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A novel obesity model: Synphilin-1 induced hyperphagia and obesity in mice

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Abstract

Aims—The pathogenesis of obesity remains incompletely understood and the exploration of the role of novel proteins in obesity may provide important insights into its causes and treatments. Here we report a previously unidentified role for synphilin-1 in the controls of food intake and body weight. Synphilin-1, a cytoplasmic protein, was initially identified as an interaction partner of alpha-synuclein, and has implications in Parkinson's disease pathogenesis related to protein aggregation.

Subjects and methods—To study the *in vivo* role of synphilin-1, we characterized a human synphilin-1 transgenic mouse (SP1) by assessing synphilin-1 expression, plasma parameters, food intake and spontaneous activity to determine the major behavioral changes and their consequences in the development of the obesity phenotype.

Results—Expression of human synphilin-1 in brain neurons in SP1 mice resulted in increased food intake, body weight and body fat. SP1 mice also displayed hyperinsulinemia, hyperleptinemia and impaired glucose tolerance. Pair-feeding SP1 mice to amounts consumed by non-transgenic mice prevented the increased body weight, adiposity, hyperinsulinemia and hyperleptinemia demonstrating that these were all the consequences of increased food intake. Transgenic expression of synphilin-1 was enriched in hypothalamic nuclei involved in feeding control, and fasting induced elevated endogenous synphilin-1 levels at these sites, suggesting that synphilin-1 is an important player in the hypothalamic energy balance regulatory system.

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Conflict of interest

The authors declare no conflict of interest.

Conclusion—These studies identify a novel function of synphilin-1 in controlling food intake and body weight, and may provide a unique obesity model for future studies of obesity pathogenesis and therapeutics.

Keywords

Synphilin-1; obesity; food intake; body weight; fat; PVN; ARC

Introduction

Synphilin-1 is a 919-amino acid protein with ankyrin-like repeats and a coiled-coil domain suggesting its protein-protein interaction function (1-3). Human synphilin-1 is expressed in various tissues and is enriched in the brain. It is expressed in neurons in many regions of the brain, including the hypothalamus and substantia nigra, suggesting it may be involved in neuronal signaling and activity (2-5). The biological functions of synphilin-1 remain incompletely understood. Synphilin-1 interacts with α -synuclein, parkin, leucine-rich repeat kinase-2, other ubiquitin ligases and proteasome subunit/regulators, and has implications in Parkinson's disease pathogenesis related to protein aggregation (1;4;6-14). We and others demonstrated recently that synphilin-1 has neurotrophic and neuroprotective effects in Parkinson's disease cell models (15;16). To further investigate the role of synphilin-1 *in vivo*, we generated synphilin-1 transgenic (SP1) mice using the mouse prion protein promoter (mPrP) to express human synphilin-1 predominantly in neurons in brain (17). Our previous studies showed that there was no evidence of any PD-like pathologies in these SP1 mice (17). Unexpectedly, SP1 mice developed obesity.

In the studies reported here, we have characterized the SP1 mice by assessing synphilin-1 expression, plasma parameters, food intake and spontaneous activity to determine the major behavioral changes and their consequences in the development of the obesity phenotype. The results of these studies demonstrate a novel function of synphilin-1 in the modulation of energy balance to promote obesity, and suggest that the SP-1 mouse may provide a useful model for future obesity pathogenesis and therapeutic studies.

Methods

Synphilin-1 transgenic mice

The synphilin-1 transgenic mice were generated using the mPrP promoter as described previously (17). Briefly, human synphilin-1 cDNA was cloned into a mPrP transgenic mouse expression vector as described (17). All mPrP-human-synphilin-1 transgenic mouse lines were established as C3H/HeJ \times C57BL6 hybrids and maintained by successive backcrossing with the C57BL6 strain. All SP1 mice and their control littermates were weaned at day 21 and, at that time, tail tips were taken for PCR-genotyping using primers to detect both the endogenous mPrP genomic sequences and transgene sequences (17). All food intake experiments used standard laboratory chow (PMI Nutrition International, Inc., Brentwood, MO). The mice were housed in cages in a temperature-controlled room on a 12-h light, 12-h dark cycle. Tap water was available *ad libitum* (*lib*). All mouse experiments

were done in accordance with the guidelines on animal care and use established by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee.

Western blot analysis and immunohistochemistry

For western blot analysis, the mouse brain homogenates were prepared in TNE buffer (10 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA) containing protease inhibitors (5 mM PMSF/10 µg/ml of aprotinin/10 µg/ml of leupeptin/10 µg/ml of pepstatin) and detergents (0.5% Nonidet P-40 and 1–2% SDS). The resulting brain homogenates were resolved on 4–12% NuPAGE Bis-Tris gels and transferred onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk and then probed with different antibodies. Proteins were detected by using enhanced chemiluminescence reagents (NEN, Boston, MA). Anti-synphilin-1 rabbit polyclonal antibodies were home made against human synphilin-1 (30-543 aa fragment), that were very specific to synphilin-1 protein (human and rat) with weak crossing immunoactivity to mouse synphilin-1 as described (1;17). Anti-actin antibody was from Santa Cruz.

