Obesity-related leptin regulates Alzheimer's Aβ

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ABSTRACT **Aβ peptide is the major proteinateous component of the amyloid plaques found in the brains of Alzheimer's disease (AD) patients and is regarded by many as the culprit of the disorder. It is well documented that brain lipids are intricately involved in Aβ-related pathogenic pathways. An important modulator of lipid homeostasis is the pluripotent peptide leptin. Here we demonstrate leptin's ability to modify Aβ levels in vitro and in vivo. Similar to methyl-β-cyclodextrin, leptin reduces β-secretase activity in neuronal cells possibly by altering the lipid composition of membrane lipid rafts. This phenotype contrasts treatments with cholesterol and etomoxir, an inhibitor of carnitine-palmitoyl transferase-1. Conversely, inhibitors of acetyl CoA carboxylase and fatty acid synthase mimicked leptin's action. Leptin was also able to increase apoE-dependent Aβ uptake in vitro. Thus, leptin can modulate bidirectional Aβ kinesis, reducing its levels extracellularly. Most strikingly, chronic administration of leptin to AD-transgenic animals reduced the brain Aβ load, underlying its therapeutic potential.—Fewlass, D. C., Noboa, K., Pi-Sunyer, F. X., Johnston, J. M., Yan, S. D., Tezapsidis, N. Obesity-related leptin regulates Alzheimer's Aβ. *FASEB J.* 18, 1870–1878 (2004)**

Key Words: leptin signaling · AD · Aβ production

Weight loss is frequently observed in Alzheimer's disease (AD) patients before the onset of dementia, supportive of an underlying metabolic syndrome (1, 2). Lipid homeostasis specifically, as exemplified in cell culture and animal models, in addition to clinical studies with statins, can affect amyloidogenic pathways (3). In this study we decided to examine whether leptin and leptin signaling pathways could be of any relevance. Leptin is a peptide hormone that controls adaptive metabolic mechanisms to energy availability, leading to storage or mobilization of fat (4). Adipocyte-derived leptin primarily exerts its central action through the arcuate nucleus neurons of the hypothalamus, but can affect other populations, including hippocampal neurons and cells of the periphery (5). Ablation of leptin or leptin signaling is sufficient to cause obesity as exemplified by ob/ob and db/db mice (and fa/fa rats), respectively, and in a few rare genetic cases (4). The leptin receptor (ObR) is widely expressed in the central nervous system (CNS) as multiple forms (6). The longest form, Ob-Rb, transduces the leptin signal by increasing Janus tyrosine kinase 2 (Jak2) activity, the signal transducer and activator 3 (STAT3), and the suppressor of cytokine signaling 3 (SOC3). SOC3 is a negative regulator of Jak2 and STAT3 (7). Overall, leptin as a hormone complements insulin or increases the target's sensitivity to insulin by facilitating lipolysis and inhibiting lipogenesis (4, 7). In the CNS these metabolic pathways serve mainly to provide the building blocks for membranes, vitamins, and second messengers and to modify proteins by acylation, as there are no main mechanisms for using triglycerides/fatty acids as energy source. Levels of cholesterol and fatty acids in cells are tightly regulated by a single family of transcription factors, named sterol regulatory element binding proteins (SREBPs), which activate relevant target genes (8). We investigated here whether cellular levels of SREBPs can influence leptin's actions.

The amount of extracellular accrual of Aβ is critical for the pathobiology of Alzheimer's disease and depends on the antagonizing rates of its production/secretion and its clearance. Based on our previous data (9), neurons depend on the interaction between presenilin 1 (PS1) and cytoplasmic linker protein 170 (CLIP-170) to generate Aβ and take it up through the lipoprotein receptor-related protein (LRP) pathway. Formation of Aβ also depends on the assembly of key proteins in lipid rafts (LRs) (10). These are membrane microdomains enriched in cholesterol, glycosphingolipids, and glucosylphosphatidyl-inositol-(GPI) -tagged proteins implicated in signal transduction, protein trafficking, and proteolysis. It is believed that within the LRs, Aβ's precursor, amyloid precursor protein (APP), is cleaved by protease β-secretase (BACE) to generate the intermediate fragment CAPPβ. The latter is subsequently processed by γ-secretase, a high molecular weight multiprotein complex containing PS1 fragments (11). Once outside, Aβ can *1)* exert a biological activity by binding and activating specific receptors [including receptor for advanced glycosylation end products

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doi: 10.1096/fj.04-2572com

 0892-6638/04/0018-1870 © FASEB

(RAGE) (12) and scavenger receptors (13)]; *2)* be removed by mechanisms of endocytosis (involving apoE and LRP or scavenger receptors); *3)* be degraded by extracellular proteases, including insulin-degrading enzyme and neprilysin (14). We specifically addressed the LRP-mediated apoE-dependent Aβ endocytosis.

Regulation of cellular levels and distribution of fatty acids and cholesterol could influence membrane fluidity, function, and localization of lipid rafts, a process that changes with aging (15). We speculated that the lipolytic actions of leptin could ramify the composition of the LRs, affecting Aβ turnover.

MATERIALS AND METHODS

Cell cultures, treatments, antibodies, and immunoassays

SY5Y cells (human neuroblastoma) were maintained in culture as described (16). Primary neural cultures were obtained from E16 rat embryonic cortex (17). These were grown either as mixed cultures (16) or under conditions that favor the isolation and proliferation of astrocytes (18).

Neuro2a (mouse neuroblastoma) stably transfected with hyg-sa134, a pcDNA3.1/Hygro plasmid (Invitrogen, San Diego, CA, USA) modified to express a fusion protein of secreted alkaline phosphatase (SEAP), and a fragment of APP consisting of the C-terminal 134 aa were maintained in culture as described (16) in the presence of 400 μg/mL of hygromycin. The SEAP-CAPP cDNA insert from hyg-sa134 was subcloned into an adenoviral vector using the Adeno Vator system (Qbiogene, Carlsbad, CA, USA).

