Original article

MOLECULAR METABOLISM

Cholinergic neurons in the dorsomedial hypothalamus regulate mouse brown adipose tissue metabolism

Jae Hoon Jeong [1], Dong Kun Lee [1], Clemence Blouet [2], Henry H. Ruiz [3,4], Christoph Buettner [3,4], Streamson Chua Jr. [1], Gary J. Schwartz [1], Young-Hwan Jo [1,5,*]

ABSTRACT

Objective: Brown adipose tissue (BAT) thermogenesis is critical in maintaining body temperature. The dorsomedial hypothalamus (DMH) integrates cutaneous thermosensory signals and regulates adaptive thermogenesis. Here, we study the function and synaptic connectivity of input from DMH cholinergic neurons to sympathetic premotor neurons in the raphe pallidus (Rpa).

Methods: In order to selectively manipulate DMH cholinergic neuron activity, we generated transgenic mice expressing channelrhodopsin fused to yellow fluorescent protein (YFP) in cholinergic neurons (choline acetyltransferase (ChAT)-Cre::ChR2-YFP) with the Cre-LoxP technique. In addition, we used an adeno-associated virus carrying the Cre recombinase gene to delete the floxed *Chat* gene in the DMH. Physiological studies in response to optogenetic stimulation of DMH cholinergic neurons were combined with gene expression and immunocytochemical analyses.

Results: A subset of DMH neurons are ChAT-immunopositive neurons. The activity of these neurons is elevated by warm ambient temperature. A phenotype-specific neuronal tracing shows that DMH cholinergic neurons directly project to serotonergic neurons in the Rpa. Optical stimulation of DMH cholinergic neurons decreases BAT activity, which is associated with reduced body core temperature. Furthermore, elevated DMH cholinergic neuron activity decreases the expression of BAT uncoupling protein 1 (*Ucp1*) and peroxisome proliferator-activated receptor γ coactivator 1 α (*Pgc1*α) mRNAs, markers of BAT activity. Injection of M2-selective muscarinic receptor antagonists into the 4th ventricle abolishes the effect of optical stimulation. Single cell qRT-PCR analysis of retrogradely identified BAT-projecting neurons in the Rpa shows that all M2 receptor-expressing neurons contain tryptophan hydroxylase 2. In animals lacking the *Chat* gene in the DMH, exposure to warm temperature reduces neither BAT *Ucp1* nor *Pgc1*α mRNA expression.

Conclusion: DMH cholinergic neurons directly send efferent signals to sympathetic premotor neurons in the Rpa. Elevated cholinergic input to this area reduces BAT activity through activation of M2 mAChRs on serotonergic neurons. Therefore, the direct DMH^{ACh}—$Rpa^{5\text{-}HT}$ pathway may mediate physiological heat-defense responses to elevated environmental temperature.

Keywords Acetylcholine; Muscarinic; Nicotinic; Neuronal tracing; Serotonin; Hypothalamus

1. INTRODUCTION

Brown adipose tissue (BAT) contributes to energy homeostasis by burning carbohydrates and lipids to generate heat using uncoupling protein-1 (*Ucp1*), a protein that uncouples electron transport from ATP production [1—3]. The hypothalamus is implicated in the regulation of BAT activity [4—10]. In particular, the dorsomedial hypothalamus (DMH) appears to be a key structure for BAT thermogenesis [4,6,8,11]. In fact, DMH neurons directly project to sympathetic premotor neurons in the raphe pallidus (Rpa) that innervate BAT sympathetic preganglionic neurons in the spinal intermediolateral nucleus [8,12,13]. Furthermore, optical or pharmacogenetic stimulation of DMH neurons increases BAT temperature [8,11], suggesting that excitatory synaptic inputs from the DMH to the Rpa activate BAT thermogenesis. Indeed, local infusion of the AMPA and NMDA glutamate receptor antagonists into the Rpa blocks the effects of optical stimulation and disinhibition of DMH neurons [8,14]. These prior studies suggest that glutamatergic neurons in the DMH are part of the neural circuitries that positively control BAT activity.

In addition, it has been recently described that the DMH expresses acetylcholine (ACh)-containing neurons [15]. Activation of central cholinergic receptors, including nicotinic and muscarinic ACh receptors oppositely regulates body temperature. For instance, central injection of ACh or muscarinic ACh receptor (mAChR) agonists, such as

[1]Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, NY 10461, USA [2]Medical Research Council Metabolic Diseases Unit, University of Cambridge Metabolic Research Laboratories, Level 4, Wellcome Trust-MRC Institute of Metabolic Science, Box 289, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK [3]Diabetes, Obesity & Metabolism Institute and Department of Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA [4]Department of Neuroscience, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA [5]Department of Molecular Pharmacology, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, NY 10461, USA

*Corresponding author. Department of Medicine, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, NY 10461, USA. Tel.: +1 718 430 3495; fax: +1 718 430 8557. E-mail: young-hwan.jo@einstein.yu.edu (Y.-H. Jo).

Received March 17, 2015 • Revision received March 27, 2015 • Accepted March 31, 2015 • Available online 11 April 2015

http://dx.doi.org/10.1016/j.molmet.2015.03.006

oxotremorine and pilocarpine induces hypothermia, which is blocked by mAChR antagonists in earlier studies [16,17]. Moreover, M2 mAChR receptor knockout mice show reduced hypothermic response induced by the mAChR agonist [18] and mice lacking M3 receptors show elevated body temperature [19]. Furthermore, intracerebroventricular (i.c.v) injection of choline reduces body temperature through activation of mAChRs [20]. In contrast, activation of the nicotinic ACh receptor (nAChR) produces hyperthermia that is blocked by nAChR antagonist administration [21]. Therefore, central cholinoceptive and cholinergic systems play a role in regulating body temperature homeostasis.

We thus sought to determine the function and synaptic connectivity of cholinergic input from the DMH to sympathetic premotor neurons in the raphe pallidus. In this study, we describe a component of the hypothalamic neural circuits that regulates interscapular brown adipose tissue (iBAT) thermogenesis and gene expression. We found that DMH cholinergic neurons directly innervate sympathetic premotor neurons in the Rpa and that elevated cholinergic input to serotonergic neurons in the Rpa reduces BAT temperature through activation of M2 mAChRs.

2. MATERIAL AND 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-Cre, tdTomato, floxed ChAT(ChAT$^{F/F}$), ChR2-YFP, and ArCH-GFP transgenic mice (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. Electrophysiological recordings

Transverse brain slices were prepared from ChAT-Cre::ChR2-YFP mice. Animals were anesthetized with isoflurane. After decapitation, the brain 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. Membrane potentials were recorded with a Multiclamp 700B in whole cell configuration.

