Role of Hypothalamic Proopiomelanocortin Neuron Autophagy in the Control of Appetite and Leptin Response

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Autophagy is a catabolic cellular process involving the degradation of the cell's own components. Although the role of autophagy of diverse tissues in body metabolism has been investigated, the importance of autophagy in hypothalamic proopiomelanocortin (POMC) neurons, key regulators of energy balance, has not been addressed. The role of autophagy in leptin sensitivity that is critical for the control of body weight and appetite has also not been investigated. We produced mice with specific deletion of *autophagy-related 7* (*Atg7*), an essential autophagy gene, in hypothalamic POMC neurons ($Atg7^{\Delta POMC}$ mice). Atg7 expression was deficient in the arcuate nucleus of the hypothalamus of $Atg7^{\Delta POMC}$ mice. p62, a specific substrate of autophagy, accumulated in the hypothalamus of $Atg7^{\Delta POMC}$ mice, which colocalized with ubiquitin. $Atg7^{\Delta POMC}$ mice had increased body weight due to increased food intake and decreased energy expenditure. $Atg7^{\Delta POMC}$ mice were not more prone to diet-induced obesity compared with control mice but more susceptible to hyperglycemia after high-fat diet. The ability of leptin to suppress fasting-elicited hyperphagia and weight gain during refeeding was attenuated in $Atg7^{\Delta POMC}$ mice. Deficient autophagy did not significantly affect POMC neuron number but impaired leptin-induced signal transducer and activation of transcription 3 activation. Our findings indicate a critical role for autophagy of POMC neurons in the control of energy homeostasis and leptin signaling. (***Endocrinology*** **153: 1817–1826, 2012)**

Macroautophagy (here referred to as autophagy) is basically a catabolic process involving the degradation of the cell's own components and involves the rearrangement of subcellular membranes to sequester cytoplasm and organelles for delivery to lysosomes, where the sequestered material is degraded and recycled (1). The main purposes of autophagy are quality control of organelles or cellular proteins in the basal state and protection of intracellular homeostasis in the case of energy failure or nutrient deficiency (2). Hence, deficiency of autophagy would render cells precarious both in static and stressed conditions.

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Abbreviations: Ab, Antibody; ARC, arcuate nucleus; *Atg7*, *autophagy-related 7*; Bip, binding immunoglobulin protein; CHOP, C/EBP homologous protein; EE, energy expenditure; eIF2α, eukaryotic initiation factor 2 α; ER, endoplasmic reticulum; HFD, high-fat diet; icv, intracerebroventricular; IPGTT, ip glucose tolerance test; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; PERK, PKR-like ER kinase; POMC, proopiomelanocortin; shRNA, short hairpin RNA; STAT3, signal transducer and activation of transcription 3.

We and others have reported that autophagy is important in the maintenance of pancreatic β-cell mass, structure, and function (3, 4). Autophagy has been shown to be involved in adipogenesis (5, 6). The effect of hypothalamic agouti-related peptide neuron-specific disruption of *autophagy-related 7* (*Atg7*), an essential autophagy gene, has also been studied, showing a lean phenotype accompanied by increases in hypothalamic proopiomelanocortin (POMC) and its cleavage product, α-MSH levels (7). In contrast, *Atg7* knockdown by short hairpin RNA (shRNA) delivery to the mediobasal hypothalamus resulted in obesity associated with activation of I κB kinase β IKKβ/nuclear factor κB (NF-κB) pathway (8). However, the role for autophagy in hypothalamic POMC neurons that are critical for the control of appetite and body weight has not been investigated. The role of autophagy in leptin sensitivity and signaling that play an important role in the central and peripheral energy homeostasis (9) has also not been explored. Here, we report that autophagy deficiency in hypothalamic POMC neurons, the major regulator of energy intake, leads to obesity and leptin resistance, suggesting that hypothalamic autophagy plays an important role in the control of appetite or body weight and leptin signaling.

Materials and Methods

Mice

Atg7$^{F/W}$ mice of C57BL/6 background were crossed to POMC-*Cre*$^{+}$ mice of C57BL/6 background (kindly provided by J. K. Elmquist) to generate mice with POMC neuron-specific *Atg7* deletion (*Atg7*$^{\Delta POMC}$ mice). *Cre*-mediated recombination was confirmed by PCR using genomic DNA from the arcuate nucleus (ARC) and primers flanking the floxed region (forward, TGGCTGCTACTTCTGCAATGATGT; reverse, CTAAGCAGGTGAGATCTACACTCA). Blood glucose levels and body weight were monitored weekly. Intraperitoneal glucose tolerance test (IPGTT) was performed as described (4). For intracerebroventricular (icv) administration of leptin, cannulae were implanted into the third ventricle of mice (1.8 mm caudal to the bregma and 5.0 mm ventral to the sagittal sinus) as described (10). After a 7-d recovery period and an overnight fast, 2 μl of 0.9% saline or 3 μg of leptin (R&D Systems, Minneapolis, MN) were administered. For some experiments, mice were fed 60% high-fat diet (HFD) (Research Diets, Inc., New Brunswick, NJ) since 3.5 wk of age.

All animal experiments were conducted in accordance with the institutional guidelines of Samsung Medical Center. This project was approved by the Institutional Animal Care and Use Committee of Samsung Medical Center.

Reverse transcription-polymerase chain reaction

RNA was prepared from punch biopsies of the ARC using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using Superscript II (Invitrogen) and oligo(dT)$_{12-18}$ primer. Atg7 expression was examined by real-time RT-PCR using total RNA from the ARC and specific primers (forward, ATGCCAGGACACCCTGTGAACTTC; reverse, CAGGACAGAGACCATCAGCTCCAC). POMC expression in the ARC was evaluated by real-time RT-PCR using specific primers (forward, CAGGTCCTGGAGTCCGAC; reverse, CATGAAGCCACCGTAACG).

