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Investigation of electrical activity of hypothalamic arcuate nucleus cholinergic neurons in fasting and fed state in ChAT-Cre transgenic mice

Yavuz Yavuz[a], Ozge Baser[a], Deniz Atasoy[b], Bayram Yilmaz[a,*]

[a] *Yeditepe University, Faculty of Medicine, Department of Physiology, Istanbul, Türkiye*
[b] *University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Department of Neuroscience and Pharmacology, Iowa City, IA, USA*

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Abstract

Aim: The hypothalamus consists of many subnuclei and contains neural pathways that play a significant role in feeding behavior. Cholinergic neurons in the basal forebrain have been shown to be involved in satiety activation during feeding, but the effect of hypothalamic cholinergic neurons on food intake is unclear. The aim of this study was to use optogenetics to examine the synaptic connections and electrophysiological features of acetyltransferase (ChAT) neurons in the hypothalamic arcuate nucleus (ARC), a region largely associated with feeding behavior, during fasting and satiety.

Materials and Methods: Fifteen (aged between 4-6 weeks) ChAT-Cre male mice were used in all experiments. Adeno-associated virus (AAV-GFP) was injected to specifically label ChAT neurons localized in the hypothalamic arcuate nucleus. In addition, AAV-ChR2 virus was injected for optogenetic experiment to determine the synaptic connection of these neurons with neighboring neurons. Then, fasting and fed mice were decapitated for electrophysiology recordings.

Results: Our results showed that the firing frequency in ARC-ChAT neuron recordings from fed mice was significantly higher than in fasted mice ($p<0.05$). We analyzed the synaptic connections of ARC CHAT neurons in neighboring cells by optogenetic technique. Our findings revealed that ARC CHAT neurons make heterogeneous (activation, inhibition, unchanged) synaptic connections to neighboring neurons.

Conclusion: The differential responses of arcuate cholinergic neurons in hunger and satiety suggest that these neurons may play an important role in modulating hypothalamic appetite circuits.

Introduction

The hypothalamus is a critical brain area for controlling appetite, energy expenditure, and overall body mass [1]. Within the hypothalamus, several populations of neurons have been identified that are involved in the regulation of feeding behavior [2]. Among these, cholinergic neurons have emerged as a particularly important population [3].

Cholinergic neurons in the hypothalamus are involved in the regulation of both appetite and satiety. These neurons release the neurotransmitter acetylcholine, which acts on other neurons in the hypothalamus to modulate feeding behavior [3, 4]. Studies have shown that stimulation of cholinergic neurons in the hypothalamus leads to increased food intake, while inhibition of these neurons leads to decreased food intake[5].

The effects of cholinergic neurons on feeding behavior in the hypothalamus are complex and multifaceted. These neurons have been shown to interact with other signaling molecules and neuronal populations involved in the regulation of feeding behavior, such as neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons [5, 6]. Cholinergic neurons are also thought to play a role in the regulation of reward and motivation associated with food intake [7].

Cholinergic neurons are known to make synapses with both excitatory and inhibitory connections [8, 9]. However, synaptic connections of cholinergic neurons in the arcuate nucleus of the hypothalamus with neighboring cells remain unclear. These synaptic connections are likely to play important roles in regulating food intake. Understanding the precise mechanisms by which cholinergic neurons in the hypothalamus regulate food intake may lead to the

*Corresponding author:
Email address: byilmaz@yeditepe.edu.tr (Bayram Yilmaz)

development of new therapies for the treatment of obesity and other eating disorders. Therefore, we examined the electrical activity of arcuate cholinergic neurons in fasted and fed states, as well as their synaptic connections with neighboring cells.

Materials and Methods

Mice

Fifteen male ChAT-Cre mice (#129S6, Jackson Laboratoires, Bar Harbor, ME, USA) were utilized throughout all experiments, with their average age falling between 4 and 6 weeks. Mice were housed under strict conditions that included a stable temperature of 21±2°C, a light/dark cycle of 12 hours per day, and unrestricted access to water and regular mouse food. Backcrossing (homozygote x homozygote) was used to keep the ChAT-Cre mouse line [5]. Mice were bred at Yeditepe University, Faculty of Medicine, Experimental Research Cente, an experimental research center affiliated with Yeditepe University's faculty of medicine in Istanbul, Turkey. The ethics committee for experimental animal research at Yeditepe University gave its approval to all of the methods.

Stereotaxic surgeries

Intracranial injection was administered as previously reported [10]. In a stereotaxic device, mice (P50-P60) were given isoflurane anesthesia (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed by an incision in the scalp, and a tiny injection hole was drilled. A drawn glass pipette (Drummond Scientific, Wiretrol, Broomall, PA, USA) with a 50 mm diameter tip was used to inject 300-600 nL of pAAV-FLEX-GFP and pAAV-ChR2 virus into either side of the brain. Virus injections were conducted on the ARC (AP -1.25 mm, DV -5.8 mm, and lateral 0.30 mm from bregma) using a micromanipulator (Narishige, East Meadow, NY, USA) at a rate of [11] 50 nL/min for 20 minutes each injection. After the removal of the pipette, the scalp was sutured. Animals were let at least 2–3 weeks to recuperate and express transgenic Green Fluorescent Protein (GFP) and Channelrhodopsin-2 (ChR2) before any further testing was performed.