2.3. 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 DMH 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-ChAT (1:1000; Millipore, AB144P), anti-c-fos (1:500; Abcam, ab7963), anti-dsRed (1:1000, Clontech, 632496), anti-cholera toxin subunit b (CTb) (1:500; Abcam, ab62429), and anti-5-HT (1:500; Immunostar, 20080) antibodies diluted in 0.5% Triton X-100 in PBS overnight at 4 °C. For ChAT staining, colchicine (1 μl of 10 mg/ml; Santa Cruz Biotech) was injected into the lateral ventricle of animals 1 day before sacrifice. After incubation in primary antibodies, sections were washed 3 times in PBS and then incubated with Alexa 488 anti-goat IgG (1:500, A11055), Alexa 568 anti-rabbit IgG (1:500, A10042), Alexa 488 anti-rabbit IgG (1:500, A11008), and Alexa 568 anti-mouse IgG for CTb staining (1:500, A11004; Life Technologies) 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 VECTASHIELD mounting media. Images were acquired using a scanning confocal microscope.

2.4. Stereotaxic surgery and bilateral injections of neuronal tracers

2-month-old ChAT-Cre mice were anesthetized with 2% isoflurane and placed into a stereotaxic apparatus. A Cre-dependent herpes simplex virus (HSV) anterograde transsynaptic tracer, *HSV129(ΔTK)-loxP-STOP-loxP-tdtomato:2a:TK* (H129ΔTK-TT) [33] was bilaterally injected into the DMH of ChAT-Cre mice (1×10^{10} pfu, 200 nl per side, stereotaxic coordinates, bregma: AP: −1.95 mm, DV: −5.0 mm, ML: ±0.25 mm) with a Hamilton syringe. For retrograde monosynaptic tracing study, CTb (0.1%) was directly injected into the rRPa (AP: −6.0 mm, DV: −6.0 mm, ML: 0 mm). In some tracing experiments, Alexa Fluor 488 conjugated to wheat-germ agglutinin [22] (10 μg; Invitrogen, W11261) or fluorescent beads [23] (100 μl, 0.04 μm diameter; Invitrogen, F10720) were directly injected into iBAT of ChAT-Cre::ChR2-YFP or ChAT-Cre::tdTomato mice 5 days prior to assays. To delete the *Chat* gene in DMH cholinergic neurons, we bilaterally injected AAV-hSyn-mCherry (control, 250 nl of 3×10^{12} pfu/ml per side) or AAV-hSyn-mCherry-Cre (250 nl of 3×10^{12} pfu/ml per side) viruses (UNC vector core) into the DMH of ChAT$^{F/F}$ mice (The Jackson Lab).

2.5. Quantitative Real-time PCR analysis

For single-cell qRT-PCR analysis, single cell samples were collected from brain slice preparations via aspiration into the patch pipette to detect M2 mAChR and Tryptophan hydroxylase 2 (*Tph2*) mRNA expression in RPa neurons. The initial reverse transcription (RT) reaction was conducted after pressure ejection of the single cell samples into a microcentrifuge tube with REPLI-g WTA single cell kit (Qiagen). Samples were incubated in a total volume of 2.5 μl at 24 °C for 5 min, followed by 95 °C for 3 min, and cooled to 4 °C. And then samples were again incubated for 10 min at 42 °C with 0.5 μl gDNA wipeout buffer prior to addition of 1.75 μl RT mix (RT mix: 0.25 μl oligodT primer, 1 μl RT buffer, 0.25 μl random primer, 0.25 μl RT enzyme mix). The tubes were incubated at 42 °C for 1 h, and at 95 °C for 3 min. The tubes then were incubated at 24 °C for 30 min with 2.5 μl ligation mix (2 μl ligase buffer, 0.5 μl ligase Mix). The reaction was stopped by incubating at 95 °C for 5 min. Samples were incubated at 30 °C for 2 h after adding the amplification mix (7.25 μl buffer, 0.25 μl DNA polymerase) and at 65 °C for 5 min. The concentration of purified single-cell whole cDNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific).

For qRT-PCR analysis of *Ucp1* and *Pgc1α* mRNA in BAT, DMH cholinergic neurons were illuminated with light at 10 Hz (10 ms pulse) with a 3 s interval for 1 h at room temperature and iBAT was harvested immediately after stimulation. Single-strand cDNAs from iBAT were synthesized using a Transcriptor First Strand cDNA synthesis kit (Roche).

Real-time qPCR was performed in sealed 96 well plates with SYBR Green I master Mix by using a Light Cycler 480 instrument (Roche Applied Science). qPCR reactions were prepared in a final volume of 20 μl containing 2 μl of single cell whole cDNA, and 10 μl of SYBR Green master mix in the presence of primers at 0.5 μM. β-2 microglobulin (B2M) was used as an internal control for quantification of each sample. A list of primer sets included: F5′-cgttccaggacccgagtcgcaga-3′ and R5′-tcagctcttgttgccgggttttg-3′ for *Ucp1*, F5′-gacagctttctgggtggatt-3′ and R5′-cgcaggctcattgttgtact-3′ for *Pgc1α*, F5′-ttcagtcgcggtcgcttc-3′ and R5′-aggccggtcagtgagacaag for *B2M*, F5′-ggagcacaacaagatccaga-3′ and R5′-ctgacagacgtggagtcgtt-3′ for *M2 mAChR*, F5′-ccatcggagaattgaagcat-3′ and R5′-cgtcctgaaaggtggtgatt-3′ for *Tph2*. The qPCR amplicon for *M2 mAChR* and *Tph2* was loaded to 2% agarose gels.

2.6. Optical manipulation of DMH cholinergic neurons and measurement of BAT and body core temperatures

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 6-week old ChAT-Cre::ChR2-YFP or ChAT-Cre::ArCH-GFP mice. Optic fibers were coupled to a 473 nm or 589 nm DPSS laser. To simultaneously measure iBAT and body core temperature, we placed a wire thermoprobe (0.23 mm in diameter, Physitemp Instruments) into the BAT pad and inserted another thermoprobe (0.81 mm in diameter) into the rectum of ChAT-Cre::ChR2-YFP mice anesthetized with isoflurane. A heat lamp was used to maintain the animal's body temperature during anesthesia. We implanted a cannula into the 4th ventricle of animals to inject pharmacological reagents. Saline (1 μl) or the cholinergic antagonists (1 μl) was injected into the 4th ventricle 30 min prior to light stimulation. In some experiments, we implanted a temperature transponder under the iBAT pad of mice (Mini-mitter, Philips Respironics), as described in the prior study [24] and measured iBAT temperature in freely moving animals.

2.7. Statistics

All statistics were performed with GraphPad Prism software. Data are expressed as mean ± SEM. Multiple comparisons were tested with an ANOVA and adjusted with Tukey's honest significant difference test or student's *t*-test as specified in the figure legends. Results with $p < 0.05$ were considered significant.