SY5Y and hyg-sa134-Neuro2a cells were treated at 80% confluency (see below). Primary neural cultures from rat embryos were allowed to grow for 6–12 days after plating and before viral infection and treatments.

Water-soluble cholesterol (5 μg/mL or 10 μg/mL) was added to cultures for 2 or 5 h: Water-soluble cholesterol (Sigma-Aldrich, St. Louis, MO, USA) is a solution made of cholesterol balanced with methyl-β-cyclodextrin (40 mg cholesterol/g CDX). Treatment with an equivalent amount of the resin alone leading to depletion of cholesterol (10) was performed for comparison.

Leptin (100 or 400 ng/mL; Harbor-UCLA, Los Angeles, CA, USA) was added in cell culture medium for 2 or 5 h. Cells were ~80% confluent at the time of treatment. Peptide YY (3–36) (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) and CNTF (Sigma-Aldrich) were added at 25 or 150 μM for the same incubation periods. TOFA, etomoxir (Research Biochemicals International) and cerulenin (Sigma-Aldrich) were used as described below.

Cell lysates were used to detect full-length APP and its C-terminal fragments generated by β-and α-secretase (10 kDa, 8 kDa, respectively) (16). This was performed by ^{35}S-[Met]/^{35}S-[Cys] metabolic labeling/immunoprecipitations or Western blots using a rabbit polyclonal antibody directed against the last 20 C-terminal amino acids of APP (16, 19). To determine Aβ, we used several methodologies. SY5Y cells in culture were metabolically labeled with ^{35}S-[Met] as described (19), followed by immunoprecipitation and densitometric analysis of the autoradiogram. Neuro2a cells were stably transfected with hyg-sa134 (K. Sambamurti, South Carolina Medical Center, Charleston, SC, USA) and Aβ40 plus Aβ42 (total Aβ) in the medium was quantified by sandwich ELISAs developed with 4G8 and 6E10 monoclonal antibodies (Signet, Dedham, MA, USA) (19). For the separate determination of Aβ40 and Aβ42/43 in the formic acid extracts of mice brains, we used commercially available ELISA kits (KMI Diagnostics, Minneapolis, MN, USA). Flotillin was detected using monoclonal anti-flotillin-1 antibodies (BD Biosciences, Palo Alto, CA, USA). Actin was detected using monoclonal anti-actin antibodies (Research Diagnostics, Inc., Flanders, NJ, USA).

For leptin, a rabbit polyclonal antibody was used (obtained from Dr. A. F. Parlow, Harbor-UCLA). Immunofluorescent confocal microscopy was performed on 2% paraformaldehyde fixed primary neural cells. Filipin staining was performed as described (20).

Preparation of ApoE and binding with ^{125}I-Aβ

ApoE was isolated from the conditioned media of HEK-293 cells stably transfected with human apoE (ε3 or ε4) cDNA (9). These preparations are usually poor in lipid, but are fully functional for uptake experiments (21). ApoE was then preincubated with ^{125}I-Aβ overnight at 37°C (Aβ/ApoE: 1/50 w/w) as described (9).

Aβ uptake by SY5Y cells

Human ^{125}I-Aβ (iodinated at Tyr-10, Amersham Biosciences, Arlington Heights, IL, USA; IM 294) uptake was measured after addition of 0.1 nM ^{125}I-Aβ to confluent SY5Y cells (60,000 cpm/mL) in the presence or absence of 100 or 400 ng/mL leptin in a 24 h preincubation period. ^{125}I-Aβ was either added alone or previously incubated with apoE3. In controls, receptor-associated protein (RAP, 1 μM) was added together with Aβ or the Aβ/apoE complex. RAP is an antagonist of lipoprotein receptors (22). After 24 h the media were collected and subjected to scintillation counting for γ-radiation (23). The amount of radioactivity was measured in trichloroacetic acid (10%) TCA pellets (representing intact Aβ) and the corresponding supernatants (representing degraded Aβ). 96.5 ± 8.2% (mean±SE, n=4 experiments, triplicate determinations) of the radioactivity found in the medium could be recovered in the TCA pellet and represented intact or oligomeric Aβ (not shown), when Aβ was preincubated with apoE. However, only 31.2 ± 5.8% (n=4) of the radioactivity was recovered in the TCA pellet in the absence of apoE, suggesting that Aβ was degraded under those conditions, consistent with reports by others. This has been suggested to be due to the activity of insulin-degrading enzyme (14). Indeed, inclusion of 1,10 phenanthroline during the uptake, abolished Aβ degradation.

We further verified that radioactivity was reduced in the media as a reflection of Aβ uptake by the cells rather than nonspecific binding to the extracellular surface of membranes or oligomerization/aggregation of Aβ. This was performed by comparing the amount of TCA precipitable radioactivity in the soluble fraction of cell lysates compared with that in the total lysates, the ratio of which was typically 0.8–0.9 (not shown).

Measurement of protein

Proteins were extracted from cells by treatment with 0.1% Igepal and brief sonication. Protein content was determined by the Bradford method (24).

SREBP cDNAs

Human SREBP-1 and SREBP-2 cDNAs were obtained by PCR from a human brain expression library using Bam*HI* and

EcoRI sites containing linker primers to allow ligation and subcloning into pcDNA3.1 vectors.

Leptin studies in mice

For our initial study, one-year-old transgenic animals with the following genotypes were used: *1)* $APP_{swe}/PS1_{M146V}$ (double transgenic (25), *2)* $PS1_{M146V}$ (26), and *3)* wild-type C57B1/6 × SJL.

Blood was withdrawn from deeply anesthetized animals (~1 mL) by cardiocentesis and mixed with 25 μL of 164 μM EDTA anticoagulant. Plasma was prepared immediately and frozen at –70C°. Plasma leptin concentrations were determined by a RIA (27) using a kit from LINCO Research, Inc. (St. Charles, MO, USA).

