Calcium-sensing beyond neurotransmitters: functions of synaptotagmins in neuroendocrine and endocrine secretion

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Synopsis

Neurotransmitters, neuropeptides and hormones are released through the regulated exocytosis of SVs (synaptic vesicles) and LDCVs (large dense-core vesicles), a process that is controlled by calcium. Synaptotagmins are a family of type 1 membrane proteins that share a common domain structure. Most synaptotagmins are located in brain and endocrine cells, and some of these synaptotagmins bind to phospholipids and calcium at levels that trigger regulated exocytosis of SVs and LDCVs. This led to the proposed synaptotagmin–calcium-sensor paradigm, that is, members of the synaptotagmin family function as calcium sensors for the regulated exocytosis of neurotransmitters, neuropeptides and hormones. Here, we provide an overview of the synaptotagmin family, and review the recent mouse genetic studies aimed at understanding the functions of synaptotagmins in neurotransmission and endocrine-hormone secretion. Also, we discuss potential roles of synaptotagmins in non-traditional endocrine systems.

Key words: calcium sensor, hormone secretion, neuropeptide, neurotransmitter, regulated exocytosis, synaptotagmin

INTRODUCTION

The classical work of Katz and colleagues (see [1–5]) more than half a century ago established the quantum nature of neurotransmitter release and the pivotal role of calcium in regulating the probability of a given quantum being released. These fundamental findings, particularly the function of calcium in exocytotic secretion, were later extended and confirmed in neuroendocrine and endocrine systems, including adrenal chromaffin cells and pancreatic β- and α-cells [6–9]. The identification of proteins that can transduce calcium signals to exocytotic machineries has been of tremendous interest among neuroscientists and cell biologists, as this would lead to the elucidation of the fundamental neuronal function, namely synaptic transmission, and the general mechanisms of regulated exocytosis. Synaptotagmin-1, which was described as a protein on SVs (synaptic vesicles) and LDCVs (large dense-core vesicles) by an antibody-binding study [10], emerged as a leading candidate, and was suggested to function as a calcium sensor for synaptic exocytosis based on molecular and structural analysis [11]. It is now known that synaptotagmin-1 belongs to a type 1 membrane-protein family of at least 15 members (Table 1), and that some synaptotagmins are expressed primarily in neurons, and neuroendocrine and endocrine cells, with calcium-binding affinities in the range of 1–40 µM, the levels at which regulated exocytosis is triggered in these cells [12]. Because of its potential role as a calcium sensor for SV and LDCV exocytosis, the synaptotagmin family has attracted considerable interest over the past 20 years. A number of bioinformatics studies and in-depth reviews have been published on various aspects of the synaptotagmin family, for example, synaptotagmin gene structures and phylogeny [13–16], protein structure and function [17–19], and molecular mechanisms of its actions in neurotransmission and SV exocytosis [20–26]. In the present review, we provide an overview of the mammalian synaptotagmin family, and focus on recent mouse genetic studies that provide an understanding of the functions of synaptotagmins in neurotransmission and endocrine-hormone secretion. In addition, we discuss potential roles of the mammalian synaptotagmin family in atypical endocrine systems and non-classical secretory-granule exocytosis.

Abbreviations used: B/K protein, brain/kidney protein; E-Syt, extended synaptotagmin-like protein; GIIP, gastric inhibitory polypeptide, also known as glucose-dependent insulino-tropic peptide; GIIP1, glucagon-like peptide 1; LDCV, large dense-core vesicle; MCTP, multiple C2-domain and transmembrane protein; RNAi, RNA interference; SNARE, soluble N-ethylmaleimide-sensitive fusion-protein attachment protein receptor; Strep14, synaptotagmin XIV-related protein; SV, synaptic vesicle.
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*The gene name for synaptotagmin-5 is synaptotagmin IX.
†The gene name for synaptotagmin-9 is synaptotagmin V.
‡Only the short form of synaptotagmin-7 is listed in the Table. Synaptotagmin-7 is expressed in multiple splicing variants, ranging from 122 amino acids for the truncated form to 687 amino acids for the long form.

THE SYNAPTOTAGMIN FAMILY

By definition, a synaptotagmin is composed of a short N-terminal sequence followed by an N-terminal transmembrane region, a linker of variable length and two functional C₂ domains: C₂A and C₂B (see Figure 1 and below for more discussion on C₂ domains) [16]. So far, at least 15 synaptotagmins have been identified and cloned, which can be divided into five groups based on their genomic and sequence similarities (see Figure 1 and Figure 2 for details) [14,16]. Several reports described or referred to Strep14 (synaptotagmin XIV-related protein) and B/K protein (brain/kidney protein) as the two latest members of the synaptotagmin family, namely synaptotagmin-16 and -17 (Figure 2 and Table 1) [14,15,27,28]. Although these two ‘synaptotagmins’ have some of the features shared by the synaptotagmins, such as two C-terminal C₂ domains, they lack an N-terminal transmembrane region and therefore they should not be classified as synaptotagmins. Two other protein families, rabphilin and Doc2 (double C₂ protein), also contain two C-terminal C₂ domains, and, similar to Strep14 and B/K protein, they lack transmembrane regions, and thus are related to synaptotagmins, but are not synaptotagmins in the strict sense of the term [29–32]. In addition to synaptotagmins, there are several other families of membrane proteins with sequence and structural similarities to synaptotagmins. E-Syts (extended synaptotagmin-like proteins) contain an N-terminal transmembrane region but more than two C₂ domains [33,34], ferlins contain three to six C₂ domains and a C-terminal transmembrane region [35], and MCTPs (multiple C₂-domain and transmembrane proteins) contain three C₂ domains and two transmembrane regions (Table 2) [36].
Functions of synaptotagmins in endocrine secretion

