Molecular Mechanisms for Active Zone Assembly at Vertebrate Synapses

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Abstract

Neurons are unique for their ability to transmit a presynaptic signal to postsynaptic target cells on a sub-millisecond timescale. This rapid and precise exchange of information relies on the ability of presynaptic nerve terminals to temporally and spatially control the exocytosis of neurotransmitter-packed vesicles in response to membrane depolarization from an action potential. Because neurotransmitter release is largely restricted to specialized areas of the presynaptic plasma membrane called active zones, it is thought that these active zones provide the necessary machinery to orchestrate rapid synaptic transmission.

Active zones are evolutionarily conserved protein networks that couple fusion competent synaptic vesicles to the core membrane fusion machinery, exactly opposite to postsynaptic receptors. Decades of research have identified many protein families specifically associated with these specialized release sites. At vertebrate synapses, these protein families include Munc13, RIM, ELKS, RIM-BP, piccolo/bassoon, and Liprin-α. While much work has been done to dissect the specific role each individual protein family plays in synaptic transmission, the role of the active zone as a complex macromolecular structure and the molecular mechanisms of its assembly are not well understood. Thus the goal of this dissertation is to address two fundamental questions: 1) whether the active zone as a
whole is necessary for synapse formation and synaptic vesicle exocytosis, and 2) what the molecular mechanisms that contribute to active zone assembly are.

To answer these questions, I generated the strongest active zone disrupted mutant to date by using mouse genetics to simultaneously remove two core protein families, RIMs and ELKS, in neuronal cultures. Using a combination of light and electron microscopy and molecular biology, I show that while the active zone is necessary for docking synaptic vesicles to the presynaptic plasma membrane, synapse formation is unaffected and a pool of releasable vesicles persists. Subsequent rescue experiments reveal RIM as the major organizer of active zone scaffolding and identify Liprin-α as a candidate upstream of RIM and ELKS in active zone assembly. Finally, I present a working model for vertebrate active zone assembly.
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—CHAPTER ONE—

Introduction
The presynaptic active zone orchestrates synaptic transmission

Synapses are specialized junctions between a presynaptic neuron and its target postsynaptic cell where an electrical signal in the form of an action potential is converted into a chemical signal via release of neurotransmitters. Within the presynaptic nerve terminal, membrane depolarization opens voltage gated Ca\(^{2+}\) channels (VGCCs) and the subsequent influx of Ca\(^{2+}\) triggers fusion of neurotransmitter-filled synaptic vesicles with the presynaptic plasma membrane. Neurotransmitters released into the synaptic cleft between presynaptic release sites and postsynaptic specializations then bind to and activate postsynaptic ion channels, generating an action potential that propagates the signal to the next target cell (Südhof, 2013, 2012).

While various cell types employ membrane fusion and vesicle exocytosis for communication or other vital biological processes, neurons are unique for their ability to do so ultrafast on the order of microseconds in response to sufficient Ca\(^{2+}\) signal (Sabatini and Regehr, 1996). Such rapid synaptic transmission must be coordinated by coupling synaptic vesicles in close proximity to Ca\(^{2+}\) channels and the core membrane fusion machinery. The minimal molecular machinery necessary for membrane fusion requires SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) and SM (Sec1/Munc18-like) proteins (Südhof, 1995; Weber et al., 1998). Remarkably, while the distribution of membrane SNARE proteins (syntaxin-1 and SNAP25) is widespread within the axonal membrane (Galli et al., 1995; Garcia et al., 1995), synaptic vesicle fusion is spatially restricted to specialized sites along the presynaptic plasma membrane, known as active zones (Couteaux and Pécot-Dechavassine, 1970). Additionally, in vitro experiments reveal that the SNARE-mediated lipid fusion reaction is by itself orders of magnitude
slower than evoked neurotransmission and vesicle fusion (Hu et al., 2003; Weber et al., 1998). Thus synaptic vesicle exocytosis must be regulated by a protein machine beyond the minimal fusion machinery, and the active zone is thought to serve this purpose (Biederer et al., 2017; Südhof, 2012).

The active zone has traditionally been defined in three distinct ways. Functionally, the active zone is a term first coined to describe sites of neurotransmitter release (Couteaux and Pécot-Dechavassine, 1970). Structurally, it is considered an electron-dense complex attached to the presynaptic plasma membrane where synaptic vesicles dock and fuse, precisely opposed to the postsynaptic density (Akert, 1972; Pfenninger et al., 1972). Finally, recent genetic and biochemical approaches have led to a biochemical definition which includes a few protein families that appear to be largely enriched and restricted to the active zone (Garner et al., 2000; Schoch and Gundelfinger, 2006; Südhof, 2012). At vertebrate synapses these protein families include: Munc13, RIM, ELKS, RIM-BP, piccolo/bassoon, and Liprin-α. It should be noted that these core active zone proteins are not the only components located at the active zone. Many additional proteins are localized to the active zone, but their localization is not restricted to it (Boyken et al., 2013; Müller et al., 2010). For this work, I will use the third definition of the active zone as a biochemical protein complex to address fundamental questions regarding the role of the active zone as a whole, complex macromolecular structure and the molecular mechanisms that contribute to its assembly at vertebrate synapses.

**The core membrane fusion machinery for synaptic vesicle exocytosis**
Membrane fusion is a fundamental process in all eukaryotic cell types and it is of particular importance for the release of neurotransmitters at presynaptic active zones. The evolutionarily conserved machinery required for intracellular membrane fusion at synapses include SNARE and SM proteins (Jahn and Fasshauer, 2012; Jahn and Südhof, 1999; Südhof and Rothman, 2009). SNARE proteins are part of a diverse superfamily that share a characteristic ~70-residue “SNARE motif” with heptad repeats (Kloepper et al., 2007). SM proteins are comprised of a conserved ~600-amino acid sequence that curves into a “clasp-like” structure (Misura et al., 2000).

At synaptic release sites, the vesicular SNARE protein synaptobrevin/VAMP (v-SNARE) interacts with SNARE proteins widely distributed on the target presynaptic plasma membrane, syntaxin-1 and SNAP-25 (t-SNAREs), to form a complex capable of fusing two membranes together (Söllner et al., 1993). In order for this complex to form, the SM protein Munc18-1 binds to the closed form of syntaxin-1, which can be induced to undergo a conformational change by priming factors that “open” syntaxin-1 for subsequent SNARE complex assembly, and then Munc18-1 remains associated with the complex to control its assembly (Dulubova et al., 2007, 1999). Initial assembly of the SNARE complex between the vesicle and target membrane results in a stable four-helix bundle, known as the “trans-SNARE” complex. Energy provided by the progressive zippering of this four-helical complex results in sufficient force to drive membrane fusion (Hanson et al., 1997; Weber et al., 1998). This process is thought to provide the molecular framework for the docking (membrane association), priming (fusion competency), and eventual release (exocytosis) of synaptic vesicles (Jahn and Fasshauer, 2012).
While SNARE and SM proteins are essential for normal synaptic transmission and synaptic vesicle exocytosis at vertebrate synapses (Bronk et al., 2007; Fujiwara et al., 2006; Gerber et al., 2008; Mishima et al., 2014; Schoch et al., 2001; Verhage et al., 2000), other protein families are also intimately involved. Of particular interest are vesicular proteins specialized for sensing Ca\(^{2+}\) signals (synaptotagmins) and for positioning vesicles close to release site hotspots (Rab GTPases). Synaptotagmins are evolutionarily conserved vesicular transmembrane proteins with two Ca\(^{2+}\) binding C2 domains, and they are the primary sensors for Ca\(^{2+}\) evoked neurotransmission (Fernández-Chacón et al., 2001; Geppert et al., 1994b; Südhof and Rizo, 1996). Although there are 16 mammalian isoforms of synaptotagmin, the main isoform involved in fast synchronous release is synaptotagmin-1, which binds to membrane phospholipids, the SNARE complex, and a small cytosolic co-factor (complexin) to trigger membrane fusion upon Ca\(^{2+}\) influx (Zhou et al., 2017). In addition to mediating synchronous release, synaptotagmin-1 has been shown to restrict spontaneous fusion events called minis (Littleton et al., 1993; Xu et al., 2009), and other synaptotagmin isoforms are involved in mediating asynchronous release (Bacaj et al., 2015). Thus synaptotagmins are essential for the timing of synaptic vesicle exocytosis at synapses.

Finally, Rabs are small GTP-binding proteins that are geranylgeranylated at their C-termini, which leads to attachment to their target membrane. At synapses, Rab3A is the major isoform associated with synaptic vesicles and undergo a cycle of membrane association and dissociation coupled to GTP binding and hydrolysis (Jahn and Südhof, 1999). Removing Rab3A from vertebrate synapses resulted in mild effects on synaptic transmission and vesicle exocytosis, suggesting that Rabs are not essential for vesicle
fusion or that there are Rabs other than Rab3A that contribute to fusion (Geppert et al., 1994a).

**Vesicle docking and priming at the active zone**

Ultrastructural analyses of synapses in various model organisms have established that vesicle docking and fusion at active zones along the presynaptic plasma membrane are conserved morphological features regardless of the types of neurons or their targets (Zhai and Bellen, 2004). Classically, docking is defined as the accumulation of vesicles that appear closely apposed to the plasma membrane on electron micrographs (Verhage and Sørensen, 2008). The criterion used to score docked vesicles differs depending on the method of membrane fixation and investigating laboratory. For instance, in samples fixed with glutaraldehyde, investigators have scored docked vesicles as those touching the membrane or as those <30 nm from the membrane (Imig et al., 2014; Verhage and Sørensen, 2008). Recent changes in fixation, namely the use of high pressure freezing, are thought to preserve membrane protein complexes in a more native state, perhaps eliminating some of the variability introduced by membrane alterations during chemical fixation (Rostaing et al., 2004; Siksou et al., 2007). For this work, I define docked synaptic vesicles as synaptic vesicles touching the presynaptic plasma membrane in high-pressure frozen, freeze-substituted thin sections imaged by transmission electron microscopy.

Proteins considered part of the minimal docking machinery vary depending on the cell type studied and the definition of docking. Vesicle docking has been extensively studied in adrenal chromaffin cells, and a picture has emerged where four core fusion proteins are involved in chromaffin granule docking. These include calcium sensor synaptotagmin-1,
SNAREs SNAP-25 and syntaxin-1, and SM protein Munc18-1 (de Wit et al., 2009). In vertebrate neurons, a more complex picture has emerged. Synapses lacking RIMs reveal about a 40% reduction in the number of vesicles touching the plasma membrane (Han et al., 2011; Kaeser et al., 2011). Additional experiments employing high-pressure freezing and tomographic analyses have uncovered more subtle functions in docking for SNARE proteins, CAPS, and Munc13s (Imig et al., 2014; Siksou et al., 2009), but glutaraldehyde fixation in these mutants did not reveal their docking functions (Augustin et al., 1999; de Wit et al., 2006; Geppert et al., 1994b). Discovering the minimal molecular mechanisms for synaptic vesicle docking at vertebrate synapses remains an interesting question, but requires the generation of an active zone mutant that strongly abolishes vesicle docking (see Chapter 3).

It is often argued that the vesicles docked to the presynaptic plasma membrane are the morphological correlates of functionally “primed vesicles,” constituting the readily releasable pool (RRP) or synaptic vesicles that can rapidly fuse upon Ca2+ signal (Südhof, 2013). Unlike vesicle docking, vesicle priming is measured electrophysiologically through methods such as using Ca2+ independent application of hypertonic solutions (Rosenmund and Stevens, 1996), or high-frequency stimulation (Schneggenburger et al., 2002; Thanawala and Regehr, 2016) to deplete and measure the size of the RRP. Initial studies suggested that the number of stably docked vesicles and the size of the RRP are well correlated (Schikorski and Stevens, 2001), but this relationship may not be so linear as more recent evidence suggests the possibility of both non-releasable “trapped” states of docking and fusible vesicles more distal to the active zone (Lai et al., 2017; Verhage and Sørensen, 2008; Wang et al., 2016) (see Chapter 2).
The active zone is an evolutionarily conserved protein complex.

In order for rapid and sustained synaptic transmission to occur, all components necessary for Ca\(^{2+}\) evoked vesicle exocytosis (the core membrane fusion machinery, docked and primed synaptic vesicles, and VGCCs) must be arranged in a way that provides temporal and spatial precision. The presynaptic active zone is an evolutionarily conserved protein network, composed of Munc13, RIM, ELKS, RIM-BP, piccolo/bassoon, and Liprin-\(\alpha\), that serves this function via three key features. First, although the core fusion machinery (SNARE and SM proteins) is distributed widely along the axon (Galli et al., 1995; Garcia et al., 1995), the active zone spatially restricts synaptic vesicle docking and fusion to specific membrane areas. Second, the active zone recruits VGCCs, coupling Ca\(^{2+}\) influx to synaptic vesicles and the release machinery, which allows for fast synchronous synaptic transmission. Finally, the active zone precisely aligns pre- and postsynaptic specializations, which facilitates immediate rapid and strong activation of postsynaptic receptors (Biederer et al., 2017; Südhof, 2012; Tang et al., 2016). Only recently, the culmination from decades of exemplary genetic, molecular, and electrophysiological work has revealed the individual role each active zone protein family plays in coordinating rapid synaptic transmission.

As one of the earliest active zone proteins identified, Munc13s were first described as phorbol ester/diacylglycerol binding proteins whose mutation in C. elegans (unc-13 gene) caused diverse defects in the nervous system (Maruyama and Brenner, 1991). Subsequent identification and characterization of mammalian unc-13 homologs (Munc-13s), for which there are four genes but only three (Munc13-1, 2, and 3) primarily expressed in brain tissue, revealed its essential role in priming, or generating fusion-
competent synaptic vesicles (Augustin et al., 1999; Brose et al., 1995; Varoqueaux et al., 2002). Further analysis in C. elegans and D. melanogaster model systems confirmed the conserved role Munc13s have in synaptic transmission (Aravamudan et al., 1999; Richmond et al., 1999), and structure-function experiments have identified the protein-protein interactions with RIMs (via the Munc13 C2A domain) and syntaxin-1 (via the Munc13 MUN domain) necessary for this priming function (Basu et al., 2005; Deng et al., 2011; Dulubova et al., 2005; Lai et al., 2017). While initial studies did not report a an ultrastructural function of Munc13s at active zones (Augustin et al., 1999), refined assessment with high pressure freezing and electron tomography revealed a complimentary role Munc13s have in docking synaptic vesicles (Imig et al., 2014; Siksou et al., 2009). Thus Munc13s are active zone proteins essential for the docking and priming of synaptic vesicles at release sites.

RIMs are central, multi-domain proteins first identified as vesicular Rab3-interacting molecules (Wang et al., 1997). While invertebrates contain a single RIM gene, vertebrates express four genes that encode several alternatively spliced variants (Wang and Südhof, 2003). Full length RIM proteins consist of five conserved domains: an N-terminal Zinc finger surrounded by \( \alpha \)-helices, a central nested PDZ domain, two C-terminal C2 domains (A and B), and a proline-rich linker sequence between the two C2 domains. Initial in vitro protein interaction assays reveal that RIM interacts with a number of other active zone and synaptic proteins, including ELKS (Ohtsuka et al., 2002; Wang et al., 2002), RIM-BPs (Hibino et al., 2002; Wang et al., 2000), and Liprin-\( \alpha \) (Schoch et al., 2002). Additionally, genetic experiments done in both C. elegans and mice reveal that RIM is functionally important for setting normal release probability and vesicle priming (Koushika
et al., 2001; Schoch et al., 2002). More complete analyses have revealed that RIM tethers Ca\(^{2+}\) channels to the active zone via a direct PDZ interaction between RIM and Ca\(^{2+}\) channels (Han et al., 2011; Kaeser et al., 2011; Müller et al., 2012), and controls vesicle docking and priming through interactions with Munc13 (Betz et al., 2001; Deng et al., 2011; Dulubova et al., 2005). Finally, RIM has recently been shown to bind the membrane lipid PIP2, an interaction that is important for targeting VGCCs and primed vesicles to PIP2 enriched areas along the presynaptic plasma membrane (de Jong et al., 2018). This body of work supports RIMs as central active zone scaffolding proteins with an essential role in mediating release probability and vesicle priming.

ELKS proteins were first linked to the active zone through protein interaction assays with the PDZ domain of RIM (Ohtsuka et al., 2002; Wang et al., 2002). *C. elegans* encodes a single gene for ELKS (Deken et al., 2005; Monier et al., 2002), while *D. melanogaster* expresses a distant homologue called bruchpilot (Monier et al., 2002; Wagh et al., 2006). Vertebrates have two ELKS genes that each express four variants (Held and Kaeser, 2018; Kaeser et al., 2009; Liu et al., 2014; Ohtsuka et al., 2002; Wang et al., 2002). Full length ELKS consists largely of predicted coiled-coil sequences and several pieces of evidence support ELKS as a scaffolding protein involved in synaptic transmission. First, in vitro protein interaction assays reveal that ELKS interact with multiple other active zone proteins, such as RIM (Ohtsuka et al., 2002), Liprin-\(\alpha\) (Ko et al., 2003b), piccolo/bassoon (Takao-Rikitsu et al., 2004; Wang et al., 2009), and more recently with b-Munc13-2 (Kawabe et al., 2017) and the \(\beta\)-subunit of VGCCs (Kiyonaka et al., 2012). Additionally, genetic experiments in *D. melanogaster* reveal that deletion of ELKS homolog bruchpilot reduces synaptic transmission to 30\% of control at the neuromuscular junction (NMJ) and
reduces the number of presynaptic active-zone like T-bar structures (Kittel et al., 2006; Wagh et al., 2006). However, further analysis of ELKS in vertebrates did not reveal a scaffolding phenotype. Instead, ELKS was shown to affect Ca\(^{2+}\) influx and the readily releasable pool (Kaeser et al., 2009; Liu et al., 2014). Subsequent analysis of ELKS at excitatory and inhibitory vertebrate synapses revealed differential, synapse specific effects on vesicle priming and release probability: boosting priming at excitatory synapses through its N-termini, but enhancing P at inhibitory synapses (Held et al., 2016). The differences seen between removing ELKS from D. melanogaster and vertebrates may be explained, in part, by the fact that bruchpilot is a fusion protein consisting of a N-terminal ELKs related domain and a C-terminal plectin-related domain with no vertebrate homolog.

