The Journal of Nutrition
Biochemical, Molecular, and Genetic Mechanisms

γ-Synuclein Is an Adipocyte-Neuron Gene Coordinately Expressed with Leptin and Increased in Human Obesity[1–3]

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Abstract

Recently, we characterized tumor suppressor candidate 5 (Tusc5) as an adipocyte-neuron PPARγ target gene. Our objective herein was to identify additional genes that display distinctly high expression in fat and neurons, because such a pattern could signal previously uncharacterized functional pathways shared in these disparate tissues. γ-Synuclein, a marker of peripheral and select central nervous system neurons, was strongly expressed in white adipose tissue (WAT) and peripheral nervous system ganglia using bioinformatics and quantitative PCR approaches. γ-Synuclein expression was determined during adipogenesis and in subcutaneous (SC) and visceral adipose tissue (VAT) from obese and nonobese humans. γ-Synuclein mRNA increased from trace levels in preadipocytes to high levels in mature 3T3-L1 adipocytes and decreased ~50% following treatment with the PPARγ agonist GW1929 ($P < 0.01$). Because γ-synuclein limits growth arrest and is implicated in cancer progression in nonadipocytes, we suspected that expression would be increased in situations where WAT plasticity/adipocyte turnover are engaged. Consistent with this postulate, human WAT γ-synuclein mRNA levels consistently increased in obesity and were higher in SC than in VAT; i.e. they increased ~1.7-fold in obese Pima Indian adipocytes ($P = 0.003$) and ~2-fold in SC and VAT of other obese cohorts relative to nonobese subjects. Expression correlated with leptin transcript levels in human SC and VAT ($r = 0.887$; $P < 0.0001$; $n = 44$). γ-Synuclein protein was observed in rodent and human WAT but not in negative control liver. These results are consistent with the hypothesis that γ-synuclein plays an important role in adipocyte physiology. J. Nutr. 138: 841–848, 2008.

Introduction

Identifying the subcellular and systemic networks that connect energy balance, tissue fuel utilization patterns, and body composition will be an important step toward understanding how metabolic homeostasis and healthy body weight are regulated. The remarkable molecular and physiological shifts that take place in response to changes in ambient temperature in rodents can be leveraged to identify genes and pathways that participate in these networks. Through transcriptome analysis of temperature-dependent gene expression changes in thermogenic mouse brown adipose tissue (BAT),[14] we previously characterized a cold-repressed white adipose tissue (WAT) and BAT gene (1) termed tumor suppressor candidate 5 (Tusc5). Rat Tusc5 has been identified as a BAT and WAT gene downregulated by cold exposure and induced during brown adipocyte maturation (2,3). Based on the presence of a CD225 cell growth-regulating protein domain, upregulation of expression by a PPARγ agonist, and induction of the gene following exit from the mitotic clonal

[1] Supported by the following: intramural USDA-Agricultural Research Service CRIS 5306-51530-016-00D (S.H.A.); Direction de la Recherche Clinique/Assistance Publique-Hopitaux de Paris, the ''Programme Hospitalier de Recherche Clinique'' (UA 5451 and CRC No.97123), Institute National de la Santé et de la Recherche Médicale Programme de Recherche Nutrition-Humaine (K.C.), and The Wellcome Trust (V.L.B.).

[2] Author disclosures: P. J. Oort, T. A. Knotts, M. Grino, N. Naour, J.-P. Bastard, K. Clément, N. Ninkina, V. L. Buchman, P. A. Permana, X. Luo, G. Pan, T. N. Dunn, and S. H. Adams, no conflicts of interest.

[3] Supplemental Figures 1–3 and Supplemental Table 1 are available with the online posting of this paper at jn.nutrition.org.

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[14] Abbreviations used: BAT, brown adipose tissue; CNS, central nervous system; DRG, dorsal root ganglia; GEO, Gene Expression Omnibus; GLUT, glucose transporter; IHC, immunohistochemistry; PNS, peripheral nervous system; SC, subcutaneous; Tusc5, tumor suppressor candidate 5; TZD, thiazolidinedione; VAT, visceral adipose tissue; VLED, very low energy diet; WAT, white adipose tissue.

Manuscript received 20 December 2007. Initial review completed 8 January 2008. Revision accepted 9 February 2008.

expansion phase of 3T3-L1 adipogenesis, we proposed a working model in which white adipocyte Tusc5 activities are associated with entry into or persistence of the fully mature, growth-arrested metabolic and structural phenotype of adipocytes (1). Identification of Tusc5 and other adipocyte candidates that modulate cell growth is important to understand mechanisms that underlie adipose malleability (plasticity) characterized by phenotypic change, cell type conversion (brown-like/white-like fat cell transdifferentiation), and/or adipocyte turnover [see, e.g. (4,5) for reviews].