3. RESULTS

3.1. Exposure to warm temperature excites DMH cholinergic neurons

It has been documented that the DMH as well as the dorsal hypothalamus (DH) express acetylcholine (ACh)-containing neurons in rodents [15,25]. To extend these previous findings, we used ChAT-Cre::tdTomato mice, in which the ChAT-Cre transgene causes cell-specific recombination to induce expression of tdTomato. We found the expression of tdTomato-positive neurons in the DMH (Figure 1A). To examine whether these neurons express the ChAT enzyme, brain sections were immunostained with an anti-ChAT antibody. There were ChAT-immunopositive neurons in the DMH. Approximately 80% of these ChAT-immunopositive neurons expressed tdTomato (Figure 1B; n = 3 animals), whereas all the tdTomato-positive neurons were ChAT-immunopositive in the DMH. These data further confirm previous findings [15,25] showing that the DMH contains ACh-expressing neurons.

To study the physiological function of DMH cholinergic neurons, we generated transgenic mice expressing channelrhodopsin fused to yellow fluorescent protein (YFP) selectively in cholinergic neurons (ChAT-Cre::ChR2-YFP). The DMH from these transgenic animals also showed the expression of cholinergic neurons as identified by YFP fluorescence (Figure 2A). As the activity of DMH neurons is regulated by ambient temperature [13,26,27], we first examined the expression of c-fos protein, an indirect index of neuronal activity, in DMH cholinergic neurons following 3 h exposure to warm (36 °C) ambient temperature. In fact, exposure to a warm (specifically 36 °C) environment induces robust c-fos expression in the central thermoregulatory system, including the dorsal part of the lateral parabrachial nucleus (LPBd) [28]. More importantly, the skin onset temperature that triggers LPBd neuronal discharge is about 35 °C [28]. In addition, it has been shown that animals exposed above 37 °C show increased serum osmolality and reduced body weight [29]. We found that exposure to warm temperature induced the expression of c-fos protein in the DMH as well (Figure 2B and C). Importantly, animals exposed to warm temperature showed a significant increase in the number of c-fos-positive cholinergic neurons (Figure 2B and D). These results suggest that a subset of DMH cholinergic neurons readily respond to warm ambient temperature.

As the activity of DMH cholinergic neurons is regulated by changes in warm ambient temperature, we examined whether DMH cholinergic neurons send projections to iBAT by direct iBAT injection of two different retrograde probes: Alexa Fluor 488 conjugated to wheat germ agglutinin (WGA-488) [22] or fluorescent microbeads [23] (Figure 3A). 5 days post injection, we found fluorescent probe-labeled neurons in the DMH (Figure 3A). Among these neurons, approximately half of DMH cholinergic neurons were also positive for these probes (range: 39–74%; n = 6 animals), indicating that DMH cholinergic neurons send efferent signals to iBAT.

3.2. DMH cholinergic neurons directly project to serotonergic neurons in the Rpa

The Rpa containing sympathetic premotor neurons plays an essential role in regulating BAT activity [30–32]. We thus examined whether

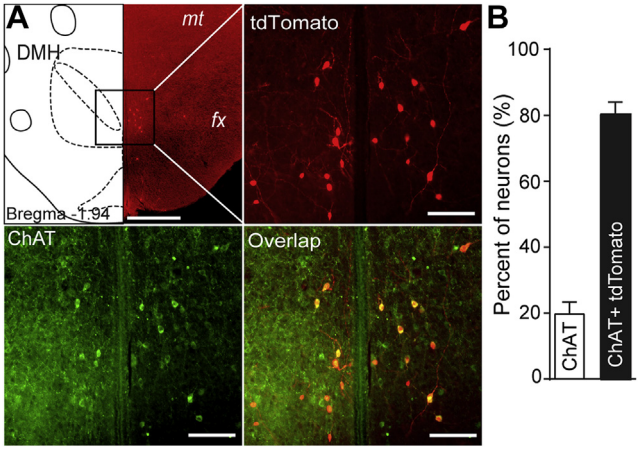

Figure 1: The DMH expresses ACh-containing neurons. A. Images of fluorescence microscopy showing tdTomato-positive neurons in the DMH of ChAT-Cre::tdTomato animals. Upper panel: tdTomato-positive neurons at Bregma -1.94 mm (left). The reference diagram was adapted from the Mouse Brain Atlas of Paxinos and Franklin (2nd edition, 2001). Upper right panel is higher magnification view of tdTomato-positive neurons. Bottom panel: tdTomato-positive neurons were immunostained with an antibody to ChAT (Green). Scale bar: 1 mm (upper left), 100 μm. **B**. Summary plot showing that approximately 80% of ChAT-positive neurons were co-labeled with tdTomato in ChAT-Cre::tdTomato animals (n = 3 animals).

