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Increased lipolysis and altered lipid homeostasis protect γ -synuclein-null mutant mice from diet-induced obesity

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Synucleins are a family of homologous proteins principally known for their involvement in neurodegeneration. γ -Synuclein is highly expressed in human white adipose tissue and increased in obesity. Here we show that γ -synuclein is nutritionally regulated in white adipose tissue whereas its loss partially protects mice from high-fat diet (HFD)-induced obesity and ameliorates some of the associated metabolic complications. Compared with HFD-fed WT mice, HFD-fed γ -synuclein-null mutant mice display increased lipolysis, lipid oxidation, and energy expenditure, and reduced adipocyte hypertrophy. Knockdown of γ -synuclein in adipocytes causes redistribution of the key lipolytic enzyme ATGL to lipid droplets and increases lipolysis. γ -Synuclein-deficient adipocytes also contain fewer SNARE complexes of a type involved in lipid droplet fusion. We hypothesize that γ -synuclein may deliver SNAP-23 to the SNARE complexes under lipogenic conditions. Via these independent but complementary roles, γ -synuclein may coordinately modulate lipid storage by influencing lipolysis and lipid droplet formation. Our data reveal γ -synuclein as a regulator of lipid handling in adipocytes, the function of which is particularly important in conditions of nutrient excess.

Understanding the link between increased adiposity and the development of metabolic disease may reveal novel therapeutic targets to counter the rising pandemic of obesity. Inhibiting adipose tissue expansion alone is likely to worsen metabolic outcome, as evidenced by human syndromes of lipodystrophy, whereby inappropriately decreased adipose mass causes severe metabolic disorders (1). Indeed, adipose tissue dysfunction and/or exceeded adipose storage capacity may underlie ectopic lipid accumulation and lipotoxicity in obesity (2). Therefore, a major challenge is to identify pathways via which adiposity can be reduced without concomitant increases in circulating lipids and attendant metabolic disease. Achieving this goal requires a better understanding of the molecular mechanisms that regulate lipid metabolism and storage in adipocytes, particularly in times of energy surplus.

γ -Synuclein belongs to the synuclein family of proteins, whose founder member α -synuclein is best known for its links with neurodegenerative diseases, most notably Parkinson disease (3). To date, no clear cellular role is attributed to γ -synuclein, and ablation of γ -synuclein causes only minor changes in the nervous system (4–7). Recently, we and others have reported high levels of γ -synuclein expression in adipose tissue of humans and other mammals (8, 9). Moreover, expression of γ -synuclein is increased in the adipose tissue of obese humans and decreased during caloric restriction (8).

Here we demonstrate that γ -synuclein-null mice display significantly reduced adiposity and fewer metabolic derangements compared with WT mice following high-fat feeding. This appears to result from increased adipocyte lipolysis coupled to enhanced whole-body lipid oxidation and energy expenditure. At a molecular level, we identify dual roles for γ -synuclein independently

regulating lipid droplet fusion and adipocyte lipolysis to coordinately regulate triglyceride (TG) storage in adipocytes. Together, our observations reveal that γ -synuclein is a regulator of lipid metabolism and, hence, a potential therapeutic target for treatment of obesity and associated metabolic diseases.

Results

γ -Synuclein Expression Is Nutritionally Regulated in Adipocytes. γ -Synuclein is abundantly expressed in adipose tissue (8, 9) (Fig. S1). Western blotting of fractionated white adipose tissue (WAT) demonstrated that γ -synuclein was expressed in the mature adipocyte but not the stromal cell fraction (Fig. 1A). When adipocytes were subjected to subcellular fractionation, γ -synuclein was recovered in the infranant, containing cytosol and membranes. No γ -synuclein was detected in the fraction containing lipid droplet-associated proteins in the presence or absence of lipolytic stimulation, which was sufficient to induce the translocation of hormone-sensitive lipase (HSL; Fig. 1B). Immunostaining of adipocytes differentiated from MEFs in vitro also revealed diffuse, partly punctuate cytoplasmic staining, and did not suggest an association of γ -synuclein with lipid droplets (Fig. 1C).

Consistent with previous observations in patients with obesity (8), γ -synuclein mRNA expression was dramatically increased in the epididymal and s.c. WAT of mice fed a high-fat diet (HFD) for 11 wk (Fig. 1D) and gradually decreased after mice fed an HFD for 10 d were switched to a low-fat diet (LFD) or fasted (Fig. 1E). Parallel changes in γ -synuclein protein levels were observed in epididymal WAT following dietary manipulation (Fig. 1F).