Like adipocytes, nerves associated with the somatosensory system and peripheral nerves in general display functional and morphological plasticity in response to external stimuli, inflammation, and neuronal damage (6–8). Interestingly, in addition to adipocytes, Tusc5 mRNA and protein are expressed at high amounts in peripheral afferent nerves that convey ambient temperature, peripheral tissue status, and other information to the brain (1). The physiological implications of robust coexpression of select proteins in adipocytes and peripheral nervous system (PNS) neurons remain to be elucidated, but these factors may provide a link connecting peripheral and environmental cues, tissue malleability, and metabolism. Identification of additional genes coexpressed in peripheral nerves and adipocytes could illuminate previously underappreciated functional aspects shared between these disparate tissues. To this end, mining of murine gene tissue expression patterns in the SymAtlas database (9) led to the novel finding that γ-synuclein, a well-established peripheral neuron marker also expressed in several central nervous system (CNS) sites (10–14) and implicated in cancer progression and cell survival (15), is strongly expressed in adipocytes. Considering its putative involvement with cancer cell progression, we hypothesized that adipocyte γ-synuclein would be downregulated in fat by PPARγ agonism and upregulated in obesity, because development of the latter involves WAT plasticity and PPARγ is critical for promoting growth arrest/terminal differentiation following the mitotic clonal expansion phase of newly differentiated fat cells. Results using PPARγ agonists in 3T3-L1 adipocytes and a survey of expression in WAT derived from nonobese and obese human cohorts support these concepts. Our findings are consistent with the hypothesis that γ-synuclein is a PPARγ target gene with an important role in fat cell physiology.

Methods

3T3-L1 adipocyte differentiation and PPARγ agonist studies. The changes in γ-synuclein mRNA expression during the course of fat cell differentiation and maturation and following agonism of PPARγ were tested in the 3T3-L1 adipogenesis model. Results herein were generated using the samples described in our recently published studies and details regarding cell culture conditions may be found in that report (1). Proof-of-principle studies tested the ability of the potent non-thiazoladinedione (TZD) PPARγ agonist GW1929 (16) to regulate γ-synuclein gene expression at various points in the 3T3-L1 adipocyte differentiation and maturation process, as indicated. For these experiments, cells were cultured for the times and doses indicated in media containing vehicle (dimethyl sulfoxide; 0.1% by volume) or GW1929 (0.1% by volume). RNA was prepared from 3T3-L1 adipocytes using TRIzol-based methods for cell culture samples (Ambion). We checked RNA abundance and integrity using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and an Agilent 2100 bioanalyzer (Agilent), respectively, per the manufacturer's instructions.

Human WAT mRNA. The effect of obesity and weight loss and differences between adipose depots with respect to γ-synuclein gene expression were analyzed using archived human WAT samples. Samples were derived from female volunteers undergoing surgery or were obtained via subcutaneous (SC) biopsy before and after a very low energy weight loss regimen, as previously described (17–19). Briefly, for comparisons between lean and obese SC WAT and visceral adipose tissue (VAT; omental), samples from experiments described by M. Grino et al. (19) derived from 10 nonobese and 12 obese (BMI 37.9 ± 1.5 kg/m^2) women were studied. VAT was collected in the course of laparoscopy or laparotomy for gastroplasty or gynecological procedures and SC fat was obtained in parallel from the abdominal region. mRNA prepared from these samples was transferred to the Western Human Nutrition Research Center for quantitative PCR analyses. WAT mRNA samples were also obtained by K. Clément et al. from a subset ($n = 21$) of the morbidly obese adult women described by Cancello et al. (18) and results from those samples were compared with nonobese control samples ($n = 9$). Matched SC and omental VAT mRNA samples were available from 20 of the obese subjects and tested to compare depot-specific gene expression. In another group of adult French obese women, SC biopsy before and after a very low energy diet (VLED) (17) enabled testing of the effects of weight loss on gene expression. Briefly, 12 obese women participating in the VLED reduced their daily energy intake by one-third (by 3940 ± 113 kJ/d with 35% protein, 20% lipids, and 45% carbohydrates) for 21 d. None of the subjects had a familial or personal history of diabetes or was taking medications and prior to the study, all volunteers were at their peak weight and were not on a restrictive diet. Subjects were not involved in an exercise program. Abdominal SC fat specimens (~1 g) from the periumbelical region were obtained by needle aspiration under local anesthesia after an overnight fast before and after the dietary intervention in obese subjects and in nonobese volunteers ($n = 7$) used for comparison. All biopsies were washed in PBS and stored in RNALater preservative solution (Qiagen) at −80°C until analysis. Total RNA was extracted using the RNeasy total RNA Mini kit (Qiagen) and quantitative real-time PCR (TaqMan) using ABI primers and probes described below was performed by the INSERM U872 laboratory. For human WAT Western blot analysis (see below), a sample of SC WAT was obtained from the upper gluteal region of an adult female volunteer via needle biopsy under local lidocaine anesthesia. The sample was washed twice in cold PBS to clean the biopsy of blood and loose connective tissue and was flash-frozen prior to storage at −80°C. Clinical investigations were approved by the ethical committees of each participating center and were performed according to the Declaration of Helsinki.

