Replacing SNAP-25b with SNAP-25a expression results in metabolic disease

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Synaptosomal-associated protein of 25 kDa (SNAP-25) is a key molecule in the soluble *N*-ethylmaleimide–sensitive factor attachment protein (SNARE) complex mediating fast Ca^{2+}-triggered release of hormones and neurotransmitters, and both splice variants, SNAP-25a and SNAP-25b, can participate in this process. Here we explore the hypothesis that minor alterations in the machinery mediating regulated membrane fusion can increase the susceptibility for metabolic disease and precede obesity and type 2 diabetes. Thus, we used a mouse mutant engineered to express normal levels of SNAP-25 but only SNAP-25a. These SNAP-25b–deficient mice were exposed to either a control or a high-fat/high-sucrose diet. Monitoring of food intake, body weight, hypothalamic function, and lipid and glucose homeostases showed that SNAP-25b–deficient mice fed with control diet developed hyperglycemia, liver steatosis, and adipocyte hypertrophy, conditions dramatically exacerbated when combined with the high-fat/high-sucrose diet. Thus, modified SNARE function regulating stimulus-dependent exocytosis can increase the vulnerability to and even provoke metabolic disease. When combined with a high-fat/high-sucrose diet, this vulnerability resulted in diabesity. Our SNAP-25b–deficient mouse may represent a diabesity model.

SNARE | insulin secretion | glucose metabolism | hypothalamus | regulated exocytosis

On-going lifestyle changes, including oversized meals with excessive amounts of sugar and fat, have led to a worldwide pandemic of obesity and type 2 diabetes (T2D) (1). These diseases and their comorbidities cause individual suffering and represent a heavy financial burden on society (2, 3). The term "diabesity" is used to define the coincidence of obesity with T2D under conditions of exaggerated intake of energy-dense diets (4–6). Moreover, genomewide association studies (GWAS) have identified polymorphisms associated with obesity and T2D, indicating that genetic factors predispose certain individuals to diabesity (7–11).

Signs of metabolic diseases include impaired regulated release of hormones, particularly insulin as in T2D (4, 12). Likewise, the secretion of inflammatory markers and other peripheral bioactive peptides is either increased (e.g., leptin, resistin, and adipsin) or decreased (e.g., ghrelin and adiponectin) (13–16). Synaptic and nonsynaptic transmission involving the release of neuropeptides and neurotransmitters, especially in hypothalamic areas controlling eating behavior and energy balance, are involved too (17–19).

In excitable cells, the release of messenger molecules is a consequence of regulated membrane fusion and involves soluble *N*-ethylmaleimide–sensitive factor attachment proteins (SNAREs) (20, 21), including synaptosomal-associated protein of 25 kDa (SNAP-25). SNAP-25 is expressed as two developmentally regulated and alternatively spliced isoforms, SNAP-25a and SNAP-25b, which differ in only 9 of 206 amino acids (22, 23). In the mouse brain, SNAP-25a precedes SNAP-25b expression during development, but by the second postnatal week SNAP-25b becomes the major splice variant, concomitantly with a dramatic increase in total SNAP-25 expression (23). In endocrine and neuroendocrine cells SNAP-25a appears to be the dominant isoform throughout life, although the expression patterns of the two splice variants are not dedicated exclusively to certain cell types (24, 25). Either SNAP-25a or SNAP-25b can participate in the core SNARE complex, which in addition comprises the two proteins syntaxin and vesicle-associated membrane protein (VAMP) (21, 26). Normal expression levels of total SNAP-25 appear to be critical for precise synaptic function (27, 28), and a reduction affects SNARE protein assembly and synaptic plasticity and even can cause neurodegeneration (28, 29). Even if both SNAP-25 splice variants mediate fast Ca^{2+}-triggered release, SNAP-25b–containing SNARE complexes are believed to have a higher degree of stability and therefore increase the pool of primed vesicles, resulting in release dynamics different from those involving SNAP-25a (26, 30).

Polymorphisms in the human *Snap25* gene have been associated with weight gain after antipsychotic treatment, with altered levels of serum triglycerides (31, 32), and also with the severity of the metabolic syndrome in T2D (33). Furthermore, polymorphisms in genes expressing proteins directly interacting with SNAP-25 have been implicated in childhood obesity, impaired glucose metabolism and obesity, age of onset of T2D, and insulin requirement in T2D (34–36). Moreover, polymorphisms in the human L-type channel (37) and the K_{ATP} channel (38) have been

Significance

Our changed lifestyle, including decreased physical activity and increased food consumption, is leading to a pandemic of obesity and type 2 diabetes, the metabolic syndrome. Impaired release of hormones, such as insulin, from excitable cells usually is considered a symptom, not a cause, of this syndrome. Here, however, we show that a small genetic modification, replacing synaptosomal-associated protein of 25 kDa (SNAP-25), SNAP-25b with SNAP-25a in the machinery mediating stimulus-dependent release of hormones and neurotransmitters is sufficient to provoke hypothalamic dysfunction, obesity, and type 2 diabetes. When combined with a Western diet, this genetic condition triggers severe diabesity. Our work expands previous knowledge supporting the notion that many individuals have an increased susceptibility to developing metabolic disease because of a genetic predisposition.

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directly associated with T2D. These channels are indispensable for glucose-stimulated insulin secretion from pancreatic β cells and thus directly influence SNARE protein function and efficiency. Thus, emerging evidence suggests that direct or indirect alterations of SNARE protein function can increase the susceptibility and predisposition to serious metabolic illness.

To test this hypothesis experimentally, we used a genetically engineered mutant mouse expressing normal levels of SNAP-25 but with the nine amino acids specific for the SNAP-25b splice variant converted, by knockout/knockin replacement, to the SNAP-25a sequence (30). Using these mice, we studied the interplay between modified SNARE function and metabolic parameters during a 7-wk diet intervention involving a control standard food diet (CoD) and a high-fat/high-sucrose diet (Western diet, WeD). Our results demonstrate that SNAP-25b deficiency alone predisposes to metabolic disease and that the progressing pathology is accelerated dramatically by a high-fat/high-sucrose diet, leading to diabesity.

Results

SNAP-25–Deficient Mice Develop an Obese Phenotype. To investigate whether SNAP-25b deficiency results in an increased risk for developing obesity and related metabolic conditions,