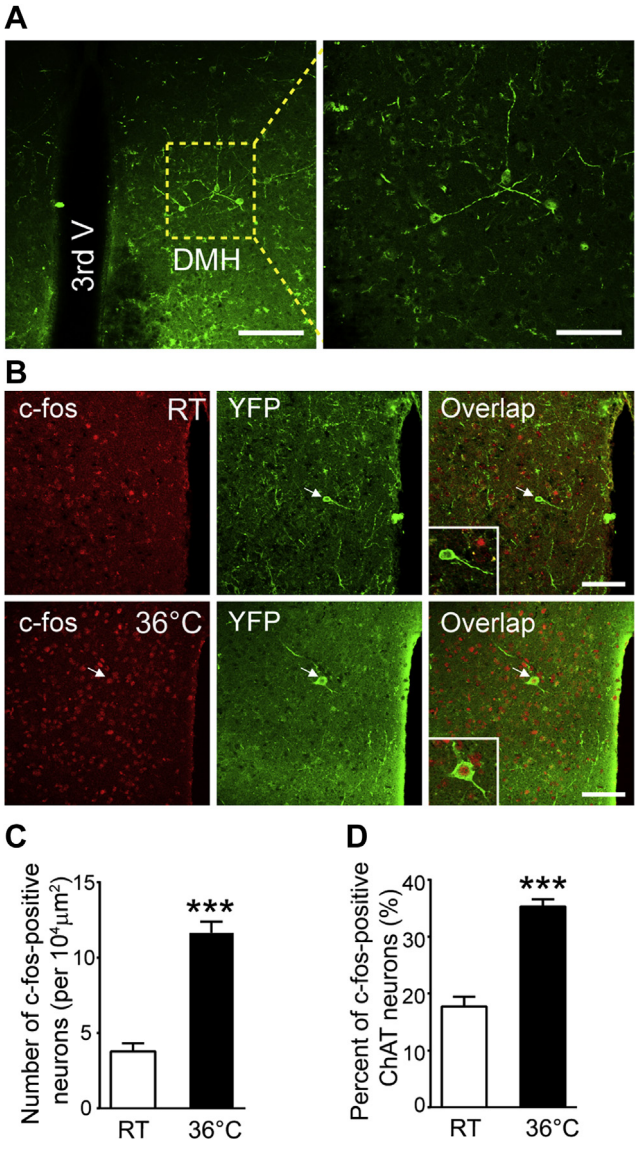

Figure 2: DMH cholinergic neurons are excited by warm ambient temperature. A. Images of fluorescence microscopy showing ChR2-YFP-expressing cholinergic neurons in the DMH of ChAT-Cre::ChR2-YFP animals. Scale bar: 200 μm (left panel), 100 μm (right panel). **B**. Images of fluorescence microscopy showing c-fos protein expression in the DMH cholinergic neurons following exposure to room (RT, upper) or warm (36 °C, bottom) temperature for 3 h. Exposure to warm ambient temperature induced c-fos protein expression in DMH cholinergic neurons. Scale bar: 100 μm. **C**. Summary plot showing the number of c-fos-positive neurons in the DMH. The number of fos-positive neurons was increased in response to exposure to warm temperature ($n = 6$ animals, respectively; $^{***}p^{t\text{-test}} < 0.001$). **D**. Percentage of c-fos-positive cholinergic neurons in response to exposure to warm temperature ($n = 6$ animals, respectively; $^{***}p^{t\text{-test}} < 0.001$). All data are expressed as mean ± SEM.

neurons in the Rpa are a downstream target of DMH cholinergic neurons. We bilaterally injected a Cre-dependent, anterograde trans-synaptic viral tracer (H129ΔTK-TT) [33] into the DMH of ChAT-Cre mice. Three to four days post viral injection, tdTomato-labeled neurons that represent virus-infected cells were found in the DMH (Figure 3B). In the same animals, tdTomato-labeled cells were found in diverse brain areas (Supplementary Figure 1). Among these structures, the Rpa showed robust expression of tdTomato-positive neurons (Figure 3B). As serotonergic neurons in the Rpa regulate BAT activity [34,35], we further determined whether tdTomato-positive cells express 5-HT. There were 5-HT-positive neurons in the Rpa (Figure 3C). Importantly, the majority of 5-HT-positive neurons were co-labeled with an anti-tdTomato antibody (Figure 3C and D), indicating that 5-HT-positive neurons receive afferent signals from DMH cholinergic neurons.

In order to investigate whether DMH cholinergic neurons directly send projections to neurons in the Rpa, we injected cholera toxin subunit-B (CTb), a retrograde monosynaptic tracer, into the Rpa (Figure 3E). There were CTb-positive neurons in the DMH in animals injected with CTb into the Rpa (Figure 3E). Approximately one-third of DMH cholinergic neurons were labeled with an anti-CTb antibody (Figure 3F and G). Taken together, our neuronal tracing studies suggest that there is a direct synaptic innervation from DMH cholinergic to Rpa serotoninergic neurons.

3.3. Optical stimulation of DMH cholinergic neurons reduces BAT activity

Given DMH cholinergic neurons directly send efferent signals to the Rpa, we examined the effect of activation of the DMH^{ACh}—$Rpa^{5\text{-}HT}$ pathway by selectively exciting DMH cholinergic neurons. In hypothalamic slices from animals of ChAT-Cre::ChR2-YFP, illumination of DMH cholinergic neurons with blue light (470 nm) readily induced action potentials (Figure 4A). We implanted an optical fiber into the area just on top of the 3rd ventricle of ChAT-Cre::ChR2-YFP mice (Figure 4B) and illuminated DMH cholinergic neurons at 10 Hz for 1 s with a 3 s interval for 1 h to study physiological responses to optical stimulation of DMH cholinergic neurons. Blue light Illumination, indeed, activated DMH cholinergic neurons as identified by staining with an anti-pS6 antibody, an indirect marker of neuronal activity (Supplementary Figure 2). Under these experimental conditions, our initial optogenetic experiments in freely moving mice revealed that stimulation of DMH cholinergic neurons at different frequencies (i.e. 0, 1, 5, and 10 Hz) reliably decreased iBAT temperature (Figure 4C and D).

As 10 ms short pulses of light stimulation at 10 Hz demonstrate high fidelity action potential output *in vitro* and reduced BAT temperature *in vivo*, we stimulated DMH cholinergic neurons at 10 Hz for 1 s with 3 s interval for 1 h in the following experiments. Under the experimental conditions where animals were anesthetized with isoflurane, we injected saline (1 μl) 30 min prior optical stimulation. Without light illumination, both iBAT and core temperatures remained stable during recordings (Figure 4E, $n = 6$ animals). However, optical stimulation of DMH cholinergic neurons significantly decreased iBAT and body core temperatures (Figure 4E, I and J; BAT, 35.85 ± 0.03 °C vs. 34.47 ± 0.12 °C, core, 35.68 ± 0.09 °C vs. 34.13 ± 0.08 °C, $n = 6$ animals, $p < 0.05$).

We then examined whether reduced BAT activity is mediated by activation of cholinergic receptors in the Rpa. Injection of the mAChR antagonist 4-DAMP (100 μM) into the 4th ventricle 30 min prior to optical stimulation completely abolished the effect of optical stimulation (Figure 4F, I and J, $n = 6$ animals). However, infusion of the nAChR antagonist mecamylamine (100 μM) did not block the hypothermic effect of optical stimulation of DMH cholinergic neurons (Figure 4G, I, and J, $n = 6$ animals). As stimulation of DMH cholinergic neurons decreases BAT activity, we further sought to determine whether inhibition of DMH cholinergic neurons increases iBAT thermogenesis. To this end, we expressed a light-activated inhibitory protein archaerhodopsin in cholinergic neurons (ChAT-Cre::ArCH-GFP). Illumination (1 Hz for 1 s) of DMH cholinergic neurons for 2 h increased BAT and body core temperatures in ChAT-Cre::ArCH-GFP animals (Figure 4H, I,

