Orexin Restores Aging-Related Brown Adipose Tissue Dysfunction in Male Mice

Dyan Sellayah and Devanjik Sikder

Metabolic Signaling and Disease Program, Diabetes and Obesity Research Center, Sanford-Burnham Medical Research Institute, Orlando, Florida 32827

The aging process causes an increase in percent body fat, but the mechanism remains unclear. In the present study we examined the impact of aging on brown adipose tissue (BAT) thermogenic activity as potential cause for the increase in adiposity. We show that aging is associated with interscapular BAT morphologic abnormalities and thermogenic dysfunction. In vitro experiments revealed that brown adipocyte differentiation is defective in aged mice. Interscapular brown tissue in aged mice is progressively populated by adipocytes bearing white morphologic characteristics. Aged mice fail to mobilize intracellular fuel reserves from brown adipocytes and exhibit deficiency in homeothermy. Our results suggest a role for orexin (OX) signaling in the regulation of thermogenesis during aging. Brown fat dysfunction and age-related assimilation of fat mass were accelerated in mice in which OX-producing neurons were ablated. Conversely, OX injections in old mice increased multilocular morphology, increased core body temperature, improved cold tolerance, and reduced adiposity. These results argue that BAT can be targeted for interventions to reverse age-associated increase in fat mass. (Endocrinology 155: 485–501, 2014)
through the use of PET-CT technology demonstrating fully functional human BAT, that this tissue gained significant attention in the context of human obesity (11, 12). Interestingly whereas the interscapular region is the predominant site for BAT thermogenesis in rodents, it is not found here in the human adult. In human adult, the regions of most BAT activity are the thyroid/tracheal, mediastinal, paracervical/supraclavicular, parathoracic, and perirenal regions (13). Despite these recent observations of multiple locations for BAT in humans, it is well established that human BAT amount and function reach a peak soon after birth and gradually deteriorate over the course of a lifetime (14). In both rodents and humans, susceptibility to weight gain and obesity increases with age (15, 16) and is accompanied by increased risk of associated complications such as diabetes (16, 17), heart disease (18), osteoporosis (19, 20), and liver disease (21).

Emerging evidence suggests that BAT is more prevalent in younger individuals. From an easily identifiable tissue in most infants, metabolically active BAT is detectable in 50% of young subjects in their 20s and disappeared in more than 90% of individuals in their 50s and 60s (2, 3). These authors also found a positive correlation between adiposity and aging in the BAT-negative group but adiposity remained unchanged in the BAT-positive group, suggesting that diminished BAT activity may be associated with adiposity. Animal studies also support a role for diminished brown fat function in aging. For example, work done by Hansen and Kristiansen (1) and McDonald et al (4) suggest that BAT thermogenic response to cold and norepinephrine declines in aging rats. Although it is unclear as to why BAT thermogenic function is compromised with aging, it is possible that less functional BAT may contribute to the increased fat accumulation in aging mammals. Given the association between obesity and age, and the influence of BAT function on the former, surprisingly little is known about the age-related functionality of rodent BAT. With the increasingly prevalent occurrence of obese and elderly populations in the western world (16, 22) and the interconnection between them, studying BAT function in the context of aging is of paramount importance, and therapies that are able to modulate BAT function have potentially attractive implications. In the present study we set out to establish the morphologic and functional changes that occur in BAT with aging in mice and address whether these deleterious changes and their metabolic consequences can be potentially reversed. Our findings indicate a progressive deterioration of BAT function with age and suggest a role of orexin (OX), a neuropeptide integral to appetite and sleep-wake cycle, in the regulation of interscapular brown fat aging (24–26).

Materials and Methods

Animals
Male C57BL6 mice were humanely destroyed at specific ages, and their BAT and WAT depots were harvested. Mice were destroyed at the following ages: 3 weeks, 4 months, 6 months, 12 months, and 24 months of age. All mice were maintained on a standard laboratory chow diet from weaning at 3 weeks of age until their euthanization. OX/Atx (ataxin) transgenic mice in a C57BL6 background were obtained from Dr Masashi Yanagisawa at University of Texas Southwestern Medical School. All fat depots were dissected and weighed and stored for either RNA extraction or histologic analysis. Mice who were harvested at 3 weeks of age were taken directly from their maternal cages in which their mothers were fed a standard laboratory chow diet. One group of C57BL6 male mice was maintained on a high-fat (60% kCal) diet (HFD) from weaning for 16 weeks. OX/Atx mice were maintained on a chow diet and euthanized at 3 weeks and 4 months of age. All experimental procedures were conducted in accordance with approved IACUC protocol at Sanford Burnham Medical Research Institute.

Cold exposure
For the cold-tolerance test mice routinely maintained at 23°C were subjected to 6 hours of cold exposure at 4°C, during which time food was withdrawn. During cold-exposure studies, the core temperature of each mouse was ascertained using the BAT-12 rectal probe (Texas Instruments) and thermal imaging camera (Flir). After 6 hours of cold exposure, mice were humanely destroyed, and fat depots were harvested, weighed, and fixed for histologic assessment.

Body composition analysis
Body composition analysis was undertaken using the LF50 Minispec Body Composition Analyzer (Bruker). The apparatus provides noninvasive measures of lean body mass, fat mass, and fluid volume.

Glucose tolerance tests (GTTs)
GTTs were performed on mice fasted for 12 hours. Glucose (20% solution, 1 mg/kg of body weight) was injected ip into the animals, and blood glucose values were measured at 0, 30, 60, 90, and 120 minutes postinjection.

Fat depots
The following fat depots were harvested for downstream analysis: back subcutaneous WAT (bsWAT), inguinal WAT (iWAT), retroperitoneal WAT (rpWAT), gonadal WAT (gWAT), and mesenteric WAT (mWAT).