Gene expression analyses. RNA abundance of γ-synuclein and other genes of interest were measured using quantitative real-time PCR. These assays utilized gene-specific TaqMan primers and FAM-MGB labeled probes (Assays-on-Demand, Applied Biosystems) and were analyzed in duplicate or triplicate for each sample using an ABI 7900HT instrument. Primers/probe ABI identifiers for human reagents were γ-synuclein (Hs00268306_m1), leptin (Hs00174877_m1), and 11β-hydroxysteroid dehydrogenase (Hs00194153_m1), and for mouse, γ-synuclein (Mm00488345_m1) and others described previously (1). Mouse and human tissue panel samples and samples derived from nonobese and obese WAT (19) were assayed using a 2-step process with preparation of cDNA from total RNA followed by quantitative real-time PCR. Briefly, for each sample, cDNA was prepared from 1–5 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) followed by RNase-H treatment per the manufacturer's instructions. Reactions were carried out in a 384-well format and contained the following in each well: cDNA corresponding to 40 ng (tissue panels) or 2 ng (obese and lean human WAT samples) of original total RNA, 1× master mix (ABI Universal PCR Master Mix, part no. 4304437), and 1× primer-probe mix (pilot studies examining the range of linearity between PCR cycle number and template amount in human WAT samples revealed that as little as 2 ng per well could be used for γ-synuclein, leptin, and 18S cDNAs). Cycle conditions were 95°C for 10 min, then 45 cycles of 95°C for 15 s/60°C for 1 min. Amplification cycle number (Ct) of 18S for each sample was determined using commercial 18S primers and probe (ABI) to correct for template loading differences across all target genes (ΔCt method: ΔCt = sample Ct − 18S Ct), and expression values were determined relative to control transcript levels using a mathematical formula as previously described (1).

γ-Synuclein Western blots and immunohistochemistry. Western blot analyses of γ-synuclein protein in murine, rat, and human WAT samples were conducted using an anti-γ-synuclein antibody E-20 (Santa Cruz Biotechnology). Briefly, tissue samples were homogenized using MPER extraction reagent (Pierce) with HALT protease and phosphatase inhibitors (Pierce). Protein concentrations were quantitated using the bicinchoninic acid assay (Pierce). Forty micrograms of total protein was separated on a 12% Bis-Tris SDS gel (Invitrogen) using 2-(*N*-morpholino)ethanesulfonic acid running buffer (Invitrogen). The proteins were transferred to polyvinylidene difluoride membrane and immunoblotted with goat anti-γ-synuclein antibody (1:100) in PBST overnight. Specific signal was detected with a horseradish peroxidase-conjugated secondary antibody (1:10,000 donkey anti-goat HRP, sc-2020, Santa Cruz Biotechnology) using Visualizer ECL reagent (Millipore). Blots were imaged using a Fluorochem 8800 instrument (Alpha Innotech). Samples of liver, retroperitoneal WAT, epididymal WAT, and SC WAT (inguinal region) for Western blot studies were obtained from 3 male B6D2F1 mice fed a high-fat diet (60% of energy from fat; D12492, Research Diets) for 8 wk. Twelve-week-old Zucker rats used for WAT harvest were fed a purified diet composed of (g/kg): casein (210), methionine (3), cornstarch (200), corn oil (80), vitamin mix (15), mineral mix (60), cellulose (392), and alphacel (40). Rats were food deprived ~4–6 h prior to tissue harvest, killed with CO_2, and tissues were flash-frozen prior to storage at −80°C. For immunohistochemistry (IHC) of murine fat tissue, dissected SC WAT from a 6-mo-old C57BL/6 female mouse was fixed, paraffin embedded, and sectioned as described previously (13). Affinity purified SK32 rabbit polyclonal anti-γ-synuclein antibody (11) in a 1:50 dilution and Vecstain Elite detection kit (Vector Laboratories) were used for immunohistochemical detection of γ-synuclein on these sections. To demonstrate specificity of immunostaining, 1 mL of diluted SK23 antibody was preincubated with 10 μg of a synthetic 15-mer C-terminal peptide of mouse γ-synuclein that was used as an immunogen for production of this antibody. All animal studies were conducted under protocols adhering to Animal Welfare Act Guidelines, approved by the Animal Care and Use Committees at UC, Davis (for Western blot samples) and Cardiff University (IHC study).

Materials. Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, and GW1929 were purchased from Sigma, DMEM and fetal bovine serum from Gibco/BRL (Invitrogen), and cell culture plates were purchased from Nunc. Murine and human total RNA tissue panels were obtained from Clontech and human preadipocyte (stromal-vascular cells from WAT biopsy) and cultured and fresh adipocyte total mRNAs were bought from Zenbio. Mouse epididymal WAT used in the mRNA tissue panel was from pooled adult C57BL/6J tissues.

Statistics. Changes in gene expression over time with and without PPARγ activation during 3T3-L1 adipogenesis were evaluated using a 2-way ANOVA testing for significant effects of time, treatment, and time × treatment (Prism 4.03 software; GraphPad). A similar approach was used to evaluate effects of obesity, WAT depot, and obesity × depot interactions, or to test obesity, sex, and obesity × sex interactions as relevant in human studies. When interactions were significant, post-hoc Bonferroni tests were employed to test for treatment effects within groups. Comparisons between 2 groups were done using a Student's *t* test. Dose-response data were tested by 1-way ANOVA with a post hoc Dunnett's test comparing groups to the vehicle-treated control. The relationship between γ-synuclein and leptin mRNA abundance was evaluated by Pearson correlation statistic (Prism 4.03). Means ± SEM are presented and differences were considered significant at $P < 0.05$.