Western blotting

Tissue lysate of the mediobasal hypothalamus was prepared as described (10), and Western blotting was conducted using anti-p62 (Progen, Heidelberg, Germany), anti-binding immunoglobulin protein (BiP) (Stressgen, Antwerp, Belgium), anti-C/EBP homologous protein (CHOP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-eukaryotic initiation factor 2 α (eIF2α) (Cell Signaling, Danvers, MA) antibodies (Ab).

Body composition and energy expenditure (EE)

Fat and lean body mass were measured by an ^{1}H minispec system (LF90II; Bruker Optik, Billerica, MA). Activity, food consumption, and EE were assessed in a metabolic monitoring system (Comprehensive Lab Animal Monitoring System; Columbus Instruments, Columbus, OH) for 4 d (2 d of acclimation followed by 2 d of measurement) as previously described (11). EE was calculated from the gas exchange data. Locomotor activity was measured by counting the number of infrared beam breaks on x- and z-axes during the measurement period.

Measurement of hormone levels

Serum levels of leptin (R&D Systems) and insulin (Shibayagi Co., Gunma, Japan) were measured using commercial ELISA kits. Fasting serum cortisol level was measured using a RIA kit (Immunotech, Marseille, France).

Confocal microscopy

Accumulation of p62 in the ARC of the hypothalamus was evaluated using a previously reported protocol with modifications (4). Briefly, frozen hypothalamic sections were incubated with anti-β-endorphin Ab (Phoenix Pharmaceuticals, Belmont, CA), followed by incubation with Alexa Fluor 555-labeld anti-rabbit IgG (Invitrogen). Sections were further incubated with anti-p62, then with fluorescein isothiocyanate-labeled anti-guinea pig IgG (Zymed, San Francisco, CA). For colocalization of p62 and ubiquitin, anti-p62 Ab-stained sections were incubated with antiubiquitin Ab (Dako, Carpinteria, CA), then with Alexa Fluor 568-labeled antirabbit IgG (Invitrogen). The number of POMC neurons was estimated using a previously reported method with modifications (12). Briefly, after immunofluorescent staining of POMC neurons using anti-β-endorphin Ab as above, sections were stained with 4′,6-diamidino-2-phenylindole. The number of β-endorphin^{+} cells in the rostral, middle, and caudal ARC was counted by confocal microscopy. Leptin signaling in POMC neurons of the ARC was examined by studying signal transducer and activation of transcription 3 (STAT3) phosphorylation. Fresh frozen hypothalamic sections were prepared 30 min after icv administration of leptin. After staining with anti-α-MSH Ab (Millipore, Billerica, MA) and then with Alexa Fluor 488-labeled antisheep IgG, hypothalamic sections were incubated with p-STAT3 Ab (Cell Signaling). Additional incubation with Alexa Fluor 546-labeled antirabbit IgG was performed for confocal microscopy. Here, anti-α-MSH Ab was used

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instead of anti-β-endorphin Ab to avoid the use of the same secondary Ab (antirabbit IgG). Accumulation of Bip or CHOP was examined by sequential immunofluorescent staining of frozen hypothalamic sections using anti-Bip Ab (Cell Signaling) and anti-α-MSH Ab or anti-CHOP Ab (Santa Cruz Biotechnology, Inc.) and anti-β-endorphin Ab as the primary Ab, followed by confocal microscopy. Activation of PKR-like ER kinase (PERK) or eIF2α was evaluated by sequential immunofluorescent staining of frozen hypothalamic sections using anti-p-PERK (Santa Cruz Biotechnology, Inc.) and anti-α-MSH Ab or anti-p-eIF2α (Cell Signaling) and anti-α-MSH Ab as the primary Ab, again followed by confocal microscopy.

Statistical analysis

Data are expressed as means ± SE from multiple measurements. Two-tailed Student's *t* test was employed to compare values between two groups. Repeated-measures ANOVA using linear mixed model was employed to compare repeated measurements between groups or interaction between variables. $P < 0.05$ was considered significant.

Results

Generation of POMC neuron-specific autophagy-deficient mice

To derive POMC-specific autophagy-deficient mice, we bred *Atg7*-floxed mice ($Atg7^{F/W}$) with POMC-Cre^{+} mice (13) that express Cre recombinase specifically in POMC neurons. Deletion of floxed *Atg7* segment in POMC-Cre^{+}; $Atg7^{F/F}$ ($Atg7^{\Delta POMC}$) mice was confirmed by PCR analysis using genomic DNA from the ARC of the hypothalamus (Fig. 1A). Floxed *Atg7* allele in $Atg7^{\Delta POMC}$ mice was from neurons other than POMC neurons in the ARC. Atg7 mRNA expression in the ARC assessed by real-time RT-PCR was significantly reduced in $Atg7^{\Delta POMC}$ mice compared with littermate control POMC-Cre^{-};$Atg7^{F/F}$ ($Atg7^{F/F}$) mice ($P < 0.001$). Residual Atg7 mRNA expression was observed in the ARC of $Atg7^{\Delta POMC}$ mice due to the expression of Atg7 mRNA in non-POMC neurons (Fig. 1B). We studied possible accumulation of p62 that is a specific substrate of autophagy (14). Western blotting showed massive accumulation of p62 in the hypothalamus of $Atg7^{\Delta POMC}$ mice (Fig. 1C). Confocal microscopy revealed p62 accumulation in both punctate and diffuse patterns that colocalized with β-endorphin in POMC neurons (Fig. 1D). p62 also colocalized with ubiquitin in the same neurons, which is consistent with the role of p62 as an adaptor for ubiquitinated proteins in autophagosome formation (Fig. 1E) (14).

