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Rapamycin Ameliorates Age-Dependent Obesity Associated with Increased mTOR Signaling in Hypothalamic POMC Neurons

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SUMMARY

The prevalence of obesity in older people is the leading cause of metabolic syndromes. Central neurons serving as homeostatic sensors for body-weight control include hypothalamic neurons that express pro-opiomelanocortin (POMC) or neuropeptide-Y (NPY) and agouti-related protein (AgRP). Here, we report an age-dependent increase of mammalian target of rapamycin (mTOR) signaling in POMC neurons that elevates the ATP-sensitive potassium (K_{ATP}) channel activity cell-autonomously to silence POMC neurons. Systemic or intracerebral administration of the mTOR inhibitor rapamycin causes weight loss in old mice. Intracerebral rapamycin infusion into old mice enhances the excitability and neurite projection of POMC neurons, thereby causing a reduction of food intake and body weight. Conversely, young mice lacking the mTOR-negative regulator TSC1 in POMC neurons, but not those lacking TSC1 in NPY/AgRP neurons, were obese. Our study reveals that an increase in mTOR signaling in hypothalamic POMC neurons contributes to age-dependent obesity.

INTRODUCTION

Obesity poses a growing risk for the middle-aged adult population. This phenomenon may have different causes including genetic predisposition, poor dietary habits, and sedentary lifestyle. As the aging population increases, obesity has become a global health issue especially in developed countries (Marcellini et al., 2009). The increased prevalence of obesity among old people has emerged as the leading cause of metabolic syndromes such as type II diabetes, heart diseases, and stroke (Mokdad et al., 2001). Hence, weight control for the elderly population is not just a cosmetic procedure; it will dramatically reduce the risk of obesity-related comorbidities, which are commonly associated with the aging process (Marcellini et al., 2009). As the obesity and diabetes epidemics continue to rise and the global population ages further, greater efforts are being devoted to understanding the mechanisms of age-dependent metabolic disorders (Freedman et al., 2002).

The hypothalamus is the control center for food intake and body weight (Berthoud and Morrison, 2008; Hill et al., 2008). Among hypothalamic neurons, the POMC neurons that express pro-opiomelanocortin (POMC) and secrete an anorexic neuropeptide melanocyte-stimulating hormone (α-MSH), a proteolytic product of POMC, and the NPY/AgRP neurons that express and secrete the orexic neuropeptides Neuropeptide-Y (NPY) and agouti-related protein (AgRP), are the key players in regulating food intake and energy homeostasis (Elias et al., 1998). Defects of this POMC-NPY/AgRP circuit cause serious abnormality in food intake and body-weight control (Elmquist, 2001). Notwithstanding our knowledge about this hypothalamic circuit in the regulation of body weight in the normal physiological setting, how this circuit might be altered with aging is an open question.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that integrates nutrients and hormonal signals to control cell growth and proliferation (Wullschleger et al., 2006). The mTOR activity is negatively regulated by the tuberous sclerosis complex (TSC) composed of TSC1 and TSC2; AKT activates mTOR by inhibiting TSC (Wullschleger et al., 2006). The mTOR inhibitor rapamycin, a Federal Drug Administration-approved drug for patients with organ transplant, has been considered for treatment of psychiatric disorders and metabolic disorders, and as a promising longevity-enhancing drug (Harrison et al., 2009). In addition to extending life span, reducing mTOR activity may improve symptoms in neurodegenerative diseases associated with aging, such as Alzheimer's disease and Parkinson's disease. This beneficial effect of reducing mTOR signaling might further improve the quality of life of the aging population (Garelick and Kennedy, 2011). Given that adult-onset obesity could result from hypothalamic neurodegeneration (Ryu et al., 2008; Xu et al., 2005) and leptin, an adipostatic hormone secreted by white adipocytes, fails to augment energy expenditure in older rodents, indicating leptin signaling may be

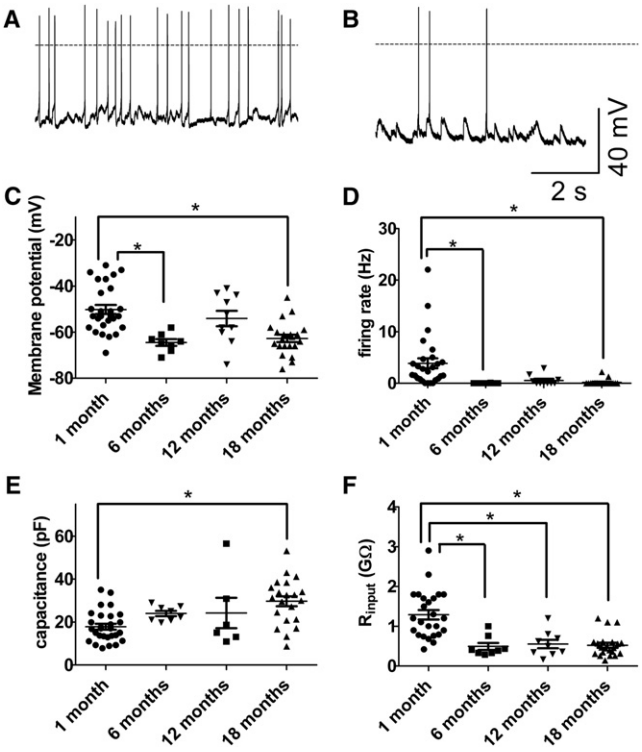

Figure 1. POMC Neurons in Old Mice Are Electrically Silent

(A and B) Representative current clamp recording traces of POMC neurons from young (1 month old) mice (A) or aged (12 months old) (B) mice are shown with the same scale. Dash lines represent 0 mV. Whereas POMC neurons from young mice fired action potentials repeatedly (A), POMC neurons from aging mice were silent (B).

(C–F) Basic biophysical characteristics of POMC neurons from 1-, 6-, 12-, and 18-month-old mice. (C) POMC neurons from mice that were 6 months old or older had more hyperpolarized resting membrane potential as compared to POMC neurons from 1-month-old young mice ($p < 0.05$, one-way ANOVA with Bonferroni post hoc test with $p < 0.05$ for 1 month versus 6- and 18-month-old mice; n = 27, 8, 7, and 21 neurons for 1-, 6-, 12-, and 18-month-old mice, respectively). (D) POMC neurons from older mice had significantly reduced action potential firing rates as compared to POMC neurons from 1-month-old young mice ($p < 0.05$ one-way ANOVA with Bonferroni post hoc test with $p < 0.05$ for 1-month versus 6- and 18-month-old mice; n = 27, 8, 7, and 21 neurons for 1-, 6-, 12-, and 18-month-old mice, respectively). (E) POMC neurons from 18-month-old mice were significantly larger as revealed by membrane capacitance measurements ($p < 0.05$ one-way ANOVA with Bonferroni post hoc test with $p < 0.05$ for 1-month versus 18-month-old mice; n = 28, 8, 6, and 21 neurons for 1-, 6-, 12-, and 18-month-old mice, respectively). (F) The input resistance was significantly reduced in POMC neurons from older mice ($p < 0.05$ one-way ANOVA with Bonferroni post hoc test with $p < 0.05$ for 1 month versus all age groups; n = 25, 7, 9, and 21 neurons for 1-, 6-, 12-, and 18-month-old mice, respectively).

See Figure S1 for the growth chart and ACTH immunostaining of GFP-positive POMC neurons from POMC-GFP mice, and examples of robust firing of hypothalamic neurons without GFP expression in old POMC-GFP transgenic mice. Error bars indicate SEM.

attenuated with aging (Li et al., 1998), we wondered whether age-dependent obesity might be associated with leptin resistance due to hyperactive mTOR signaling in the hypothalamic neuronal circuit. In particular, whereas in young mice only ~10% of POMC neurons show active mTOR signaling and this fraction is increased to 18% upon leptin stimulation (Reed et al., 2010), we wondered whether mTOR signaling is increased in POMC neurons of aging mice. If so, suppressing this excessive mTOR signaling via rapamycin administration may reestablish the hypothalamic circuit and ameliorate age-dependent obesity.

