Minor differences in the molecular machinery mediating regulated membrane fusion has major impact on metabolic health

Ismael Valladolid-Acebes, Teresa Daraio, Kerstin Brismar, Tomas Hökfelt, and Christina Bark

Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

ABSTRACT
The exocytosis of signaling molecules from neuronal, neuroendocrine and endocrine cells is regulated by membrane fusion involving SNAP-25 and associated SNARE proteins. The importance of this process for metabolic control recently became evident by studies of mouse mutants genetically engineered to only express one of 2 closely related, alternatively-spliced variants of SNAP-25. The results showed that even minor differences in the function of proteins regulating exocytosis are sufficient to provoke metabolic disease, including hyperglycaemia, liver steatosis, adipocyte hypertrophy and obesity. Thus, an imbalance in the dynamics of hormonal and/or neurotransmitter release can cause obesity and type 2 diabetes. This recent discovery highlights the fact that metabolic health requires a perfectly operating interplay between the SNARE protein machinery in excitable cells and the organs responding to these messengers.

CONTACT
Ismael Valladolid-Acebes ismael.valladolid.acebes@ki.se; Christina Bark christina.bark@ki.se
Department of Molecular Medicine and Surgery, The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska Institutet, Karolinska University Hospital L1:01 SE-171 76 Stockholm, Sweden

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Figure 1. Regulated exocytosis and role of SNAP-25 (synaptosomal-associated protein of 25 kDa). (A) Regulated exocytosis, or regulated membrane fusion, is the process by which excitable cells release signaling molecules such as hormones or neurotransmitters through the fusion of intracellular vesicles with the plasma membrane. The final stages of exocytosis are classified into docking, priming and fusion. Vesicles are recruited and tethered to the plasma membrane, where they are docked. During priming, SNARE complexes of proteins including SNAP-25 are formed, after which the vesicles are pulled closer to the plasma membrane bilayers and fuse with them; the vesicle contents are then released into the extracellular space. Fusion is initiated by an influx of calcium via specific channels. (B) The gene for SNAP-25 is located on chromosome 20 p12-12p11.2. (C) This gene encodes a presynaptic protein with two differentially expressed splicing variants, SNAP-25a and SNAP-25b. The two mutually exclusive splice variants result from a duplication of exon 5 in the Snap25 gene. Four single nucleotide polymorphisms (SNPs) or mutations in the human Snap25 gene have been found to be associated with metabolic traits (C). The rs363050 polymorphism in exon 1 of the Snap25 gene results in reduced expression of SNAP-25 protein, and when this SNP coincides with T2D, patients demonstrate higher levels of fasting glucose and HbA1c (39). Diabetic individuals carrying the rs363050 polymorphism also exhibit lower insulinaemia, possibly due to an impaired exocytosis machinery in beta cells (39). The rs3746544 and TaiI SNPs located in the 3’ UTR region of the human SNAP-25 mRNA have been associated with weight gain after antipsychotic treatment (37,38). Furthermore, SNP genotypes of rs362551 located 7 kb downstream of exon 8 of the Snap25 gene have been associated with the severity of the syndrome (40). (D) SNAP-25a and SNAP-25b differ only in 9 out of 206 amino acids. The two alternative exon 5 sequences both encode 39 amino acids spanning positions 56 to 94 in the SNAP-25 polypeptide. Abbreviations: VGCC, voltage-gated calcium channel; 3’UTR, 3’ untranslated region.
are not part of the α-helical structure of the protein and include both a Lys to Thr change at amino acid 84, and also the reorganization of a quartet of cysteines within the protein sequence. This quartet includes the only cysteines present in both SNAP-25a and SNAP-25b and these cysteine are substrates for the post-translational palmitoylation events that are required for membrane targeting of the protein (Fig. 1D).

SNAP-25 is essential for stimulated neuroexocytosis. Targeted disruption of the mouse Snap25 gene abolishes evoked synaptic transmission and mutant mice die at birth. Indeed, other mouse mutants of SNAP-25 have revealed that alterations in the total levels of SNAP-25 protein expression appear to affect neuronal function, especially if the SNAP-25a/SNAP-25b ratio is changed. The functional difference between SNAP-25a and SNAP-25b is still not fully understood, since the splice variants appear to be interchangeable in SNARE-mediated vesicle fusion. In neuronal cells, SNAP-25a seems to be the splice variant which is predominantly expressed during early development, whereas SNAP-25b is the major variant in adult neuronal populations. In other excitable cells, such as endocrine and neuroendocrine cells, SNAP-25a appears to be the dominant isoform throughout life. However, even in adulthood, the neuroanatomical distribution of SNAP-25a and SNAP-25b appears not to be strictly and permanently distributed among cell populations. Overexpression of the SNAP-25 isoforms in primary embryonic adrenal chromaffin cells from SNAP-25 null embryos demonstrated that SNAP-25b has a greater capacity to stabilize primed vesicles than SNAP-25a, leading to changes in the burst of Ca2+-evoked release. Taken together, it appears that alternative splicing between SNAP-25a and SNAP-25b fine-tunes the kinetics of regulated membrane fusion.