Results

Tissue expression profile of γ-synuclein. Mouse gene chip tissue expression surveys cataloged in SymAtlas, a public database constructed by the Genomics Institute of the Novartis Research Foundation (9), were queried to identify genes displaying predominant expression in somatosensory nervous system ganglia [dorsal root ganglia (DRG) and trigeminal ganglia], WAT, and/or BAT. γ-Synuclein, a marker of peripheral neurons and select CNS neurons (10,11,13,14), emerged as 1 of 2 genes fitting this pattern (**Supplemental Table 1**); the other identified gene was Tusc5. High expression of the gene in murine WAT was confirmed by real-time quantitative PCR, whereas γ-synuclein mRNA was found to be negligible in other tissues tested (**Supplemental Fig. 1**). In humans, γ-synuclein mRNA abundance was highest in the DRG and mRNA prepared from isolated SC adipocytes, with trace to negligible γ-synuclein mRNA in most other tissues (including preadipocytes) with the exception of spinal cord, brain, cerebellum, and adrenals where levels were ~36, 43, 33, and 42% of adipocytes, respectively (Supplemental Fig. 1). γ-Synuclein protein was readily detected by Western blot analysis in murine and human WAT samples but not in negative control mouse liver (**Fig. 1***A*); γ-synuclein protein was also observed in retroperitoneal WAT and DRG, but not liver samples, derived from 12-wk-old female Zucker rats (data not shown; $n = 2$). Consistent with our observation that γ-synuclein mRNA resides in adipocytes in humans (Supplemental Fig. 1; see Results below for Pima Indian SC adipocytes), γ-synuclein protein appears to be located in mouse adipocytes as judged by strong IHC staining patterns (Fig. 1*B*) that were well above background (Fig. 1*C*).

γ-Synuclein mRNA expression patterns in differentiating 3T3-L1 adipocytes. Determining the time frame over which γ-synuclein is expressed during the 3T3-L1 adipocyte differentiation and maturation program will be useful to understand when γ-synuclein function affects fat cell physiology. We have

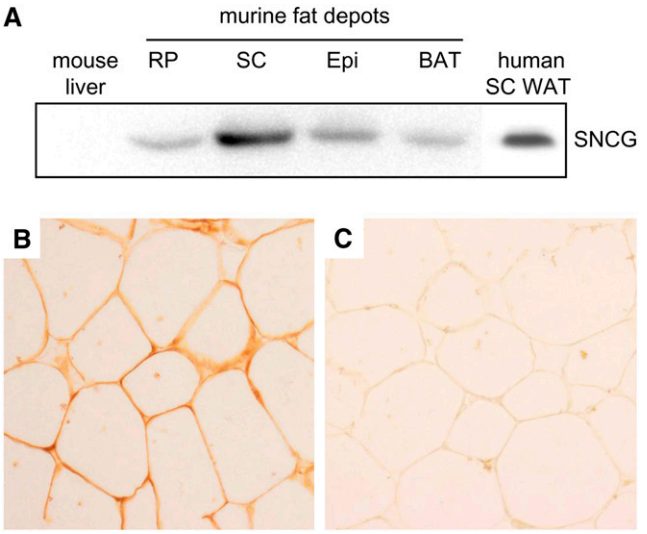

FIGURE 1 γ-Synuclein protein expression in adipose tissue. (*A*) Western blot revealed γ-synuclein protein expression in mouse WAT, BAT, and human SC WAT and no detectable expression in murine liver negative control. Mouse adipose depots: RP, Retroperitoneal; SC, inguinal SC; Epi, epididymal; BAT, intrascapular BAT. Two additional mice were assayed and displayed a qualitatively similar pattern of γ-synuclein protein in SC, RP, and BAT depots; however, epididymal WAT γ-synuclein protein level was highly variable across animals (data not shown). (*B*,*C*) Immunohistochemical evidence for γ-synuclein protein expression in murine adipocytes. Paraffin sections (8 μm) of the SC WAT from a 1-mo-old C57BL/6 female mouse was stained with anti-γ-synuclein SK23 antibody (*B*) or the same antibody preincubated with an excess of immunization peptide (*C*) as described in "Methods." A similar block of immunostaining was achieved by preincubation of antibody with recombinant mouse γ-synuclein (data not shown).

previously outlined gene expression clusters in this system for Tusc5 and 24 other adipocyte differentiation and functional markers and found that the pattern of Tusc5 expression clustered with a group of metabolically relevant mature adipocyte markers (1). γ-Synuclein mRNA expression was assayed using the samples from those experiments and had a pattern consistent with that observed for a gene cluster that included Tusc5, phosphoenolpyruvate carboxykinase 1, leptin, adipsin, and glucose transporter (GLUT) 4 (**Fig. 2**). This suggests that these factors share gene regulatory elements.

