MOLECULAR
METABOLISM

Cholinergic neurons in the dorsomedial hypothalamus regulate food intake

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ABSTRACT

Objective: Central cholinergic neural circuits play a role in the regulation of feeding behavior. The dorsomedial hypothalamus (DMH) is considered the appetite-stimulating center and contains cholinergic neurons. Here, we study the role of DMH cholinergic neurons in the control of food intake.

Methods: To selectively stimulate DMH cholinergic neurons, we expressed stimulatory designer receptors exclusively activated by designer drugs (DREADDs) and channelrhodopsins in DMH cholinergic neurons by injection of adeno-associated virus (AAV) vectors into the DMH of choline acetyltransferase (ChAT)-IRES-Cre mice. We also generated transgenic mice expressing channelrhodopsins in cholinergic neurons with the Cre-LoxP technique. To delete the *Chat* gene exclusively in the DMH, we injected an AAV carrying a Cre recombinase transgene into the DMH of floxed ChAT mice. Food intake was measured with and without selective stimulation of DMH cholinergic neurons.

Results: Mice lacking the *Chat* gene in the DMH show reduced body weight as compared to control. Chemogenetic activation of DMH cholinergic neurons promotes food intake. This orexigenic effect is further supported by experiments of optogenetic stimulation of DMH cholinergic neurons. DMH cholinergic neurons innervate pro-opiomelanocortin neurons in the arcuate nucleus of the hypothalamus (ARC). Treatment with acetylcholine (ACh) enhances GABAergic inhibitory transmission to ARC POMC neurons that is blocked by the muscarinic receptor antagonist. Direct activation of cholinergic fibers in the ARC readily stimulates food intake that is also abolished by the muscarinic receptor antagonist.

Conclusion: ACh released from DMH cholinergic neurons regulates food intake and body weight. This effect is mediated in part through regulation of ARC POMC neurons. Activation of muscarinic receptors on GABAergic axon terminals enhances inhibitory tone to ARC POMC neurons. Hence, this novel $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway plays an important role in the control of food intake and body weight.

1. INTRODUCTION

The cholinoceptive system in the hypothalamus is important in the control of food intake. Activation of nicotinic acetylcholine receptors (nAChRs) reduces food intake via modulation of melanocortinergic neurons in the arcuate nucleus of the hypothalamus (ARC) [1,2]. Nicotine excites anorexigenic pro-opiomelanocortin (POMC) neurons through activation of β4-containing nAChRs [1]. In addition, ARC POMC neurons receive cholinergic input [2], suggesting that endogenous acetylcholine (ACh) can directly or indirectly influence POMC neuron activity as well. Interestingly, it has been shown that mice lacking the M3 muscarinic acetylcholine receptor (mAChR) consume less food and have lower body weight [3,4]. Hence, activation of hypothalamic nicotinic and muscarinic receptors can induce opposing effects on food intake by differentially regulating the melanocortinergic system in the ARC.

Despite accumulated evidence for the physiological roles of the cholinoceptive system in the control of food intake, the origin of cholinergic neurons remains elusive. Recently, it has been shown that optogenetic stimulation of cholinergic neurons in the diagonal band of Broca reduces food intake through activation of ARC POMC neurons [5], indicating that cholinergic basal forebrain regulates feeding. It has been also shown that the hypothalamus contains cholinergic cell groups [6,7]. In particular, there exist cholinergic neurons in the dorsomedial hypothalamus (DMH) [6,8—10]. The DMH has long been considered the appetite-stimulating center as lesions of the DMH reduce food intake [11]. Our prior study describes that overnight fasting induces expression of c-fos protein, an indirect marker of neuronal activity, in DMH cholinergic neurons [9], consistent with the fact that DMH cholinergic neurons have the ability to respond to changes in nutrient status. These prior findings suggest that DMH cholinergic neurons are part of the neural circuitry that regulates feeding behavior.

DMH cholinergic neurons project to diverse brain structures, including the ARC [10]. We thus examined whether stimulation of the $DMH^{ACh} \rightarrow ARC$ pathway is able to regulate food intake. We manipulated cholinergic neuronal activity *in vivo* using chemogenetic and optogenetic methods. We found that increased cholinergic neuron activity promoted food intake. DMH cholinergic neurons sent

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projections to ARC POMC neurons. ARC POMC neurons received GABAergic input that was positively regulated by muscarinic receptors. Direct stimulation of cholinergic axon terminals in the ARC also increased food intake. This orexigenic effect was completely abolished by the muscarinic receptor antagonist. Hence, the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway may play a key role in regulating energy intake.