γ -Synuclein-Null Mice Are Resistant to Diet-Induced Obesity. The nutritional regulation of γ -synuclein expression in adipose tissue led us to investigate whether it could play a role in the development of obesity. WT and γ -synuclein^{-/-} mice (9 wk old) were fed an LFD or HFD for a period of 11 wk. LFD-fed WT and γ -synuclein^{-/-} mice showed no differences in weight gain. In contrast, during HFD-feeding, γ -synuclein^{-/-} mice gained substantially less weight than their WT counterparts (Fig. 2A). Quantitative MRI analysis of body fat volume during the study

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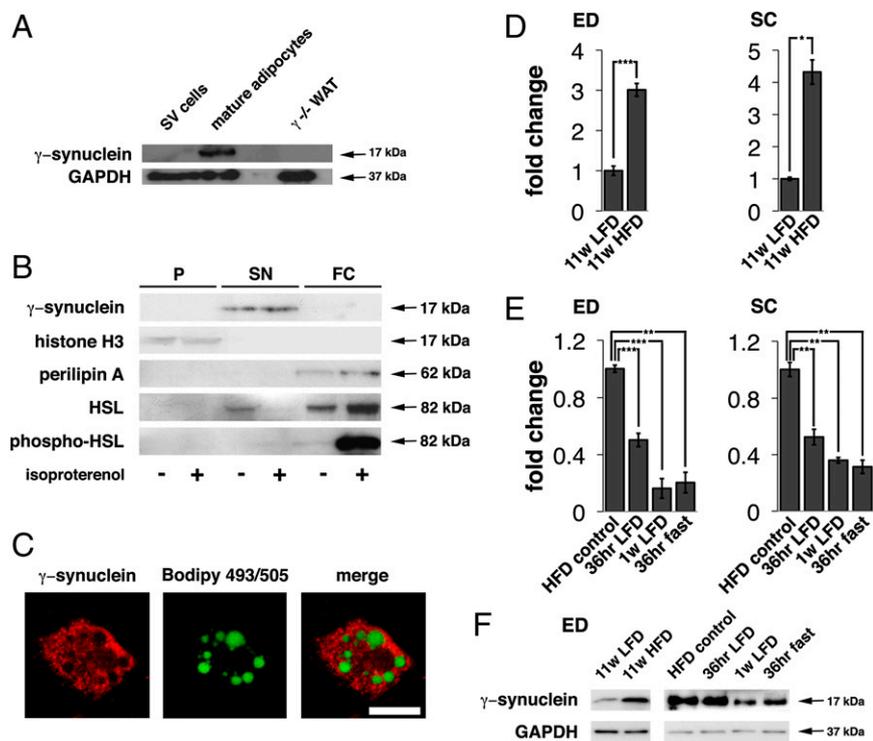


Fig. 1. Expression of γ -synuclein in murine WAT. (A) Western blot analysis of γ -synuclein expression in the total cell lysates of stromal-vascular (SV) and mature adipocyte fractions from epididymal WAT of WT mice. A lysate of epididymal WAT from a γ -synuclein^{-/-} mouse (γ ^{-/-} WAT) was included as a control. GAPDH was used as a loading control. (B) Subcellular localization of γ -synuclein in mature white adipocytes isolated from epididymal WAT of WT mice with 30 min incubation in the presence or absence of 10 μ M isoproterenol. Proteins in the pellet (marked "P"), supernatant (SN), and fat cake (FC) fractions were Western-blotted by using antibodies to γ -synuclein and protein markers for each fraction. (C) Immunofluorescent staining of MEFs differentiated into adipocytes with SK23 antibodies (29) against γ -synuclein (red) and counterstained with the neutral lipid stain Bodipy 493/505 (green). (Scale bar: 20 μ m.) (D) Quantitative RT-PCR analysis of γ -synuclein mRNA expression in epididymal (ED; $n = 8$) and s.c. ($n = 4$) WAT of WT mice fed an LFD or HFD for 11 wk. Results are shown as mean \pm SEM of fold change compared with LFD-fed mice. (E) Quantitative RT-PCR analysis of γ -synuclein mRNA expression in epididymal and s.c. WAT of mice fed an HFD for 10 d and then given LFD for 36 h or 1 wk, fasted for 36 h, or maintained on HFD. Results are shown as mean \pm SEM fold change compared with HFD-fed controls ($n = 3-5$). (F) Western blot analysis of γ -synuclein expression in epididymal WAT of the same mice used in D and E. GAPDH was used as a loading control. (In D and E, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Mann-Whitney U test.)

revealed significantly reduced WAT accumulation in HFD-fed γ -synuclein^{-/-} mice compared with WT mice (Fig. 2B and Fig. S2A). This reduction was confirmed postmortem by determining the mass of dissected epididymal, s.c., and retroperitoneal depots, as well as fat pad mass to body mass ratios for all three depots (Fig. 2C, Fig. S2B and C, and Table S1).

Observed differences in WAT accumulation between HFD-fed WT and γ -synuclein^{-/-} mice were not explained by altered food intake (Fig. S2D) or absorption of lipids from the diet (Fig. S2E). White adipocytes were of similar size in LFD-fed mice of both genotypes; upon HFD feeding, the average adipocyte size increased by ~ 4.5 fold in s.c. WAT of WT mice but only ~ 2.4 fold in γ -synuclein^{-/-} mice (Fig. 2D and E). To investigate whether

the lack of γ -synuclein affected adipocyte size in a cell-autonomous manner, we injected lentiviruses containing a bicistronic construct encoding GFP and γ -synuclein locally into femoral s.c. fat pads of γ -synuclein^{-/-} mice before the 11-wk HFD feeding protocol. This allowed us to specifically identify adipocytes reexpressing γ -synuclein by GFP staining (Fig. S2G). Following 11 wk on an HFD, we observed a statistically significant increase in size of adipocytes transduced with γ -synuclein-expressing lentivirus when compared with adjacent nontransduced cells or cells transduced with a control lentivirus expressing only GFP in the contralateral WAT depot of the same animal (Fig. 2F). Overall, these data strongly suggest that regulation of γ -synuclein within fat cells specifically and cell-autonomously affects adipocyte size