Obesity and type 2 diabetes (T2D) are complex pathophysiological disorders that can be associated with impairments in both energy metabolism and feeding behavior. These conditions possess a multifactorial pathogenesis and are most frequently not caused by single genetic mutations, but instead are triggered by ‘external risk factors’, such as increased calorie intake and decreased physical activity. Physiological mechanisms regulating feeding behavior are integral to the pancreas-gut-adipose tissue-brain axis (Fig. 2A). Therefore, adiposity and circulating hormones such as leptin, cholecystokinin, ghrelin and insulin are involved in the regulation of both energy balance and appetite/satiety via their direct or indirect actions on the brainstem and hypothalamic nuclei, as well as in peripheral metabolic organs. However, as it is considered that regulated membrane fusion is at the heart of the stimulus-activated information flow from excitable cells, we have speculated that even small differences in the dynamics of signaling molecule release can predispose to metabolic disease, particularly if combined with increased calorie intake.

To test our hypothesis, we placed male and female SNAP-25b-deficient mutant mice on a Western Diet (WD) rich in fat and simple carbohydrates. We discovered that, by interfering with the alternative splicing of SNAP-25, this mutation alone triggered dyslipidemia, adipocyte hypertrophy, impaired glucose homeostasis, hepatic steatosis and hypothalamic dysfunction, all of which are features of metabolic syndrome. Indeed, we found that in the hypothalamus of mutant mice, the active form of AMP-activated protein kinase (AMPK), a master metabolic regulator, was decreased compared to WT mice. We also noticed that activation of molecules involved in the leptin and insulin signaling pathways such as STAT3 and ERK1/2, was impaired in mutants, suggesting hypothalamic leptin and insulin resistance. Interestingly, the hypothalamic leptin receptor was found to be downregulated only in WD-fed animals.

Furthermore, we observed dramatic sex differences between male and female mice, both due to the mutation but also in the response to WD. In mutant females on control diet (CD), the body weight gain and increases in triglyceride levels appeared earlier than in males, most likely due to increased food intake. In male mutants, however, obesity was dependent on the failure of circadian feeding behavior, which resulted in constant eating throughout the day. Sex hormones are likely playing an important role in the susceptibility and development of the metabolic syndrome, and further scientific investigations should address this question.

A critical issue is, of course, the nature of the ‘weakest point’ that initially triggers the metabolic phenotype in our mouse mutants. Our data support the idea that it is a defect in the exocytosis machinery in those neurons, neuroendocrine and endocrine cells which regulate energy balance, rather than the diet itself, that drives dyslipidemia and derangements in glucose homeostasis. If this condition is originating from such excitable cells, then there are many possibilities: for example, a direct effect on insulin secretion from pancreatic β cells, or on other circulating hormones that are also released by regulated membrane fusion, such as cholecystokinin or ghrelin (Fig. 2A). Also, deleterious processes could be
Figure 2. (A) SNAP-25b deficiency and/or Western Diet (WD) intervention can impair the peripheral organ-brain circuitry in glucose-sensing organs. SNAP-25b deficiency and/or a WD intervention impair hypothalamic function, disrupting the balance between satiety and hunger signals which are integrated in this brain area (1). These pathophysiological conditions are exacerbated when combining a WD intervention with genetic manipulations affecting the alternative splicing of SNAP-25 (1). These events can also have an impact on sympathetic and parasympathetic efferents. Thus, we speculate that both SNAP-25b-deficiency and WD have a negative effect on sympathetic efferents from the hypothalamus that project to the intermediolateral cell column (in blue) and also impair the inputs from the PBN and the BLM. Parasympathetic efferents (in red) comprise projections from the BLM and the NTS. In our model, we hypothesize that alterations in the parasympathetic efferents affect the network of interactions between the hypothalamus, the BLM and the NTS. Sympathetic innervation triggers glucagon secretion from α cells, inhibits insulin secretion from β cells, and activates epinephrine secretion from the adrenal gland. By contrast, parasympathetic efferents stimulate insulin secretion and inhibit hepatic glucose production (50). It is also possible that SNAP-25b-deficiency, by directly affecting insulin secretion from pancreatic β cells, initiates the metabolic phenotype. Also note a possible involvement of sensory neurons. Abbreviations: BLM, basolateral medula; CMGP, celiac/mesenteric ganglion plexus; HTH, hypothalamus; NTS,
triggered by impaired signals from cells in the brainstem and hypothalamic nuclei that normally regulate energy balance and appetite/satiety (Fig. 2A). Another possibility is the presence of defects in peripheral nerves innervating target organs such as the pancreas, liver and adipose tissue (Fig. 2A).