Similar to ELKS, RIM-BPs (RIM-binding proteins) were first associated with the active zone through the finding that RIM-BPs biochemically interact with RIMs (Wang et al., 2000). Subsequent protein interaction assays reveal that RIM-BPs also bind to Ca\(^{2+}\) channels (Hibino et al., 2002). Specifically, all RIM-BPs contain three SH3 domains and three fibronectin II domains (Mittelstaedt and Schoch, 2007), and the SH3 domains can bind to proline rich motifs in both RIM and Ca\(^{2+}\) channels, potentially recruiting Ca\(^{2+}\) channels to the active zone together with RIM in a tripartite complex. This hypothesis is supported by genetic experiments in D. melanogaster that reveal that removal of Drosophila RIM-BP leads to impaired Ca\(^{2+}\) channel clustering and Ca\(^{2+}\) influx, as well as drastically reduced release probability (Liu et al., 2011). In vertebrates, there are three genes for RIM-BP and deletion of RIM-BPs primarily expressed in brain tissue result in relatively mild effects on synaptic transmission but increased the coupling distance between Ca\(^{2+}\)
channels to release sites (Acuna et al., 2015). It is likely that this mild phenotype is the result of functional redundancy between RIMs and RIM-BPs (see Chapters 3, 5).

Piccolo and bassoon are massive > 400 kDa multi-domain proteins initially linked with the active zone via immunogold labeling and protein biochemistry (Cases-Langhoff et al., 1996; tom Dieck et al., 1998). Unlike other active zone proteins, they are thought to be specific to vertebrate synapses, although more recent work has identified putative homologs in both *D. melanogaster* and *C. elegans* (Bruckner et al., 2012; Xuan et al., 2017). In vitro protein interaction experiments reveal that piccolo/bassoon are closely interconnected to a number of other active zone proteins, including RIMs, ELKS, Munc13, and RIM-BP (Davydova et al., 2014; Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004; Wang et al., 2009), and genetic studies suggest that the major function of piccolo/bassoon may be to guide synaptic vesicles from further within the presynaptic nerve terminal to the presynaptic plasma membrane (Hallermann et al., 2010; Mukherjee et al., 2010). It is possible that this role in more peripheral active zone functions is of particular importance for specialized synapses, such as retinal ribbon synapses, whose high frequency activity relies on synaptic vesicle replenishment.

Liprin-α’s remain the most enigmatic active zone protein family. They were first identified as LAR (leukocyte common antigen related) protein tyrosine phosphatase interacting proteins (Serra-Pagès et al., 1995) and eventually linked to the active zone when a mutation in invertebrate Liprin-α homologs resulted in changes in the size of the active zone and disruptions in synaptic vesicle accumulation (Kaufmann et al., 2002; Zhen and Jin, 1999). While invertebrates encode a single gene for Liprin-α, vertebrates express four genes with multiple alternatively spliced isoforms, whose roles at the active zone are
not well understood (Zürner et al., 2011). Structurally, Liprin-α consists of two highly conserved N-terminal coiled-coil domains, referred to as Liprin homology domains (LH1 and LH2), while C-terminal of Liprin-α consists of three sterile-α-motif (SAM) domains. Various in vitro protein interaction assays revealed that Liprin-α interacts with a number of proteins both specific to the active zone and more ubiquitously expressed. Specific to the active zone, The N-terminal LH domains of Liprin-α bind to themselves, forming homodimers (Taru and Jin, 2011), as well as the RIM C2B domain (Schoch et al., 2002) and the ELKS N-terminal coiled-coil domain (Ko et al., 2003b). While work in invertebrate models suggest Liprin-α’s are involved in active zone assembly (Dai et al., 2006; Patel et al., 2006), their role at vertebrate synapses are not well understood, perhaps due to the complexity of their expression and localization. More rigorous genetic studies are necessary to provide insight on the function of Liprin-α’s, particularly at vertebrate synapses (see Chapter 4).

**Structural redundancy within the active zone protein network**

Although decades of work have gone into understanding the role each individual active zone protein family plays in orchestrating the rapid and precise nature of synaptic transmission, it is abundantly clear that many of these proteins have redundant functions, particularly at vertebrate synapses where there exist many alternatively spliced variants of each gene. Such redundancy makes understanding the structural organization and scaffolding of the active zone protein complex as a whole challenging to address and there remain many open questions: how is the active zone assembled, what mechanisms hold the active zone protein complex together, how does the active zone target to specific sites
along the presynaptic plasma membrane, and is active zone structure itself necessary for synapse formation and vesicle fusion?

Findings from invertebrate studies provide some insight into these questions. In *D. melanogaster*, removing the homologs of ELKS (*bruchpilot*) and RIM-BP (DRBP) disrupts the active-zone like T-bar structure at the neuromuscular junction (Kittel et al., 2006; Wagh et al., 2006), and removing the homolog of Liprin-α (Dliprin) increases the size of the active zone complex (Kaufmann et al., 2002). In *C. elegans*, removing Liprin-α’s similarly increased the length of the active zone (Zhen and Jin, 1999) and there is evidence suggesting that Liprin-α’s have a role in active zone assembly upstream of ELKS (Dai et al., 2006; Patel et al., 2006), but there still appears to be a great amount of redundancy between core conserved active zone components, as RIM, ELKS, and Munc13s can localize independently of one another (Deken et al., 2005).

At vertebrate synapses, previous reports have demonstrated that removing a single active zone protein family only results in mild structural effects, and none that have significantly disrupted the active zone ultrastructure as seen in the *D. melanogaster* *bruchpilot* and DRBP mutants. Specifically, no major structural disruptions have been reported for Munc13, ELKS, RIM-BP, or Liprin-α knockouts (Acuna et al., 2015; Liu et al., 2014; Varoqueaux et al., 2002; Wong et al., 2018). Removing RIM leads to a reduction of Munc13, a slight increase in ELKS solubility, and a reduction in Ca$^{2+}$ channels (Deng et al., 2011; Kaeser et al., 2011; Schoch et al., 2002), and removing Bassoon resulted in disrupted RIM-BP localization at synapses (Davydova et al., 2014). It is likely that the tightly interconnected nature of the active zone protein complex makes disassembly possible only when targeting multiple active zone protein families simultaneously.
The goal of this dissertation is to better understand the mechanisms of active zone assembly at vertebrate synapses. Here I present the work I have done remove two core active zone protein families, RIM and ELKS, which led to a disruption of active zone structure. With structure-function rescue experiments, I identify the specific molecular mechanisms holding the active zone together by rebuilding its structure within this mutant. Finally, I provide insight on Liprin-α as a putative mechanism for active zone assembly upstream of RIM and ELKS that may play a role in synapse formation and targeting the active zone to the presynaptic plasma membrane. These findings support a stepwise working model for active zone assembly at vertebrate synapses and provide the groundwork for future experiments dissecting the structure-function interplay between multiple active zone proteins.
Fusion competent synaptic vesicles persist upon active zone disruption and loss of vesicle docking
Contributions
Shan Shan H. Wang*, Richard G. Held*, Man Yan Wong, Changliang Liu, Aziz Karakhanyan
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Ca^{2+}-triggered fusion of synaptic vesicles is mediated by soluble NSF-attachment protein receptors (SNAREs) and is restricted to release sites called active zones (Couteaux and Pécot-Dechavassine, 1970; Südhof, 2012). The active zone is a highly organized structure that docks synaptic vesicles close to release machinery and presynaptic Ca^{2+} channels (Figure 2.1 A). This establishes the tight spatial organization required for fast synaptic vesicle fusion upon Ca^{2+} entry and it provides molecular machinery to set and regulate synaptic strength (Kaeser and Regehr, 2014). Functionally, synaptic strength is determined by two parameters that are set at the active zone. First, only a subset of vesicles can be released upon arrival of an action potential. This pool of vesicles is generated through a priming reaction and is called the readily releasable pool (RRP). Second, an action potential releases RRP vesicles with a certain probability, called vesicular release probability (P). Synaptic strength, the amount of release from a given synapse, is proportional to the product of RRP and P (Zucker and Regehr, 2002).

The active zone matrix consists of multi-domain proteins that control RRP and P, and their localization is restricted to the presynaptic plasma membrane area opposed to the postsynaptic density (PSD). These proteins include RIM, ELKS (also known as ERC/CAST/Rab6IP2), Munc13, Bassoon/Piccolo, RIM-BP, and Liprin-α (Schoch and Gundelfinger, 2006; Südhof, 2012). Many additional proteins including SNAREs, ion channels, receptors, cytoskeletal proteins and adhesion molecules are also present (Boyken et al., 2013; Morciano et al., 2009; Müller et al., 2010) but are not restricted to the active zone matrix. Removing individual proteins of the active zone matrix has effects on release that vary in extent, and there are differences between synapses in vertebrates, *C. elegans,*
and *D. melanogaster* in the reliance on specific active zone proteins (Acuna et al., 2015; Kaeser et al., 2011; Kittel et al., 2006; Koushika et al., 2001; Liu et al., 2014; Müller et al., 2012). At vertebrate synapses, release strongly depends on synaptic vesicle priming activities of Munc13s such that loss of Munc13 leads to loss of all fusion competent vesicles (Augustin et al., 1999; Varoqueaux et al., 2002). RIMs contribute to priming through anchoring and activation of Munc13 (Andrews-Zwilling et al., 2006; Betz et al., 2001; Deng et al., 2011; Lu et al., 2006), and they tether primed vesicles to presynaptic Ca\(^{2+}\) channels to enhance release probability (Han et al., 2011; Kaeser et al., 2012, 2011). ELKS (Held et al., 2016; Kaeser et al., 2009; Liu et al., 2014), Bassoon/Piccolo (Davydova et al., 2014; Hallermann et al., 2010), and RIM-BPs (Acuna et al., 2015) are also present, but knockouts for these proteins show milder impairments in release. The domain structure and the *in vitro* interactions of RIM, ELKS and Bassoon/Piccolo further predicted strong scaffolding roles (Schoch and Südhof, 2006; Südhof, 2012), but except for a partial loss of Munc13 in RIM knockout mice (Schoch et al., 2002) or of RIM-BP in Bassoon knockout mice (Davydova et al., 2014), the active zone protein complex was intact in knockout mice for individual protein families (Davydova et al., 2014; Deng et al., 2011; Held et al., 2016; Liu et al., 2014). Together with the notion that synaptic vesicle docking and fusion are spatially restricted to the active zone, these studies led to the hypothesis that the active zone is required to translate the incoming action potential into neurotransmitter release (Schoch and Gundelfinger, 2006; Südhof, 2012).

Synaptic vesicle tethering and docking are thought to precede fusion and have been studied using various methods and definitions. Docking is often defined as synaptic vesicles that are attached to the plasma membrane in electron microscopic images of
glutaraldehyde fixed tissue such that the electron densities of the vesicle membrane and target membrane merge (Acuna et al., 2015; Augustin et al., 1999; Bronk et al., 2007; Kaeser et al., 2011). Using glutaraldehyde fixation only RIMs participate in synaptic vesicle docking without affecting total numbers of synaptic vesicles in a nerve terminal (Augustin et al., 1999; Bacaj et al., 2015; Bronk et al., 2007; Kaeser et al., 2011). Recently, the use of high pressure freezing and tomography have allowed for distinction of tight vesicle docking with a resolution of a few nanometers. This has led to the discovery that SNARE proteins and Munc13 contribute to the tight attachment of synaptic vesicles to the presynaptic plasma membrane. Their genetic removal leads to a loss of tightly docked vesicles when observed in high pressure frozen tissue (Imig et al., 2014; Siksou et al., 2009), but these phenotypes are too subtle to be uncovered using glutaraldehyde fixation (Augustin et al., 1999; Bronk et al., 2007). Overall, there is a good correlation between the number of docked vesicles observed with either fixation method, the size of the active zone, and the size of the RRP, which suggested, along with the observation that docked vesicles fuse upon stimulation, that docked vesicles are the RRP (Holderith et al., 2012; Imig et al., 2014; Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001; Watanabe et al., 2013). Here, we measure the RRP as vesicles that are released by the application of hypertonic sucrose, and we assume that the same vesicles are accessible to action potentials although differences may exist (Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001; Thanawala and Regehr, 2016; Zucker and Regehr, 2002). We use the term ‘fusion competent’ for vesicles in the RRP and vesicles that are released through spontaneous miniature events (Augustin et al., 1999).
To date, no knockout mutation has led to a strong structural disruption of the vertebrate active zone matrix. We set out to generate such a mutation with the goal to test whether the active zone is necessary for the structural assembly of synapses, whether it is required for fusion, and how it participates in setting RRP size and P. We produced conditional mouse mutants to simultaneously remove all active zone isoforms of RIM and ELKS in cultured hippocampal neurons. This led to loss of Munc13, Bassoon, Piccolo, RIM-BP, and Ca\textsubscript{v}2.1 Ca\textsuperscript{2+}channels. The overall synaptic assembly including the postsynaptic densities, the synaptic vesicle cluster, and the levels of SNARE proteins remained unaffected. However, we observed a near complete loss of vesicle docking and release probability was strongly decreased. Surprisingly, a pool of fusion competent vesicles, released as spontaneous miniature events or during stimulation with action potential trains or hypertonic sucrose, persisted upon strong disruption of the active zone and vesicle docking.

**Experimental Procedures**

The quadruple homozygote floxed mice for RIM1\textsubscript{αβ}, RIM2\textsubscript{αβγ}, ELKS1\textsubscript{α} and ELKS2\textsubscript{α} were generated by crossing single conditional knockout mice (Kaeser et al., 2011, 2009, 2008; Liu et al., 2014). All experiments were performed in cultured hippocampal neurons infected at day in vitro 5 (DIV) with lentiviruses expressing cre recombinase or an inactive mutant of cre under a synapsin promoter, and experiments were performed at DIV 15-19. Biochemical, confocal, electron microscopic and electrophysiological analyses were performed as described (Deng et al., 2011; Kaeser et al., 2011, 2008). Quantitative Western blotting was performed using fluorescently tagged secondary antibodies.
Electrophysiological recordings were done in whole cell patch clamp configuration, and action potentials were triggered by a focal stimulation electrode. For pHluorin imaging, the neurons were infected with lentiviruses expressing SypHy and SV2-TdTomato at DIV 3 in addition to the cre and control lentiviruses supplied at DIV 5. Experiments were performed and analyzed by an experimenter blind to the genotype and significance was determined using Student’s t-tests unless otherwise noted. Detailed descriptions of the methods are provided in the supplemental materials (Appendix Two).

Results

Genetic disruption of the presynaptic active zone.

We generated mice to simultaneously and conditionally remove all presynaptic RIM and ELKS proteins. We targeted RIM and ELKS proteins because they are expressed at all synapses and they interact with all major active zone proteins (Figure 2.1 A). We crossed conditional knockout mice for presynaptic RIM proteins (Kaeser et al., 2011, 2008), encoded by the genes Rims1 and Rims2, to conditional knockout mice for both genes encoding ELKS proteins (Kaeser et al., 2009; Liu et al., 2014), Erc1 and Erc2, to generate quadruple conditional knockout mice (Supplemental Figure 1 A). All analyses were done in primary hippocampal neurons from these mice or the double conditional knockout mice for either RIM or ELKS proteins. Lentivirus expressing cre recombinase or an inactive mutant of cre in neurons (Liu et al., 2014) was supplied at 5 days in vitro (DIV) to generate knockout neurons (cKO^{R+E}) or control neurons (control^{R+E}). In cKO^{R+E} neurons, we remove RIM and ELKS proteins as assessed by confocal microscopy (Figures 2.1 B, C) and quantitative Western blotting using fluorescent secondary antibodies (Figures 2.1 D,
**Figure 2.1. Genetic removal of RIM and ELKS leads to disruption of the active zone.**

(A) Schematic of the protein complex at the active zone and its connections to other important presynaptic protein families (marked in red). SAM: sterile alpha motif, MUN: Munc13 homology domain, PDZ: PSD-95/Dlg1/ZO-1 homology domain, SH3: Src homology 3 domain, PxxP: proline rich motif, FN3: fibronectin 3 repeat. (B, C) Sample images (B) and quantitation (C) of protein levels at RIM and ELKS knockout (cKO\textsuperscript{R+E}) and control (control\textsuperscript{R+E}) synapses using confocal microscopy. The synaptic vesicle marker Synaptophysin-1 (Syp-1) was used to define the region of interest (ROI). The black dotted line indicates control levels and the grey dotted line non-specific staining as assessed for RIM. Example images for RIM-BP2, Piccolo, Liprin-α2, Liprin-α3, SNAP-25, and quantitation of puncta number and size are in Supplemental Figure 1 (control\textsuperscript{R+E} n = 3 independent cultures, cKO\textsuperscript{R+E} n = 3, 10 images per culture). (D, E) Quantitative Western blotting for presynaptic proteins using fluorescent secondary antibodies. Some cultures were fractionated into pellet and supernatant (sup.) using Triton X-100 solubilization and ultracentrifugation. Quantitation (E) of total protein levels in cKO\textsuperscript{R+E} neurons normalized to protein levels in control\textsuperscript{R+E} neurons (black dotted line) are shown. Black and grey dotted as in C (control\textsuperscript{R+E} n = 6 independent cultures, cKO\textsuperscript{R+E} n = 6, except for Bassoon where n = 3 for both conditions, Syb-2: synaptobrevin/VAMP-2). (F, G) Sample images (F) and quantitation (G) of the fraction of Bassoon puncta containing Munc13-1 or RIM-BP2. The fraction of Bassoon pixels and the fraction of Synaptophysin-1 puncta containing Munc13-1 or RIM-BP2 are in Supplemental Figure 1 (control\textsuperscript{R+E} n = 6 independent cultures, cKO\textsuperscript{R+E} n = 6, 10 images per culture). All data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as determined by Student's t test.
Figure 2.1 (Continued)
In immunostaining, 22% of RIM and 23% of ELKS signal remained upon knockout of these proteins (grey dotted line, Figure 2.1 C) despite much stronger reductions in Western blotting (Figure 2.1 E), establishing that this signal is non-specific (see Supplemental Figure 1 D for background from secondary antibodies only). Genetic removal of RIM and ELKS led to a very strong reduction of interacting active zone proteins. Munc13-1 was eliminated from synaptic puncta in an extent similar to RIM and ELKS, and synaptic Bassoon and Piccolo were reduced nearly as strongly (Figures 2.1 B, C), whereas the total protein levels of Munc13-1 and Bassoon were reduced by ~80% (Figures 2.1 D, E). Synaptic and total RIM-BP2 was reduced by 31% and 42% respectively (Figures 2.1C-E, S1 B) and synaptic CaV2.1 Ca^2+ channel levels were reduced by 29% (Figures 3.1 B, C). Because synaptic Munc13-1 levels (Figure 2.1 C) were more strongly reduced than total Munc13-1 levels (Figure 2.1 E), we tested whether the remaining Munc13-1 was clustered at synapses. The Munc13-1 protein that was left in the cKO_R+E neurons was insoluble as measured in a fractionation experiment of the cultured neurons (Figure 2.1 D). Furthermore, when we evaluated whether the remaining Munc13 clusters co-localized with the remaining Bassoon (Figure 2.1 G) or with synaptic vesicles (Supplemental Figures 1 E, F), we found that many Bassoon puncta and synapses did not contain Munc13-1, whereas the remaining RIM-BP2 co-localized well with the same markers (Figure 2.1 G and Supplemental Figures 1 E, F).