In this study, we have found that mTOR signaling is elevated in the hypothalamic POMC neurons of old mice, causing silencing of these neurons due to upregulation of K_{ATP} channel activity accompanied with an aging-associated expression of the Kir6.2 pore-forming subunit of K_{ATP} channels. In support of the critical role of enhanced mTOR signaling in causing obesity, removal of the mTOR-negative regulator TSC1 in POMC neurons of young mice elevated K_{ATP} channel activity, leading to silencing of POMC neurons and obliteration of leptin-induced release of the anorexic hormone α-MSH. Whereas TSC1 deletion in POMC neurons resulted in obesity of young mice, TSC1 deletion in NPY/AgRP neurons had no effect on neuronal excitability or body weight. Remarkably, infusion of the mTOR inhibitor rapamycin into the brain of aging mice caused a reduction of body weight. This intracerebral rapamycin infusion reduced food intake without altering the blood glucose level. It also suppressed K_{ATP} channel activity to increase repetitive firing of POMC neurons, and expanded the POMC neuronal projection into the paraventricular nucleus (PVN) involved in controlling food intake and body weight. Taken together with our finding that systemic rapamycin injection also reduces body weight of old mice, this study raises the prospect of potential therapeutic application of rapamycin to reduce midlife obesity.

RESULTS

POMC Neurons of Aged Mice Are Silent

Studies of POMC neurons from young rodents (typically < 3 months old) have shown that these neurons fire action potentials repeatedly so as to cause α-MSH secretion; elimination of action potential firing in POMC neurons abolishes α-MSH secretion (Bunel et al., 1990). It is an open question whether POMC neurons are still active in older rodents, which tend to display obesity and increased-adiposity. Our recording of green fluorescent protein (GFP)-labeled POMC neurons from transgenic mouse hypothalamic slices revealed that POMC neurons from young (1 month old) mice were electrically active (Figures 1A and 1D). In contrast, POMC neurons from aging (>6 months old) mice, which had gained more weight (Figure S1C available online), were silent (Figures 1B and 1D). As control for the health of brain slices from aging mice, recordings from neurons without GFP labeling from 12-month-old POMC-GFP mice revealed that these neurons fired action potentials repeatedly, and some displayed rhythmic bursting characteristic of tuberoinfundibular neurons in the arcuate nucleus (Figures S1A and S1B) (Lyons et al., 2010). By surveying four different age groups of mice, we found a significant reduction of input resistance of POMC neurons from 6-, 12-, or 18-month-old mice as compared to those from 1-month-old mice ($p < 0.05$ one-way ANOVA with Bonferroni post hoc test) (Figure 1F), leading to a reduction of action potential firing (Figure 1D). This silencing of POMC neuronal activity was accompanied with a more hyperpolarized

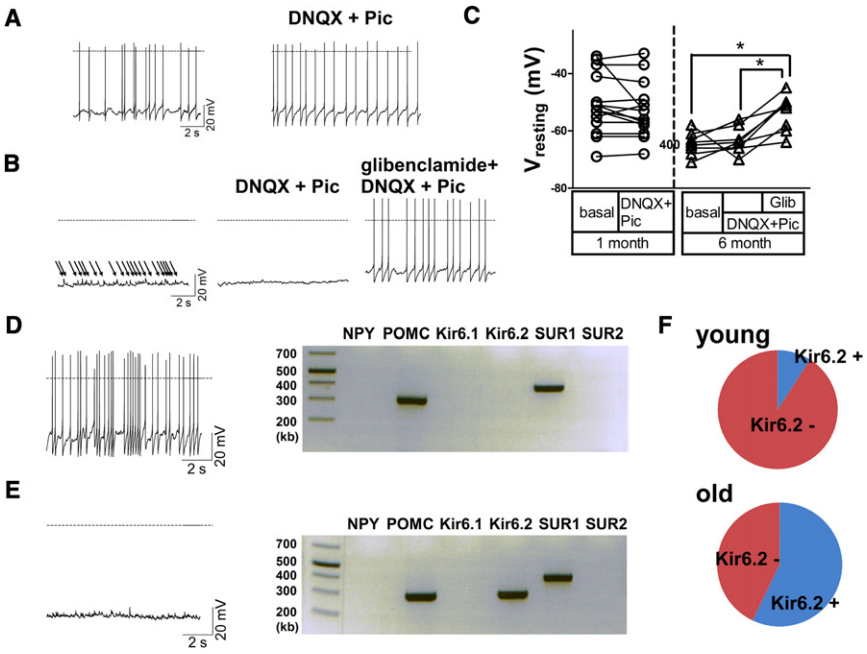

Figure 2. Increased K_{ATP} Channel Activity Silences POMC Neurons in Old Mice

(A) Blocking transmitter receptors with 10 μM DNQX, an AMPA receptor antagonist, and 50 μM picrotoxin (Pic), a $GABA_A$ receptor antagonist, did not alter the resting membrane potential of POMC neurons from young (1 month old) mice.

(B) DNQX and Pic abolished postsynaptic potentials (arrows) without affecting the resting membrane potential of POMC neurons (from 6-month-old mice) that remained silent. Further addition of 10 μM glibenclamide, a specific K_{ATP} channel blocker, significantly depolarized the resting membrane potential and triggered spontaneous action potential firing.

(C) Statistical analysis of the effect of DNQX plus Pic on the resting membrane potentials of young (1 month old, data sets on the left; n = 14) and older (6 months old, data sets on the right; n = 8) mice. Glibenclamide (10 μM) significantly depolarized the silenced POMC neurons from 6-month-old mice (ANOVA with Bonferroni post hoc test with $p < 0.05$ for glibenclamide vs. other two groups).

(D) Left: A representative current clamp recording of a POMC neuron from a young (1 month old) mouse. Right: mRNA expression profiling of the POMC neuron with its firing patterns shown on the left; this single-cell RT-PCR analysis verified that this neuron contained mRNA for POMC but not NPY, and further showed that it had mRNA for SUR1, an auxiliary K_{ATP} channel subunit, but lacked mRNA for the K_{ATP} channel pore-forming subunit Kir6.1 or Kir6.2.

(E) Left: A representative current clamp recording of a POMC neuron from an old (12 month old) mouse. Right: mRNA expression profiling of the POMC neuron with its recordings shown on the left; this single-cell RT-PCR analysis confirmed that this neuron contained mRNA for POMC but not NPY, and further showed that it expressed mRNA for both K_{ATP} channel subunits SUR1 and Kir6.2.

(F) Statistical analysis of Kir6.2 expression in young (1 month old; top pie chart; n = 11) and old (12 months old; bottom pie chart; n = 7) mice. A significantly larger fraction of POMC neurons from old mice showed a strong expression of Kir6.2, the pore-forming subunit of K_{ATP} channel, as compared with POMC neurons from young mice ($p < 0.05$, Fisher's exact test).

See Figure S2 for high mTOR activity of NYP/AgRP neurons from young mice. Error bars indicate SEM.

resting potential (Figure 1C). We also found that the membrane capacitance, which provides a measure of the cell surface area, was significantly increased in POMC neurons from 18-month-old mice (Figure 1E).