Electrophysiology recording and optogenetic stimulations

Slice preparation was performed as described previously [12]. Briefly, mice were sacrificed and brains were immersed in N-Methyl-D-glucamine (NMDG)-HEPES containing artificial cerebrospinal fluid (aCSF) cutting solution (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2 \bullet 2H_2O$, and 10 $MgSO_4 \bullet 7H_2O$. Brain tissue was kept in 95% O_2 / 5% CO_2 aerated ice-cold cutting solution and 250 µm fresh slices containing the hypothalamus were obtained with vibratome and transferred to 95% O_2 / 5% CO_2 aerated and HEPES containing aCSF incubation solution containing (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2 \bullet 2H_2O$, and 2 $MgSO_4 \bullet 7H_2O$. The sections were incubated in this solution for at least 1h and placed in the recording chamber containing the recording aCSF (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 12.5 glucose, 5 HEPES, 2 $CaCl_2 \bullet 2H_2O$, and 2 $MgSO_4 \bullet 7H_2O$. Cell-attached loose-seal recordings were performed in voltage clamp mode and action currents were recorded from ARCChAT neurons using electrodes with 6-8 MΩ tip resistances. Recording aCSF was used as the pipette solution for cell-attached recordings. Blue light (470nm) generated by CoolLED were used to find synaptic connections. Electrophysiology cell-attach recordings were taken from cells without random hypothalamus ARCChAT neurons. MultiClamp 700B Amplifier (Molecular Devices, San Jose, CA) and Axon™ pCLAMP™ 11.3 software (Molecular Devices, San Jose, CA) were used to obtain and analyze the data.

Imaging

The mice were transcardially perfused with a 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer under anesthesia and decapitated. Brains were fixed in the same solution for 4 hours at 4°C and transferred to 30% sucrose solution and incubated overnight. Brain slices at a thickness of 70 µm were obtained by using a vibratome. Slices were then permeabilized in permeabilization buffer (% 0.1 Triton-X in PBS) for 1 h at 4°C, blocked in blocking buffer (3% BSA and 0.05% Triton-X in PBS) for 1 hour at room temperature. Slices were incubated with anti-c-Fos (1:2000, Abcam, ab190289) antibody diluted in 1% BSA and 0.05% Triton-X in PBS overnight at 4°C. The slices were washed three times for 10 minutes with PBS, incubated with the secondary antibody (anti-rabbit IgG Fab2 AlexaFlour 594, 1:2000, #8889, Cell Signalling) at room temperature. After the slices were washed with PBS, they were mounted on microscope slides using Fluoromount mounting medium (Sigma, F4680, St. Louis, MO, USA). Imaging was conducted by using a confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis

The statistical analysis were conducted by using GraphPad Prism v. 8.0 (GraphPad Software, CA, USA). All results were represented as mean ± standard deviation. Data of two independent groups were analyzed by using Student's unpaired t-test. $P<0.05$ was considered statistically significant.

Results

Electrical activity of ARC ChAT neurons associated with hunger and satiety

We investigated whether ARC^{ChAT} neurons have a role in hunger and satiety. Therefore, ARC^{ChAT} neurons in mice were first infected with FLEX-GFP virus. Mice with expected infection times (2-3 weeks) were decapitated by fasting for 16 hours. Afterwards, electrophysiology cell-attach recordings were taken in ARC^{ChAT} neurons from fasted and fed mice. Our results showed that the firing frequency in ARC^{ChAT} neuron recordings from fed mice was significantly higher than in fasted mice ($p<0.05$; Figure 1).

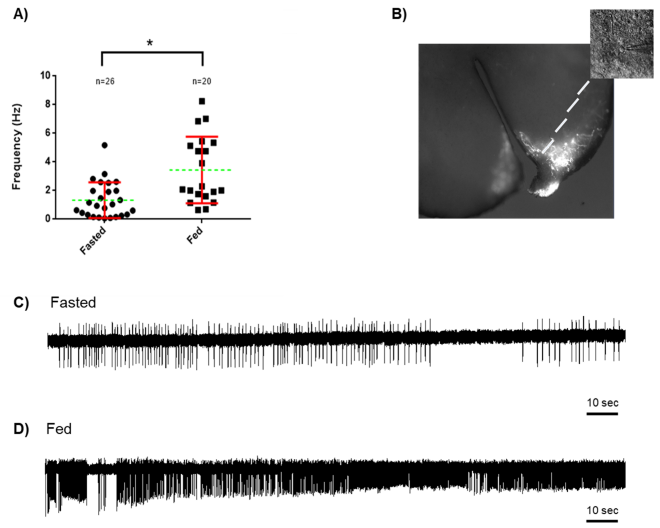

Figure 1. A) Electrophysiology recording analysis from ARC^{ChAT} neurons from fasted and fed mice. (Fasted: n=26, Fed: n=20; four mice were used in each group). All results were represented as mean ± standard deviation. Data of two independent groups were analyzed by using Student's unpaired t-test. B) Confocal image of ARC^{ChAT} neurons labeled with GFP virus (Scale bars: 100 µm). C-D) Representative electrophysiology recording traces from fasted and fed mice.