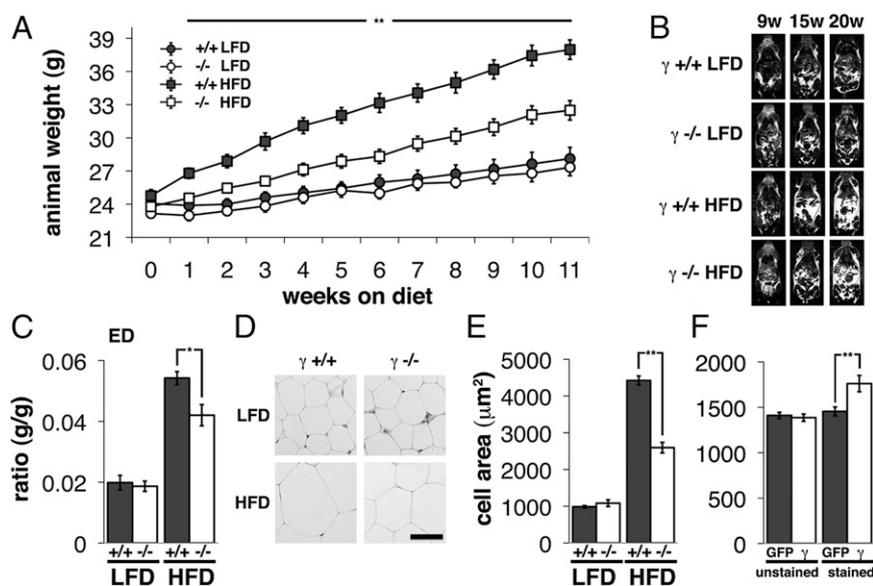


Fig. 2. Effect of γ -synuclein deficiency on WAT of mice challenged with HFD. (A) Body weight changes of WT (+/+), γ -synuclein^{-/-} mice fed an LFD or HFD for 11 wk ($n = 12-15$). (B) MRI scans (fat-only images, fat shown in white) of WT and γ -synuclein^{-/-} mice at the beginning (9 wk), middle (15 wk), and end (20 wk) of LFD or HFD feeding. (C) Total epididymal (ED) WAT mass to body mass ratios of WT and γ -synuclein^{-/-} mice fed an LFD or HFD for 11 wk ($n = 12-14$). (D) H&E-stained sections through s.c. WAT taken from WT and γ -synuclein^{-/-} mice fed an LFD or HFD for 11 wk. (Scale bar: 50 μ m.) (E) Average white adipocyte area for these groups was quantified by using ImageJ ($n = 3$ animals/12 sections, per group). (F) Average adipocyte area was measured on GFP antibody-stained sections of s.c. WAT of γ -synuclein^{-/-} mice that received local injection of γ -synuclein-expressing lentivirus (γ) or control GFP-only-expressing lentivirus (GFP) into s.c. WAT followed by feeding HFD for 11 wk ($n = 3$ animals/12 sections per group, minimum of 600 adipocytes). Bar charts represent mean \pm SEM (* $P < 0.05$, ** $P < 0.01$, Mann-Whitney U test).

in vivo. We observed no difference in the induction of several markers of adipogenesis in γ -synuclein-null and WT MEFs induced to differentiate in culture (Fig. S2 H–J), suggesting that the effects observed in vivo do not reflect decreased adipogenic capacity of γ -synuclein precursor cells.

Loss of γ -Synuclein Ameliorates HFD-Induced Hyperinsulinemia and Hepatosteatosis and Increases Lipid Oxidation and Energy Expenditure. Restricting adiposity in the setting of nutrient excess may lead to an increased circulating lipids and ectopic lipid accumulation in nonadipose tissues, causing or exacerbating insulin resistance and metabolic disorders (1, 2). Whereas HFD feeding resulted in an approximately twofold increase in hepatic and plasma triacylglycerol (TAG) levels in WT mice, no such increases were observed in γ -synuclein^{-/-} mice despite their reduced adiposity (Fig. 3 A and B). Histological examination of H&E-stained sections revealed that the absence of γ -synuclein protected the liver from the development of the extensive hepatosteatosis observed in HFD-fed WT mice (Fig. 3C). Similarly, whereas HFD feeding dramatically altered the morphology of brown adipose tissue (BAT) in WT mice with the appearance of much larger lipid droplets, this was significantly attenuated in γ -synuclein^{-/-} mice (Fig. 3C). γ -Synuclein^{-/-} mice also appeared protected against the significant increase in fasting plasma insulin levels observed in WT mice following HFD feeding (Fig. 3D). Commensurate with their altered adiposity, plasma leptin levels were also lower in γ -synuclein^{-/-} vs. WT mice fed an HFD (Fig. 3E). The absence of γ -synuclein had no effect on the levels of plasma free fatty acids, ketone bodies, or adiponectin on LFD or HFD (Table S2). i.p. glucose and insulin tolerance tests also revealed no significant differences between γ -synuclein^{-/-} and WT mice in the area under the curve (Table S2), although glucose levels in both tests were lower in γ -synuclein-deficient mice at all time points tested (Fig. S3). Taken together, these data suggest that the reduced adiposity observed in γ -synuclein-deficient mice is accompanied by a partly improved, rather than worsened, metabolic profile compared with WT mice when both are fed an HFD. This is a result of significantly improved aspects of lipid metabolism, and any effects on glucose metabolism are more modest and may be secondary.