Histology
The iBAT, iWAT, and gWAT depots were dissected and placed into a petri dish containing PBS for cleanup. Tissues were fixed in 10% formalin and were paraffin embedded. Multiple sections were prepared and stained with hematoxylin and eosin for general morphologic observation. Immunofluorescent UCP1 staining was undertaken using antibody no. SC-6529 (Santa Cruz Biotechnology). For UCP1 immunohistochemistry, sections were stained with UCP1 primary antibody no. AB23841.
Primary culture and differentiation

iBAT from 3-week- and 24-month-old mice was dissected and placed into a petri dish containing prewarmed high-glucose DMEM. The tissues were cut into small slices using surgical scissors and placed in medium containing 20% BSA and 0.2% collagenase for enzymatic digestion and left on an orbital shaker for 1 hour at 37°C. Digested tissue from both bsWAT and iWAT were placed in individual tubes and filtered using a sterile mesh for 10 minutes. The resultant pellet (stromal vascular fraction) was removed and resuspended in prewarmed sterile tissue culture medium. The filtrate was centrifuged at 350 x g for 10 minutes. The resultant pellet (stromal vascular fraction) was removed and resuspended in prewarmed high-glucose DMEM containing 10% fetal bovine serum and plated. Preadipocytes were cultured to 70% confluence and then treated with fresh medium (high-glucose DMEM), or medium containing adipogenic cocktail of dexamethasone (0.5 μM), 3-isobutyl-1-methylxanthine (250 μM) indomethacin (60 μM), and insulin (0.5 μg/mL) for 7 days to induce differentiation. To ascertain whether OX would induce differentiation in 24-month-old mice, at 70% confluence cells were treated with fresh adipogenic medium as described above together with OX (100 ng/mL) for 7 consecutive days to induce differentiation. For visualization of lipid accumulation adipocytes were stained with Oil Red O. Stained cells were visualized using a light microscope at 20× magnification, and representative images were captured.

Statistical analysis

GraphPad Prism software was used for statistical analysis. Statistical significance was determined by two-tailed Student’s t test when 2 groups were analyzed. A one-way ANOVA followed by post hoc Student-Newman-Keuls test was employed to test a pair-wise comparison of every combination of group pairs, when multiple groups were compared. Multiple time-point measurements (such as GTT over time) were analyzed using two-way ANOVA for repeated measures followed by Bonferroni correction. P < .05 was considered significant.

Results

Effect of aging on body weight, adiposity, and glucose tolerance

To examine how aging process altered adiposity and impaired glucose homeostasis, we conducted phenotypic assessments of Chow-fed C57BL6 mice of 5 different age groups: 3 weeks, 4 months, 6 months, 12 months and 24 months (n = 6 animals per group). We observed that body size varied considerably depending on age (Figure 1A) with a noticeable elevation of both body size and visible visceral fat with increasing age, particularly in 12- and 24-month-old mice (Figure 1A). In terms of total body mass the largest difference was observed between ages 3 weeks and 4 months in which 18 g of mass was gained (Figure 1B). Little weight change was observed between 4 and 6 months of age. However, there was almost a 10-g difference in total body mass between the 6-month and 12-month age groups, with the former weighing 32.4 ± 1.1 g. There was also a significant difference in body weight between the 12-month and 24-month age groups with the latter exhibiting a substantially heavier weight of 53.3 ± 1.4 g (Figure 1B).

We next measured the total adiposity in different age groups of mice. We observed very little fat mass in the 3-week-old mice as expected (0.9 ± 0.1 g of fat). This equated to a body fat percentage of just 7.3% (Figure 1B). The 4- and 6-month-old mice exhibited similar fat mass
(13% fat mass). Body fat increased by more than 2-fold and, strikingly, 5-fold in 12- and 24-month-old animals, respectively, relative to 1-month-old animals. At 3 weeks of age iBAT weighed an average of 0.1 g and represented the largest depot by weight (Figure 1C). iBAT reached a peak of 0.43 g at 6 months of age but remained relatively constant until 24 months of age (Figure 1C). By 24 months of age iBAT represented the smallest depot by weight. gWAT mass, on the other hand, increased incrementally with age to reach a maximum at 24 months, constituting about one third of total body fat. The largest increase in inguinal WAT (iWAT) mass was observed between the 4-month and 6-month ages during which time its mass weight tripled. Thereafter iWAT mass did not increase significantly (Figure 1C). The bsWAT, which is located in the interscapular region, also expanded considerably with age. In 3-week-old mice bsWAT was hardly evident. It expanded, however, by some 20-fold from 3 weeks to 4 months of age and continued to accumulate mass up to the age of 24 months. These observations reveal a negative correlation between iBAT mass and adiposity during the aging process.

We next compared glucose tolerance in different age groups of mice to examine how brown and white fat mass correlated with glucose metabolism. Mice in the 24-month-old cohort were diabetic because they exhibited high fasting glucose levels (more than 225 mg/dL; Figure 1D). Fasting blood glucose was slightly, but significantly, higher in 6-month and 12-month-old groups relative to 3-week-old mice. We next tested the ability of aging mice to clear injected glucose. Repeated measures two-way ANOVA with post hoc Bonferroni correction indicated marked glucose intolerance in the 24-month-old group as demonstrated by a blood glucose peak of more than 550 mg/dL at 30 minutes postinjection, when compared with 3-week-old mice (P < .005; Figure 1D). The 24-month-old group exhibited defective glucose clearance even at later time points (60, 90, and 120 minutes postinjection; **, P < .005). Mice in the 12-month group were also compromised in their ability to clear glucose relative to 3-week-old mice (*, P < .05). Together these observations indicate that although adiposity increases and glucose sensitivity declines in aging mice, these features do not correlate with iBAT mass.