POMC neurons in the arcuate nucleus receive multiple synaptic inputs and display synaptic plasticity (Liu et al., 2012) in response to physiological changes (Dicken et al., 2012; Pinto et al., 2004). To ask whether synaptic plasticity may contribute to the POMC neuron silencing in aged mice, we applied the glutamate receptor antagonist DNQX (10 μM) and the GABA receptor antagonist picrotoxin (50 μM) to the bath solution. Blocking these transmitter receptors did not affect the resting potential or reduce action potential firing of POMC neurons from young (1 month old) mice (Figures 2A and 2C), nor did the antagonists for these transmitter receptors alter the resting potential of the silent POMC neurons from older (6 months old) mice (Figures 2B and 2C). Interestingly, glibenclamide, a specific K_{ATP} channel blocker, induced significant depolarization and caused these silent POMC neurons from aged mice to fire action potentials even in the presence of the transmitter receptor antagonists (Figures 2B and 2C). To test for the possibility that POMC neurons from old mice have increased expression of K_{ATP} channels composed of the pore-forming subunit Kir6.2 (KCNJ11) and the auxiliary sulfonylurea receptor subunit SUR1, we performed single-cell RT-PCR of POMC neurons from young mice (Figure 2D) and old mice (Figure 2E). We found an age-dependent upregulation of Kir6.2 but not SUR1 mRNA (Figures 2D–2F). Thus, aging is accompanied with Kir6.2 expression and K_{ATP} channel-mediated silencing of POMC neurons.

mTOR Signaling Is Elevated in POMC Neurons of Aged Mice

Previous studies have shown that increased mTOR activity causes hypertrophy in numerous cell types including neurons (Meikle et al., 2008). Given the suggestion that aging brains may have elevated mTOR activity (Garelick and Kennedy, 2011), we wondered whether the hypertrophy of POMC neurons in aged mice (Figure 1E) might be caused by increased mTOR signaling in POMC neurons. Consistent with previous findings of low mTOR activity in POMC neurons from young mice (Reed et al., 2010; Villanueva et al., 2009), our immunostaining of hypothalamic sections from the POMC-GFP transgenic mice with antibody against GFP to label POMC neurons and antibody against phospho-S6 (p-S6), an endogenous reporter of mTOR activity, revealed that only a small fraction of the GFP-labeled POMC neurons in 1-month-old mice showed phospho-S6 immunoreactivity (Figures 3A–3F), unlike NPY/AgRP neurons that displayed robust mTOR signaling in young mice (Figure S2).

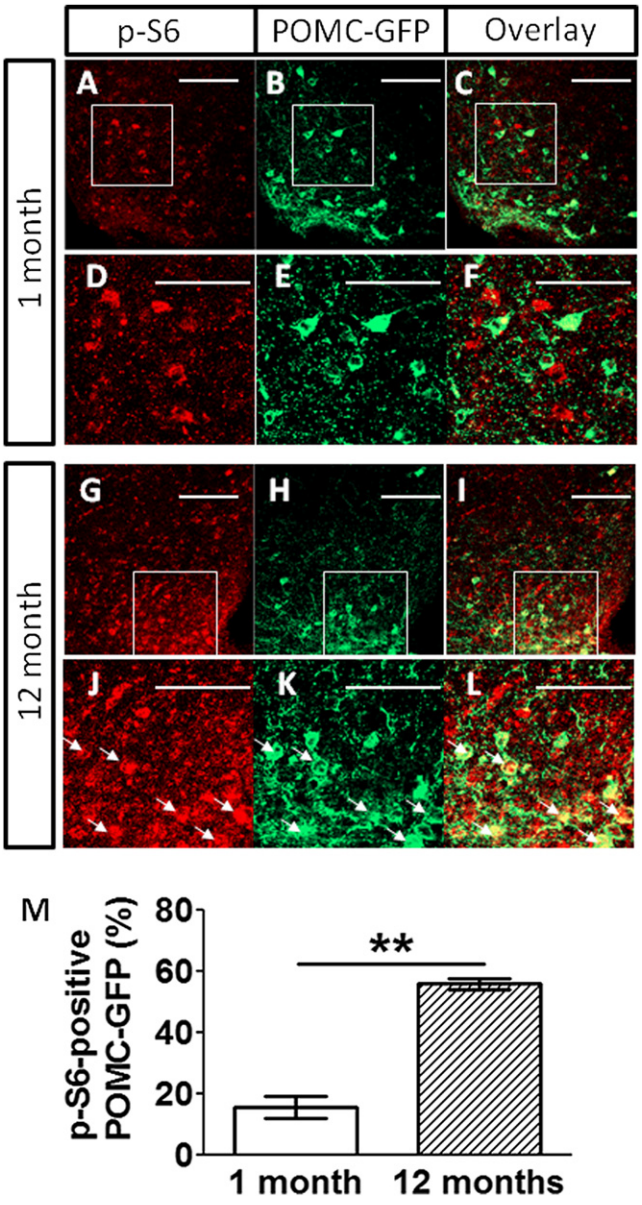

Figure 3. mTOR Signaling Is Upregulated in POMC Neurons from Aged Mice

(A) Phosphorylated S6 (red, p-S6) (A, D, G, and J), a downstream effector of mTOR and hence a reliable endogenous reporter for mTOR activity, in POMC-GFP neurons (green, B, E, H, and I) from 12-month-old mice (bottom panels, G–L) was at much higher levels than those from 1-month-old mice (A–F). (C), (F), (I), and (L) show the merged images of p-S6 and GFP. A significantly greater fraction of POMC-GFP neurons from old mice showed p-S6 staining indicative of mTOR signaling (G–L). (D) to (F) and (J) to (L) are higher magnification images corresponding to areas in the white squares shown in (A) to (C) and (G) to (I), respectively. (M) Compared to POMC neurons in young mice, a larger fraction of hypothalamic POMC neurons from old mice were positive for p-S6 ($p < 0.01$, Student's t test; five mice for each age group), indicative of increased mTOR signaling. Scale bars are 100 μm for (A)–(C) and (G)–(I), and 50 μm for (D)–(F) and (J)–(L). In contrast to POMC neurons, NPY/AgRP neurons displayed high levels of mTOR signaling in young (1 month old) mice (Figure S2). Error bars indicate SEM.

In contrast, the mTOR activity as indicated by phospho-S6 immunoreactivity was significantly elevated in POMC neurons of 12-month-old mice (Figures 3G–3M).

Genetic Manipulation to Increase mTOR Activity in POMC Neurons Causes Obesity in Young Mice

Given that POMC neurons and NPY/AgRP neurons are the two major appetite-controlling neurons in the hypothalamus, we tested whether obesity could be induced by activating mTOR signaling via conditional knockout of its upstream negative regulator TSC1 (Meikle et al., 2008) in either POMC neurons (Figure 4) or NPY/AgRP neurons (Figures 4 and S3). Consistent with a previous study (Mori et al., 2009), we found deleting *Tsc1* in POMC neurons via Pomc-cre (Figure S4) but not in NPY/AgRP neurons via Agrp-cre caused obesity (Figures 4A and 4B). Moreover, we found that TSC1 is essential for maintaining the excitability of POMC neurons but not NPY/AgRP-neurons; conditional knockout of *Tsc1* in POMC neurons silenced these neurons (Figure 4C), which could be induced to fire action potential via current injection (Figure S4), whereas conditional knockout of *Tsc1* in NPY/AgRP neurons had no effect on their firing pattern (Figure 4C), resting membrane potential (Figure 4G) or neuronal size (Figure 4E). Recapitulating features of POMC neurons in aged mice (Figure 1), removal of the mTOR-negative regulator TSC1 in POMC neurons resulted in hypertrophic soma (Figure 4D), hyperpolarized resting membrane potential (Figure 4F) and reduced excitability (Figure 4H). Since the PI3K signaling pathway has been proposed to silence POMC neurons through activation of K_{ATP} channels (Plum et al., 2006) and mTOR is downstream of PI3K in the signaling pathway, we wondered whether the elevated mTOR signaling caused silencing of POMC neurons by upregulating their K_{ATP} channel activity. To test this possibility, we dialyzed the neuron under patch-clamp whole-cell recording with an internal solution containing low (0.5 mM) MgATP and treated the hypothalamic slice with 300 μM diazoxide, a K_{ATP} channel opener, to estimate the total K_{ATP} channel activity in POMC neurons (Speier et al., 2005). We found that removing the mTOR-negative regulator TSC1 indeed caused a significant increase of the total K_{ATP} channel conductance (Figure 4I). These results lend further support to the notion that elevation of mTOR signaling causes silencing of POMC neurons mainly by increasing the K_{ATP} channel activity.