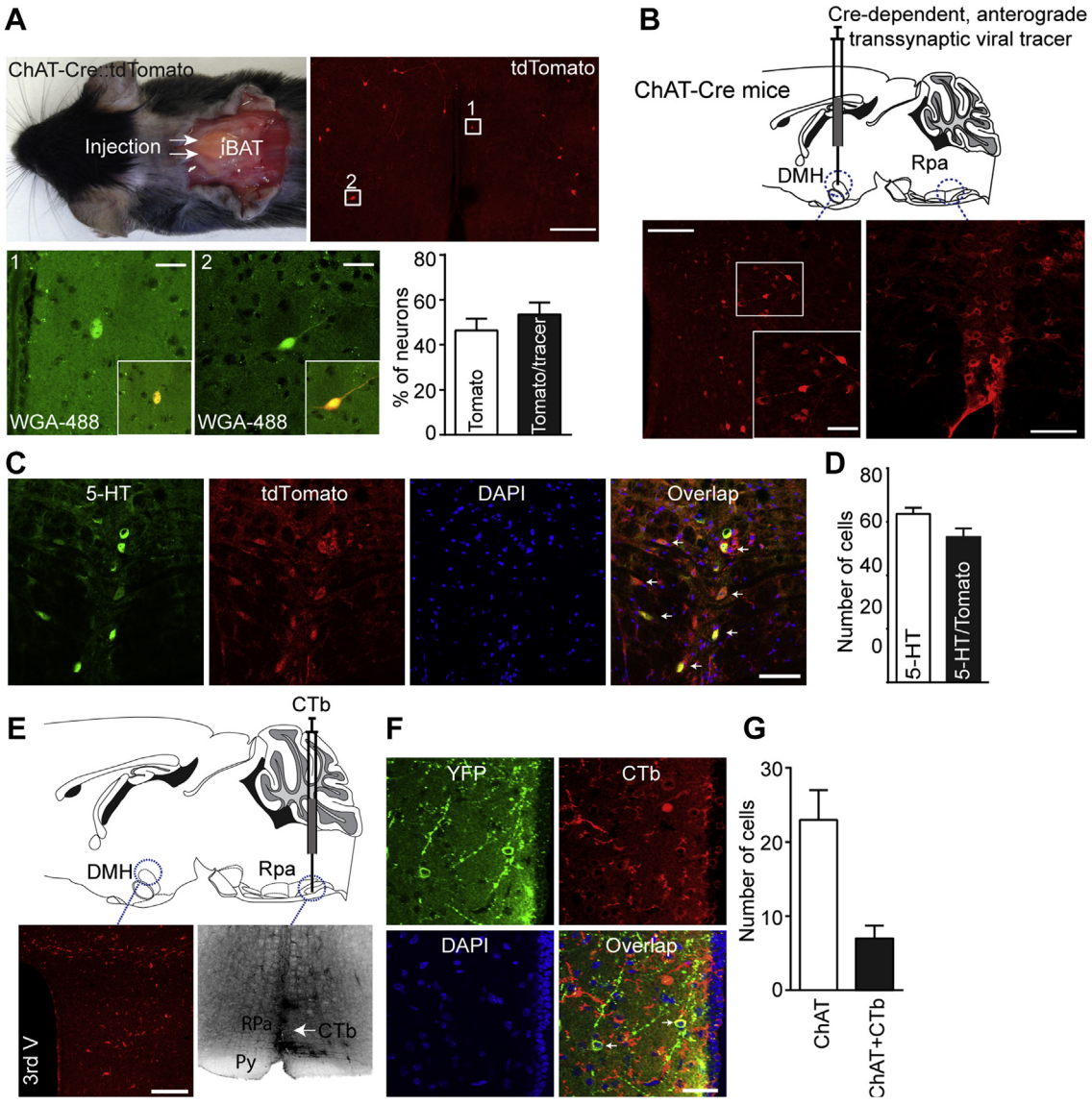

Figure 3: DMH Cholinergic neurons send efferent projections to serotonergic neurons in the Rpa. A. Images of fluorescence microscopy showing WGA-labeled cholinergic neurons in the DMH following bilateral injections of retrograde WGA-488 into iBAT of the ChAT-Cre::tdTomato animal. Upper panel: WGA-488 was directly injected into iBAT (left) and tdTomato-positive neurons in the DMH (right). Scale Bar: 500 μm. Bottom panel: examples of WGA-488-positive cholinergic neurons in the DMH (left and middle). Summary plot showing that approximately half of cholinergic neurons were labeled with fluorescent tracing probes (right; n = 6 animals). Scale Bar: 50 μm. **B**. Images of fluorescence microscopy showing tdTomato-positive neurons in the DMH and Rpa from ChAT-Cre animals injected with a Cre-dependent, transsynaptic anterograde viral tracer. Schematic drawing of the experimental configuration (upper panel). Bottom panel: tdTomato-positive neurons in the DMH (left) and Rpa (right). Scale bars: 100 μm, 50 μm (inset). **C and D**. Images of fluorescence confocal microscopy showing tdTomato-labeled serotonergic neurons in the Rpa (C). 85.5% of serotonergic neurons were also positive to tdTomato (D; n = 3 animals). Scale bar: 100 μm. **E**. Images of microscopy of CTb-positive neurons in the DMH. Schematic illustration of the experimental condition (upper panel). Bottom panel: CTb-positive neurons were observed in the DMH/DH areas (left panel) and CTb was injected into the Rpa (right panel). Scale bar: 200 μm. **F and G**. Images of fluorescence microscopy showing CTb-positive cholinergic neurons in the DMH from animals injected with CTb (0.1%, 1 μl) into the Rpa. About one-third of DMH cholinergic neurons were stained with an anti-CTb antibody (n = 3 animals). Scale bar: 50 μm. All data are expressed as mean ± SEM.

and J; iBAT, 35.32 ± 0.07 °C vs. 35.88 ± 0.13 °C, $p < 0.05$, core, 34.98 ± 0.19 °C vs. 35.58 ± 0.22 °C, $p > 0.05$, n = 5 animals). Importantly, decreased BAT temperature was associated with reduced expression of iBAT *Ucp1* and *Pgc1α* mRNAs (Figure 5A and B). This was further supported by western blot analysis of iBAT showing that light stimulation significantly decreased *Ucp1* protein levels (Supplementary Figure 3). Moreover, reduced expression of iBAT *Ucp1* and *Pgc1α* mRNAs was significantly abolished by the mAChR, but not nAChR, antagonist (Figure 5A and B). In contrast, increased BAT temperature due to inhibition of DMH cholinergic neurons was associated with elevated *Ucp1* and *Pgc1α* mRNA expression (Figure 5A and B). It has been described that Gi/o-coupled M2 receptors inhibit neurons in the raphe [36]. We thus determined the contribution of the M2 receptor to the reduction of iBAT activity. Injection of the M2 selective antagonist AF DX-116 (50 nM) blocked the effect of optical stimulation of DMH cholinergic neurons on *Ucp1* and *Pgc1α* mRNA expression (Figure 5A and B). Moreover, single-cell qRT-PCR analysis of retrogradely identified iBAT-projecting neurons in the Rpa (Figure 5C) showed that almost all iBAT-projecting neurons were tryptophan hydroxylase (*Tph2*)-positive (n = 18 out of 19 neurons) and

