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Article

Exploring a new mechanism controlling thermogenesis of adipose tissue

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SUMMARY

Adipose tissue dissipates energy for thermogenesis, a heat-generating process that helps maintain body temperature. Sympathetic nerves release norepinephrine (NE) to increase thermogenesis of adipose tissue during cold stimulation. However, other factors that regulate adipose tissue thermogenesis should not be ignored. We hypothesized that some neuroactive factors also participate in regulating adipose tissue thermogenesis. We exposed mice to ambient temperatures of 4°C, 22°C, and 30°C and found that the thermogenesis of inguinal white adipose tissue (iWAT) increased after moving from 22°C to 4°C for 3 days, and decreased after moving from 22°C to 30°C for 3 days. We observed that iWAT is mainly innervated by three nerve bundles and verified that these nerves contained adrenergic nerves but no cholinergic nerves. While NE levels increased at 4°C as expected, they did not decrease at 30°C, which does not correlate with the decrease in thermogenesis observed. Further analysis revealed the expression of multiple receptors for hypothalamus/pituitary neuroendocrine hormones in adipocytes. Among these pituitary hormones, arginine vasopressin (AVP) decreased the expression of uncoupling protein 1 (UCP1) for thermogenesis, whereas oxytocin (OXT) increased UCP1 expression. Our results suggested that in addition to the sympathetic nerves, some neuroendocrine hormones regulate adipose tissue thermogenesis. Therefore, the observed changes in thermogenesis may be a result of synergic regulation of neurotransmitters and neuroendocrine hormones.

INTRODUCTION

Adipose tissue is an important organ for maintaining the body's energy homeostasis (1,2). Adipose tissue stores energy in the form of lipids when nutrients are abundant and mobilizes these lipids to provide energy when the body needs nutrients. The storage and mobilization of lipids provide an energy buffer, which helps keep the body in a state of energy homeostasis (3). Additionally, adipose tissue is capable of consuming energy and producing heat in a process called thermogenesis, which can be used to maintain body temperature (4). Activating thermogenesis of adipose tissue could be a promising way to combat obesity.

Adipose tissues are generally classified as white, brown, or beige. White adipose tissue (WAT) is critical for energy storage and comprises the largest adipose tissue volume (5). Brown adipose tissue is found in the interscapular area of the human neonate and disappears in adulthood (6,7). This tissue has a high thermogenic activity and is important for maintaining normothermia and survival in newborns (6,7). In depots of WAT in human and rodent adults, high thermogenic activity in select adipocytes is induced under certain conditions, such as cold stimulation (8,9). Beige adipocytes exhibit morphology and function midway between the classic brown and white adipocytes and are abundant at subcutaneous depots around the neck in humans (4). The existence of beige adipocytes was recognized due to their high glucose uptake on PET-CT (positron emission tomography integrated with computed tomography), suggesting their high metabolic activity (8,10,11). Mouse subcutaneous adipose tissues at inguinal depots are easily induced to beiging and thermogenesis under cold conditions and are commonly used to study thermogenesis (12).

In cold environments, the body produces heat to maintain body temperature through both shivering and non-shivering thermogenesis. Shivering thermogenesis is accomplished by the rapid contractions of skeletal muscles (13,14), an acute response to cold. Shivering thermogenesis by muscles decreases after days of cold acclimation, accompanied with an increase in non-shivering thermogenesis by adipose tissues (15,16). Both cold stimuli and food can induce adipose tissue thermogenesis (17,18,19).

The thermogenic function of brown or beige adipose tissue is due to the mitochondrial uncoupling protein 1 (UCP1) (1). Sugars, lipids, and amino acids are metabolized to produce acetyl-coA, which enters the tricarboxylic acid (TCA) cycle to produce reducing equivalents, the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenosine dinucleotide ($FADH_2$). When the electrons donated by NADH and FADH2 go through the electron-transport chain, the molecular oxygen is reduced to water. Meanwhile, the protons are pumped from the mitochondrial matrix into the mitochondrial intermembrane space, establishing a proton gradient across the mitochondrial inner membrane (20). The energy conserved in the proton gradient drives adenosine diphosphate (ADP) and inorganic phosphate (Pi) to synthesize adenosine triphosphate (ATP) by ATP synthase (20). Proton leak mediated by UCP1 partially dissipates the proton gradient, releasing some energy as heat (20,21).

Activation of adipose tissue thermogenesis during cold is caused by the activation of the sympathetic nervous system (22,23). Cold signals felt by the skin are transmitted via afferent nerve impulses to the central nervous system, which eventually activate efferent sympathetic activity through multi-neurons across brainstem and paravertebral

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ganglions (24,25). The sympathetic nerve endings release norepinephrine (NE), which binds to adrenergic receptors on the surface of adipocytes to activate adenylyl cyclase, increasing the intracellular level of cyclic adenosine monophosphate (cAMP) (26,27). cAMP activates protein kinase A (PKA), which increases the transcription of UCP1 and the mobilization of lipids by phosphorylating regulatory factors (26,27). By using immunohistochemistry (IHC), the sympathetic nerve fibers can be detected based on the expression of tyrosine hydroxylase (TH), an enzyme that participates in NE synthesis. In addition to sympathetic NE, other neuronal involvement in the regulation of thermogenesis of adipose tissue has been less extensively explored. We hypothesized that other neurons may participate in regulating thermogenesis of adipose tissue by releasing neuroactive factors. Thus, we aimed to investigate other potential neurotransmitters and neuroendocrine hormones that may be signaling to adipocytes. By studying mouse models exposed to different ambient temperatures, we examined the innervation of iWAT and the nature of nerve fibers from an anatomical perspective. We further examined the expression of hormone receptors on the surface of adipocytes in C57BL/6 mouse model exposed to differing ambient temperatures. We detected receptor expression for neuroendocrine hormones secreted by the hypothalamic-pituitary gland such as arginine vasopressin (AVP), oxytocin (OXT) and prolactin (PRL), indicating their potential function for adipocytes. Finally, we tested the regulatory effects of AVP and OXT on thermogenesis of adipocytes *ex vivo*. The activating effect of OXT and inhibitory effect of AVP indicate complex regulation of adipose tissue thermogenesis by diverse neurons.