Table 2 Other C2-containing membrane proteins

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Figure 2 Phylogenetic tree for mouse synaptotagmins (mSyts), Syt14l and B/K protein

Synaptotagmins are grouped into five subfamilies. Note that Syt14l and B/K protein are grouped together with synaptotagmin-12 to -15. However, these two proteins are not part of the synaptotagmin family due to their lack of N-terminal transmembrane regions. Syt14l, synaptotagmin 14-like protein (also known as synaptotagmin 14-related protein).

The precise tissue distribution, cellular localization and functions of these other protein families are mostly unknown, although certain members of the ferlins are linked to muscular dystrophy, which may be caused by impaired membrane fusion and consequent defects in plasma-membrane repair [33,35,36].

The C2 domain is a widely occurring conserved sequence motif of 130–140 amino acid residues, which was first defined as the second constant sequence in protein kinase C isoforms [11,17,18]. The C2 domain was first shown to bind to calcium in synaptotagmin-1 [17,18,37,38]. Subsequent atomic-structure analysis of synaptotagmin-1 at 1.9 Å (1 Å = 0.1 nm) resolution indicated that its C2 domains are composed of a stable eight-stranded β-sandwich with flexible loops emerging from the top and bottom (Figure 3) [18,39]. NMR studies of synaptotagmin-1 revealed that calcium binds exclusively to the top loops, and the binding pockets are coordinated by five conserved aspartate residues: three calcium ions bind to C2A via Asp172, Asp238, Asp230, Asp178, and Asp232, and two calcium ions bind to C2B via Asp303, Asp309, Asp365, Asp363 and Asp371 (Figure 3) [40–42]. Not all synaptotagmin C2 domains bind to calcium. In fact, based on sequence similarities and subsequent confirmation by biochemical analyses, only eight synaptotagmins bind to calcium, namely synaptotagmin-1, -2, -3, -5, -6, -7, -9 and -10. The lack of critical residues involved in calcium binding accounts for the majority of the failure of the other synaptotagmins to bind to calcium. This applies to both of the C2 domains of synaptotagmin-8, -12, -13, -14 and -15, and the C2A domain of synaptotagmin-4 and -11 (Figure 3) [16,43,44]. Although the C2B domains of synaptotagmin-4 and -11 possess all five acidic residues in the top loops, they do not bind to calcium due to the spatial orientation of the calcium ligands, which fail to form proper calcium-binding sites (Figure 3) [45]. For calcium-binding synaptotagmins, although amino acid residues in the top loops other than those mentioned above are not directly involved in coordinating calcium binding, they affect calcium-binding affinity, such as Arg233 in synaptotagmin-1 [40,41,46,47]. The diversity of sequences and structures flanking the calcium-coordinating amino acid residues enables the eight synaptotagmins to bind to calcium with various affinities, covering the full range of calcium requirements for regulated exocytosis [11,12,16,48–51].

THE SYNAPTOTAGMIN–CALCIUM-SENSOR PARADIGM

As alluded to earlier, the regulated exocytosis of SVs in neurons, and of LDCVs in neuroendocrine and endocrine cells, is triggered by calcium. To function as a calcium sensor in regulated exocytosis, a protein must satisfy the following criteria: first, it must have proper calcium affinity to detect a rise in intracellular calcium levels during the release processes; secondly, it must show a calcium cooperativity that is consistent with the calcium dependence of triggered exocytosis. In the case of SV exocytosis, neurotransmitter release is composed of two kinetically distinct components: a major synchronous component and a delayed asynchronous component [20,52,53]. For the synchronous component, the required calcium concentrations are in
Figure 3  For legend see facing page
the range of \( \sim 10-40 \ \mu M \) and the calcium-cooperativity value is \( \sim 5 \), as determined by the photolysis of caged calcium in the calyx of Held giant synapse, the best and most systematically analysed preparation to date [24,54–58], whereas the calcium requirement for the asynchronous component is estimated to be in the range of several \( \mu M \) to several tens of \( \mu M \), with a calcium cooperativity value of 2–3 [24,58]. Remarkably, synaptotagmin-1, which was previously proposed to function as a calcium transducer for neurotransmitter release based on biochemical studies [11], meets the requirements for the synchronous calcium sensor, an idea that is supported and confirmed by two important mouse genetic studies.