Given that the observed decreases in adiposity and ectopic lipid storage are not attributed to decreased caloric intake, we next examined substrate utilization and energy expenditure in γ -synuclein^{-/-} mice. Analysis of HFD-fed mice by indirect calorimetry using a Comprehensive Lab Animal Monitoring System (CLAMS) revealed that γ -synuclein^{-/-} mice had a significantly

lower respiratory exchange ratio (RER), consistent with increased lipid oxidation and reduced carbohydrate oxidation (Fig. 3F and Fig. S4 A and B). HFD-fed γ -synuclein^{-/-} mice also exhibited increased energy expenditure during the light cycles (Fig. 3G). This was despite lower levels of physical activity (Fig. S4C).

Analysis of the expression of mRNA encoding proteins involved in the regulation of lipid oxidation (PPAR γ coactivator 1 α , uncoupling protein 3, PPAR α , peroxisomal acyl-CoA oxidase 1, acyl-CoA synthetase long-chain family member 1, and carnitine palmitoyltransferase 1) revealed that these were increased in BAT but not in WAT of HFD-fed γ -synuclein^{-/-} mice compared with corresponding control mice (Fig. S5). This strongly implies that the increased lipid oxidation observed in the γ -synuclein^{-/-} mice at least partly occurs in the BAT and that the associated energy is likely to be dissipated as heat.

Loss of γ -Synuclein Increases Lipolysis in Vivo and in Vitro. We next investigated whether reduced lipid storage and increased lipid utilization in γ -synuclein^{-/-} mice fed an HFD could be associated with altered adipocyte lipolysis. Glycerol release was approximately threefold higher in epididymal adipocytes isolated from HFD-fed γ -synuclein^{-/-} mice compared with adipocytes from HFD-fed WT mice under basal conditions. Moreover, although isoproterenol dramatically stimulated glycerol release in adipocytes of both genotypes, this remained significantly higher in adipocytes from γ -synuclein^{-/-} mice (Fig. 4A). Conversely, lentiviral reexpression of γ -synuclein reduced the rate of basal lipolysis in MEFs derived from γ -synuclein^{-/-} mice and differentiated into adipocyte-like cells (Fig. 4B). We did not observe altered expression of perilipin A or HSL in the WAT of γ -synuclein^{-/-} mice fed HFD compared with HFD-fed WT mice. Moreover, we did not observe increased phosphorylation of HSL at serine 563 or serine 660, although levels of ATGL were increased in the WAT of HFD-fed γ -synuclein^{-/-} mice (Fig. 4C and Table S3).

To further investigate the mechanism via which γ -synuclein loss might increase lipolysis, we used siRNA to knock down γ -synuclein in mature 3T3-L1 adipocytes. This led to an ~90% decrease in γ -synuclein mRNA levels with no effect on any adipocyte marker genes tested (Fig. S6A). Levels of γ -synuclein protein were undetectable in these cells, and expression of the adipocyte marker aP2 was unchanged (Fig. S6A). Consistent with the effects of gene KO in vivo, γ -synuclein knockdown significantly increased basal and isoproterenol-stimulated rates of lipolysis (Fig. S6A). Also consistent with the effects of altering γ -synuclein levels in adipocytes in vivo, lipid droplet size was

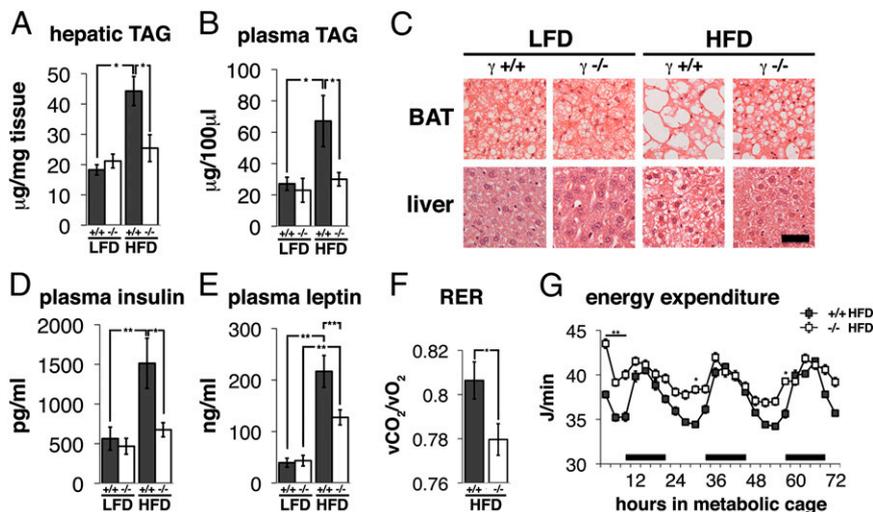


Fig. 3. Effect of γ -synuclein deficiency on non-WAT lipid accumulation, hormonal status, and metabolism. (A and B) Levels of TAG in liver ($n = 5-8$) and plasma ($n = 5-7$) of WT (+/+) and γ -synuclein^{-/-} mice (-/-) fed an LFD or HFD for 11 wk. (C) H&E-stained sections through BAT and liver of WT and γ -synuclein^{-/-} mice fed an LFD or HFD for 11 wk. (Scale bar: 50 μ m.) (D and E) Levels of insulin and leptin in plasma of WT and γ -synuclein^{-/-} mice fed an LFD or HFD for 11 wk ($n = 11-14$). (F) RER calculated by indirect calorimetry using CLAMS over a period of 72 h in WT (+/+) and γ -synuclein^{-/-} (-/-) mice fed an HFD for 8 wk at the time of the trial ($n = 8$ per genotype). (G) Energy expenditure by these animals over the same time period. Black bars indicate periods of dark cycles. Individual values represent mean \pm SEM ($*P < 0.05$, $**P < 0.01$, Mann-Whitney U test).

