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

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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 (Pgc1α) 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 Pgc1α 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→Rpa 5-HT pathway may mediate physiological heat-defense responses to elevated environmental temperature.

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 hypothalamic nucleus (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...
oxotremorine and pilocarpine induces hyperthermia, which is blocked by mAChR antagonists in earlier studies [16,17]. Moreover, M2 mAChR receptor knockout mice show reduced hyperthermic 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 cholinceptive 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<sup>F</sup>), 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 ± 1 °C) with a 12:12 h light–dark cycle and fed a standard chow diet with <i>ad libitum</i> 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<sub>2</sub>/5% CO<sub>2</sub> and maintained at 37 ± 1 °C. This solution contained the following (in mM): 113 NaCl, 3 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 2.6 NaHCO<sub>3</sub>, 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 ± 1 °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<sub>2</sub>PO<sub>4</sub>, 2.6 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 5 glucose in 95% O<sub>2</sub>/5% CO<sub>2</sub>. Brain slices were placed on the stage of an upright, infrared-differential interference contrast microscope mounted on a Gibraltar X/C21 microscope equipped with infrared (i.e., 633 nm) interference contrast microscope. For whole-cell recordings, the holding current was −200 pA.

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; Abbcam, ab7963), anti-dsRed (1:1000, Clontech, 632496), anti-cholera toxin subunit b (CTb) (1:500; Abbcam, 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, cholchicine (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, A10108), and Alexa 568 anti-mouse IgG for 2 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<sub>ΔTK</sub>-loxp<sup>-</sup>-STOP-loxp<sup>-</sup>-tdTomato:2aTK (H129ΔTK-TT) [33] was bilaterally injected into the DMH of ChAT-Cre mice (1 × 10<sup>12</sup> 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 rPpA (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<sup>12</sup> pfu/ml per side) or AAV-hSyn-mCherry-Cre (250 nl of 3 × 10<sup>12</sup> pfu/ml per side) viruses (UNC vector core) into the DMH of Chat<sup>F/F</sup> 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 rPpA 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. Then samples were again incubated for 10 min at 42 °C with 0.5 μl qDNA wipework 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 were then 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'-cgctcaggcccc-gagtcgcaga-3' and R5'-tcagctcgtggtcgcttc-3' for Ucp1, F5'-gacagctttcgggtggatt-3' and R5'-ccagctcgtattgtgctact-3' for Pgc1a, F5'-tcagctcggtagcttcttc-3' and R5'-aggccgctaacacagaa-3' for B2M, F5'-ggagcaacacagttcaga-3' and R5'-ctgagagcgtttggtgatt-3' for M2 mACHr, F5'-ccatcggagaattgacgac-3' and R5'-ctgccggaaagtggt-3' for Tph2. The qPCR ampiclon 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 thermocouple (0.23 mm in diameter, Physitemp Instruments) into the BAT pad and inserted another thermocouple (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 to 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-neutral volume of fluorescent microbeads [23] (Figure 3A). 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...
neurons in the Rpa are a downstream target of DMH cholinergic neurons. We bilaterally injected a Cre-dependent, anterograde transynaptic viral tracer (H129 ΔTK-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 serotonergic 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 → Rpa 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 hypothalamic 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 archerhodopsin 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, and J).
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 decreases 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; ap < 0.001: excitation vs. excitation + drugs or inhibition; bp < 0.05: excitation vs. excitation + drugs. All data are expressed as mean ± SEM.
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<sup>fl/fl</sup>, 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<sup>a</sup> nor Pgc1<sup>a</sup> mRNA expression in animals lacking ChAT in the DMH, although cold challenge robustly increased iBAT activity in these animals (Figure 6C, n = 6 animals, respectively). However, inhibition of DMH cholinergic neurons significantly increased the expression of Ucp1 and Pgc1<sup>a</sup> mRNAs in iBAT (n = 5 animals). *p < 0.01, **p < 0.001: control vs. excitation or excitation + drugs or inhibition; "p < 0.001: excitation vs. excitation + drugs or inhibition. All data are expressed as mean ± SEM.

**Figure 6A.** Single-cell qRT-PCR analysis of iBAT-projecting neurons shows the co-expression of M2 mAChRs and Tph2. Scale bars: 100 mm, 25 mm (inset).

**Figure 5: Serotonergic neurons in the Rpa express M2 mAChRs.** A and B. Summary plots showing Ucp1 and Pgc1<sup>a</sup> mRNA expression in iBAT following optical manipulation of DMH cholinergic neuron activity. The expression of Ucp1 (A) and Pgc1<sup>a</sup> (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 Pgc1<sup>a</sup> mRNAs in iBAT (n = 5 animals). *p < 0.01, **p < 0.001: control vs. excitation or excitation + drugs or inhibition; "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 (B). 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).

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
Microinjection of the GABAA receptor agonist muscimol into the Rpa sites of AAV viruses. AAV viruses were bilaterally injected into the DMH of ChAT MOLECULAR METABOLISM 4 (2015) 483 activity of serotonergic neurons is negatively regulated by ambient sympathetic premotor neurons in the Rpa in our preparations. The DMH cholinergic neurons directly sent efferent signals to serotonergic neurons polysynaptically, suggesting that iBAT activity was still sensitive to cold challenge. These results strongly support the fact that ACh in the DMH is an important neurotransmitter that regulates iBAT activity. Moreover, mice lacking Chat/C211 Cre viruses (n = 6 animals, respectively, p < 0.001: control (AAV-control) vs. AAV-Cre. All data are expressed as mean ± SEM.

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/fl mice. B. The expression of Chat mRNAs was robustly reduced by this manipulation (n = 6 animals, respectively, p < 0.001: control (RT) vs. cold or warm; *p < 0.01 and **p < 0.001: control (AAV-control) vs. AAV-Cre. C. A and B. Plots shows that exposure to warm temperature did not alter the expression of Ucp1 and Pgc1α in BAT from Chat/fl 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; *p < 0.01: 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 GABAA 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 G/o proteins [40]. It has been described that activation of M2 mAChRs inhibits neurons in the raphe [36]. Furthermore, the hypothemic 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/Chat-Rpa/Ucp1/Pgc1α 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/Chat-PVN pathway may be part of the neural circuits involved in regulating iBAT activity. In addition, stimulation by light or leptin of DMH neurons 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 serotonergic neuron activity decreases iBAT temperature. Therefore, our present study demonstrates a novel neurochemically specific circuit modulating iBAT thermogenesis and gene expression. This novel DMH/Chat-Rpa/Ucp1 pathway could contribute to physiological heat-defense responses to elevated environmental temperature.
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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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