The first genetic study was performed on hippocampal neurons of synaptotagmin-1 knockout mice, which showed a complete absence of the major synchronous component of neurotransmitter release, demonstrating that synaptotagmin-1 is required for synchronous neurotransmitter release [24,59,60]. It is worth noting that the essential role of synaptotagmin-1 in neurotransmission was also shown in synaptotagmin-1 null mutants of Drosophila and Caenorhabditis elegans [61–64]. The second genetic study was carried out on hippocampal neurons of a synaptotagmin-1 knockin mouse harbouring a point mutation (R233Q) in the synaptotagmin-1 C2A domain that alters the overall apparent calcium affinity of synaptotagmin, which showed an identical shift in the apparent calcium affinity of SV exocytosis [46]. Besides these genetic studies, two other studies that used synaptotagmin-1 knockin (D232N and D238N) mice [47,65] and two studies that used the viral expression of various synaptotagmin-1 mutants in synaptotagmin-1 knockout neurons [66,67] provide further support for the conclusion of the R233Q knockin study: the overall calcium affinity of synaptotagmin-1 determines the calcium affinity of SV exocytosis. Together with the calcium-binding properties, these genetic studies of synaptotagmin-1 established its function as a calcium sensor for synchronous neurotransmitter release [24].

Subsequent genetic analyses of synaptotagmin-2 and -9 knock-out mouse lines provided unequivocal evidence that these two synaptotagmins also function as synchronous calcium sensors for neurotransmitter release, synaptotagmin-2 for neurons in the caudal brain regions and synaptotagmin-9 for neurons in the limbic system, which complement synaptotagmin-1 as the fast calcium sensor for neurons in the rostral brain regions [24,46,48,58,59,68–71]. The differential distribution and distinct properties of the three neuronal calcium sensors support the emerging synaptotagmin–calcium-sensor paradigm, namely that synaptotagmins serve as individually acting \( \text{Ca}^{2+} \) sensors in neurotransmitter release [24,46,48,58,59,68–71]. However, this also raises several important questions. What are the identities of the calcium sensors for asynchronous neurotransmitter release? What is the function of non-calcium-binding synaptotagmins?

**CALCIAL SENSORS FOR ASYNCHRONOUS NEUROTTRANSMITTER RELEASE**

Synchronous neurotransmitter release is a highly specialized process and the fastest in cell biology. Thousands of vesicles can be released with a lag time as short as 60 \( \mu s \) in certain synapses [57,72–74]. This process is estimated to require calcium in the 10–40 \( \mu M \) range and a calcium-binding cooperativity of 5 [24,54–58]. In comparison, asynchronous neurotransmitter release is considerably slower, and requires slightly lower levels of calcium and a lower calcium-binding cooperativity [58]. It is clear that synaptotagmin-1, -2 and -9 function as the principal calcium sensors for synchronous neurotransmitter release in their respective synapses [46,58,59,68,69,75,76]. For calcium-sensing during asynchronous neurotransmitter release, additional proteins must be involved. One possibility is that SVs for fast and slow release are molecularly distinct, i.e. SVs possessing fast calcium sensors are destined for fast synchronous release and those with slow sensors for delayed asynchronous release. However, there is no evidence that these different pools of SVs with different molecular compositions exist. Another possibility, which also assumes the existence of SV pools of distinct molecular identities, is that different ratios and/or combinations of proteins on SVs determine how a particular SV is released. A third possibility, which is supported by experimental data and mathematical modelling, is the dual-sensor model: calcium sensors for synchronous and asynchronous release operate in competition with each other, with the slower asynchronous sensor binding to calcium at lower concentrations and cooperativity, whereas the faster synchronous sensor binds at higher concentrations and cooperativity [58,77,78]. This model, which predicts that the synchronous sensor dominates during pulses of high calcium concentrations, while the asynchronous sensor dictates release events during sustained phases of lower calcium concentrations, is supported by the demonstration in the calyx of Held synapse that synchronous and asynchronous release operate on the same vesicle pool [58].

As only synaptotagmin-1, -2 and -9 are principal calcium sensors for neurotransmitter release in their respective brain

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**Figure 3** Sequence alignment for mouse synaptotagmin (mSyt)-family proteins

C2A (A) and C2B (B) of synaptotagmins were aligned using the AlignX® program of Vector NTI® (Invitrogen). Red bars labelled \( \beta \)-1–\( \beta \)-8 mark the eight \( \beta \)-strands in the C2 domains. Blue bars mark the two \( \alpha \)-helices in the C2B domain. Inverted filled triangles denote the acidic residues that form the calcium-binding pockets. Colour highlighting of the sequences: yellow and blue denote identical amino acids in all (yellow) or most (blue) synaptotagmins, and green indicates conserved amino acid substitutions.
Table 3 Comparison of neurotransmitter release with peptide secretion

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<th>Peptide secretion</th>
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<td>Peptides/hormones (e.g. insulin, catecholamines)</td>
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<td>Synaptic vesicles</td>
<td>LDCVs or secretory granules</td>
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<tr>
<td>Speed</td>
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<td>Slow (up to tens of s)</td>
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<tr>
<td>Duration of action</td>
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<tr>
<td>Calcium requirement</td>
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<td>Lower (several to tens of μM)</td>
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*SVs may be recycled locally or regenerated through the endosomal pathway.
†Hot spots, analogous to the synaptic active zone, were reported to be present on the plasma membrane of neuroendocrine cells [189].

regions, other calcium-binding synaptotagmins (synaptotagmin-3, -5, -6, -7 and -10) may function as slow sensors in cooperation with one of the principal sensors. Support for this hypothesis comes from the observations that these five other synaptotagmins bind to calcium at higher affinities [12,24,79]. Of these five synaptotagmins, only synaptotagmin-7 was investigated for its role as a calcium sensor in GABAergic neurons from a mouse knockout model: deletion of synaptotagmin-7 did not change fast synchronous release, slow asynchronous release or short-term synaptic plasticity of release of neurotransmitters [80]. This study indicated that synaptotagmin-7 is unlikely to be a slow sensor for inhibitory neurons in the cortex, but may function in other forms of calcium-dependent synaptic exocytosis or peptide-hormone secretion [80].