RESULTS

Ambient Temperature Affects Thermogenesis of Adipose Tissue

We split 18 mice into 3 groups and housed them for three days at cold (4°C), room temperature (22°C), or warm (30°C) conditions. Thermal camera photos showing the average mouse body surface temperature at inguinal depots was the highest in the 4°C treatment group, followed by 22°C, then 30°C (**Figure 1A**). Therefore, cold exposure at 4°C likely activated the thermogenesis of subcutaneous adipose tissue, while warm conditions at 30°C likely decreased thermogenesis. Dissected inguinal white adipose tissue (iWAT) of 4°C group appeared reddish, while that of 30°C group was whiter than the 22°C group (**Figure 1B**). The oxidative capacity detected by oxygen electrodes showed that the oxygen consumption rate (OCR) of adipose tissue at 4°C was upregulated (two-tailed student's *t*-test, p=0.0449), whereas OCR in adipose tissue of mice at 30°C was downregulated (two-tailed student's *t*-test, p=0.0199) when compared with that of mice at 22°C (**Figure 1C**). Meanwhile, the UCP1 expression detected by quantitative polymerase chain reaction (qPCR) showed over a 100-fold change between the 4°C and 22°C groups and between the 22°C and 30°C groups (two-tailed student's *t*-test, 4°C vs 22°C, p=0.0017; 22°C vs 30°C p=0.0049) (**Figure 1D**). These results indicated that ambient temperature affected the activity of fat thermogenesis.

Anatomic Analysis of iWAT Innervation

Considering the role of neurotransmitters in activating the thermogenesis function of fat, the nerves innervating iWAT were anatomically analyzed (**Figure 2A, B**). We found that nerve bundles accompanied blood vessels entering the adipose tissue at two sites: the head of the fat pad near the waist and the middle near the thigh. In addition, there were some nerves underneath the skin that ran across the surface of the adipose tissue (**Figure 2B**). We dissected