This was followed by this strategy: APP_{swe} expressing mice (Tg2576) or wild-type littermates were maintained in pathogen-free environment at 25°C on a 12–12 h light-dark cycle. Mice were killed between the ages of 31 and 40 wk. They were provided ad libidum access for up to 9 wk (1 wk before leptin treatments and 8 wk during) to a high-fat diet (D12451) containing 45% of the total calories from fat (Research Diets, New Brunswick, NJ, USA) or a low-fat diet (D12450B) containing 10% of the total calories from fat. An equal number of male and female Tg2576 mice under each diet from the age of 32 wk were administered leptin or placebo (PBS) for up to 40 wk. Mice were anesthetized with intraperitoneal injection of ketamin (55 mg/mL) and xylazine (7–10 mg/mL), then surgically fitted with an Alzet miniosmotic pump (model 2004, Durect Corp., Cupertino, CA, USA) placed subcutaneously. Local subcutaneous injection of 0.5 mL of 0.5% lidocaine ensured postoperative relief. Half received daily 20 μg leptin in PBS (0.25 μL/h of 3.33 mg/mL recombinant murine leptin); the other half were infused with PBS. Four from each group (two males and two females) were killed after 4 wk treatment. Osmotic pumps were replaced in the rest and treated for a total of 8 wk. Wild-type littermates were also treated with leptin under high- or low-fat diet regimens.

The animal protocol was reviewed and approved by the IACUC at Columbia University Medical Center.

Statistical analysis

All values are the mean ± SE of each group. Variations between pairs of groups was evaluated with *t* test and differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

We first investigated the effects of leptin on Aβ production in vitro. Human (SY5Y) or mouse neuroblastoma cell lines (Neuro2a), the latter stably transfected with hyg-sa134 (a plasmid driving expression of a recombinant fusion protein containing the human C-terminal fragment of APP of 134 amino acids, $CAPP_{134}$), were treated for 2 or 5 h with 100 or 400 ng/mL leptin (**Fig. 1*a, b***). Primary neurons from embryonic rat brain infected with an adenovirus to direct the expression of $CAPP_{134}$ were tested. In transfected Neuro2a cells, leptin caused a dose- and time-dependent decrease in the levels of Aβ detected in the media (56±5% after 5 h with 400 ng/mL, Fig. 1*b*). Leptin was almost as efficient as CDX in lowering Aβ (Fig. 1*a, b*). In agreement with published data (28), inclusion of water-soluble cholesterol in the culture media increased Aβ production (205±6% after 5 h with 10 μM, Fig. 1*b*). Leptin was able to partially revert the amyloidogenic potency of cholesterol when coadministered (150±4% after 5 h with the highest concentrations, Fig. 1*b*). None of these treatments caused significant differences in the degradation of Aβ in the medium as assessed by measuring the % of ^{125}I-Aβ converted to TCA soluble radioactivity when included in the media, during treatments in the presence or absence of 1 mM 1,10-phenanthroline (see "Aβ uptake by SY5Y cells"). This is a general metalloprotease inhibitor that effectively inhibits degradation of secreted Aβ in vitro (29). The inhibitor treatment did not cause a significant difference in the tracer's uptake by the cells either (see below).

To investigate whether these changes in Aβ production were concomitant with fluctuations in β-secretase activity, we used two approaches. First, we treated our cultures in the presence of the γ-secretase inhibitors L-685,458 or Z-VL-CHO to allow accumulation of 10 kDa CAPPβ (C99) and 8 kDa CAPPα (C83), the C-terminal fragments of APP generated by β- and α-secretase, respectively. Under these conditions, 5 h treatment with 10 μM cholesterol caused an increase in C99 but not C83 (Fig. 1*c*, lanes 2, 4), consistent with an increase in β-secretase activity. This increase was abolished in the presence of 400 ng/mL leptin (Fig. 1*c*, lane 3, 4). APP levels detected by Western blot were unchanged; ^{35}S-Met metabolic labeling confirmed that APP synthesis was not affected (Fig. 1*c*, bottom lanes 5–8), nor was proliferation as detected by actin Western blots (Fig. 1*c*, top lanes 5–8). Leptin's effect on C99 levels through possible inhibition of β-secretase was observed in the absence of cholesterol (Fig. 1*c*, lanes 1, 2).

Second, we measured BACE activity using a fluorescence quenching assay (QTL Biosystems, NM, USA) in fractionated cell extracts (Fig. 1*e*). LRs were prepared from a Triton X-100-insoluble membrane fraction further resolved onto a sucrose gradient (30). In agreement with others (30), BACE activity in extracts from control cells was detected in a low density fraction containing flotillin (Fig. 1*d*), a marker for neuronal LRs (31). The bulk of BACE activity was detected outside LRs at higher density fractions. In addition, distribution of APP immunoreactivity was similar to that of BACE activity in gradient fractions. Only a small fraction comigrated with the flotillin peak (Fig. 1*d*). Leptin treatment resulted in a subtle change of the composition and/or density of LRs, as determined by the distribution of BACE activity, APP, and flotillin on sucrose gradient fractions. Flotillin migrated at heavier subcellular fractions compared with controls and the activity of BACE in the low density fractions was almost absent. A similar shift in the elution position for flotillin and BACE was observed when cells were treated with CDX (not shown). Our data are consistent with the notion that a prerequisite for BACE to generate Aβ from APP is its association within LRs and that the disruption of the lipid composition of those structures