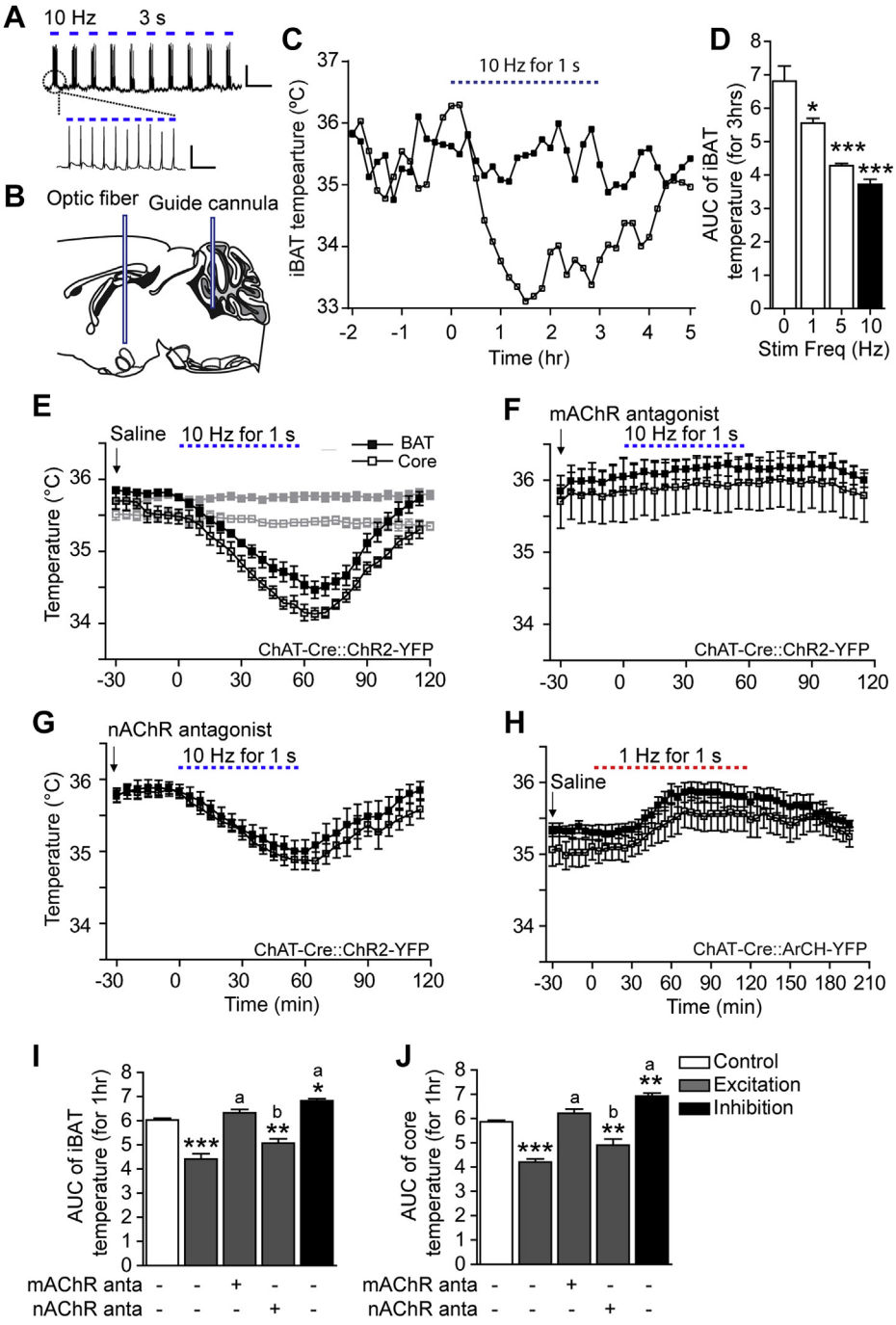

Figure 4: Elevated DMH cholinergic neuron activity decreases BAT activity. A and B. Representative traces showing that optical stimulation of DMH cholinergic neurons readily induced action potential discharge in hypothalamic slices of ChAT-Cre::ChR2-YFP animals. DMH cholinergic neurons were illuminated at 10 Hz (10 ms pulse) for 1 s with a 3 s interval (upper panel). Bottom panel: expanded scale. Scale bars: upper panel, 20 mV, 5 s; bottom panel: 20 mV, 200 ms. **B**. Schematic drawing of the experimental configuration. **C and D**. Elevated DMH cholinergic neuron activity reduces iBAT temperature in freely moving mice. Graph showing changes in iBAT temperature during optical stimulation of DMH cholinergic neurons for 3 h at 10 Hz for 1 s with a 3 s interval. iBAT temperature was continuously measured by telemetry (filled box: no stimulation, open box: 10 Hz optical stimulation). **D**. Plot showing AUC of iBAT temperature during optical stimulation of DMH cholinergic neurons for 3 h. iBAT temperature was changed in a frequency-dependent manner ($n = 5$ animals). *$p < 0.05$, ***$p < 0.001$. **E**. Graph showing changes in iBAT and body core temperatures during optical stimulation of DMH cholinergic neurons from ChAT-Cre::ChR2-YFP animals. Although iBAT and core temperatures remained stable without light illumination (Gray), 10 Hz stimulation for 1 h significantly reduced iBAT and core temperatures ($n = 6$ animals). This effect of optical stimulation was completely reversible. Saline (1 μl) was injected into the 4th ventricle through cannula 30 min prior to light illumination. **F and G**. Graphs showing changes in iBAT and core temperatures during optical stimulation of DMH cholinergic neurons in the presence of mAChR (4-DAMP) or nAChR (mecamylamine) antagonists. Pretreatment with the mAChR antagonist completely abolished the effect of optical stimulation (F; 100 μM, $n = 6$ animals), whereas the nAChR antagonist did not block the effect (G; 100 μM, $n = 6$ animals). **H**. Graph showing changes in iBAT and core temperatures during optical inhibition of DMH cholinergic neurons from ChAT-Cre::ArCH-GFP mice. Optical inhibition (1 Hz for 1s, 1 s pulse, 3 s interval) of DMH cholinergic neurons for 2 h elevated iBAT and core temperatures ($n = 5$ animals). **I and J**. Summary plots showing AUC of iBAT and core temperatures during optical stimulation of DMH cholinergic neurons for 1 h with or without the cholinergic antagonists. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: control vs. excitation or excitation + drugs; $^{a}p < 0.001$: excitation vs. excitation + drugs or inhibition; $^{b}p < 0.05$: excitation vs. excitation + drugs. All data are expressed as mean ± SEM.