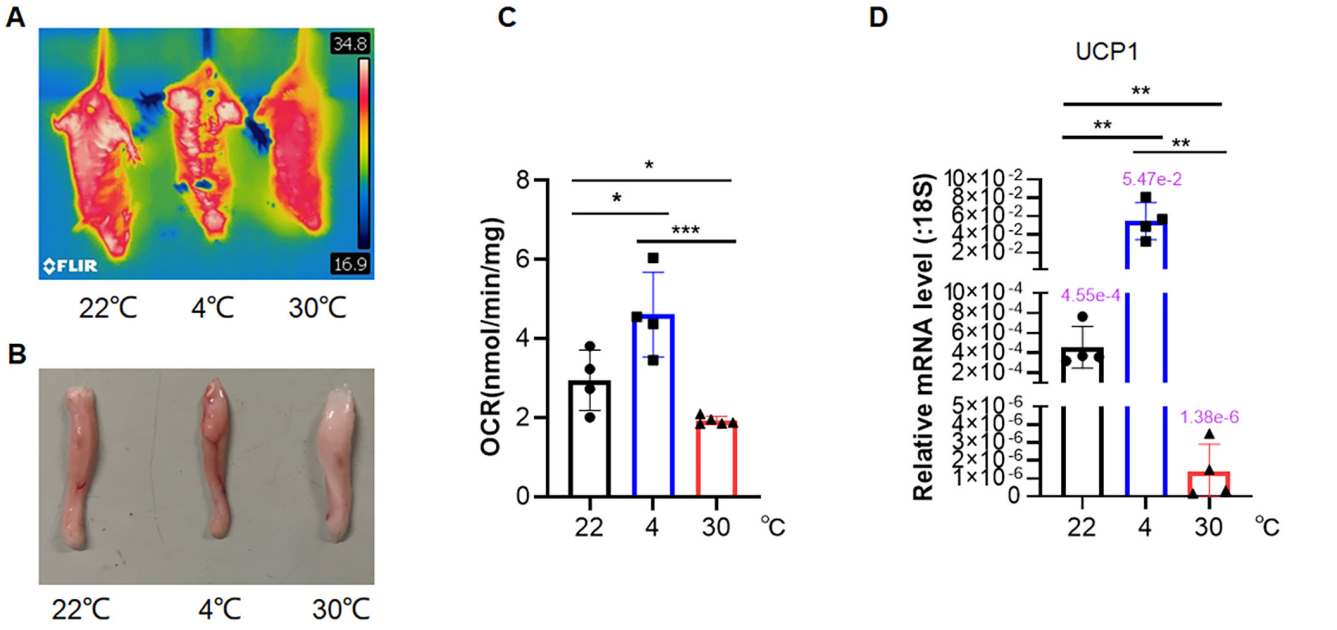

Figure 1: Thermogenesis of iWAT. A: Photos of mice under different ambient temperatures taken with an infrared camera. B: Photos of dissected iWAT. C: The levels of OCR measured with an oxygen electrode. n=4-5/group. D: The expression of UCP1 examined by qPCR. n=4/group. iWAT, inguinal white adipose tissue. OCR, Oxygen consumption rate. UCP1, uncoupling protein 1. Data in C&D are represented as mean values ± SD. *P*-values were determined by a two-tailed student's *t*-test for comparing two groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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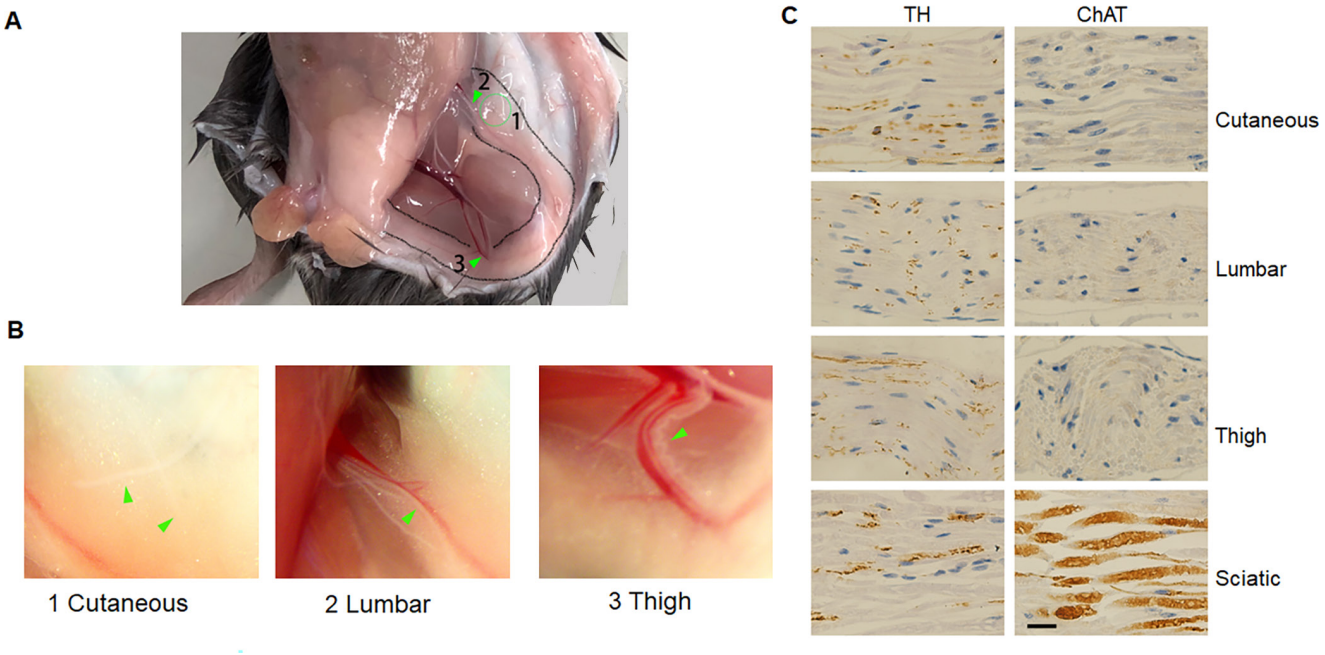

Figure 2: Anatomy of iWAT innervation. A: Photo of the iWAT. Black lines outline the adipose tissue, while green arrows and numbers mark the nerve fibers. B: Zoomed-in pictures of the nerve fibers marked by arrows (2&3) and circle (1) in A. C: Images showing immunohistochemistry (IHC) staining of TH and ChAT of nerve fibers included in A&B. The sciatic nerve was used as the positive control for ChAT. Scale bar: 20μm. iWAT, inguinal white adipose tissue. TH, tyrosine hydrogenase. ChAT, choline acetyltransferase.

these nerves and analyzed with IHC the expression of tyrosine hydroxylase (TH; an enzyme that synthesizes NE), as well as choline acetyltransferase (ChAT; the enzyme that synthesizes acetylcholine). We found that the nerves at these three sites contained TH-positive fibers but no ChAT-positive fibers, indicating that these nerve fibers included sympathetic nerves but not cholinergic nerves (**Figure 2C**). However, not all nerves in these bundles expressed TH, suggesting that some nerves innervating iWAT may release other neurotransmitters.

TH and NE Levels Do Not Correlate Well with Thermogenesis

Although adipose tissue has been shown to be innervated by noradrenergic nerves (23,28,29), it is unclear whether the activity of NE independently accounts for the changes in UCP1 and thermogenesis induced by different ambient temperatures. We examined the protein levels of TH in adipose tissue by Western blot (**Figure 3A, B**). The results showed that the TH level in iWAT after cold stimulation at 4°C was higher than that at 22°C (two-tailed student's *t*-test, p=0.0013) but did not decrease at 30°C compared to 22°C (two-tailed student's *t*-test, p=0.0584) (**Figure 3A, B**). The amount of NE in tissues was further detected by enzyme linked immunosorbent assay (ELISA), and we saw an approximate 1.5-fold increase from 22°C to 30°C (two-tailed student's *t*-test, p=0.0157) (**Figure 3C**). Upon normalization to protein amount, we observed a 1.7-fold increase in the amount of NE per unit of protein (two-tailed student's *t*-test, p=0.0016) (**Figure 3C**). We found no correlation between TH and UCP1 expression (Pearson's correlation, r=-0.2395, p=0.3234) (**Figure 3D**).