**Effects of aging on iBAT morphology and gene expression**

To determine whether aging altered iBAT morphology, we conducted histologic analysis of the tissue obtained from mice with varying ages. iBAT of 3-week-old mice displayed very small adipocytes with numerous lipid droplets all stored in the characteristic multilocular formation (Figure 2A). iBAT of 4-month and 6-month-old animals resembled similar characteristics to that of 3-week-old animals, albeit with generally larger lipid droplets (Figure 2A). In contrast, iBATs from the 2 oldest age groups (12 and 24 months) exhibited a mixed population of multilocular and unilocular adipocytes, with the latter significantly outnumbering in almost all the microscopic fields examined (one-way ANOVA with post hoc analysis; *, P < .05; **, P < .005).
Morphometric examination indicated a significant increase in adipocyte size with age, in agreement with visual appearance from histologic sections (Figure 2B).

Loss of multilocular organization during the aging process suggested that brown fat thermogenic process might be diminished in iBAT. We therefore conducted a PCR analysis to examine the impact of morphologic alteration on thermogenic gene program. We observed that UCP1 expression was markedly reduced in 12-month and 24-month-old mice relative to younger animals (3 weeks, 4 months, and 6 months of age; Figure 2C). Expression of other brown fat regulators such as deiodinase 2 (DEIOD2), mitochondrial transcription factor A, and peroxisomal proliferator-activated receptor-γ coactivator 1α were also significantly reduced. Expression of lipoprotein lipase, a critical regulator of lipolysis, exhibited a signif-
icant reduction in the 12-month and 24-month-old mice relative to the younger groups (3 weeks, 4 months, and 6 months of age) (Figure 2C). OX serves as an extracellular regulator of brown fat thermogenesis. By binding to OXR1, OX induces preadipocyte recruitment as well as brown fat differentiation (6, 7). We therefore examined whether OXR1-expression was diminished with aging. OXR1 mRNA expression was relatively stable over the course of the lifetime examined here. We noticed, however, that expression of OXR2, the second receptor to which OX couples, progressively decreased with age (Figure 2C). Together, these observations show that the unique multilocular brown morphology is progressively replaced by white-like unilocular disposition. This, along with aberration in the expression of brown adipose functional markers, suggests that thermogenic function may be compromised during the aging process.

Effects of aging on cold tolerance

Thermogenesis in brown fat during acute cold exposure contributes to thermal homeostasis. Given the morphologic and transcriptional aberrations in iBAT in aging mice, we examined their ability to defend core temperature in acute cold (6 hours/4°C), relative to younger animals. Younger mice (3 weeks, 4 months, and 6 months of age) successfully defended their core temperature for the duration of the 6-hour cold exposure (Figure 3A). Measurement of skin surface temperature confirmed that these mice were able to maintain a relatively constant temperature when shifted from room temperature to cold for 6 hours exposure, as assessed by thermal imaging (Figure 3B). In contrast, both the 12- and 24-month-old animals showed significant sensitivity to cold, exhibiting marked reductions in both core and skin surface temperatures (Figure 3, A and B). These results indicate that older mice are compromised in their ability to defend homeothermy.

Brown adipocytes rapidly mobilize a considerable amount of intracellular stored fat in order to produce heat during exposure to acute cold (7). During this process brown adipocytes and the stored intracellular fat shrink in size, which can be easily visualized in histologic sections. As expected, microscopic examination of iBAT histologic sections from a younger group (3 weeks, 4 months, and 6 months of age) subjected to acute cold showed considerable reduction in lipid droplet and adipocyte size. In contrast, acute cold failed to reduce adipocyte and lipid droplet size in iBAT of older mice (12 and 24 months of age) (Figure 3C). Moreover, intracellular lipids in the iBAT of old mice remained fairly constant despite increased thermogenic need. Heightened cold sensitivity in aging animals, along with reduced thermogenic marker expression and brown fat morphologic aberrations, suggest that iBAT function is compromised in older mice.

We next asked whether unilocular lipid deposition and failure to mobilize them during cold exposure were the consequences of elevated body weight and fat mass in aging mice. To address this, we investigated whether diet-induced obese mice also failed to mobilize brown fat fuel reserves during thermogenic need. We observed that upon feeding an HFD to 4-week-old mice for 16-weeks (final age, 5 months), they exhibit body weight and adiposity comparable to those of 12-month-old mice on standard chow. When exposed to acute cold, these high-fat-fed animals rapidly mobilized intracellular lipids stored in brown adipocytes (Figure 3D). These results indicate that the inability to mobilize brown fat fuel is not a consequence of increased adiposity but is related to the aging process. Remarkably, we also observed significant lipid mobilization from inguinal white adipocytes, as interpreted from reduction in lipid droplets and adipocyte size in iWAT following cold exposure in young animals. However, as in iBAT, we did not observe a visible reduction in lipid droplet or adipocyte size in iWAT of older mice (12 and 24 months of age) following cold exposure (Figure 3E).

Given that body weight loss has been shown in as little as 2 hours of cold exposure in mice, we studied the effects of aging on cold-induced body weight loss. Thermogenesis during acute cold has been shown to induce weight loss (27). However, it remains unclear whether cold can induce weight loss in older animals. Given the deficit in brown fat-morphologic features, dampened UCP1 expression, and defective thermogenesis in older animals, we reasoned that sympathetic activation via cold exposure might fail to induce significant weight loss in older animals. We therefore sought to measure the weights of young and old animals before and after exposure to acute cold. Food was withdrawn during the course of the experiment. After exposure to cold, 3-week-old mice exhibited more than 8% total body weight loss. This cold-induced weight loss was significantly attenuated in 12-month-old and completely abolished in the 24-month-old group (Figure 3F). These observations demonstrate that acute cold exposure is efficient in triggering weight loss in young animals but is ineffective in doing so in aged animals.