For immunohistochemical analysis, mice were perfused with paraformaldehyde. Cryosections from flash-frozen tissue through the arcuate (ARC) and the paraventricular (PVN) nucleus were subjected to immunohistochemical staining as previously described (17;18). Briefly, frozen sections were fixed in 4% paraformaldehyde. All tissue sections were treated with 0.6% H₂O₂ in methanol for 10 min at room temperature to exhaust endogenous peroxidases and were subsequently treated with 5% normal goat serum (Sigma) to block non-specific reactions. Sections were then sequentially incubated in polyclonal home-made synphilin-1 primary antibody (1: 50–1:200 dilution overnight at 4 C, biotinylated anti-rabbit secondary antibody (Vector Laboratories) and avidin–biotin–peroxidase complex (ABC Elite kit, Vector Laboratories). Immunoreactivity was visualized by peroxidase reaction using diaminobenzidine (DAB, Sigma) as chromogen. In some case, sections were subsequently counterstained with hematoxylin. The specificity of the immunostaining was checked by incubating adjacent sections with preabsorbed anti-synphilin-1 antibody. For this, antibody against synphilin-1 was incubated with excess synphilin-1 antigen (GST-30-543 aa fragment of synphilin-1). The images were captured using a Zeiss Axioskop 2 plus microscope connected with a Zeiss Axiocam camera. The digital images were processed in Adobe Photoshop (v.VII). Negative controls, omitting primary antibody, were performed in each case and no significant staining was seen.

Food intake and open field activity measurements

Cohorts of non-transgenic and synphilin-1 mice were adapted to test cages containing computerized feeding devices (Coulbourn Instruments), which deliver 20-mg chow pellets. Data of daily food intake were recorded and calculated. Novelty-induced activity was assessed in the open field over 24 hours using activity chambers with infrared beams (San Diego Instruments, Inc., San Diego, CA, USA) as described previously (17;19). Testing took place in a square open field, 50 × 50 cm, with 20-cm high opaque plastic walls. The floor of the open field was divided into 36 sections of approximately equal area by a series of solid lines forming small squares. There were 8 mice (half male and half female) in each

experimental group for this experiment. Total daily activity was automatically recorded. Mice were monitored at 4 and 12 months of age. The data are presented as the means (beams broken) \pm SEM.

Pair feeding experiments

The experiment had three groups: *ad lib* fed non-transgenic control mice, *ad lib* fed SP1 mice and pair-fed SP1 mice. The pair-fed SP1 mice were fed to match the food intake of the non-transgenic control mice. The experiment was started when mice were 6 weeks old, an age at which weights of SP1 mice did not differ from control mice. Body weights were monitored weekly for 10 weeks. There were 12 male mice in each experimental group.

Glucose tolerance tests, plasma assays and fat mass assays

At the end of pair-feeding, mice were food deprived overnight for 16 hours, with access to water only. A baseline blood sample for glucose was taken before the glucose injection. Mice were injected with glucose (1.5 mg/g body weight) via I.P. administration. Blood samples were taken at 15, 30, 45, 60, and 120 minutes for determination of blood glucose and plasma insulin levels. Blood glucose was measured using a glucometer (Freestyle, Alameda, CA). Plasma insulin and leptin were measured using a commercial RIA kit (Linco, St. Charles, Missouri). Mice were euthanized with Euthasol. Brain, adipose tissue, blood and carcass were harvested. Retroperitoneal, subcutaneous and brown adipose fat depots were dissected and weighed (20).

Real time RT-PCR

Two cohorts of animals were examined. The first cohort of 6-wk-old non-transgenic mice (12 mice) was food-deprived for 48 hours with water available *ad lib*. The second group (12 mice) was maintained with *ad lib* food access. Mice were sacrificed at end of the 48 h fast and the dorsal (containing PVN) and ventral hypothalamus (containing ARC) were dissected. Total RNA was extracted from each region and subjected to real time RT-PCR to detect endogenous mouse synphilin-1 mRNA by a standard protocol as previously described (21). The primers were 5'-CTCAAGACCATCCCAGCACT-3' and 5'-TCAGTGGAGAAACTCGCTTCA -3'.

Data Analysis

Quantitative data were expressed as arithmetic means \pm SEM. The difference among groups was statistically analyzed by analyses of variance (ANOVA) using Sigstart 3.1 statistical software (Aspire Software International, VA). A *P* value of <0.05 was considered significant.