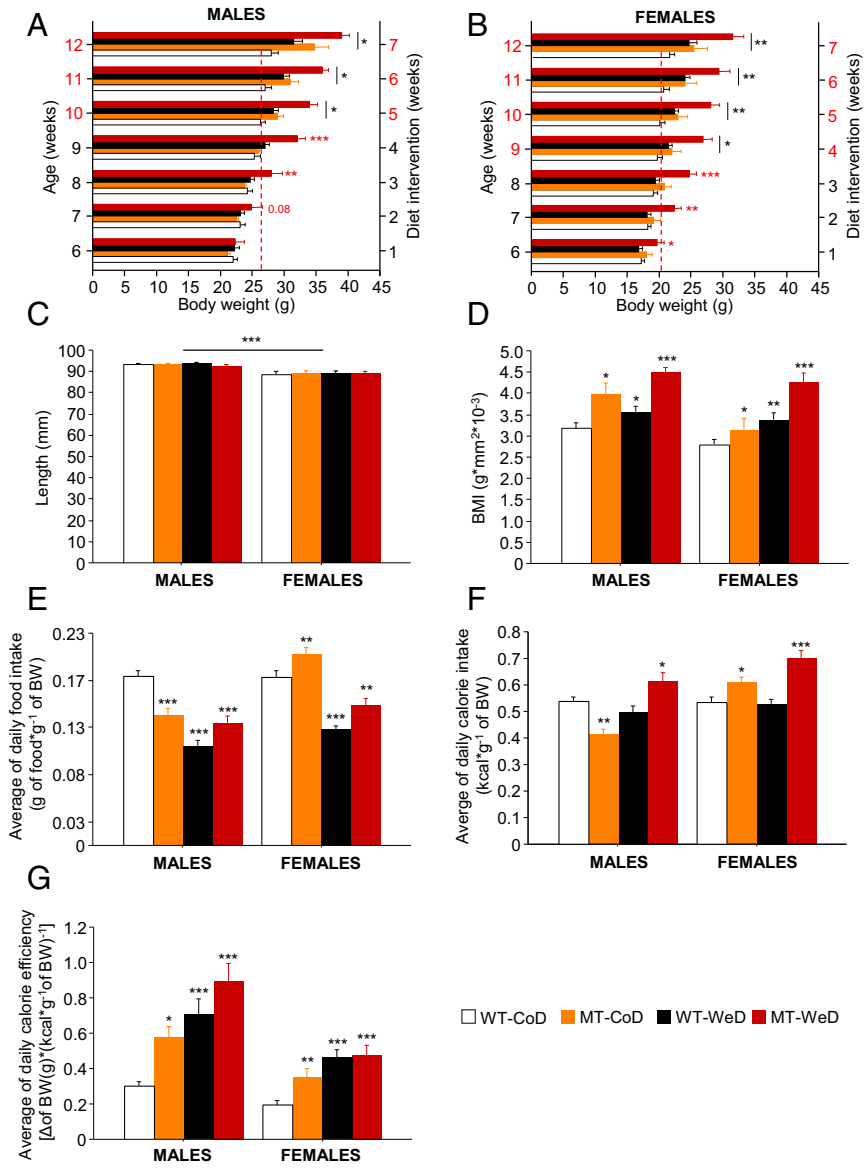

Fig. 1. Overweight and impaired eating habits. (*A* and *B*) Monitoring of body weight. Compared with other groups, a significant increase in body weight is seen in male WeD-fed SNAP-25b–deficient MT mice after 3 wk of diet intervention (*A*) and in females after 1 wk of diet intervention (*B*). CoD-fed MT mice become significantly heavier than CoD-fed WT mice after 5 wk of the diet intervention in males (*A*) and after 4 wk in females (*B*), as indicated by red numbering on the *y* axes of *A* and *B*. Discontinuous red line in *A* and *B* marks the body weight of male (*A*) and female (*B*) WT-CoD mice at the time point of the diet study when all experimental groups became overweight. (*C*) Nose-tip to tail-root length was measured at the end of the 7-wk diet intervention and demonstrated no significant differences between experimental groups. (*D*) All experimental groups demonstrate a higher BMI than CoD-fed WT mice. (*E*) The average weight-matched daily food intake (grams of food consumed per gram of body weight) is significantly lower in male CoD-fed MT mice than in male CoD-fed WT mice, whereas female MT mice are hyperphagic. All animals on the CoD show a decrease in food ingested independent of genotype and sex. (*F*) Male CoD-fed MT mice exhibit lower calorie intake than CoD-fed WT mice but become hypercaloric when fed the WeD. Female CoD- or WeD-fed MT mice show a higher calorie intake than CoD-fed WT animals. (*G*) Average calorie efficiency is increased in all experimental groups compared with CoD-fed WT mice. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with CoD-fed WT mice. See also Figs. S2 and S3.

SNAP-25b–deficient (MT) and WT mice were subjected to a 7-wk diet intervention starting in the fifth postnatal week (Fig. S1 and Table S1). The body weights of MT and WT mice in the different experimental groups were similar before the start of the diet intervention, although sex differences were observed (Table S2). The CoD-fed male SNAP-25b–deficient mice developed a significant increase of body weight compared with WT controls around week 5 of the diet intervention ($P < 0.05$) (Fig. 1*A*). In CoD-fed female SNAP-25b–deficient mice, differences in body weight increase, as compared with WT females ($P < 0.05$), were already statistically significant after 4 wk of diet intervention (Fig. 1*B*). The increased body weight in male and female CoD-fed MT mice, as compared with their WT controls, was sustained until the end of the study ($P < 0.05$) (Fig. 1 *A* and *B*; also see Fig. S3 *D* and *E*). Interestingly, the increase in body weight in both male and female CoD-fed MT mice followed a pattern similar to that observed in WeD-fed WT mice (Fig. 1 *A* and *B*). WeD-fed SNAP-25b–deficient MT mice exhibited a dramatic increase in body weight compared with all other experimental groups ($P < 0.001$) (Fig. 1 *A* and *B*), the difference being statistically significant in males after 3 wk on the WeD ($P < 0.01$) (Fig. 1*A*) and in females after only 1 wk on the WeD ($P < 0.01$) (Fig. 1*B*). All differences increased progressively in both sexes until the end of the study. After 7 wk of diet intervention, the length (nose to tail root) of the mice in the experimental groups did not differ from that of control mice (Fig. 1*C*); however, the body mass index, BMI, was increased significantly ($P < 0.01$ to $P < 0.001$) (Fig. 1*D*).

To investigate possible differences in eating habits, food and calorie intake were measured and corrected by the body weight of each individual animal (Fig. 1*E* and Fig. S2 *A* and *B*). The average food and calorie intake of male CoD-fed MT mice was significantly lower than that of CoD-fed WT mice ($P < 0.01$ to $P < 0.001$) (Fig. 1 *E* and *F* and Figs. S2 *A* and *C* and S3 *A* and *B*). In contrast, CoD-fed female SNAP-25b–deficient MT mice demonstrated an increased food and calorie intake relative to WT controls ($P < 0.05$ to $P < 0.01$) (Fig. 1 *E* and *F* and Fig. S2 *B* and *D*). Moreover, the average calorie efficiency in CoD-fed MT mice was greater than in their corresponding WT controls (males, $P < 0.05$; females, $P < 0.01$) (Fig. 1*G* and Figs. S2 *E* and *F* and S3*C*), possibly as a result of increased adiposity (see below). The daily food consumption of WeD-fed WT mice was reduced compared with that of CoD-fed WT animals ($P < 0.001$) (Fig. 1*E* and Fig. S2 *A* and *B*). Thus, both male and female WeD-fed WT mice were isocaloric (Fig. 1*F* and Fig. S2 *C* and *D*). In any case, the WeD induced significantly increased calorie efficiency ($P < 0.05$ to $P < 0.0010$ (Fig. 1*G* and Fig. S2 *E* and *F*) in WT mice of both sexes. The food intake of WeD-fed SNAP-25b–deficient MT mice was reduced significantly compared with their control CoD-fed WT counterparts ($P < 0.01$ to $P < 0.001$) (Fig. 1*E* and Fig. S2 *A* and *B*). Furthermore, both male and female WeD-fed MT mice were hypercaloric ($P < 0.05$ to $P < 0.001$) (Fig. 1 *E* and *F* and Fig. S2 *B* and *D*) and presented an increased calorie efficiency compared with corresponding WT controls.