FUNCTIONS OF NON-CALCIUM-BINDING SYNAPTOTAGMINS

Seven of the 15-member synaptotagmin family (synaptotagmin-4, -8, -11, -12, -13, -14 and -15) do not bind to calcium, and some of these non-calcium-binding synaptotagmins are present at high levels in neurons and endocrine cells (see below for a discussion on particular non-calcium-binding synaptotagmins in endocrine secretion) [12,16,23,24,81,82]. As they do not bind to calcium, these synaptotagmins cannot function as individually acting calcium sensors for various release processes. They may, however, regulate calcium-independent vesicle exocytosis, such as calcium-independent voltage-dependent secretion in dorsal root ganglia neurons [83], or partner with a calcium-binding synaptotagmin to regulate calcium-dependent neurotransmitter release.

Among the seven non-calcium-binding synaptotagmins, very little information is available for synaptotagmin-13, -14 and -15 beyond descriptions of molecular cloning and basic characterizations [28,43,84]. Synaptotagmin-12 forms a stable complex with synaptotagmin-1 on SVs, but regulates spontaneous vesicle release independent of synaptotagmin-1 [85]. Synaptotagmin-11 has been linked to neurological and neurodegenerative diseases, but its molecular functions are unclear [86,87]. Synaptotagmin-8 is present in kidney epithelia and sperm acrosomes, but is absent or present at very low levels in the brain [82,88,89]. Synaptotagmin-4 is the best-studied non-calcium-binding synaptotagmin, but it is expressed at rather low levels (lower than, for example, its close relative, synaptotagmin-11 [82]), and its tissue distribution, cellular localization and function remain inconclusive or even controversial. For example, synaptotagmin-4 was reported to be located on LDCVs to regulate secretory-granule maturation and fusion-pore kinetics during regulated exocytosis [90–93], on SVs in hippocampal neurons but without a role in regulating fusion-pore kinetics or calcium-dependent SV exocytosis [94], on Golgi and non-LDCV vesicular structures in PC12 cells (a tumour cell line derived from rat adrenal medulla) [95], in the postsynaptic compartment [96,97], and on astrocytes to regulate calcium-dependent vesicle exocytosis [98,99]. More studies are needed to understand whether and how the non-calcium-binding synaptotagmins function in regulated exocytosis.

CALCIUM SENSORS IN NEUROENDOCRINE AND ENDOCRINE SECRETION

Although neurotransmitter release and peptide-hormone secretion have distinct characteristics (Table 3), the fundamental mechanisms of regulated exocytosis in neuronal, neuroendocrine and endocrine cells are quite similar, in that they all depend on a conserved core machinery for vesicle fusion at the plasma membrane. The proteins involved in regulated exocytosis in synapses and neuroendocrine cells, and the distinct characteristics of these two types of regulated exocytosis, have been extensively studied and reviewed (for reviews, see [22–25,100–107]). This section of the review focuses on recent studies, particularly mouse genetic studies on calcium-sensing in primary neuroendocrine and
endocrine cells, with the occasional mention of relevant studies using derived tumour cell lines.

Adrenal chromaffin cells
Adrenal chromaffin cells and the PC12 tumour cell line derived from them are one of the most widely used cell systems in studying regulated exocytosis [2,6,100–103,105,108–112]. Pancreatic β-cells are another popular cell model, which will be discussed below [113–115]. Many fundamental studies regarding regulated exocytosis and the identities of calcium sensors were performed in PC12 cells (e.g. [12,79,116–122]), some of which have been confirmed by subsequent genetic studies [123–129]. However, there are discrepancies between studies performed on PC12 and adrenal chromaffin cells in certain cases, due to different molecular compositions of protein isoforms in these two cell types [130–133]. Regarding calcium-sensing in adrenal chromaffin cells, the first functional genetic analysis was carried out in synaptotagmin-1 knockout mice [134]. Similar to neurotransmitter release, catecholamine release from adrenal chromaffin cells also follows rapid and delayed phases [135–137]. Using a combination of calcium-uncaging and membrane-capacitance measurements, Voets et al. [134] showed that synaptotagmin-1 deletion specifically abolished the fast burst of exocytosis that is supported by the readily releasable pool of vesicles in chromaffin cells. Consistent with SV exocytosis studies of synaptotagmin-1 knockout, this report demonstrated that synaptotagmin-1 was required for the rapid calcium-dependent fusion of LDCVs, and suggested that synaptotagmin-1 functions as a calcium sensor during the rapid phase of LDCV exocytosis in chromaffin cells [134].