Obesity of $Atg7^{\Delta POMC}$ mice

Development of $Atg7^{\Delta POMC}$ mice was apparently indistinguishable from littermate control $Atg7^{F/F}$ mice. However, body weight of male $Atg7^{\Delta POMC}$ mice was increased compared with male $Atg7^{F/F}$ mice since 5 wk of age, and the difference persisted throughout the observation period ($P < 0.001$) (Fig. 2A). In contrast, body weight of female $Atg7^{\Delta POMC}$ mice appeared higher compared with female $Atg7^{F/F}$ mice, but the difference was not statistically significant (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Thus, we analyzed male $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice throughout the study. Nuclear magnetic resonance analysis of body composition showed that the increase in body weight of $Atg7^{\Delta POMC}$ mice was mostly due to an increase in fat mass ($P < 0.001$) (Fig. 2B). Lean body mass was not significantly increased in male $Atg7^{\Delta POMC}$ mice compared with $Atg7^{F/F}$ mice ($P > 0.05$) (Fig. 2B). To study the mechanism underlying the increased body mass in $Atg7^{\Delta POMC}$ mice, we measured food intake. Food intake was significantly higher in $Atg7^{\Delta POMC}$ mice compared with $Atg7^{F/F}$ mice at 6–16 wk of age ($P < 0.05$) (Fig. 2C). We also determined EE that is regulated by hypothalamic hormones (9). EE was significantly reduced in $Atg7^{\Delta POMC}$ mice compared with $Atg7^{F/F}$ mice ($P < 0.05$) (Fig. 2D), indicating that both increased food intake and decreased EE contributed to the increased body mass of $Atg7^{\Delta POMC}$ mice. In contrast, locomotor activity was not different between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice ($P > 0.1$) (Fig. 2E), thereby eliminating the possibility that a reduction in locomotor activity contributes to the decreased EE.

Despite the increased body fat mass, nonfasting blood glucose level was not different between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice ($P > 0.1$) (Fig. 2F). IPGTT also revealed no difference in glucose tolerance between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice ($P > 0.1$) (Fig. 2G).

Because POMC and its cleavage products are also produced in corticotrophs of the pituitary gland (15), we examined whether function of corticotrophs is affected by $POMC^{+}$ cell-specific autophagy deficiency by measuring serum cortisol level. Fasting serum cortisol level was not different between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice ($P > 0.1$) (Fig. 2H), suggesting no alteration of pituitary corticotroph function in $Atg7^{\Delta POMC}$ mice and no role of pituitary-adrenal hormone axis in the weight gain of $Atg7^{\Delta POMC}$ mice.

We next investigated whether leptin resistance of $Atg7^{\Delta POMC}$ mice predisposes the mice to diet-induced obesity. Repeated-measures ANOVA using linear mixed model showed that body weight of $Atg7^{\Delta POMC}$ mice fed HFD since 3.5 wk of age was significantly higher compared with $Atg7^{F/F}$ mice fed HFD ($P < 0.05$) (Fig. 3A). However, linear mixed model analysis showed that weight gain of $Atg7^{\Delta POMC}$ mice after HFD was not statistically different from that of $Atg7^{F/F}$ mice ($P > 0.1$), showing no interaction between diet-induced obesity and hypothalamic autophagy status (Fig. 3B). We next analyzed the

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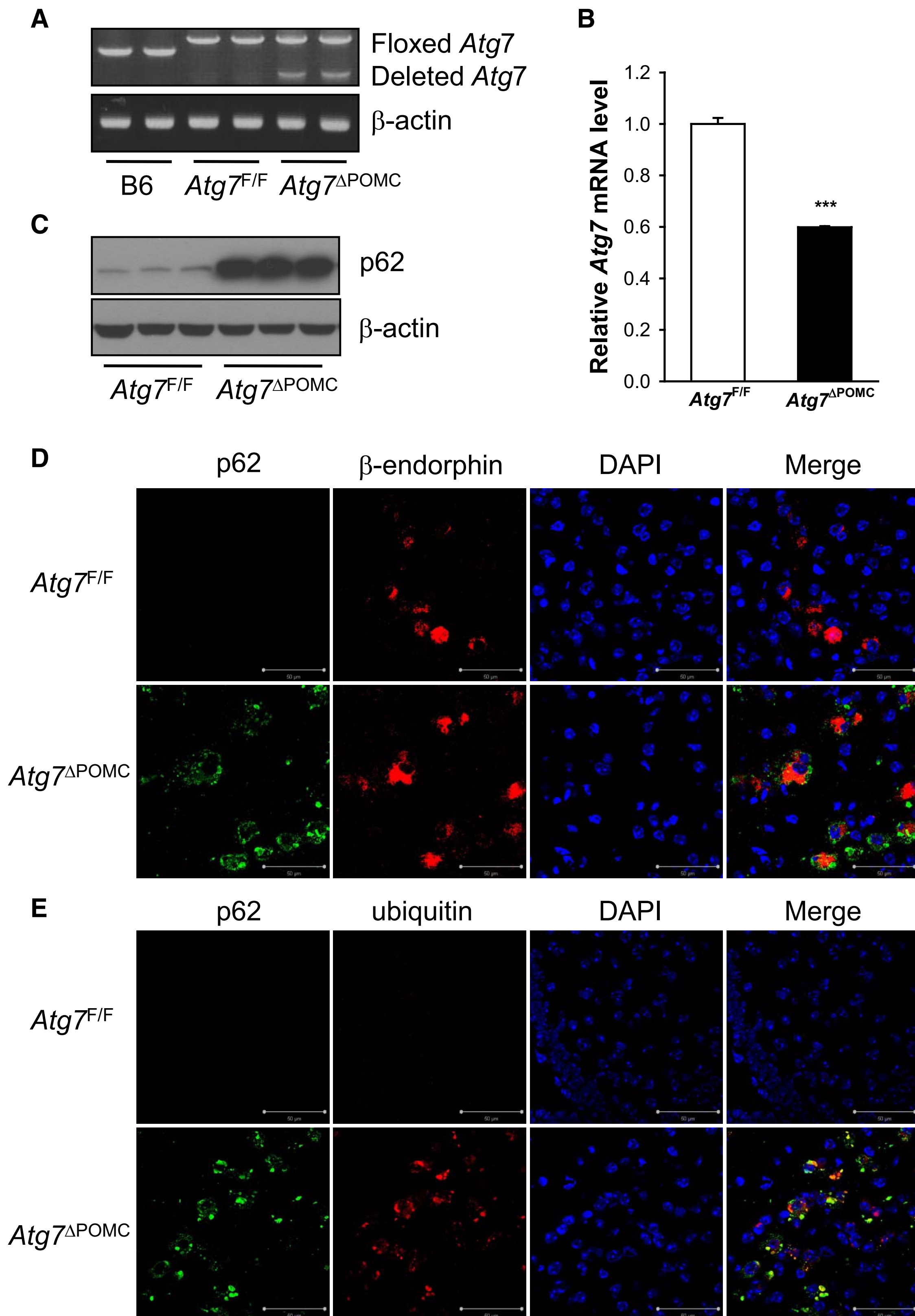