2. MATERIALS METHODS

2.1. Animals
All mouse care and experimental procedures were approved by the institutional animal care research advisory committee of the Albert Einstein College of Medicine. Mice used in these experiments included ChAT-IRES-Cre (stock# 006410), floxed ChAT (stock# 016920), floxed channelrhodopsin (ChR2)-YFP (stock# 012569), and POMC-GFP mice (stock# 009593) (The Jackson Laboratory). 2-month-old male and female mice on a mixed C57BL6/129SVJ background were used for all experiments. Animals were housed in groups in cages under conditions of controlled temperature (22 °C) with a 12:12 h light—dark cycle and fed a standard chow diet with *ad libitum* access to water.

2.2. Stereotaxic surgery and bilateral injections of AAV vectors
2-month-old ChAT-IRES-Cre mice were anesthetized with 2% isoflurane and placed into a stereotaxic apparatus. To delete the *Chat* gene in DMH cholinergic neurons, we bilaterally injected AAV5-hSyn-mCherry (control) or AAV5-hSyn-mCherry-Cre viruses (250 nl of 3×10^{12} pfu/ml per side; UNC vector core) into the DMH (AP: −1.95 mm, DV: −5.0 mm, ML: ±0.25 mm) of a floxed mutant that possesses loxP sites flanking exons 3 and 4 of the *Chat* gene (ChAT^*flox/flox*; The Jackson Lab). To map the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway, we bilaterally injected a Cre-dependent anterograde long-distance viral tracer with spaghetti monster (sm) fluorescence protein (smFP) (AAV1.CAG.Flex.GFPsm-Flag.WPRE.SV40 (AAV1-Flex-GFPsm); UPenn Vector core) into the DMH of the ChAT-IRES-Cre mice (200 nl of 1.36×10^{13} pfu/ml per side).
An AAV5 carrying a Cre-dependent ChR2 transgene (AAV5-EF1-dflox-hChR2(H134R)-mCherry, 200 nl of 1.0×10^{12} pfu/ml per side) or an AAV5 carrying a Cre-dependent $hM_3D(Gq)$-mCherry transgene (AAV5-hSyn-DIO-hM_3D(Gq)-mCherry, 200 nl of 1.8×10^{12} pfu/ml per side; UNC vector core) was injected into the DMH of the ChAT-IRES-Cre animals to selectively stimulate DMH cholinergic neurons. Experiments were performed at 4 weeks post viral injection.

2.3. Manipulation of DMH cholinergic neuron activity and measurement of food intake
A mono fiber-optic cannula was implanted into the area just above the 3rd ventricle (AP: −1.95 mm, DV: −4.5 mm, ML: 0 mm) of ChAT-Cre::ChR2-YFP mice. Optic fibers were coupled to a 473 nm DPSS laser. We also used an optic-fluid cannula that guides the optogenetic fiber and restricts liquid delivery around the fiber tip (Doric lenses, Inc). With this cannula, we optogenetically stimulated cholinergic fibers and applied drugs directly into the ARC (AP: −1.4 mm, DV: −5.8 mm, ML: 0 mm). The cholinergic antagonists (1 μl; 4-DAMP and mecamylamine from Sigma—Aldrich) were administered into the ARC 30 min prior to optogenetic stimulation. Clozapine-N-oxide (CNO, 0.3 mg/g [13]) or saline was intraperitoneally administered 1 h prior to measurement of feeding. To measure food intake, animals were separated individually in single cages for 5 days and fasted for overnight. Food intake was measured on a scale with 0.1 g accuracy.

2.4. Electrophysiological recordings
Animals were anesthetized with isoflurane. After decapitation, the brain from POMC-GFP mice was transferred into a sucrose-based solution bubbled with 95% O_2/5% CO_2 and maintained at ∼3 °C. This solution contained the following (in mM): 248 sucrose, 2 KCl, 1 $MgCl_2$, 1.25 KH_2PO_4, 26 $NaHCO_3$, 1 sodium pyruvate, and 10 glucose. Transverse coronal brain slices (200 μm) were prepared using a vibratome. Slices were equilibrated with an oxygenated artificial cerebrospinal fluid (aCSF) for >1 h at 32 °C before transfer to the recording chamber. The slices were continuously superfused with aCSF at a rate of 1.5 ml/min containing the following (in mM): 113 NaCl, 3 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 5 glucose in 95% O_2/5% CO_2.
Brain slices were placed on the stage of an upright, infrared-differential interference contrast microscope mounted on a Gibraltar X-Y table and visualized with a 40× water-immersion objective by infrared microscopy. The internal solution contained the following (in mM): 130 KCl, 5 $CaCl_2$, 10 EGTA, 10 HEPES, 2 MgATP, 0.5 Na_2GTP, and 5 phosphocreatine. All recordings were made at 30 ± 2 °C. GABAergic synaptic currents were recorded with a Multiclamp 700B in whole cell configuration in the presence of CNQX (10 μM) and DL-APV (50 μM).