Previous studies indicate that hypothalamic K_{ATP} channels regulate the blood glucose homeostasis: local application of glibenclamide to the arcuate nucleus reduces the ability of glucagon-like peptide 1 (GLP-1) to suppress hepatic gluconeogenesis (Sandoval, 2008), and hypothalamic K_{ATP} channel activation by infusing diazoxide, a specific K_{ATP} channel opener, to the third ventricle suppresses glucose production thereby lowering blood glucose (Pocai et al., 2005). Having found that the increased mTOR signaling in POMC neurons from Pomc-cre;*Tsc1*-f/f mice caused K_{ATP} activation (Figure 4I), we asked whether the increased K_{ATP} currents in POMC neurons affect glucose homeostasis. To our surprise, at 3 months of age, those Pomc-cre;*Tsc1*-f/f mice that were about 50% heavier than their heterozygous littermates (Figure 4B) did not show signs of glucose intolerance; instead, they displayed a slight but significant improvement in glucose tolerance based on intraperitoneal

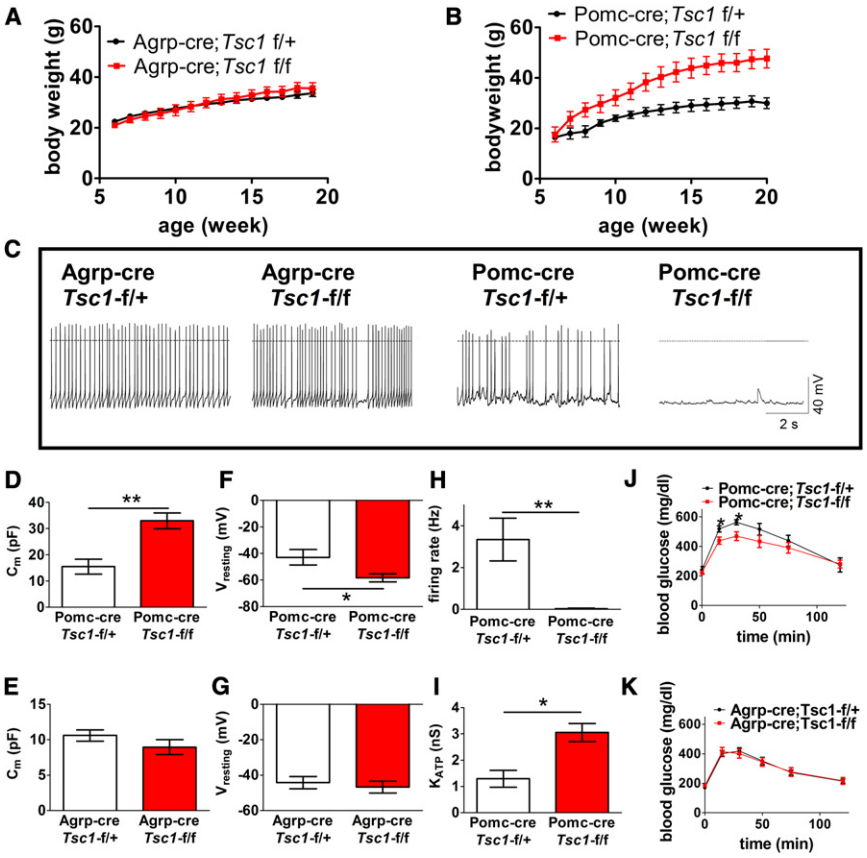

Figure 4. Obesity Can Be Induced by Targeted Deletion of *Tsc1* in Hypothalamic POMC Neurons, but Not in NPY/AgRP Neurons

(A) Deleting *Tsc1* in NPY/AgRP neurons did not change body weight ($p > 0.05$, two-way repeated-measures ANOVA; n = 14 for AgRP-cre;*Tsc1*-f/+ and n = 7 for AgRP-cre;*Tsc1*-f/f). The efficiency of TSC1 removal was confirmed by immunostaining to detect TSC1, the protein product of the *Tsc1* gene. TSC1 were absent in NPY/AgRP neurons in Agrp-cre;*Tsc1*-f/f;ai14 but not Agrp-cre;*Tsc1*-f/+;ai14 mice (Figure S3).

(B) Deleting *Tsc1* in POMC neurons significantly increased body weight shortly after weaning ($p < 0.001$, two-way repeated-measures ANOVA; n = 10 for Pomc-cre;*Tsc1*-f/+ and n = 6 for Pomc-cre;*Tsc1*-f/f).

(C–G) Representative current clamp traces from 1 months old (left to right): Agrp-cre;*Tsc1*-f/+, Agrp-cre;*Tsc1*-f/f, Pomc-cre;*Tsc1*-f/+, Pomc-cre;*Tsc1*-f/f mice (C). To fluorescently label NPY/AgRP neurons, mice were crossed to the ai14 td-tomato line and NPY/AgRP neurons were identified as red neurons. To fluorescently label POMC neurons, mice were crossed to the ZeG-GFP line and POMC neurons were identified as green neurons. Activating mTOR by removing *Tsc1* increased the size of POMC neurons ($p < 0.01$, Student's t test; n = 11 for Pomc-cre;*Tsc1*-f/+ and n = 28 for Pomc-cre;*Tsc1*-f/f) (D) but not NPY/AgRP neurons ($p > 0.05$, Student's t test; n = 4 for each group) (E). Activating mTOR by removing *Tsc1* hyperpolarized POMC neurons ($p < 0.05$, Student's t test; n = 4 for Pomc-cre;*Tsc1*-f/+ and n = 7 for Pomc-cre;*Tsc1*-f/f) (F) but not NPY/AgRP neurons ($p > 0.05$, Student's t test; n = 4 for each group) (G).

(H and I) Activating mTOR by removing *Tsc1* reduced action potential firing ($p < 0.01$, Student's t test; n = 4 for Pomc-cre;*Tsc1*-f/+ and n = 7 for Pomc-cre;*Tsc1*-f/f) (H) and increased K_{ATP} currents in POMC neurons ($p < 0.005$, n = 12 for Pomc-cre;*Tsc1*-f/+ and n = 27 for Pomc-cre;*Tsc1*-f/f) (I).

(J and K) Intraperitoneal glucose tolerance tests of 3-month-old mice revealed an improved glucose tolerance of Pomc-cre;*Tsc1*-f/f mice compared to heterozygous littermates ($p < 0.05$, Student's t test, n = 6 for Pomc-cre;*Tsc1*-f/+ and n = 10 for Pomc-cre;*Tsc1*-f/f) (J), whereas Agrp-cre;*Tsc1*-f/+ and Agrp-cre;*Tsc1*-f/f mice had comparable glucose tolerance ($p > 0.05$, Student's t test, n = 11 for Agrp-cre;*Tsc1*-f/+ and n = 8 for Agrp-cre;*Tsc1*-f/f) (K).

See Figure S3 for control of *Tsc1* conditional knockout from NPY/AgRP neurons and Figure S4 for control of Pomc-cre expression in POMC neurons and the ability of silent POMC neurons from old mice to fire action potentials upon depolarization. Error bars indicate SEM.

glucose tolerance test (Figure 4J). Similar tests revealed no alterations of glucose tolerance in Agrp-cre;*Tsc1*-f/f mice (Figure 4K).