Results

Synphilin-1 increased mouse body weight and fat deposition

We generated human synphilin-1 transgenic mice (SP1 mice) using mPrP promoter to lead to expression of human synphilin-1 predominantly in neurons in brain as described previously (17). Body weight of non-transgenic and SP1 mice was measured monthly

though the lifetime. Male and female SP1 mice displayed significantly increased body weight after 3 months of age compared with non-transgenic mice (Fig. 1A-C). Male SP1 mice had significantly increased body weight by 3 months of age (Fig.1B) and female (at SP1 mice had increased body weight by 4 months of age (Fig.1C). SP1 mice from line-A4 that had lower expression level of synphilin-1 than that of line-A6, also displayed increased body weight but to a lesser degree than line-A6 SP1 mice (data not shown). All the data shown in this study were obtained from line A6 SP1 mice. Fat depots of SP1 mice at 4 months of age were significantly heavier than those of non-transgenic mice (Fig. 2A-C). There were roughly two-fold increases in the weight of retroperitoneal, subcutaneous and brown adipose fat depots in SP1 mice.

Synphilin-1 was highly expressed in hypothalamus

Human synphilin-1 was expressed in whole brain and hypothalamus homogenates as demonstrated by western blot analysis using home-made synphilin-1 antibodies (Fig. 3A and 3C). This anti-human synphilin-1 antibody has very strong immunoreactivity to human synphilin-1 protein with one single band by western blot analysis. However, it has only moderate immunoreactivity to rat synphilin-1 and very weak immunoreactivity to mouse synphilin-1 as described previously (2-5;17). To date, there is no specific antibody available to detect mouse synphilin-1 protein. Thus, the levels of endogenous mouse synphilin-1 were not reflected in Figure 3C. Using this anti-synphilin-1 antibody to perform immunohistochemical staining, we found that human synphilin-1 was overexpressed in multiple brain regions including cortex, hippocampus, cerebellum, brainstem, thalamus and hypothalamus compared with non-transgenic control mice (Fig. 3B). Synphilin-1 was predominantly expressed in the cytosol of neurons. The cytoplasm of glial cells was negative for synphilin-1. Interestingly, synphilin-1 immunoactivity was dense and particularly evident in the PVN and ARC (Fig. 3D), two hypothalamic nuclei involved in feeding and body weight control. Synphilin-1 immunostaining in the PVN and ARC was specific since adjacent sections incubated with anti-synphilin-1 antibody preabsorbed with antigen followed hematoxylin counterstaining yielded no DAB signal (Fig. 3D left panel). Endogenous mouse synphilin-1 expression as measured by endogenous RT-PCR was not affected by the overexpression of human synphilin-1 (data not show). Thus, the total synphilin-1 expression in the SP-1 mice (mouse + human) was greater than in the wild type mice.

Synphilin-1 induced hyperphagia

Both food intake and activity influence body weight (22). In order to investigate whether alteration in either or both of these contribute to the increased body weight in SP1 mice, we measured the daily food intake and 24-hour activity of SP1 mice. SP1 mice consumed significantly more chow than age-matched non-transgenic mice and did so throughout the 15 week period (Fig 4A). Overall activity levels were assessed using computerized open field activity monitors. There were no significant differences in locomotor activity between SP1 and control mice at 4 (Fig 4B) or 12 (data not shown) months of age, suggesting that altered activity is unlikely to contribute to the obesity phenotype.

To further assess whether the increased food intake accounted for the obesity of SP1 mice, we performed a pair-feeding experiment in which one cohort of SP1 mice was pair-fed to the intake of the non-transgenic control mice. Thus, this experiment had three groups: *ad lib*-fed non-transgenic control mice, *ad lib*-fed SP1 mice, and SP1 mice pair-fed to amounts consumed by the control mice. The experiment began when mice were 6 weeks old, an age at which the weight of the SP1 mice did not differ from that of the control mice. Body weights were monitored for 16 weeks. Pair-feeding completely prevented the excess body weight gain of the synphilin-1 mice (Fig. 5A). The body weight trajectory of pair-fed SP1 mice was identical to that of the *ad lib*-fed control mice.

Expression of synphilin-1 caused hyperinsulinemia and hyperleptinemia in SP1 mice and these were also prevented by pair-feeding (Fig. 5B and 5C). Plasma levels of insulin and leptin of pair-fed SP1 mice did not differ from those of non-transgenic mice. To further characterize the consequences of obesity in SP1 mice, a glucose tolerance test was performed at the end of the 16 weeks of the pair-feeding experiment described above. SP1 mice had impaired glucose tolerance, which was prevented by pair-feeding (Fig. 5D).

