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*JB Review*

# CAST and ELKS proteins: structural and functional determinants of the presynaptic active zone

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**Cytomatrix at the active zone-associated structural protein (CAST) was first purified from rat brain. It belongs to a protein family with the protein ELKS being its close relative. In nerve terminals, these proteins are specifically localized in the active zone (AZ). They have been shown to directly interact with other AZ proteins, including RIM1, Piccolo and Bassoon, and indirectly with Munc13-1 through RIM1, forming a large molecular complex at AZ. Moreover, the direct interaction of CAST with RIM1 and Bassoon appears to be involved in the release of neurotransmitters. However, it still remains elusive how CAST and ELKS regulate the assembly and function of AZ during synapse maturation. This review focuses on recent findings about the ELKS/CAST family revealed by biochemical strategies and genetic studies, and discusses the potential roles of this protein family in the function and organization of the presynaptic AZ.**

*Keywords*: active zone/CAST/ELKS/neurotransmitter release/RIM1.

*Abbreviations*: AZ, active zone; BRP, Bruchpilot; CAST, cytomatrix at the active zone-associated structural protein; CC, coiled-coil; KO, knock-out; liprin-α, LAR-interacting protein related protein-α; NMJs, neuromuscular junctions; PSD, postsynaptic density; SVs, synaptic vesicles.

The active zone (AZ) is a slightly electron-dense region beneath the presynaptic plasma membrane where the release of neurotransmitters occurs in a spatially and temporally coordinated manner (*1*). Synaptic vesicles (SVs) containing neurotransmitters dock to AZ and undergo a prefusion step, so called priming. Upon the arrival of an action potential to the nerve terminal and the subsequent rise in intracellular $Ca^{2+}$ concentration, SVs fuse with the membrane and release their neurotransmitters from AZ into the synaptic cleft (*2*). Thus, AZ plays a crucial role in the release of neurotransmitters by determining the timing and site of SV docking and fusion (*3*). The AZ cytomatrix is thought to contain various proteins implicated in signal transduction, cytoskeletal organization and cell–cell adhesion, as well as the release of neurotransmitters.

Recent advances in biochemistry and molecular biology have allowed researchers to identify and characterize several AZ-specific proteins including Bassoon (*4*), Piccolo/Aczonin (*5–7*), RIM1 (*8*), Munc13-1 (*9*), ELKS (*10–13*) and cytomatrix at the active zone-associated structural protein (CAST)/ERC2 (*12*, *14*). All these proteins are relatively large and contain various domains such as coiled-coil (CC), PDZ, zinc finger, C1 and C2. However, the mechanism by which these AZ proteins regulate the formation, maintenance and function of AZ remains unclear. This review focuses on the biochemical properties of ELKS/CAST family members and protein–protein interactions among AZ-specific proteins. It also attempts to correlate these interactions with AZ structure and function.

## The AZ cytomatrix

Higher brain functions such as learning, memory, emotion and consciousness rely on the precise regulation of complicated neural networks in the brain. A synapse is the fundamental unit underlying the networks where neurotransmitter release occurs in a spatially and temporally coordinated manner and is generally composed of three structures: presynapse, synaptic cleft and postsynapse. In the presynapse, the AZ cytomatrix is recognized as a slightly electron-dense region beneath the presynaptic plasma membrane (*2*, *3*). The AZ cytomatrix is known to be a principal site for $Ca^{2+}$-dependent exocytosis of neurotransmitters. SVs dock to AZ and fuse with the plasma membrane, resulting in the exocytosis of neurotransmitters as intracellular $Ca^{2+}$ concentration rises. Thus, AZ plays a pivotal role in the release of neurotransmitters by determining the timing and site of SV docking and fusion in presynaptic nerve terminals (*3*). Considering its electron density, the AZ cytomatrix has been thought to contain various proteins involved in signal transduction, cytoskeletal organization and cell–cell adhesion, as well as neurotransmitter release. However, since its discovery in the 1960 s, the exact molecular composition and structure of the AZ cytomatrix has remained unknown.