Effects of the PPARγ agonist GW1929 on expression profiles in 3T3-L1 adipocytes. Many metabolically relevant adipocyte genes are regulated by PPARγ activities and PPARγ actions promote entry of developing fat cells into the growth-arrested mature adipocyte phenotype. Thus, should γ-synuclein activity be important to adipocyte development and/or metabolism, it is reasonable to consider that the gene would be responsive to PPARγ agonism. In proof-of-concept studies, γ-synuclein mRNA was measured in fully differentiated 3T3-L1 adipocytes treated for 18 h with the potent non-TZD GW1929, provided at a concentration just above those reported to stimulate in vitro adipocyte glucose uptake and GLUT1 expression (20) and lipogenesis in C3H10T1/2 cells (16) and at lower concentrations to establish a dose response. In response to short-term GW1929, γ-synuclein mRNA abundance decreased by up to ~50% (**Fig. 3**). This pattern was paralleled qualitatively by that of leptin mRNA (Fig. 3), consistent with the notion that γ-synuclein and leptin could share gene regulatory elements that confer repression following PPARγ activation in fat cells. γ-Synuclein and leptin mRNA levels were also decreased by the less potent thiazolidinedione troglitazone, but the effective doses required were at least 5 times higher compared with GW1929 (data not shown). In separate studies, the ability of a single dose of GW1929 to decrease γ-synuclein expression following short-term (18 h) exposure to the compound was most apparent in

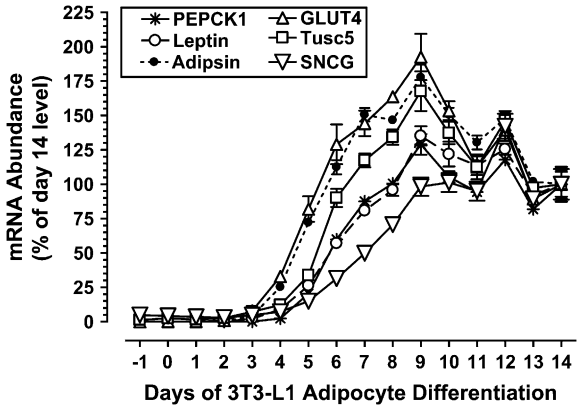

FIGURE 2 Transcript levels of a suite of differentiation stage and functional markers were determined by quantitative real-time PCR in 3T3-L1 cells at various times before and after initiation of the adipocyte differentiation program. Results for phosphoenolpyruvate carboxykinase 1 (PEPCK1), GLUT4, leptin, adipsin, and Tusc5 [adapted with permission from (1)] are shown for comparison. Differentiation of 2 d confluent 3T3-L1 fibroblasts was triggered at d 0 by 48 h exposure to differentiation medium. Transcript levels for each gene are expressed relative to the mean for that gene on d 14 postdifferentiation. Values are means ± SEM, $n = 3$ (some error bars are within the symbol). The effect of time was significant for all genes, $P < 0.0001$.

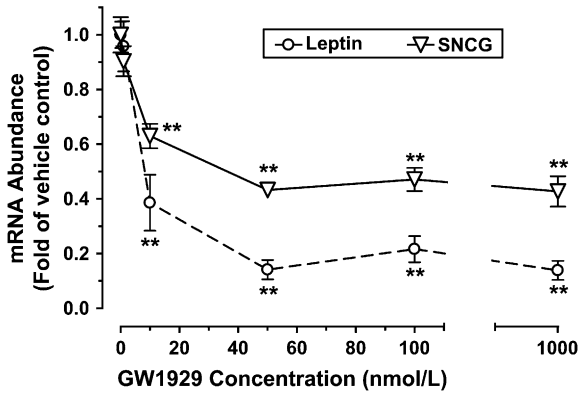

FIGURE 3 Reduction of γ-synuclein and leptin mRNA levels by short-term treatment with the PPARγ agonist GW1929 in mature 3T3-L1 adipocytes. RNA from mature (12 d differentiated) 3T3-L1 adipocytes was isolated from cells exposed to various doses of the non-TZD GW1929 and relative transcript abundances measured by quantitative real-time PCR. Values are means ± SEM, $n = 4$ per dose. Transcript level in vehicle-treated cells was considered 1.0, $P < 0.001$, 1-way ANOVA; $^{**}P < 0.01$, significantly different vs. 0 dose control expression.

mature fat cells, in contrast to preadipocytes and immature adipocytes in which γ-synuclein mRNA modestly increased, similar to what was observed for leptin (**Supplemental Fig. 2**).

Effects of human obesity and WAT depot site on γ-synuclein mRNA expression. γ-Synuclein has been implicated as a cancer marker and might be involved in cell survival mechanisms based on its overexpression in some cancers and studies examining cell death upon titration of γ-synuclein expression in vitro (see "Discussion"). Thus, we reasoned that in obesity, WAT γ-synuclein expression and activity would increase, because this condition, at least at some stages of its development, is characterized by increased adipocyte proliferation and, potentially, enhanced adipocyte plasticity/turnover to adapt to a high energy load. Consistent with this hypothesis, WAT γ-synuclein transcript levels significantly increased in obesity in several independent groups of human subjects. The pattern was initially revealed when the National Center for Biotechnology Information Gene Expression Omnibus (GEO) gene chip database was queried to identify studies in which γ-synuclein was differentially expressed in obesity. The γ-synuclein mRNA level was upregulated in isolated SC adipocytes derived from obese nondiabetic adult Pima Indians compared with nonobese, nondiabetic Pima Indians [GEO accession no. GSE2508, derived from studies by Permana et al. (21)]. Follow-up analysis using the gene chip raw data from male and female subjects confirmed that WAT γ-synuclein mRNA levels increased in obesity ($P < 0.01$), an outcome that is largely influenced by differences between obese and nonobese males ($P < 0.05$, obesity × sex interaction); signal intensities were 44,817 ± 2142 in obese males and 29,629 ± 3927 in nonobese males, respectively ($P < 0.01$). Signal intensities did not differ between obese (38,021 ± 1549) and nonobese (35,770 ± 2474) females. Consistent with our quantitative RT-PCR human tissue expression profile (Supplemental Fig. 1), a GEO query of a 2nd Pima Indian microarray study indicated trace to no expression of γ-synuclein in cultured preadipocytes/stromal vascular cells (GEO accession no. GSE2510).