This is the most extensive genetic disruption of the vertebrate active zone protein complex to date with a near complete loss of most of the vital components. Somewhat surprisingly, synapse number and size were unchanged (Supplemental Figure 1 C), and the levels and localization of SNARE proteins and synaptic vesicle markers (Syntaxin-1, SNAP-
25 and Synaptobrevin-2/VAMP-2, and Synaptophysin-1) were not affected (Figures 2.1 B-E, Supplemental Figure 1 B).

At invertebrate synapses, Liprin-α controls presynaptic assembly upstream of ELKS and RIM (Dai et al., 2006; Kaufmann et al., 2002; Patel et al., 2006; Zhen and Jin, 1999). Vertebrates express Liprin-α proteins from four genes (Zürner and Schoch, 2009). Although it is not well understood which Liprin-α isoforms localize to the active zone, and post- and extra-synaptic localization has also been observed, Liprin-α2 and Liprin-α3 are likely the prominent synaptic Liprin-α isoforms (Spangler et al., 2011; Wyszynski et al., 2002; Zürner et al., 2011). In cKO<sup>R+E</sup> neurons, Liprin-α2 and Liprin-α3 localization (Figure 2.1 C), and Liprin-α3 levels (Figure 2.1 E) and biochemical solubility (Figure 2.1 D) were unaffected. Notably, Liprin-α2 and Liprin-α3 antibodies reveal relatively widespread labeling (Supplemental Figure 1 B), compatible with additional roles for Liprin-α outside active zones (Miller et al., 2005). In summary, simultaneous deletion of RIM and ELKS reveals strong, redundant, and active zone specific scaffolding functions for these proteins that were not detected when a single protein family was deleted.

**Loss of synaptic vesicle docking but normal postsynaptic assembly upon active zone disruption.**

To characterize effects on presynaptic and postsynaptic structure, we fixed cultures by high-pressure freezing and analyzed them using transmission electron microscopy. In agreement with the immunostainings, cKO<sup>R+E</sup> synapses had normal bouton size and synaptic vesicle numbers (Figures 2.2 A, B). At cKO<sup>R+E</sup> synapses, we observed a massive, 92% reduction of docked vesicles (Figure 2.2 B). We repeated this analysis in
**Figure 2.2. Disruption of the active zone leads to loss of synaptic vesicle docking but PSDs appear normal.**

(A, B) Sample images (A) and quantification (B) of synaptic morphology of high-pressure frozen neurons analyzed by electron microscopy of cKO\textsuperscript{R+E} and control\textsuperscript{R+E} synapses. For an identical analysis using glutaraldehyde fixed tissue, see Supplemental Figure 2 (control\textsuperscript{R+E} n = 50 synapses, cKO\textsuperscript{R+E} n = 50). (C) Distribution of synaptic vesicles relative to the presynaptic plasma membrane area opposed to the PSD. Vesicle distribution is shown in 100 nm bins (left) in cKO\textsuperscript{R+E} and control\textsuperscript{R+E} synapses. Gaussian fits were used to model the vesicle distribution. The two genotypes were significantly different (* p < 0.05) and could not be fit with a single distribution, requiring individual fits. Distribution of synaptic vesicles within the first 100 nm in 10 nm bins and the number of tethered vesicles (defined as vesicles within 100 nm of the presynaptic plasma membrane) are shown in the middle and on the right, respectively (control\textsuperscript{R+E} n = 50 synapses, cKO\textsuperscript{R+E} n=50). (D, E) Sample images (D) and quantification (E) of postsynaptic protein synaptic fluorescence levels at cKO\textsuperscript{R+E} and control\textsuperscript{R+E} synapses using confocal microscopy as described in Figures 2.1 B, C. The black dotted line indicates control levels (control\textsuperscript{R+E} n = 3 independent cultures, cKO\textsuperscript{R+E} n = 3, 10 images per culture). (F, G) Quantitative Western blotting for PSD proteins using fluorescent secondary antibodies. Sample images (F) and quantification (G) of total postsynaptic protein levels in cKO\textsuperscript{R+E} neurons normalized to protein levels in control\textsuperscript{R+E} neurons (black dotted line) are shown (control\textsuperscript{R+E} n = 3 independent cultures, cKO\textsuperscript{R+E} n = 3). All data are means ± SEM; *** p ≤ 0.001 as determined by Student’s t test (B) or * p < 0.05 by extra sum of squares F test (C).
Figure 2.2 (Continued)
glutaraldehyde fixed tissue (Supplemental Figures 2 A-C) and saw a similarly robust 85% reduction of docked vesicles. This effect was much stronger than loss of RIMs alone, as assessed with glutaraldehyde fixed tissue (Kaeser et al., 2011). Furthermore, in cKO<sup>R+E</sup> neurons we observe a 50% loss of vesicles within 100 nm of the target membrane (which we refer to as tethered vesicles) using high pressure frozen tissue (Figure 2.2 C) and a similar reduction using glutaraldehyde fixed tissue (Supplemental Figure 2 C). Knockout mutations for Munc13 and the SNAREs, SNAP-25 and Syntaxin-1 do not have a reduced number of docked vesicles using glutaraldehyde fixed tissue nor a reduction in tethered vesicles, but reveal their functions in tight vesicle docking only with high-pressure freezing and/or tomography (Augustin et al., 1999; Bronk et al., 2007; de Wit et al., 2006; Imig et al., 2014; Siksou et al., 2009). Thus, because the cKO<sup>R+E</sup> docking deficit is easily detected in glutaraldehyde fixed tissue and extends to distances up to 100 nm away from the target membrane, we conclude that these neurons have a very strong deficit in docking and tethering synaptic vesicles to the presynaptic plasma membrane, establishing a requirement for the active zone for these processes.

Because the active zone protein complex interacts through trans-synaptic protein complexes with the PSD (Kaeser et al., 2012), it is possible that a strong disruption of the active zone affects the integrity of the PSD. In cKO<sup>R+E</sup> neurons, the length of the postsynaptic density was not affected (Figures 2.2 B, Supplemental Figure 2 B), and levels and localization of PSD-95, N-methyl-D- aspartate (NMDA) receptors, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors were not changed (Figures 2.2 D-G). Thus, we conclude that structural effects of RIM and ELKS deletion are largely restricted to the active zone. We monitored synaptic transmission electrophysiologically in
cultured neurons and found that single action potential evoked excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs, respectively) were strongly reduced (by 90% and 81%, respectively) but not abolished (Figures 2.3 A, B, E, F). The rise time of the synaptic response was slowed (Figures 2.3 C, D, G, H), and the variability in amplitude and rise time was strongly increased, suggesting increased asynchrony in cKO<sup>R+E</sup> synapses.

We next stimulated the synapses with pairs of stimuli at closely spaced time intervals and calculated the paired-pulse ratios (PPRs). PPRs are inversely correlated with initial vesicular release probability P (Zucker and Regehr, 2002) and can be used as a relative measure of P when comparing genotypes. Consistent with a strong reduction in release probability, PPR was strongly increased at short inter-stimulus intervals at excitatory (Figures 2.3 I, J) and inhibitory synapses (Figures 2.3 K, L). This decrease is reminiscent of the decrease in release probability observed in RIM knockout synapses (Kaeser et al., 2011, 2008; Schoch et al., 2002), which is caused by reduced tethering of presynaptic Ca<sup>2+</sup> channels (Kaeser et al., 2011).

To test whether Ca<sup>2+</sup> influx is reduced upon disruption of the active zone, we imaged single action potential evoked presynaptic Ca<sup>2+</sup> transients (Figures 2.4 A-C). Briefly, individual neurons were patched and filled with an Alexa594 dye to identify the axon and the presynaptic boutons, and with the Ca<sup>2+</sup> indicator Fluo5F that increases fluorescence upon Ca<sup>2+</sup> binding (Figure 2.4 A). After dye filling, a brief somatic current injection was used to induce a single action potential, and Ca<sup>2+</sup> transients were recorded in individual boutons and secondary dendrites (Figure 2.4 B). We found a 44% reduction in the peak amplitude of the Ca<sup>2+</sup> influx in boutons, but dendritic Ca<sup>2+</sup> transients remained unaffected (Figure 2.4 C). These data match well with the observation of a loss of Ca<sub>γ</sub>2.1 Ca<sup>2+</sup> channels
Figure 2.3. Single action potential evoked synaptic transmission and release probability are strongly decreased upon disruption of the active zone. (A, B) NMDAR-EPSCs were evoked by a focal stimulation electrode. Example traces (A) and quantitation of EPSC amplitudes (B) and their coefficient of variation (C.V.) in cKO<sup>R+E</sup> and control<sup>R+E</sup> neurons are shown (control<sup>R+E</sup> n = 24 cells/4 independent cultures, cKO<sup>R+E</sup> n = 26/4). (C, D) Sample traces (C) and quantitation (D) of EPSC rise times and their C.V. (n as in B). Individual sweeps are shown in grey and the average of all sweeps is shown in black. Traces are normalized to the average response. (E – H) Same analysis as in A-D for IPSCs (control<sup>R+E</sup> n = 19/3, cKO<sup>R+E</sup> n = 19/3). (I, J) Analysis of NMDAR-EPSC paired pulse ratios (PPRs) in cKO<sup>R+E</sup> and control<sup>R+E</sup> neurons. Sample traces (I, traces normalized to the first response) and quantitation (J) of the PPR at 100 ms interstimulus interval (control<sup>R+E</sup> n = 23/4 independent cultures, cKO<sup>R+E</sup> n = 26/4). (K, L) Scaled sample traces (K, traces normalized to the first response) and summary data (L) of IPSC PPRs at variable interstimulus intervals (control<sup>R+E</sup> n = 19/3, cKO<sup>R+E</sup> n = 19/3). All data are means ± SEM; **p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test in A-H, or by two-way ANOVA in J (genotype, interstimulus interval, and interaction p ≤ 0.001, p values of post-hoc Holm-Sidak tests are shown).
Figure 2.3 (Continued)
Figure 2.4. Impaired Ca\textsuperscript{2+} influx in active zone disrupted neurons. (A) Sample images of cKO\textsuperscript{R+E} and control\textsuperscript{R+E} neurons filled via patch pipette with Fluo5F and AlexaFluor594 (red, top) and enlarged view of boutons (bottom) analyzed in B. (B) Somatic action potentials (top) and presynaptic Ca\textsuperscript{2+} transients imaged via Fluo5F fluorescence (bottom) of the color coded boutons shown in A. (C) Summary plots of single action potential-induced Ca\textsuperscript{2+} transients in boutons, inset: same plot for dendrites. Data are shown as mean (line) ± SEM (shaded area). *** p < 0.001 for Ca\textsuperscript{2+} transients during the first 60 ms after the action potential as assessed by two-way ANOVA for genotype and time; interaction n.s. (boutons: control\textsuperscript{R+E} n = 202 boutons/16 cells/3 independent cultures, cKO\textsuperscript{R+E} n = 157/13/3; dendrites: control\textsuperscript{R+E} n = 148 dendrites/16 cells/3 independent cultures, cKO\textsuperscript{R+E} n = 100/13/3).
Figure 2.4 (Continued)
(Figure 2.1 C), with the strong reduction in vesicular release probability (Figures 2.3 I-L), and with the previously described roles for RIM and ELKS proteins in enhancing presynaptic Ca\textsuperscript{2+} influx in hippocampal neurons (Kaeser et al., 2011; Liu et al., 2014).

**Persistence of release upon active zone disruption.**

We next stimulated the cKO\textsuperscript{R+E} neurons with short action potential trains (50 stimuli at 10 Hz). Surprisingly, we detected a strong buildup of release at excitatory cKO\textsuperscript{R+E} synapses starting with the second action potential, and the increase was sustained throughout the action potential train (Figures 2.5 A, B; Supplemental Figure 3 A). Similarly, vesicles were released quite efficiently throughout an action potential train at inhibitory synapses (Figures 2.5 E, F; Supplemental Figure 3 C). When we quantified the synchronous charge component throughout the train, we observed a reduction of 50% at excitatory cKO\textsuperscript{R+E} synapses (Figure 2.5 C) and of 62% at inhibitory synapses (Figure 2.5 G). The total charge, the tonic component during the train, and delayed charge starting 100 ms after stimulation ended were affected to a similar extent (Supplemental Figures 3 B, D). The steady state amplitude at the end of the train was reduced by 41% and 33%, for EPSCs and IPSCs, respectively (Figures 2.5 D, H). Thus, despite loss of docked vesicles, disruption of the active zone and a strong impairment of single action potential induced release, release persisted during trains of action potentials. This finding is consistent with a strong reduction in P, but suggests that an increase in P due to Ca\textsuperscript{2+} buildup during the stimulus train releases synaptic vesicles quite efficiently. This is reminiscent of a similar electrophysiological phenotype upon deletion of RIM-BP in *D. melanogaster* (Liu et al., 2011).
**Figure 2.5. Release during action potential trains and mini release are sustained upon disruption of the active zone.** (A-D) Sample traces (A) and quantitation of amplitudes (B), synchronous charge (C), and steady state EPSC amplitude (D, average of the last ten EPSCs) of NMDAR-EPSCs evoked by stimulation trains (10 Hz, 50 stimuli) in cKO^{R+E} and control^{R+E} neurons (control^{R+E} n = 17/3, cKO^{R+E} n = 18/3). (E-H) Analysis as in A-D but for IPSCs evoked by stimulation trains (10 Hz, 50 stimuli, control^{R+E} n = 19/3, cKO^{R+E} n = 19/3). (I-K) Example traces (I) of action potential evoked IPSCs at [Ca^{2+}]_{ex} of 0.5, 1, 2, 5, and 7 mM in cKO^{R+E} and control^{R+E} neurons. Absolute IPSC amplitudes (J) and amplitudes normalized to the response at 7 mM [Ca^{2+}]_{ex} (K) are shown (control^{R+E} n = 8/3, cKO^{R+E} n = 8/3). (L-N) Recordings of mEPSCs in synapses lacking RIM (cKO^R), ELKS (cKO^E), or both (cKO^{R+E}). In each experiment, control neurons are identical to the respective cKO neurons except that the cre lentivirus is inactive in the control neurons. Sample traces (L) and quantitative analysis of mEPSC frequencies (M) and amplitudes (N) are shown (control^R n = 20/3, cKO^R n = 21/3; control^E n = 25/3, cKO^E n = 24/3; control^{R+E} n = 32/5, cKO^{R+E} n = 31/5). For expanded mEPSC traces and mIPSC data, see Supplemental Figure 3.

All data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 as determined by Student’s t test (A-H, L-N) or two-way ANOVA in J (genotype: *** p < 0.001; [Ca^{2+}]_{ex}: *** p < 0.001, interaction: *, p ≤ 0.05. p values of post-hoc Holm-Sidak tests are shown in J). For analyses of release components during trains, see Supplemental Figures 3 A-D.
We next varied the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{ex}\)) and measured the IPSC amplitude. Remarkably, despite the reduction of presynaptic Ca\(^{2+}\) channels and Ca\(^{2+}\) influx, increasing [Ca\(^{2+}\)]\(_{ex}\) from 2 mM to 5 mM strongly enhanced the IPSC in cKO\(^{R+E}\) neurons by a factor of 2.7 to 4.5 nA (Figures 2.5 I, J). In both conditions, release saturated above 5 mM [Ca\(^{2+}\)]\(_{ex}\) likely because Ca\(^{2+}\) influx itself saturates at 5 mM (Ariel and Ryan, 2010). When we expressed these data normalized to the largest response (Figure 2.5 K), we observed a right-shift in the dependence of release on [Ca\(^{2+}\)]\(_{ex}\) (EC50 values as obtained through fitting each cell: control\(^{R+E}\) EC50 = 1.55 ± 0.238, cKO\(^{R+E}\) EC50 = 2.33 ± 0.197, *, p < 0.05), confirming a reduction in P. Thus, the active zone per se is not required for fusion of synaptic vesicles, and when P is increased, for example during action potential trains or by increasing [Ca\(^{2+}\)]\(_{ex}\), vesicles can be quite efficiently released. Because release is proportional to the product of RRP size and P, these data suggest that RRP vesicles remain in cKO\(^{R+E}\) neurons, despite the loss of RIM, Munc13, and vesicle docking.

We next assessed the frequency and amplitude of miniature excitatory and inhibitory PSCs in the presence of tetrodotoxin (mEPSCs, mIPSCs, Figures 2.5 L-N, Supplemental Figures 3 E, G, H, I) in cKO\(^ {R+E}\) synapses, and compared these data with synapses that either lack only RIM (cKO\(^ R\)) or ELKS (cKO\(^ E\)). Simultaneous removal of RIM and ELKS led to surprisingly mild, 47% and 49% reductions in mEPSC (Figure 2.5 M) and mIPSC (Supplemental Figure 3 H) frequencies, respectively. The effect on spontaneous release after disruption of the active zone in cKO\(^ {R+E}\) synapses was comparable to the deletion of ELKS alone, and was milder than the reduction upon loss of RIM (78%, Figures 2.5 M, Supplemental Figure 3 F) or Munc13 (Augustin et al., 1999; Varoqueaux et al., 2002) alone. Consistent with the normal architecture of the PSD (Figures 2.2 B, Supplemental
Figure 2 B), mini amplitudes were not affected. Direct comparison of the miniature frequencies in the three lines confirmed a statistically significantly stronger reduction of mini frequency in the synapses that lack only RIM compared to the synapses in which the entire active zone is disrupted (Supplemental Figure 3 F).