## AZ-specific proteins

In the 1990 s, advances in biochemistry and molecular biology allowed researchers to identify and characterize AZ-specific proteins including Bassoon, Piccolo, RIM1 and Munc13-1.



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Bassoon and Piccolo/Aczonin are very large proteins and are structurally homologous to each other, except for the presence of additional PDZ and C2 domains in Piccolo/Aczonin (Fig. 1A) (*5–7*). These proteins are expressed in most regions of the brain with overlapping localization. Their zinc-finger domains bind to the prenylated Rab3 A-associated protein-1 (Pra1) *in vitro*. The physiological significance of this interaction is currently unknown. However, because Bassoon and Piccolo are major components of precursor vesicles in AZ (*15*, *16*), it is believed that their interactions with Pra1 may regulate the trafficking of these vesicles to the axon during early synapse formation. As mentioned later in the text, CAST and ELKS are Rab6-binding proteins and both proteins are also components of precursor vesicles in AZ, along with Bassoon and Piccolo (*17*). Thus, the elucidation of the links between these trafficking proteins and AZ proteins would shed new light on the understanding of the molecular and cellular mechanisms underlying the specific transport of AZ proteins to the nerve terminals.

RIM1 and Munc13-1 directly interact with each other, and are known to be essential factors for the priming of SV cycling (*18*). RIM1 was originally identified as a Rab3 A-binding protein (*8*). RIM1 also contains various domain structures, such as zinc finger, PDZ and C2 domains, suggesting that it functions as a scaffold protein at AZ. Munc13-1 is a mammalian orthologues of UNC-13 found in *Caenorhabditis elegans* (*9*). It regulates the release of neurotransmitters through an interaction with syntaxin, a component of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complex (*19–21*).

## Identification and molecular structures of CAST and ELKS

Before the discovery of CAST in 2002, Bassoon, Piccolo/Aczonin, RIM1 and Munc13-1 were known as AZ-specific proteins, but the molecular mechanisms underlying their interactions and AZ localizations were poorly understood. However, scientists now have a much better understanding of the protein–protein interactions that occur among the network of AZ proteins.

CAST was first purified and identified from the biochemically isolated postsynaptic density (PSD) fraction of rat brain (*14*). Since the biochemically isolated PSD fraction contains not only PSD components but also AZ proteins, it is reasonable to assume that CAST would be found in the PSD fraction. CAST is also known as ERC2, which was subsequently isolated using yeast two-hybrid screening as a RIM1-binding partner (*12*). In vertebrates, CAST belongs to a protein family that also includes ELKS (Fig. 1A) (*10–13*) and importantly, the genes for CAST and ELKS are located on different chromosomes in mice, rats and humans (Table I). ELKS was originally identified as a gene whose 5′-end is fused to the RET tyrosine kinase oncogene in papillary thyroid carcinomas (*10*). ELKS also has several other names, including Rab6IP2 (*11*), ERC1 (*12*) and CAST2 (*13*). Since this protein was first tagged as ELKS, the same is used