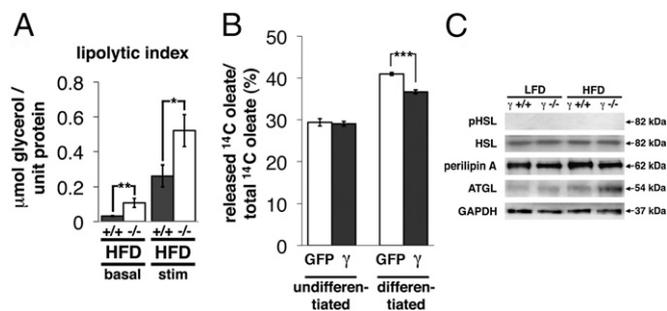


Fig. 4. γ -Synuclein affects lipolysis in mouse adipocytes. (A) Measurement of lipolysis in adipocytes isolated from epididymal WAT of WT ($+/+$) and γ -synuclein $^{-/-}$ ($-/-$) mice fed an HFD for 11 wk. Lipolysis was determined by measuring glycerol released from adipocytes incubated for 2 h in the absence (basal) or presence (stim) of 10 μ M isoproterenol ($n = 8$ animals per group; * $P < 0.05$, ** $P < 0.01$, Mann–Whitney U test). (B) Radioactive oleate release was used to assess basal lipolysis in undifferentiated or differentiated γ -synuclein $^{-/-}$ MEFs infected with lentiviruses encoding GFP or γ -synuclein. Data are means \pm SEM of [14 C]oleate released during 4 h, expressed as percentage of total incorporated [14 C]oleate (*** $P < 0.001$, Mann–Whitney U test; $n = 12$). (C) Western blot analysis of protein expression in epididymal WAT of WT and γ -synuclein $^{-/-}$ mice fed an LFD or HFD for 11 wk. GAPDH was used as a loading control.

significantly reduced in 3T3-L1 adipocytes in which γ -synuclein had been knocked down (Fig. 5B).

Despite increased ATGL protein expression in the adipose tissue of γ -synuclein $^{-/-}$ mice, we did not detect a consistent alteration in ATGL expression following knockdown of γ -synuclein in 3T3-L1 adipocytes, suggesting that this may represent a secondary effect. However, γ -synuclein knockdown did result in a dramatic redistribution of ATGL in 3T3-L1 adipocytes, resulting in a distinctive patterning of ATGL staining around lipid droplets in the basal state (Fig. 5C). This pattern was similar to that seen when cells transfected with control siRNA are stimulated with isoproterenol (Fig. 5C). In vitro pull-down assays indicated that γ -synuclein and ATGL are capable of interacting (Fig. S6B). However, we did not observe interaction of γ -synuclein with perilipin A or CGI-58 in the same assay (Fig. S6C). These observations suggest that γ -synuclein could be involved in

sequestering ATGL away from the lipid droplets in the basal state. Together, our data strongly argue that the altered lipolysis and decreased adipocyte size observed in the γ -synuclein $^{-/-}$ mice results from a cell-autonomous effect of γ -synuclein loss in adipocytes whereby the pool of ATGL targeted to lipid droplets is increased, promoting its involvement in basal and hormonally stimulated TG hydrolysis.

γ -Synuclein Promotes Assembly of SNARE Complexes in WAT and Cultured Cells. α - and γ -Synuclein are closely related proteins, with potential functional redundancy (5, 10). In neuronal synapses, α -synuclein is involved in regulation of synaptic vesicle fusion with the cell membrane by promoting the assembly of SNARE complexes (11). In adipocytes, SNARE complexes have been implicated in lipid droplet formation and growth by fusion between neutral TAG packaged within amphipathic lipoproteins and the lipid droplet phospholipid monolayer (12–15). To determine whether γ -synuclein may regulate the efficiency of this fusion mechanism, we quantified the assembled SNARE complexes in epididymal WAT lysates by measuring the amount of VAMP-4 (vSNARE) that coimmunoprecipitated with syntaxin-5 (tSNARE). Although we found no differences between genotypes for LFD-fed mice, the number of SNARE complexes was reduced by $\sim 35\%$ in γ -synuclein $^{-/-}$ mice fed HFD compared with HFD-fed WT mice (Fig. 6A and B). This difference was not caused by reduced expression of VAMP-4, syntaxin-5, or SNAP-23 in WAT of γ -synuclein $^{-/-}$ mice (Fig. S7A and B).