Adipocytes Express Receptors for Some Neuroendocrine Hormones

To explore other factors contributing to adipocyte thermogenesis, we used qPCR to examine the expression of receptors for neurotransmitters and hormones in mature adipocytes isolated from adipose tissues of mice maintained at 22°C. We found that mature adipocytes expressed the adrenergic receptors, *Adra1a, Adra1d*, *Adrb1*, and *Adrb3*. Moreover, cholinergic receptors m3 type (*Chrm3*), glutamate receptors (*Grik2*) and γ-aminobutyric acid (GABA) receptors (*Gabra3*) were also expressed (**Figure 4A**). The expression of *Tshr* (thyroid stimulating hormone receptor), *Prlr* (prolactin receptor), *Oxtr* (oxytocin receptor), *Avpr2* (arginine vasopressin receptor 2), and *Avpr1a* were measured (**Figure 4A**).

Since transmitters and hormones must bind to receptors to signal, the number of receptors also affects their function. We tested the differences in the expression levels of adrenergic receptors, AVP receptors, and OXT receptors at the three different temperatures: 4°C, 22°C, and 30°C. We found that the expression of *Adrb1* was the highest at 4°C (two-tailed student's *t*-test, 22°C vs 4°C p=0.0003; 4°C vs 30°C p=0.0005), while *Adrb3* was highest at 30°C (two-tailed student's *t*-test, 22°C vs 4°C p=0.0506; 4°C vs 30°C p=0.0091) (**Figure 4B, C**). Two AVP receptors (*Avpr2* and *Avpr1a*) were most highly expressed at 30°C (two-tailed student's *t*-test, *Avpr2*: 4°C vs 30°C p=0.0363; *Avpr1a*: 4°C vs 30°C p=0.0195), whereas *Oxtr* had the lowest level of expression at 30°C. There was a more than 20-fold decrease from 22°C to 30°C (two-tailed student's *t*-test, p=0.0026) and a 10-fold decrease from 4°C to 30°C (two-tailed student's

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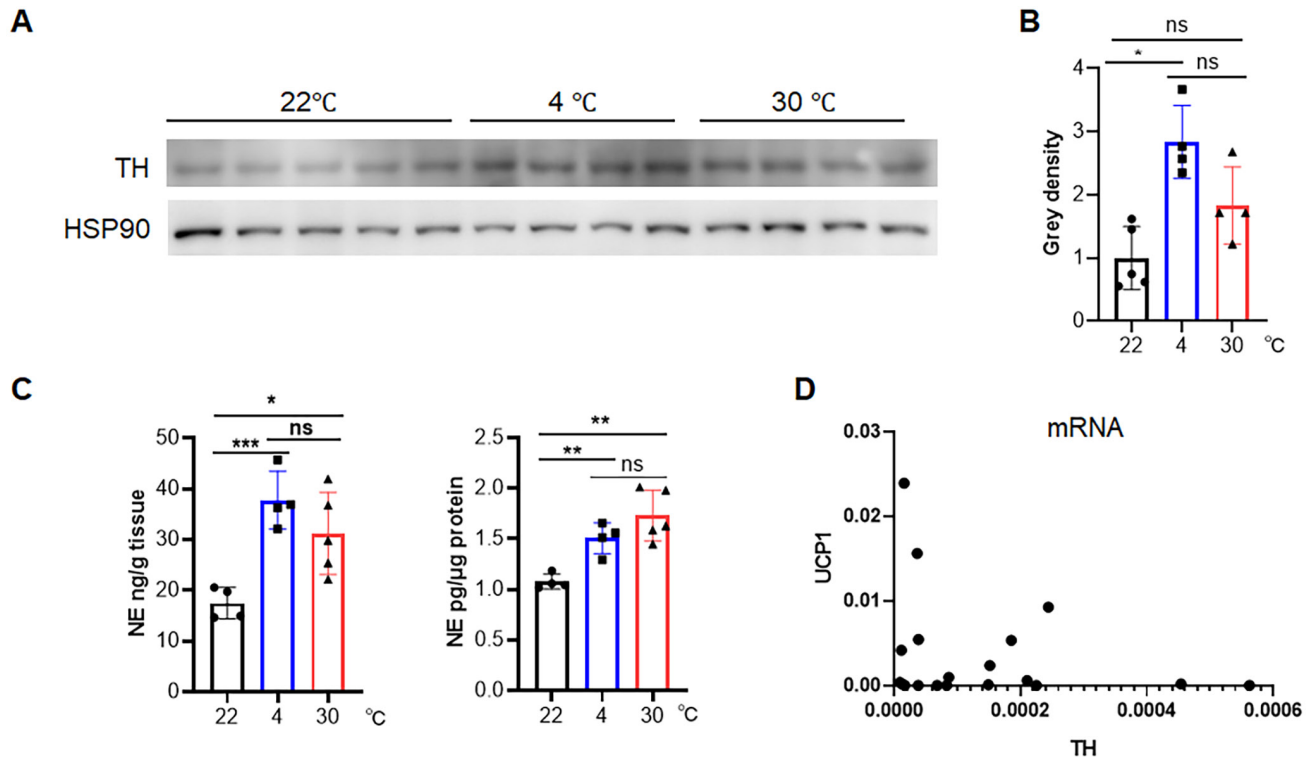