FIG. 1. Generation of *Atg7*$^{\Delta POMC}$ mice. A, *Atg7*$^{F/W}$ mice were crossed to POMC-*Cre*$^{+}$ mice to generate *Atg7*$^{\Delta POMC}$ mice. Genomic DNA prepared from the ARC was analyzed by PCR to detect floxed and deleted *Atg7* alleles (B6, C57BL/6). B, RNA was prepared from the ARC, and real-time RT-PCR was done using primers specific for *Atg7* (n = 2–4). C, Tissue lysates of the mediobasal hypothalamus were subjected to Western blotting using anti-p62 Ab. D and E, Colocalization of p62 and β-endorphin (D) or that of p62 and ubiquitin (E) was evaluated as described in *Materials and Methods* (***, $P < 0.001$). *Scale bar*, 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.

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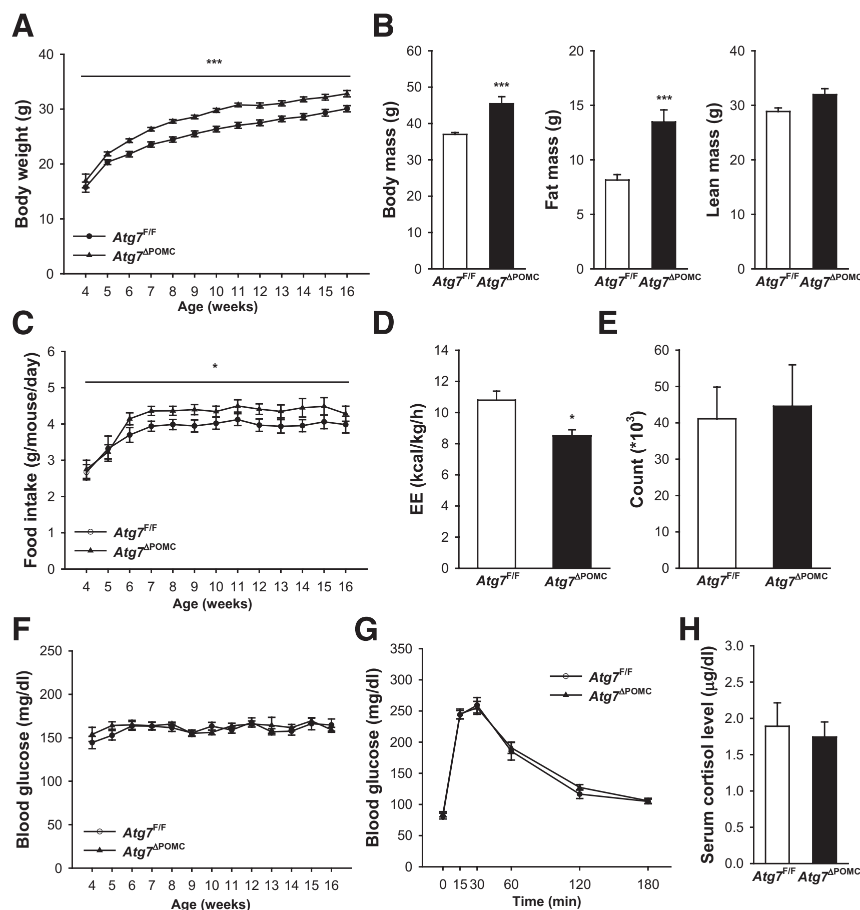

FIG. 2. Obesity of *Atg7*$^{\Delta POMC}$ mice. A, Body weight of male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice fed normal chow diet was monitored up to 16 wk of age, and repeated-measures ANOVA was done (n = 22–30). B, Total body weight, fat mass, and lean body mass were evaluated at 20 wk of age using nuclear magnetic resonance analysis (n = 8 each). C, Food intake was measured up to 16 wk of age, and repeated-measures ANOVA was done (n = 23–31). D and E, EE (D) and locomotor activity (E) were assessed at 20 wk of age in metabolic cages (n = 8 each). F, Nonfasting blood glucose level of male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice fed normal chow diet was monitored up to 16 wk of age (n = 23–31). G, IPGTT was done at 16 wk of age (n = 6–7). H, Serum cortisol level of fasted 20-wk-old mice was measured by RIA (n = 11–14; *, $P < 0.05$; ***, $P < 0.001$).