2.5. Immunohistochemistry
Mice were anesthetized with isoflurane and perfused transcardially with PBS. Brains were incubated in 4% paraformaldehyde overnight at 4 °C. The rostral to caudal extension of the hypothalamus was cut in 50 μm coronal sections with a vibratome. Sections were blocked with 5% bovine serum albumin at room temperature and then incubated with anti-POMC (1:1000; Phoenix pharmaceuticals, H-029-30), anti-GFP (1:1000, AbCam, ab13970) and anti-DsRed or anti-RFP (1:1000, Clontech, 632496; 1:1000; ThermoFisher, MA5-15257) antibodies diluted in PBS containing 0.5% Triton X-100 at 4 °C. After incubation in primary antibodies, sections were washed 3 times in PBS and then incubated with Alexa 568 anti-rabbit IgG (1:500, Life Technologies, A10042), Alexa 568 anti-mouse IgG (1:500, Life Technologies, A10037), Alexa 488 anti-rabbit IgG (1:500, Life Technologies, A11008), Alexa 488 anti-chicken IgG (1:500, AbCam, ab150169) diluted in 0.5% Triton X-100 in PBS for 3 h at room temperature. Tissues were then washed in PBS, dried and mounted with VECTA-SHIELD mounting media. Images were acquired using a scanning confocal microscope.

2.6. Statistics
Statistical analyses were performed using a paired or unpaired t-test as noted (GraphPad Prism software). Data are expressed as mean ± SEM. Results with $p < 0.05$ were considered significant.

3. RESULTS

3.1. ACh in the DMH regulates body weight
We first examined whether ACh in the DMH regulates body weight. To this end, we deleted the *Chat* gene exclusively in the DMH by bilateral injection of AAV5-Cre into the DMH of ChAT^*flox/flox* mice (Figure 1A and Supplementary Figure 1) as described in our recent study [10]. We found that male mice lacking the *Chat* gene in the DMH significantly gained less weight than did control mice on a chow diet (Figure 1B, AAV5-con, n = 12 mice; AAV5-Cre, n = 12 mice). Although female mice showed a trend for decreased body weight as well, there was no significant difference between mice infected with AAV5-control or AAV5-Cre (Figure 1B, AAV5-con, n = 11 mice; AAV5-Cre, n = 11
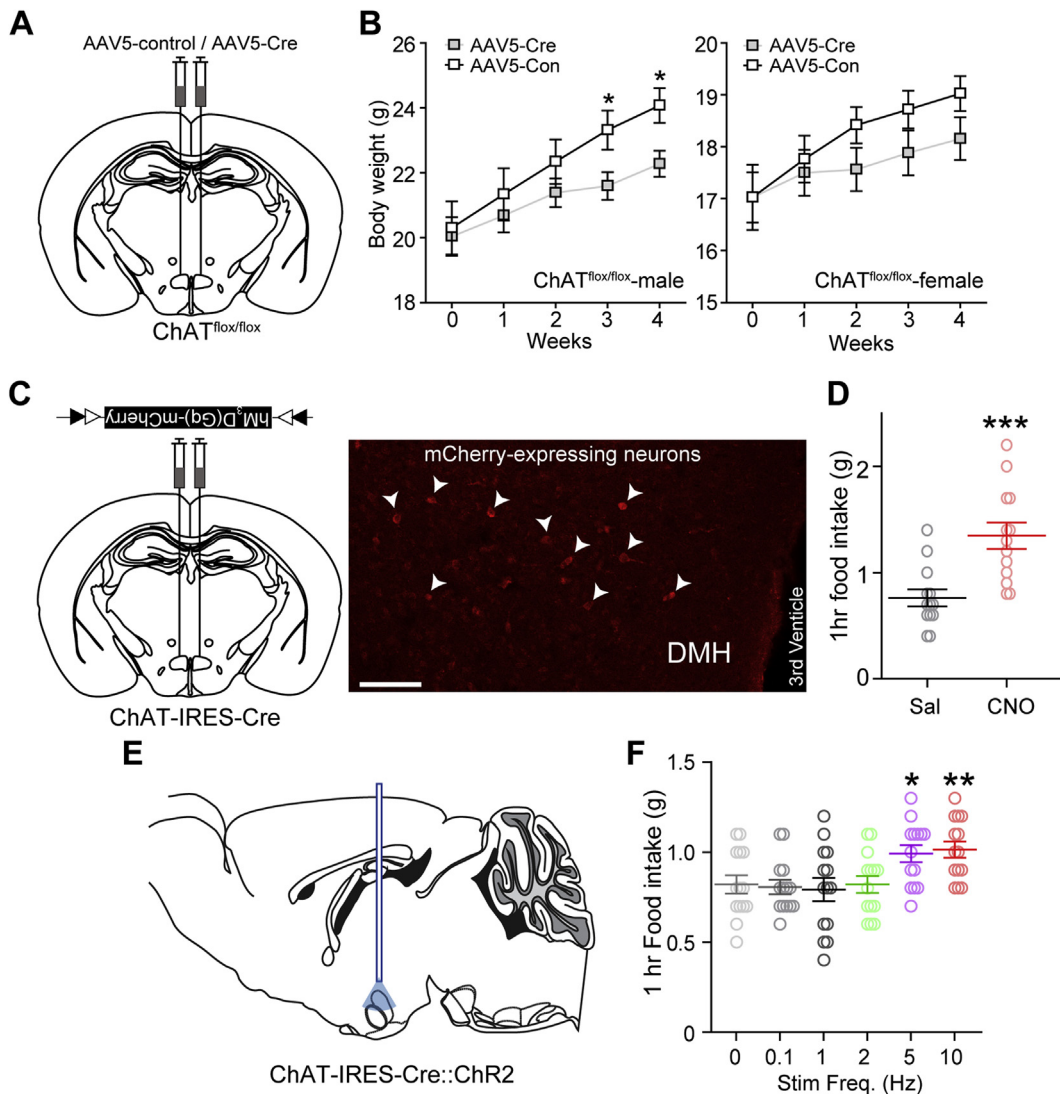
Figure 1: Activation of DMH cholinergic neurons promotes food intake. **A**. Schematic drawing showing injection of AAV vectors into the DMH of ChAT$^{flox/flox}$ mice. **B**. Summary plots showing effect of loss of DMH cholinergic function on body weight in mice. Male mice lacking the *Chat* gene in the DMH showed a significant reduction in body weight (AAV5-control, n = 12 mice, AAV5-Cre, n = 12 mice, *p < 0.05, unpaired *t*-test). There was no significant difference in female mice (AAV5-control, n = 11 mice, AAV5-Cre, n = 11 mice, p > 0.05, unpaired *t*-test). **C**. Expression of G$_q$-DREADDs in the DMH cholinergic neurons. Left panel: Schematic drawing showing injection of AAV5-hM$_3$Dq-mCherry viruses into the DMH of the ChAT-IRES-Cre animals. Image of fluorescence microscopy showing expression of mCherry in DMH cholinergic neurons (right panel, arrowheads). Scale bar: 100 μm. **D**. Pooled data from 14 mice showing effect of chemogenetic activation of DMH cholinergic neurons on food intake. Treatment with CNO significantly increased food intake (n = 14 animals; ***p < 0.001, unpaired *t*-test). **E**. Schematic drawing showing optogenetic stimulation of DMH cholinergic neurons of ChAT-IRES-Cre::ChR2 mice. **F**. Summary plots showing that optogenetic stimulation of DMH cholinergic neurons significantly promoted food intake for 1 h (n = 14 animals for each frequency, *p < 0.05, **p < 0.01, unpaired *t*-test).