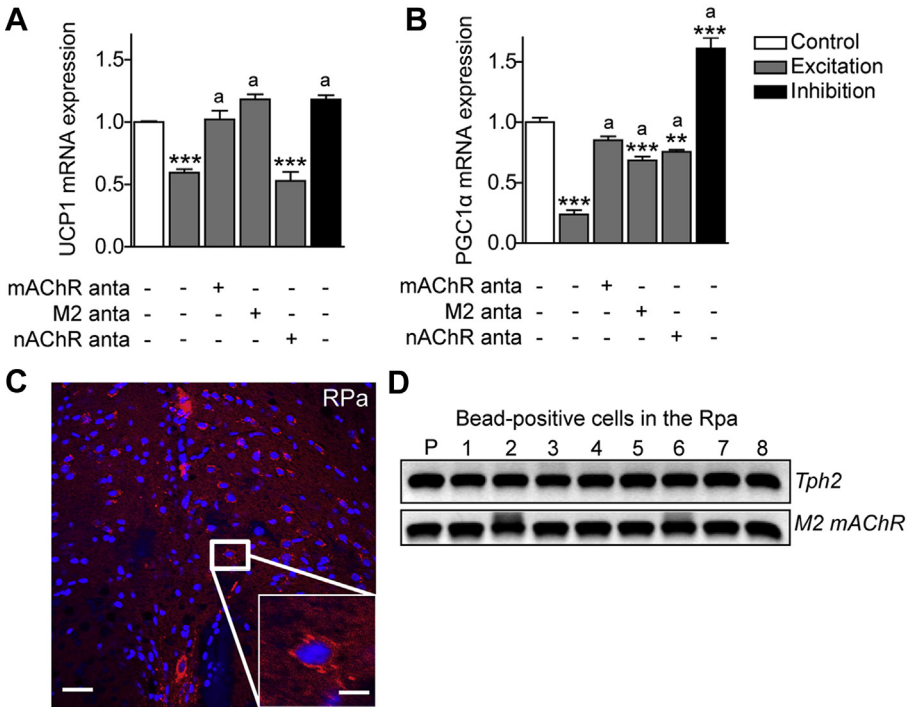

Figure 5: Serotonergic neurons in the Rpa express M2 mAChRs. A and B. Summary plots showing *Ucp1* and *Pgc1α* mRNA expression in iBAT following optical manipulation of DMH cholinergic neuron activity. The expression of *Ucp1* (**A**) and *Pgc1α* (**B**) mRNAs was significantly reduced with optical excitation of DMH cholinergic neurons (n = 6 animals, respectively), which was completely blocked by pretreatment with the mAChR and the M2 specific antagonists (n = 6 and 4 animals, respectively). However, inhibition of DMH cholinergic neurons significantly increased the expression of *Ucp1* and *Pgc1α* mRNAs in iBAT (n = 5 animals). **p < 0.01, ***p < 0.001: control vs. excitation or excitation + drugs or inhibition; [a]p < 0.001: excitation vs. excitation + drugs or inhibition. All data are expressed as mean ± SEM. **C and D**. Image of fluorescence microscopy of microbead-labeled neurons in the Rpa (**C**). Single-cell qRT-PCR analysis of iBAT-projecting neurons shows the co-expression of M2 mAChRs and *Tph2* (**D**). Scale bars: 100 μm, 25 μm (inset).

that all M2 receptor-expressing neurons expressed *Tph2* (Figure 5D; n = 8 out of 8 neurons).

In order to define the contribution of ACh to the regulation of iBAT activity, we selectively deleted the *Chat* gene by bilateral injections of AAV-Cre-viruses into the DMH of floxed ChAT animals (ChAT$^{F/F}$, Figure 6A). This manipulation induced a robust decrease in *Chat* mRNA expression in the DMH (Figure 6B, n = 6 animals, respectively). Exposure to warm temperature reduced neither iBAT *Ucp1* nor *Pgc1α* mRNA expression in animals lacking ChAT in the DMH, although cold challenge robustly increased iBAT activity in these animals (Figure 6C and D). Taken together, DMH cholinergic neurons directly innervate sympathetic premotor neurons in the Rpa and elevated cholinergic input to this area reduces BAT activity through activation of M2 mAChRs on serotonergic neurons.

4. DISCUSSION

Our present work provides cellular evidence for a neurochemically specific hypothalamic neural circuit that negatively regulates brown adipose tissue thermogenesis. Hence this neuronal circuit appears to be the first inhibitory pathway between the DMH and Rpa to be identified. It has been demonstrated that there are ACh-containing neurons in the DMH [15]. In light of the effects of ACh on the regulation of body temperature [16–20], this cholinergic cell group would be a suitable candidate to regulate BAT thermogenesis. Immunocytochemical analysis with an anti-ChAT antibody further confirms the expression of cholinergic neurons in the DMH. We demonstrated that these DMH cholinergic neurons have the ability to respond to warm ambient temperature and to directly send efferent signals to sympathetic premotor neurons in the Rpa. Elevated DMH cholinergic neuron activity reduces rather than increases BAT activity. We further showed that ACh-mediated hypothermia is due to activation of M2 mAChRs in serotonergic neurons in the Rpa, consistent with the earlier behavioral and genetic studies showing that activation of central mAChRs causes hypothermia [16–19].

Recent extensive studies have identified neuronal circuits that are involved in iBAT thermogenesis, including the median preoptic nucleus (MnPO), dorsolateral preoptic area (DLPO), lateral parabrachial nucleus (LPB), DMH, and Rpa [12,28,37,38]. Both cool and warm cutaneous thermosensory signals are transmitted from the spinal dorsal horn neurons to the preoptic area (POA) via glutamatergic neurons in the LPB [32]. The rostral medullary raphe (RMR), including the Rpa and raphe magnus nucleus contains the principal BAT sympathetic premotor neurons [32] and inhibition of these neurons decreases body temperature in freely moving rodents [39]. In this regard, the DMH is an important relay site between the POA and RMR [32]. Indeed, optical stimulation of DMH neurons at 10 Hz activates the DMH–Rpa pathway, which increases BAT thermogenesis [8]. Moreover, activation of leptin receptor-expressing neurons in the DMH elevates BAT activity [4,11]. Although the phenotypical identity of leptin receptor-expressing neurons in the DMH has remained undetermined, at least a subset of these neurons appear to be glutamatergic [8] or prolactin-releasing peptide neurons [6]. Therefore, non-ChAT-positive neurons in the DMH appear to be involved in driving BAT thermogenesis.

It has been well documented that DMH neurons are excited by both cold and warm exposure [13,26,27]. In line with these previous