To assess whether increasing γ -synuclein expression could affect the number of SNARE complexes in cells, we infected undifferentiated 3T3 cells, which do not express detectable level of this protein, with control or γ -synuclein-expressing lentivirus. At 48 h after infection, assembled SNARE complexes were more abundant in γ -synuclein-expressing cells than in control cells (Fig. 6C).

In differentiating 3T3-L1 cells, we observed that γ -synuclein and SNAP-23 colocalized within the cytoplasm (Fig. S7C). However, acute treatment of cells with oleic acid to induce the formation of lipid droplets led to partial loss of this colocalization (Fig. S7C and D). This is consistent with the hypothesis that γ -synuclein interacts with a cytoplasmic form of SNAP-23 and chaperones the assembly of SNARE complexes in conditions of

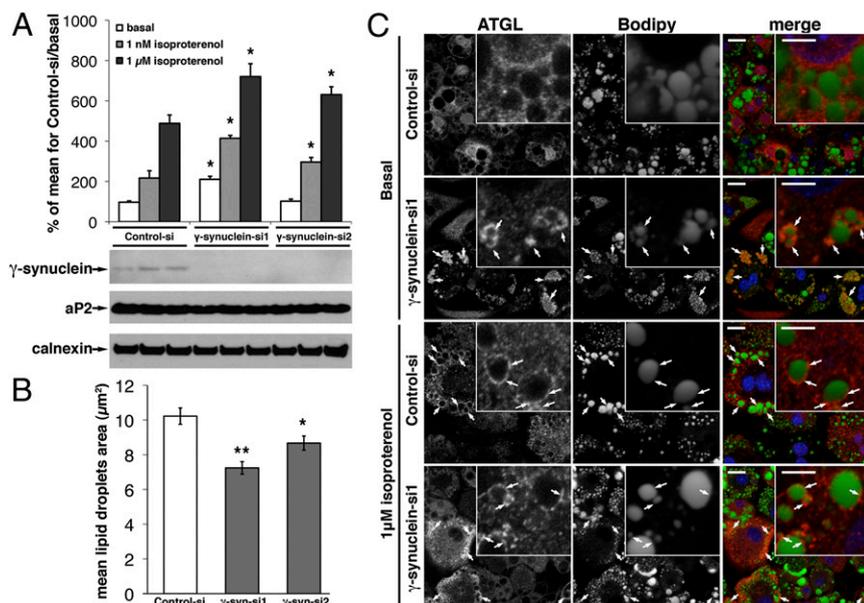


Fig. 5. γ -Synuclein affects lipolysis in differentiated 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with control siRNA (Control-si) or siRNA targeting γ -synuclein (γ -synuclein-si1 or γ -synuclein-si2). (A) Cells were labeled for 16 h with [14 C]oleate before incubation on day 8 of differentiation in the absence (Basal) or presence of 1 nM or 1 μ M isoproterenol as indicated for 30 min (Upper). Carbon 14 release as a proportion of total incorporated 14 C was determined. Results are means \pm SEM compared with identically treated cells transfected with control siRNA (* $P < 0.05$, Mann–Whitney U test; $n = 5$). Identically transfected 3T3-L1 adipocyte cultures were lysed, and the expression of γ -synuclein, aP2, and calnexin was determined by Western blotting (Lower). (B) Lipid droplet area in untreated cells was determined as described in Methods, and shown as means \pm SEM. (C) At day 8 of differentiation, cells were incubated for 30 min in the absence (Basal) or presence of 1 μ M isoproterenol before fixing and staining with antibody to endogenous ATGL (red), Bodipy to reveal neutral lipid droplets (green), and DAPI to visualize nuclei (blue). Individual grayscale images are shown for ATGL and Bodipy as indicated. White arrows highlight regions of ATGL interaction with lipid droplets. (Scale bars: 20 μ m; Insets, 5 μ m.)

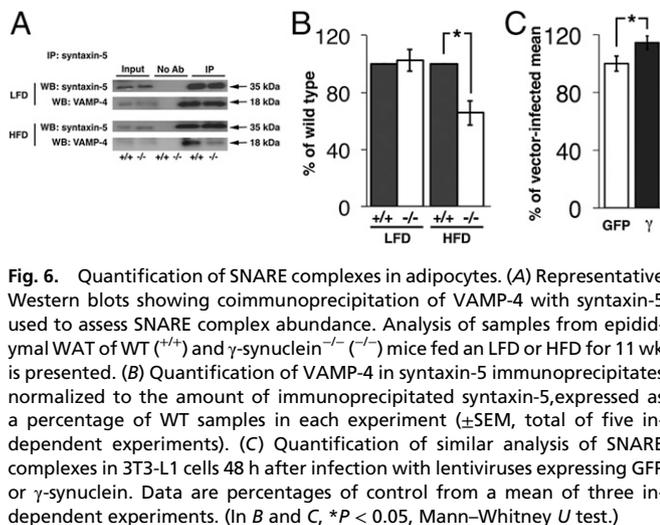


Fig. 6. Quantification of SNARE complexes in adipocytes. (A) Representative Western blots showing coimmunoprecipitation of VAMP-4 with syntaxin-5 used to assess SNARE complex abundance. Analysis of samples from epididymal WAT of WT (+/+) and γ -synuclein^{-/-} (-/-) mice fed an LFD or HFD for 11 wk is presented. (B) Quantification of VAMP-4 in syntaxin-5 immunoprecipitates normalized to the amount of immunoprecipitated syntaxin-5, expressed as a percentage of WT samples in each experiment (\pm SEM, total of five independent experiments). (C) Quantification of similar analysis of SNARE complexes in 3T3-L1 cells 48 h after infection with lentiviruses expressing GFP or γ -synuclein. Data are percentages of control from a mean of three independent experiments. (In B and C, * $P < 0.05$, Mann-Whitney U test.)

increased lipid availability, despite not being a constituent of these complexes itself.