Food deprivation increased endogenous hypothalamic synphilin-1 expression

To be to assess whether endogenous synphilin-1 is involved in energy balance, we compared levels of synphilin-1 expression in *ad lib* fed and food-deprived normal non-transgenic mice. We found that endogenous synphilin-1 mRNA expression was significantly increased in the dorsal and ventral hypothalamic region (containing the PVN and ARC respectively) after a 24 h fast (Fig. 6A and 6B). After a 48h fast synphilin-1 mRNA levels remained elevated in the dorsal hypothalamic region (containing the PVN) but were normalized in the ventral hypothalamic area (containing the ARC)(Fig.6C and 6D). Food deprivation did not change the synphilin-1 mRNA levels in other non-hypothalamic regions, such as cortex and cerebellum (Fig. 6E and 6F).

Discussion

Synphilin-1 is a cytoplasmic protein that was initially cloned over a decade ago. However, the functions of synphilin-1 have not been well characterized. We and others previously found that synphilin-1 has implications in Parkinson's disease (1;4;6-8). Recent reports show that synphilin-1 does not cause Parkinson's disease-like phenotypes but has neuroprotective effects (15-17;23). Our recent studies showed that the life span of our SP1 mice was not different from that of non-transgenic mice (17). There were no increases in anti-active caspase-3 immunoreactivity or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, a cell death marker) in brains of SP1 mice compared with non-transgenic mice (17). Moreover, SP1 mice also did not have altered astroglial reaction (another marker of neurodegeneration) compared with non-transgenic control animals (17). Taken together, these results suggest that overexpression of human synphilin-1 in mice did not induce neuronal degeneration. There are several reports of synphilin-1 mouse models from other groups but the results have been inconsistent (24-26). This inconsistency may be due to the use of various approaches and promoters resulting in various expression patterns and levels (24-26). In our SP1 mice, human synphilin-1 was expressed in the cytosol of

neurons. The cytoplasm of glial cells was negative for synphilin-1. Synphilin-1 positive neurons were in multiple brain regions including cortex, hippocampus, cerebellum, brainstem, thalamus and hypothalamus. The expression pattern of human synphilin-1 in SP1 mice was similar to endogenous synphilin-1 expression in human and in rat brain tissues as reported previously (2-5). Interestingly, synphilin-1 immunoactivity was dense and particularly evident in the PVN and ARC, two hypothalamic nuclei involved in feeding and body weight control, which may provide the molecular and anatomical foundation for synphilin-1's effects on energy balance.

Our data showed that SP1 mice displayed striking obese phenotypes including progressive increases in body weight, fat depots, hyperleptinemia, hyperinsulinemia and impaired glucose tolerance. The increased body weight in SP1 mice was expressed as increased body fat. There were roughly two-fold increases in the weight of adipose depots in SP1 mice. Moreover, SP1 mice consumed significantly more food compared with non-transgenic mice. The increase of food intake was evident prior to increases in body weight in SP1 mice. In contrast, there were no significant differences in locomotor activity between SP1 and control mice up to one year of age, suggesting that the obesity is primarily due to the increased food intake rather than to changes in energy expenditure. Furthermore, pair-feeding SP1 mice with an amount of food equal to that consumed by non-transgenic mice prevented the increases in body and fat depot weights. Interestingly, pair-feeding SP1 mice also prevented the hyperleptinemia, hyperinsulinemia and impaired glucose tolerance. These results indicate that synphilin-1 induced obesity is secondary to hyperphagia, and suggest that the hyperleptinemia, hyperinsulinemia and impaired glucose tolerance were a consequence of the obesity rather than a direct effect of the synphilin-1 expression. These findings are consistent with supplemental data of a report showing that expression of synphilin-1 in mice resulted in increased body weight (26). However, a comprehensive examination of the metabolic phenotype was not included in that report (26).

Demonstrating that overexpression of synphilin-1 in SP1 mice induces increases in food intake and body weight does not necessarily indicate a role for endogenous synphilin-1 in the controls of energy balance. However, the evidence that endogenous synphilin-1 expression is modulated by energy status provides further support for such a role. Food deprivation results in a compensatory increase in food intake, a refeeding hyperphagia (27;28). Our data showed that food deprivation significantly increased endogenous synphilin-1 mRNA expression in the hypothalamic segments (containing PVN and ARC) in normal mice. This result indicates that endogenous synphilin-1 is modulated by energy status in the PVN and ARC, brain regions that control food intake and energy balance. This increase of endogenous synphilin-1 expression suggests that synphilin-1 may be involved in the compensatory food increase in response to food deprivation. This finding is consistent with our data showing that overexpression of synphilin-1 in SP1 mice induced hyperphagia. Synphilin-1 may alter various aspects of hypothalamic signaling to increase food intake, and this remains to be investigated.