Thus, SNAP-25b deficiency, without the WeD, was sufficient to induce an obese phenotype. In WeD-fed WT animals the increased body weight apparently was related directly to diet composition, whereas SNAP-25b deficiency generated an impaired energy balance with elevated caloric efficiency, resulting in higher body weight. Additionally, CoD-fed male MT mice demonstrated a changed eating behavior which most likely contributed to obesity (*SI Results*).

SNAP-25b Deficiency Impairs Insulin Secretion and Glucose Homeostasis. To characterize insulin secretion and glucose homeostasis, we performed standard glucose tolerance tests (GTT) by i.p. injection of glucose (Fig. 2 *A* and *B*). During the time course of the experiment both blood glucose and serum insulin levels were measured (Fig. 2 *C* and *D*). In addition, we monitored blood glucose in nonfasting mice during the entire diet intervention (Fig. 2 *E* and *F*).

There were significant differences in fasting basal glucose levels between SNAP-25b–deficient MT and WT mice (Fig. 2 *A* and *B* and Fig. S4*A*). In females, all groups demonstrated a significant increase in basal blood glucose compared with CoD-fed WT mice ($P < 0.05$ to $P < 0.001$) (Fig. 2*B* and Fig. S4*A*), but the effect was significant only in WeD-fed MT mice ($P < 0.001$) (Fig. 2*B* and Fig. S4*A*). Both male and female CoD-fed MT mice exhibited significantly lower blood glucose levels 15 min after the glucose challenge compared with CoD-fed WT mice ($P < 0.001$) (Fig. 2 *A* and *B*). When fed the WeD, both WT and MT mice had severely increased blood glucose levels at every time point measured, in a sex-independent manner ($P < 0.05$ to $P < 0.001$) (Fig. 2 *A* and *B*). The differences in blood glucose concentrations during the GTT also were obvious when the increase in total blood glucose was analyzed as area under the curve (AUC) (Fig. S4*B*).

The influence of SNAP-25b deficiency and/or diet on basal serum insulin levels was evaluated in animals starved for 12 h after 7 wk of diet intervention (Fig. 2 *C* and *D* and Fig. S4*C*). Here SNAP-25b deficiency combined with the WeD resulted in a dramatic increase in basal serum insulin levels in males (males, $P < 0.01$; females, $P = 0.08$) (Fig. 2 *C* and *D* and Fig. S4*C*), but significant changes were not seen in the other groups (Fig. 2 *C* and *D* and Fig. S4*C*). Furthermore, the pattern of insulin secretion appeared to be affected both by genetics and diet (Fig. 2 *C* and *D*), with a significant genotype–diet interaction that was increased dramatically, especially in males ($P < 0.001$) (Fig. 2*C*). This interaction also was obvious when the AUC for serum insulin levels was analyzed during the entire GTT ($P < 0.05$) (Fig. S4*D*).

Preprandial blood glucose levels were normal in all experimental groups before the diet intervention (nonsignificant, NS) (Table S2), but MT mice developed hyperglycemia compared with CoD-fed WT mice ($P < 0.01$ to $P < 0.001$) (Fig. 2 *E* and *F*). Both primary (genetic) and secondary (dietary) preprandial hyperglycemia became significant after 3 wk of diet intervention in males ($P < 0.001$) (Fig. 2*E*) and at 6 wk in females ($P < 0.01$) (Fig. 2*F*). This increase continued until the end of the study ($P < 0.01$ to $P < 0.001$) (Fig. 2 *E* and *F*).

Total pancreas insulin content was determined in all experimental groups (Fig. 2*G*). SNAP-25b deficiency in itself increased the insulin content, resulting in insulin levels similar to those observed in WT mice at the end of the 7-wk-long diet intervention ($P < 0.001$) (Fig. 2*G*). It is noteworthy that the WeD in combination with SNAP-25b deficiency did not result in significantly higher insulin levels than seen with the WeD or SNAP-25b deficiency alone (Fig. 2*G*). Calculation of the insulin resistance index (the homeostatic model assessment of insulin resistance, $HOMA_{IR}$) revealed a tendency (NS) to increase in male CoD-fed MT and WeD-fed WT mice, reaching significance in WeD-fed MT male mice ($P < 0.05$) and in all female groups as compared with CoD-fed WT female mice ($P < 0.05$) (Fig. 2*H*).

In summary, although CoD-fed SNAP-25b–deficient mice initially appeared to handle a glucose challenge during a standard GTT better than WT controls, the basal and preprandial blood glucose levels showed that SNAP-25b deficiency alone results in a state similar to T2D diabetes. In males, SNAP-25b deficiency plus the WeD resulted in a severe inability to maintain normal blood glucose levels despite a 16-fold increase in insulin secretion during the GTT, suggesting insulin resistance (*SI Results*).

Impaired Hypothalamic Feeding Signals in SNAP-25b–Deficient Mice. The discrepancy between preprandial blood glucose levels and those found in SNAP-25b–deficient MT mice after 12-h starvation indicated uncharacteristic eating habits, possibly resulting from disturbed feeding signals from the brain and in particular from the hypothalamus. Hypothalamic dysfunction associated with obesity and T2D is currently an important area of investigation (18). A diagnostic marker for metabolic impairment is the activation/phosphorylation of a master metabolic regulator,