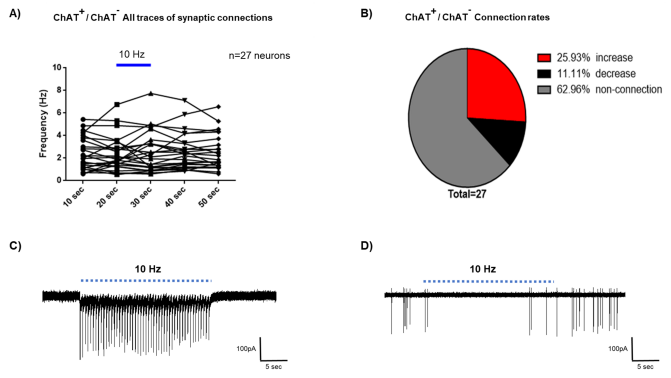

Figure 2. A) Electrophysiology recording traces from non-ChATneurons and optogenetic stimulated ChAT neurons. (n=27 neurons / 7 mice). B) Synaptic connection ratio graph of ARC^{ChAT} neurons with neighboring cells. C) Representative traces of cell-attach recordings from ARC non-ChAT neurons during ARC^{ChAT} axonal photostimulation. C-D) Representative electrophysiology recordings for activation and inhibition recordings.

Optogenetic analysis of synaptic connection of ARCChAT neurons to neighboring cells

The electrical activities of ARC^{ChAT} neurons changed during the fasting and satiety phases. We also investigated the synaptic connections of these neurons with neighboring cells. Therefore, ARC^{ChAT} neurons in mice were first infected with FLEX-ChR2 virus. Likewise, mice were decapitated after the infection period. Electrophysiology recordings were taken from non-ChAT neurons from the arcuate nucleus of the hypothalamus. After sealing non-ChAT neurons, the ChAT neuron was stimulated (470nm) by using optogenetic technique. Electrophysiology recording was taken from each neuron for 1 minute using the loose recording technique, with 10 Hz optogenetic stimulation between 10 and 20 seconds. We showed whether there was a change in firing frequency in ARC non-ChAT neurons during optogenetic stimulation of ARC-ChAT neurons (Figure 2A). Our results showed that ARC^{ChAT} neurons make heterogeneous synaptic connections to neighboring neurons. Synaptic activation was measured in these connections (Figure 2B), which increased by 26%, decreased by 12%, and remained unchanged by 63%.

Discussion

In this study, we investigated the electrical and synaptic properties of ARC^{ChAT} neurons during hunger and satiety. Our study showed that the electrical activity of ARC^{ChAT} neurons changed during hunger and satiety. The firing frequency of these neurons was higher in fed mice than in fasted mice. Recent studies have shown that hypothalamic ChAT neurons overlap more with anorexigenic POMC neurons than orexigenic AgRP neurons [13]. The high electrical activity of these neurons in the state of satiety may be the effect of the leptin hormone secreted from the adipose tissue. In addition, it has been shown to modulate the activity of cholinergic neurons indirectly through its effects on other neurons and signaling pathways involved in the regulation of feeding behavior [14]. Leptin is thought to reduce hunger by inhibiting AgRP and stimulating POMC neurons in the hypothalamus [15]. Increased ARC^{ChAT} neuron firing frequency in fed mice may be directly or indirectly affected by hormones, peptides or neurotransmitters. Cholinergic neurons are found in a number of distinct areas of the brain, and the activation of their receptors plays a significant part in a variety of processes and actions involving the brain [7]. In addition, the synapses of cholinergic neurons are known to have both excitatory and inhibitory connections [8]. A study showed that optogenetic stimulation of axon terminals of dorsomedial hypothalamic ChAT neurons to the ARC nucleus increases food intake by enhancing GABAergic transmission to ARC POMC neurons [5]. In contrast, stimulation of diagonal band of Broca ChAT neuron terminals in the hypothalamus suppresses food intake [3]. These studies show that cholinergic neurons and their projections in the brain have different effects on food intake. We investigated the synaptic effects of ARC^{ChAT} neurons with neighboring neurons. According to the findings, synaptic connections between ARC^{ChAT} neurons and nearby cells are formed in a variety of ways.

Taken together, these findings suggest that cholinergic neurons in the arcuate nucleus can be an important component of the hypothalamic appetite circuits, playing a crucial role in the regulation of feeding behavior. Understanding these mechanisms could lead to the development of novel therapeutic strategies for the treatment of obesity and other eating disorders.

Conclusion

The firing frequency in ARC^{ChAT} neuron recordings from fed mice was significantly higher than in fasted mice. In addition, these neurons make heterogeneous synaptic connections to neighboring neurons. Included in the control of food behavior, cholinergic neurons in the arcuate nucleus may be a significant part of the hypothalamic appetite circuits.

Ethical approval

Experimental procedures were approved by the Yeditepe University experimental animal research ethics committee (19.02.2018-650).

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