The Blunted Leptin Responsiveness of POMC Neurons with Elevated mTOR Signaling Can Be Restored by Blocking K_{ATP} Channels

Activation of mTOR in POMC neurons causes an attenuation of sensitivity to leptin (Mori et al., 2009), which exerts its anorexic effect by stimulating α-MSH secretion from POMC neurons (Forbes et al., 2001). To test whether removal of TSC1 from POMC neurons attenuates the ability of leptin to induce α-MSH release, we measured α-MSH secretion from hypothalamic tissue explants from Pomc-cre;*Tsc1*-f/+ mice and Pomc-cre;*Tsc1*-f/f mice. Indeed, leptin failed to stimulate α-MSH secretion from Pomc-cre;*Tsc1*-f/f hypothalamic explants (Figure 5), while leptin applied together with 10 μM glibenclamide caused an increase of α-MSH secretion (Figure 5). These results indicate that the elevated K_{ATP} channel activity in POMC neurons lacking TSC1 reduced the ability of leptin to stimulate α-MSH secretion likely by silencing those POMC neurons.

Intracerebral Rapamycin Infusion Causes Body-Weight Loss in Aged Mice

As mTOR signaling in POMC neurons was significantly increased in aged mice, we next tested whether suppressing mTOR signaling by rapamycin can cause weight loss. Indeed, daily intraperitoneal injection of rapamycin at 5 mg/kg of body weight, the dose that has been shown previously to be effective for rapamycin to cross blood-brain barrier without causing body-weight change in young adult mice (Meikle et al., 2008) (Figure 6A), reduced the body weight of 12-month-old mice (Figure 6B). Because chronic systemic administration of rapamycin causes glucose intolerance and hypoinsulinemia (Yang et al., 2012), we infused rapamycin into the lateral ventricle in the brain through an osmotic pump to avoid potential complications of

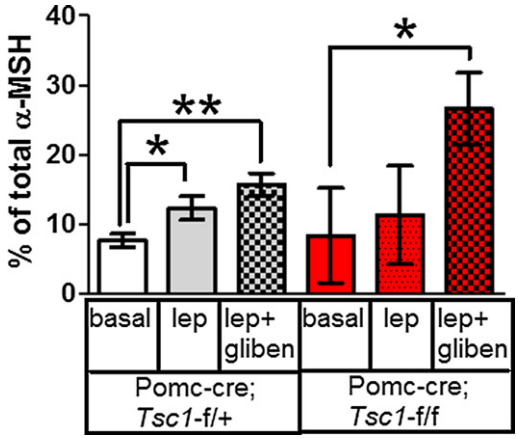

Figure 5. Activating mTOR via *Tsc1* Deletion Suppresses α-MSH Secretion from Hypothalamic Tissue Explants Treated with Leptin

Basal levels of α-MSH secretion were comparable for explants from Pomc-cre;*Tsc1*-f/+ (n = 5) and Pomc-cre;*Tsc1*-f/f (n = 3) mice. Leptin (50 nM) stimulated α-MSH secretion from hypothalamic explants from Pomc-cre;*Tsc1*-f/+ mice ($p < 0.05$, Student's t test; n = 5) but failed to trigger α-MSH secretion from hypothalamic explants from Pomc-cre;*Tsc1*-f/f ($p > 0.05$, Student's t test; n = 4). Hypothalamic explants from both heterozygous and homozygous littermates responded to 50 nM leptin administered together with 10 μM glibenclamide with α-MSH secretion ($p < 0.01$ for Pomc-cre;*Tsc1*-f/+, n = 5; $p < 0.05$ for Pomc-cre;*Tsc1*-f/f, n = 4). See Figure S4 for control showing Pomc-cre driving GFP expression in ACTH-positive neurons and control for the ability of current injection to cause the silent POMC neurons from Pomc-cre;*Tsc1*-f/f mice to fire action potential. Error bars indicate SEM.

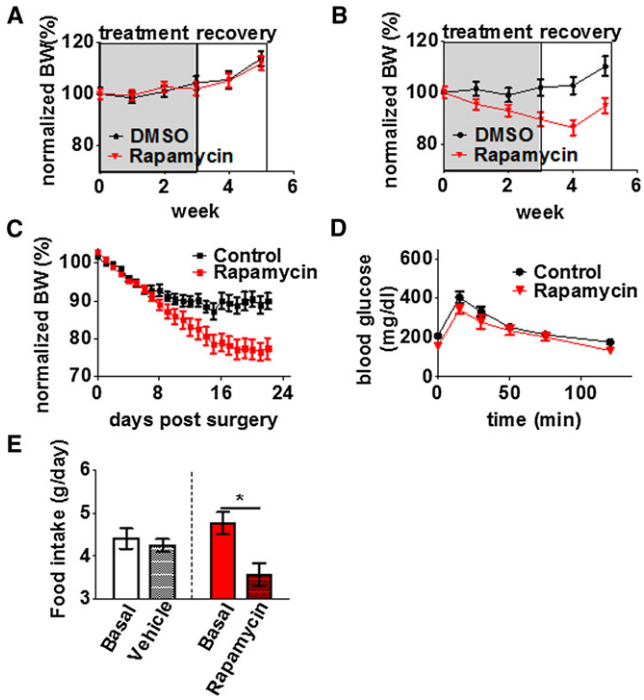

Figure 6. Rapamycin Caused Weight Loss in Old—12 Months Old—but Not Young—2 Months Old—C57B/6 Mice

(A and B) Mice received daily intraperitoneal injection of rapamycin (5 mg/kg) or DMSO as vehicle-only control for 3 weeks (gray area; treatment) and recovered for 2 weeks after rapamycin treatment (white area; recovery). Their weekly averaged body weights were normalized to the initial value (as 100%) before the onset of the treatment. Two-month-old mice receiving daily rapamycin injection did not show any weight change (A), whereas 12-month-old mice receiving rapamycin treatment showed a significant weight loss ($p < 0.01$ at third week; Student's t test) (B). Ten mice were used for each group.

(C) Intracerebral rapamycin infusion caused weight loss in old (12 months old) mice ($p < 0.001$, two-way repeated-measures ANOVA; n = 13 for each group).

(D) Mice receiving intracerebral infusion of either rapamycin or vehicle-only control had similar glucose tolerance as revealed by the intraperitoneal glucose tolerance test ($p > 0.05$, two-way repeated-measures ANOVA; n = 9 for control and n = 12 for rapamycin group).

(E) Mice receiving intracerebral rapamycin infusion showed a reduction in food intake ($p < 0.05$, Student's t test, n = 13 for each group).

See Figure S5 for the reduction of mTOR signaling in POMC-GFP transgenic mice following intracerebral rapamycin infusion. Error bars indicate SEM.

rapamycin actions in the periphery. Similar to systemic rapamycin injection, chronic intracerebral infusion of rapamycin significantly suppressed mTOR signaling in POMC neurons from 12-month-old mice (Figure S5). Moreover, intracerebral rapamycin caused weight loss of 12-month-old mice (Figure 6C). Those mice receiving intracerebral rapamycin infusion had normal glucose tolerance (Figure 6D), indicating that rapamycin had largely been confined within the central nervous system. Thus, the weight loss was due to reduced mTOR signaling in the central nervous system. Old mice receiving rapamycin infusion into the brain also exhibited a reduction in food intake (Figure 6E). Whereas rapamycin suppressed mTOR signaling in NPY/AgRP neurons as well (Figure S2), it did not alter their biophysical properties nor did it halt the action potential firing (Figure S6), in contrast to the ability of rapamycin to enhance the excitability of POMC neurons (Figure 7). These findings provide further support for our hypothesis that the age-dependent elevation of mTOR signaling inactivates POMC neurons to cause excessive food consumption and body-weight gain, raising the possibility that suppressing mTOR activity by rapamycin can restore POMC neurons' anorexic effect in exerting weight control.