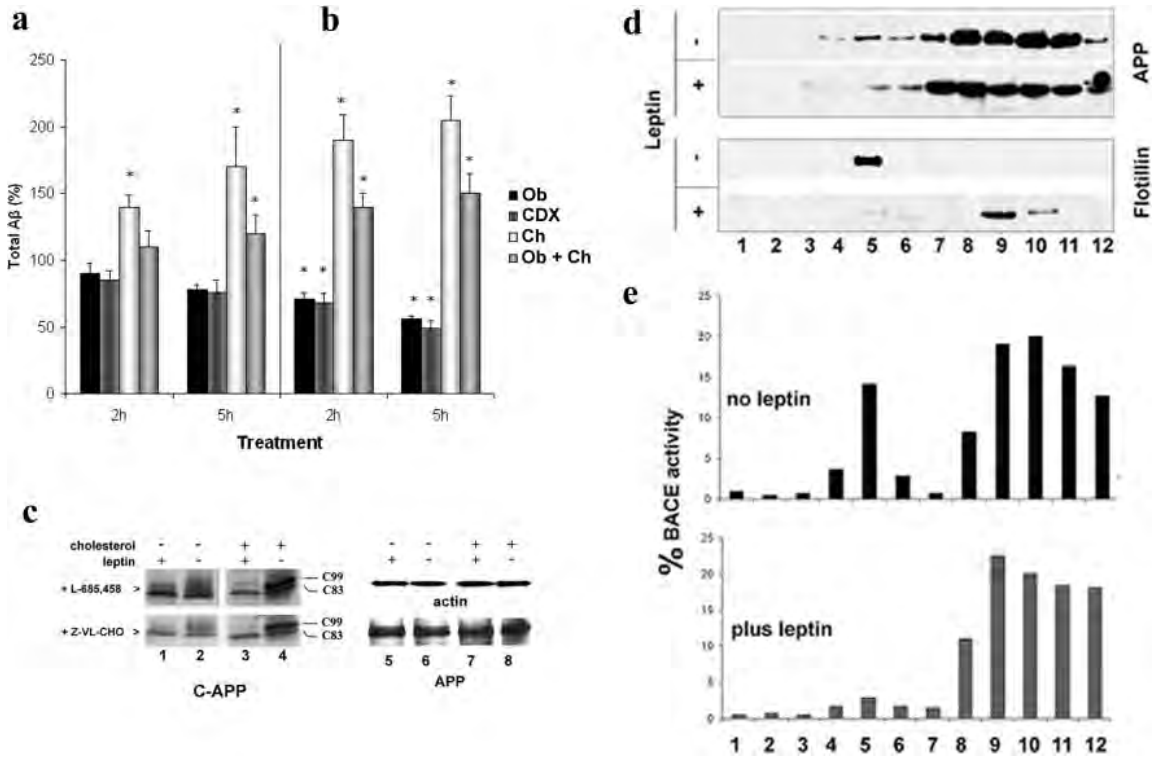

Figure 1. Leptin affects Aβ production through BACE in rafts. *a)* Neuro2a cells stably transfected with hyg-sa134 were treated for 2 or 5 h with 100 ng/mL leptin, Ob (black); 125 μg/mL cyclodextrin, CDX (gray stripe); 5 μg/mL cholesterol, Ch (pale gray); leptin plus cholesterol, Ob+Ch (medium gray). Media were collected and assayed for total Aβ by ELISAs (19). Results are expressed as a % of the corresponding controls that did not receive drug treatment measured at 2 and 5 h, respectively. Water soluble cholesterol (Sigma-Aldrich) is a solution made of cholesterol balanced with CDX (40 mg cholesterol/g CDX). *b)* Same as panel *a* except 400 ng/mL leptin, Ob (black); 250 μg/mL cyclodextrin, CDX (gray stripe), 10 μg/mL cholesterol, Ch (pale gray) and leptin plus cholesterol, Ob+Ch (medium gray) were used. *c)* SY5Y cells in culture were treated with 400 ng/mL leptin or 10 μg/mL cholesterol or in the presence of the γ-secretase inhibitors L-685,458 or Z-VL-CHO for 5 h. Extracts prepared from harvested cells were analyzed by SDS-PAGE and Western blot using an antibody directed against the C-terminal fraction of APP (C-APP, lanes 1–4) or actin (top lanes 5–8) or full-length APP (bottom lanes 5–8). Immunoreactive bands C99 and C83 correspond to β- and γ-secretase-generated fragments. *d)* Extracts from SY5Y cells treated with and without leptin as above were solubilized in the presence of Triton X-100 and the insoluble fraction was applied to a sucrose gradient as described (30). Fractions collected from the bottom of the gradient were analyzed by SDS-PAGE and Western blot for the detection of APP and flotilin (marker for lipid rafts). A shift of the flotilin peak to more dense fractions of the gradient is observed after leptin treatment. *e)* Fractions collected as above were assayed for β-secretase activity using a fluorescence-quenching assay (QTL Biosystems, NM). The results are expressed as the % distribution of BACE activity within the gradient derived from cell cultures in the absence (black) or presence (gray) of leptin in the medium. *Value significantly different from that of the corresponding control (set at $P<0.05$).

by leptin is sufficient to block the activity, presumably by hindering its encounter with the substrate. Consistent with its ability to modulate the lipid composition of membranes, leptin treatment of primary neurons (**Fig. 2*a–d***) and astrocytes (Fig. 2*e–h*) diminished the filipin labeling (Fig. 2*d, h*). Filipin is a fluorescent polyene antibiotic that binds to plasma membrane cholesterol (20). The presence of leptin in cultures prohibited an increase in filipin labeling by cholesterol (Fig. 2*b, f*) in both cell types (Fig. 2*c, g*).

Leptin's ability to lower the production of Aβ was mimicked by *1)* TOFA (32) [a long chain fatty acid analog, 5-(tetradecyloxy)-2-furancarboxylic acid], an inhibitor of acetyl CoA carboxylase, and *2)* cerulenin, a fatty acid synthase inhibitor (33, 34). In contrast, etomoxir (35) increased Aβ production (**Table 1**). This is consistent with an association between leptin's prolipolytic/antilipogenic properties and APP metabolism. Similar results were obtained with SY5Y cells and adenovirus vector-infected primary neurons derived from embryonic rat brains (Table 1).