Adrenergic stimulation during cold increases lipolysis in white adipocytes, resulting in release of nonesterified fatty acids (NEFA) to support thermogenesis (8, 9). We measured serum NEFA before and after cold exposure as a marker of lipolysis. NEFA levels were significantly elevated in the serum of younger groups (3 weeks, 4 months, and 6 months of age), indicative of efficient lipolysis within the adipose tissue of these animals (Figure 3G). In
contrast, cold failed to increase NEFA in old animals, likely due to impaired lipolysis in WAT to mobilize stored fat during thermogenic need. To directly examine this postulate, we dissected various fat depots and compared their weights in animals exposed to ambient temperature or following acute cold. Measurements revealed that
3-week-old mice had a marked reduction in mass of most fat depots after cold, indicating highly efficient lipolysis. The back subcutaneous WAT, iBAT, gonadal WAT, inguinal WAT, and retroperitoneal white depots of younger mice group (3 weeks, 4 months, and 6 months of age) showed marked reductions in mass (Figure 3H). iBAT mass reduced by 20%-36% following cold exposure (Figure 3H). This ability to mobilize intracellular response was considerably dampened in older animals (Figure 3H). We next examined whether the inability to mobilize intracellular lipids was a consequence of reduced β3-adrenergic receptor (3AR) expression. Surprisingly, measurement of β3AR-expression did not reveal significant differences between young and old animals (data not shown). These results indicate that 1) lipolysis is attenuated in older mice, 2) sympathetic stimulation via cold exposure does not reduce fat mass or body weight in older mice, and 3) thermogenic dysfunction in aging mice is not due to diminished β3AR-expression.

### Aging impairs brown preadipocyte differentiation

Given the diminished expression of differentiation factors such as Cebp and Prdm16 (Figure 1H), we examined whether brown differentiation was compromised in older animals. We dissected iBAT, isolated brown preadipocytes from different age groups, and differentiated them in the presence of adipogenic cocktail (Figure 4, A and B). As expected, primary brown preadipocytes isolated from the younger age group differentiated efficiently in vitro. Upon differentiation these adipocytes expressed the characteristic brown fat markers (Figure 4C). In contrast, preadipocytes isolated from older animals differentiated poorly as assessed by Oil Red O staining for adipogenesis (Figure 3D). Preadipocyte markers known to inhibit adipogenesis, such as Necdin, Pref-1, and Wnt10a (10–13), were expressed at higher levels (Figure 4C), whereas terminal differentiation marker, UCP1, was poorly expressed (Figure 4D). To examine the functional consequence of low UCP1 expression on respiration, we plated an equal number of cells from in vitro differentiated cells from young and old animals and measured their basal as well as uncoupled O2 consumption rates. As expected, these in vitro differentiated adipocytes exhibited low levels of basal O2 consumption rate as measured by Extracellular Flux analyzer. Consistent with low UCP1 expression in differentiated adipocytes from old animals, these cells exhibited poor uncoupled respiration relative to adipocytes derived from younger animals (Figure 4E). These observations indicate that defective thermogenic differentiation of brown preadipocytes may be at the core of diminished thermogenic capacity of older animals.

### OX restores function and induces differentiation in 24-month-old iBAT

Neuropeptide OX serves as an extracellular regulator of brown fat differentiation and thermogenesis (6), a function that is mediated via G protein-coupled OX receptor 1 (OXR1) (7). Measurement of OXR1 mRNA expression in young and old mice indicated that its expression did not decrease with age (Figure 2C). Given that OXR1 was expressed in preadipocytes, we investigated whether OX could induce thermogenic differentiation in preadipocytes isolated from aged animals. We isolated preadipocytes from iBAT and cultured them in the presence of adipogenic cocktail for 7 days. Following differentiation in the presence of adipogenic media, brown preadipocytes from 3-week-old animals accumulated intracellular lipids (Figure 5A) and expressed UCP1, relative to vehicle control cells (Figure 5C). OX did not further augment adipogenesis or UCP1 expression in these cells (Figure 5, A and B). In contrast, adipogenic cocktail alone failed to induce adipogenesis in 24-month-old brown preadipocytes, as assessed by Oil Red O staining at the end of the differentiation protocol (Figure 5A). Remarkably, addition of OX to the media induced a significant amount of adipogenesis in the 24-month-old preadipocytes (Figure 5A). Measurement of respiration indicated that these adipocytes exhibit higher O2 consumption rate (Figure 5C). To directly examine whether OX had increased thermogenic potential of adipocytes, we measured their O2 consumption in the presence of oligomycin. As seen in Figure 5C, OX-differentiated adipocytes displayed significant increase in uncoupled respiration, relative to control cells. OX, however, did not increase adipogenesis (Figure 5A) or UCP1 expression (Figure 5B) in brown preadipocytes from 3-week-old mice in the presence of adipogenic inducing media, suggesting that the adipogenic cocktail was sufficient to saturate the adipogenic drive. In contrast, adipogenic cocktail alone failed to induce UCP1 expression in preadipocytes isolated from 24-month-old mice (Figure 5A). The combination of both OX and adipogenic cocktail, however, did induce UCP1 expression considerably from baseline (Figure 5B). Together, these findings indicate that OX not only rescues the adipogenic defect in preadipocytes of old mice but also rescues their thermogenic differentiation to a large extent.