In summary our results provide evidence for synphilin-1 in the positive regulation of energy balance: brain overexpression of synphilin-1 induced hyperphagia resulting in obesity and acute food-deprivation increased endogenous synphilin-1 expression in hypothalamic

neurons critical to energy balance. Thus, our findings identify a novel biological function for synphilin-1 and provide a new genetic mouse model that may allow a greater understanding of the pathogenesis of obesity and contribute to the development of novel therapeutics.

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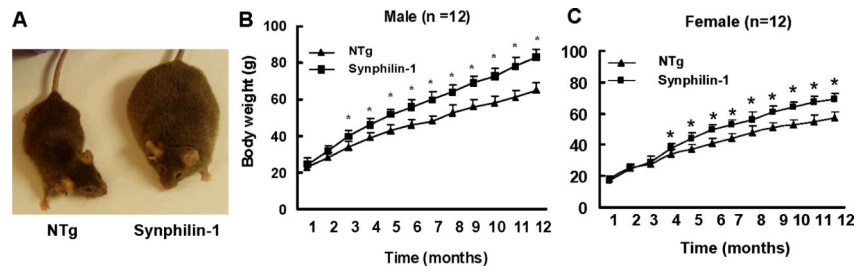


Fig.1. Synphilin-1 increases mouse body weight

A. An adult synphilin-1 transgenic mouse and a non-transgenic littermate at 1 year of age.

B. Body weights of male synphilin-1 mice and control mice at indicated time points. **C.** Body weights of female synphilin-1 mice and control mice at indicated time points. Data are means \pm SEM. There were 12 mice in each group. Significant differences between SP1 mice and non-transgenic control mice as indicated, * $p < 0.05$ by ANOVA. NTg: Non-transgenic mice

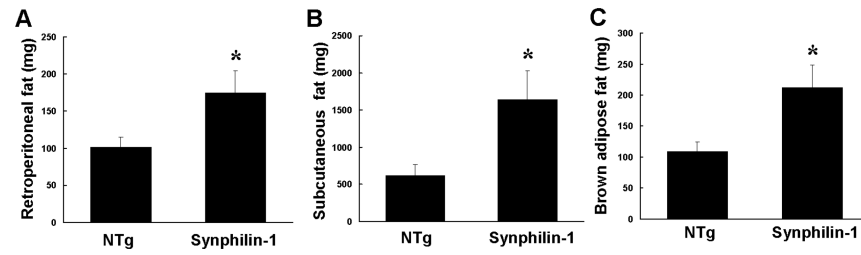


Fig. 2. Synphilin-1 increases mouse body fat

Male mice at 4 months of age were sacrificed and body fat was measured. Data are means \pm SEM. There were 8 mice in each group. Significant differences between SP1 mice and non-transgenic control mice as indicated, * $p < 0.05$ by ANOVA.

