-mCherry; and bregma 1.5 mm, lateral ±0.5 mm, ventral 5.2 mm and 4.9 mm; bregma 1.5 mm, lateral ±0.9 mm, ventral −5.0 mm, for AAV-CMV-FLEX-DTA. Four weeks after the AAV injection, mice were analyzed using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA).

2.5. Analysis of feeding behaviors and metabolism

Locomotion, food intake, water intake, and the RER were concurrently recorded using CLAMS. CLAMS is a set of live-in cages for automated, non-invasive, and simultaneous monitoring of horizontal and vertical activity, feeding and drinking, oxygen consumption, and CO_2 production. Twelve-week-old mice were individually placed in CLAMS cages and monitored for more than 5 days. The first 3 days were used as an acclimation period. Food and water consumption were measured directly