mice). These findings suggest that ACh in the DMH cholinergic neurons is required to maintain body weight.

3.2. Increased DMH cholinergic neuron activity promotes food intake

Given loss of the *Chat* gene in the DMH resulted in reduced body weight, we sought to determine whether increased cholinergic neuron activity promotes feeding. Stimulatory designer receptors exclusively activated by designer drugs (DREADDs) were expressed in DMH cholinergic neurons by injection of AAV5-hM$_3$D(Gq)-mCherry into the DMH of the ChAT-IRES-Cre animals (Figure 1C). At 4 weeks post viral injection, DMH cholinergic neurons were labeled with an anti-mCherry antibody, consistent with expression of DREADDs in DMH cholinergic neurons (Figure 1C). To activate DMH cholinergic neurons, mice infected with AAV5-hM$_3$D(Gq)-mCherry in the DMH were administrated with CNO. We found that treatment with CNO significantly increased food intake for 1 h (Figure 1D, n = 14 mice).

In the next set of experiments, we expressed light-activated proteins in DMH cholinergic neurons by crossing the ChAT-IRES-Cre mice with the floxed channelrhodopsin animals (Figure 1E) [10]. Bursts of light pulses were applied for 1 s followed by a 3 s break that repeated continuously for 1 h. In the burst, frequency (Hz) was varied (0, 0.1, 1, 2, 5, 10 Hz). We found that optogenetic stimulation of DMH cholinergic neurons promoted food intake in a frequency-dependent manner (Figure 1F). In other words, low-frequency stimulation (i.e. less than 2 Hz) had no effect on food intake during stimulation. However, mice showed a

significant increase in food intake during stimulation when DMH cholinergic neurons were optogenetically stimulated at 5 and 10 Hz (Figure 1F and Supplementary Figure 2, n = 14 mice). These chemogenetic and optogenetic results indicate that DMH cholinergic neurons have the ability to drive feeding in mice.