**Uniform disruption of synaptic composition and release in cKO\(^{R+E}\) neurons.**

Thus far, our data reveal a strong reduction in release probability at cKO\(^{R+E}\) synapses and show that mini release and release in response to stimulus trains is more mildly impaired than one would predict from the strong structural disruption of the active zone. This suggests that fusion competent vesicles are present despite loss of Munc13, RIM, and vesicle docking. An alternative explanation is that only a subset of synapses is affected in cKO\(^{R+E}\) neurons, and that a population of near normal synapses confounds our analysis. Such heterogeneity could be due to the presence of different neuronal subtypes in our cultures and could arise at the molecular or functional level. We first excluded that the heterogeneity is derived from a population of cells that does not express cre recombinase. Consistent with the analysis of protein levels (Figure 2.1 E), all cells expressed cre (Supplemental Figures 4 A, B). We then tested whether the active zone components of synapses showed a distribution consistent with heterogeneity. We plotted the data presented in Figures 2.1 B, C and Supplemental Figures 1 B-G as a frequency distribution of the fluorescence intensity. Peaks for Munc13, Bassoon (Figure 2.6 A), Piccolo, and RIM-BP (Supplemental Figure 4 C) shifted uniformly to lower intensities. These data argue against strong molecular heterogeneity upon active zone disruption.
**Figure 2.6. Uniform disruption of active zone composition and function in cKO^{R+E} neurons.** (A) Histograms of the distribution of fluorescence intensity levels in cKO^{R+E} and control^{R+E} synapses (normalized to the average fluorescence in control). Data are from the experiments shown in Figures 2.1 B, C. For histograms of RIM, ELKS, Piccolo, RIM-BP2, Liprin-α3, and PSD-95 see Supplemental Figure 4 C. (B) Pseudocolored images of SypHy expressing cultures stimulated with 40 and 200 action potentials (APs), and dequenched with NH4Cl. Images represent peak fluorescence change. (C-E) Quantification of fluorescence changes in cKO^{R+E} and control^{R+E} neurons stimulated with 40 APs, including the time course of the mean fluorescence change in active synapses as a percentage of the fluorescence increase upon NH4Cl application (C), the peak response (D) of active synapses, and frequency distribution of the % response in active synapses at the end of the stimulus train (E; control^{R+E} n = 3493 NH4Cl responsive synapses/2486 active synapses/9 coverslips/3 independent cultures, cKO^{R+E} n = 2192/1272/9/3, the number of coverslips is used as a basis for statistics). (F-H) Quantification as in C-D but for neurons stimulated with 200 APs (control^{R+E} n = 3493/2640/9/3, cKO^{R+E} n = 2291/1405/9/3). All data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as determined by Student's t test.
Figure 2.6 (Continued)
We next turned to presynaptic imaging in cultures expressing SypHy (Granseth et al., 2006), a version of synaptophysin coupled to an intravesicular pHluorin tag. In brief, neuronal cultures were infected with two independent lentiviruses expressing SypHy (for imaging exocytosis) and SV2-TdTomato (to identify synapse-rich areas in the cultures) at DIV3 in addition to the cre and control lentiviruses (which were supplied at DIV5). At DIV15-18, a synapse dense area was chosen based on SV2-TdTomato expression, and the neurons were stimulated with a focal stimulation electrode for 40 or 200 action potentials at 20 Hz. In this experiment, exocytosis is identified as an increase in fluorescence due to unquenching of the pHluorin when it is exposed to the neutral extracellular pH. For the analysis, only puncta that showed at least a 2-fold increase in fluorescence upon application of NH₄Cl (which uniformly raised the intravesicular pH to unquench pHluorin fluorescence) were included. We first determined the fraction of synapses responsive to electrical stimulation at both frequencies, and observed a small but significant decrease in active synapses in the cKO⁹+E neurons (Supplemental Figure 4 D). We next characterized release at active synapses in both genotypes and found a 67% and 68% decrease in the peak response at the end of stimulation at 40 or 200 action potentials, respectively (Figures 2.6 B-H). When we plotted a frequency histogram of the % of the total pool released at the end of the stimulus train for all active synapses, there was a prominent increase in synapses with smaller pHluorin fluorescence changes in cKO⁹+E synapses. This experiment supports that active synapses in cKO⁹+E neurons have impaired release and establishes that the secretory deficit cannot be explained by inactive synapses only. Furthermore, it establishes that vesicle recycling, which contributes strongly to the response to 200 action
potentials, is reduced in the cKO$^{R+E}$ synapses. These experiments exclude that a large fraction of synapses has normal active zones and operates at essentially normal levels.

**Comparison of docking and RRP size upon active zone disruption.**

Our data thus far suggest that there may be a sizable RRP left in cKO$^{R+E}$ synapses even though such a pool is thought to be reflected in docked vesicles at hippocampal synapses (Imig et al., 2014; Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001; Watanabe et al., 2013). We measured the RRP at excitatory synapses using the application of 500 mM hypertonic sucrose and compared the RRP with vesicle docking and distribution at cKO$^R$, cKO$^E$, and cKO$^{R+E}$ synapses (Figure 2.7). RIM deletion resulted in a 46% reduction in docked synaptic vesicles paralleled by a 75% reduction in the RRP, but the distribution of vesicles within a nerve terminal was normal (Figures 2.7 A-D, Supplemental Figures 2 A, B). Removal of ELKS alone did not result in a detectable effect on vesicle docking or vesicle distribution (albeit there was a non-significant trend towards a small reduction in docked vesicles), but induced a 34% reduction in RRP at excitatory synapses (Figures 2.7 E-H, Supplemental Figures 5 C, D). At cKO$^{R+E}$ synapses the RRP was more mildly affected (Figures 2.7 I-L) than one would predict from the effects observed in cKO$^R$ or cKO$^E$ synapses, from the loss of docking, and from the massive reduction in RIM, Munc13, and other active zone proteins (Figure 2.1). 42% of RRP vesicles remained despite the strong reduction in vesicle docking (89% in glutaraldehyde fixed tissue, Figure 2.7 J; 92% in high pressure frozen tissue, Figure 2.2 B). Direct comparison of the three genotypes revealed a significantly stronger loss of docking in cKO$^{R+E}$ synapses compared to cKO$^R$ synapses.
Figure 2.7. Persistence of a readily releasable pool upon loss of synaptic vesicle tethering and docking. (A-D) Sample images (A) and quantitative analyses of synaptic vesicle docking (B) in glutaraldehyde fixed neuronal cultures, and sample traces (C) and quantitation (D) of RRP of RIM deficient cKO\textsuperscript{R} and corresponding control\textsuperscript{R} synapses. Focal application of hypertonic sucrose for 10 s was used to deplete the RRP (D). (E-H) Analyses as outlined in A-D, but of ELKS deficient cKO\textsuperscript{E} and control\textsuperscript{E} synapses. (I-L) Analyses as outlined in A-D, but of RIM/ELKS deficient cKO\textsuperscript{R+E} and control\textsuperscript{R+E} synapses. For analyses of vesicle numbers, bouton size, PSD length, and vesicle distribution, see Supplemental Figures 5 A-F. All data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test (analysis of vesicle docking and tethering: control\textsuperscript{R} n = 25 synapses, cKO\textsuperscript{R} n = 25; control\textsuperscript{E} n = 25, cKO\textsuperscript{E} n = 25; control\textsuperscript{R+E} n = 25, cKO\textsuperscript{R+E} n = 25; analysis of RRP: control\textsuperscript{R} n = 20 cells/3 independent cultures, cKO\textsuperscript{R} n = 20/3; control\textsuperscript{E} n = 17/3, cKO\textsuperscript{E} n = 17/3; control\textsuperscript{R+E} n = 20/3, cKO\textsuperscript{R+E} n = 20/3). (M) Schematic of synaptic architecture and function upon disruption of the active zone. Structures and processes that are strongly disrupted upon RIM and ELKS deletion are labeled in yellow (active zone, docking, single action potential mediated release). Synaptic structures and functions that remain at least partially intact are labeled in green (the synaptic vesicle cluster, the postsynaptic density containing neurotransmitter receptors, mini release, and release in response RRP depleting stimuli such as action potential trains or hypertonic sucrose). Our experiments indicate that at least some vesicles can be recruited from vesicle pools distant from the presynaptic plasma membrane for release, and that these vesicles may be released immediately or undergo a transient docking state (dotted arrow) that is initiated after the onset of stimulation.
Conversely, RRP was more strongly reduced in cKOR synapses than in cKORE or cKO E synapses (Supplemental Figures 5 G, H). These data suggest that at least some RRP vesicles can be recruited over distance and do not have to be stably docked at the active zone before the application of hypertonic stimulus.

Discussion

We here establish a conditional mouse mutant that strongly and specifically disrupts the active zone matrix and synaptic vesicle docking in cultured hippocampal neurons (Figures 2.1 and 2.2). We find that disruption of the active zone results in a strong impairment of vesicular release probability, but surprisingly >40% of RRP vesicles remained (Figures 2.3 to 2.7).

Redundant scaffolding functions of RIM and ELKS.

The multi-domain structure of RIM and ELKS and their extensive biochemical binding activities with other active zone proteins suggested that they operate as scaffolds (Ohtsuka et al., 2002; Schoch et al., 2002; Takao-Rikitsu et al., 2004; Wang et al., 2002). However, loss of function approaches thus far provided mixed support for this hypothesis. Knockout mutants for ELKS1 and/or ELKS2 showed no changes in active zone composition at hippocampal synapses (Held et al., 2016; Liu et al., 2014), except for a small increase in the biochemical solubility of RIM (Kaeser et al., 2009). Similarly, RIM1 and/or RIM2 knockout mice revealed isolated changes in the clustering and levels of Munc13-1 (Deng et al., 2011; Schoch et al., 2002). Beyond these changes in individual active zone proteins however, the active zone protein complex was intact. Here, we reveal a strong, redundant
scaffolding role for RIM and ELKS: simultaneous removal leads to disruption of the active zone with a loss of three out of four additional active zone protein families. Our morphological and functional analyses further strongly support redundant scaffolding roles for RIM and ELKS that are similar at excitatory and inhibitory synapses. Thus, we establish an important presynaptic clustering role for RIM and ELKS that is shared across synapses and that tethers Piccolo, Bassoon, Munc13-1, and RIM-BP2. Because these proteins cannot be anchored and maintained in levels at mutant synapses, RIM and ELKS are necessary and thus upstream for their tethering to the active zone.

Interestingly, levels and localization of Liprin-α2 and Liprin-α3, the two Liprin-α isoforms that are strongly expressed in brain and are thought to be localized at the active zone (Spangl et al., 2011; Zürner et al., 2011; Zürner and Schoch, 2009), are not affected in our mutants. This suggests that Liprin-α2/3 are either upstream in active zone assembly and can be tethered independent of RIM and ELKS, or that Liprin-α2/3 are not part of the same protein complex. Genetic experiments have firmly established presynaptic roles for Liprin-α/syd-2 in synapse assembly in C. elegans and D. melanogaster (Kaufmann et al., 2002; Zhen and Jin, 1999). Although the localization of individual vertebrate Liprin-α proteins has not been conclusively solved and the available data support pre-, post-, or extra-synaptic localization of Liprin-α proteins (Spangl et al., 2011; Wyszynski et al., 2002; Zürner et al., 2011), a recent study employed knockdowns for Liprin-α2 and supported presynaptic scaffolding functions (Spangl et al. 2013). In vitro binding of Liprin-α1 through 4 to RIM and/or ELKS (Ko et al., 2003b; Schoch et al., 2002) provide further support for a presynaptic scaffolding role. One possible explanation for these and our data is that Liprin-α is upstream of RIM and ELKS in vertebrate active zone assembly.
Importantly, invertebrate Liprin-α/syd-2 mutant synapses also have reduced vesicle numbers in the nerve terminal (Kaufmann et al., 2002; Patel et al., 2006; Zhen and Jin, 1999) suggesting that the active zone may recruit vesicles to a presynaptic nerve terminal. Our experiments at vertebrate synapses reveal that synaptic vesicle numbers are unchanged upon active zone disassembly, establishing that the active zone protein complex downstream of Liprin-α is not required for recruitment of vesicles to the nerve terminal. Our data are consistent with additional roles for Liprin-α outside of the active zone, for example in trafficking of vesicles or active zone material, as has been shown for D. melanogaster Liprin-α (Miller et al., 2005). Further genetic experiments will be necessary to dissect the roles of Liprin-α in the vertebrate brain.

Recent studies support that synaptic and network activity contribute to active zone protein turnover (Lazarevic et al., 2011; Sugie et al., 2015; Weyhersmüller et al., 2011). It is thus possible that loss of synaptic activity in the cultured neurons contributes to the strong active zone disruption that we observe upon RIM/ELKS deletion. However, reduced activity is unlikely to play an important causative role for active zone disruption in our experiments because knockouts for only RIM (Deng et al., 2011) or Munc13 (Varoqueaux et al., 2002), which have similar or more severe reductions in activity, do not lead to strong active zone disruption. In the long-term, it will be important to test causes and effects of the active zone disruption we describe here in a system that allows manipulation and characterization of a specific synapse in a defined circuit to better understand how cell-type specificity and activity contribute to the phenotypes.

The role of the active zone in synaptic vesicle docking.
Because synaptic vesicles are only docked at the active zone (Couteaux and Pécot-Dechavassine, 1970; Imig et al., 2014; Siksou et al., 2009; Südhof, 2012), it has been proposed that the active zone provides the molecular mechanism for docking of synaptic vesicles to the target membrane. Consistent with this hypothesis, RIM1/2 double knockout synapses have an approximately 50% reduction in the number of docked vesicles in cultured hippocampal neurons in glutaraldehyde fixed tissue. Importantly, using the same method, no other presynaptic protein, including Munc13 (Augustin et al., 1999; Varoqueaux et al., 2002), synaptobrevin-2 (Deák et al., 2004), SNAP-25 (Bronk et al., 2007), or CAPS (Jockusch et al., 2007) have a role in synaptic vesicle docking. Compellingly, disruption of the active zone in the cKO\textsuperscript{R+E} neurons leads to a near complete loss of vesicle docking in glutaraldehyde fixed tissue (Supplemental Figure 2 B, Figure 2.7 J). Recent experiments have used high-pressure freezing and tomography, which improved the resolution in the analysis of docking to two nanometers. Using this method, it has been found that loss of function mutations for Munc13, CAPS, and the SNAREs syntaxin-1, synaptobrevin-2, and SNAP-25 have a strong reduction in synaptic vesicles within 0-5 nm of the target membrane, but normal or increased vesicle numbers in bins at 10 and 20 nm away from the active zone (Imig et al., 2014; Siksou et al., 2009). The reduction of docked vesicles at cKO\textsuperscript{R+E} synapses is apparent with both fixation methods. With high-pressure freezing, the 92% reduction at cKO\textsuperscript{R+E} synapses (Figure 2.2 A-C) is similar to Munc13 null mutants, which have an 96% reduction in docked vesicles when using the same method combined with electron tomography (Imig et al., 2014). Furthermore, unlike Munc13 deficient synapses, cKO\textsuperscript{R+E} synapses fail to accumulate vesicles 10-20 nm away from the target membrane, but show a 50% reduction in numbers of tethered vesicles within 100
nm of the target membrane. Thus, we conclude that the loss of docking and tethering of synaptic vesicles in the cKO^{R+E} mutant is stronger than in previous mutants because the loss of docking is readily detected in glutaraldehyde fixed tissue and there is a shift of the entire vesicle cluster away from the target membrane that has not been seen in other mutants. We conclude that the active zone is required for synaptic vesicle docking and tethering.

**The relationship between synaptic vesicle docking, priming, and release.**

The strong decrease in single action potential mediated release (Figure 2.3) corresponds well with the loss of vesicle docking (Figure 2.2) and is consistent with the hypothesis that single action potentials release docked vesicles (Rosenmund and Stevens, 1996). Release from a single synapse is proportional to the RRP size and P (Zucker and Regehr, 2002). Our analysis revealed a strong reduction in P upon disruption of the active zone (Figures 2.3 I-L, 2.4, 2.5 I-K). This observation is supported by a strong increase in PPRs, a right-shift in the $[\text{Ca}^{2+}]_{\text{ex}}$ dependence of release, an increase in C.V. of the PSC amplitude, and a loss of presynaptic Ca$_{V2.1}$ Ca$^{2+}$ channels and Ca$^{2+}$ influx. Surprisingly, manipulations that enhanced P (increasing $[\text{Ca}^{2+}]_{\text{ex}}$ and action potential trains) or bypassed the need for Ca$^{2+}$ (stimulation with hypertonic sucrose) demonstrated that a significant pool of vesicles is available for release in cKO^{R+E} synapses.

Many studies have defined the RRP as vesicles that are either released during the transient response to hypertonic sucrose (Augustin et al., 1999; Deng et al., 2011; Rosenmund and Stevens, 1996; Varoqueaux et al., 2002) or during brief trains of action potentials (Schikorski and Stevens, 2001) and RRP size estimates determined by these
methods are well correlated with the number of morphologically docked vesicles. Using hypertonic sucrose in cKO<sup>R+E</sup> neurons, 42% of the RRP remained (Figure 2.7 L), which is significantly larger than the RRP left after deletion of RIM (Supplemental Figure 5 H and (Kaeser et al., 2011)) or Munc13 (Augustin et al., 1999; Varoqueaux et al., 2002) despite the more severe docking deficit at cKO<sup>R+E</sup> synapses. This challenges the notion that the priming functions of these proteins are identical to their functions in vesicle docking. Furthermore, spontaneous miniature release (Figures 2.5 L-N, Supplemental Figures 3 G, H) and release during action potential trains (Figures 2.5 A-H, 2.6 C-H) are also more mildly reduced than expected. Due to the strong reduction in P at cKO<sup>R+E</sup> synapses, it was not possible to measure RRP size using high frequency action potential stimulation (Thanawala and Regehr, 2016). Nevertheless these data reveal that fusion competent vesicles can be recruited over distance and do not require a persistently docked state. RRP vesicles may therefore be stored away from the presynaptic plasma membrane, at least in the absence of an active zone, as has been proposed based on experiments that labeled RRP vesicles after recycling (Rizzoli and Betz, 2004).