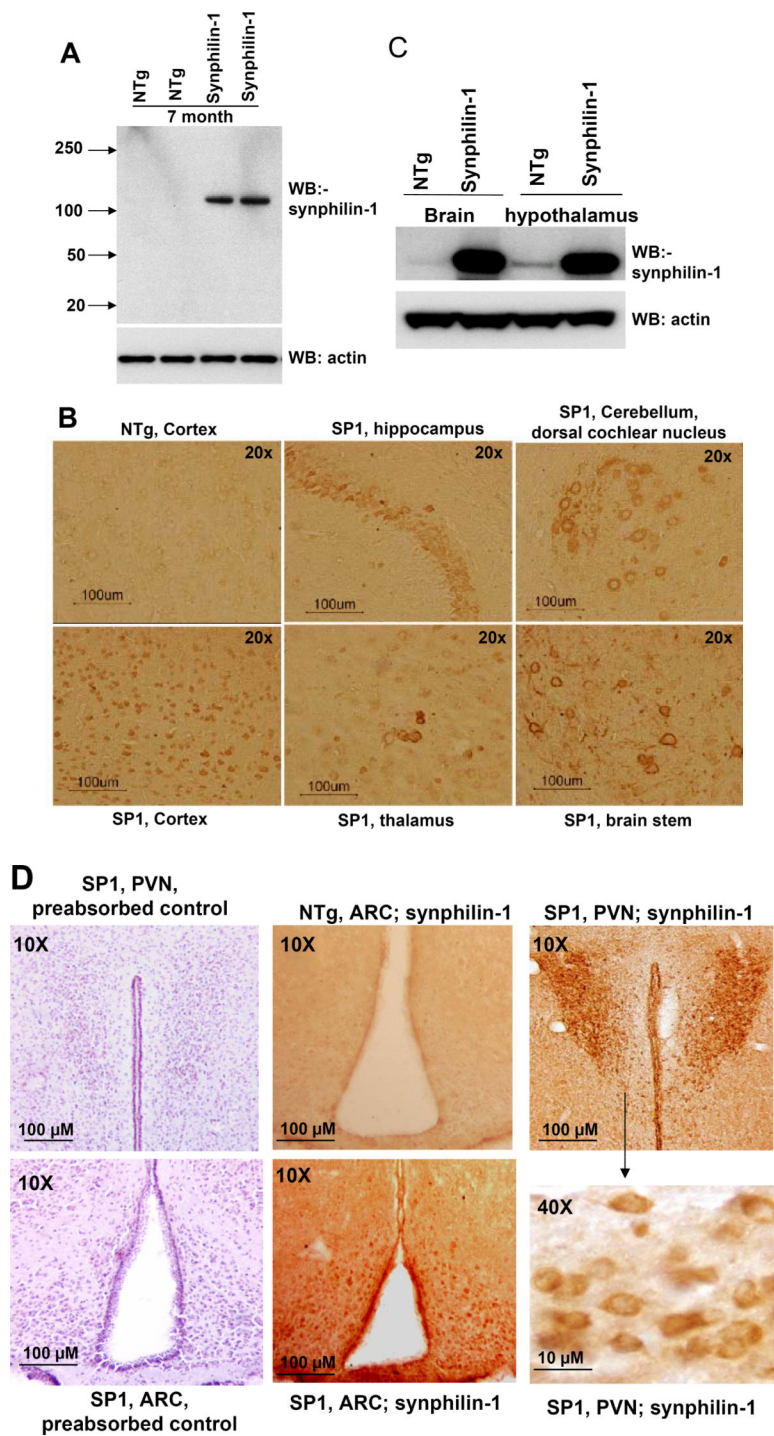


Fig.3. Human synphilin-1 is highly expressed in hypothalamus

A, Whole brain homogenates from SP1 and non-transgenic mice at 7 months of age were subjected to Western blot analysis using home-made anti-human-synphilin-1(17) and anti-actin antibodies (1 second exposure for western blot film). **B**, Mouse brain sections from SP1 and non-transgenic mice were subjected to immunohistochemical analysis using anti-synphilin-1 polyclonal antibody. Representative images of immunohistochemical staining

showing that human synphilin-1 expressed in neurons in multiple brain regions including cortex, hippocampus, cerebellum, brainstem, and thalamus. Scale bar is indicated as μM . **C**, Whole brain and hypothalamic homogenates from SP1 and non-transgenic mice at 4 months of age were subjected to western blot analysis using home-made anti-human-synphilin-1(17) and anti-actin antibodies. The endogenous mouse synphilin-1 levels in non-transgenic mice in this blot did not reflect the real levels in brains since the band was very weak with 10 min exposure of film and 50 μg proteins/per lane. **D**, Mouse brain sections from SP1 and non-transgenic mice were subjected to immunohistochemical analysis using anti-synphilin-1 polyclonal antibody. Left panel showing that synphilin-1 immunostaining in the PVN and ARC was specific since adjacent sections incubated with anti-synphilin-1 antibody preabsorbed with antigen followed hematoxylin counterstaining yielded no DAB signal; middle and right panels showing the human synphilin-1 was highly expressed in the PVN and ARC in SP1 mice. The lower right panel is the high magnification of neurons in the PVN in SP1 mice, showing that human synphilin-1 was predominantly expressed in the cytosol in neurons.