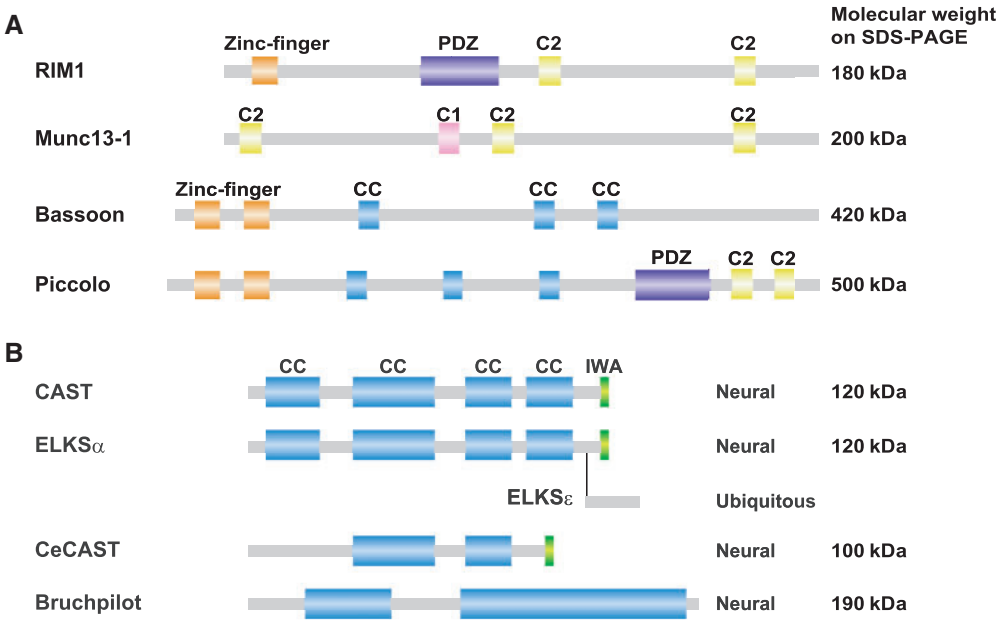


**Fig. 1 Molecular structure of AZ proteins.** (A) RIM1 is composed of an $NH_2$-terminal zinc-finger domain, a PDZ domain and two C2 domains. Munc13-1 contains three C2 domains and a C1 domain involved in the binding of diacyl-glycerol and phorbol esters. Bassoon and Piccolo are structurally related proteins composed of a zinc-finger domain and three CC regions. (B) ELKS/CAST proteins contain four CC regions and a COOH-terminal IWA motif, which is required for binding to the RIM1 PDZ domain. ELKS has at least five alternatively spliced isoforms (*10*): ELKSα, ELKSβ, ELKSγ, ELKSδ and ELKS*ε*. ELKSα is expressed mainly in the brain. Note that the longer ELKS*ε* isoform lacks the IWA motif (*10*). The $NH_2$-terminal region of *Drosophila* BRP resembles the $NH_2$-terminal region of mammalian ELKS/CAST, but the COOH-terminal region is homologous to cytoskeletal proteins.

Table I. Gene symbol and locus of ELKS/CAST.

| | | Gene locus | | |
|---|---|---|---|---|
| Symbol | Aliases | Rat | Mouse | Human |
| ELKS *(10)* | Rab6IP2A *(11)*<br>Erc1 *(12)*<br>CAST2 *(13)* | 4 q42 | 6 F1 | 12 p13.3 |
| CAST *(14)* | Erc2 *(12)*<br>ELKS2 *(30)* | 16 p16 | 14 A3 | 3 p14.3 |

throughout this review to avoid confusion. In order to avoid further confusion, Table I presents a clarification for these naming conventions.

Both CAST and ELKSα are proteins of ~120 kDa with four CC regions and a unique COOH-terminal amino acid motif, IWA (Fig. 1B). Only one gene product of the ELKS/CAST family has been identified and characterized in each of *C. elegans* (*22*) and *Drosophila*; these are CeCAST and Bruchpilot (BRP), respectively (Fig. 1B) (*23*, *24*).

CAST is mainly expressed in the brain (*14*), whereas ELKS is ubiquitously expressed in various tissues and displays a number of different splice isoforms (*25*). Among these isoforms, ELKSα is predominantly expressed in the brain (*12*, *13*, *25*) and shows a relatively high homology with CAST at the protein level (~70% identical). In the mouse hippocampus, CAST is localized close to the presynaptic plasma membrane (*14*). Similar to CAST and Bassoon, ELKSα is localized in the vicinity of the presynaptic plasma membrane in the mouse cerebellum (*13*). In mammalian retina, there is an area equivalent to the AZ cytomatrix called the synaptic ribbon, where photoreceptor cells and bipolar cells release glutamate as a neurotransmitter (*26*). At the immunoelectron microscopic level, CAST is localized at the base of the ribbon, whereas ELKSα is localized around the ribbon (*27*). These observations suggest that CAST and ELKS have similar but distinct functions at the synapse in spite of their high degree of homology. In addition, a ubiquitous isoform of ELKS appears to regulate the exocytosis of insulin from pancreatic β cells (*28*) and also appears to be involved in microtubule stabilization through complex formation with the microtubule-binding proteins, CLASP and LL5β, in non-neural fibroblasts (*29*). Further, ELKS is also involved in vesicle transport regulated by the small G protein, Rab6 (*12*). Indeed, ELKS is a known Rab6-binding protein (*11*) and it would be of interest to determine whether Rab6 regulates the transport of CAST- and ELKS-containing vesicles in axons in the brain.