POMC Neurons from Old Mice Treated with Rapamycin Are More Excitable

To directly assess the impact of intracerebral rapamycin infusion on neuronal activity, we recorded from POMC neurons of 12-month-old mice that had rapamycin infused into the brain for 3 weeks. Similar to untreated old mice, POMC neurons from control old mice receiving vehicle only from the pump were silent (Figure 7A). Applying 10 μM glibenclamide to block K_{ATP} channels restored neuronal excitability and action potential firing (Figure 7A). In contrast, POMC neurons from old mice receiving rapamycin infusion were more excitable and fired action potentials repeatedly (Figure 7B) and had reduced K_{ATP} channel activity (Figure 7C) and more depolarized resting membrane potential (Figure 7D), thus recapitulating the features of POMC neurons from young mice (Figure 1). Interestingly, we found rapamycin not only restored the excitability but also reduced the soma size of POMC neurons (Figure 7E), consistent with previous reports of the mTOR effects on neuronal morphology (Jaworski et al., 2005).

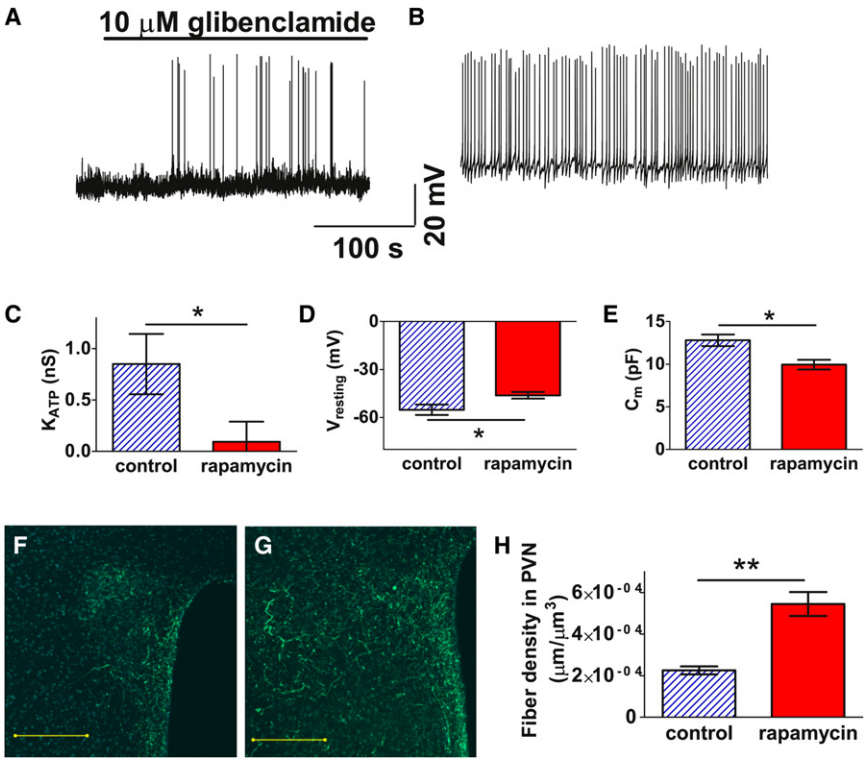

Figure 7. Intracerebral Rapamycin Infusion Restores Hypothalamic POMC Neuronal Excitability and Increased POMC Neurite Projection

(A) Representative current clamp recording from POMC neurons in mice receiving intracerebral vehicle-only infusion. Application of 10 μM glibenclamide restored its excitability and caused repetitive action potential firing. Intracerebral rapamycin infusion significantly reduced mTOR level in the POMC neurons (Figure S5).

(B) Representative current clamp recording from POMC neurons in mice receiving intracerebral rapamycin infusion. Unlike POMC neurons from control old mice, POMC neurons from rapamycin-treated old mice were highly excitable with repetitive action potential firing.

(C) Intracerebral rapamycin infusion significantly reduced K_{ATP} currents in POMC neurons (defined as glibenclamide-sensitive current) ($p < 0.05$, Student's t test; n = 5 for control and n = 7 for rapamycin group).

(D) Intracerebral rapamycin infusion significantly reduced the resting membrane potential in POMC neurons ($p < 0.05$, Student's t test; n = 11 for control and n = 8 for rapamycin group).

(E) Intracerebral rapamycin infusion significantly reduced POMC neuronal size as revealed by capacitance measurement ($p < 0.05$, Student's t test; n = 6 for control and n = 9 for rapamycin group). In contrast to POMC neurons, NPY/AgRP neurons displayed high levels of mTOR signaling, which was suppressed by daily intraperitoneal injection of 5 mg/kg rapamycin (Figure S2) but the basic biophysical characteristics of NPY/AgRP neurons were unaltered (Figure S6).

(F–H) Representative images of GFP-positive POMC neurite projections in the PVN region from old (12 months old) mice that had received intracerebral vehicle-only control (F) or rapamycin (G) infusion. Rapamycin-treated mice had a significantly greater amount of POMC neuronal projections in the PVN region as compared to mice receiving vehicle-only control treatment. GFP-positive POMC neurite densities were quantified in (H) ($p < 0.01$, Student's t test; n = 3 for each group).

Scale bars in (F) and (G) are 100 μm. Error bars indicate SEM.

Rapamycin Increases POMC Neurite Projection to the PVN

Previous study has shown that removal of TSC1 from POMC neurons reduces their projection to the target areas such as the PVN that mediates the control of food intake and body weight (Mori et al., 2009). To quantify the POMC neuronal projection, we measured the GFP-labeled POMC neurite density within the PVN region from 12-month-old POMC-GFP transgenic mice that had received intracerebral rapamycin infusion for 3 weeks. Indeed, we found that mice with rapamycin infusion had more extensive POMC neurites in the PVN region than control mice with vehicle-only infusion (Figures 7F–7H).

DISCUSSION

Our study shows that mTOR signaling in hypothalamic POMC neurons is elevated in aged mice, and we propose that this heightened mTOR activity in POMC neurons contributes to midlife obesity via two mechanisms: silencing POMC neurons by activating K_{ATP} channels and reducing POMC neurite projection to its downstream target such as the PVN (Figure 8).

As POMC neurons exert their anorexic effect via α-MSH secretion, and secretion of the anorexic α-MSH is highly dependent on the POMC neuronal excitability, modulating POMC neuron electrical activity can have a dramatic effect on body weight. Previous study has shown that nutrient overload such as high fat diet silences the POMC neurons via activation of peroxisome proliferator-activated receptor γ (PPAR-γ) thereby reducing reactive oxygen species. Pharmacological block of PPAR-γ reactivates those silent POMC neurons and reduces food intake (Diano et al., 2011). In our study of aging-dependent obesity, we found that elevating mTOR signaling causes K_{ATP} channel activation to silence POMC neurons (Figures 1, 2, 3, and 4). It would be interesting to test whether the PPAR-γ-mediated neuronal silencing involves K_{ATP} channel activation.