3.3. DMH cholinergic neurons project to ARC POMC neurons

We then examined the cellular mechanisms underlying the orexigenic effect of DMH cholinergic neuron stimulation. Our prior study showed that DMH cholinergic neurons sent projections to the ARC [10]. These findings suggest that neurons in the ARC are a downstream target of
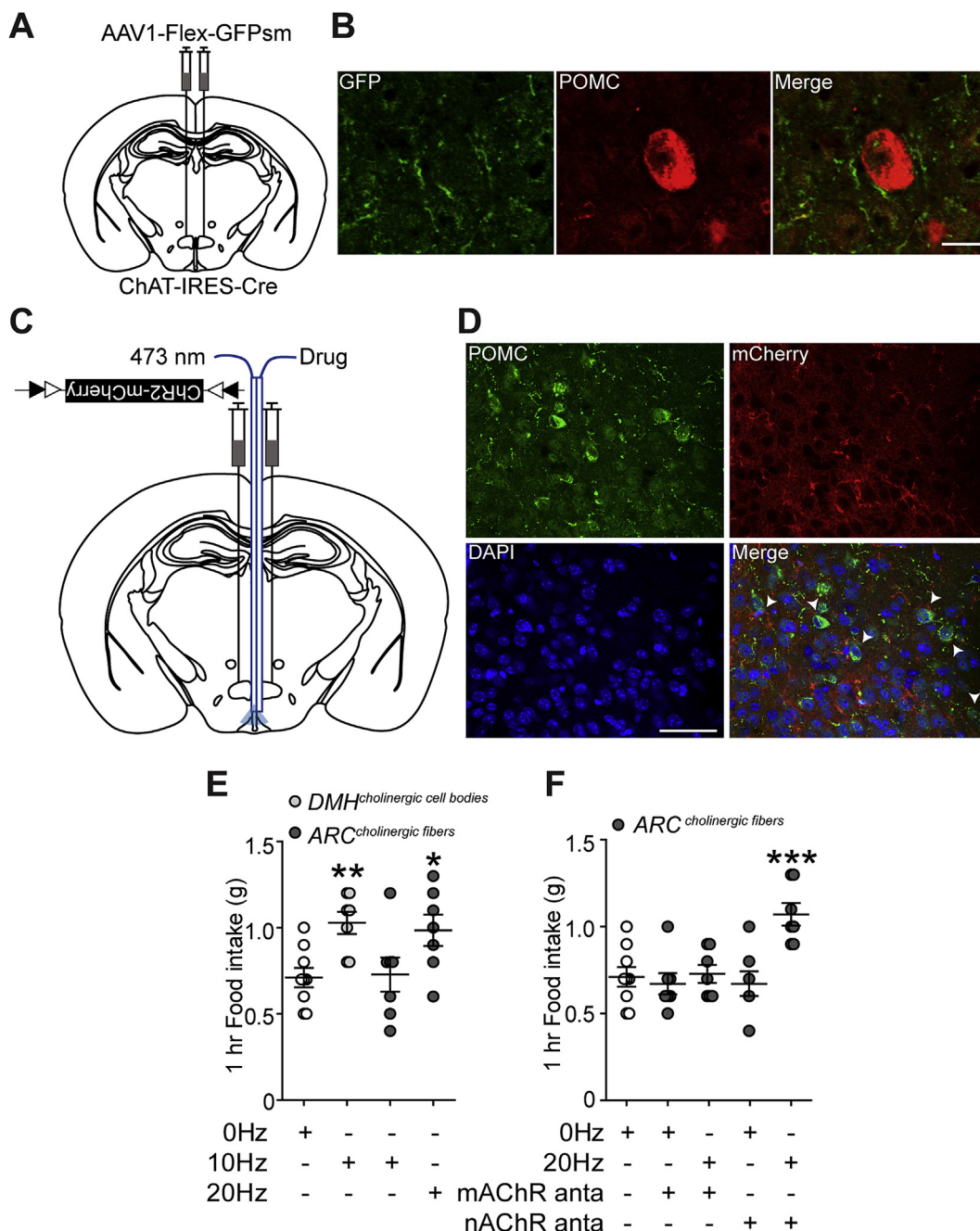
Figure 2: Stimulation of cholinergic fibers in the ARC increases feeding. A. Schematic drawing of our experimental configuration. AAV1-FLEX-GFPsm viruses were bilaterally injected into the DMH of ChAT-IRES-Cre mice. B. Images of fluorescence microscopy showing that POMC (red) neurons in the ARC received cholinergic input form DMH cholinergic neurons. Scale bar: 10 μm. C. Schematic drawing of our experimental configuration. AAV5-ChR2-mCherry viruses were bilaterally injected into the DMH of ChAT-IRES-Cre mice. An optic-fluid cannula was implanted to the ARC to optogenetically stimulate cholinergic fibers and apply drugs directly to the ARC. D. Images of fluorescence microscopy showing that mCherry (red)-positive fibers innervate ARC POMC (green) neurons, implying that a subset of POMC neurons receives cholinergic input from the DMH (arrowheads). Scale bar: 50 μm. E. Pooled data from n = 7 animals showing effect of optogenetic stimulation of cholinergic fibers in the ARC. Although 10 Hz stimulation of cholinergic fibers in the ARC had no effect, 20 Hz stimulation readily promoted food intake for 1 h (*p < 0.05, **p < 0.01, unpaired t-test). F. Pooled data from 7 mice showing blockade of the effect of cholinergic fiber stimulation by the muscarinic receptor antagonist. Local infusion of the muscarinic, but not nicotinic, receptor antagonist to the ARC completely blocked the effect of optogenetic stimulation of ARC cholinergic fibers (food intake for 1 h; n = 7 animals). (***p < 0.001, unpaired t-test).