Similar to neurotransmitter-release studies, the specific effects of synaptotagmin-1 deletion on the rapid phase of LDCV exocytosis prompted strong interest in the search for an additional calcium sensor that complements synaptotagmin-1 to regulate the delayed phase of LDCV exocytosis. Besides synaptotagmin-1, synaptotagmin-7, but not synaptotagmin-2 or -9, is also expressed in adrenal chromaffin cells [132]. Synaptotagmin-7 is ubiquitously expressed during early stages of development, but becomes restricted to secretory cells with regulated exocytosis after birth [138]. Among the eight calcium-binding synaptotagmins, synaptotagmin-7 shows maximal calcium-dependent phospholipid binding in the low-micromolar calcium range [12], which fits well with the calcium requirement during delayed exocytosis in chromaffin cells [134]. Schonn et al. [132] studied the role of synaptotagmin-7 in chromaffin-granule exocytosis on three genetically modified mice and found that: (i) deletion of synaptotagmin-7 alone impaired calcium-triggered exocytosis by ∼50%; (ii) calcium-binding to synaptotagmin-7 C2B was essential for the normal function of synaptotagmin-7, as the impaired exocytosis observed in synaptotagmin-7 knockout mice was reproduced in synaptotagmin-7 knockin mice that expressed normal levels of synaptotagmin-7 with a mutated C2B domain that resulted in the complete loss of calcium binding; (iii) both the fast and the delayed LDCV exocytosis were nearly abolished in synaptotagmin-1 and -7 double-knockout mice. These results established that synaptotagmin-7 is a major calcium sensor for chromaffin-granule exocytosis, and functions in complement with synaptotagmin-1 in the regulation of calcium-dependent LDCV exocytosis in adrenal chromaffin cells [132].

Pancreatic islet β-cells
Among endocrine cells, pancreatic β-cells are probably the most extensively studied cell model because of the great importance of insulin secretion in normal physiology and in diabetes [113–115]. Insulin is stored and secreted from β-cells by a complex, highly regulated process. Under physiological conditions, an elevation of blood glucose triggers rapid uptake of glucose into pancreatic β-cells. Glucose metabolism inside β-cells results in an increased ATP/ADP ratio, which leads to K$_{ATP}$-channel closure, membrane depolarization and the subsequent opening of voltage-gated calcium channels and a rise in cytoplasmic calcium concentration [114]. Glucose-stimulated insulin secretion has a biphasic pattern, consisting of a 10–15-min rapid first phase and a less prominent but sustained second phase [139]. The first phase of insulin secretion requires a rapid and marked elevation of intracellular calcium concentration, whereas the second phase requires amplifying signals from glucose metabolism in addition to oscillatory intracellular calcium [140]. Insulin secretion by calcium-dependent LDCV exocytosis is probably executed by SNARE (soluble N-ethylmaleimide-sensitive fusion-protein-attachment protein receptor) proteins, similar to neurotransmitter release in central neurons and neuropeptide secretion from chromaffin cells [7,22]. In addition to SNARE proteins, numerous exocytotic proteins that may be involved in insulin-secretion regulation have been identified [104,113], although functional roles of these proteins in insulin secretion remain to be established.

Regarding calcium-sensing in insulin secretion, most of the studies were performed using insulin-secreting cell lines, for example, INS-1, RINm5F and MIN6 cells. These studies on the potential roles of individual synaptotagmins and other proteins, such as calpain and calcium channels, in insulin secretion in insulin-secreting cell lines were recently reviewed or reported [113,141,142]. The rest of this section will focus on two calcium-binding synaptotagmin isoforms that show the highest transcript levels in pancreatic islets: synaptotagmin-7 and -9 [81]. Synaptotagmin-7 is expressed in LDCVs of pancreatic β-cells [81,143]. Although no consensus has been reached in terms of the precise calcium levels that support insulin-granule exocytosis, it is estimated to be in the low-micromolar range, similar to that in neuroendocrine cells, such as adrenal chromaffin cells [144–147]. Studies have shown that synaptotagmin-7 is present and appears to regulate insulin secretion in insulin-secreting cell lines [143,148]. Physiological analysis of synaptotagmin-7 knockout mice demonstrates that deletion of synaptotagmin-7 leads to glucose intolerance and impaired insulin secretion in vivo, as well as markedly reduced insulin secretion from isolated islets [81]. Synaptotagmin-7 knockout mice exhibit normal insulin sensitivity, insulin production, islet architecture and β-cell ultrastructural organization [81,149]. Furthermore, the knockout mice display normal metabolic and calcium responses after glucose stimulation [81]. These results show that reduced insulin secretion in
synaptotagmin-7 knockout mice is not caused by cellular defects or impaired signalling upstream of calcium triggering of insulin-granule exocytosis, and reveal that synaptotagmin-7 functions as a positive regulator of insulin secretion, consistent with its proposed role as a high-affinity calcium sensor regulating insulin secretion.

As synaptotagmin-7 deletion only reduces insulin secretion by ∼40–50%, other protein(s) must be involved in regulating the remaining LDCV exocytosis in pancreatic β-cells. One probable candidate is synaptotagmin-9, which is expressed at similar levels to synaptotagmin-7 in mouse islets, and at least 6–8-fold higher than the two next-highest-expressed calcium-binding synaptotagmins, synaptotagmin-2 and -3 [81]. Synaptotagmin-9 binds to calcium and phospholipids with an affinity of 10–30 μM [51]. Moreover, synaptotagmin-9 is present in β-cells, and knockdown of synaptotagmin-9 by adeno-viral-mediated RNAi (RNA interference) results in impaired insulin secretion in rat islet cells [113,150]. The in vitro results appear to support synaptotagmin-9 as another calcium sensor in regulating insulin-granule exocytosis. However, RNAi often has off-target effects [151,152], and a more specific method, such as the analysis of synaptotagmin-9 knockout mice, is needed to test the functions of synaptotagmin-9 in regulating insulin secretion at the final calcium-dependent exocytotic steps.