effect of HFD on blood glucose profile of *Atg7*$^{\Delta POMC}$ mice. Although nonfasting blood glucose level was not different between *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice fed normal chow diet (Fig. 2F), nonfasting blood glucose level of *Atg7*$^{\Delta POMC}$ mice on HFD was higher compared with *Atg7*$^{F/F}$ mice on HFD by repeated-measures ANOVA using linear mixed model ($P < 0.05$) (Fig. 3C). Further, linear mixed model analysis showed that the effect of HFD on nonfasting blood glucose level was significantly higher in *Atg7*$^{\Delta POMC}$ compared with *Atg7*$^{F/F}$ mice ($P < 0.05$), showing interaction between glucose profile after HFD and hypothalamic autophagy status (Fig. 3D). These results suggest that autophagy deficiency in POMC neurons does not render animals more prone to HFD-induced weight gain but render them more susceptible to hyperglycemia after HFD. However, blood glucose level of *Atg7*$^{\Delta POMC}$ mice on HFD did not reach diabetic level (Fig. 3C).

Resistance to leptin in *Atg7*$^{\Delta POMC}$ mice

Increased food intake and body mass of *Atg7*$^{\Delta POMC}$ mice may be due to an impaired response of autophagy-deficient POMC neurons to leptin, because POMC neurons are the major targets of leptin. We thus studied changes in food intake and body weight after leptin challenge to overnight-fasted mice. In control *Atg7*$^{F/F}$ mice, icv leptin treatment dramatically reduced cumulative food intake during the 24-h postinjection period compared with saline injection as expected ($P < 0.05$). In contrast, cumulative food intake was not changed by icv leptin administration to *Atg7*$^{\Delta POMC}$ mice between 1 and 24 h after treatment ($P > 0.1$), suggesting that anorectic response to leptin was impaired in these mice relative to *Atg7*$^{F/F}$ mice that exhibited an intact response to leptin (Fig. 4A). Thus, food intake after leptin treatment was significantly higher in *Atg7*$^{\Delta POMC}$ mice compared with *Atg7*$^{F/F}$ mice between 1 and 24 h ($P < 0.05$) (Fig. 4A). Further, gain of body weight during the 24-h postinjection period was significantly reduced by icv administration of leptin compared with saline injection in control mice ($P < 0.05$) but not in *Atg7*$^{\Delta POMC}$ mice ($P > 0.05$); thus, leptin failed to suppress weight gain in *Atg7*$^{\Delta POMC}$ mice (Fig. 4B). Accordingly, weight gain after refeeding was significantly higher in leptin-treated *Atg7*$^{\Delta POMC}$ mice compared with control mice ($P < 0.05$) (Fig. 4B). During the refeeding period, food intake and body weight gain in saline-injected *Atg7*$^{\Delta POMC}$ mice did not differ from those in saline-injected *Atg7*$^{F/F}$ mice, which may be due to stress from icv administration. ELISA showed that serum leptin concentration was markedly elevated in *Atg7*$^{\Delta POMC}$ mice compared with *Atg7*$^{F/F}$ mice ($P < 0.01$), probably due to increased fat mass of *Atg7*$^{\Delta POMC}$ mice after impaired leptin sensitivity. In contrast, serum insulin level appeared elevated in *Atg7*$^{\Delta POMC}$ mice compared with *Atg7*$^{F/F}$ mice, also probably due to increased fat mass; however, the difference was not statistically significant ($P > 0.05$) (Fig. 4C).

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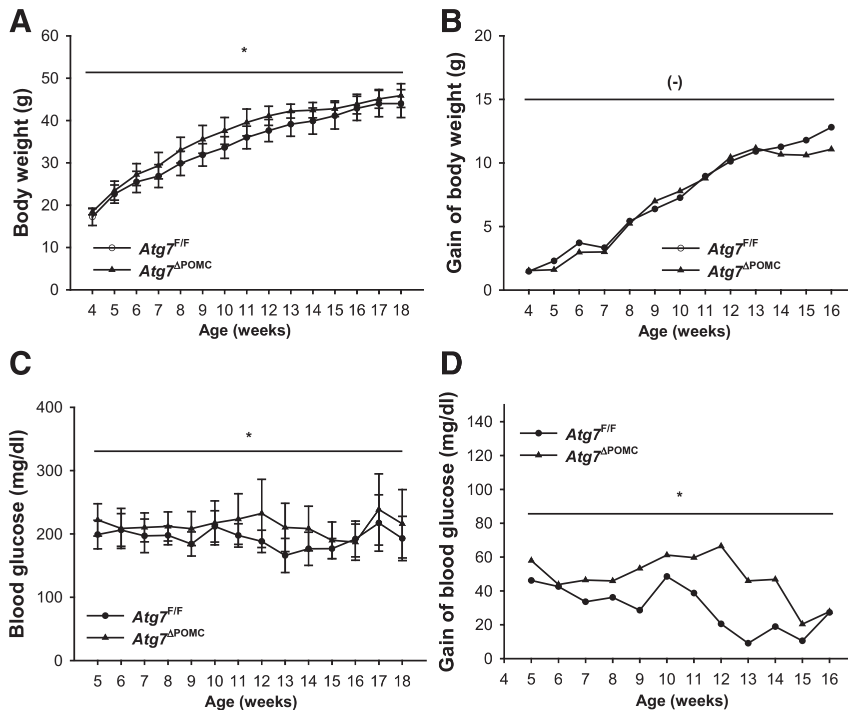

FIG. 3. Changes of body weight and glucose profile in *Atg7*$^{\Delta POMC}$ mice fed HFD. A, Body weight of male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice fed HFD since 3.5 wk of age was monitored up to 18 wk of age, and repeated-measures ANOVA was done (n = 8–9). B, Gain of body weight after HFD was plotted and compared between male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice (n = 8–30). C, Nonfasting blood glucose level of male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice fed HFD since 3.5 wk of age was monitored up to 16 wk of age (n = 8–10). D, Increases of nonfasting blood glucose level after HFD were plotted and compared between male *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice (n = 8–31; *, $P < 0.05$).