DMH cholinergic neurons. To obtain evidence for the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway, we used a Cre-dependent anterograde viral tracer with spaghetti monster (sm) fluorescence protein (smFP) (AAV1-Flex-GFP2sm; Figure 2A). This viral vector induces intense cytoplasmic staining at the injection sites and labels long-range projections, which permits mapping of local neural circuits [12]. Mice injected with AAV1-Flex-GFPsm into the DMH of the ChAT-IRES-Cre mice showed GFP-positive fibers in the ARC (Figure 2B). Importantly, ARC POMC neurons received synaptic input from DMH cholinergic neurons (Figure 2B).

To further examine the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway, we bilaterally injected a Cre-dependent ChR2 (AAV5-ChR2-mCherry) into the DMH of ChAT-IRES-Cre mice (Figure 2C). As shown in Figure 2D, mCherry-positive fibers were present in the ARC of mice infected with AAV5-ChR2-mCherry. Importantly, double immunostaining revealed that ARC POMC neurons received cholinergic input originated in the DMH (Figure 2D), suggesting that the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway is part of the DMH cholinergic neural circuitry that regulates feeding behavior.

3.4. Activation of muscarinic receptors in the ARC increases food intake

To examine the effect of the $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway on food intake, we directly stimulated cholinergic fibers originated in the DMH. We injected an AAV carrying a Cre-dependent ChR2 transgene into the DMH of ChAT-IRES-Cre mice and implanted an optic fiber into the ARC (Figure 2C). In contrast to the effect of stimulation of DMH cholinergic cell bodies (Figure 2E), we found that optogenetic stimulation of cholinergic axon terminals at 10 Hz for 1 h had no effect on food intake (Figure 2E). However, increased frequency to 20 Hz from 10 Hz readily promoted feeding (Figure 2E; n = 7 mice). To further examine the cellular mechanisms underlying the orexigenic effect, we locally infused drugs via an optic-fluid cannula prior to optogenetic stimulation. Although the cholinergic receptor antagonists (4-DAMP and mecamylamine) alone did not alter food intake (Figure 2F), we found that the orexigenic effect of optogenetic stimulation of cholinergic axon terminals was completely abolished by treatment with the muscarinic receptor antagonist (4-DAMP, 10 μM), while the nicotinic receptor antagonist had no effect (mecamylamine, 10 μM; Figure 2F). These findings suggest that activation of muscarinic receptors by ACh released from DMH cholinergic fibers drive feeding.