It is possible that hypertonic sucrose stimulation leads to a transient increase in vesicle docking that is not captured in our electron-microscopic images. This may also be the case for some of our other manipulations, for example high [Ca<sup>2+</sup>]<sub>ex</sub> or prolonged stimulus trains. The small amount of remaining Munc13 in the cKO<sup>R+E</sup> neurons may rapidly add vesicles to the RRP, but this hypothesis implies that Munc13 dependent priming in the cKO<sup>R+E</sup> neurons occurs upstream or simultaneous to contact with the target membrane and is transient, since there are no stably docked vesicles. The relatively mild reduction in mini frequency supports that docking in cKO<sup>R+E</sup> neurons is transient and not a stable state of a
primed vesicle. Alternatively, massive disruption of the active zone may bypass the need for Munc13 to prime vesicles before fusion.

A long-standing question has been whether the partial assembly of SNARE complexes is required for synaptic vesicle docking and priming (Jahn and Fasshauer, 2012; Südhof and Rothman, 2009). Our experiments reveal that SNARE proteins are present in the nerve terminal upon disruption of the active zone, and that synaptic vesicle fusion, which is mediated by SNARE proteins, is not abolished. However, SNARE proteins are not sufficient to drive docking at synapses in the absence of an active zone, suggesting that not all fusion competent vesicles require stable preassembly of SNARE complexes.

Finally, the molecular mechanisms that underlie docking upstream of SNARE complex assembly are poorly understood. With a gene mutation that disrupts docking, but leaves synaptogenesis and presynaptic vesicle clustering intact, analysis of the minimal protein interactions between synaptic vesicles and release sites required for docking will now be possible.
—CHAPTER THREE—

Rebuilding the active zone: the essential role of RIM in vertebrate active zone assembly
Contributions

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S.S.H.W. performed all mouse genetic experiments, molecular biology, immunostaining, super-resolution microscopy, western blotting, and electron microscopy experiments. C.T. performed and analyzed all electrophysiological experiments.
Introduction

The presynaptic active zone is an evolutionarily conserved and highly interconnected protein complex composed of Munc13, RIM, ELKS, RIM-BP, piccolo/bassoon, and Liprin-α (Schoch and Gundelfinger, 2006; Südhof, 2012) that is essential for orchestrating rapid synaptic transmission (see Chapters 1 and 2). Changes in both the molecular composition and structural organization of the active zone scaffold due to genetic manipulations or synaptic plasticity (Glebov et al., 2017; Gundelfinger and Fejtova, 2012; Matz et al., 2010; Tang et al., 2016) can greatly impact synaptic function. Thus, it is important to have a detailed understanding of the mechanisms coordinating active zone assembly and maintaining its structural organization. While decades of genetic studies have been instrumental towards understanding the functional role of individual active zone proteins, redundant scaffolding mechanisms between these proteins have made deciphering the process of active zone assembly challenging, particularly at vertebrate synapses. Questions that remain unclear are: how do active zone proteins target to active zone sites along the presynaptic plasma membrane and what specific molecular mechanisms maintain its structure?

Active zone assembly is a complex and hierarchical process. From initiation, the active zone first needs to target to specific presynaptic membrane sites of newly forming synapses. In both C. elegans and D. melanogaster, genetic experiments have revealed that Liprin-α may be involved in this process, as loss of Liprin-α led to aberrations in active zone size and synaptic vesicle clustering (Dai et al., 2006; Kaufmann et al., 2002; Miller et al., 2005; Patel et al., 2006). Following site selection, active zone components next need to be transported and captured at synapses. It has been proposed that active zone
components are pre-assembled in precursor transport vesicles that traffic to release sites from the golgi (Dresbach et al., 2006a; Shapira et al., 2003; Zhai et al., 2001). These 80-nm dense core vesicles are positive for piccolo/bassoon and thought to transport other active zone components (ELKS, RIM, and Munc13) during synapse formation (Ohtsuka et al., 2002; Shapira et al., 2003). More complete analysis using light microscopy and live cell imaging revealed that active zone precursor vesicles are not completely assembled at the golgi, but rather are thought to undergo various maturation steps en route to nascent synapses (Maas et al., 2012). However the specific mechanisms that mature and capture active zone cargo at synapses remains an open question.

Finally at synapses, active zone components must scaffold and arrange themselves in such a way to maintain structure and support a diverse range of functions. For this, much of what we know about the molecular mechanism involved in assembling the active zone scaffold comes from extensive genetic experiments and biochemical protein-protein interaction assays. For example, RIM and Munc13 proteins are functionally related priming factors at the active zone (Augustin et al., 1999; Deng et al., 2011; Kaeser et al., 2008; Schoch et al., 2002; Varoqueaux et al., 2002). The N terminal Zn finger of RIM interacts with the C2A domain of Munc13 (Betz et al., 1998; Dulubova et al., 2005), but while synaptic levels of Munc13 are strongly reduced in RIM mutants (Deng et al., 2011; Schoch et al., 2002), RIM levels are not reduced in Munc13 mutants (Andrews-Zwilling et al., 2006). Further protein biochemical assays revealed that a point mutation in the Munc13 C2A domain (I121N) strongly interferes with its capacity to bind RIM, and the Munc13_{I121N} mutant is unable to target to synapses when expressed in Munc13-1/2 DKO neurons (Andrews-Zwilling et al., 2006). This finding establishes that the RIM/Munc13 interaction
is an important mechanism for targeting Munc13 to the active zone. However the ability to address such questions for the active zone, as a whole, has been limited because genetically targeting individual active zone proteins does not generally cause strong structural impairments.

Active zone assembly relies on redundant scaffolding mechanisms and only by targeting multiple active zone proteins simultaneously are strong structural disruptions achieved (Acuna et al., 2016; Wang et al., 2016). Specifically, at vertebrate synapses, removing both RIM and RIM-BP led to a further reduction in Munc13 and Bassoon levels, as well as an increase in expression of the postsynaptic density protein PSD-95. Structurally, RIM and RIM-BP knockout resulted in a loss of vesicle tethering, and a reduction in the hexagonal grid of dense projections seen with phosphotungstic acid (PTA) that mark the active zone (Acuna et al., 2016). Similarly, even though deletion of either RIM or ELKS alone has not uncovered extensive scaffolding functions at mammalian synapses (Deng et al., 2011; Kaeser et al., 2011; Liu et al., 2014), removing both RIM and ELKS from cultured hippocampal neurons leads to the strongest active zone disrupted mutant to date (Wang et al., 2016) (Chapter 2). In neurons lacking RIM and ELKS, while synapses form normally and there is no change in PSD, there is are profound defects in active zone assembly, illustrated by reductions in the synaptic and total expression levels of Munc13, piccolo/bassoon, RIM-BP, and Ca^{2+} channels. Furthermore, while there is no change in presynaptic bouton size or vesicle recruitment as assessed by electron microscopy, there is an almost complete loss of vesicle docking and tethering to the presynaptic plasma membrane.

Thus the RIM and ELKS knockout model presents a unique opportunity: the ability to study active zone assembly by rebuilding the active zone at mutant synapses. This
mutant background provides two major advantages. First, because RIM and ELKS knockout neurons do not exhibit more widespread impairments in synapse formation, such as a change in the PSD or vesicle recruitment, rescue experiments in this background will provide insight on assembly and functional mechanisms specific to the core active zone complex. Second, the extensive active zone disruption seen in the RIM and ELKS mutant provides the opportunity to study the assembly mechanisms for the majority of core active zone proteins simultaneously.

In order to identify the specific protein domains capable of restoring active zone structure and function, we use lentiviruses to express RIM or ELKS rescue constructs at active zone disrupted synapses. Here, we establish that RIM1α and ELKS1α localize to mutant synapses independent of one another. In contrast, while ELKS1α can recruit bassoon back to active zone disrupted synapses, RIM1α can restore all interaction partners to mutant synapses in a concentration-titrated manner, as well as restore vesicle docking and synaptic transmission. These data establish that RIM is essential for holding the protein complex together and for vesicle docking. Using structure-function rescue experiments and STED imaging, we then identify that the PDZ domain of RIM is necessary for its active zone localization while the Zn finger of RIM is necessary to restore vesicle docking. When expressed alone, the Zn finger of RIM co-localizes with the synaptic vesicle cloud and recruits priming factor Munc13 to vesicles distal from the presynaptic plasma membrane. Together, these results establish that RIM is a master organizer of the active zone.

Experimental Procedures
The quadruple homozygote floxed mice for RIM1αβ, RIM2αβγ, ELKS1α, and ELKS2α were previously characterized (Chapter Two). All experiments were performed in cultured hippocampal neurons infected at DIV 5 with lentiviruses expressing cre recombinase or an inactive mutant of cre under a synapsin promoter and then assessed at DIV 15-19, as previously described (Chapter Two and Appendix Two). For lentiviral expression of HA-tagged RIM and ELKS rescue constructs, neurons were infected at DIV3. For details on the production of lentivirus, HPF electron microscopy, confocal imaging, Western blotting see Appendix Two. All experiments were performed and analyzed by an experimenter blind to the genotype and significance was determined using Student’s t-tests unless otherwise noted. Statistical significance was set at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Materials and methods specific to this chapter are described below.

**RIM and ELKS rescue constructs.** For full length RIM1α, the open reading frame (ORF) was subcloned into a lentiviral backbone driven by either the human synapsin promoter (RIM1α\text{low}) or the human ubiquitin promoter (RIM1α\text{high}). The human synapsin promoter is used to drive all other rescue constructs. A Human influenza hemagglutinin (HA) tag was inserted between residues E1378-S1379 for all RIM1α rescue constructs. For ELKS1α an HA tag was inserted at the N terminus of the protein. For the RIM1α domain specific rescue constructs, the Zn finger is defined from residues M1-D213, the PDZ is defined from residues H597-R705, the C2A domain is defined from residues Q754-E856, and the C2B domain is defined from residues G1446-S1615. All individual RIM domain and Δ RIM domain rescue constructs span or are missing these corresponding domains, except for the ΔZn finger construct, which spans H597-S1615.
**Antibodies.** The antibodies and concentrations used in this study are as follows.

- Monoclonal mouse anti-Bassoon (1:1000; RRID: AB_11181058),
- Guinea pig anti-Bassoon (1:1000, RRID: AB_2290619),
- Rabbit anti-Cav2.1 (1:1000; RRID: AB_887699),
- Monoclonal mouse anti-ELKS1/2α (1:500; RRID: AB_869944),
- Rabbit anti-ELKS1/2 (serum 1083, 1:5000; custom made),
- Rabbit anti-ELKS2α (serum 1029, 1:100; custom made),
- Monoclonal mouse anti-HA (1:1000; RRID: AB_2565006),
- Rabbit anti-Liprin-α3 (1:2000; a gift from Dr. T. Südhof),
- Monoclonal mouse anti-MAP2 (1:500, RRID: AB_477193),
- Rabbit anti-MAP2 (1:1000, RRID: AB_2138183),
- Rabbit anti-Munc13-1 (1:1000, RRID: AB_887733),
- Monoclonal mouse anti-PSD-95 (1:500, RRID: AB_10698024),
- Rabbit anti-RIM1 (1:1000, RRID: AB_887774),
- Rabbit anti-RIM-BP2 (1:500, RRID: AB_2619739),
- Rabbit anti-synaptophysin (1:1000, RRID: AB_887905),
- Guinea pig anti-synaptophysin (1:1000, RRID: AB_1210382).

**STED imaging.** Neurons cultured on #1.5 thickness 12 mm coverslips were rinsed twice with warm PBS, then fixed for 10 minutes in 2% PFA. After fixation, coverslips were rinsed twice in PBS, then permeabilized in PBS + 0.1% Triton X-100 + 3% BSA (TBP) for 1 hour. Primary antibodies were diluted in TBP and stained for 48 hours at 4C. Coverslips were then washed 5 times for 3 minutes in TBP. Alexa Fluor 488, 555, and 633 were used as secondary antibodies at 1:250 (488 and 555) and 1:500 (633) dilution in TBP and stained for 24 hours at 4C. Following secondary incubation, coverslips were washed 5 times for 3 minutes and then post-fixed for 10 minutes with 4% PFA. Finally, coverslips were rinsed.
twice in PBS, then once in deionized water, and mounted on glass slides with ProLong ® Diamond Antifade Mountant.

STED images were acquired with a Leica SP8 Confocal/STED 3X microscope with an oil immersion 100X 1.44 numerical aperture objective at the Harvard NeuroDiscovery Center Enhanced Imaging Core. 46.51 x 46.51 μm² areas were taken at a pixel density of 4096 x 4096 (11.358 nm/pixel). Alexa Fluor 633, Alexa Fluor 555, and Alexa Fluor 488 were excited with 633 nm, 555 nm, and 488nm white light lasers respectively at 2-5% of 1.5 mW laser power in sequence by frame. The Alexa Fluor 633 channel was acquired first in confocal mode using 3x frame averaging. Subsequently, the Alexa Fluor 488 channel was acquired in STED mode using 2x frame averaging and depleted with the 592 nm (80% of max power) depletion laser. Finally, the Alexa Fluor 555 channel was acquired in STED mode using 2x frame average and depleted with the 660 nm (50% of max power) depletion laser. Line accumulation (1-10x) was applied during scanning.

Synapses within STED images were selected in ‘side view’, defined as a synaptic vesicle cluster directly opposed to an elongated postsynaptic ‘bar’ structure along its edge, using only the synaptophysin and PSD-95 channels. For line profile analysis, a ~1.2 μm long x ~200 μm wide rectangle was drawn perpendicular to the elongated PSD ‘bar’ structure and an intensity profile was obtained for all three channels along this region. To align individual profiles, the PSD-95 signal was first smoothed using a 5 pixel moving average and the smoothed signal was used to define the point of peak intensity. All three channels were aligned to this peak position and averaged. To normalize the signal within individual channels, the average of the maximum signal in each channel for the control condition was used.
Results

**RIM titrates synaptic active zone protein levels in active zone disrupted mutants**

Removing both RIM and ELKS proteins from cultured hippocampal neurons (cKO^{R+E}) strongly disrupts the active zone protein complex compared to control (control^{R+E}) (Figure 2.1). To test whether RIM and ELKS can localize to active zone disrupted synapses and rescue active zone assembly, we infected cKO^{R+E} neurons at 3 days in vitro (DIV3) with lentiviruses expressing HA-tagged full-length RIM1α or ELKS1α (Figure 3.1). For RIM1α, two different lentiviruses were used: one driven by the human synapsin promoter (RIM1α_{low}) for lower expression, and one driven by the human ubiquitin promoter (RIM1α_{high}) for higher levels of RIM1α expression (Figure 3.1 B). Synaptic expression levels of RIM1α and ELKS1α were assessed by confocal microscopy (Figures 3.1 A, C), which revealed that both full-length RIM1α and ELKS1α can localize to active zone disrupted synapses independent of one another. Compellingly, higher levels of RIM1α protein expression resulted in increased synaptic levels of RIM (Figure 3.1 C).

Having established that RIM1α and ELKS1α can localize to mutant synapses, we next investigated whether RIM1α or ELKS1α can recruit their active zone protein interaction partners back to cKO^{R+E} synapses (Figure 3.2). Both RIM1α and ELKS1α were capable of partially restoring synaptic Bassoon levels (Figure 3.2 D). RIM1α could further rescue Munc13-1, RIM-BP2, and Cav2.1 to active zone disrupted synapses in a concentration dependent manner (Figures 3.2 A-C). That is to say, increasing the level of RIM1α at synapses increased the synaptic levels of these interaction partners. Interestingly, Liprin-α is the only core active zone protein whose synaptic levels appear unaffected by the absence or presence of RIM or ELKS (Figure 3.2 E), perhaps because Liprin-α localizes to active
Figure 3.1. Expressing RIM1α and ELKS1α at active zone disrupted synapses. (A)

Sample images of RIM1α (top) and ELKS1α (bottom) (green channel) along with synaptophysin (red channel) and MAP2 (blue channel) in controlR+E, cKO R+E, RIM1α rescue (low), RIM1α rescue (high), and ELKS1α rescue conditions. (B) Levels of RIM and ELKS protein in controlR+E, cKO R+E, and rescue conditions as assessed by western blotting. (C) Quantification of RIM (left) and ELKS (right) fluorescence levels at synapses. Synaptophysin (red) was used to define ROIs (for all conditions n = 3 cultures, 10 images were averaged per culture). All data are means ± SEM; ***p ≤ 0.001 as determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc test comparing each condition to cKO R+E.
**Figure 3.2. Synaptic rescue of the active zone protein scaffold.** (A-E) Sample images (left) and quantification (right) of Munc13-1 (A), RIM-BP2 (B), Cav2.1 (C), Bassoon (D), and Liprin-α3 (E) (green channel) in control$_{R+E}$, cKO$_{R+E}$, RIM1α rescue (low), RIM1α rescue (high), and ELKS1α rescue conditions. Synaptophysin (red channel) was used to define ROIs (for all conditions n = 3 cultures, 10 images were averaged per culture). All data are means ± SEM; ***p ≤ 0.001 as determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc test comparing each condition to cKO$_{R+E}$. 
Figure 3.2 (Continued)
zones by a mechanism independent of RIM or ELKS. Altogether, these data reveal that RIM is a central scaffolding molecule for active zone assembly that controls the synaptic levels of most of its interaction partners.