Since synaptotagmin-7 knockout and synaptotagmin-9 knockdown each reduces insulin secretion by ∼40–50%, the question remains as to whether synaptotagmin-7 and -9 act independently from each other, each being responsible for a subset of insulin secretion, or whether synaptotagmin-7 and -9 work as a team and together are responsible for the ∼40–50% secretion of insulin. To address this issue, it will be necessary to generate synaptotagmin-7 and -9 double-knockout mice to test: (i) whether deletion of both synaptotagmin-7 and -9 causes further inhibition of insulin secretion beyond the 40–50% level, as observed in synaptotagmin single-knockout mice. If the inhibition effect is additive, i.e. the deletion of both synaptotagmin-7 and -9 abolishes insulin-granule exocytosis, then synaptotagmin-7 and -9 function independently. If the inhibition remains at 40–50%, then synaptotagmin-7 and -9 function as partners to regulate the same pool of LDCVs. The situation is complicated greatly if the inhibition falls in between the two scenarios, as partial compensation by other synaptotagmins or proteins may be involved; (ii) whether there is any residual calcium-dependent insulin secretion in the absence of synaptotagmin-7 and -9. The presence of a residual calcium-dependent component would indicate the existence of a third calcium sensor.

**Pancreatic islet α-cells**

Glucagon is stored in LDCVs of pancreatic α-cells, and is secreted in response to low blood-glucose levels to stimulate glucose production and glucose release in the liver to restore normal glucose levels [153]. Although both insulin and glucagon regulate glucose homeostasis, far fewer studies have been devoted to the regulation of glucagon secretion than that of insulin release: currently, there is only one glucagon-secretion study for every five insulin-secretion reports. It is no wonder that after almost fifty years since the description of the glucagon-quantification method [154–156], many aspects of glucagon-secretion regulation remain unclear or are just beginning to be unravelled [9,157]. The control of glucagon secretion is very complicated, and involves direct calcium-mediated stimulus-secretion coupling [158], paracrine regulation by insulin, γ-aminobutyric acid, zinc and other factors released from neighbouring β- and δ-cells, and circulating hormones and the autonomic nervous system [9]. An in-depth and comprehensive review on many aspects of glucagon secretion and α-cell biology was published recently [9].

Although the precise cellular mechanism of glucagon-granule exocytosis remains incompletely defined, it is clear that K<sub>ATP</sub> channels and N-type calcium channels are intimately involved in the process [158–162], and calcium provides the trigger for LDCV exocytosis in α-cells [8,9]. Very recently, it was reported that synaptotagmin-7 functions as an individually acting calcium sensor that mediates nearly all calcium-triggered glucagon-granule exocytosis [163]. To date, this remains the only study on calcium sensors for glucagon secretion. This study showed that deletion of synaptotagmin-7 nearly abolished (a reduction of ∼80%) glucagon secretion induced by hypoglycaemia in vivo, by low glucose in isolated islets or by membrane depolarizations in single α-cells, whereas all other physiological and morphological parameters, for example, glucagon content, glucagon-granule number and distribution, and α-cell electrical properties, are unchanged, and thus identified synaptotagmin-7 as the principal synaptotagmin isoform required for glucagon secretion [163].

The deletion of synaptotagmin-7 results in ∼40% reduction in insulin secretion, an effect that is reminiscent of neuropeptides and catecholamine release in synaptotagmin-7 knockout chromaffin cells, which rely on two synaptotagmin isoforms: synaptotagmin-1 and -7 [81,132]. This raised the question of the relative importance of synaptotagmin-7 in regulated exocytosis. On the one hand, synaptotagmin-7 is essential for calcium-dependent LDCV exocytosis in chromaffin and pancreatic β-cells, as synaptotagmin-7 deletion causes significant impairment to regulated exocytosis [81,132]. On the other hand, it appears that synaptotagmin-7 always relies on another calcium sensor, for example, synaptotagmin-1 in chromaffin cells, to exert its actions in calcium sensing, thus making synaptotagmin-7 an accessory calcium sensor to the principal calcium sensor [132]. These observations raised further questions: whether the synaptotagmin–calcium-sensor theory applies only to neuronal calcium sensors, i.e. synaptotagmin-1, -2 and -9, but not to neuroendocrine or endocrine secretion; and whether regulated exocytosis in neuroendocrine and endocrine cells always involves the collaboration of two synaptotagmin isoforms. The glucagon-secretion study in synaptotagmin-7 knockout mice identified synaptotagmin-7 as an individually acting calcium sensor and the principal synaptotagmin isoform regulating glucagon secretion, and thus validates the synaptotagmin–Ca<sup>2+</sup>-sensor paradigm, and more importantly, extends the theory beyond the established neuronal Ca<sup>2+</sup>-sensors for synaptic vesicle
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Figure 4 Comparison of adipocyte models
(A) Differentiated 3T3-L1 cells. Lipid droplets are shown in red. (B) Differentiated mouse stromal vascular cells. (C) Primary adipocytes from mouse epididymal fat pad. Arrows denote adipocyte nuclei. Scale bars for (A), (B) and (C) are 40 μm, 20 μm and 40 μm respectively. Images of adipocytes were kindly provided by Dr Parasuraman Padmanabhan, Dr Wenjie Qiu and Mr Chun-Yan Lim, Laboratory of Metabolic Medicine, Singapore Bioimaging Consortium, A*STAR.