3.5. Activation of muscarinic receptors enhances GABAergic inhibitory tone to ARC POMC neurons

It has been described that GABAergic input to ARC POMC neurons plays a crucial role in the regulation of food intake [14]. We examined the electrophysiological impact of ACh on POMC neurons in slice preparations. Recordings from ARC POMC neurons revealed that ARC POMC neurons received GABAergic synaptic input that was regulated by ACh. Exogenous application of ACh (1 mM) strongly increased the frequency of spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) to POMC neurons (Figure 3A; n = 9 neurons). This GABAergic enhancement was completely blocked by the muscarinic receptor antagonist (Figure 3B; n = 8 neurons). In addition, treatment with muscarine (10 μM) increased the frequency of miniature GABAergic IPSCs (mIPSCs) in the presence of tetrodotoxin (TTX, 500 nM; Supplementary Figure 3). These results indicate that activation of presynaptic mAChRs elevates GABAergic inhibitory tone to ARC POMC neurons, thereby indirectly controlling the excitability of POMC neurons. Therefore, both *in vitro* and *in vivo* experiments suggest the involvement of muscarinic rather than nicotinic receptors in the control of feeding (Figure 3C).
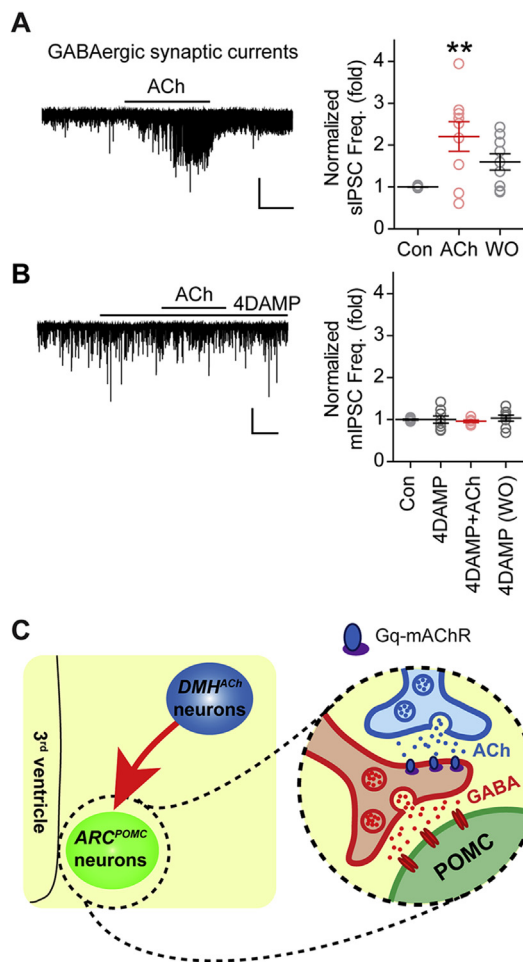
Figure 3: Activation of muscarinic receptors enhances GABAergic transmission to ARC POMC neurons. A. Representative recording sample showing effect of ACh on GABAergic IPSCs. Exogenous application of ACh (1 mM) robustly increased GABA transmission to POMC neurons (left panel). Right panel: Pooled data from 9 neurons showing effect of ACh on GABAergic sIPSCs (mean frequency, control 1.6 ± 0.3 Hz, ACh, 3.7 ± 0.7 Hz, **p < 0.01, paired *t*-test). Wo: wash-out. Scale bars: 200 pA, 2 min. B. Representative recording sample showing blockade of the effect of ACh by the muscarinic receptor antagonist (left panel). The mAChR antagonist (4-DAMP, 1 μM) completely blocked the effect of ACh (right panel; n = 8 neurons, p > 0.05, paired *t*-test). Scale bars: 100 pA, 2 min. C. Schematic illustration of our proposed model. DMH cholinergic neurons innervate ARC POMC neurons. ACh released from DMH cholinergic neurons enhances GABAergic inhibitory tone to ARC POMC neurons through activation of presynaptic muscarinic receptors. Activation of the direct $DMH^{ACh} \rightarrow ARC^{POMC}$ pathway drives feeding in mice.

4. DISCUSSION

Our present study outlined the cellular mechanisms underlying the orexigenic effect of ACh released from DMH cholinergic neurons in mice. We found that mice lacking the *Chat* gene in the DMH cholinergic neurons had lower body weight, consistent with the necessity of ACh to maintain body weight. We also demonstrated that elevated DMH cholinergic neuron activity promoted food intake, suggesting the sufficiency of DMH cholinergic neurons to recapitulate a hunger state. In addition, we found that DMH cholinergic neurons sent projections to anorexigenic POMC neurons in the ARC. Activation of muscarinic receptors on GABAergic axon terminals enhanced inhibitory tone to ARC POMC neurons. Optogenetic stimulation of cholinergic fibers in the ARC

promoted food intake that was completely blunted by the muscarinic receptor antagonist. Therefore, the ACh-induced increase in food intake would be explained in part by muscarinic receptor-mediated GABAergic enhancement onto ARC POMC neurons.

Early lesion studies describe a critical role of the DMH in the control of body weight and food intake [11]. In addition, it has been shown that optogenetic activation of glucose-responsive GABAergic neurons in the DMH increases food intake [15]. These studies suggest that the DMH contains distinct cell groups that drive feeding. Also, it has been described that there are ACh-, prolactin-releasing peptide (PrRP)-, neuropeptide Y (Npy)-, and thyrotropin-releasing hormone (TRH)-containing neurons in the DMH [9,10,16—18]. Interestingly, ACh, Npy, PrPR, and leptin receptor-expressing neurons in the DMH mainly contribute to brown adipose tissue thermogenesis [10,16,17,19] and TRH neurons in the DMH control circadian rhythms of sleep-wake behavior in rodents [18].