RIM rescues synaptic vesicle docking and fusion at cKO^{R+E} mutant synapses

Disruption of the active zone protein complex leads to the loss of synaptic vesicle docking, as defined by synaptic vesicles touching the presynaptic plasma membrane (Figure 2.2). Expressing full-length RIM1α or ELKS1α at cKO^{R+E} synapses is sufficient to rescue multiple components of the active zone protein complex (Figure 3.2). To test which aspects of the active zone protein scaffold are necessary to restore synaptic vesicle docking, we fixed cKO^{R+E} neurons expressing RIM1α or ELKS1α with high pressure freezing and used electron microscopy to compare their ultrastructure to control^{R+E} neurons (Figure 3.3 A). While there was no difference in the total number of vesicles, the length of the postsynaptic density, or the size of the presynaptic bouton in any of the conditions, RIM1α was able to partially restore both synaptic vesicle docking and synaptic vesicle tethering, defined as the number of vesicles within 100nm of the presynaptic active zone (Figures 3.3 B-H). Expressing higher levels of RIM1α did not further increase the number of docked vesicles, and ELKS1α did not have a significant effect on synaptic vesicle docking or tethering (Figures 3.3 C, D).

Because RIM1α is sufficient to restore vesicle docking (Figure 3.3), and much of the active zone scaffold (Figure 3.2) we hypothesized that RIM1α would also be sufficient to rescue vesicle fusion. To test this hypothesis, we next turned to electrophysiological recordings to assess whether RIM1α can restore synaptic transmission and we found that
Figure 3.3. Ultrastructural analysis of restored active zone protein scaffolds. (A)

Sample images of high-pressure frozen neurons analyzed by electron microscopy of control\textsuperscript{R+E}, cKO\textsuperscript{R+E}, RIM1\textalpha  rescue (low), RIM1\textalpha  rescue (high), and ELKS1\textalpha  rescue conditions. (B-F) Quantification of the number of total vesicle per bouton (B), the number of docked vesicles as defined by vesicles touching the presynaptic membrane (C), and the number of tethered vesicles within 100 nm of the presynaptic membrane (D), as well as bouton size (E) and PSD length (D). (G, H) Distribution of synaptic vesicles relative to the presynaptic plasma membrane opposed to the PSD within 100nm bins for the first 1000nm (left) and 10 min bins for the first 100nm (right). (For all conditions n = 50 synapses). All data are means ± SEM; ***p ≤ 0.001 as determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc test comparing each condition to cKO\textsuperscript{R+E}. 
Figure 3.3 (Continued)
RIM1α expression partially rescues the amplitude and rise time of single action potential evoked inhibitory postsynaptic currents compared to mutant cKO_{R+E} synapses (Figures 3.4 A-C). Next we stimulated synapses with pairs of stimuli at closely spaced time intervals to calculate the paired-pulse ratio (PPR), which is inversely proportional to vesicular release probability (P) (Zucker and Regehr, 2002). RIM1α partially restored the PPR and thus partially rescues the strongly impaired release probability in RIM and ELKS mutants (Figures 3.4 D, E). These results are consistent with what we see upon stimulation with short action potential trains (50 stimuli at 10 Hz), where RIM1α expression leads to higher amplitudes and greater depression compared to mutant synapses (Figure 3.4 F, G). Finally, while we have established that RIM1α partially restores P, to test if RIM is sufficient to rescue the size of the readily releasable pool (RRP) of primed vesicles, we applied hypertonic sucrose (Rosenmund and Stevens, 1996) and saw that RIM1α partially restored the size of the RRP (Figures 3.4 H, I). It is possible that simultaneous expression of RIM1α and ELKS1α is necessary to fully rescue synaptic transmission, even though ELKS1α by itself is not sufficient to restore vesicle release (data not shown). Taken together, these experiments establish that in the absence of ELKS, RIM1α is capable of restoring the active zone protein complex at cKO_{R+E} synapses, as well as synaptic vesicle docking and fusion. It still remains unclear, however, exactly where within mutant synapses RIM localizes and what the molecular mechanisms are that direct this localization.

**RIM recruits active zone proteins to the active zone**

RIM1α recruits all active zone interaction partners to mutant synapses (Figure 3.2), and this restores synaptic vesicle docking and fusion (Figures 3.3, 3.4). However, it remains
Figure 3.4 RIM restores synaptic transmission at cKOR+E mutant synapses. (A-C).
IPSCs were evoked by a focal stimulation electrode. Sample traces (A) and quantification of
IPSC amplitudes (B) and their rise times (C) (controlR+E n = 20 cells/3 cultures, cKOR+E n =
21/3, and cKOR+E + RIM1α n = 22/3). (D, E). Analysis of IPSC paired pulse ratios (PPR).
Sample traces (D) and quantification (E) of the PPR at 100 ms interstimulus interval
(controlR+E n = 15 cells/3 cultures, cKOR+E n = 15/3, and cKOR+E + RIM1α n = 15/3). (F, G)
Sample traces (F) and quantification of amplitudes (G) of IPSCs evoked by train stimulation
(50 stimuli, 10 Hz) (controlR+E n = 13 cells/2 cultures, cKOR+E n = 12/2, and cKOR+E + RIM1α
n = 14/2). (H, I). Analysis of sucrose IPSC to assess the size of the RRP. Sample traces (H)
and quantification (I) (controlR+E n = 18 cells/3 cultures, cKOR+E n = 18/3, and cKOR+E +
RIM1α n = 15/3). All data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as
determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc
test comparing each condition to cKOR+E.
Figure 3.4 (Continued)
unclear whether RIM1α and its interaction partners correctly target to active zones, or alternatively whether they are localized more broadly within the presynaptic nerve terminal. Because diffraction limited confocal microscopy does not provide the resolution sufficient to distinguish protein localization within the synaptic vesicle cloud, to the presynaptic active zone, or to the postsynaptic density (PSD), we turned to stimulated emission-depletion (STED) super-resolution microscopy to address this question. We imaged synapses stained for active zone proteins along with antibodies against the PSD marker PSD-95 and the synaptic vesicle marker synaptophysin in the control (controlR+E), active zone disrupted (cKO<sup>R+E</sup>), and RIM1α rescue (cKO<sup>R+E</sup> + RIM1α) conditions (Figure 3.5). For analysis, we selected ‘side-view’ synapses that exhibited an elongated PSD-95 ‘bar’ like signal along on the edge of the vesicle cloud of > 500nm diameter for analysis. In brief, we plotted the intensity profile from a ~1.2 μm long x ~200 μm rectangle drawn perpendicular from the center of the PSD-95 ‘bar’ of each active zone protein with respect to the peak localization of PSD-95, determined by a 5-pixel rolling average. We normalized these data to the average maximum signal in the control<sup>R+E</sup> condition (de Jong et al., 2018) (see experimental procedures for more details).

We found that RIM1α rescue localizes to the same peak position as endogenous RIM, directly opposed the PSD-95 signal along the edge of the synaptic vesicle cloud (Figure 3.5 A). Similarly, RIM1α was able to recruit Munc13-1, RIM-BP2, Cav2.1, and Bassoon to their respective endogenous active zone localizations (Figures 3.5 B-D, Supplemental Figure 6 B). Unexpectedly, although synaptic Liprin-α3 levels appeared unchanged in control<sup>R+E</sup>, cKO<sup>R+E</sup>, and RIM1α rescue conditions as assessed by confocal microscopy (Figure 3.2 E),
Figure 3.5. STED imaging of rescued active zone components. (A) Sample images (left) and line profiles (RIM, middle; PSD-95, right) of ‘side view’ synapses stained for RIM (green channel, STED), PSD-95 (red channel, STED), and the vesicle marker synaptophysin (blue channel, confocal) in controlR+E, cKOR+E, RIM1α rescue conditions. Scale bar is 500nm. (B-E) Sample images (left) and line profiles (right) of ‘side view’ synapses stained for Munc13-1 (B), CaV2.1 (C), Bassoon (D), and Liprin-α3 (E) (green channel) along with PSD-95 (red channel) and the synapse marker synaptophysin (blue channel). All line profiles were taken from ~200 nm wide ROIs drawn perpendicular to the center point of PSD-95 'bars' and aligned to the peak of PSD-95. Values were normalized to the max average of the controlR+E condition (see experimental methods for details). Abscissa indicate the distance from the peak of PSD-95 (marked with a dashed line). For all conditions n = 60 synapses/3 independent cultures.
Figure 3.5 (Continued)

A

B

Munc13-1

cKO^{+/+}

PSD-95

merge

+ synapse

control^{+/+}

RIM

cKO^{+/+}

+ RIM1α

cKO^{+/+}

+ RIM1α

C

Cav2.1

cKO^{+/+}

control^{+/+}

+ RIM1α

control^{+/+}

+ RIM1α

D

Bassoon

cKO^{+/+}

control^{+/+}

+ RIM1α

control^{+/+}

+ RIM1α

E

Liprin-α3

cKO^{+/+}

control^{+/+}

+ RIM1α

control^{+/+}

+ RIM1α

profile aligned to PSD peak (μm)
STED microscopy revealed an increase in the active zone localization of Liprin-α3 at active zone disrupted synapses, which was rescued upon RIM1α expression (Figure 3.5 E). It is possible that Liprin-α3 redistributes closer to the presynaptic plasma membrane in a compensatory manner upon active zone disruption, and that confocal microscopy did not originally reveal this.

**Molecular mechanisms targeting RIM to the active zone**

Upon establishing that RIM1α localizes to the active zone of cKO<sup>R+E</sup> synapses, we next used STED microscopy to determine the molecular mechanisms by which RIM targets to the presynaptic plasma membrane of active zone disrupted synapses. To address this question, we infected cKO<sup>R+E</sup> neurons with two sets of lentiviral HA-tagged RIM domain specific rescue constructs: RIM Δ domain constructs each missing one of RIM1α’s conserved domains (ΔZn finger, ΔPDZ, ΔC2A, ΔC2B) (Figures 3.6 A, B), and RIM individual domain constructs each expressing a single RIM domain (Zn finger, PDZ, C2B) (Figures 3.6 D, E). All rescue constructs were expressed at levels comparable to full-length RIM1α as assed by Western blotting (Figures 3.6 B, E), however the C2A domain is not included in the individual domain dataset because its expression is unstable on its own, unless linked to the long profile-rich linker between the C2A and C2B domains (data not shown).

Interestingly the PDZ domain of RIM, which interacts with <sup>Ca</sup>V<sub>2.1</sub> channels and ELKS (Kaeser et al., 2011; Ohtsuka et al., 2002; Wang et al., 2002), was essential for its active zone localization because full-length RIM1α that lacks only the PDZ domain (ΔPDZ) is unable to scaffold to the active zone protein complex (Figure 3.6 C). Line profile analysis revealed that unlike ΔZn, ΔC2A, and ΔC2B truncations, which all peak around ~100nm
**Figure 3.6 Molecular mechanisms of RIM localization.** (A, D) Sample images of ‘side view’ synapses stained for HA (green channel), PSD-95 (red channel), and synaptophysin (blue channel) in RIM Δ domain rescue conditions (A; ΔZn RIM, ΔPDZRIM, ΔC2ARIM, ΔC2BRIM) and RIM individual domain rescue conditions (D; Zn RIM, PDZRIM, and C2BRIM) compared to cKO R+E and full-length RIM rescue (RIM WT). (B, E) Expression level of rescue constructs as assessed by western blotting (top) and schematic representation of rescue conditions (bottom) for RIM Δ rescue conditions (B) and RIM individual domain rescue conditions (E). Deleted sequences are illustrated as dashed lines. (C, F) Line profiles of RIM Δ domain rescue (C) and RIM individual domain rescue (F) conditions. Line profiles were taken from ~200 nm wide ROIs drawn perpendicular to the center point of PSD-95 ‘bars’ and aligned to the peak of PSD-95. Values were normalized to the max average of the control R+E condition (see experimental methods for details). Abscissa indicate the distance from the peak of PSD-95 (marked with a dashed line). For all conditions n = 60 synapses/3 independent cultures.
Figure 3.6 (Continued)

A

B

C

D

E

F

RIM Individual Domain Localization

HA fluorescent intensity (normalized to max average)

profile aligned to PSD peak (μm)

- cKO^{RTE}
- RIM1α
- ΔZn
- ΔPDZ
- ΔC2A
- ΔC2B

RIM Δ domain localization

HA fluorescent intensity (normalized to max average)

profile aligned to PSD peak (μm)

- cKO^{RTE}
- RIM1α
- ΔZn
- ΔPDZ
- ΔC2A
- ΔC2B
from the PSD with full-length RIM1α, the ΔPDZ does not peak but rather appears widespread throughout the presynaptic nerve terminal (Figure 3.6 C). Interestingly, although the PDZ domain is essential for targeting RIM to mutant synapses, it is not sufficient to localize to the active zone when expressed in isolation (Figure 3.6 F). In fact, none of the individual domains of RIM were sufficient to localize to the active zone, and it is likely that more than one domain is necessary to target RIM to the active zone. Compellingly, while the PDZ and C2B domains appear widespread within and outside of synapses, the Zn finger alone is enriched strongly within the presynaptic nerve terminal (Figure 3.6 F). In fact, the pattern of its expression is strikingly similar to that of the vesicle marker synaptophysin (blue channel). Because RIM is known to interact with vesicular Rab3-GTPases via its alpha-helical region included in the Zn finger construct (Wang et al., 1997), it is likely that the Zn finger of RIM associates with synaptic vesicles when expressed by itself. Taken together, these data provide an assessment of the molecular mechanisms targeting RIM to the active zone. While the PDZ domain is essential for RIM’s active zone localization, there is not single domain that is sufficient to for this function. Rather, the Zn finger of RIM alone appears to associate with synaptic vesicles.

**The Zn finger of RIM is necessary for synaptic vesicle docking and priming**

In addition to associating with synaptic vesicles via Rab3, the Zn finger of RIM is known to interact with the vesicle priming protein Munc13 (Betz et al., 2001; Deng et al., 2011; Dulubova et al., 2005). Specifically, in its inactive form Munc13 tightly homodimerizes via its C2A domain and the Zn finger of RIM is capable of displacing this homodimer to recruit and activate Munc13 priming (Deng et al., 2011). While it is likely
that the Zn finger of RIM is thus necessary to restore both synaptic vesicle docking and priming at active zone disrupted synapses, it is less clear whether the Zn finger must be located at the active zone to confer these functions. To address this question, we infected cKO<sup>R+E</sup> synapses with lentiviruses expressing either the Zn finger of RIM alone, which localizes to the vesicle cloud (Figure 3.6 F), or RIM missing the Zn finger (ΔZn finger), which localizes to the active zone (Figure 3.6 C).

Analysis of synapse ultrastructure with high pressure freezing and electron microscopy revealed that neither the Zn finger of RIM nor the ΔZn finger construct were capable of restoring synaptic vesicle docking and tethering at active zone disrupted synapses (Figures 3.7 A, C, F). Similarly, the total number of synaptic vesicles per bouton, presynaptic bouton size, PSD length, and distribution of vesicles from the presynaptic plasma membrane remained unchanged in all conditions (Figures 3.7 B-H). These data suggest that the Zn finger of RIM must be tethered to the rest of the presynaptic plasma membrane as a minimal docking mechanism. Hypothetically, because docked synaptic vesicles are thought to be the morphological correlates of primed synaptic vesicles (Imig et al., 2014; Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001), this further suggests that neither the Zn finger nor ΔZn finger construct of RIM are capable of restoring vesicle priming. However, previous experiments demonstrating that the Zn finger of RIM alone is sufficient to rescue vesicle priming in neurons lacking RIM1/2αβ imply otherwise (Deng et al., 2011). To address this discrepancy, we next tested the capacity of vesicle associated RIM Zn to prime vesicles by first using STED microscopy to assess the localization of priming protein Munc13-1 and then measuring the size readily releasable pool of primed vesicles (RRP) using a hypertonic sucrose solution in the Zn finger or ΔZn
Figure 3.7. The Zn finger of RIM is essential for vesicle docking. (A) Sample images of high-pressure frozen neurons analyzed by electron microscopy of controlR+E, cKO\textsuperscript{R+E}, RIM1\textalpha Zn finger rescue (ZN\textsubscript{RIM}), and RIM1\textalpha Δ Zn finger rescue (ΔZN\textsubscript{RIM}). (B-F) Quantification of the number of total vesicle per bouton (B), the number of docked vesicles as defined by vesicles touching the presynaptic membrane (C), and the number of tethered vesicles within 100 nm of the presynaptic membrane (D), as well as bouton size (E) and PSD length (D). (G, H) Distribution of synaptic vesicles relative to the presynaptic plasma membrane opposed to the PSD within 100nm bins for the first 1000nm (left) and 10 min bins for the first 100nm (right). (For all conditions n = 100 synapses/2 independent cultures). All data are means ± SEM; **p ≤ 0.001 as determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc test comparing each condition to cKO\textsuperscript{R+E}. 


Figure 3.7 (Continued)
finger rescue conditions. Compellingly, we found that the Zn finger of RIM is capable of enhancing presynaptic Munc13-1 levels (Figure 3.8 A), as well as endogenous Munc13-1 protein levels (Figure 3.8 B). Remarkably, however, Munc13-1 was not localized to the active zone, but was distributed in a pattern similar to the Zn finger of RIM throughout the presynaptic nerve terminal (Figures 3.8 A, C). Hence, the RIM Zn finger likely recruits Munc13-1 to synaptic vesicles. This raises the possibility that we reconstituted a mechanism for vesicle priming on vesicles, away from the active zone. If true, this would mean that vesicle priming and docking are molecularly distinct processes. To test this hypothesis, we turned to electrophysiological experiments. Preliminary data suggest that, while the Zn finger of RIM alone was unable to restore synaptic vesicle docking, it enhanced the size of the RRP compared to cKO<sup>R+E</sup> neurons alone (Figures 3.8 D, E). Furthermore, while expressing the ΔZn finger construct of RIM at active zone disrupted synapses had no effect on synapse ultrastructure, there appeared to be a further suppression of the RRP size as assessed by sucrose (Figures 3.8 D, E). Altogether, these data suggest that while the Zn finger of RIM must be localized to the active zone to rescue synaptic vesicle docking, it can recruit Munc13-1 to the synaptic vesicle cloud and this may be sufficient to enhance priming.