## Physical and functional interactions of AZ proteins

The AZ cytomatrix is highly complex and resistant to extraction with nonionic detergents, such as Triton X-100, which makes it difficult to biochemically analyse the structure and function of AZ. However, extraction with SDS, followed by dilution with Triton X-100, and subsequent immunoprecipitation with anti-CAST antibody revealed that all known AZ proteins are co-immunoprecipitated with CAST. This was the first evidence suggesting that a network of protein–protein interactions exists among AZ proteins (*14*). CAST and ELKS directly bind to Bassoon, Piccolo and RIM1, and indirectly to Munc13-1, forming a large molecular complex at AZ (Fig. 2). Exogenously expressed RIM1 without the PDZ domain, a CAST-binding domain, shows a diffuse distribution of axons in primary cultured rat hippocampal neurons, whereas CAST lacking the IWA motif, a RIM1-binding motif, is correctly localized to AZ (*14*). These observations suggest that CAST may play a role in anchoring RIM1 to AZ. Also, the finding that RIM1 is more soluble in CAST/ERC2 knockout (KO) mice than wild-type mice (*30*) is consistent with the aforementioned observations. In spite of their tight interactions, both CAST and Bassoon can independently localize to AZ in primary cultured neurons (*17*). At present, the signal targeting Bassoon to AZ appears to reside in several regions of Bassoon, including amino acids 1692–2087, 2565–2714 and 3015–3263, none of which include a CC region that binds to CAST (*17*, *31*).

Although such large AZ proteins may often be the cause of non-specific binding, it is assumed that CAST-dependent complex formation is specific due to the following lines of evidence: (i) colocalization of these proteins at AZ; (ii) highly specific binding of CAST and RIM1; (iii) binding specificity of CAST and Bassoon; and (iv) the fact that these bindings are implicated in synaptic transmission, as shown by electrophysiological studies. As of today, the complete molecular structure of AZ still remains unknown, but the CAST-dependent large protein complex may be the molecular basis for the electron density observed at AZ. At the core of AZ, CAST and ELKS may serve as a 'nucleation site' for the assembly of AZ by capturing Bassoon, Piccolo, RIM1 and Munc13-1 (Fig. 2). More recently, new binding regions of AZ proteins have been discovered and characterized (*32*). For example, in addition to RIM1 and CAST, Bassoon and Piccolo/Aczonin have been shown to interact with the $NH_2$-terminal region of Munc13-1 (*32*). Therefore, molecular interactions among AZ proteins might be much more complicated than previously envisaged. Future research should be directed towards elucidating how these protein–protein interactions observed *in vitro* have physiological relevance *in vivo*; for example, how these interactions affect neurotransmitter release and synaptic plasticity.

## Invertebrate homologues of CAST implicated in the formation of AZ

The specific localization of CAST and ELKS to AZ and their interactions with other AZ proteins suggest that the ELKS/CAST family is involved in the formation and/or maintenance of AZ. This conclusion is supported by the results of recent genetic studies in *Drosophila* (*23*, *24*). The monoclonal antibody NC82 has long been known to label AZs of almost all of