Figure 3: Sympathetic activity and UCP1 expression in iWAT. A: Western blot of TH expression in iWAT from mice exposed to different temperatures. Each lane represents the sample from one individual mouse. B: Grey density of bands in A calculated with Image J. The individual value was the grey density of TH normalized by that of the corresponding internal control HSP90. C: The amount of NE in adipose tissue detected with ELISA, which was normalized to tissue weight or protein amount. n=4-5/group. Data in B&C are represented as mean values ± SD. *P*-values were determined by a two-tailed student's t-test for comparing two groups D: Correlation of TH and UCP1 expressions in iWAT as determined by qPCR. Pearson correlation, r=-0.2395, p=0.3234. iWAT, inguinal white adipose tissue. NE, norepinephrine. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

t-test, p=0.0033) (**Figure 4D-F**).

Functions of AVP and OXT on Thermogenesis of Adipocytes

The expression pattern of receptors in adipocytes at differing temperatures suggested that OXT and AVP may regulate the function of adipocytes. To explore whether OXT and AVP directly regulate the thermogenesis of adipocytes, we tested cultured adipocytes *ex vivo*. We differentiated primary preadipocytes into mature adipocytes and added exogenous AVP and OXT to examine their effects on UCP1 expression. We found that OXT had promoted UCP1 expression by more than 10-fold at 1μM (one-way ANOVA, p=0.0001) (**Figure 5A**), while AVP inhibited UCP1 expression to 50% at 1μM (one-way ANOVA, p=0.0107) (**Figure 5B**).

DISCUSSION

The thermoneutral temperature of the mice is 30°C, at which thermogenesis of adipose tissue is at a low level (30). However, thermogenic activity is increased when mice are exposed to a 4°C environment, which was confirmed by our experiments (**Figure 1**). Since sympathetic nerves in adipose tissue regulate fat thermogenesis, it can be inferred that sympathetic activity of adipose tissue is highest at 4°C and lowest at 30°C (29). However, neither TH expression, nor the amount of local NE decreased at 30°C as expected in our study. Prior research has found that the density of sympathetic nerves in adipose tissue increased after cold exposure at 4°C and decreased when subsequently warmed to 30°C (29). This result might have been caused by the gradual temperature shift from 4°C to 30°C, which is different from our experimental procedure. In addition, because adipocytes increase lipolysis when they are stimulated by cold, the volumes of both individual adipocytes and entire tissues shrink. Conversely, the volumes increase when they are in thermoneutral environment at 30°C. Therefore, sympathetic density per unit area might not represent a change in the density of nerve fibers innervating to individual adipocytes. In our experiment, the protein load was the same for all samples when performing Western blotting, and the expression of TH tended to increase at 30°C when compared with that at 22°C after being normalized with heat shock protein 90 (HSP90), which was relatively stable in adipose tissue. Similar to the 4°C group, NE in iWAT detected by ELISA was increased at 30°C relative to that at 22°C. Therefore, sympathetic innervation and the amount of NE did not explain the decrease in adipose tissue oxygen consumption and the almost undetectable UCP1 expression at 30°C (**Figure 1D**).

In addition to the adrenoceptor subtypes, we found receptors in adipocytes for many other neurotransmitters, including acetylcholine, GABA, and glutamate. However, there are currently no reports on whether adipose tissues

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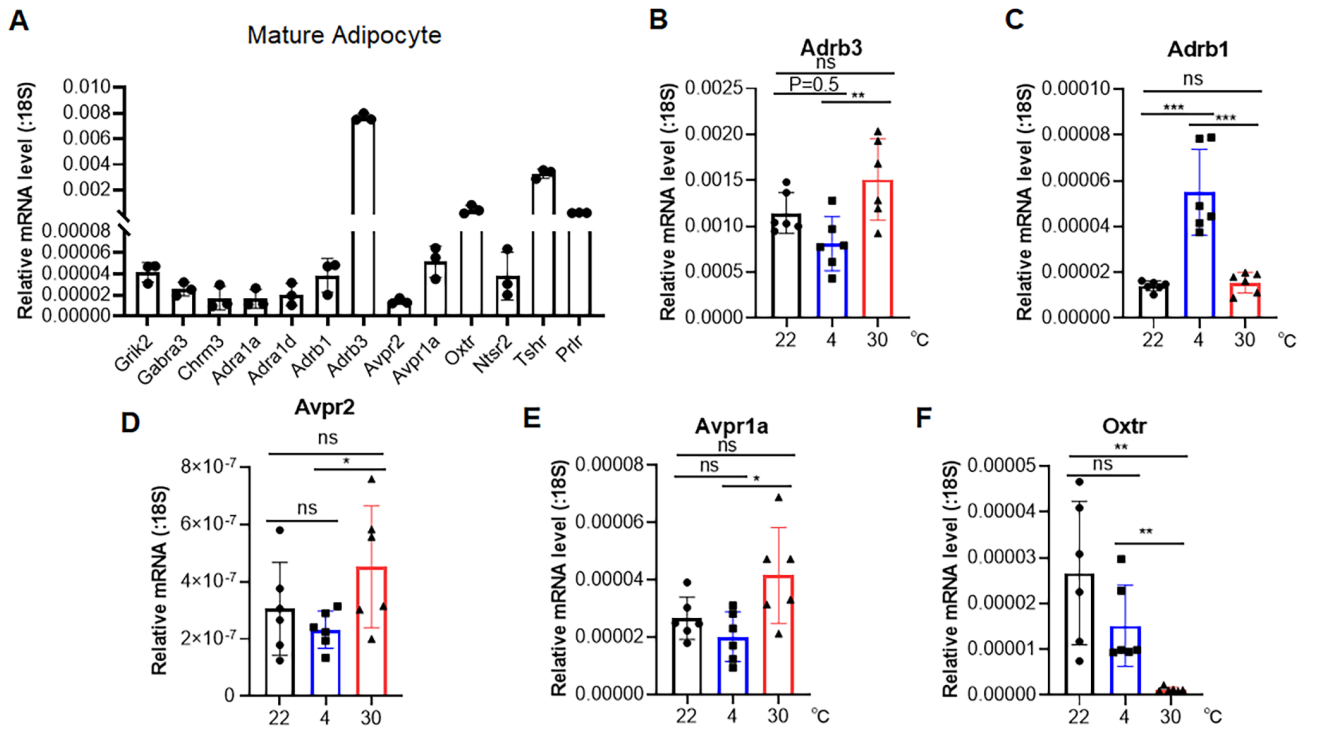