The cholinoceptive system in the hypothalamus, in particular in the ARC, plays a role in the regulation of feeding behavior [1,20]. The neurons in the ARC appear to be a downstream target of central cholinergic cell groups [2,5,10]. Central cholinergic cell groups are found predominantly in the basal forebrain and brainstem regions [7]. Thus, it is possible that neurons in the ARC receive cholinergic inputs from these areas. Indeed, a recent study clearly demonstrated that mice lacking the *Chat* gene in the basal forebrain exhibit reduced *Pomc* transcript levels and hyperphagia [5]. More importantly, selective activation of cholinergic fibers originating in the basal forebrain reduces food intake possibly via activation of nAChRs on POMC neurons in the ARC [5]. This is consistent with the prior study showing the anorexigenic effect of nicotine [1]. It appears, therefore, that both endogenous ACh and exogenous nicotine have the ability to reduce food intake through nAChRs on ARC POMC neurons [1,5]. In addition to the expression of nAChRs, there also exists expression of mAChRs in the ARC [21]. Muscarinic receptors, particularly the type 3 (M3), appear to be important in the control of feeding as M3 receptor-deficient mice show hypophagia [3]. Thus, It is possible that, in contrast to the anorexigenic effect of nicotine, endogenous ACh could promote food intake by activating muscarinic receptors in the ARC. Our study supports this possibility as ACh released from DMH cholinergic neurons promoted feeding through activation of muscarinic receptors.

The ARC appears to be one of the downstream targets of DMH cholinergic neurons [10]. Importantly, our current study showed that DMH cholinergic neurons innervated ARC POMC neurons. Since cholinergic cell groups in the basal forebrain send projections to ARC POMC neurons as well [5], it appears that ARC POMC neurons are key players implicated in the cholinergic regulation of feeding. Interestingly, the anorexigenic effect of nicotine is mediated by α3β4-containing postsynaptic nicotinic receptors on POMC neurons [1]. In fact, activation of presynaptic nicotinic receptors on glutamatergic terminals does not modulate glutamatergic excitatory transmission to ARC POMC neurons [2]. These prior findings suggest that nicotine depolarizes ARC POMC neurons directly rather than indirectly. In addition to direct action of nicotine, we found that activation of presynaptic muscarinic receptors on GABAergic axon terminals enhanced spontaneous GABAergic inhibitory transmission onto POMC neurons, consistent with the fact that POMC neuron activity can be regulated by either postsynaptic nicotinic or presynaptic muscarinic receptors, which may differently regulate POMC neuron activity.

These findings raise the question of how ACh released from cholinergic axon terminals activates either nicotinic or muscarinic receptors, or both, at synapses on ARC POMC neurons. It has been shown that central cholinergic neurotransmission predominantly alters presynaptic

release of neurotransmitters [22—25]. Importantly, phasic release of ACh exerts its effects at some distance from the release sites by "volume transmission" [26,27]. In this case, only high levels of ACh can directly activate postsynaptic ACh receptors. In this regard, the degree and/or pattern of cholinergic neuron activity would be a critical component that determines the nature of cholinergic signaling. Indeed, 20 Hz phasic stimulation of cholinergic axon terminals for 1 s was sufficient to increase food intake in our current study. However, 20 Hz stimulation of cholinergic fibers for 5 s, which may result in higher levels of ACh at synapses, induces the opposite effect on food intake [5]. Interestingly, this anorexigenic effect was only partially abolished by the nicotinic receptor antagonist [5], suggesting the involvement of receptors other than nicotinic. In our preparations, the orexigenic effect was completely blocked by the muscarinic receptor antagonist, whereas the nicotinic receptor antagonist had no effect. Therefore, levels of ACh at synapses to POMC neurons appear to play a critical role in determining the nature of cholinergic outflow (nicotinic vs. muscarinic components). In addition, it is also possible that distinct subsets of ARC POMC neurons receive different cholinergic inputs. In fact, ARC POMC neurons are neurochemically heterogenous [21,28] and activation of POMC neurons either promotes or reduces feeding [29,30].

5. CONCLUSION

Our previous study showed that overnight fasting increased DMH cholinergic neuron excitability [9], consistent with the fact that DMH cholinergic neurons have the ability to respond to the availability of nutrients. Our current study further demonstrated that increased DMH cholinergic neuron activity was able to drive feeding in mice. This orexigenic effect was mediated in part through elevated inhibitory tone to ARC POMC neurons. Muscarinic rather than nicotinic receptors contributed to the control of GABAergic transmission to ARC POMC neurons. The melanocortin system was a critical downstream target of DMH cholinergic neurons. As we showed that the DMH^{ACh} → Raphe pallidus^{5-HT} pathway was important in regulating energy expenditure [10], ACh released from DMH cholinergic neurons could regulate both energy intake and expenditure through two independent neural pathways. Therefore, DMH cholinergic neurons may play a critical role in the regulation of overall energy balance.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.01.001.

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