Thus, these findings confirm that metabolic pathways involving neuronal lipids and their distribution in membrane compartments influence Aβ production and establish that these can be controlled partially by exogenous leptin. As Aβ and lipid homeostasis are the result of their production and clearance/uptake, we investigated the effect of leptin on the uptake of extracellular Aβ by SY5Y cells in culture. We and others have demonstrated that this process is facilitated by apoE, which binds to Aβ and directs its capture via the low density LRP and subsequent endocytosis/degradation of the protein-lipid complex by endosomes/lysosomes (only LRP is recycled). This may be the primary mechanism by which neurons absorb lipids from circulating HDL-like lipoproteins from the brain interstitial

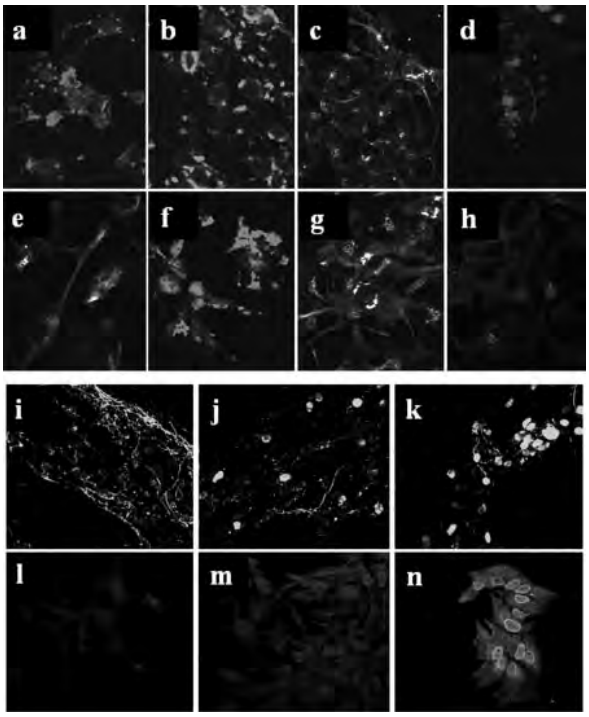

Figure 2. Leptin can modulate free cholesterol-rich membrane domains and surplus cholesterol may trigger leptin production. Neural cultures from E15 rat cerebral cortex were processed for enrichment in neurons (*a–d*) or astrocytes (*e–h*) as described (51). After 7–10 days in culture, cultures were treated for 5 h with 10 μg/mL cholesterol (*b, f*) or 400 ng/mL leptin plus cholesterol (*c, g*) or leptin alone (*d, h*). Controls (*a, e*) were treated with media alone. Filipin staining was performed as described (20). Neurons (*i–k*) and astrocytes (**l-*n***) prepared as above were treated with 0 μM (**i, l**), 5 μM (*j, m*) or 10 μM cholesterol (*k, **n***) for 5 h. Immunostaining was performed for leptin (A. F. Parlow, Harbor-UCLA, CA).

space (36). For the purpose of our experiments, however, lipid-poor apoE was used (37) (**Fig. 3*a***). Leptin increased in a dose-dependent fashion uptake of apoE-Aβ (Fig. 3*a*, striped and white bars for apoE3 and apoE4, respectively). The ε3 allele of apoE was more efficient than ε4 in delivering Aβ to the cell. This indicates that the apoE isoform associated with increased risk for AD may be more resistant to the beneficial action of leptin in promoting lipid delivery to neurons and degradation of Aβ. Next, we preloaded SY5Y cells with cholesterol, introducing a preincubation step with cholesterol/CDX and compared it to controls preincubated with medium. Only 22 ± 6% of apoE3-Aβ was taken up by cholesterol-loaded SY5Y cells compared with controls (Fig. 3*b*, black bars, first two pairs). Addition of 400 ng/mL leptin during the cholesterol preincubation period and during the uptake almost completely reverted the phenotype to that of controls (Fig. 3*b*, striped bars with leptin, black bars without leptin). These results suggest that leptin increases the capacity of neurons to take up apoE-Aβ (and presumably lipids), which may be of paramount importance under conditions of remodeling and/or repair. LRP-mediated apoE-lipoprotein internalization is arbitrated through clathrin-coated pits, suggesting that Aβ uptake may not involve membrane microdomains. However, there is increased awareness that LRs and clathrin-coated pits may not be exclusive concepts.

For insight into the specificity of leptin's ability to modulate Aβ production, peptide YY (3–36, 38) and ciliary neurotrophic factor (CNTF) (39), peptides with a physiological profile similar to leptin, were tested on cells for 5 h treatments. At equimolar concentrations (25 μM), neither peptide caused a statistically significant change (Table 1), and this was the case at higher (150 μM) concentrations (not shown). This was particularly surprising for CNTF, which has a mode of action similar to leptin at the receptor level and postreceptor binding signaling events (39).

Three SREBP isoforms, SREBP-1a, -1c, and -2 are known. The first two are transcribed from the same gene under different promoters. SREBP-2 is more selective in activating the transcription of cholesterol biosynthetic genes whereas SREBP-1 preferentially regulates fatty acid synthesis, but there is considerable overlap. SREBP-1c mRNA and protein were shown to be increased in the ob/ob mouse (40), suggesting that leptin could regulate

TABLE 1. *The effect of metabolic regulators on Aβ production from transfected Neuro2a cells, SY5Y cells or primary embryonic rat neurons infected with adenovirus*[a]

Inhibitor or agent	Target or action	Neuro2a/SEAP-APP Aβ (% control)	SY5Y Aβ (% control)	Neurons/SEAPP-APP Aβ (% control)
TOFA, 200 μM	ACC	40 ± 15	58 ± 12	35 ± 4
Cerulenin, 200 μM	FAS	52 ± 12	65 ± 9, NS	66 ± 5
Etomoxir, 40 μM	CPT-1	154 ± 14	142 ± 14	158 ± 14
Peptide YY (3-36), 25 μM	Anti-obesity	92 ± 9, NS	96 ± 7, NS	98 ± 5, NS
Ciliary neurotrophic factor, 25 μM	Anti-obesity, neurotrophin	95 ± 4, NS	96 ± 8, NS	89 ± 12, NS
Leptin, 400 ng/mL	Anti-obesity, energy balance, immunomodulation	56 ± 5	38 ± 7	35 ± 4

[a] Results are expressed: as mean ± SE from 4 experiments, each with 3 determinations. Values are expressed as a percentage of total Aβ found in the conditioned media of cells not receiving treatment. In 5 h SY5Y cells produced 252 ± 50 pM, Neuro2a-SEAP-APP produced 820 ± 210 pM, and Neurons/SEAPP-APP produced 131 ± 83 pM. Student's *t* test was used and statistical significance was set at $P \leq 0.05$. NS: statistically nonsignificant; TOFA: 5-(tetradecyloxy)-2-furancarboxylic acid; ACC: acetyl CoA carboxylase; FAS: fatty acid synthase; CPT-1: carnitine palmitoyl transferase-1.