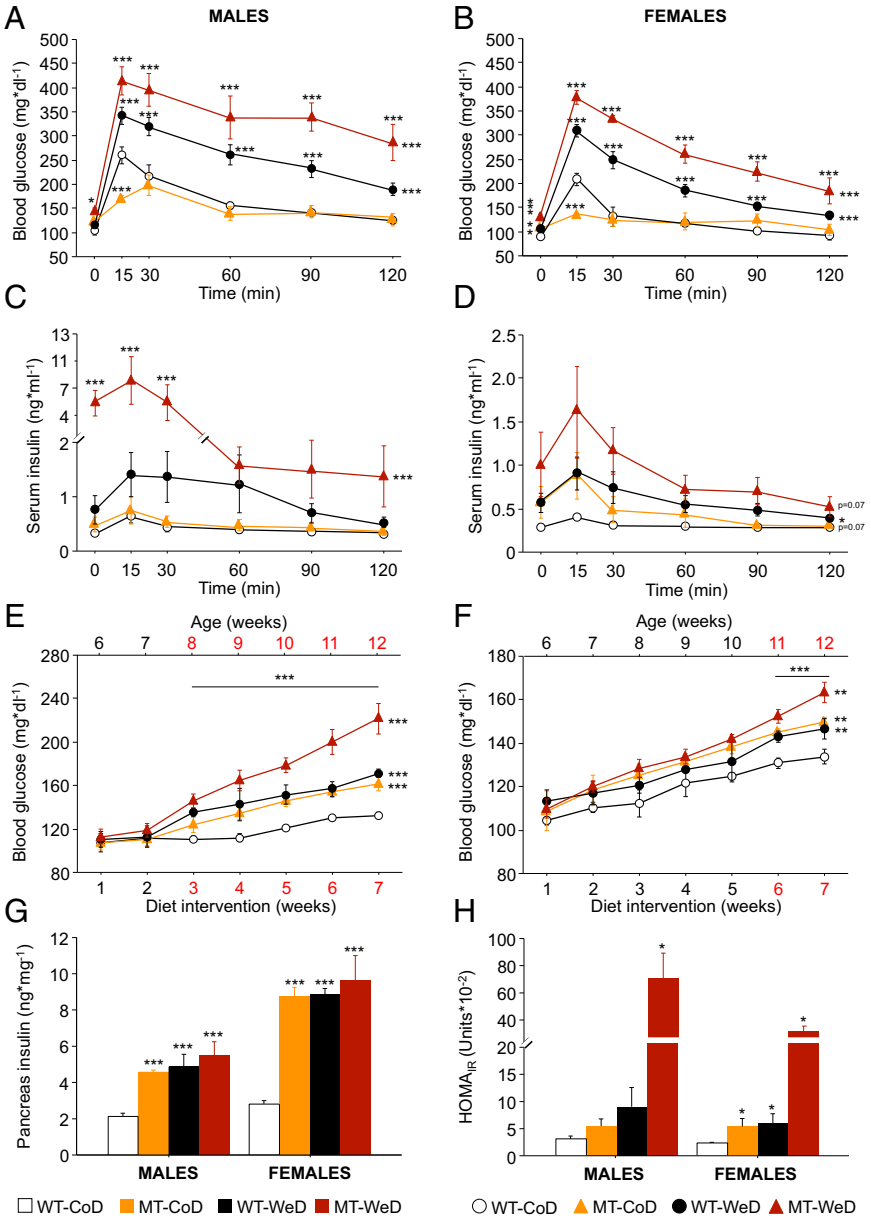

Fig. 2. Impairment of glucose homeostasis and insulin secretion. (*A* and *B*) GTT analyses show decreased blood glucose levels in both male (*A*) and female (*B*) CoD-fed MT mice after 15 min. Both male and female WeD-fed WT and MT mice exhibit hyperglycemia at the time points analyzed. (*C* and *D*) Serum insulin levels evaluated at the same time points as blood glucose are increased significantly only in WeD-fed male MT (*C*) and female WT (*D*) mice. (*E* and *F*) SNAP-25b deficiency elicits hyperglycemia that is more pronounced and debuts earlier in males. (*G*) Total pancreas insulin content is increased significantly in all experimental groups compared with CoD-fed WT mice. (*H*) The $HOMA_{IR}$ is statistically significant only in WeD-fed male MT mice but is significantly higher in all female groups as compared with CoD-fed WT female mice. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with CoD-fed WT controls. See also Fig. S4.

AMP-activated protein kinase (AMPK). We evaluated this switch in the hypothalamus by monitoring AMPK-$\alpha_{1/2}$ (39, 40). Immunoblotting demonstrated that AMPK activation was compromised in a sex-independent manner in all experimental groups as compared with CoD-fed WT mice ($P < 0.001$) (Fig. 3 *A* and *B*). It is well known that both genetic- and diet-induced obesity account for increasing leptin levels and elicit leptin resistance in a tissue-specific manner (41). Immunoblotting experiments were performed on the leptin receptor, ObR, in hypothalamic lysates (Fig. 3 *C* and *D*). As shown in Fig. 3 *C* and *D*, the long isoform of hypothalamic ObR, ObRb, was severely down-regulated in WeD-fed animals compared with CoD-fed WT mice, independent of sex and genotype ($P < 0.05$ to $P < 0.01$). The WeD-fed SNAP-25b–deficient MT mice at 7 wk of diet intervention displayed the most dramatic ObRb deficiency ($P < 0.01$) (Fig. 3 *C* and *D*). However, ObRb expression remained unchanged in CoD-fed MT mice, independent of the sex (nonsignificant, NS) (Fig. 3 *C* and *D*). To characterize further the hypothalamic intracellular signaling pathways regulated by leptin and insulin, we investigated hypothalamic STAT3 and ERK1/2 activation by phosphorylation of these proteins in animals after 7 wk of diet intervention. As shown in Fig. 3 *E* and *F*, STAT3 phosphorylation was reduced significantly in all experimental groups compared with CoD-fed WT mice, except for male CoD-fed MT mice ($P < 0.05$ to $P < 0.001$) (Fig. 3 *E* and *F*). Moreover, there was an increased protein content of hypothalamic phospho-ERK1/2 in all experimental groups, independent of sex ($P < 0.05$ to $P < 0.01$) (Fig. 3 *G* and *H*). Phosphorylation of ERK1/2 has been described as a response to