K_{ATP} channels can be activated under injury such as hypoxia (Ballanyi, 2004). Since brain slice preparations from old mice are more fragile, we need to consider the possibility that neurons with higher K_{ATP} channel density may have been more resistant to the insults during the slice preparation. This possibility seems unlikely for the following reasons. First, slices from old mice treated with rapamycin had less K_{ATP} channel activity in POMC neurons, which were also more excitable than POMC neurons from old mice receiving vehicle-only infusion (Figures 7A and 7B). Second, recording from neurons without GFP expression, which are hence not POMC neurons, in the arcuate nucleus of

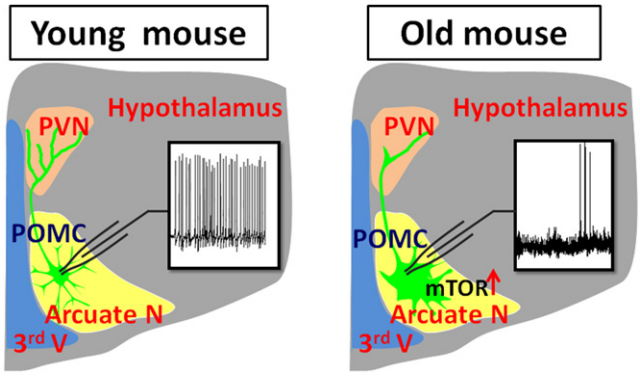

Figure 8. Summary of the Role of mTOR in Aging POMC Neurons
mTOR signaling in hypothalamic POMC neurons is relatively low in young mice, those POMC neurons are electrically active and fire action potentials repeatedly, and they have smaller soma with extensive neurite projections to downstream targets such as PVN (left). In contrast, mTOR is elevated in aged mice. This heightened mTOR activity silences POMC neurons by increasing the K_{ATP} channel activity and reduces POMC neurite projection to its downstream target such as the PVN (right).

old POMC-GFP mice revealed that those neurons showed excitability similar to that seen in young mice (Figure S1). These controls provide validation for our conclusion that aging is associated with reduced excitability of POMC neurons.

POMC neurons in the hypothalamic arcuate nucleus are heterogeneous; they may exhibit different responses to hormones and neurotransmitters. Thus, altering mTOR activity will likely change the responsiveness of POMC neurons to a number of different inputs (Williams et al., 2010). mTOR can be activated by multiple ways such as branch-chained amino acids (Cota et al., 2006), high-protein diet (Ropelle et al., 2008), and leptin and insulin (Blouet et al., 2008). Previous studies have found that short-term activation of mTOR by acute leucine infusion (Cota et al., 2006) or through inhibiting the AMP-activated protein kinase (AMPK), an endogenous mTOR suppressor, reduces food intake and body weight (Ropelle et al., 2008). However, other studies have shown that genetic manipulation to increase mTOR activity by deleting the *Tsc1* gene in POMC neurons results in hyperphagic obesity in mice (Mori et al., 2009). In our study, we found that sustained elevation of mTOR activity due to conditional knockout of *Tsc1* in POMC neurons resulted in obesity of young mice (Figure 4). It takes at least 2 weeks of rapamycin treatment to cause body-weight loss. Moreover, chronic mTOR inhibition by intracerebral rapamycin infusion suppressed appetite and reduced body weight of old mice (Figure 6). It should be noted that POMC neurons of young healthy mice rarely show any elevated mTOR activity (Reed et al., 2010; Villanueva et al., 2009). Whereas leptin increases mTOR activity in the basomedial hypothalamus including the arcuate nucleus (Blouet et al., 2008; Cota et al., 2006), leptin only modestly activates mTOR in POMC neurons (Reed et al., 2010). Our finding of elevated mTOR signaling in POMC neurons of old mice accounts for the effect of rapamycin on midlife obesity and is further substantiated by recapitulating the effects of elevated mTOR signaling on silencing POMC neuronal activity and causing body-weight gain in young mice with conditional knockout of *Tsc1* in POMC neurons.

It is important to consider various scenarios to reconcile the discrepancy that short-term mTOR activation causes weight loss while chronic activation of mTOR causes obesity. Depending on the dosage and duration, rapamycin may affect glucose homeostasis in different ways (Blagosklonny, 2011b). Rapamycin has also been proposed to have a hormetic effect on aging and aging-related diseases (Blagosklonny, 2011a). Short-term rapamycin transiently affects intracellular signaling activated by nutrient overload; it does not affect body weight but abrogates the anorexia induced by nutrients such as leucine (Cota et al., 2006). On the other hand, long-term rapamycin treatment has a profound impact on neuronal morphology and functions. Ion channels typically have slow turnover rates (Crane and Aguilar-Bryan, 2004), and any loss of neurite projection may require a few days to be restored (Keck et al., 2011). Similarly, Mori and colleagues have shown that activating mTOR in POMC neurons by deleting the *Tsc1* gene also results in enlarged POMC neuron soma and reduced neurite projections to the PVN, and chronic intraperitoneal injection of high doses of rapamycin restores the projection (Mori et al., 2009). In our study, we have identified that aging is one of the physiological factors to activate mTOR in POMC neurons and the genetic activation of mTOR in conditional knockout mice recapitulates the physiological consequences of aging by activating mTOR in POMC neurons.

Glucose intolerance and diabetes are commonly associated with obesity (Mokdad et al., 2001). Paradoxically, we did not observe such a deleterious metabolic dysfunction in Pomc-cre;*Tsc1*-f/f mice, despite the fact that they were obese and hyperphagic. Instead, these mice had an improved glucose tolerance (Figure 4J). Interestingly, a recent study has also revealed that direct leptin action in POMC neurons regulates glucose homeostasis independent of body weight (Berglund et al., 2012). Other than regulating body weight and appetite, the arcuate nucleus is well documented as a central regulator for glucose homeostasis. To keep a constant glucose supply to the brain, those hypothalamic neurons that are located outside the blood-brain barrier can monitor the glucose level in the periphery and send feedback commands to visceral organs such as the liver and pancreas through the descending autonomic system to counterbalance any fluctuation of the glucose supply to the brain (Parton et al., 2007). Moreover, activating K_{ATP} channels in hypothalamic neurons has been shown to improve glucose metabolism as infusing diazoxide to the third ventricle suppresses hepatic gluconeogenesis (Pocai et al., 2005) and central GLP-1 reduces peripheral glucose levels by activating K_{ATP} channels in POMC neurons (Sandoval et al., 2008). As obesity impairs glucose tolerance, we suspect that the improved glucose tolerance in the Pomc-cre;*Tsc1*-f/f mice might be more significant in pair-feeding experiments that ensure matched body weight.

Previous study has shown that conditional knockout of Phosphatase and Tensin Homolog (PTEN), a lipid phosphatase that antagonizes the function of PI3K, in POMC neurons causes hyperphagic obesity, raising the possibility that removal of PTEN results in an increase of PIP3, which could interact with

K_{ATP} channels on the plasma membrane to increase their activity (Plum et al., 2006). In our study, we found that elevated mTOR signaling in POMC neurons increased K_{ATP} current, and this heightened K_{ATP} channel activity silenced POMC neurons and reduced leptin-stimulated α-MSH secretion; Pomc-cre;*Tsc1*-f/f mice also exhibited a hyperphagic obese phenotype (Figures 4 and 5). Since increasing PIP3 level on the plasma membrane activates PI3K, a canonical activator of mTOR (Wullschleger et al., 2006), it is expected that deleting PTEN in POMC neurons may also activate mTOR, and this elevated mTOR activity could activate K_{ATP} channels, as we have shown in our study. Whereas deleting PTEN increases plasma PIP3 thereby prolonging the opening time of K_{ATP} channels (Plum et al., 2006), the elevated mTOR signaling in POMC neurons lacking TSC1 likely causes an increase in K_{ATP} channel density, because the maximum K_{ATP} current level is doubled in the presence of diazoxide (Figure 4I). Since PIP3 and diazoxide share the same common mechanism for K_{ATP} channel activation due to increased open time (Koster et al., 1999), the maximum K_{ATP} current in POMC neurons without TSC1 should remain unchanged if activating mTOR were to increase K_{ATP} current by generating PIP3. It is of interest to note that deleting PTEN in POMC neurons also results in hypertrophic soma as in POMC neurons with an elevated mTOR activity (Mori et al., 2009). It thus seems likely that activation of the PI3K pathway will have effects similar to those caused by elevating mTOR activity, likely an increase of K_{ATP} channel density, in addition to an increase of channel open time due to an increase of phosphoinositides such as PIP3.