### Disruption of OX-producing neurons accelerates age-related iBAT morphologic impairment

We previously reported that loss of OX function induces thermogenic dysfunction in OX-deficient mice. This thermogenic dysfunction is due to failure of brown fat to properly differentiate during in utero development. The
The ability of OX to induce differentiation in cultured preadipocytes from old animals suggests that OX may have a role in thermogenesis that is independent of its role during the developmental period. To directly test this hypothesis, we used OX/Atx transgenic mice, in which OX is progressively depleted with age due to polyglutamine repeat accumulation in orexinergic neurons. On postnatal day 3, no apparent neuronal death is noticed; by 3–4 months of age, these mice lose more than 99% of OX-producing neurons and accumulate excess weight despite consuming 30% less calories (5). At birth, brown tissue morphology was indistinguishable from wild-type control mice (data not shown). Brown fat morphology also appeared normal in 3-week-old OX/Atx transgenic mice and comparable to wild-type mice. By 4 months of age wherein the most of OX-producing neurons were ablated, OX/Atx mice exhibited abnormalities in iBAT morphology. Most of the brown adipocytes in OX/Atx transgenic mice stored large unilocular lipid droplets, whereas unilocular adipocytes were hardly observed in age-matched wild-type mice (Figure 6A, bottom panel). Three-week-old OX/Atx mice display body weight and adiposity similar to that of wild-type mice of the same age (Figure 6B). After 4 months of age, with 99% OX neuron ablation, OX/Atx mice exhibited a

Figure 4. Aging induces aberration in thermogenic differentiation of brown preadipocytes. A, Protocol for brown fat differentiation is shown. B, Representative images of Oil Red O staining following differentiation in preadipocytes isolated from 4 month (4M), 12 month (12M), and 24-month-old (24M) mice. C, Relative abundance of adipogenesis-inhibitory factors in adipocytes prior to and post differentiation. Target genes were corrected via reference 18S rRNA and calculated on the basis of the median of triplicates. D, Relative UCP1-expression adipocytes relative to that in undifferentiated cells is shown. Gene expression was normalized to reference 18S rRNA and calculated on the basis of the median of triplicates. Asterisks represent statistical significance relative to UCP1 expression relative to differentiated adipocytes from control 4-month-old group. E, Basal and uncoupled respiration following differentiation of preadipocytes isolated from indicated age groups. O2 consumption rates in differentiated adipocytes were measured by Seahorse Extracellular Flux Analyzer relative to untreated (basal) and oligomycin-treated mature differentiated adipocytes from 4-week-old mice. Oligomycin was added to assess uncoupled respiration. One-way ANOVA with Students-Newman-Keuls test was used to analyze the differences between groups; *, P < .05. Equal number of cells was plated for the measurement of O2 consumption rate. Each data point is the mean of 9–12 wells. All values are expressed as mean ± SEM. *, P < .05. Dex, dexamethasone; IBMX, isobutylmethylxanthine.
significant increase in both body weight and fat mass percent (Figure 6B). The increase in adiposity was not due to overconsumption, because OX/Atx mice consumed significantly fewer calories, relative to wild-type control mice (Figure 6C), confirming the findings of Hara and colleagues (5).

Unilocular disposition of brown adipocytes in several genetic models tracks with BAT hypoactivity (14–17). Because most brown adipocytes in OX/Atx mice were packed with unilocular lipid droplets, we assessed the thermogenic function of OX/Atx mice by exposing them to acute cold. OX/Atx mice exhibited enhanced cold sensitivity as demonstrated by an inability to maintain core body temperature in response to acute exposure to acute cold (Figure 6C). Their body temperature rapidly dropped and continued to do so until the end of the experiment. Microscopic examination of hematoxylin and eosin-stained iBAT histologic sections suggested that, unlike wild-type control mice, the brown tissue in OX/Atx mice failed to significantly mobilize intracellular lipid reserves during exposure to cold (Figure 6E), similar to observations made in brown tissue of aging animals (12- and 24-month-old rodents; Figure 1E). We next investigated whether OX replacement in OX-depleted OX/Atx transgenic mice could improve their cold-tolerance phenotype. We observed that a single injection had no effect on cold sensitivity (data not shown). However, chronic OX administration (daily × 2-weeks × 1 mg/kg) rescued cold insensitivity in OX/Atx mice. In contrast, chronic OX treatment failed to rescue the cold insensitivity in OXR1-null mice. Together these results indicate that loss of OX-producing neurons correlate with 1) emergence of white-like unilocular adipocytes in brown fat, and 2) impaired thermogenic capacity, cold sensitivity, and increased adiposity paradoxically in the face of hypophagia. However, OX-peptide, rather than other factors/proteins produced by orexinergic neurons, is required for driving the brown fat thermogenic role in mice.

**OX-peptide administration improves cold tolerance and rescues the brown differentiation defect in old animals**

In view of accelerated iBAT dysfunction in young OX-neuron ablated mice, we investigated whether administration of exogenous OX would improve iBAT function in old mice. Based on our previous experience (6), we first determined the pharmacologic dose required for increasing core body temperature (10 mg/kg, 1 mg/kg, 1 ng and 0.1 ng, ip) in 3-week-old chow-fed C57BL6 wild-type mice. A single injection of 10 mg/kg significantly increased core body temperature after 6 hours (data not shown). We next tested the ability of 1 mg/kg OX-peptide in elevating the core temperature of OX/Atx mice, relative to 3-week-old wild-type control mice. As observed in Figure 6, OX increased the core body as well as iBAT skin temperature, in wild-type...
control mice. However, this effect was not observed in 24-month-old mice (Figure 7, A and B).