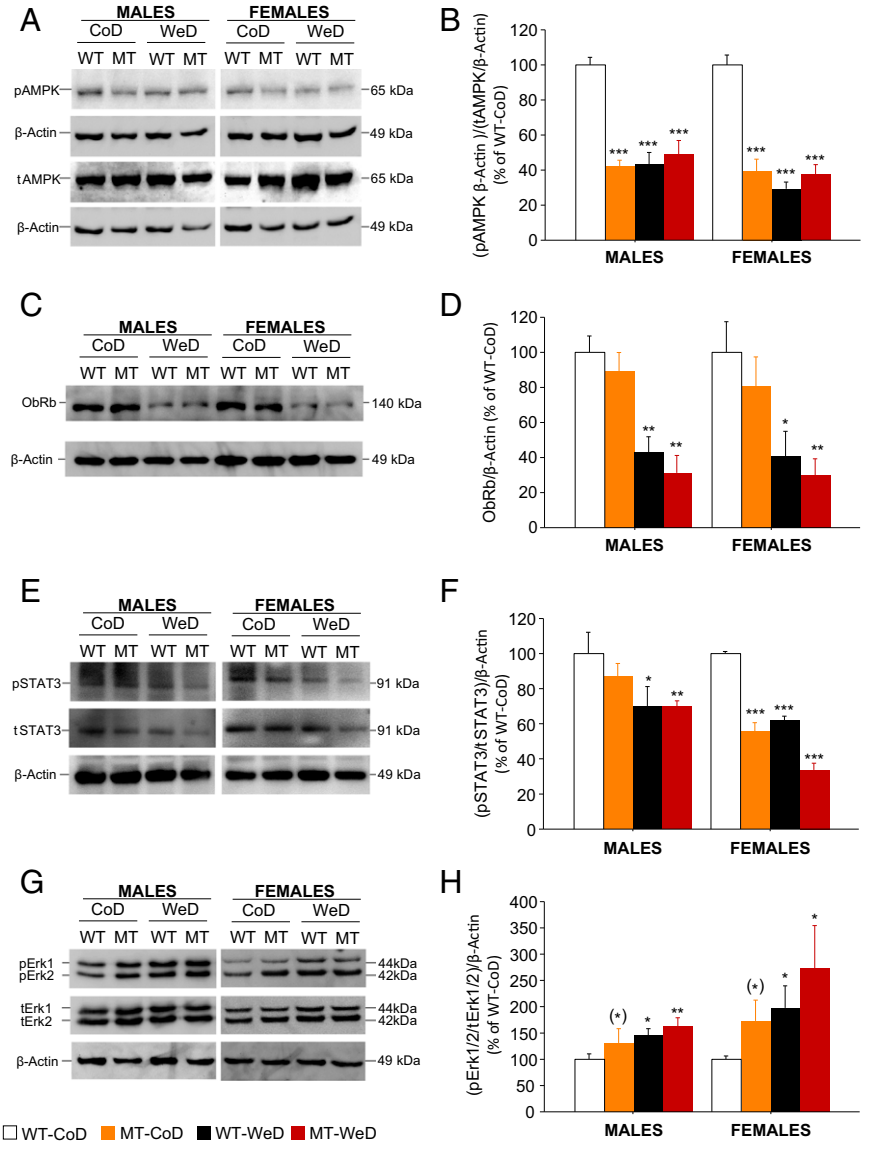

Fig. 3. Hypothalamic function. Representative semiquantitative Western blots and densitometry analyses of hypothalamic homogenates. (*A* and *B*) AMPK-$\alpha_{1/2}$ phosphorylation is significantly compromised in all experimental groups compared with CoD-fed WT mice, independent of sex. (*C* and *D*) Both WeD-fed male and female mice develop ObRb deficiency compared with CoD-fed WT mice. (*E* and *F*) Hypothalamic pSTAT3 is decreased in all experimental groups compared with CoD-fed WT mice, except for CoD-fed MT mice, in which only a tendency to decreased pSTAT3 levels was found. (*G* and *H*) ERK1/2 phosphorylation was significantly increased in all experimental groups compared with CoD-fed WT animals. β-Actin served as a loading control. (*)$P = 0.05$–0.09; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with CoD-fed WT mice.

low-grade inflammation in the hypothalamus of individuals with obesity and T2D (42).

Taken together, our results show that SNAP-25b deficiency alone was sufficient to inactivate the regulatory subunit of AMPK partially but did not significantly affect the levels of ObRb expression in the hypothalamus. However, in combination with the WeD, the absence of SNAP-25b expression resulted in both a significant decrease of AMPK phosphorylation and reduced expression of ObRb. These changes were accompanied by defective hypothalamic leptin signaling caused by STAT3 dephosphorylation. The increased phosphorylation of ERK1/2 suggested the presence of a low-grade inflammatory process within the hypothalamus.

Enhanced Accumulation of White Adipose Tissues. Abdominal obesity, also known as "central obesity," is the accumulation of excessive abdominal fat around the stomach and abdomen. Visceral (intra-abdominal) white adipose tissue (WAT) is located inside the peritoneal cavity and torso and includes mesenteric adipose tissue (MsAT), peri-gonadal AT (PgAT), and retroperitoneal AT (RpAT), as opposed to the subcutaneous adipose tissue (ScAT) located underneath the skin. SNAP-25b–deficient MT mice exhibited a significant increase in all visceral and nonvisceral fat depots ($P < 0.05$ to $P < 0.001$) (Fig. 4*A* and Fig. S5) in both sexes as compared with CoD-fed WT mice. The increase in WAT accumulation in MT mice was even greater than in WeD-fed WT mice ($P < 0.05$ to $P < 0.001$) (Fig. 4). Consequently, all types of WAT were increased in all WeD-fed SNAP-25b–deficient MT mice as compared with the other experimental groups ($P < 0.05$ to $P < 0.001$) (Fig. 4).

In summary, SNAP-25b deficiency resulted in central obesity, a condition aggravated when mice were fed the WeD.

Dyslipidemias and Adipocyte Hypertrophy. Hyperlipidemia, the most common form of dyslipidemia, involves abnormally elevated levels of triglycerides, cholesterol, and/or lipoproteins in the blood (43).

These lipid parameters are prognostic/diagnostic markers for metabolic disturbances. Analyses of triglycerides (Fig. 5*A*) and cholesterol (Fig. 5*B*) after 12 h of starvation demonstrated a significant increase ($P < 0.01$ to $P < 0.001$) in all groups except in WeD-fed female WT mice (NS) (Fig. 5*A*). Moreover, preprandial monitoring of lipid homeostasis parameters revealed the time of primary hyperlipidemia debut (Fig. 5 *C* and *D*). Thus, at 5 wk of age, before the diet intervention, male and female MT mice demonstrated normal triglycerides and cholesterol levels compared with age-matched WT controls (Table S2). During the diet intervention signs of disturbances in lipid homeostasis developed in CoD-fed SNAP-25b–deficient MT mice but did so later in males than in females. Thus, males exhibited hypertriglyceridemia in week 5 of the diet intervention ($P < 0.001$) (Fig. 5*C*), vs. week 3 for females ($P < 0.001$) (Fig. 5*D*), and higher blood cholesterol levels were observed at week 3 for the males ($P < 0.001$) (Fig. 5*E*), vs. week 4 for females ($P < 0.001$) (Fig. 5*F*). The differences in triglycerides and cholesterol levels were maintained and increased progressively until the end of the study. Interestingly, the dyslipedemias seen after SNAP-25b deficiency alone followed the same pattern as observed in WT mice on WeD (NS) (Fig. 5 *C*–*F*). Finally, the primary dyslipidemias detected in the MT mice caused by the mutation, together with the secondary ones caused by WeD, became severe when WeD concurred with SNAP-25b deficiency ($P < 0.001$) (Fig. 5 *C*–*F*).

Adipocyte size and turnover are important factors underlying the onset of obesity and metabolic disorders (44). Here we observed that both genetics and diet contributed to adipose cell hypertrophy. Male and female CoD-fed MT mice exhibited enlarged adipose cell size in the ScAT ($P < 0.001$) (Fig. 6 *A* and *B*) and PgAT ($P < 0.001$) (Fig. 6 *C* and *D*) compared with WT mice receiving the same diet. In addition, WeD feeding increased the size of ScAT and PgAT adipocytes compared with CoD-fed WT animals ($P < 0.001$) (Fig. 6), independent of sex and genotype.

In summary, SNAP-25b deficiency elicited dyslipidemias that were severely aggravated in combination with the WeD. In addition, a pronounced increase in the size of adipocytes accompanied obesity.

Impaired Leptin Levels, Liver Lipotoxicity, and Hepatic Leptin Receptor Deficits. Obesity and its associated metabolic disorders are characterized by changes in the release of humoral factors such as leptin and ghrelin (41). We analyzed basal serum leptin and ghrelin levels after diet intervention (Fig. 7 *A* and *B*). Female, but not male, CoD-fed MT mice demonstrated increased leptin levels compared with WT controls ($P < 0.01$) (Fig. 7*A*). WeD-fed MT mice of both sexes demonstrated a strong genetic–diet synergistic interaction with an approximately eightfold increase in basal leptin levels ($P < 0.01$) (Fig. 7*A*). Ghrelin was decreased in all experimental groups compared with CoD-fed WT mice ($P < 0.01$ to $P < 0.05$), except for male WeD-fed MT mice ($P = 0.08$) (Fig. 7*B*). Because increased leptin levels can elicit leptin resistance in a tissue-specific manner (41), we also studied ObRs in liver lysates with immunoblotting (Fig. 7*C*). SNAP-25b deficiency by itself was sufficient to down-regulate liver ObRb, independent of sex and diet ($P < 0.05$) (Fig. 7 *C* and *D*). Interestingly, ObRa, a short isoform of the ObR, was detectable only in MT mice (Fig. 7*C*). The role of the short isoforms of the ObR is not fully understood but possibly could compensate for a deficiency in ObRb expression.