Rapamycin has been found to affect the expression of Kv1.1 and Kv4.2 in dendrites of hippocampal neurons (Lee et al., 2011; Raab-Graham et al., 2006). Here we provide another example of how mTOR regulates neuronal activity by controlling ion channel density. Under physiological conditions, the ion channel density in neurons is tightly regulated (Ma and Jan, 2002). For example, when Parton et al. (2007) expressed in transgenic mice a mutant form of Kir6.2 under the POMC promoter, POMC neurons in these transgenic mice nonetheless exhibit normal levels of K_{ATP} channel density (Parton et al., 2007). Ion channel density may be controlled at several different levels including transcription, translation, trafficking, and quality control of the endoplasmic reticulum (ER) (Ma et al., 2001). We found that POMC neurons from old mice express the transcripts for K_{ATP} channel subunits Kir6.2 and SUR1, for the most common K_{ATP} channel composition in neurons (van den Top et al., 2007). As functional K_{ATP} requires the coassembly of Kir6.2 and SUR1 (Schwappach et al., 2000; Zerangue et al., 1999), the upregulation of Kir6.2 in POMC neurons in older mice is likely to increase the expression of K_{ATP} channels, leading to hyperpolarization and neuronal silencing (Figure 2). Moreover, constitutive mTOR activation that results in excessive protein translation could lead to ER stress (Reiling and Sabatini, 2006), and ER stress may silence brain endothelial cells by increasing the activity of Kir2.1 channels (Kito et al., 2011). Interestingly, multi-unit recording in the hypothalamic suprachiasmatic nucleus of aging rats has revealed a reduction in the amplitude of the electrical rhythm (Nakamura et al., 2011). The aging process has also been shown to modulate ion channels such as the expression of Kv1.1 and Kv1.2 in Purkinje neurons (Zhang et al., 2010). It would be of interest to test in future studies whether the age-dependent elevation of mTOR signaling causes ER stress in POMC neurons, and if ER stress or other aspects of mTOR signaling would unleash K_{ATP} channel trafficking or in some other ways increase K_{ATP} channel density, and ultimately reduce POMC neuron excitability.

We have shown that inhibiting mTOR by infusing rapamycin can promote POMC neuronal projections to their target region, the PVN (Figure 7). POMC neurons exert their anorexigenic effects on neurons expressing melanocortin 4 receptor (MC4R), a mandatory receptor for mediating the α-MSH effect in vivo (Vaisse et al., 1998). The expansion of POMC neuronal projection to the PVN with MC4R expression is likely one of the mechanisms for rapamycin to reduce midlife obesity. Multiple studies have revealed the impact of mTOR signaling on neuronal morphology. For example, rapamycin injection restores axon projection in Pomc-cre;*Tsc1*-f/f mice (Mori et al., 2009). Other studies have shown that the AKT-TSC-mTOR pathway plays a pivotal role in axon/dendrite polarity, axon/dendrite growth and projection (Choi et al., 2008). Activating mTOR by the AKT-TSC pathway upregulates SAD kinase, a kinase that determines the fate of neurite development by phosphorylating tau protein (Kishi et al., 2005; Wildonger et al., 2008). In the visual system of fruit flies, increased TSC-TOR signaling cell autonomously affects photoreceptor axon guidance (Knox et al., 2007). Recent study also has shown that deleting the autophagy gene 7 (Atg7) in POMC neurons reduces neurite projection to the PVN (Coupé and Bouret, 2012). Interestingly, Atg7 is inhibited by mTOR (Wyttenbach et al., 2008). Hence, the elevated mTOR signaling in POMC neurons of aged mice may suppress Atg7 and reduce neurite projection. Another study has found that deleting the LKB1 kinase, another suppressor of mTOR, in POMC neurons also reduces POMC neuronal projections to the PVN (Claret et al., 2011). Interestingly, aging is associated with numerous alterations of neuronal morphology, such as the hypertrophic soma of projection neurons with impoverished neuropiles in the limbic system (Machado-Salas and Scheibel, 1979) and the disruption of synaptic circuits of olfactory sensory neurons (Richard et al., 2010). Another fertile ground for future studies would be the age dependence of neuronal morphology regulation.

In summary, our study has demonstrated an important role of mTOR signaling in POMC neurons in developing age-dependent obesity. Upregulation of mTOR signaling in the hypothalamic POMC neurons causes an increase of K_{ATP} channel activity to silence POMC neurons and reduce their anorexigenic output, by suppressing leptin-stimulated α-MSH secretion and by reducing POMC neuronal projections to the target regions (Figure 8). Moreover, rapamycin may provide beneficial effects on aging-related metabolic disorders, by reducing midlife obesity.

EXPERIMENTAL PROCEDURES

Animals

This study was approved by the IACUC of the UCSF. At least three animals were used for every single experiment. Details about the mouse strain origins and drug preparation are described in the Supplemental Information.

Intracerebral Rapamycin Infusion

A brain infusion kit (Alzet) was implanted into the right lateral ventricle attached to an osmotic minipump (Model1004 osmotic pump, Alzet). Minipumps and tubing was filled with rapamycin (10 mg/ml) or vehicle only (60% PEG400, 30% cremaphor and 10% DMSO). Detailed procedures are described in the Supplemental Information.

Intraperitoneal Glucose Tolerance Test

Experimental procedure for intraperitoneal glucose tolerance test is described in the Supplemental Information.

Electrophysiology

Brain slices (250 μm thick) containing arcuate nucleus were prepared as described previously (Sternson et al., 2005). K_{ATP} currents in POMC neurons were measured as the glibenclamide-sensitive slope conductance of a voltage ramp as described previously (Plum et al., 2006; Speier et al., 2005). Briefly, POMC neurons were dialyzed with the same intracellular solution with low Mg_2ATP and simultaneously the slices were perfused with diazoxide (Sigma) for ten minutes, and then glibenclamide was added to block K_{ATP} currents. Detailed experimental procedures are described in the Supplemental Information.

Single cell RT-PCR: cDNA synthesis single-cell PCR were prepared as described earlier (Liss et al., 1999). An RT-PCR kit (Superscript III, Invitrogen) was used to generate first strain cDNA using random hexamers and the cDNA library for each sample was used for multiplex PCR and nested PCR. Primer sequences were adapted as described earlier (Liss et al., 1999; Price et al., 2008).

Ex Vivo α-MSH Secretion Assay

The hypothalamic explants containing the arcuate nucleus were incubated for 45 min in 250 μl aCSF then transferred to solutions containing 50 nM leptin (Sigma) or 50 nM leptin plus 10 μM glibenclamide. At the end of each period, the aCSF was frozen until it was assayed for α-MSH by a fluorescent immunoassay (Phoenix Pharmaceuticals). Detailed experimental procedures are described in the Supplemental Information.

Immunostaining

Brain sections (20 μm) were stained with primary antibodies against GFP (chicken 1:200, Aves) and phospho-S6 (rabbit, 1:200, Cell Signaling) followed by Alexa dye-tagged secondary antibodies (donkey 1:500, Invitrogen). See Supplemental Information for detailed experimental procedures.

Statistical Analysis

Statistical analyses were performed with Prism software (Graphpad Software) using the Fisher's exact test, one-way ANOVA or two-way repeated-measures ANOVA with Bonferroni post hoc multicomparison test and Student's t test for pair-wise comparisons. $p < 0.05$ was considered statistically significant. GFP-positive neurite densities within the PVN region were first converted to binary file then further quantified by Image J (NIH).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.03.043.

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