The effect caused by the small mutation introduced into the mouse Snap25 gene is remarkable indeed. It is tempting to speculate that many individuals carrying an even more trivial genetic mutation that directly or indirectly affects hormonal or transmitter release in excitable cells have a predisposition for metabolic disease. In this respect it is intriguing that most genes that were identified as risk genes for T2D in GWAS were genes affecting β cell function and/or insulin release. Such a minor genetic vulnerability might be ‘silent’ and not noticed, until the individual increases the disease risk by combining it with an unhealthy lifestyle; this may include lack of physical activity or increased food intake. Many of the features of metabolic illness are manifested in cells such as adipocytes and hepatocytes that do not express SNARE proteins, including SNAP-25, that are the trademarks for neurons, neuroendocrine and endocrine cells. Therefore these injuries have to be secondary to the mutation, and a result of the intricate interplay between the liver, pancreas, adipose tissue, hypothalamus and/or nerves innervating these target organs (Fig. 2).

Numerous polymorphisms have been identified in the human gene for Snap25, and they have so far been associated with neuropsychiatric disorders and/or cognitive function. However, little is known about how these polymorphisms affect transcription of the Snap25 gene, or if they interfere with splicing, micro-RNA binding or otherwise affect mRNA stability. Interestingly, recent findings show that some of these polymorphisms are associated with ‘non-neuronal’ traits, such as weight gain after antipsychotic treatment, and altered levels of serum triglycerides, as well as with glycaemic parameters or with the severity of the metabolic syndrome in T2D. Furthermore, genes encoding proteins which directly or indirectly interact with SNAP-25 have been linked to childhood obesity, impaired glucose metabolism and obesity in adults, the requirement for insulin treatment, or the age of T2D onset. As neurons and endocrine cells are closely related, it is not surprising that, for example, mutations found in the Snap25 gene are also linked to metabolic traits such as weight gain. These mutations, which are located in the 3′ untranslated region (UTR) of the mRNA and which have previously been identified as being associated with ADHD, and possibly span the binding site for a micro-RNA, mir-641 (Fig. 2). In addition, a polymorphism previously associated with IQ has now been linked to higher levels of fasting glucose and lower serum insulin levels in T2D patients. It is intriguing that the same mutations in the gene for SNAP-25 demonstrate both central and peripheral phenotypes. This further strengthens our hypothesis that mutations in SNARE or SNARE interacting proteins can cause metabolic disease.

As T2D and obesity are increasing worldwide, there is an urgent need to find additional therapies to combat these disorders. Today, almost 382 million people are afflicted by T2D, and, in 2014, approximately 600 million people were obese, with almost 2 billion adults worldwide being overweight, according to the WHO. Obesity in itself causes a general, chronic low grade inflammation which not only increases the risk for T2D but also predisposes for other complications, such as cardiovascular disease and cancer. Other risk factors for T2D are physical inactivity, ethnicity, increasing age, high blood pressure and genetic predispositions. Metabolic disorders usually develop when several risk factors coincide, although there are exceptions. One example of such an exception is monogenic diabetes, or “Maturity Onset Diabetes of the Young” (MODY), which mainly affects β cell function. We have now demonstrated that manipulating the expression of the splicing variants of SNAP-25, a protein controlling the efficacy of regulated membrane fusion in neurons, neuroendocrine and endocrine cells, can create a phenotype which includes all of the features of metabolic syndrome. Further research will...
reveal, if there are corresponding monogenic mutations in SNAPRE or SNAPRE-regulating proteins in humans that can cause similar conditions. In conclusion, our recent discoveries have revealed additional mechanisms that may underlie the onset of metabolic disease. This seems encouraging for the future, as it opens up new avenues for therapy.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We would like to thank Dr. Neil Portwood for comments on the manuscript.

**Funding**

This work was supported by grants from The Swedish Research Council, The Family Erling-Persson Foundation, Karolinska Institutet Funds, Magnus Bergvall’s Foundation, Gun and Bertil Stohne’s Foundation, Längmanska Kulturfonden, Fogelström’s Foundation and Sven Mattsson's Foundation.

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