To investigate further whether metabolic organs were affected, we performed qualitative (Fig. 7*E*) and quantitative (Fig. 7*F*) analyses of total liver triglycerides. SNAP-25b deficiency alone elicited hepatic steatosis that in WT mice was secondary to diet-induced diabesity ($P < 0.001$) (Fig. 7 *E* and *F*). When SNAP-25b deficiency coincided with WeD administration, the hepatic

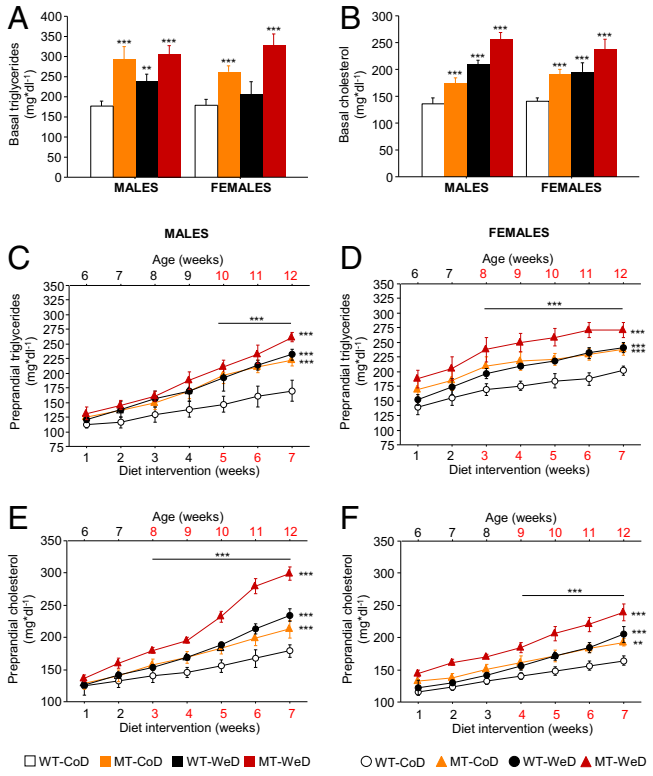

Fig. 5. Lipid homeostasis. (*A*) Basal blood triglycerides levels after 7 wk of diet intervention are increased significantly in all experimental groups compared with CoD-fed WT animals, except for WeD-fed female WT mice. (*B*) All experimental groups demonstrate hypercholesterolemia compared with levels in CoD-fed WT mice after 7 wk of diet intervention. (*C* and *D*) Preprandial blood triglycerides levels are significantly increased in all groups as compared with CoD-fed WT mice, being most pronounced in the WeD-fed MT mice, and starting after 5 wk of diet intervention in males (*C*) and after 4 wk of diet intervention in females (*D*). (*E* and *F*) Preprandial blood cholesterol levels show development similar to that of triglycerides but are statistically significant after 3 wk of diet intervention in males (*E*) and after 4 wk of diet intervention in females (*F*). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with CoD-fed WT mice.

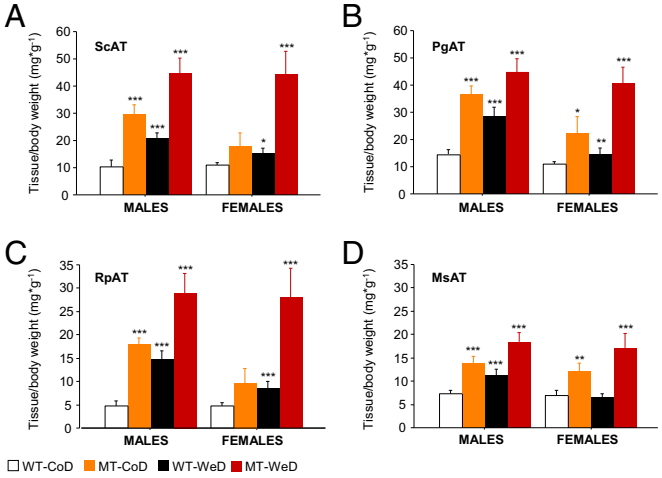

Fig. 4. Body fat distribution. All experimental groups show increased amounts of ScAT (*A*), PgAT (*B*), RpAT (*C*), and MsAT (*D*) fat (corrected by body weight). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with CoD-fed WT controls. See also Fig. S5.

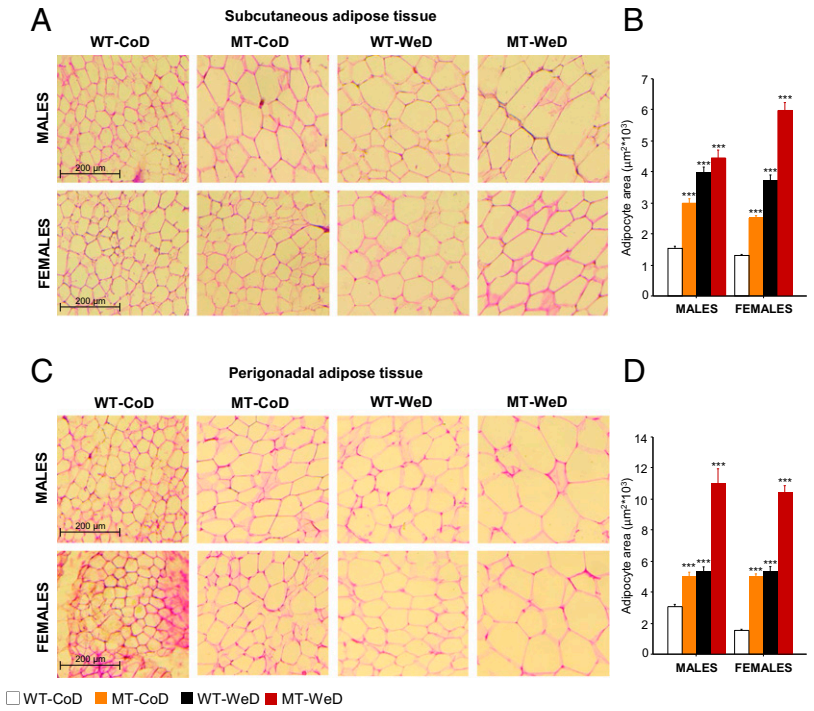

Fig. 6. Adipocyte hypertrophy. Qualitative (*A* and *C*) and quantitative (*B* and *D*) analyses of adipocyte area are carried out on H&E-stained cryosections of ScAT (*A*) and PgAT (*C*). Both male and female CoD-fed MT mice and WeD-fed WT mice exhibit enlarged adipocytes compared with CoD-fed WT animals, with the strongest effect seen in WeD-fed MT mice (female ScAT and male and female PgAT). (Scale bars: 200 µm.) *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with CoD-fed WT mice.

steatosis turned into severe fatty liver disease ($P < 0.001$) (Fig. 7 *E* and *F*).

In summary, SNAP-25b deficiency resulted in elevated serum leptin levels and impaired expression of ObRb in liver and also in an increase in liver triglycerides and hepatic steatosis. Serum ghrelin levels were decreased in all groups as compared with CoD-fed WT mice. All effects observed in SNAP-25b–deficient MT mice were exaggerated by the WeD (Table S3).