Discussion

Our data show that γ -synuclein is an important regulator of adipocyte function in vivo that can influence whole-body energy balance. KO of γ -synuclein, which is highly expressed in WAT, leads to decreased weight gain in mice fed an HFD, and this is associated with increased energy expenditure and lipid oxidation. Adipocytes from γ -synuclein^{-/-} mice exhibit increased basal and isoproterenol-stimulated lipolysis whereas adipocyte size is decreased in vivo. Together, this suggests that γ -synuclein loss promotes mobilization of TG from adipocytes and increases lipid oxidation, most likely in BAT, but potentially also in other tissues. In support of this hypothesis, we observe increased basal and stimulated rates of lipolysis in cultured adipocytes in which γ -synuclein has been knocked down. This is associated with a significant redistribution of ATGL within the cell to lipid droplets similar to that observed in control cells treated with isoproterenol to stimulate lipolysis. Our data strongly argue that γ -synuclein acts as a cell-autonomous regulator of lipolysis in adipocytes, particularly affecting ATGL function, leading to decreased adipocyte size in γ -synuclein^{-/-} mice.

It has previously been demonstrated that, in transgenic mice, increased ATGL activity like γ -synuclein loss—specifically in adipose tissue—leads to obesity resistance with increased energy expenditure and lipid oxidation, decreased adipocyte size, and reduced hepatic steatosis following HFD feeding, without affecting food intake (16). Conversely, adipose-selective disruption of ATGL leads to increased adiposity and a more WAT-like phenotype in BAT (17). The lipolytic action of ATGL has been shown to be important for inducing the expression of genes favoring lipid oxidation genes in hepatocytes, macrophages, cardiac muscle, and BAT, as well as increased thermogenesis in the latter (17–20). As γ -synuclein is clearly detectable in BAT and appears able to regulate ATGL function, activation of ATGL in the BAT of γ -synuclein^{-/-} mice may also increase lipolysis in this tissue and drive the increased lipid oxidation profile we observe. The exact mechanism whereby γ -synuclein influences ATGL localization and lipolysis, if this also occurs in brown adipocytes, and whether this underlies the induction of genes controlling lipid oxidation, are key questions we will seek to answer in our future studies. However, the evidence from studies of ATGL suggests that potentiating the lipolytic function of ATGL in adipocytes could alone provide ample means via which the loss of γ -synuclein could bring about the attenuated obesity and metabolic phenotype we have observed.

Although γ -synuclein expression is particularly high in adipocytes, we cannot completely exclude a role for γ -synuclein in nonadipose tissues, primarily in the nervous system, that could contribute to other aspects of the phenotype observed in mice lacking this protein. However, we do show that specifically reexpressing γ -synuclein in the fat pads of null mutant mice reverses the observed decrease in adipocyte size. Critically, this was seen in only those adipocytes reexpressing γ -synuclein, and not in nontransduced cells in the same depot. This, coupled to our data showing that γ -synuclein knockdown in cultured adipocytes alters lipid handling and lipid droplet size, strongly implies that this aspect of the in vivo phenotype is fat cell-autonomous.

It has been shown previously that γ -synuclein mRNA is a late marker of adipocyte differentiation (8), and our data also suggest that γ -synuclein does not play a major role in adipocyte differentiation, indicating that the reduced adiposity of γ -synuclein^{-/-} mice does not reflect impaired capacity to expand adipose depots when required. This is important, as human syndromes of lipodystrophy and mouse models of restricted adipose expandability demonstrate that merely constraining adipose mass leads to dyslipidemia, insulin resistance, and metabolic disease (21, 22). As the loss of γ -synuclein appears to selectively affect mature adipocytes, it may prove a useful therapeutic target for improving obesity-related metabolic disease. It is also encouraging that no adverse phenotypes were apparent in neuronal or other tissues, suggesting that altering γ -synuclein function therapeutically might not require tissue-selective targeting; however, this clearly requires further confirmation.

In addition to regulating lipolysis, we have also identified a further, distinct function for γ -synuclein in adipocytes: regulating the formation of SNARE complexes that are involved in lipid droplet fusion, contributing to lipid accumulation in the adipocyte and a consequent increase in its size (13–15). This provides an intriguing echo of the function of α -synuclein in neuronal synapses, where it enhances neurotransmitter exocytosis by promoting the assembly of SNARE complexes, with a pivotal role in synaptic vesicle docking and fusion pore formation at the plasma membrane (11). Hence, our data imply that, in very different cellular systems, α -synuclein and γ -synuclein share a fundamental function, namely potentiating SNARE-mediated fusion selectively