**Discussion**

Our findings reveal that RIM is an important organizer of the presynaptic active zone at vertebrate synapses. We demonstrate that beyond the role RIM has in recruiting Ca<sup>2+</sup> channels and priming synaptic vesicles (Deng et al., 2011; Kaeser et al., 2011), RIM is necessary and sufficient to rebuild the active zone protein scaffold and to dock and release
Figure 3.8. The Zn finger of RIM recruits priming factor Munc13 to synaptic vesicles. (A) Sample images of ‘side view’ synapses stained for Munc13 (green channel), PSD-95 (red channel), and synaptophysin (blue channel) in control\textsuperscript{R+E}, cKO\textsuperscript{R+E}, full-length RIM rescue (RIM\textsubscript{WT}), RIM\textsubscript{1α} Zn finger rescue (ZN\textsubscript{RIM}), and RIM\textsubscript{1α} Δ Zn finger rescue (ΔZN\textsubscript{xRIM}) conditions. (B) Expression level of Munc13 assessed by western blotting (top) and schematic representation of RIM rescue conditions (bottom). Deleted sequences are illustrated as dashed lines. (C) Line profiles of Munc13 in control\textsuperscript{R+E}, cKO\textsuperscript{R+E}, and RIM rescue conditions. Line profiles were taken from ~200 nm wide ROIs drawn perpendicular to the center point of PSD-95 ‘bars’ and aligned to the peak of PSD-95. Values were normalized to the max average of the control\textsuperscript{R+E} condition (see experimental methods for details). Abscissa indicate the distance from the peak of PSD-95 (marked with a dashed line). For all conditions n = 60 synapses/ 3 independent cultures. (D, E) Sample traces of EPSCs in response to superfusion with 500 mOsm sucrose (D) and quantification (E) of EPSC charge transfer during the first ten seconds of the response (for all conditions n = 9 cells/ 2 cultures). Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test.
Figure 3.8 (Continued)
synaptic vesicles along the presynaptic plasma membrane of active zone disrupted synapses (Figures 3.1 to 3.5). We further find that the PDZ domain of RIM is essential for its active zone localization in the absence of ELKS and the Zn finger of RIM becomes vesicle associated when expressed alone (Figure 3.6). Finally, our data suggest that the Zn finger of RIM is vesicle associated when expressed alone, co-recruits Munc13-1 to vesicles, and may enhance priming without enhancing vesicle docking (Figures 3.7, 3.8)

**RIM rebuilds the active zone**

We found that expressing full-length RIM1α at active zone disrupted cKO^{R+E} neurons is sufficient to restore the entire active zone scaffold, and for proteins that directly interact with RIM (Munc13s, RIM-BPs, and Cav2.1) this happens in a concentration-dependent manner (Figures 3.1, 3.2). On the other hand, although full-length ELKS1α is capable of localizing to the active zone independent of RIM, ELKS1α only rescues bassoon. These results are consistent with previous in vitro protein interaction assays (Betz et al., 2001; Dulubova et al., 2005; Kaeser et al., 2011; Ohtsuka et al., 2002; Wang et al., 2000). However, because active zone assembly is a complex, multi-step process that involves the production of synaptic proteins at the Golgi, trafficking and trapping these active zone precursor materials to newly forming synapses, and then finally stabilizing and scaffolding active zone materials at specific sites along the presynaptic plasma membrane (Dresbach et al., 2006b; Maas et al., 2012; Torres and Inestrosa, 2018; Zhai et al., 2001), it is difficult to say which step of this process our manipulations target. It is possible that rescuing RIM1α or ELKS1α changes the molecular content of active zone precursor vesicles before they are trafficked to synapses (Maas et al., 2012). It is equally possible that the presence of RIM1α
or ELKS1α at synapses provides a stabilizing force for their protein interaction partners, thus protecting them from higher rates of protein turnover (Alvarez-Castelao and Schuman, 2015; Bingol and Sheng, 2011). While the exact mechanisms for transporting, capturing, and stabilizing active zone content at synapses is an important avenue for future investigation, our results establish that the presence of RIM1α is necessary for active zone assembly and maintenance as a whole.

**Redundant scaffolding mechanisms of RIM**

By performing structure-function rescue experiments with RIM Δ domain (ΔZn, ΔPDZ, ΔC2A, ΔC2B) and RIM individual (Zn, PDZ, C2B) constructs at active zone disrupted cKOΔ synapses, we identify that the PDZ domain of RIM is essential for its active zone localization (Figure 3.6). The PDZ domain of RIM stoichiometrically interacts with the C-terminal tail of ELKS and CaV2.1 channels (Deng et al., 2011; Ohtsuka et al., 2002; Wang et al., 2002). Our finding that truncated RIM lacking the PDZ domain (RIM ΔPDZ) is unable to target to the active zone at cKOΔ synapses is consistent with previous work showing that RIM ΔPDZ overexpressed at wildtype hippocampal neurons fails to cluster at synapses and is instead diffusely distributed within neuronal processes (Ohtsuka et al., 2002). However, other studies done in both *C. elegans* and cultured hippocampal neurons have demonstrated that RIM ΔPDZ is able to localize to RIM knockout synapses, suggesting that the PDZ domain is actually not necessary for RIM localization (Deken et al., 2005; Kaeser et al., 2011). Taken together, it is likely that the highly interconnected nature of RIM’s protein-protein interactions with other active zone proteins provides multiple, redundant scaffolding mechanisms. At wildtype synapses or at synapses lacking only RIM, where
much of the active protein scaffold remains intact, the PDZ domain is likely not essential for RIM localization. However at active zone disrupted cKO\textsuperscript{R+E} synapses, where many of these redundant protein interactions are lost, we show that PDZ domain of RIM is necessary to target RIM to the presynaptic plasma membrane.

**Minimal molecular mechanisms for docking and priming**

We show that while RIM1\(\alpha\) rescues both synaptic vesicle docking and tethering, ELKS1\(\alpha\) does not appear to have a significant effect (Figure 3.3). It is likely that RIM docks vesicles through its Munc13/Rab3 interaction via the Zn finger region (Betz et al., 2001; Deng et al., 2011; Dulubova et al., 2005). To identify the minimal molecular mechanism by which RIM docks vesicles, we performed structure-function rescue experiments comparing full length RIM with the Zn finger of RIM alone (Zn) or with truncated RIM lacking the Zn finger (RIM \(\Delta\)Zn). STED imaging revealed that full length RIM1\(\alpha\) is localized to active zones and restores docking. However, the RIM Zn finger associates to synaptic vesicles when expressed alone, and the RIM \(\Delta\)Zn localizes to the active zone (Figure 3.6). However, vesicle associated RIM Zn finger is able to recruit Munc13-1 to vesicles distal from the presynaptic plasma membrane and may enhance RRP size (Figure 3.8).

These results raise two intriguing possibilities. First, although synaptic vesicle docking is considered the morphological correlate of vesicle priming (Imig et al., 2014; Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001), we demonstrate here that while the RIM Zn finger does not rescue vesicle docking, it is capable of further enhancing the size of the sucrose pool (Figure 3.8). These data imply that soluble Munc13-1 or Munc13-1 that is not active zone localized may play a role in vesicle priming and further

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dissociates the linear docking-priming-fusion dogma in the field (Verhage and Sørensen, 2008). In the future, it will be important to parse apart the roles of soluble Munc13 in docking and priming to determine whether they are separate functions. Second, although vesicle associated RIM Zn finger is not capable of docking vesicles to the presynaptic plasma membrane, it likely boosts release. This leads to a working model whereby the priming mechanism can be reconstituted on a synaptic vesicle. In future experiments, we will target this mechanism through Zn finger chimeric proteins linked directly to Ca$^{2+}$ channels. We predict that these chimeric proteins are capable of restoring rapid release in the absence of much of the active zone scaffold. This would be equal to artificially rebuilding three key active zone functions: docking, priming, and spatial coupling of Ca$^{2+}$ sensors to Ca$^{2+}$ channels. If successful, this experiment would essentially establish that these three functions are the fundamental roles of active zone scaffolding, and that the active zone scaffold itself is not necessary to restore vesicle release.
CHAPTER FOUR

Roles of Liprin-α proteins at vertebrate synapses
Introduction

Liprin-α proteins remain one of the most enigmatic components of the presynaptic active zone complex. They were first identified by their interaction with leukocyte common antigen related (LAR) receptor-like protein tyrosine phosphatases (RPTPs) (Serra-Pagès et al., 1998, 1995), which are transmembrane cell adhesion molecules implicated in synapse development (Um and Ko, 2013). Liprin-α was eventually linked to the active zone protein complex when a genetic screen done in *C. elegans* revealed that Liprin-α homolog Syd-2 loss of function mutation resulted in diffuse localization of presynaptic proteins, including RIM homolog unc-10 and ELKS homolog elks, disrupted active zone structure, and impaired vesicle accumulation (Zhen and Jin, 1999). Further support for Liprin-α’s role at the active zone came from genetic experiments done in *D. Melanogaster*, where removing the Liprin-α homolog dlinprin-α decreased the number and size of synapses at the NMJ, while increasing the range and variability of active zone size (Kaufmann et al., 2002). From these initial genetic studies, Liprin-α proteins are hypothesized to be involved in active zone assembly.

More in-depth analyses in invertebrates revealed that Liprin-α proteins play a hierarchical role in active zone assembly. Specifically in *C. elegans*, a hypermorphic gain of function mutation of Liprin-α homolog Syd-2 was found to promote synaptogenesis at Syd-1 deficient synapses (Dai et al., 2006). Syd-1 is a scaffolding protein required for vesicle clustering at HSN motorneuron synapses and likely has no direct vertebrate homolog, although one study disputes this (Wentzel et al., 2013). Syd-1 is proposed to work upstream of Syd-2 because overexpression of Syd-2 in *C. elegans* was found to rescue synapse development in Syd-1 mutants, but the same was not true for overexpression of
Syd-1 in Syd-2 mutants (Patel et al., 2006). In addition to working downstream of Syd-1, Syd-2 is proposed to work upstream of unc-10 and elks. While removing Syd-2 from synapses disrupts localization of unc-10 and elks, Syd-2 localization is not disrupted at either unc-10 or elks mutants (Dai et al., 2006). Additionally, Syd-2 rescues the syd-1 phenotype in an elks dependent manner (Dai et al., 2006). Taken together, these data from invertebrates provide evidence for Liprin-α as an active zone scaffold upstream of other active zone proteins. These findings are also consistent with my data from active zone disrupted vertebrate synapses (see Chapters 2 and 3). Removal of both RIM and ELKS from hippocampal neurons leads to a strong disruption of all core active zone components, except for Liprin-α (Figure 2.1). In fact, super-resolution imaging revealed that Liprin-α3 becomes more enriched at the active zone upon loss of RIM and ELKS, perhaps as a compensatory response to active zone disruption (Figure 3.5).

Roles for Liprin-α proteins at vertebrate synapses have been more challenging to address because of two complicating factors. First, unlike invertebrates, which have a single gene for Liprin-α, vertebrates express four genes (Ppfia1-Ppfia4) for Liprin-α1-4 with multiple alternatively spliced isoforms (Zürner and Schoch, 2009). Although all vertebrate Liprin-α proteins are expressed in the brain, Liprin-α1 is most strongly expressed in non-neuronal tissue and Liprin-α4 is only present at low levels, while both Liprin-α2 and Liprin-α3 are specifically and strongly enriched in neurons (Spangler et al., 2011; Zürner et al., 2011). The complexity in expression and localization of Liprin-α proteins in vertebrates suggests that different Liprin-α isoforms may play both redundant and complementary roles to each other in neurons. Second, unlike other core active zone proteins, in vitro biochemical assays revealed that Liprin-α has a diverse range of
interactions with both pre- and postsynaptic proteins. In addition to interacting with RIM and ELKS (Ko et al., 2003b; Schoch et al., 2002), Liprin-α interacts with actin nucleator mDiaphanous during stress fiber formation (Sakamoto et al., 2012), the neuron-specific kinesin motor protein KIF1A potentially for the transport of synaptic cargo along microtubules (Shin et al., 2003), G protein-coupled receptor kinase interaction protein (GIT1) perhaps during the trafficking of AMPA receptors (Ko et al., 2003a), and finally with calmodulin-dependent serine protein kinase (CASK) found both pre- and postsynaptically potentially for synaptic development (Olsen et al., 2005). Such a broad range of protein interactions primes Liprin-α for a multitude of diverse roles in neurons.

In light of Liprin-α proteins’ complex expression and protein interactions, an important step towards understanding the role of Liprin-α proteins at synapses is to carefully assess their subcellular localization. A recent study looking at Liprin-α2 and Liprin-α3 localization in cultured hippocampal neurons with stimulated emission-depletion (STED) super-resolution microscopy revealed that while both Liprin-α2 and Liprin-α3 are widely distributed within the presynaptic nerve terminal, Liprin-α3 has a component that strongly co-localized with active zone proteins (Wong et al., 2018). To address the function of Liprin-α proteins at vertebrate synapses, this study generated Liprin-α3 KO mice. Interestingly, upon loss of Liprin-α3, Liprin-α2 translocated to the active zone, suggesting that Liprin-α2 may at least partially compensate for Liprin-α3 at synapses. However, this compensation was limited because loss of Liprin-α3 still led to ultrastructural impairments in synaptic vesicle docking and tethering, as well as reduced exocytosis (Wong et al., 2018). This study complements another recent assessment of Liprin-α2 knockdown in cultured hippocampal neurons, which resulted in ultrastructural
defects and mild changes in the active zone protein complex (Spangler et al., 2013). Taken together, these results establish that vertebrate Liprin-α proteins, although distributed widely in the nerve terminal, have an active zone localized component that is structurally and functionally important for synaptic transmission. However, because of the partial compensation between the main neuronal Liprin-α isoforms (-α2 and -α3), the full extent of their function may be obscured.

In order to more completely address the role of Liprin-α proteins at vertebrate synapses, we generated Liprin-α2/3 KO mice. We targeted both Liprin-α2 and Liprin-α3 because, in addition to being the two isoforms strongly and specifically expressed in brain tissue (Schoch et al., 2002; Spangler et al., 2011; Zürner et al., 2011; Zürner and Schoch, 2009), we now know that Liprin-α2 translocates to the active zone upon loss of Liprin-α3 (Wong et al., 2018). Here, we found that removing Liprin-α2/3 from cultured hippocampal neurons resulted in the further disruption of RIM and ELKS at synapses, as well as a surprising increase in the transsynaptic Liprin binding partner LAR. Interestingly, unlike knockout of other active zone protein families, Liprin-α2/3 KO may disrupt synaptogenesis, with an increase in both the size of the presynaptic bouton and an increase in the length of the postsynaptic density (PSD). This preliminary assessment reveals that Liprin-α’s play functionally important roles in synapse ultrastructure and active zone assembly upstream of RIM and ELKS, which was not seen in the KO of individual Liprin-α isoforms alone.

Experimental Procedures
All experiments were done in primary hippocampal culture and assessed at day in vitro (DIV) 15-19 as previously described (Chapter two, Chapter three, Appendix two). For a detailed description of confocal imaging, HPF confocal microscopy, and Western blotting see Appendix two. Experiments were performed and analyzed by an experimenter blind to the genotype and significance was determined using Student’s t-tests unless otherwise noted. Statistical significance was set at *p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. Materials and methods specific to this chapter are described below.

**Mouse Lines.** The Liprin-α3 constitutive knockout (-/-) mice are viable and fertile and were previously characterized (Wong et al., 2018). Briefly, exon 18 of Liprin-α3 was targeted with CRISPR/Cas9 gene editing in one-cell zygotes, which were then transplanted into foster mothers. A frame-shift mutation deleting 8 bp was selected from a founder offspring, and removal of Liprin-α3 was confirmed by Western blotting. The allele was outbred to 129S2/SvPasCrl mice and maintained as a heterozygous (Het) line. The Liprin-α2 conditional knockout (cKO) mice were obtained from the IMPC (Name: C57BL/6N-Ppfia2tm1a(EUCOMM)Hmgu/H, RRID: IMSR_EM:09631) and crossed to transgenic mice expressing Flp recombinase (Dymecki, 1996) to remove the neo cassette and generate a conditional allele. To generate Liprin-α2/3 KO mice, we crossed Liprin-α3+/– mice with conditional Liprin-α2fl/fl mice. We maintained the line with Liprin-α2fl/flα3–/– and Liprin-α2fl/flα3+/– breeders to generate appropriate Liprin-α2 conditional KO and Liprin-α3 constitutive KO and Het littermate controls.

**Cell culture and lentiviral infection.** Primary hippocampal cultures were prepared as previously described (Chapter two, Chapter three, Appendix two), with one modification.
Single pups were cultured individually and then genotyped before DIV5 to determine Liprin-α3 homo- or heterozygosity (-/- or +/-). Liprin-α2^{fl/fl}α3^{−/−} cultures were infected with lentiviral cre recombinase expressed under a synapsin promoter on DIV5 and Liprin-α2^{fl/fl}α3^{+/−} cultures were infected with an inactive mutant of cre to generate Liprin-α2/3 knockout (cKO^{L23}) and control (control^{L23}) cultures respectively.

**Antibodies.** The antibodies and concentrations used in this study are previously described (Appendix two), with the notable addition of the rabbit anti-Liprin-α1 (1:200), -α2 (1:100), and -α4 antibodies (1:200), a generous gift from Dr. S. Schoch.