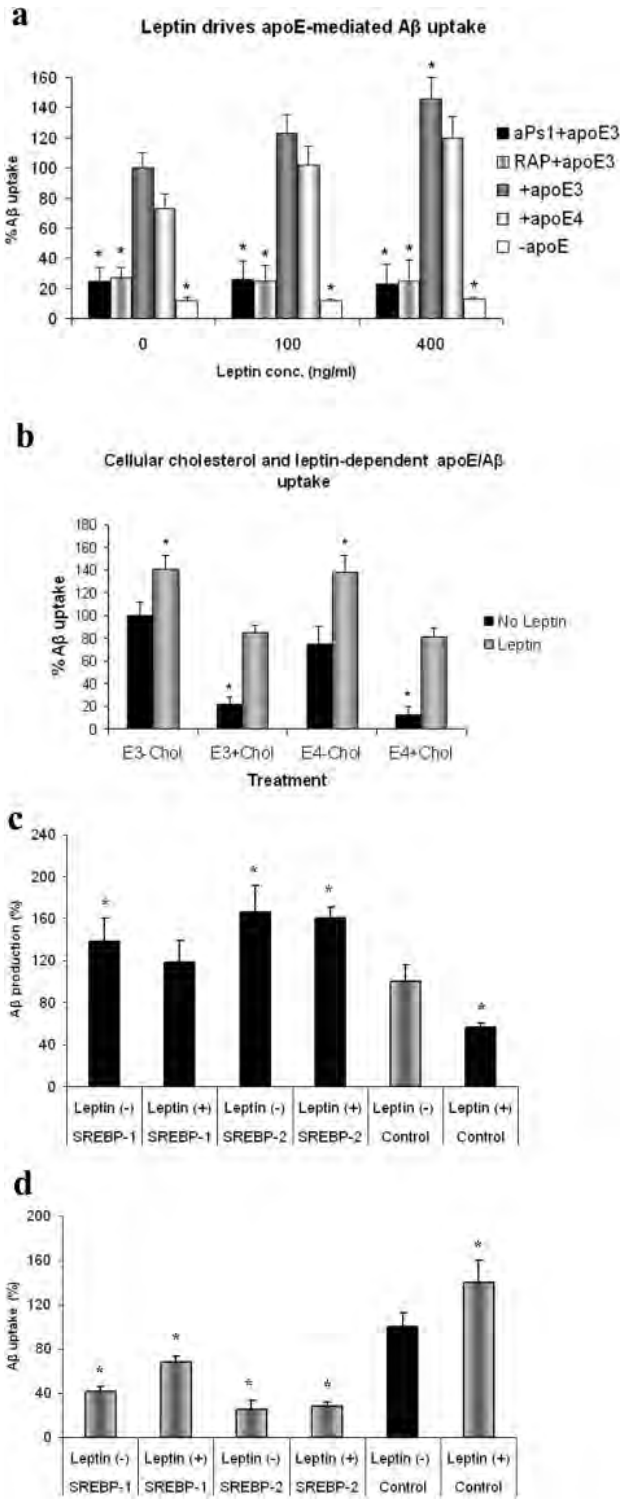

Figure 3. Leptin affects apoE-dependent Aβ uptake and this possibly involves SREBPs. *a)* Aβ uptake was measured in SY5Y cells after their treatment at 0, 100, or 400 ng/mL leptin. Uptake was performed in cells transfected with antisense DNA for PS1 as described (9) (black). Uptake is expressed as % of that observed with Aβ preincubated with apoE3 (medium gray) in the absence of leptin (first set of columns). Inclusion of RAP abolished uptake (gray stripe) and so did omission of apoE (white). Leptin induced a dose-dependent increase in Aβ uptake with a preference for apoE3 (medium gray) over apoE4 (light gray). *b)* SY5Y cells were pretreated with 10 μg/mL cholesterol (+Chol) or normal medium (–Chol). Aβ uptake was measured after its preincubation with apoE3 (E3) or apoE4 (E4) in the absence (black) or presence (gray) of 400 ng/mL leptin. Cells were more resistant in taking-up Aβ when loaded with cholesterol. *c)* SY5Y cells were transiently transfected with SREBP-1 or SREBP-2 cDNA or empty vector (control). Then Aβ was measured in the medium by ELISAs (19) after treatment with (+) or without (–) leptin. Results are expressed as % of the Aβ produced in cells transfected with empty vector that did not receive leptin treatment, set at 100% (gray bar). *d)* Aβ uptake was measured in SY5Y cells prepared as in panel *c*. Uptake was performed using Aβ/apoE3 complexes. Results are expressed as % of the Aβ taken up by cells transfected with empty vector that did not receive leptin treatment, set at 100% (black bar). *Value significantly different from that set as 100% (set at $P<0.05$).