We next examined WAT mRNA samples from a cohort of obese and nonobese French women previously described in an expression profile study of adipose 11β-hydroxysteroid dehy-

drogenase that demonstrated significantly increased expression of the latter in obesity (19). γ-Synuclein mRNA abundance significantly increased in the SC and VAT of obese compared with nonobese women (**Fig. 4***A*), a pattern also observed for leptin (Fig. 4*B*). Visceral fat γ-synuclein transcript levels were significantly lower compared with SC in both nonobese and obese groups. Visual inspection of the raw data for γ-synuclein and leptin expression in all 44 samples (nonobese and obese, VAT, and SC) suggested coordinate expression of leptin and γ-synuclein and this was confirmed through correlation analysis that indicated a correlation between expression of these genes ($r = 0.887$; $P < 0.0001$), regardless of sample source (**Fig. 5**). The abundance of γ-synuclein mRNA was next assayed in SC WAT samples derived from a different cohort of obese and nonobese French women. Consistent with the results discussed above, expression significantly increased in obese SC WAT, with a qualitatively similar pattern for leptin mRNA (**Fig. 6***A*). Also consistent was the finding that γ-synuclein and leptin mRNA levels were significantly lower in VAT relative to SC WAT in obese women (Fig. 6*B*). In a study examining the effect of a weight loss regimen in obese women, γ-synuclein mRNA levels were initially higher in obese SC WAT vs. nonobese controls (202 ± 26% vs. 100 ± 38% of nonobese expression, respectively; $P < 0.05$), and were reduced following the VLED intervention (131 ± 18% of nonobese controls; $P > 0.05$).

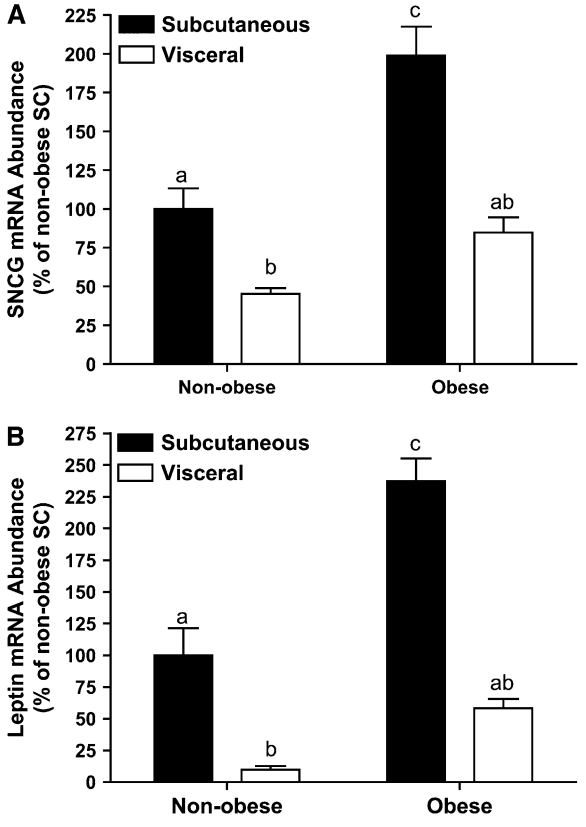

FIGURE 4 γ-Synuclein transcript level is highest in SC adipose tissue and significantly increased in obesity, a pattern similar to leptin, in adult women. Samples from a cohort of obese ($n = 12$) and nonobese ($n = 10$) adult women (19) were used to assay mRNA abundance for γ-synuclein (*A*) and leptin (*B*) by quantitative RT-PCR. Values are means ± SEM with the nonobese SC mRNA level for each gene considered 100%. Obesity ($P < 0.0001$), fat depot ($P < 0.0001$), and their interaction ($P < 0.05$) affected both genes. Means without a common letter differ, γ-synuclein (SNCG): $P < 0.05$, a vs. b; $P < 0.001$, c vs. a,b. Leptin: $P < 0.0001$, a vs. b; $P < 0.001$ c vs. a,b.

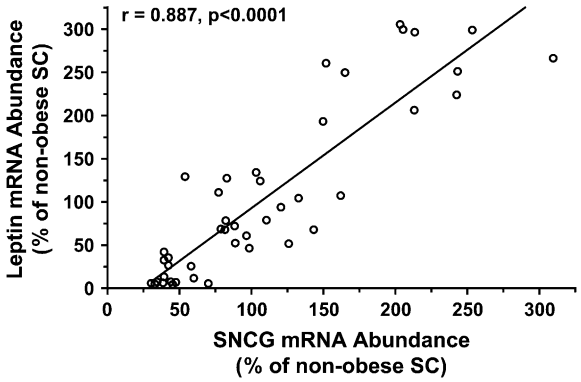

FIGURE 5 Human adipose tissue γ-synuclein mRNA levels are correlated with leptin mRNA levels. Utilizing the individual sample data used to generate results depicted in Figure 4 (SC and VAT from obese and nonobese women, $n = 44$ samples), the relationship between γ-synuclein and leptin mRNA abundance was evaluated by correlation analysis (Pearson correlation).