Mechanism of leptin resistance of autophagy-deficient POMC neurons

We next studied the mechanism underlying leptin unresponsiveness of autophagy-deficient POMC neurons. POMC mRNA expression in the ARC of *Atg7*$^{\Delta POMC}$ mice was not different from *Atg7*$^{F/F}$ mice in fasted state ($P > 0.1$) (Fig. 5A). In refed state, POMC mRNA expression in the ARC of *Atg7*$^{F/F}$ mice was significantly increased compared with fasted state ($P < 0.01$), similar to a previous paper (16–18). However, postprandial increase in ARC POMC mRNA expression was absent in *Atg7*$^{\Delta POMC}$ mice ($P > 0.1$). Thus, postprandial POMC mRNA expression in *Atg7*$^{\Delta POMC}$ mice was significantly lower compared with *Atg7*$^{F/F}$ mice ($P < 0.05$) (Fig. 5A), suggesting functional impairment of POMC neuron response to dietary changes. Next, we determined the number of hypothalamic POMC neurons in *Atg7*$^{\Delta POMC}$ mice to examine the role of autophagy in the viability of POMC neurons. Confocal microscopy showed that the number of POMC neurons in the hypothalamus of *Atg7*$^{\Delta POMC}$ mice was not significantly different from that in *Atg7*$^{F/F}$ mice ($P > 0.05$) (Fig. 5B). TUNEL staining of hypothalamic sections from *Atg7*$^{\Delta POMC}$ mice did not reveal a detectable number of apoptotic POMC neurons (Supplemental Fig. 2), consistent with the absence of the change in the POMC neuron number. Because these results suggested functional impairment of POMC neurons rather than a decrease in POMC neuron number, we studied intracellular signal transduction after leptin administration. Phosphorylation and nuclear translocation of STAT3 after icv leptin administration were markedly reduced in the hypothalamic POMC neurons of *Atg7*$^{\Delta POMC}$ mice compared with *Atg7*$^{F/F}$ mice ($P < 0.001$) (Fig. 5C), suggesting that impaired cellular response to leptin was responsible for hyperphagia and increased body weight of *Atg7*$^{\Delta POMC}$ mice.

To unveil the mechanism underlying the impaired response to leptin, we studied activation of endoplasmic reticulum (ER) stress markers in the hypothalamus that could be affected by autophagy and influence leptin signaling (19, 20). Western blotting showed no differences in Bip and CHOP expression or eIF2α phosphorylation in the hypothalamus of *Atg7*$^{\Delta POMC}$ mice compared with control mice (Supplemental Fig. 3), suggesting that ER stress is not a cause of leptin resistance in *Atg7*$^{\Delta POMC}$ mice. Because Western blot analysis might not detect changes of ER stress markers that occur only in POMC neurons, we next conducted immunofluorescent staining of ER stress markers in the hypothalamus. Confocal microscopy revealed no induction of ER stress marker genes such as Bip or CHOP in the ARC of *Atg7*$^{\Delta POMC}$ mice compared with *Atg7*$^{F/F}$ mice, whereas strong induction of Bip and CHOP was observed in the liver of C57BL/6 mice 72 h after *in vivo* administration of 2 mg/kg tunicamyin (Sigma, St. Louis, MO) (Supplemental Fig. 4A). Further, accumulation of p-PERK or p-eIF2α, markers of ER stress response, in the ARC was also not different between *Atg7*$^{\Delta POMC}$ and *Atg7*$^{F/F}$ mice, whereas massive accumulation of p-PERK and p-eIF2α was noted in the liver 72 h after tunicamyin administration (Supplemental Fig. 4B), These results suggest that causes other than ER stress leads to leptin resistance associated with autophagy deficiency.

Discussion

Using *Atg7*$^{\Delta POMC}$ mice, we demonstrated that autophagy is necessary for the physiologic function of POMC neu-

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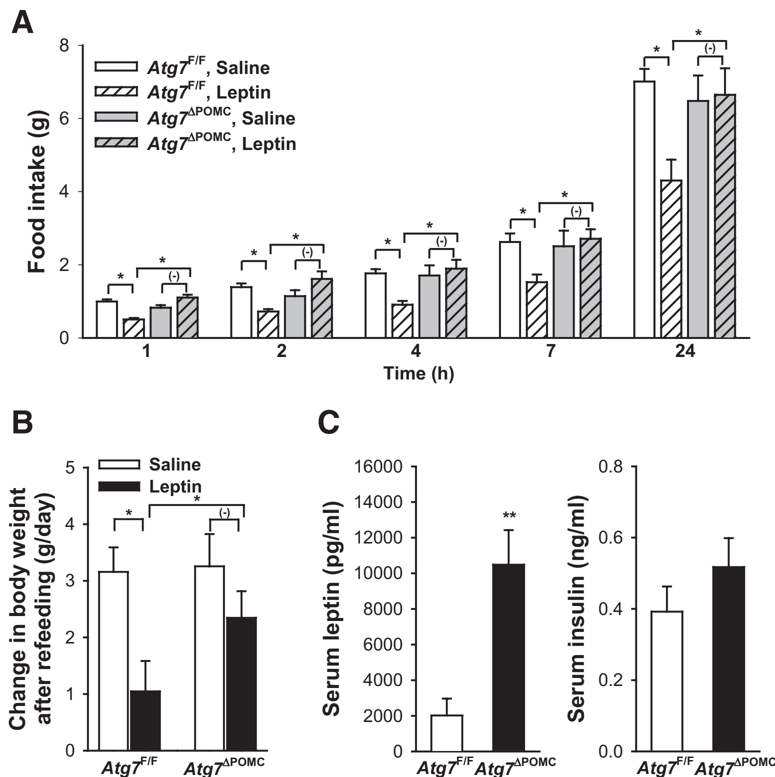