SREBP-1c levels. To test this, we transfected SY5Y cells with modified pcDNA3.1 vectors to drive the expression of SREBP-1 or SREBP-2 under the CMV promoter and repeated some of the experiments of Aβ production or uptake in the presence or absence of leptin. SREBP-2 transfected cells were more resistant to the inhibition of Aβ production by leptin than SREBP-1 transfected cells (Fig. 3*c*). SREBP-2 cells were resistant to the increase of apoE/Aβ uptake by leptin (Fig. 3*d*). Transient expression of SREBP-1 increased the production of Aβ to 138 ± 22% compared with controls (Fig. 3*c*) and reduced the uptake of apoE/Aβ to 41 ± 5% (Fig. 3*d*). SREBP-2 expression increased production of Aβ to 166 ± 25% and inhibited uptake of apoE/Aβ to 25 ± 8%. At least two different scenarios could explain these results: *1)* leptin limits the availability of a common precursor for fatty acids and cholesterol (i.e., acetyl-CoA) or *2)* postleptin receptor signaling events somehow turn-off SREBP-1, causing a reduction in cholesterol that is important for Aβ turnover. The minor changes observed in SREBP-1 transfected cells in the presence of leptin support the second possibility; however, both may be working in cohort.

In agreement with previous reports (41), we detected leptin by immunocytochemistry (Fig. 2*i–n*) and Western blot (not shown) in dispersed neural cultures prepared from rat embryonic brain. We detected the leptin receptor (not shown) (6). Cholesterol treatment enhanced in a dose-dependent fashion levels of leptin-like immunoreactivity in both neurons (Fig. 2*i–k*) and astrocytes (Fig. 2*l–n*). We speculate that leptin serves as a local feedback signal to inhibit further cholesterol synthesis and uptake, which in turn affects Aβ production and uptake. Consequently, deficiencies in leptin or transduction of its signal in neural cells, could be contributory to AD-related pathways. Within the CNS, glia are the cell group prominently synthesizing apoE, cholesterol, and phospholipids-rich HDL-like lipoprotein particles (42). Lipids are required by neurons during plasticity-related neuritic arborization/outgrowth or during neural progenitor cell proliferation. Nonetheless, excess cholesterol and Aβ can be harmful. Thus, bidirectional communication between neurons and glia, based on local leptin (rather than leptin derived from the circulation) and leptin signaling pathways, may serve to balance local lipid requirement.

It has been demonstrated that leptin can modulate hippocampal excitability (5), supporting a link between endocrine factors and AD.

We subsequently measured plasma leptin levels (**Fig. 4*a***) in 12-month-old transgenic mice engineered to express either or both mutants linked to familial AD: APP with the Swedish mutation (APP_{Swe}) and PS1 with the M146V substitution ($PS1_{M146V}$). We determined that in both males and females, circulating leptin levels were approximately half those in littermates not expressing the APP_{Swe} regardless of the expression of PS1mut (Fig. 4*a*).

Based on leptin's antiamyloidogenic activity in vitro described above and the apparent leptin deficiency in the APP_{Swe}-expressing mice, we investigated the effect of chronic peripheral administration of leptin to ani-