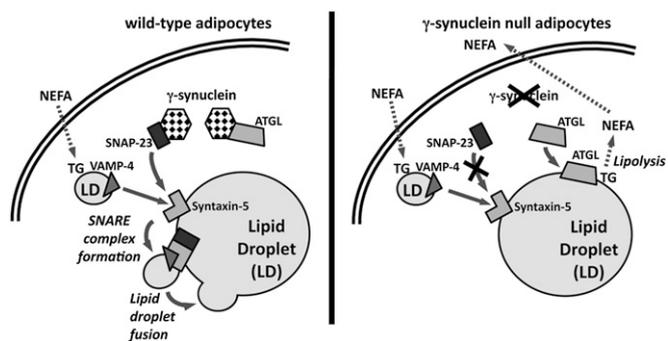


Fig. 7. Scheme of proposed role of γ -synuclein in lipid metabolism in WAT adipocytes. In WT adipocytes, γ -synuclein promotes efficient delivery of SNAP-23 to the SNARE complex as it forms. Nutrient excess in vivo, or lipid loading in culture, increases delivery of SNAP-23 to the SNARE complexes, thereby decreasing the association of SNAP-23 with γ -synuclein. These SNARE complexes facilitate the fusion of lipid droplets (LD) containing TG generated from the esterification of nonesterified fatty acids (NEFA), favoring TG storage. In the absence of lipolytic stimulation, γ -synuclein also retains the adipose TG lipase, ATGL, in a cytosolic location, possibly via protein–protein interaction. In adipocytes lacking γ -synuclein, SNARE complex formation, droplet fusion and thus TG accumulation is reduced. Loss of γ -synuclein also leads to a redistribution of ATGL to the lipid droplet, increasing basal lipolysis and priming the lipolytic machinery for hormonal stimulation of lipolysis.

under physiological and/or environmental conditions that require this process to occur with increased efficiency.

We have demonstrated that, in conditions of increased lipid supply, i.e., in HFD-fed mice, the lack of γ -synuclein substantially attenuates of SNARE complex assembly in adipocytes. We propose that γ -synuclein delivers SNAP-23 to the forming SNARE complex, and that this delivery is increased at times of nutrient excess and increased lipid droplet formation or enlargement. Conversely, overexpression of γ -synuclein in preadipocytes increases the abundance of SNARE complexes. It is therefore plausible that the decreased SNARE complex formation seen in γ -synuclein-deficient adipocytes reduces TAG incorporation into the lipid droplet. Ultimately, this involves γ -synuclein in two key, independent aspects of adipocyte biology regulating lipid droplet formation and TG lipolysis (Fig. 7). Hence, these twin roles for γ -synuclein may act in a concerted manner, converging to regulate the size of lipid droplets and thereby adipocytes.

In conclusion, our study reveals an important role of γ -synuclein in adipocyte lipid metabolism and suggests that increased γ -synuclein expression could contribute to the development of obesity-related metabolic disease. In addition, inhibiting γ -synuclein expression or function may represent a novel potential therapeutic avenue for the treatment of this prevalent condition.

Methods

Animals. Production, genotyping, and initial characterization of γ -synuclein-null mutant mice on a C57Bl6J genetic background have been described previously (4). The colony was maintained by backcross breeding, and intercrosses were used for production of experimental cohorts (details provided in *SI Methods*). From the age of 9 wk, animals were fed a 10% energy-from-fat LFD or a 45% energy-from-fat HFD (23). Body mass changes and food consumption were measured weekly. All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Ethical Review Committee of Cardiff University.

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Metabolic Analysis. Metabolic rate in WT and γ -synuclein^{-/-} mice in the eighth week of HFD feeding was measured by indirect calorimetry with the use of a CLAMS (Columbus Instruments). RER (volume of CO₂/volume of O₂) and energy expenditure [using constants according to a previous publication (24)] was determined over a 72-h period.

Isolation of Cells from WAT and Adipocyte Lipolysis. Mature adipocytes and the preadipocyte-containing stromal cell fraction were isolated from epididymal fat depots as described previously (25). Mature WAT adipocyte lipolysis was quantified by glycerol release under basal [i.e., 200 nM (R)-(-)-N⁶-(2-phenylisopropyl)adenosine] and catecholamine-stimulated [i.e., 200 nM (R)-(-)-N⁶-(2-phenylisopropyl)adenosine plus 10 μ M isoproterenol] conditions. In 3T3-L1 adipocytes and MEFs, lipolysis was determined by ¹⁴C release from cells loaded overnight with [¹⁴C]oleate, serum-deprived for 2 h, then stimulated for 30 min with or without 1 nM or 1 μ M isoproterenol as indicated, essentially as described previously (26).

Expression Analysis. Levels of RNA were measured by using quantitative RT-PCR and proteins by quantitative Western blotting as described previously (27). Oligonucleotide primers, primary antibodies and dilutions used are shown in *SI Methods*.

In Vitro Cell Culture Experiments. Primary cultures of MEFs from embryonic day 12.5 embryos were maintained and differentiated essentially as described previously (28). 3T3-L1 cells were cultured, transfected, and differentiated as previously described (29). Cells were transfected by siRNA at days 3 and 6 of differentiation by using Lipofectamine RNA iMAX transfection reagent (Invitrogen). Lipid droplet areas were calculated by using Volocity 6.1 (Perkin-Elmer). Analysis was performed on >2,400 droplets in randomly selected fields. For reexpression studies, lentiviruses were added to cells for 48 h (10⁶ virus transducing units per milliliter of medium). Additional methods are described in *SI Methods*.

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