exocytosis, i.e. synaptotagmin-1, -2 and -9, to include synaptotagmin-7 as an individually acting high-affinity Ca^{2+}-sensor for LDCV exocytosis [163].

Clearly, synaptotagmin-7 does not always function as an individually acting calcium sensor, as in the case of adrenal chromaffin and pancreatic β-cells, where multiple synaptotagmins function together to regulate neuropeptide and hormone secretion [81,132]. Besides a calcium-sensing role in endocrine secretion, synaptotagmin-7 was also proposed to regulate Ca^{2+}-dependent lysosome fusion during wound repair [164–166], glucose transporter 4 trafficking [149], and osteoclast and osteoblast secretion [167], although alternative interpretations were offered regarding some of the proposed functions [168,169]. Nonetheless, these studies suggest that synaptotagmin-7 may function as a non-selective regulator for various membrane-fusion events outside neuroendocrine and endocrine systems.

Adipocytes

Neuroendocrine and endocrine systems perform their regulatory functions in maintaining physiological homeostasis by releasing signalling molecules, such as adrenaline, growth hormone and insulin, to act on target sites, often far away from the release sites. These include the well characterized adrenal chromaffin cells and insulin-secreting β-cells. Neuroendocrine and endocrine systems are distinguished from exocrine and paracrine systems by at least three major characteristics: (i) the signalling molecules are released directly into the blood or interstitial fluids; (ii) the signalling molecules act on remote targets; and (iii) the signalling molecules are released via regulated exocytosis of LDCVs.

After the discovery of leptin in 1994, adipocytes, from which leptin is secreted, have also been classified as part of the endocrine family [170–174]. Besides leptin, several other hormones and cytokines are also secreted from adipocytes, including adiponectin, tumour necrosis factor α and interleukin-6 [173,175,176]. These hormones and cytokines that are released from adipocytes are collectively referred to as adipokines [177]. Similarly to neuroendocrine and endocrine systems, adipokines secreted from adipocytes are also transported via the blood and act on remote targets, for example, leptin regulates hypothalamic neurons after passing through the blood–brain barrier [178–180]. However, it is not clear whether adipokines are secreted through the constitutive pathway or regulated exocytosis, another common feature of neuroendocrine and endocrine systems. Moreover, it has not yet been determined how adipokines are stored inside adipocytes prior to their release, and whether different adipokines share common storage systems or secretory pathways. Finally, there has not yet been a systematic analysis of the molecular regulation of adipokine secretion in primary adipocytes or a mouse model. Previous studies relied on differentiated 3T3-L1 and other derived cells as model systems for adipocytes; however, these cell models have dramatically different morphology and molecular composition from primary adipocytes (Figure 4). Therefore, it is necessary to examine the following in primary adipocytes: (i) how adipokines are stored (are they stored in LDCVs or similar subcellular structures, or simply produced when needed?); (ii) whether various adipokines are secreted through distinct pathways (leptin and adiponectin secretion follows very different patterns, particularly in obese animals), and whether there is regulation beyond the levels of transcription and translation; (iii) whether adipokine secretion follows regulated exocytosis and/or the constitutive pathway, which cellular signals trigger the release and whether calcium is involved; (iv) the nature of the precise molecular machinery that is used in the transport and fusion of adipokine-containing structures.

At present, none of these questions have been addressed. Interestingly, similar questions on cellular and molecular mechanisms and the regulation of cytokine secretion in macrophages were also proposed, and recent progress in cytokine secretion was reviewed...
by Stow et al. [181]. Although adipocytes and macrophages are quite different, the secretion mechanisms of adipokines from adipocytes may be more similar to cytokine secretion in macrophages than to insulin/glucagon secretion from pancreatic β- and α-cells. Thus, the cytokine studies in macrophages may offer clues on understanding adipokine-secretion mechanisms.

Regarding a potential role of calcium in leptin secretion, one study found that blocking calcium entry inhibits acute leptin secretion in cultured adipocytes [182], whereas another study using rat adipocytes showed that calcium elevations inhibited insulin-induced leptin secretion [183]. This discrepancy may reflect differences between the cultured and freshly isolated primary adipocytes. Nonetheless, calcium appears to have an active role in leptin secretion. Interestingly, several synaptotagmins, including non-calcium-binding synaptotagmin-13, are expressed in adipocytes, and the expression of some synaptotagmins is developmentally regulated during adipocyte differentiation, for example, the long form of synaptotagmin-7 is significantly up-regulated at 3–7 days after adipocyte differentiation (Y. Wang and W. Han, unpublished work). Further analysis is needed to establish whether calcium is a triggering signal for adipokine secretion, and whether synaptotagmins play a role in adipokine exocytosis.