We next tested whether chronic OX treatment had any effect on iBAT morphology and function; we injected OX (1 mg/kg) or vehicle daily for 2 weeks in 24-month-old mice. At the end of the experiment, we dissected iBAT and assessed the effects of the treatment on brown adipose morphology. We observed that large unilocular adipocytes had largely been replaced with multilocular cells in animals receiving OX injections, suggesting a considerable improvement in morphologic features of brown tissue in old animals. We also noticed a marked reduction in

Figure 6. Ablation of OX-producing neurons accelerates BAT dysfunction A, Representative histologic images of hematoxylin and eosin (H&E)-stained iBAT are shown. B, Body weight and percent body fat in 3-week and 4-month-old male OX/Atx mice maintained on a regular chow-diet from weaning, relative to control wild-type siblings (two-tailed Student’s t test; *, P < .05). C, Food intake of OX/Atx transgenic mice compared with wild-type control (two-tailed Student’s t test; *, P < .05). D, Core body temperature of acute cold-exposed wild-type control, OX/Atx transgenic, and OXR1-null mice injected with saline or OX, is shown. Significant differences in mutants relative to wild-type control are shown by asterisks by a two-way ANOVA with repeated measures followed by Bonferroni correction; *, P < .005; **, P < .005. Rescue of cold sensitivity in OX/Atx mice by OX-replacement is shown compared with vehicle-injected OX/Atx mice (repeated measure two-way ANOVA with Bonferroni correction; *, P < .05; **, P < .005). E, Histologic images of H&E-stained iBAT, in response to acute cold (6-hours at 4°C) in 4-month-old male wild-type and OX/Atx mice maintained on a regular chow diet from weaning. All values are expressed as mean ± SEM (n = 6 animals in each group). KO, knockout; Tempt, temperature.
the abundance of cytoplasmic intracellular lipids, suggesting an elevation in thermogenic activity in OX-injected 24-month-old animals. Consistent with this, immunohistochemistry on brown tissue sections demonstrated marked increase in UCP1 protein abundance, suggesting improved iBAT functionality and thermogenic capability (Figure 7C).

To test the functional impact of improved iBAT features, we compared the cold tolerance of vehicle and OX-injected 24-month-old animals. We exposed 24-month-old mice receiving OX or vehicle injections for 2 weeks to acute cold (4°C for 6 hours). We observed that cold tolerance was greatly improved in OX-injected 24-month-old mice, relative to vehicle-treated control (Figure 7D). The positive changes in iBAT morphology and function were accompanied by an improvement in glucose tolerance compared with the vehicle-injected group (Figure 7E), indicating that OX not only improved iBAT function but...
also restored some of the metabolic consequences of aging in older mice.

To examine whether functional changes in brown fat were associated with weight loss in OX-injected mice, we compared body composition of individually housed experimental group relative to saline-injected control cohort. We observed that OX-injected group lost about 10% of their body weight (Figure 7F), with significant alteration in food consumption (data not shown). This loss in body weight was primarily due to reduction in fat mass. On an average, the experimental group lost about 22% of fat mass (Figure 7F). Changes in the lean body mass were not statistically significant. These results demonstrate a clear association between OX therapy, brown fat activation, and improvement in glucose sensitivity and weight loss.

Inducible brown fat in white fat (also called browning) is thought to provide resistance to metabolic disturbances and obesity. These brown-like cells in white depots are recruited in response to prolonged cold and β3AR-and PPARα agonists. We therefore investigated whether, in driving weight loss and improving glucose sensitivity, OX had also induced the emergence of brown-like cells in white fat depots. To address this, we dissected various white fat depots (gWAT, iWAT, rpWAT, and mWAT) and microscopically analyzed the histologic sections for multilocular, UCP1-expressing cells. We did not detect any multilocular or UCP-expressing cells in white depots, in 24-month-old control cohorts (Figure 7G). In contrast, we noticed brown-like cells in the inguinal sc depot of OX-injected mice. These cells with multilocular disposition exhibited brown-like features as they also expressed UCP1. We did not observe these brown-like cells in gWAT, iWAT, rpWAT, or mWAT. However, these multilocular UCP1-positive cells in the OX-injected group were rare in occurrence, constituting only about 0.5% of white inguinal adipocytes, and therefore these brown-like cells are less likely to significantly drive OX’s metabolic effects.

**Discussion**

Aging is inevitable and associated with several metabolic ailments including obesity (18, 19), suggesting that age-related pathways overlap with metabolic signaling. The reason why aging predisposes one to weight gain remains an intriguing topic of discussion. Environmental factors, chronic imbalance in energy homeostasis, sleep deficit, behavioral factors, genetic attributes, DNA damage, and accumulation of reactive oxygen species, are some of the aspects that likely contribute to age-related increase in adiposity, but the predisposition mechanisms are not understood and are only beginning to be addressed (20). Elucidating the mechanisms by which extracellular and cellular age-related pathways cross talk with metabolic signaling will provide fundamental insights relevant to therapeutic intervention.

Heat dissipation mechanism in brown fat is considered to be an important contributor to energy expenditure in rodents and arguably in humans (9, 21–23). Brown fat mass and/or its metabolic activity diminish with age in both rodents and humans (2–4). This raises an important question: could declining brown fat mass/activity tip the balance in favor of calorie storage? Surprisingly, only a handful of studies have shed any light on the involvement of BAT dysfunction with age-related weight gain. These studies show a reduction in UCP1 expression and its inducibility in response to cold or β-adrenergic receptor activation (4, 24, 25). However, the age-dependent structural alterations in the brown tissue, the molecular reasons for its declining thermogenic activity, and the nature of its regulation have largely remained unaddressed. Neither have there been attempts to activate brown function in aging animals, despite the prevalence of obesity and obesity-related metabolic dysfunction in the aging population.