Discussion

PNS functions profoundly influence metabolic homeostasis. Somatosensory circuits, for instance, transmit environmental and tissue temperature information to the brain, thus enabling fine-tuning of downstream thermoregulatory and food intake responses emanating from the CNS. The recent body of work by Bartness et al. [see reviews (22,23)] has shown that partial chemical ablation of somatosensory afferents in WAT increases the size of select WAT depots in rats, indicating that the somatosensory system transmits information related to adiposity to the CNS, which in turn provides efferent signals that influence depot growth. Interestingly, sensory nerves have also been implicated in cold-related brown adipocyte development in WAT depots (24) and may modulate BAT thermogenic responses to norepinephrine (25,26). The sympathetic nervous system also regulates adipose function via lipolytic signaling and through modulation of proliferative events in WAT (27,28) and BAT (29,30). More recently, Yamada et al. (31) demonstrated that afferent signals from murine WAT helped control leptin sensitivity and food intake phenotypes. Our discoveries of Tusc5 (1) and γ-synuclein (this study) as proteins most strongly expressed in PNS neurons and adipocytes add to this body of knowledge by illustrating that in addition to anatomical connectivity between the PNS and WAT, there are apparently certain functional pathways shared by these otherwise disparate systems. To our knowledge, an association between γ-synuclein activities and metabolism, obesity, or fat cell physiology has not been reported previously.

γ-Synuclein mRNA expression was induced as 3T3-L1 adipocytes developed into mature fat cells in culture and mRNA levels for this gene were high in isolated human SC adipocytes, suggesting an important function primarily in maturing-to-mature adipocytes. However, the role of γ-synuclein in adipocytes remains to be elucidated. One possible function could be participation in events associated with exit from the growth-arrested, terminally differentiated mature fat cell phenotype and/or support of adipocyte cell survival. Support for these views comes from the oncology literature [see (15) and refs. therein].

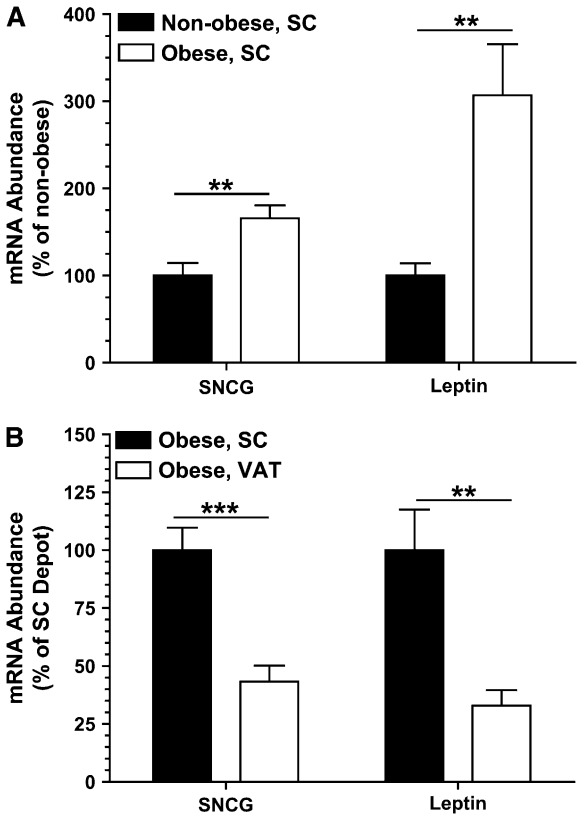

FIGURE 6 Adipose γ-synuclein transcript levels are increased in human obesity and highest in SC fat of adult women. (*A*) γ-Synuclein mRNA abundance in SC WAT samples derived from a cohort of adult women (9 nonobese, 21 obese; different from those depicted in Fig. 4) confirmed that γ-synuclein mRNA expression is increased in obesity, qualitatively similar to results observed for leptin. Values are means ± SEM. **Obese and nonobese differ, $P<0.001$. (*B*) γ-Synuclein mRNA levels in a matched set of WAT from the obese subjects in *A* were higher in SC WAT than in VAT, similar to leptin transcript abundance. Values are means ± SEM, $n = 20$ matched sample pairs. Asterisks indicate that SC and VAT differ: $^{**}P < 0.001$;$^{***}P < 0.0001$.

These studies highlight that despite low to nondetectable levels in most normal tissues, γ-synuclein is overexpressed in several hyperplastic tissues, including ovarian cancer and advanced breast cancer tissue. Furthermore, ectopic overexpression of γ-synuclein in breast cancer lines and experimental knockdown of γ-synuclein in the T47D breast cancer cell line led to increased and decreased cell growth, respectively. Our preliminary results using siRNA knockdown of γ-synuclein in several breast cancer cell lines (**Supplemental Fig. 3**) are consistent with these results. As reviewed by Ahmad et al. (15), the effect of γ-synuclein to promote cancer cell proliferation or cell survival is reportedly via actions that reduce the activities of mitotic growth arrest proteins and enhance cell cycle progression. There is evidence that γ-synuclein activates MAP kinase as part of its pro-proliferation activities and induces several matrix metalloproteinases that support cell migration and metastasis [see (15)].