FIG. 4. Leptin unresponsiveness of $Atg7^{\Delta POMC}$ mice. A, After overnight fasting, 3 μg of leptin or saline was administered icv. Cumulative food intake between 1 and 24 h after leptin injection was measured (n = 5–7). B, After injection of leptin or saline as in A, changes in body weight 24 h after injection were measured (n = 5–7). C, Serum levels of leptin (n = 20–31) and insulin (n = 11–13) in fasted 20-wk-old mice were determined by ELISA (*, $P < 0.05$; **, $P < 0.01$).

rons controlling energy balance. Given the important role of hypothalamic leptin signaling in the control of appetite and EE (9, 21), changes of body weight, food intake, and EE in $Atg7^{\Delta POMC}$ mice may be largely due to leptin unresponsiveness of autophagy-deficient POMC neurons. Increased body weight of $Atg7^{\Delta POMC}$ mice is similar to a report showing weight gain after delivery of shRNA against Atg7 to the mediobasal hypothalamus (8), whereas Atg7 shRNA was not specifically directed to POMC neurons in that paper. Weight gain in $Atg7^{\Delta POMC}$ mice with autophagy deficiency in POMC neurons that produce anorectic hormones is also in line with a decrease of body weight in mice with autophagy deficiency in agouti-related peptide neurons that produce orectic hormones (7). A significant increase in body weight was observed in male $Atg7^{\Delta POMC}$ mice but not in female $Atg7^{\Delta POMC}$ mice, which is probably due to the expression of estrogen receptors in hypothalamic neurons (22) and differential effect of estrogen on the hypothalamus (23). Despite the increase in body weight, glucose tolerance was not significantly changed in $Atg7^{\Delta POMC}$ mice on normal chow diet, which is similar to the absence of glucose intolerance in mice lacking leptin receptors in POMC neurons (13) and could be due to a mild degree of obesity in $Atg7^{\Delta POMC}$ mice. Although there was no difference in the nonfasting blood glucose level between $Atg7^{\Delta POMC}$ mice and $Atg7^{F/F}$ mice fed normal chow diet, nonfasting blood glucose level of $Atg7^{\Delta POMC}$ mice fed HFD was significantly higher than that of $Atg7^{F/F}$ mice fed HFD, and the increase of nonfasting blood glucose level after HFD was higher in $Atg7^{\Delta POMC}$ mice compared with $Atg7^{F/F}$ mice. In contrast, weight gain after HFD was not different between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice. These results suggest that autophagy deficiency in POMC neurons leads to increased susceptibility to hyperglycemia but not to weight gain after HFD.

Mammalian target of rapamycin (mTOR) signaling is a well-known negative regulator of autophagy (1). Thus, our data showing the role of hypothalamic autophagy in the control of energy balance are consistent with a previous report showing that POMC neuron-specific mTOR activation by deletion of tuberous sclerosis complex 1 (TSC1), an inhibitor of mTOR, induces hyperphagia (24). In contrast, another paper reported that mTOR inhibition blunted the anorectic effect of leptin (25). Thus, mTOR/S6K1 signaling or nutrients inducing mTOR activation in the hypothalamus may affect other aspects of energy balance besides autophagy.

There was no significant effect of *Atg7* deletion on the number of POMC neurons, which differs from previous reports showing increased death of autophagy-deficient islet β-cells or cortical neurons (4, 26). Instead, abrogation of autophagy in POMC neurons led to impaired leptin-induced STAT3 phosphorylation. Attenuated POMC mRNA expression after refeeding in $Atg7^{\Delta POMC}$ mice might also be due to impaired STAT3 phosphorylation, because POMC induction depends on the intact leptin signaling (27). The mechanism of the impaired leptin-induced STAT3 phosphorylation is unclear. ER stress is not likely to be a cause of leptin resistance of autophagy-deficient POMC neurons, because we observed no induction of ER stress markers such as Bip or CHOP, and no accumulation of p-PERK or p-eIF2α that are critical mediators of ER stress responses (28). NF-κB activation has been suggested as a mechanism linking hypothalamic autophagy deficiency to the reduced EE and increased body weight (8). Because NF-κB activation occurs in obesity (29, 30) and NF-κB activation has been reported to repress