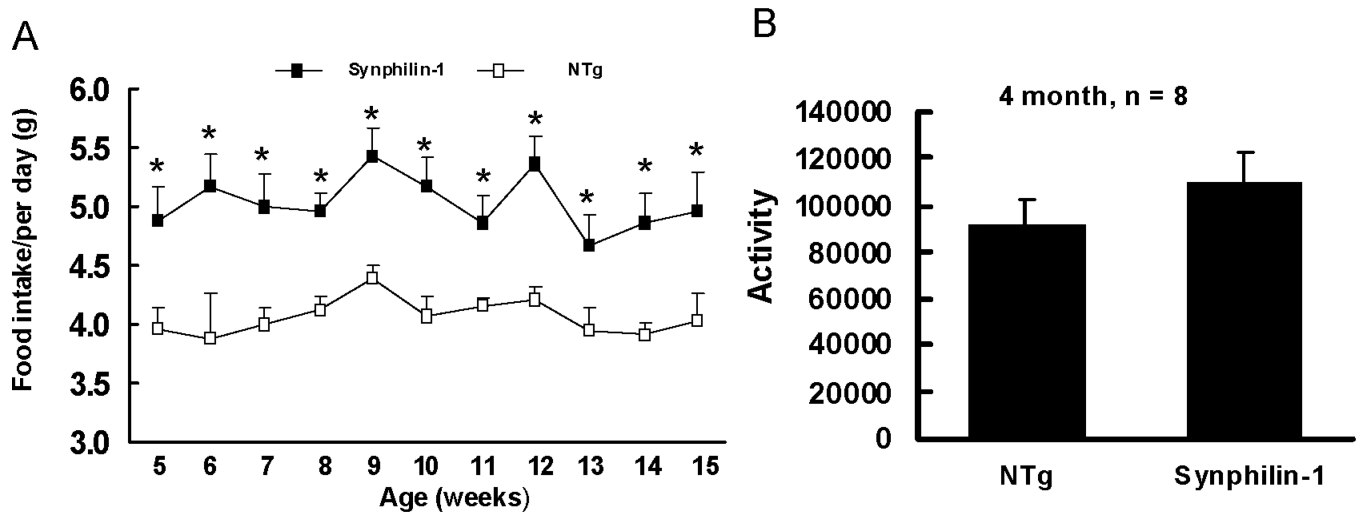


Fig 4. SP1 mice have increased food intake but no change in activity

A. Twenty four hr chow intake from 5-15 weeks old mice. There were 8 male mice in each group. **B.** Overall activity of SP1 mice and non-transgenic mice at 4 month of age were monitored using computerized VersaMax open field activity monitors. Shown are the average of daily open field total activity in SP1 mice and non-transgenic mice. Data are means \pm SEM. There were 8 mice (half male and half female) at 4 months of age in each group. Significant differences between SP1 mice and non-transgenic control mice as indicated, * $p < 0.05$ by ANOVA.