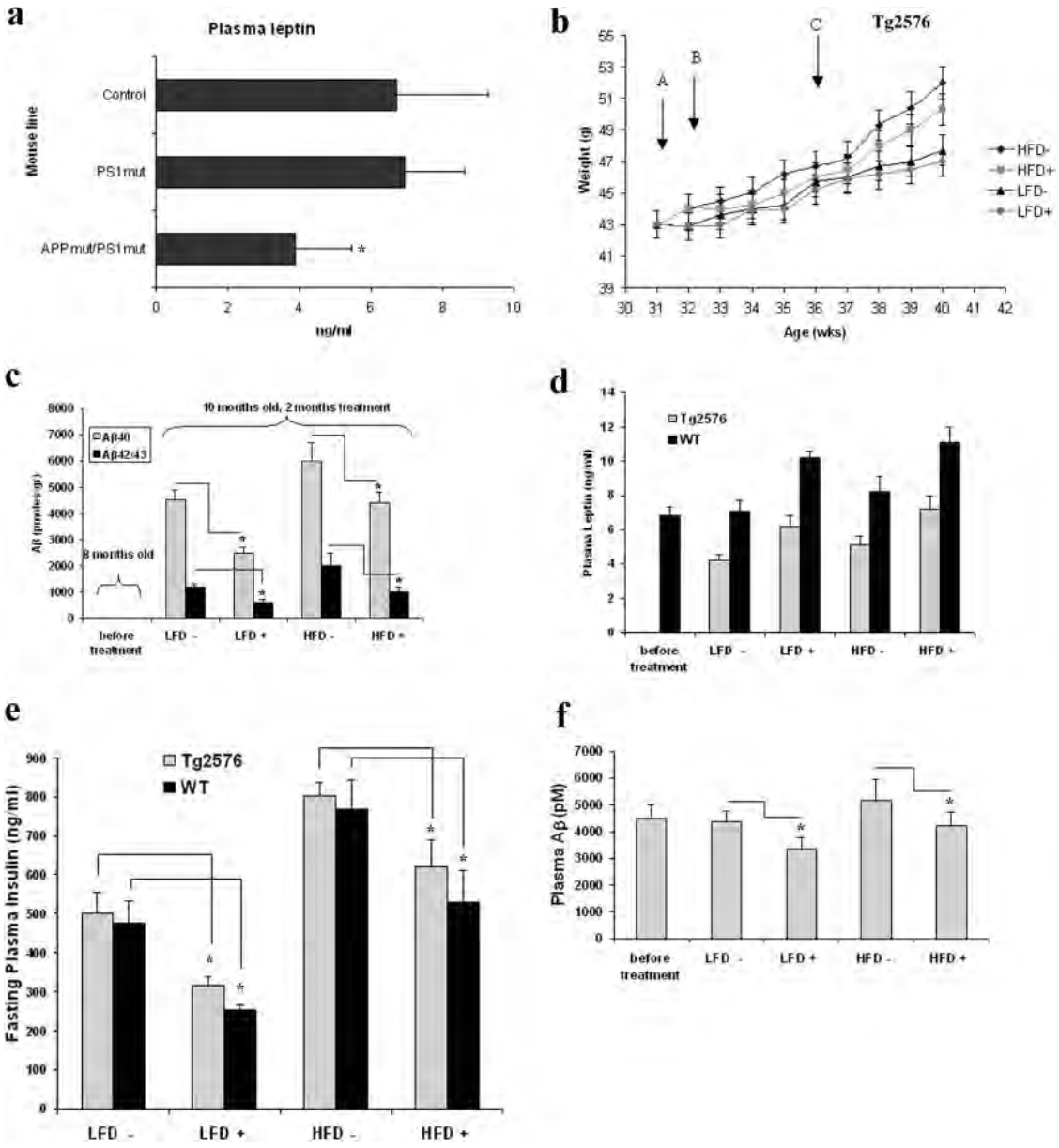

Figure 4. Deficiency of leptin in AD transgenic mice and the effect of leptin supplementation on amyloid load. *a)* Plasma leptin was quantified in 1-year-old mice with the following genotypes: *1)* double mutant $APP_{Swe}/PS1_{M146V}$, *2)* single mutant $PS1_{M146V}$, and *3)* wild-type (a cross between C57BL/6Ntac and B6SJLF1). *Value significantly different from that of nontransgenic controls (set at $P<0.05$). *b)* Tg2576 mice under high (HFD) and low (LFD) diets were weighed every week from 1 wk before (*A*) implantation of the Alzet pump s.c (*B*) for constant delivery of leptin (+) or vehicle PBS (–). Pump was replaced after 4 wk (C). *c)* Formic acid extracts of brains obtained as described (43) were used to determine the Aβ40 and Aβ42 content by commercially available ELISA kits (KMI Diagnostics), as described by the manufacturer. Only APP_{Swe}-expressing mice (Tg2576) contained detectable amounts of Aβ species. At 8 months of age the Tg2576 mouse has very low levels of Aβ. *d)* Plasma leptin was determined by RIA (LINCO Research) in 10-month-old Tg2576 and WT littermate mice after treatments as described in panel *b*. Leptin was measured in WT but not Tg2576 mice before treatment. *e)* Plasma insulin was determined by RIA (LINCO Research) in 10-month-old WT and Tg2576 mice after a 2 month LFD or HFD with (+) or without (–) leptin infusion. *f)* Plasma total Aβ (Aβ40 plus Aβ42/43) was measured in 10 month Tg2576 mice after a 2 month LFD or HFD with (+) or without (–) leptin infusion. Plasma Aβ was measured in these mice before treatment.

mals under a high- or low-fat diet (Fig. 4*b–f*). Constant subcutaneous infusion of leptin (or PBS as placebo) for up to 8 wk was administered to Tg2576 or wild-type littermate mice from ~8 months of age under the two different dietary regimens (described in Materials and Methods). Brain Aβ levels of the APP_{Swe} hemizygous mouse rise between 6 and 9 months and lead to the appearance of the first thioflavin S positive amyloid plaques in the hippocampus and cerebral cortex ~2 months later. Tg2576 mice under the high-fat diet had higher levels of Aβ40 and Aβ42 in formic acid extracts of brain homogenates than those under the low-fat diet (Fig. 4*c*), in agreement with others (28). Neuropathological examination was not performed because amyloid deposits of cored or diffuse plaques in the 10-month-old Tg2576 brains are too few (43) to allow statistically significant correlative studies. We measured plasma leptin and insulin levels. Leptin was confirmed to be lower in APP_{Swe}-expressing mice at 10 months than controls irrespective of diet and weight (Fig. 4*b, d*). In contrast, fasting insulin levels in mice of both genotypes fluctuated and were elevated by a high-fat diet and lowered by a low-fat diet. Leptin treatment decreased fasting insulin levels in all groups, consistent with its ability to increase insulin sensitivity (Fig. 4*e*). Finally, quantification of total Aβ in the plasma (Fig. 4*f*) of the Tg2576 mouse revealed that leptin treatment was able to lower the levels of circulating Aβ under both diets. Again, it is not known whether this reflects the lowering of the CNS amyloid load shown in Fig. 4*c* or is due to changes in peripheral Aβ production.

As the APP_{Swe} transgene in the Tg2576 mouse is under the control of the Prion-protein promoter (44), allowing its expression in the CNS and periphery (45, 46), and leptin is primarily produced in adipocytes, we decided to examine the adipose tissue extracted from these mice under high- or low-fat diets plus or minus leptin treatment, as described (47). We detected higher levels of APP expression in the adipocytes derived from the transgenic compared with wild-type animals, with no apparent influence by the leptin treatment (not shown). Transgenic adipocytes were less responsive to insulin-induced expression of leptin and glucose uptake (not shown). This was similar to the changes associated with senescence that develop with normal aging in adipocytes (48).

CONCLUSIONS

Whether early leptin administration to these mice affects CNS amyloid deposition, synaptic function, and behavioral profile remains to be established. Studies are needed to determine whether a low-fat diet in combination with leptin supplementation could be a potential palliative treatment for certain AD cases, perhaps those with cardiovascular or diabetic complications or individuals carrying the apoE4 allele. Further adjustment of dosing and direct delivery to the CNS may provide additional useful information regarding the action of the peptide.

The association between leptin/leptin signaling and AD-like pathobiology reported here in a mouse model perhaps is complementary to or works in parallel with pathways involving insulin, as reviewed recently (49). Plasma leptin levels decrease with aging in a more profound manner in postmenopausal women (50) and leptin receptors are present throughout the brain, including the hippocampus and olfactory bulb, domains affected early in the course of the disease. Even though there is no comprehensive clinical data to establish any relationship between leptin and incidence and/or severity of dementia in AD, our underlying hypothesis is that dysregulation of pathways associated with leptin may have a critical role. FJ

We would like to thank Dr. Kumar Sambamurti (Medical University of South Carolina) for the SEAP-CAPP cDNA plasmid and Dr. Karen Duff (Nathan Kline Institute/New York University) for the plasma samples in Fig. 4*a*. Funding was provided by the Alzheimer's Association.

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Received for publication June 16, 2004.
Accepted for publication August 25, 2004.