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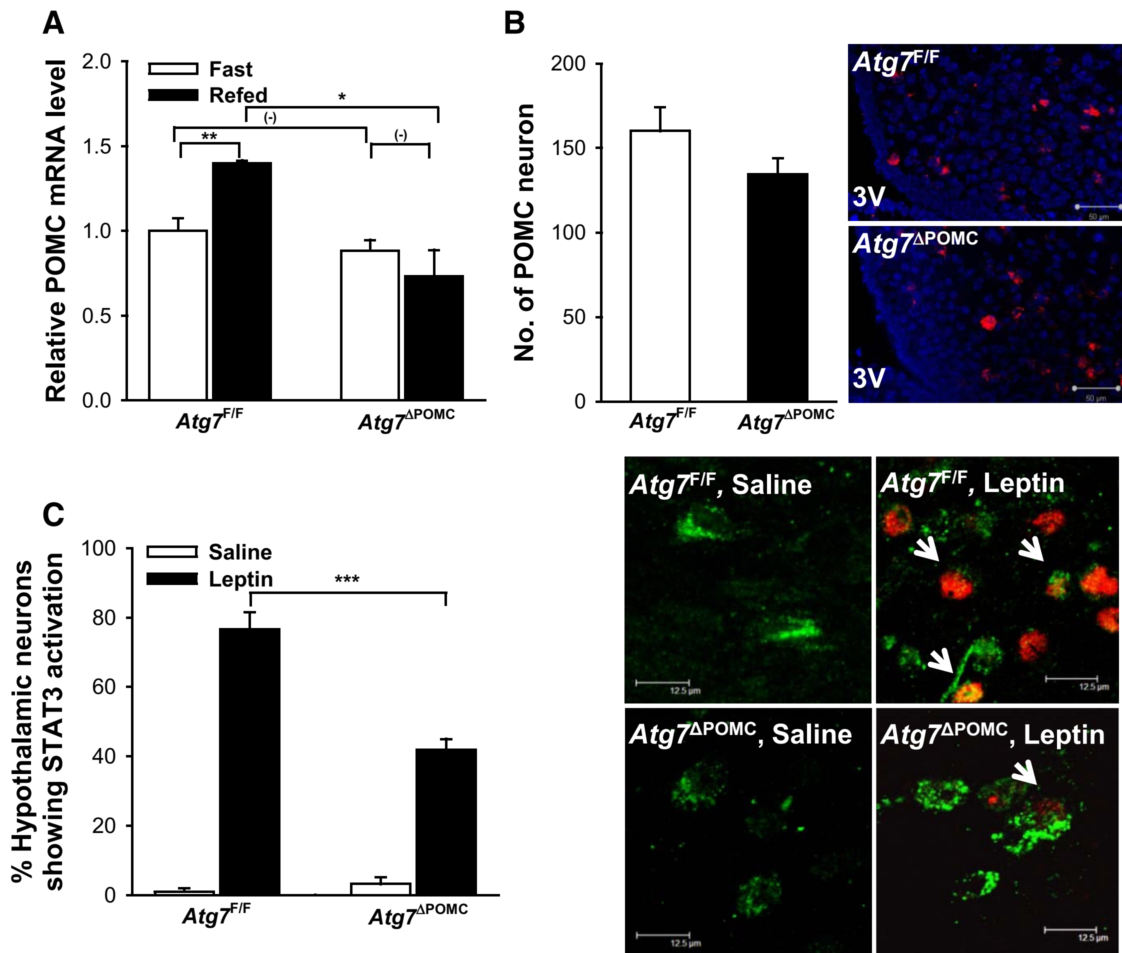

FIG. 5. Impaired leptin signaling in autophagy-deficient POMC neurons. A, RNA was prepared from the ARC of 16-wk-old mice after overnight fasting with or without refeeding for 3 h, and real-time RT-PCR was done (n = 3 each). B, After staining with anti-β-endorphin Ab and 4′,6-diamidino-2-phenylindole staining, POMC neuron numbers in the rostral, middle, and caudal ARC were counted and summed (n = 4 each) (*left*). Representative images are shown (*right*). 3V, Third ventricle. *Scale bar*, 50 μm. C, After sequential immunofluorescent staining with anti-α-MSH Ab and anti-p-STAT3 Ab as the primary Ab, the percentage of POMC neurons showing nuclear p-STAT3 among total POMC neurons was calculated (n = 6–10) (*left*). Representative images are shown (*right*). *Arrows* indicate POMC neurons with nuclear p-STAT3. *, $P < 0.05$; ***, $P < 0.001$. *Scale bar*, 12.5 μm.

autophagy (31, 32), NF-κB activation could also be an upstream event of autophagy deficiency and metabolic derangement after chronic HFD feeding. Although we presented evidence showing that autophagic process is abrogated by *Atg7* deletion in POMC neurons, such as accumulation of autophagic substrate p62, ubiquitin, and their colocalization, it is possible that nonautophagic function of *Atg7* might also be involved, because previous papers have shown nonapoptotic function of autophagy genes such as *Ulk1* in the neuronal signal transduction and axonal development (33). Because p62 is involved in diverse intracellular signal transduction systems (34, 35), massive accumulation of p62 observed in autophagy-deficient POMC neurons might also affect leptin signaling through unidentified signal pathways or disturbance of intracellular environment.

Although we focused on the leptin sensitivity of POMC neurons in the ARC of the hypothalamus, POMC and its cleavage products are also produced in corticotrophs of the pituitary gland (15); however, serum cortisol level was not different between $Atg7^{\Delta POMC}$ and $Atg7^{F/F}$ mice, suggesting no significant effect of autophagy deficiency on the function of corticotrophs and no contribution of altered adrenocorticotrphic hormone axis in the weight gain of $Atg7^{\Delta POMC}$ mice. Although not addressed in this investigation, POMC neurons in the nucleus tractus solitarius of the hindbrain that have been reported to respond to leptin and participate in the regulation of energy homeostasis (36, 37) might also be affected by POMC neuron-specific autophagy deficiency and contribute to the altered leptin sensitivity and energy homeostasis of $Atg7^{\Delta POMC}$ mice.

We observed that constitutive autophagy is important for proper function of POMC neurons, and autophagy of POMC neurons plays an important role in the control of appetite or EE and leptin sensitivity or signaling. Autophagy deficiency in anorectic hypothalamic neurons, such as POMC neurons, may be a cause of obesity. Be-

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cause autophagy may also affect viability and function of diverse hypothalamic neurons, further studies will be required to clarify the interaction between constitutive/adaptive autophagy in hypothalamic neurons and its role in hypothalamic control of appetite and energy balance.

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