Here, we characterized structural and molecular alterations in brown tissue in young vs old mice in an effort to examine the contribution of the tissue in aging-related weight gain. Our findings show that body weight increases systematically with age, mostly because of an increase in fat mass, particularly in the gonadal and sc inguinal white adipose depots. This is associated with progressive decline in brown tissue function. We observed that aging was associated with striking disappearance of multilocular brown adipocytes; instead brown adipocytes stored lipids as large unilocular lipid droplets, indicative of reduced lipolytic activity. Consistent with this, old animals were unable to defend their core and skin temperature to cold. In aged mice, intracellular lipids were hardly mobilized during cold exposure, signifying impairment in BAT lipolysis. On the other hand, young rodents were able to efficiently utilize BAT lipids during cold exposure to support homeothermy.

Cold activation drives lipolytic activity in both white and brown fat depots (9). The resultant increased availability of free fatty acids is rapidly taken up by BAT to replenish its triglyceride reserves that are used to drive thermogenesis (26, 27). This elevated lipolytic and thermogenic activity, although acute, was significant enough to evoke substantial weight loss in young rodents (Figure
2F), as has also been noted previously (28). Two fat depots that particularly contributed to weight loss were gonadal and inguinal sc fat pads in young animals (Figure 2, E and I). For example, 3-week-old mice were able to lose a staggering 8% of body weight in just 6 hours’ cold exposure, demonstrating unequivocal lipolytic capability. Older animals (especially 24-month-old animals), failed to mobilize fat reserves from these depots and lose weight during acute cold exposure, confirming a dramatic impairment of lipolysis with advanced age, as has been previously shown in sheep (33). To this end we observed an attenuation of cold-induced elevations in circulating free fatty acids in old mice (Figure 2G). Conversely, circulating triglycerides were significantly lowered following cold exposure in younger mice, consistent with recent findings that BAT thermogenesis drives triglyceride clearance (27). Taken together, these results point to a greatly reduced lipolytic capacity of both WAT and BAT. This, coupled with thermogenic inadequacies, may contribute to the age-associated adiposity observed in older mice. Whereas it is well established that obesity and elevated adiposity are associated with increased BAT lipid accumulation and greater unilocular lipid droplet appearance, increased body weight and adiposity in elderly mice cannot account for the morphologic changes we see in BAT because wild-type mice on HFD with comparable weight and adiposity to 12-month-old mice did not display a similar degree of morphologic impairment in their BAT. Moreover, the BAT of HFD-fed wild-type mice was able to efficiently utilize lipids during cold exposure, suggesting superior BAT function. These observations argue that the aging process, and not an indirect effect of obesity, attenuates BAT function in older mice. Thus, BAT dysfunction and age-related obesity seem to have a cause-and-effect relationship rather than the inverse scenario.

In addition to morphologic defects in BAT structure, we also observed abnormalities in thermogenic and lipolytic gene expression with increased age. We also observed a significant reduction in OX receptor-2 (OXR2) gene expression in BAT. Our previous studies have demonstrated that OX, a neuropeptide produced in the lateral hypothalamic neurons, is essential for BAT development (24), whereas others have demonstrated its ability to induce thermogenesis (34). Ablation of OX neurons post development accelerated the rate of BAT morphologic and functional disturbance. We observed that 3-week-old OX/Atx mice display morphologically normal BAT; however, by 4 months of age, at which point most OX-producing neurons have been ablated, these mice exhibit severe morphologic abnormalities such as greatly increased unilocular lipid appearance and larger lipid droplets. BAT from 4-month-old OX/Atx mice appear strikingly similar to that of 12-month-old wild-type mice, suggesting a loss in thermogenic capacity of the tissue. Consistent with this notion, acute cold exposure in OX/Atx mice fails to promote BAT lipolysis unlike that observed in wild-type siblings. The diminished lipolytic activity likely plays a significant part in elevated cold sensitivity and obesity in OX/Atx transgenic mice (Figure 5D). Interestingly there are a number of studies that document a decline in OX neurons with advanced age in rodents (29, 30). OX-neuronal loss has been shown to be preferentially susceptible to age-related neurodegeneration compared with similarly distributed neurons such as melanin-concentrating hormone neurons of the lateral hypothalamus (30). Moreover, postmortem analyses of brains in human patients suffering from age-related neurodegenerative disorders such as Alzheimer’s disease (a disease commonly associated with obesity, diabetes, and cardiovascular disease (32)) show a marked reduction in OX neurons (by as much as 40%) relative to age-matched controls (31). Whether or not the metabolic syndrome in Alzheimer’s disease is also associated with brown fat defect remains to be investigated.

The contribution of brown fat thermogenesis to whole-body energy expenditure is considered to be substantial. It has been suggested that thermogenically stimulated brown fat can account for about one quarter of daily metabolic activity (33). This prospect has motivated numerous studies documenting therapeutic advances in the obesity control through BAT manipulation. Yet, the therapeutic potential of activating BAT has not been achieved in humans. Drugs that induce BAT activity via β-adrenergic agonism have been inadequate in inducing weight loss or laced with detrimental side effects due to β3-adrenergic activation in other tissues. At least a part of the molecular reasons rendering the β3-stimulators ineffective in inducing weight through BAT-activation could be the relative lack of mature brown adipocytes in obese subjects, either as a consequence of metabolic dysfunction or aging. Indeed, studies have revealed that obese and diabetic human subjects harbor immature brown preadipocytes, (34) that lack functional β3-adrenergic receptors (34, 35). Therefore, there is a burning need to design strategies that activates brown fat selectively and also induces maturation in preadipocytes of obese aging subjects. It is important to realize that most brown fat-related studies have exploited young animals in demonstrating weight loss by thermogenic activation (36). In our opinion, aged mice with their relatively dysfunctional brown fat provide an alternate preclinical model suitable for testing thermogenic potential of candidate drugs. Our efforts in this direction show that a single dose activates thermogenesis in
young adults but not in 24-month-old mice. Chronic OX treatment however, increased colonic and skin temperature, while greatly resolving the age-related morphologic abnormalities in BAT. Upon OX administration for 2 weeks, prevalence of unilocular lipids was markedly reduced and replaced by multilocular adipocytes. Moreover, OX treatment increased UCP1 protein in BAT as evidenced by immunohistochemical analysis. These positive changes in BAT morphology resulted in improved cold tolerance and glucose homeostasis in older animals, consistent with recent findings highlighting a role for BAT in glucose homeostasis and insulin sensitivity (37).