Discussion

SNAP-25 isoforms fine-tune the kinetics of regulated membrane fusion. Here, we took advantage of a SNAP-25b–deficient mouse mutant (30) and explored whether small modifications in SNARE function in excitable cells predispose to obesity and metabolic disease. In CoD-fed MT mice multiple impairments were observed, such as increased body weight, a pronounced effect on calorie efficiency and WAT mass, adipocyte hypertrophy, steatohepatitis, and hypothalamic dysfunction. As expected, WT animals fed the WeD for 7 wk also developed impairments in the parameters analyzed. Moreover, when SNAP-25 deficiency was combined with the WeD a dramatic, synergistic effect was observed. The results we observed are all established features of the human metabolic syndrome (1, 45) and diet-induced diabesity (4–6). Indeed, it appeared that the genetic mutation in combination with the WeD triggered a vicious circle of increased lipid accumulation and impaired glucose homeostasis. Interestingly, although SNAP-25b deficiency and diet generally affected both sexes equally dramatically, there were certain distinct sex-based differences (*SI Discussion*).

Deficiencies of hormonal secretion, e.g., the impaired release of insulin from pancreatic β cells, usually are considered secondary signs and consequences of the progressing pathology in metabolic disease, such as in T2D (12). Here we asked instead if a minor alteration in membrane fusion dynamics can lead to obesity and metabolic disease, a hypothesis not previously tested experimentally, to our knowledge. SNAP-25, together with VAMP/synaptobrevin and syntaxin, form a core complex essential for mediating regulated membrane fusion. These proteins exist as several splice variants or isoforms, some of which also operate at intracellular membrane-trafficking steps. However, because many of them appear to be functionally interchangeable, the physiological differences, if any, are yet not fully understood (21).

SNAP-25 is essential for stimulus-dependent exocytosis, and disruption of the gene results in death at birth (46). However, SNAP-25 exists as two splice variants, SNAP-25a and SNAP-25b, both of which can support insulin release (24). SNAP-25b–deficient mice are viable and live until adulthood (30). SNAP-25b forms a SNARE complex with a higher degree of stability than SNAP-25a, thereby increasing the pool of primed vesicles (26). Indeed, when Sørensen et al. (26) separately overexpressed either SNAP-25a or SNAP-25b in embryonic adrenal chromaffin cells from SNAP-25–null mice, the burst of Ca^{2+}-evoked catecholamine release differed. Furthermore, impairment of vesicle trafficking and reduced insulin secretion was observed in the blind-drunk mouse, a mutant with a dominant mutation in the SNAP-25b–specific exon, resulting in a further increased stability of the SNARE complex (47). Although SNAP-25a has a lower capacity to keep vesicles in a primed state, little is known how this molecular difference between the splice variants influences animal physiology.

We started the diet intervention at 5 wk of age, a developmentally critical window in the life of mice (i.e., puberty), and assessed different metabolic parameters before, during, and after the intervention. First we characterized glucose homeostasis and insulin secretion. Surprisingly, we detected an apparently improved ability to handle a glucose challenge in CoD-fed SNAP-25b–deficient MT mice. When injected with glucose, starved MT mice did not demonstrate the typical rise in blood glucose, usually peaking after 15 min. The lack of increase in blood glucose was accompanied by facilitated insulin release, suggesting that the presence of SNAP-25a alone does favor insulin exocytosis, perhaps

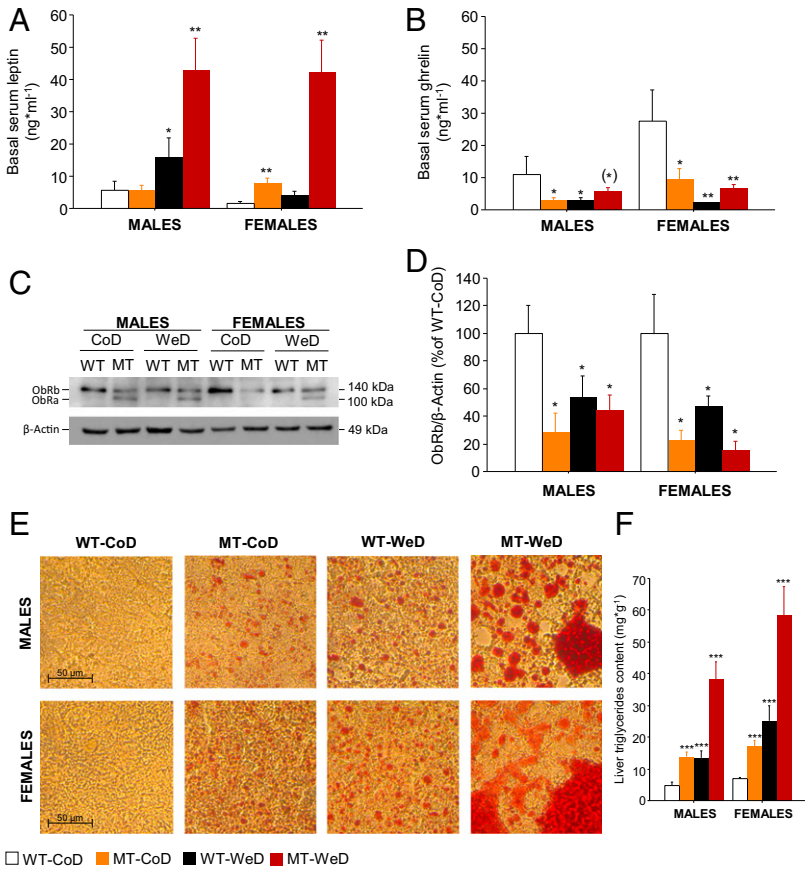

Fig. 7. Increased serum leptin levels and liver disease. (*A*) Basal serum leptin levels are markedly increased (<10-fold) in male and female WeD-fed MT mice compared with CoD-fed WT mice. Significant effects also are detected in CoD-fed MT females and in WeD-fed WT males. (*B*) Basal serum ghrelin levels are decreased significantly in all experimental groups compared with CoD-fed WT mice, except for WeD-fed male WT mice ($P < 0.05$). (*C*) The ObRa isoform is present only in MT mice liver homogenates. β-Actin served as a loading control. (*D*) There is a significant decrease in liver ObRb protein content in all experimental groups compared with CoD-fed WT mice. (*E*) ORO-stained liver cryosections show steatohepatitis in all experimental groups compared with CoD-fed WT mice (both sexes). (Scale bars: 50 μm.) (*F*) Spectrophotometry analyses confirm data obtained with ORO staining. (*)$P = 0.05$–0.09; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with CoD-fed WT mice.

at a lower stimulatory threshold (24, 47). In sharp contrast, in males, SNAP-25b deficiency in combination with the WeD resulted in a 16-fold increase in insulin secreted during GTT. Nonetheless, the preprandial measurements of blood glucose demonstrated a severe inability to maintain normal blood glucose levels, suggesting insulin resistance. However, a closer investigation of basal blood glucose levels during the diet intervention showed that CoD-fed MT mice had preprandial levels comparable with those in WeD-fed WT mice. This finding suggests that the increased nonfasting blood glucose levels in both CoD- and WeD-fed SNAP-25b–deficient mice likely were caused by impaired eating behavior and peripheral insulin resistance.