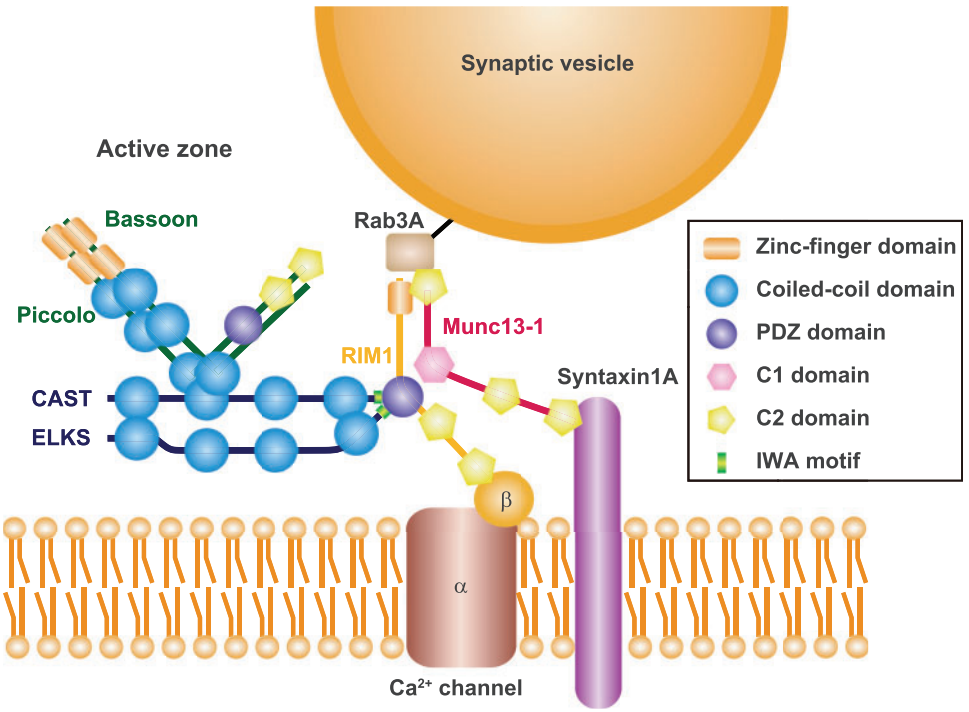


**Fig. 2 A network of protein–protein interactions among AZ proteins.** CAST and ELKS can form homo- and hetero-oligomers. In addition, the second CC region of ELKS/CAST binds the third CC region of Bassoon/Piccolo. The COOH-terminal IWA motif of ELKS/CAST is required for binding the PDZ domain of RIM1. RIM1 and Munc13-1 bind to each other via the zinc-finger domain of RIM1 and the $NH_2$-terminal C2 domain of Munc13-1.

the synapses in *Drosophila* (*33*). Groups led by Drs Buchner and Sigrist have identified a genetic product, Bruchpilot (Fig. 1B), that is recognized by NC82 (*23*). BRP is a ~190 kDa protein containing an $NH_2$-terminal domain with significant homology to the vertebrate ELKS/CAST family domains and a large COOH-terminal domain rich in CC structures. *brp* mutants are defective in locomotor activities and show unstable flight behaviours (hence the name Bruchpilot, which means a crash pilot). At the electron microscopic level, the neuromuscular junctions (NMJs) of *brp* mutants appear to have normal density of synapses and proper pre- and postsynaptic contacts. However, the electron-dense matrix (T-bar) is completely lost and clustering of $Ca^{2+}$ channels is reduced at the nerve terminal (*23*, *24*). This is the first example that shows that the deletion of one molecule results in the disappearance of the AZ cytomatrix or T-bar in *Drosophila* (Fig. 3). At the NMJ of *brp* mutants, the amplitude of the evoked postsynaptic currents is reduced to ~25% of the normal level. However, spontaneous neurotransmitter release is unaffected, suggesting that the efficacy of synaptic exocytosis is impaired but the fundamental mechanism of exocytosis is still preserved in these mutants. Therefore, it is believed that *Drosophila* BRP may play a role in the clustering of $Ca^{2+}$ channels in AZ, which would explain the deficiency of evoked synaptic transmission in *brp* mutants; however, it does not appear to be essential for synapse formation or normal spontaneous neurotransmitter release. Recently, BRP has been shown to directly interact with the α-subunit of $Ca^{2+}$ channels in *Drosophila* (*34*).