What is the mechanism whereby OX reverses the functional and morphologic deterioration of BAT? Observations indicate that this may be mediated through the induction of brown adipogenesis in preadipocytes in aging mice. Whereas adipogenic cocktail was sufficient to promote adipogenesis in 3-week-old primary brown preadipocytes, it had little impact on 24-month-old primary brown preadipocytes. Treatment of primary brown preadipocytes with OX for 10 consecutive days in the presence of adipogenic cocktail induced significant adipogenesis in 24-month-old primary brown preadipocytes, albeit to a lesser extent than in 3-week-old mice. The combined adipogenic effects of OX and adipogenic cocktail in 3-week-old primary brown preadipocytes did not exceed what was observed with addition of adipogenic cocktail alone, suggesting that adipogenic induction was maximal even without OX treatment in these young cells. OX-induced adipogenesis in 24-month-old mice promoted the induction of UCP1 mRNA expression. This, together with the findings of increased UCP1 protein expression in BAT, elevated core body temperature as well as improved cold tolerance in OX-treated 24-month-old mice support the assertion of greater thermogenic potential of BAT following OX treatment in older mice. Our previous work has indicated that OXR1 signaling mediates OX’s effects on brown adipocyte differentiation (6, 7); thus it is likely that OX’s ability to improve BAT function in 24-month-old mice is due to OXR1 signaling, the expression of which is not affected by age.

An important question is whether OX’s thermogenic differentiation function is mediated by central or peripheral mechanisms? Thermogenic dysfunction in orexnergic neurons-ablated mice suggests a central mechanism. On the other hand, rescue of differentiation defect by OX in isolated brown adipocytes from aged animals indicates that at least part of the thermogenic defect in aging brown fat cells resides within the preadipocytes and is independent of neuronal signals and innervations (Figure 5, A–C). Support for central mechanism in mediating OX-dependent uncoupled thermogenesis is rather weak, if any, and often contradictory. For example, centrally injected OX does not increase UCP1-expression in BAT (38). Furthermore, disruption of central OX signaling in mice by central delivery of OXR-antagonist increases UCP1 expression, suggesting that brain OX inhibits UCP1-mediated thermogenic process (39). In view of these findings, the thermogenic dysfunction and the impending cold intolerance and obesity in OX/Atx mice appear to be caused by depletion of peripheral OX rather than the loss of hypothalamic mechanisms. The rescue of thermogenic defect in OX neuron-ablated OX/Atx mice by OX administration demonstrates that OX is necessary and sufficient to drive thermogenesis in this model; other factors/hormones/proteins produced by the orexigeric neurons appear to be dispensable for brown differentiation and uncoupling (Figure 6D). This observation, however, does not preclude a role for OXR1-expressing neurons, because peripheral OX may cross the blood brain barrier and engage OXR in driving brown thermogenesis. But this possibility remains less likely given that central OX does not augment UCP1 expression in BAT in any model or species studied thus far (38–40). Although evidence strongly indicates that direct OX ligand-receptor interaction drives brown adipocyte thermogenic differentiation (6, 7), it remains unclear as to the mechanism of receptor activation in vivo. In vitro, brown adipogenesis is stimulated at low nanomolar OX concentrations. Given that the Kd of OX receptor is around 7 nM, physiological level of circulating OX is unlikely to trigger differentiation signaling effectively. However, a low degree of receptor occupancy cannot be ruled out. Neither can we ignore the possibility that OX released by the local nerve terminals innervating the interscapular depot may engage OXR1 in triggering brown differentiation. Further studies would be required to decipher the exact mechanism.

The results from the present study show that aging is associated with morphologic impairment of BAT structure and reduced thermogenic gene expression. These detrimental characteristics lead to BAT dysfunction, which is likely, a major contributor to aging-related cold sensitivity, obesity, and metabolic complications. The age-related decline in BAT thermogenic function can be restored, to a considerable degree, by OX treatment. This improvement in BAT function in older mice may be due to an increase in both BAT activation and differentiation. Taken together, our data suggest that OX therapy may have therapeutic implications in the treatment of aging-associated obesity and related metabolic abnormalities. Our results also raise the important question of whether older mice would serve as appropriate models with which to study the effects of agents that modulate BAT function. It is clear that, with a growing population of elderly individuals in
current society and an elevated risk of obesity with age, the study of BAT function in older rodents would be of considerable therapeutic importance.

Acknowledgments

We thank the histology core at Sanford Burnham Medical Research Institute for assistance with histologic processing. Professor Masashi Yanagisawa generously gifted OX/Atx mice.

Address all correspondence and requests for reprints to: Devjanik Sikder, DVM, PhD, Assistant Professor, Sanford Burnham Medical Research Institute-Metabolic Signaling and Disease, 6400 Sanger Road, Orlando, FL 75019. E-mail: dev@sanfordburnham.org.

This work was supported in part by funds made available by Marilyn Swan in the memory of John D’ Alessio.

Author Contributions: D. Sikder conceived the concept. D. Sikder and D. Sellayah designed the experiments. D. Sikder performed the experiments. D. Sikder and D. Sellayah analyzed the data and wrote the manuscript.

Disclosure Summary: The authors do not have any conflict of interest

References

35. Bronnikov G, Bengtsson T, Kramarova L, Golozezoubova V, Cannon