Figure 4: Expression of receptors of select neurotransmitters and hormones in mature adipocytes. A: The expression of receptors of select neurotransmitters and hormones in mature adipocytes isolated from adipose tissue by qPCR. Values were from 3 experimental replicates. In each experiment, mature adipocytes were pooled from 4-5 mice. B-F: Bar graphs showing the changes in receptor expressions in iWAT of mice under different ambient temperatures. n=6/group. iWAT, inguinal white adipose tissue. Data are represented as mean values ± SD. *P*-values were determined by a two-tailed student's t-test for comparing two groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

are innervated by nerves containing such neurotransmitters. In addition to the neurotransmitters released from the nerve endings that directly enter the adipose tissue, bloodstream hormones could reach the tissue through the capillary network. Therefore, we cannot exclude the possibility that factors originating in other parts of the body could act on the adipocytes to regulate thermogenesis. As the hypothalamus is the temperature controlling center for the body, we speculated that the neuroendocrine hormones released into the blood by the hypothalamus/pituitary gland may change along with temperature. Therefore, we screened for receptors for endocrine hormones released by the pituitary gland and found expression of the receptors for the pituitary hormones TSH, PRL, AVP, and OXT. The presence of those receptors suggests pituitary hormones released into circulation might also regulate adipocyte thermogenesis. While there has been prior research on the relationship between these hormones and fat thermogenesis, the subjects and experimental conditions were different among studies, so more research is needed to systematically elucidate the function of these hormones and the physiological conditions in which they act (31,32). In our study, AVP inhibited UCP1 expression, while OXT promoted UCP1 expression in a model of *ex vivo* cultured adipocytes, suggesting OXT and AVP could increase and inhibit thermogenesis *in vivo*, respectively. Therefore, the function of AVP and OXT in thermogenesis might explain the contradiction between sympathetic activity and thermogenesis at 30°C as shown in **Figures 1 and 3**. In this study, we did not analyze the levels of AVP and OXT in circulation after exposure to different ambient temperatures, therefore confirmation of these values is needed in the future. The endocrine regulation of adipose tissue thermogenesis might represent a mechanism of cold acclimation. Comparing levels of neuroendocrine hormones under different durations of cold exposure may further elucidate endocrine regulation of adipose tissue thermogenesis.

In conclusion, our results that iWAT has no cholinergic innervation still cannot exclude the possibility of undefined innervation participating in thermogenesis. However, the existence of receptors for pituitary hormones in the adipocytes suggests an alternative mechanism involving endocrine hormones, which may also reach the adipose tissue to exert potential regulatory roles on thermogenesis (**Figure 6**). This study suggests the synergic regulation of neurotransmitters and neuroendocrine hormones in thermogenesis, especially at thermoneutral temperatures in mice. More extensive studies are needed to explore the ligands and receptors, their functions, and their physiological regulation in the future. Continuation of this work would be of important for understanding the functions of adipose tissue, as well as metabolic disorders related to adipose tissue, such as obesity.

MATERIALS AND METHODS

Mice

All mouse experiments were approved by the ethics

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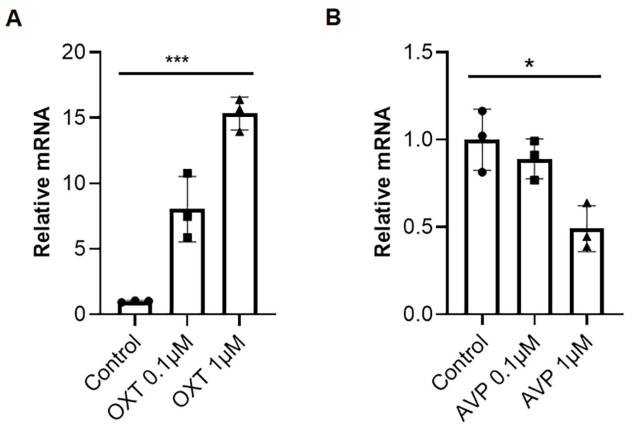