Altered eating habits usually involve dysfunction in the hypothalamic areas involved in the maintenance of metabolic homeostasis, and it is likely that SNAP-25b deficiency introduces changes in peptidergic/neurotransmitter signaling in those brain centers (18, 19). Therefore, we investigated the expression of the long isoform of the leptin receptor, ObRb, and the phosphorylation status of AMPK, a key switch in leptin signaling in the hypothalamus. To explore further hypothalamic intracellular cascades involving leptin and insulin signaling, we also investigated the phosphorylation status of STAT3 and ERK1/2. Our results indicate that the dramatic synergistic effects of combined SNAP-25b deficiency and the WeD likely are the result of cross-talk between periphery and the brain, in particular hypothalamic dysfunction. This notion is in line with previous studies reporting that the onset of diet-induced obesity and insulin resistance induces permanent effects in both the periphery and the brain (48–50). The impaired eating habits demonstrated by the SNAP-25b–deficient MT mice also included increased feeding during the light hours, suggesting that the circadian systems regulating sleep and/or feeding cycles did not follow a normal pattern. Indeed, dysregulation of these hypothalamic-dependent rhythms alone can induce an obesogenic development (51).

Surprisingly, SNAP-25b deficiency in itself significantly increased baseline serum cholesterol and triglyceride levels and liver triglycerides. Previously, elevated serum triglyceride levels and body weight gain have been described in patients with polymorphism in the human *Snap25* gene, although, notably, these patients had received antipsychotic treatment for schizophrenia (31, 32). The hypothalamus controls hepatic lipid metabolism and storage via the autonomic nervous system (52, 53). It is believed that increased sympathetic activity augments the production of triglycerides and that increased parasympathetic activity enhances lipid accumulation in liver (53, 54). Thus, it is plausible that in SNAP-25b–deficient mice neuroexocytosis from autonomic nerves innervating the liver is less well controlled and thus contributes to the phenotype with increased serum triglycerides and liver steatosis (51, 54).

We propose that our SNAP-25b–deficient mice might represent a model for studies of the mechanisms associated with obesity and metabolic conditions. At 5 wk of age SNAP-25b–deficient mice

are similar to their WT littermates in body weight and blood triglycerides and cholesterol levels. However, a more detailed characterization of young mice is necessary to determine if differences exist that could explain the mechanism of obesity. Unlike most other rodent obesity models used in diabetes research (e.g., *ob*/*ob* and *db*/*db* mice and certain fa/fa rats) (55–57), our model does not have a mutation in the genes for leptin or its receptor, but leptin signaling is affected, nevertheless. Only a handful of mutations have been identified in human genes encoding leptin or the leptin receptor in patients with obesity and insulin resistance; however, the majority of patients lack such mutations but still demonstrate defects in leptin signaling (58).

The severe diabese phenotype found in WeD-fed SNAP-25b–deficient mice suggests that any polymorphism in genes directly or indirectly regulating Ca^{2+}-dependent membrane fusion potentially could be associated with an increased risk of developing metabolic disease, especially in combination with increased calorie intake and insufficient physical activity. Our mutant mouse expresses only SNAP-25a, and thus those excitable cells that normally express SNAP-25b in the exocytosis process are affected (23, 25, 26, 59). In this respect, it is intriguing that several genes identified in GWAS or in functional studies as carrying a risk for susceptibility for T2D encode proteins that are important for insulin secretion from β cells, including potassium channels, voltage-gated Ca^{2+} channels, and G protein-coupled receptors (34–38). These genes also are expressed in other excitable cells, including neurons, and therefore it is possible to envisage a scenario similar to that of our SNAP-25b–deficient mice in which a small imbalance in stimulus-dependent exocytosis mechanisms in excitable cells deregulates the interplay of metabolic signals and is followed by obesity and T2D. A recent publication also links the severity of T2D to an SNP in the human *Snap25* gene (33). Therefore it would be interesting to investigate mouse mutants targeting other genes identified in GWAS as susceptibly genes for T2D and obesity and directly or indirectly involved in the control of regulated membrane fusion. Would such mice develop diabesity when exposed to a diet intervention as the SNAP-25b–deficient mice does, as shown in this study? In contrast to conditional knockouts, our model is in agreement with most humans who have a genetic predisposition to metabolic disease; i.e., they have the same mutation in all cells in the body (7–11).

In conclusion, our data suggest that mutations or minor alterations in the expression of the proteins regulating membrane fusion can increase the vulnerability to develop obesity and precede T2D. We hypothesize that in humans, also, such mutations may participate in the initial phase of developing a metabolic disease and, in combination with a hypercaloric diet, may in fact be a factor initiating the development of diabesity.

Materials and Methods

Animals and Diets. The generation of SNAP-25b–deficient MT mice and their breeding, maintenance, and genotyping were performed as previously described (30). Weight-matched daily food and calorie intake, as well as calorie efficiency, were determined during the entire diet-intervention study (Fig. S1). Body fat distribution was determined in all genotypes after 7 wk of diet intervention. Animals were killed by decapitation, and ScAT, PgAT, RpAT, and MsAT WAT were dissected and weighed. Tissue weight was corrected by the body weight of each individual animal. All animal studies were done in accordance with the guidelines from local authorities and ethical committees, i.e., the Stockholm Northern Animal Experiments Ethics Board, and in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes.

GTTs and Serum and Pancreas Insulin Levels. Intraperitoneal GTTs were carried out after 12-h overnight starvation. Blood glucose levels were determined using a FreeStyle Glucometer (Abbot Diabetes Care). Serum and total pancreas insulin levels were analyzed using an ultrasensitive mouse insulin ELISA kit (Crystal Chem Inc.). A separate cohort of animals was used to evaluate hyperglycemia, defined as nonfasting blood glucose ≥250 mg/dL. Nonfasting blood glucose was determined in blood obtained from the tail vein (*SI Materials and Methods*).

Leptin and Ghrelin Measurements in Serum. Basal leptin and ghrelin levels were determined after 12-h overnight starvation in serum samples of age-matched mice belonging to all experimental groups at the end of the diet-intervention study, using multiplex immunoassays specific for mouse serum samples (BioPlex Pro) (*SI Materials and Methods*).

Adipocyte Size Quantification. Adipocyte size quantification was carried out as previously described (*SI Materials and Methods*) (60).

Triglycerides Content and Oil Red "O" Staining in Liver. Hepatic triglycerides content were determined in liver samples (61) and lipid visualization by Oil Red "O" (ORO) staining (*SI Materials and Methods*) (62).

Monitoring Blood Triglycerides and Cholesterol Levels. A separate cohort of animals was used to analyze basal blood levels of triglycerides and cholesterol. Nonfasting blood triglycerides and cholesterol were monitored in the same cohort used for hyperglycemia studies. Blood lipid levels were measured using a multiparameter diagnostic device for triglycerides and cholesterol (multiCare-in; Biochemical Systems International Srl) (*SI Materials and Methods*).

Western Blotting. Western blotting was performed as described previously (*SI Materials and Methods*) (30).

Statistical Analysis. Quantifications of the data are presented in bar and line graphs created with StatView 5.0 (SAS Institute Inc.). Data represent mean values ± SEM. We used two-way ANOVA and repeated measures ANOVA with a corrected Bonferroni multiple comparison test to calculate statistical significance in all our experiments (*SI Materials and Methods*).

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