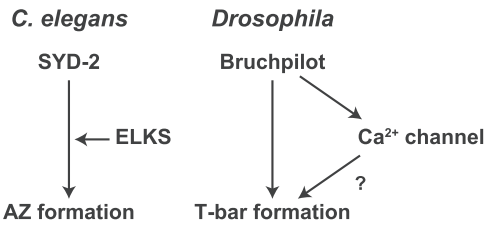


**Fig. 3 Summary of ELKS/CAST function in *C. elegans* and *Drosophila*.** In *C. elegans*, SYD-2 regulates AZ formation through ELKS, although ELKS itself is not essential for AZ formation. In *Drosophila*, the CAST/ELKS homolog BRP is essential for the assembly of the AZ cytomatrix, T-bar.

BRP is expressed exclusively in the nervous system, suggesting that BRP may be functionally more similar to CAST than ELKS since CAST expression is restricted to the brain whereas that of ELKS is ubiquitous. A *C. elegans* null mutant for *elks*, which encodes the only ELKS/CAST homologue known in worms intriguingly shows no significant changes in either the synaptic ultrastructure or synaptic transmission (*22*). However, recent genetic studies in *C. elegans* have revealed another role of ELKS in AZ formation (Fig. 3). SYD-2, a binding partner of ELKS, is the only homologue of members of a conserved protein family known as LAR interacting protein-related protein-α (liprin-α) (*35*, *36*). In *syd-2* null mutants, SVs are diffusely distributed and the size and morphology of AZ is altered (*35*). The group led by Dr Jin recently identified a gain-of-function mutation that promotes the assembly and maturation of AZ (*37*). Importantly, this activity depends on ELKS but not on the RIM homologue, UNC10. Although the

functional roles of ELKS in *C. elegans* are only revealed under conditions where the activity of SYD-2 is increased, these observations suggest that there might be functionally redundant proteins of ELKS in synapse formation. One such candidate is SYD-1 (*38*). Supporting this conclusion is the finding that the overexpression of SYD-2, or introduction of a gain-of-function mutation in *syd-2*, rescues the synaptic defects observed in *syd-1* mutants (*37*, *39*). Moreover, SYD-1 directly interacts with ELKS and promotes the binding of ELKS to SYD-2 (*40*).

## CAST implicated in the release of neurotransmitters

The role of CAST in the release of neurotransmitters has been previously examined using rat superior cervical ganglion neurons, in which well-characterized cholinergic synapses are formed (Table II) (*41*). This is an ideal system because proteins can be introduced into relatively large (30–40 μm) presynaptic cell bodies by microinjection. The injected proteins then rapidly diffuse to nerve terminals, and the effect of these proteins on the release of acetylcholine by nerve stimulation can be accurately monitored. This can be achieved by recording excitatory postsynaptic potentials evoked by action potentials in the presynaptic neurons. Microinjection of the RIM1-binding domain of CAST or the CAST-binding domain of RIM1 significantly impairs neurotransmission (*17*). Since the direct binding of RIM1 and Munc13-1 is involved in the priming of SVs (*18*) and the localization of RIM1 at AZ appears to be CAST dependent, as described above (*14*), it follows that inhibition of the binding of RIM1 to CAST probably affects the RIM1–Munc13-1 pathway. It is presumed that this may occur as a result of the mislocalization of RIM1 at AZ, resulting in a reduction of neurotransmitter release. Consistent with this hypothesis, is the observation that ELKS regulates $Ca^{2+}$-dependent exocytosis of human growth hormone through the RIM–Munc13-1 pathway in PC12 cells (CAST is not expressed in PC12 cells) (*42*). Moreover, inhibition of CAST–Bassoon binding significantly impairs neurotransmission (*17*); however, it is currently unclear whether the impairment of synaptic transmission by the inhibition of CAST–Bassoon binding is due to the mislocalization of CAST and/or Bassoon at AZ, or because of the possibility that localization to AZ might be independent. Also, a functional linkage between RIM1 and Bassoon is currently unclear; nonetheless, it is hypothesized that by forming a ternary complex with RIM1 and Bassoon, CAST may play a role as a 'platform' on which the signalling pathways downstream of Bassoon and RIM1 can be molecularly coupled. Piccolo is structurally homologous to Bassoon but has additional PDZ and C2 domains (*6*, *7*), suggesting that Piccolo could play similar but non-identical roles as Bassoon at the presynaptic site. Thus, it is also possible that the impairment of synaptic transmission is due to the disruption of the binding of Piccolo to CAST since Bassoon and Piccolo share the same binding site on CAST (the third CC region of Bassoon/Piccolo directly binds to the second CC region of CAST).