Figure 5: Effect of the hormones OXT and AVP on the expression of UCP1 in cultured adipocytes. Mature adipocytes were differentiated from stromal vascular fraction of iWAT. The cells were treated with OXT (A) or AVP (B) at 0.1μM and 1μM for 8 hours. UCP1 expression was detected with qPCR. The experiment was repeated 3 times. Values were normalized to the control. iWAT, inguinal white adipose tissue. UCP1, uncoupling protein 1. OXT, oxytocin. AVP, arginine vasopressin. Data are represented as mean values ± SD. *P*-values were determined by one-way ANOVA. * $p < 0.05$, *** $p < 0.001$.

committee of the School of Basic Medical Sciences of Fudan University (No.20200306-146). All mice were C57BL6/J and purchased from Nanjing University Model Animal Research Center. For experiments at 22°C (room temperature), mice were housed in an individually ventilated caging system. For experiments at 4°C and 30°C, mice were housed individually in cages that were put inside temperature-controlled chambers (Friocell 404) set to the indicated temperature. Mice had free access to food and water under 12h light-dark cycles. All mice were fed with a lab mice maintenance diet (calories provided by protein 21.5%, fat 11.1%, carbohydrates 67.4%; Cat. 1010001, Xietong Pharmaceutical Bio-engineering Co., Ltd. Nanjing, China). At the end of challenge, the mice were euthanized with isoflurane, and iWAT and nerve fibers were dissected. Fresh iWATs were immediately subjected to the OCR experiment. Tissues for histology examination were fixed with 4% paraformaldehyde (PFA), and those for ELISA, mRNA extraction, and Western blot were snap-frozen with liquid nitrogen and stored at -80°C.

Tissue Oxygen Consumption Experiment

Oxygen electrodes (Hansatech, Oxygraph+ system) were installed and calibrated. About 50mg of iWAT from each mouse was weighed and placed into 500μL of buffer (225.2mg glucose (Sigma-Aldrich, Cat. G7021), 1mM pyruvate (MedChemExpress, Cat.HY-Y0781), 1g of bovine serum albumin (Yeasen Shanghai, Cat. 36101ES), in 50mL PBS) and minced, and the mixture was added to the reaction tube and measured for 1 minute. The data of the first 10 seconds after adding the sample mixture were processed, and values were normalized by tissue weight (unit: nmol/ml/min).

Western Blot

The tissue was homogenized in Tris-HCL buffer (2% SDS, 60 mM Tris-cl PH 6.8, protease inhibition) and centrifuged. This was repeated several times to fully remove residual lipid. The amount of total protein in the supernatant was determined with the BCA (Bicinchoninic acid) method (Cat.23227, Thermo Scientific). The supernatant was added to 5X loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The amount of protein loaded was the same among samples. After electrophoresis, the proteins on the gel were transferred to a 0.22μm polyvinylidene fluoride (PVDF) membrane, after which the membrane was blocked with 5% milk for 1 hour at room temperature. TH antibody (1:1000 dilution, ab1542, Merck) prepared with 3% BSA in Tris-buffered saline with Tween-20 (TTBS) was subsequently added and incubated overnight at 4°C. The next day, the membrane was washed 3 times for 15 minutes with TTBS at room temperature. The HRP-conjugated secondary antibody of the corresponding species (1:10000 dilution, Cat. 33801ES60, Yeasen Biotechnology, Shanghai) was added to the membrane and incubated for 45 minutes at room temperature. The chemiluminescent substrate was added to the membrane after unbound antibody was washed away to show specific protein bands. The bands were later scanned with a LAS4000 chemiluminescent imager (GE Healthcare), and the grey density of each band was calculated with Image J software.

Real-Time qPCR

Adipose tissue was homogenized in Trizol (Invitrogen, Cat. 15596026), and the supernatant was taken after centrifugation at 12000g for 10 minutes. Total RNA was extracted and transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622). The cDNA was used as a template for fluorescent PCR amplification (instrument: ABI Viia7 thermal cycler; reagent: AceQ® qPCR SyBR Green, Vazyme, Q121). The relative expression of target gene to internal control 18S was calculated with comparative *Ct* method, and compared between groups.

Measurement of NE Levels

The NE levels in adipose tissue were detected and quantified by an ELISA kit (Labor Diagnostika Nord GmbH & Co. KG, Cat. BA E-5200R) according to the manufacturer instructions. iWAT was homogenized in a chilled homogenization buffer (0.01N HCl (SinoPharm, Cat. 10011018), 1mM EDTA (Sigma-Aldrich, Cat. E6758), 4mM Na2O5S2 (Sigma-Aldrich, Cat. S9000) in PBS) with protease inhibitors. After centrifugation at 4°C and removal of the fat cake at the top, the supernatant was collected and subjected to NE analysis. NE levels were normalized to the tissue weight or the protein concentration determined by BCA method.

Isolation of Preadipocyte and Induction to Differentiation

Freshly dissected adipose tissue was finely minced with scissors and incubated in PBS containing 0.075% collagenase Type VIII (Sigma-Aldrich, Cat. C2193) for 45 minutes at 37°C with shaking. The suspension was filtered through a 100 μm strainer, then centrifuged at 500g for 5 minutes. The preadipocytes were in the stromal vascular fraction (SVF) in the pellet and mature adipocytes floated at the surface. The pellet was resuspended with ammonium chloride lysis buffer (1.5M NH4Cl (Sigma-Aldrich, Cat. A9434), 100nM KHCO3 (Sigma-Aldrich, Cat. 60339), 10nM Na2EDTA (Sigma-Aldrich,