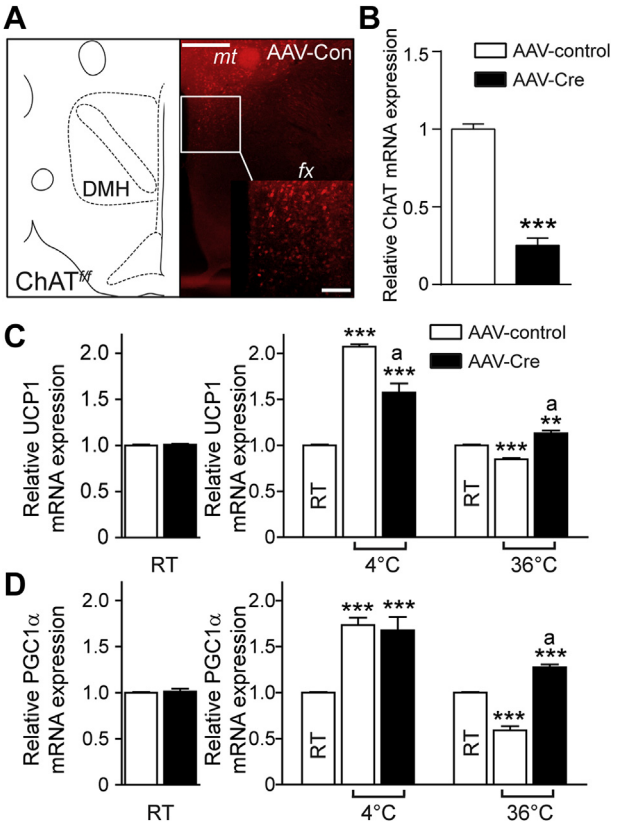

Figure 6: Effect of selective deletion of the *Chat* gene in the DMH on *Ucp1* and *Pgc1α* mRNA expression. A. Image of fluorescence microscopy showing the injection sites of AAV viruses. AAV viruses were bilaterally injected into the DMH of ChAT$^{F/F}$ mice. The reference diagram was adapted from the Mouse Brain Atlas of Paxinos and Franklin (2nd edition, 2001). Scale Bar: 1 mm, 100 μm (inset). **B**. The expression of *Chat* mRNAs was robustly reduced by this manipulation ($n = 6$ animals, respectively, $^{***}p^{t\text{-test}} < 0.001$). **C and D**. Plots shows that exposure to warm temperature did not alter the expression of *Ucp1* and *Pgc1α* in iBAT from ChAT$^{F/F}$ mice injected with AAV-Cre viruses ($n = 6$ animals, $p < 0.05$). However, iBAT activity from animals injected with AAV-Cre viruses was still regulated by cold exposure ($n = 6$ animals, $p < 0.05$). $^{**}p < 0.01$, $^{***}p < 0.001$: control (RT) vs. cold or warm; $^{a}p < 0.001$: control (AAV-control) vs. AAV-Cre. All data are expressed as mean ± SEM.

studies, DMH cholinergic neuron activity was increased by warm ambient temperature. This may imply that these neurons have the ability to reduce body temperature in response to elevated ambient temperature. Indeed, a subset of DMH cholinergic neurons innervated iBAT polysynaptically, suggesting that iBAT is a downstream target of DMH cholinergic neurons. Moreover, mice lacking *Chat* in DMH cholinergic neurons failed to respond to warm exposure, although they were still sensitive to cold challenge. These results strongly support the fact that ACh in the DMH is an important neurotransmitter that regulates iBAT activity.

DMH cholinergic neurons directly sent efferent signals to serotonergic sympathetic premotor neurons in the Rpa in our preparations. The activity of serotonergic neurons is negatively regulated by ambient temperature and is positively correlated with BAT temperature [35]. Microinjection of the $GABA_A$ receptor agonist muscimol into the Rpa decreases body temperature [39]. As stimulation of DMH cholinergic neurons decreased elevated iBAT temperature, it is possible that DMH cholinergic neurons provide inhibitory input to neurons in the Rpa. Endogenous ACh binds to both ligand-gated nicotinic receptors and G protein-coupled muscarinic receptors. In our preparations, the ability of DMH cholinergic neurons to reduce iBAT activity was intact in the presence of the nAChR antagonist. However, excitation of DMH cholinergic neurons was no longer effective in decreasing iBAT temperature following injection of mAChR antagonists, suggesting that released ACh activates mAChRs rather than nAChRs in the Rpa.

Among muscarinic receptor subtypes, M2 and M4 receptors are preferentially coupled to inhibitory Gi/o proteins [40]. It has been described that activation of M2 mAChRs inhibits neurons in the raphe [36]. Furthermore, the hypothermic response induced by mAChR agonists is impaired in mice lacking the M2 receptor [18], suggesting the potential contribution of the M2 receptor to hypothermia. In line with these previous studies, local injection of the M2-selective antagonist blocked the effect of optical stimulation of DMH cholinergic neurons on *Ucp1* and *Pgc1α* mRNA expression. More importantly, our single-cell qRT-PCT analysis of BAT-innervating neurons in the Rpa showed the co-expression of *Tph2* and M2 mAChRs, further supporting the fact that DMH cholinergic neurons send cholinergic input to serotonergic neurons expressing the M2 mAChR. As a result, activation of the M2 receptor hyperpolarizes serotonergic sympathetic premotor neurons, thereby reducing iBAT activity.

Although our current study supports the importance of the DMH^{ACH}—Rpa$^{5\text{-}HT}$ pathway in the regulation of iBAT activity, it is plausible that there are alternative DMH cholinergic circuits regulating iBAT thermogenesis. In fact, neurons in the paraventricular nucleus (PVN) in the hypothalamus appear to be an essential component for the regulation of BAT activity. For instance, both NPY [7] and GABAergic RIP-Cre [9] neurons in the ARC project to neurons in the PVN and stimulation of NYP and GABAergic RIP-Cre neurons oppositely regulates iBAT temperature. In our studies, we found that DMH cholinergic neurons also project to neurons in the PVN, suggesting that the DMH^{ACH}—PVN pathway may be part of the neural circuits involved in regulating iBAT activity. In addition, stimulation by light or leptin of DMH neurons [4,6,8,11] increases iBAT temperature. Thus, it is also possible that a local interneuronal circuit within the DMH could alter BAT activity.

In physiology, the neural circuit that we described in this study downregulates BAT activity and body core temperature. However, iBAT as well as other organs can control body core temperature. For instance, animals have the ability to increase endogenous heat production through shivering thermogenesis on cold exposure [41]. Moreover, if the ambient temperature were below the thermoneutral zone such as ~22 °C, loss of iBAT function would not necessarily lead to an immediate body core temperature drop, as other thermogenesis mechanisms can partly compensate for iBAT function, even at cold exposure [42,43]. Therefore, it is likely that DMH cholinergic neurons would also have an influence on other thermogenesis mechanisms and/or essential vital functions as they send cholinergic projections to diverse areas.

5. CONCLUSIONS

DMH cholinergic neurons positively respond to warm ambient temperature. Elevated cholinergic neuron activity in the DMH directly transfers to sympathetic premotor neurons in the Rpa, which express serotonin. Released ACh activates inhibitory M2 mAChRs in serotonergic neurons and consequently reduces the activity of serotonergic neurons. Suppression of serotoninergic neuron activity decreases iBAT temperature. Therefore, our present study demonstrates a novel neurochemically specific circuit modulating iBAT thermogenesis and gene expression. This novel DMH^{ACH}—Rpa$^{5\text{-}HT}$ pathway could contribute to physiological heat-defense responses to elevated environmental temperature.

ACKNOWLEDGMENTS

We thank Althea Cavanaugh and Licheng Wu for technical supports. This work was supported by NIDDK (RO1DK092246) to Y.-H.J. and New York obesity nutrition research center to J.H.J.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.molmet.2015.03.006.

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