**Enteroendocrine cells**

Enteroendocrine, or gut endocrine, cells refer mainly to two cell lineages, K cells, found in mucosa of the duodenum and the jejunum of the gastrointestinal tract, and L cells, located at the distal gut, predominantly the ileum and colon. K cells secrete GIP (gastric inhibitory polypeptide, also known as glucose-dependent insulinotropic peptide) and L cells secrete GLP-1 (glucagon-like peptide 1). GIP and GLP-1 belong to a family of molecules called incretins, which potentiate glucose-stimulated insulin secretion, suppress glucagon secretion, inhibit gastric emptying, and reduce appetite and food intake [184]. Owing to the potential significant health benefits and implications in diabetes/obesity treatment, the incretins have received attention from pharmaceutical industries and academic researchers, with pharmaceutical companies focusing on developing long-lasting analogues of incretins and inhibitors of the enzyme that degrades incretins, and academic researchers studying the cellular and molecular mechanisms of the effects of incretins on the brain, liver, muscle, adipose tissue and pancreas.

The fact that K and L cells are scattered in the gastrointestinal tract without distinct morphology from neighbouring cells makes it very difficult to study cell biology and molecular mechanisms of secretion regulation in primary cells. Thus, most of the previous studies to understand the cellular regulation of incretin secretion were performed on cell lines, such as GLUTag cells, a stable immortalized murine enteroendocrine cell line that expresses the glucagon gene and secretes GLPs in a regulated manner [185]. Recently, Reimann et al. [186] used a transgenic approach to specifically label L cells by expressing a fluorescent protein under the control of the proglucagon promoter, and found that GLP-1 secretion is regulated by the activity of sodium–glucose cotransporter 1 and ATP-sensitive K+-channels. When considering this finding and a previous report from the same group that GLP-1 secretion requires membrane depolarization and calcium entry through L-type calcium channels [187], it is tempting to speculate that GLP-1 secretion may operate in a similar fashion to insulin secretion from pancreatic β-cells: both insulin and GLP-1 secretion are stimulated by glucose, and require a glucose transporter and glucose metabolism, ATP-sensitive K+-channels, and membrane depolarizations and Ca2+ entry through L-type Ca2+-channels.

There is no report on the calcium-sensing mechanisms governing GIP or GLP-1 secretion. We have identified the expression of several synaptotagmins in GLP-1-secreting cells. In particular, we found that synaptotagmin-7, the high-affinity calcium sensor for insulin secretion, is expressed in mouse L cells (N. Gustavsson and W. Han, unpublished work). Further studies are necessary to understand whether synaptotagmins, particularly synaptotagmin-7, regulate GLP-1 secretion.

**CONCLUDING REMARKS AND FUTURE PERSPECTIVES**

Since the initial cloning and biochemical characterizations [11, 71, 188] that led to the proposed synaptotagmin–calcium-sensor paradigm, intensive research studies, particular those on genetically modified knockout and knockin animals, have established the roles of synaptotagmin-1, -2 and -9 as principal calcium sensors for neurotransmitter release in their respective synapses [46, 59, 68–70]. Here, we have reviewed mouse genetic studies that provide clear evidence on the functions of several synaptotagmins in SV and LDCV exocytosis, particularly for synaptotagmin-7 and its function in insulin and glucagon secretion [46, 59, 81, 163]. These studies in pancreatic α- and β-cells of synaptotagmin-7 knockout mice, along with a number of in vitro and cell-line reports (reviewed in [113]), validate and further extend the synaptotagmin–calcium-sensor theory beyond the three established fast calcium sensors in neurotransmitter release, namely synaptotagmin-1, -2 and -9, to include synaptotagmin-7 as an individually acting calcium sensor in hormone secretion [46, 59, 68–70, 81, 113, 163].

It is an exciting time to witness the rapid progress of the molecular dissection of regulated exocytosis, in particular the molecular understanding of calcium sensing in neurotransmitter, neuropeptide and hormone secretion. There remain many important unanswered questions, and future studies are needed to address: (i) the significance of co-existing calcium-sensing mechanisms (individually acting versus collaborating/competing calcium sensors); (ii) the identities of all of the calcium-sensing proteins involved in regulated exocytosis, for example, slow sensors in neurotransmitter release and minor sensors for glucagon secretion; (iii) whether non-calcium-binding synaptotagmins participate in calcium sensing in collaboration with a calcium-binding synaptotagmin, perhaps for a regulated exocytosis that
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requires low calcium concentration and/or cooperativity; (iv) the involvement of synaptotagmins in less-studied or atypical endocrine systems, such as GLP-1-secreting L cells, and leptin and adiponectin secretion from adipocytes.

ACKNOWLEDGEMENTS

We thank Sir George Radda for advising on and supporting our studies, Dr Tom Südfhof for advice and encouragement for many of our studies and the present review, Dr Jianyuan Sun and Dr Zheping Pang for enjoyable discussions and constructive suggestions, and members of the Laboratory of Metabolic Medicine, Singapore Bioimaging Consortium, A*STAR, Singapore for discussions.

FUNDING

This work was supported by an intramural program of Singapore A*STAR Biomedical Research Council.

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