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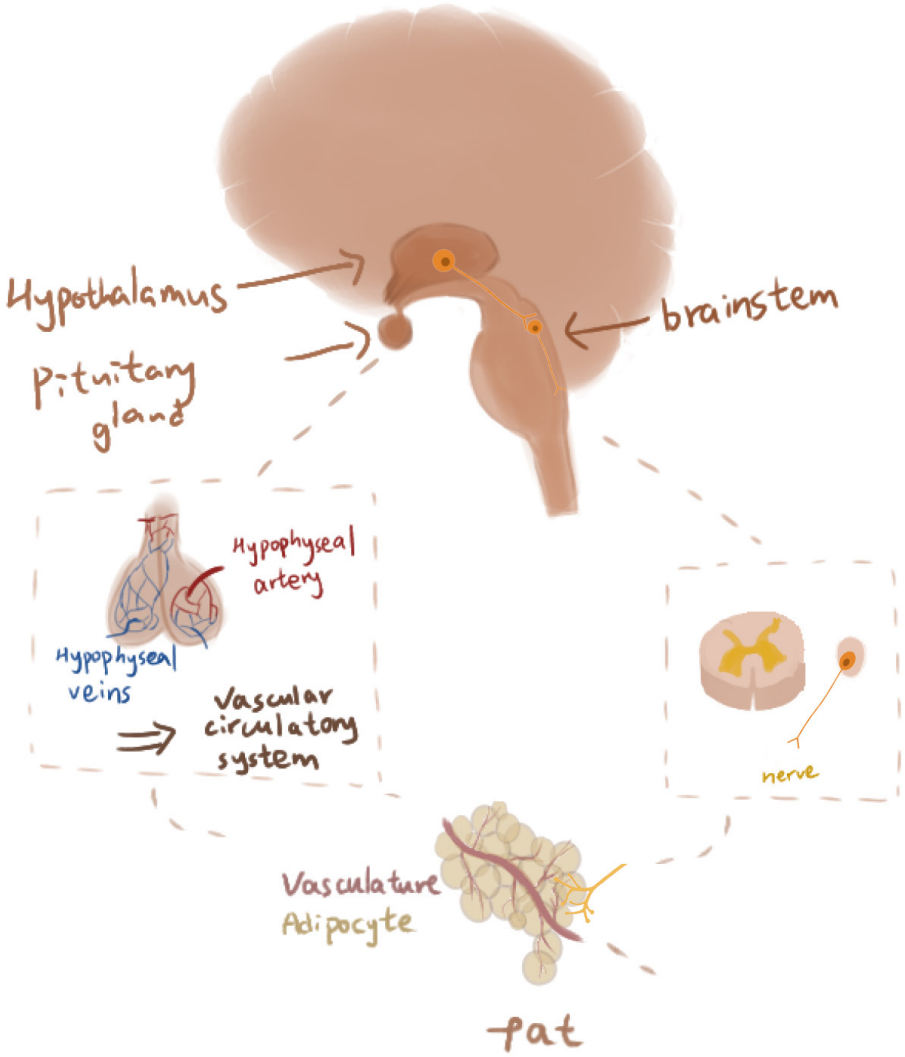

Figure 6: Schematic for sympathetic nervous system and neuroendocrine hormones regulating thermogenesis of adipose tissue. For sympathetic nervous system, the efferent neural circuit to adipose tissue involves the multiple neurons in the hypothalamus, brain stem, and paravertebral ganglions. Pituitary neuroendocrine hormones are released and transported to adipose tissue through the circulatory system. The picture was drawn by Siyun Yang with MediBang Paint software.

Cat. E5134) to lyse red blood cells. The remaining cells were washed and resuspended with culture medium and plated into dishes. Primary adipocytes were grown to confluence (37°C, 10% CO2) followed by induction of differentiation for 2 days using 0.5mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Cat. I7018), 1μM dexamethasone (Sigma-Aldrich, Cat. D4902), 1μg/mL insulin (Roche, Cat. 11376497001) and 1μM rosiglitazone (Sigma-Aldrich, Cat. R2408) in growth medium containing DMEM/F12 (HyClone, Cat. SH30023.01), 10% fetal bovine serum (Gibco, Cat. A5669701) and 100U/mL penicillin and streptomycin (Thermo Scientific, Cat. 15140122). The cells were then maintained in growth medium with 1μg/mL insulin and 1μM rosiglitazone added in. On day 4 of differentiation, the cells were cultured in growth medium without those chemical compounds, and the growth medium was replaced every other day until day 6, when the adipocytes were used for experiments.

Histology

Dissected adipose tissue was fixed in 4% paraformaldehyde for 24 hours at room temperature. Fixed tissues were embedded in paraffin, sectioned at 5μm thickness, deparaffinized, and rehydrated through graded concentrations of ethanol in water. For IHC staining, sections were probed with primary antibody against TH (Abcam, ab112, 1:500) or ChAT (Merck, ZRB1012, 1:500) and then subjected to the IHC workflow using VECTASTAIN ABC Kit (Vector laboratories, PK-8200) according to the manufacturer's instructions.

Statistics and Reproducibility

All data were analyzed using GraphPad Prism v8.0.2.263 and are represented in the figures as mean values ± SD.

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P-values were determined by a two-tailed student's t-test for comparing two groups and one-way ANOVA for multiple groups. *P*-values are indicated as follows in the figures: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Where graphs are labeled with ns, any differences between the test groups and the control groups were non-significant. 'n' in the figure legends indicate the number of mice in each group or biologically independent replicates. Western blot and micrographs results are representative experiments of more than three biologically independent replicates.

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