## KO mice of AZ proteins and CAST

Analyses of RIM1, Munc13-1 and Bassoon in KO mice have revealed that these proteins regulate the release of neurotransmitters at the priming step, although the gross synaptic structures, including the AZ structure, in these KO mice is still intact (*43–46*).

However, in the retinas of Bassoon KO mice, the synaptic ribbon cannot attach to the presynaptic plasma membrane; instead, the ribbon floats in the cytoplasm forming ectopic synapses (*47*). Proper ribbon formation requires the interaction of Bassoon and the ribbon-specific protein, RIBEYE (*48*).

CAST/ERC2 KO mice have also been recently reported (*30*). Unlike BRP, no gross changes in the size or morphology of AZ in CAST/ERC2 KO mice (*30*) are evident, which is similar to that of CAST in *C. elegans*. One reasonable explanation for this phenomenon is that ELKS may have some functional redundancies with CAST in mice. In addition, CAST/ERC2 deletion in these mutant mice causes no significant changes in the phenotype of excitatory synapses; instead, it induces a large increase in inhibitory neurotransmitter release and potentiates the size, but not the properties, of the readily releasable pool of vesicles at inhibitory synapses. These results seem to be opposed to the findings from previous studies using cultured neurons, which suggested that CAST/ERC2 regulates excitatory synaptic transmission (*17*). Currently, there are no reasonable explanations for these discrepancies. However, it seems likely that a remaining short isoform of CAST/ERC2 (~95 kDa) in mutant mice may compensate for defects in excitatory synapses caused by the deletion of CAST/ERC2 (*30*). This isoform is produced by an internal promoter in the CAST/ERC2

**Table II. Summary of ELKS/CAST function in exocytosis.**

| Protein | Preparation | Methods | Results | References |
|---|---|---|---|---|
| CAST | SCG neuron | Peptide inhibition | Reduced EPSPs | *(17)* |
| | Mouse | knockout | Increased neurotransmitter release at inhibitory synapses | *(30)* |
| ELKS | PC12 cell | Overexpression | Increased $Ca^{2+}$-dependent Exocytosis | *(42)* |
| | MIN6 β cell | RNAi | Decreased insulin exocytosis | *(28)* |
| | Mast cell | Overexpression | Increased exocytosis | *(49)* |
| | | RNAi | Decreased exocytosis | *(49)* |

SCG, superior cervical ganglion; EPSPs, excitatory postsynaptic potentials.

gene that drives expression of the isoform-specific 5′-exon located 400 bp upstream of exon 6 (*30*). It remains unknown whether or how CAST/ERC2 regulates the release of excitatory and/or inhibitory neurotransmitters in the brain.

## Closing remark

Over recent decades, advances in biochemistry and molecular biology have identified AZ-specific proteins and the interactions underlying the structure of AZ. However, scientists are still far from understanding the complete molecular composition of AZ and the physiological roles of AZ in synaptic functions such as basic neurotransmitter release, synaptic plasticity and learning and memory mechanisms. Moreover, there are significant discrepancies between the phenotypes caused by CAST/ELKS deletion in worms, flies and mice. Thus, it is quite a challenge, but a necessary one, to reveal the exact protein composition of AZ, analyse their interactions step-by-step and then correlate these interactions with the functions of AZ at the molecular, cellular and organism levels.

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**Conflict of interest**
None declared.

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