Should tissue plasticity and cell survival properties of γ-synuclein manifest in adipocytes, it is anticipated that the protein plays a role in regulating such events as net fat cell turnover, transdifferentiation, and/or exit from the fully differentiated, mature fat cell phenotype. There is growing support that WAT is malleable, or plastic, in terms of its component adipocyte phenotypes and fat cell numbers [see reviews by (4,5)]. The positive energy balance associated with obesity development, for instance, leads to increased adipocyte size coupled to an expansion of adipocyte numbers to accommodate the larger energy load. Phenotypic and morphological conversions (transdifferentiation events) in subpopulations of adipose cells enable these cells to acquire white adipocyte- or brown adipocyte-like characteristics, even without proliferation from preadipocyte precursors per se. Some have suggested that mature, fully differentiated adipocytes can "de-differentiate" such that these cells revert phenotypically to resemble adipocytes derived from earlier stages of the adipogenic program (32–35) and/or acquire proliferative potential (35). We have consistently observed increased adipose expression of γ-synuclein mRNA in obese human subjects, which hypothetically may signal increased WAT plasticity or engagement of antiapoptotic pathways in fat cells as adaptational responses to a chronically high energy storage load. WAT γ-synuclein mRNA expression was reduced following 3 wk of weight loss in our obese subjects, a period in which energy storage is reduced and WAT energy stores are mobilized. Furthermore, it is interesting that γ-synuclein expression was consistently higher in SC fat than in internal VAT and the former is typically considered to manifest a greater phenotypic and morphologic plasticity, i.e. in response to TZD. Additional studies are required to assess whether observed differences in transcript abundance in obesity and following dieting also occur the protein level, but it is notable that we have readily detected γ-synuclein protein in WAT from multiple species.

Treatment with antidiabetic TZD drugs leads to profound changes in WAT, including an increased prevalence of small- to medium-sized adipocytes, higher numbers of apoptotic adipocytes, changes indicative of increased metabolism including emergence of brown adipocyte-like cells in WAT depots, and enhanced insulin sensitivity (36–39). Our finding that short-term treatment with the PPARγ agonists GW1929 and troglitazone significantly reduced γ-synuclein mRNA levels in differentiated adipocytes raises the interesting possibility that WAT changes after in vivo TZD treatment might involve regulation of adipocyte γ-synuclein expression, an idea that awaits experimental validation. Notably, using an algorithm to predict PPAR response elements across the human genome, the γ-synuclein gene was identified as containing this regulatory motif [see supplemental tables in (40)].

In addition to a putative role in cell survival and cell cycle regulation, our results and information from the literature suggest that other functions for γ-synuclein are possible, including chaperone activities for secreted and nonsecreted proteins [i.e. (41–44) and refs. therein] and involvement with metabolically relevant events in the cell. With respect to the latter, we have observed a remarkable correlation between γ-synuclein and leptin mRNA levels in human WAT samples and following PPARγ agonist treatment of mature 3T3-L1 adipocytes; this suggests that γ-synuclein shares gene promoter elements with leptin. Leptin expression is induced by insulin (45–48) via stimulation of glucose utilization (49–51) and expression is typically correlated with fat cell size/adiposity, but the influence of these metabolic and hormonal factors on γ-synuclein expression remain to be determined. Recently, using large-scale analysis of protein-protein interactions in human embryonic kidney 293 cells, Ewing et al. (52) found that γ-synuclein protein binds fatty acid binding protein 4 and hepatocyte nuclear factor 4, important proteins regulating fat and glucose metabolism. γ-Synuclein also bound tissue inhibitor of metalloproteinase-2 (52), a protein recently found to be increased ~2-fold in the SC WAT of obese

humans (53). Although these associations are interesting, it remains to be seen if γ-synuclein interacts with these proteins in adipocytes and whether such protein-protein interactions influence metabolism or adiposity.

In summary, our results are consistent with the hypothesis that γ-synuclein is a PPARγ-regulated gene with an important role in fat cell physiology. The functional relevance of robust coexpression of select proteins such as Tusc5 and γ-synuclein in adipocytes and peripheral neurons remains mysterious. Neurons, like adipocytes, display morphologic and functional plasticity and both cell types are sensitive to changes in metabolic status and insulin action. Compromised PNS function in obesity, prediabetes, and type 2 diabetes is an early event related to system-wide metabolic dysregulation and, intriguingly, TZD seem to improve diabetes-associated PNS functional indices (54), which may be independent of changes in blood glucose status. Because previous phenotyping experiments in γ-synuclein knockout mice focused on neurophysiology and neuroanatomical outcomes (13), it would be interesting to extend these efforts to focus on metabolism and adiposity phenotypes to help clarify the physiological roles of this protein in adipose tissue.

Acknowledgments

We thank Drs. Steven C. Griffen and Marta van Loan for supporting human WAT biopsy work at UC Davis, Drs. Daniel Hwang and Ling Zhao for mouse tissues used in Western blots, Drs. Judith S. Stern and John W. Newman for access to rat tissues, Gabriel Paulino for technical assistance, and Dr. Craig H. Warden for helpful discussions.

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