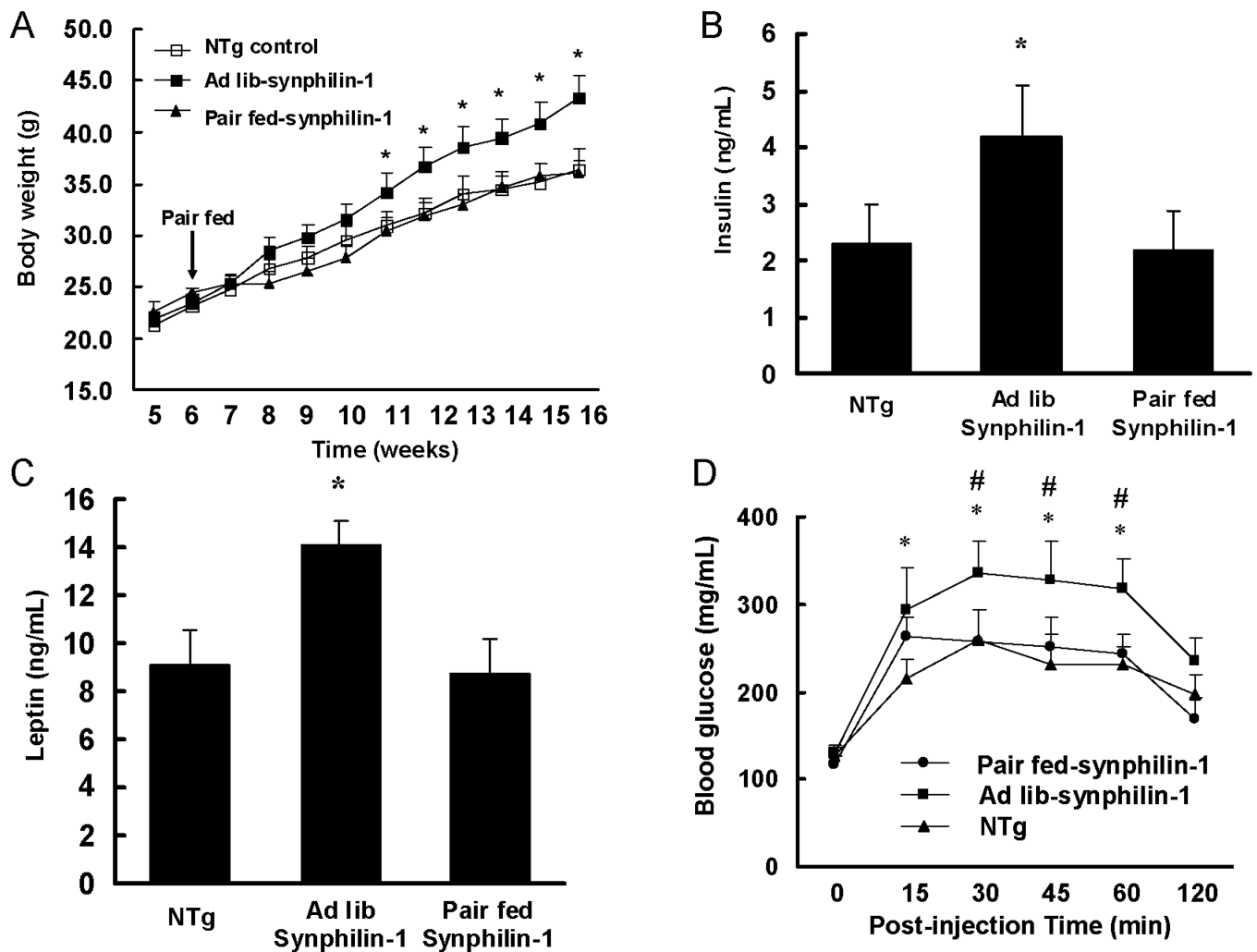


Fig. 5. Effect of pair feeding on body weight in SP1 mice

A. *Ad lib*-fed SP1 mice gained more weight than non-transgenic controls. Pair-feeding completely prevented the excess body weight gain of SP1 mice. **B and C,** Pair-feeding prevented synphilin-1-induced hyperinsulinemia and hyperleptinemia. *Ad lib*-fed SP1 mice were hyperinsulinemic (**B**) and hyperleptinemic (**C**). Pair-feeding resulted in normal insulin (**B**) and leptin (**C**) levels. **D.** Glucose tolerance test. Mice at the end of pair-feeding were injected IP with 1.5 mg/g glucose. Blood glucose was measured at indicated time points. All data are mean \pm SEM. There were 12 male mice in each experimental group. * $p < 0.05$ by ANOVA, significant differences between *ad lib* SP1 mice and non-transgenic controls; # $p < 0.05$ by ANOVA, significant differences between *ad lib* SP1 mice and pair-feeding SP1 mice.

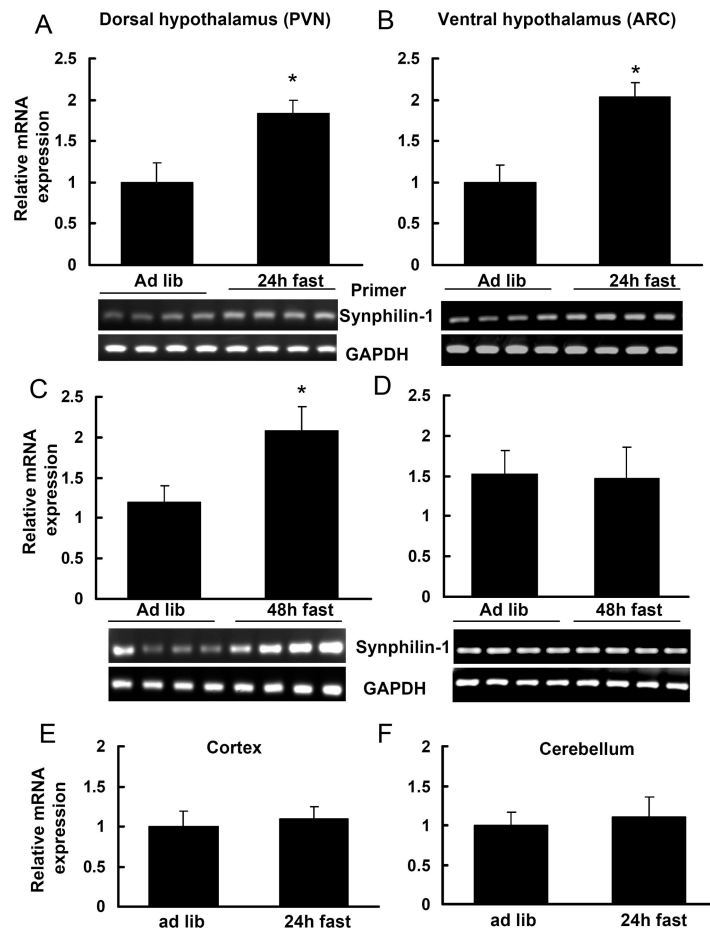


Fig. 6. Food deprivation increased endogenous synphilin-1 mRNA expression in dorsal hypothalamus

Cohorts of 4 month old normal (non-transgenic) mice (12 mice per group) were deprived of food for 24h or 48 h with water available *ad lib*. An additional group (12 mice) was maintained with *ad lib* food access. Mice were sacrificed at the end of 24 or 48 h fast. The hypothalamus was dissected into dorsal (**A and C**, containing PVN) and ventral hypothalamic blocks (**B and D**, containing ARC). The cortex and cerebellum from 24 h fast mice were dissected (**E and F**). Total RNA from each sample was extracted and subjected to real time RT-PCR to assess endogenous mouse synphilin-1 mRNA expression levels. The housekeeping gene GAPDH served as an internal control. Lower panel in A-D: the representative DNA gel of real time RT-PCR product. * $p < 0.05$, statistically significant differences between *ad lib* and fasted groups are indicated.