**Results**

**Genetic removal of Liprin-α2/3 partially disrupts the active zone protein scaffold**

We generated Liprin-α2/3 KO mice by crossing Liprin-α3 constitutive KO mice (Wong et al., 2018) with Liprin-α2 conditional KO mice, in order to determine the role of Liprin-α’s in vertebrate active zone assembly (Figures 4.1 A-F). We targeted both Liprin-α2 and Liprin-α3 because they are the primary Liprin-α isoforms enriched in the brain and may structurally compensate for one another (Spangler et al., 2011; Wong et al., 2018; Zürner et al., 2011). Breeding pairs were maintained with Liprin-α2^{fl/fl}α3^{−/−} and Liprin-α2^{fl/fl}α3^{+/−} crosses to generate Liprin-α2^{fl/fl}α3^{−/−} and Liprin-α2^{fl/fl}α3^{+/−} littermate pairs. All experiments were done in primary hippocampal cultures of individual pups. Lentiviruses expressing either cre recombinase were used to infect Liprin-α2^{fl/fl}α3^{−/−} cultures to generate (cKO^{L23}) neurons and a mutant form of cre was used to infect Liprin-α2^{fl/fl}α3^{+/−}...
Figure 4.1. Genetically targeting Liprin-α2 and Liprin-α3. (A) CRISPR/Cas9 gene editing in single-cell zygotes to generate Liprin-α3 KO mice previously characterized in (Wong et al., 2018) (adapted from Figure 2). The sgRNA-targeting sequence is shown in bold, the protospacer-adjacent motif is in blue, and the dashed line indicates the deletion. (B) Liprin-α3 expression in brain tissue assessed by Western blotting (left) and survival analysis of offspring of heterozygous matings (right). The dashed line represents a Mendelian distribution (n = 418 animals/54 litters). (C) Gene targeting strategy for the generation of Liprin-α2 conditional KO mice. (D) Liprin-α2 expression in hippocampi assessed by Western blotting (left) and survival analysis of offspring of heterozygous matings (right) from Liprin-α2 mutant allele before (top) and after (bottom) Flp recombination. The dashed line represents a Mendelian distribution (top: n = 69 animals/10 litters, bottom: n = 52 animals/9 litters). (E) Confocal assessment of synaptic Liprin-α2 fluorescence levels (green) in control and conditional KO cultures, co-stained with synaptophysin (red) and MAP2 (blue). (F) Synaptic protein levels assessed by Western blotting in control and Liprin-α2 cKO cultures.
Figure 4.1 (Continued)

A

wild-type: AAGAGCAGAGAGCATAGCTGTCCCATAGGCCG
mutant: AAGAGCAGAGAGCATAGCTTGCCCATAGGCCG

B

Liprin-α3
+/+  +/−  −/−
Liprin-α2
+/+  +/−  −/−
β-actin
+/+  +/−  −/−

offspring ratio
0%  25%  50%

C

wild-type Liprin-α2
Liprin-α2 mutant allele
Liprin-α2 floxed allele
Liprin-α2 KO allele

D

Liprin-α2 mutant
+/+  +/−  −/−
Liprin-α2 floxed
+/+  +/−  −/−
β-actin
+/+  +/−  −/−

offspring ratio
0%  25%  50%

E

Lu2  Syp-1  MAP2+merge
control
cko

F

control  kKO
Liprin-α2
Liprin-α3
RIM
ELKS
RIM-BP2
PSD-95
β-actin
cultures to generate (control\textsuperscript{1,2}) neurons. Western blotting was used to confirm Liprin-\(\alpha_2\) and Liprin-\(\alpha_3\) removal in this culture system (Figures 4.1 B, D, F).

To test whether removal of Liprin-\(\alpha_2\) and Liprin-\(\alpha_3\) (cKO\textsuperscript{1,2}) changed the active zone protein scaffold, we used confocal microscopy to assess the synaptic fluorescence levels of multiple presynaptic and postsynaptic proteins (Figure 4.2) and fluorescent Western blotting to assess the total expression levels of these proteins (Figure 4.3). Unlike removal of Liprin-\(\alpha_3\) alone, both RIM and ELKS levels were reduced at cKO\textsuperscript{1,2} synapses (Figures 4.2 A, B). Compellingly, we observed an increase in the synaptic fluorescence levels of the Liprin-\(\alpha\) binding partner LAR, as well as a trend towards increased synapsin-1 and Ca\textsubscript{\(\text{V}_{2.1}\)} channel levels, which was not seen upon strong active zone disruption when RIM and ELKS are removed (Figure 4.2 B, Chapter 2). The loss of Liprin-\(\alpha_2/3\) did not reveal a change in the fluorescence levels of postsynaptic proteins PSD-95 for GluA1 receptors. Furthermore, the changes in synaptic protein levels as assessed by confocal microscopy were not reflected in the assessment of total protein levels assessed by fluorescent Western blotting (Figures 4.3 A, B). It is likely, then, that loss of Liprin-\(\alpha\)’s does not disrupt the total protein expression levels of various active zone proteins, but rather it impairs their proper targeting to synapses.

These results are striking because genetically targeting single active zone protein families rarely leads to changes in the active zone protein scaffold, due to the highly interconnected nature of its protein interactions conferring redundant scaffolding mechanisms. The fact that loss of Liprin-\(\alpha_2/3\) is sufficient to disrupt the synaptic levels of both RIM and ELKS suggests that Liprin-\(\alpha\)’s may function at a level upstream of other core
Figure 4.2. Synaptic protein composition of neurons lacking Liprin-α2/3. (A) Sample images of control neurons (control\textsuperscript{1,23}) and neurons lacking Liprin-α2/3 (cKO\textsuperscript{1,23}) stained with antibodies against active zone and postsynaptic proteins (green channel), along with synaptophysin (red channel), and MAP2 (blue channel). (B) Quantification of synaptic proteins within ROIs defined by synaptophysin (for all conditions n = 3 independent cultures/ 10 images averaged per culture). Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test.
Figure 4.2 (Continued)

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control 23</th>
<th>cKO 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liprin-α2</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>Liprin-α3</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>RIM</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>ELKS</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>Bassoon</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>Ca, 2.1</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>LAR</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>PSD95</td>
<td>control</td>
<td>cKO</td>
</tr>
<tr>
<td>GluA1</td>
<td>control</td>
<td>cKO</td>
</tr>
</tbody>
</table>

B

Synaptic Fluorescence Levels (normalized to control)

- Liprin-α2
- Liprin-α3
- RIM
- ELKS
- Bassoon
- Synapsin
- LAR
- Ca, 2.1
- PSD95
- GluA1

* and ** indicate significant differences.
Figure 4.3. Total protein composition of neurons lacking Liprin-α2/3. (A) Quantitative Western blotting for synaptic proteins using fluorescent secondary antibodies for control\textsuperscript{L23} and cKO\textsuperscript{L23} neurons. (B) Quantification of total protein levels in control\textsuperscript{L23} neurons compared to cKO\textsuperscript{L23} neurons. Values normalized to synaptic protein content (Synapsin-1) as a fraction of total protein content (β-actin) (for all conditions n = 3 independent cultures). Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001 as determined by ratio t-test.
active zone protein families, which would be consistent with several invertebrate studies (Dai et al., 2006; Patel et al., 2006).

**Ultrastructural analysis of Liprin-α2/3 KO synapses**

To determine whether loss of Liprin-α2/3 changed synapse structure, we used high pressure freezing followed by freeze-substitution to preserve cultured control and cKO neurons and performed electron microscopy to assess synapse ultrastructure (Figure 4.4 A). In a preliminary dataset, while there was no change in vesicle recruitment and the total number of vesicles per bouton, we observed a downward trend in both synaptic vesicle docking and tethering (Figures 4.4 B, C, F, G, H), similar to what is seen upon removal of Liprin-α3 alone (Wong et al., 2018). Interestingly, we observed a significant increase in the presynaptic bouton size and the length of the postsynaptic density that was not seen in Liprin-α3 KO neurons or active zone disrupted neurons (Figures 4.4 D, E, Chapter 2). These results are reminiscent of the changes in synapse ultrastructure seen in invertebrate synapses (Kaufmann et al., 2002; Zhen and Jin, 1999) and imply that Liprin-α2/3 are involved in synapse formation, that is independent of its role in active zone assembly upstream of RIM and ELKS. However, because these results are preliminary (n=1), we wait to draw conclusions for mild effects until the repeated experiments are completed.

**Discussion**

Here we generate Liprin-α2/3 KO mice (Figure 4.1) and establish that Liprin-α proteins play a role in active zone assembly upstream of RIM and ELKS at vertebrate synapses, as well as in synapse formation independent of RIM and ELKS. We show that loss
Figure 4.4 Ultrastructural analysis of neurons lacking Liprin-α2/3. (A) Sample images of high-pressure frozen neurons analyzed by electron microscopy of control and cKO conditions. (B-F) Quantification of the number of total vesicle per bouton (B), the number of docked vesicles as defined by vesicles touching the presynaptic membrane (C), and the number of tethered vesicles within 100 nm of the presynaptic membrane (D), as well as bouton size (E) and PSD length (D). (G, H) Distribution of synaptic vesicles relative to the presynaptic plasma membrane opposed to the PSD within 100nm bins for the first 1000nm (left) and 10 min bins for the first 100nm (right). (For all conditions n = 50 synapses). All data are means ± SEM; ***p ≤ 0.001 as determined by one-way ANOVA followed by Holm-Sidak multiple comparisons post-hoc test comparing each condition to cKO R+E.
Figure 4.4 (Continued)
of Liprin-α2/3 impairs RIM and ELKS localization at synapses, but does not change the total expression level of these proteins (Figures 4.2, 4.3). Furthermore, we show that Liprin-α’s may be involved in synapse formation because loss of Liprin-α2/3 leads to an increase in the synaptic localization of its transsynaptic binding partner LAR and changes in synapse ultrastructure (Figures 4.2, 4.4).

**A hierarchical role for Liprin-α’s in active zone assembly**

Consistent with invertebrate literature, our data establish that Liprin-α proteins are involved in a hierarchical process of active zone scaffolding upstream of RIM and ELKS. While loss of RIM and ELKS at active zone disrupted synapses does not reduce the levels of Liprin-α2/3 at synapses (Chapter 2) and in fact enhances Liprin-α3 localization at the active zone (Chapter 3), loss of Liprin-α2/3 reduces the synaptic levels of RIM and ELKS, as well as increases the synaptic levels of Liprin-α binding partner LAR (Figure 4.2). There are a number of possible explanations for this. On the one hand, because Liprin-α binds the kinesin motor protein KIF1A (Shin et al., 2003), it is possible Liprin-α plays a role in trafficking synaptic material to synapses. On the other hand, because Liprin-α interacts directly with RIM (Schoch et al., 2002), ELKS (Ko et al., 2003b), and LAR (Serra-Pagès et al., 1998, 1995) it is possible that Liprin-α acts as a scaffold to target RIM and ELKS to active zone sites along the presynaptic plasma membrane. Because *in vivo* live imaging experiments at the *D. Melanogaster* NMJ have revealed that Liprin-α arrives at newly forming active zones several hours before the ELKS homolog *bruchpilot* (Fouquet et al., 2009; Owald et al., 2010), it is likely that Liprin-α does not directly traffic ELKS to synapses, but rather loss of Liprin-α impairs ELKS scaffolding at the active zone. These results are
consistent with our data from fluorescent Western blotting, which suggest loss of Liprin-α does not disrupt the total protein expression levels of RIM or ELKS, but rather it impairs their proper scaffolding to synapses. From these data, we would hypothesize that there is an increased soluble fraction of RIM and ELKS at Liprin-α2/3 mutant synapses.

**Targeting the active zone to the presynaptic plasma membrane**

Although Liprin-α proteins have a role in active zone assembly upstream of RIM and ELKS, the complexity of its expression and protein interactions implies Liprin-α’s has functions independent of RIM and ELKS as well. For example, the increase seen in synaptic expression levels of LAR upon removal of Liprin-α2/3, and the similar trend seen in synapsin-1 and Cav2.1 channel levels (Figure 4.2), is not observed upon strong active zone disruption and loss of RIM and ELKS (Figure 2.1, 4.2). Furthermore, loss of Liprin-α2/3 leads to changes in synapse ultrastructure not seen in active zone disrupted mutants, specifically an increase in presynaptic bouton size a lengthening of the postsynaptic density (Figure 2.1, 4.4). These results suggest that Liprin-α’s have RIM and ELKS independent roles in synapse formation.

Synapse formation is a complex, multi-step process from initiation, likely mediated by transsynaptic cell-adhesion molecules (CAM), to maturation and the development of pre- and postsynaptic specialization (Südhof, 2018). One candidate transsynaptic CAM is LAR/RPTP whose functions are to mediate cell to cell adhesion at synapses, as well as to trigger presynaptic and postsynaptic differentiation (Takahashi and Craig, 2013). Liprin-α’s were first identified as LAR interacting proteins (Serra-Pagès et al., 1998) and it has been hypothesized that one function of Liprin-α’s is to promote the local recruitment of active
zone proteins along specific LAR enriched sites along the presynaptic plasma membrane. Support for this hypothesis comes from invertebrate work, where loss of *D. Melanogaster* LAR (*Dlar*) leads to changes in active zone ultrastructure nearly identical to those seen upon loss of *D. Melanogaster* Liprin-α (*Dliprin-α*) (Kaufmann et al., 2002). Furthermore, an epistasis test that simultaneously removes *Dliprin-α* function while overexpressing *Dlar*, suggests that *Dliprin-α* is necessary for *Dlar* function in determining NMJ bouton number at synapses (Kaufmann et al., 2002). While the functional association between Liprin-α’s and LAR/RPTPs remains unclear at vertebrate synapses, the increase in synaptic LAR expression upon loss of Liprin-α2/3 and the subsequent disruptions in synapse structure seen here provide evidence that Liprin-α’s are involved with LARs for vertebrate synapse formation. In the future, a more complete genetic analysis of presynaptic LAR/RPTPs will be necessary to dissect these functions.

**Redundant functions for vertebrate Liprin-α isoforms**

Removing Liprin-α3 from cultured hippocampal neurons revealed that Liprin-α2, normally widely distributed within the presynaptic bouton, translocates to the active zone (Wong et al., 2018). This structural compensation implies that various Liprin-α isoforms may also be able to functionally compensate for one another, albeit partially because Liprin-α2 is not fully able to compensate for loss of Liprin-α3 (Wong et al., 2018). Similarly, Liprin-α3 is not fully capable of compensating for loss of Liprin-α2 (Spangler et al., 2013). In this study, we set out to test the general function of Liprin-α proteins in presynaptic active zone assembly and synapse formation. We decided to target the two most prominent Liprin-α isoforms in brain tissue to generate a strong disruption. However, although we did
not observe a compensatory increase in expression levels for the remaining Liprin-α isoforms (-α1, and -α4) (Figure 4.3), we cannot rule out the possibility that the presence of Liprin-α1 and Liprin-α4 may mask further functions of Liprin-α’s at vertebrate synapses. A more complete genetic study of all four Liprin-α isoforms will be necessary to address these concerns. Similarly, future studies of the role of individual Liprin-α isoforms alone are needed to parse apart their diverse and differential roles.
—CHAPTER FIVE—

Conclusion and Future Directions
A working model for vertebrate active zone assembly

The overarching goal of my thesis work is to better understand the molecular mechanisms that govern assembly of the protein scaffold at vertebrate presynaptic active zones. In the past, these mechanisms have been difficult to study because the active zone is a complex molecular machine with multiple, redundant interactions and its overall structure is resilient to genetic knockout of any single active zone protein family. In this body of work, we have addressed these challenges by generating a compound gene knockout for two central active zone protein families, RIM and ELKS.

In Chapter 2, we show that loss of RIM and ELKS results in the further reduction Munc13s, RIM-BPs, and piccolo/bassoon, but not Liprin-α’s. This is the strongest biochemical disruption of the active zone yet described and leads to a near complete loss of synaptic vesicle docking and tethering, as well as strong reductions in synaptic vesicle priming (RRP) and release probability (P). However, despite loss of vesicle docking and reductions in RRP and P, fusion still persisted in response action potentials at high extracellular calcium and as spontaneous miniature events. These data suggest that while the active zone is necessary for synaptic vesicle docking and to enhance release probability, releasable vesicles can be localized distant from the presynaptic plasma membrane. Interestingly, synapses still form normally upon active zone disruption and there is no change in the total number of vesicles recruited to the nerve terminal or the structure of the postsynaptic density (PSD). Our data indicate that by removing RIM and ELKS we disrupt the mechanisms holding the active zone protein scaffold together, subsequently impairing its function without disrupting synapse formation.
The active zone disrupted mutant provides a unique opportunity: the ability to rebuild the active zone and thus identify the molecular mechanisms involved in assembling its protein scaffold. In Chapter 3, using structure-function rescue experiments at active zone disrupted synapses, we show that while RIM1α and ELKS1α can localize to synapses independent of one another, RIM1α is the major organizer. RIM1α is capable of rescuing several active zone interacting partners in a concentration-dependent manner, as well as restore synaptic vesicle docking and tethering, while ELKS1α only restores bassoon levels. From these data, it is clear that in order to understand the specific molecular mechanisms scaffolding core active zone components together and docking synaptic vesicles to the presynaptic plasma membrane, it is necessary to dissect the protein domains through which RIM functions. Detailed analyses of RIM recruitment revealed that RIM localizes to the active zone disrupted synapses via its PDZ domain, while its N-terminal Zn finger associates with synaptic vesicles when expressed on its own. The Zn finger is further capable of recruiting Munc13 to the nerve terminal. These structure-function experiments established specific aspects of the mechanisms through which RIM assembles the active zone, as well as provided insight on what might be upstream of RIM in targeting the active zone to specific sites along the presynaptic plasma membrane.

Liprin-α’s are important core active zone proteins that are not reduced upon loss of RIM and ELKS. With structure-function rescue experiments we show that although Liprin-α3 total protein levels did not change upon active zone disruption, Liprin-α3 becomes more enriched at the presynaptic plasma membrane of mutant synapses, and this enrichment is reversed by RIM1α expression. Together with a rich body of literature from invertebrate work, these results suggest that Liprin-α’s may be upstream of RIM and ELKS in active zone
assembly. To test this hypothesis at vertebrate synapses, we decided to use mouse genetic experiments to remove Liprin-α proteins from cultured neurons. Liprin-α3 KO mice were produced for a previous study (Wong et al., 2018). In thus study, Liprin-α synaptic localization was assessed with stimulated emission depletion (STED) super-resolution microscopy and it was established that Liprin-α3 had a prominent component that strongly co-localizes with other active zone proteins. On the other hand Liprin-α2, the other Liprin-α isoform specifically enriched in the brain, is distributed widely in hippocampal nerve terminals. Compellingly, Liprin-α2 translocates to the active zone upon removal of Liprin-α3, structurally compensating for Liprin-α3. This compensation is only partial functionally, because loss of Liprin-α3 still results in impairments in synaptic vesicle docking, tethering, and fusion. Thus, to more comprehensively address the role of Liprin-α’s in active zone assembly, we decided to genetically target both Liprin-α2 and Liprin-α3. In Chapter 4, we show that loss of both Liprin isoforms results in reduced levels of RIM and ELKS at synapses, as well as elevated levels of transsynaptic Liprin binding partner LAR. We also observe changes in synapse structure, with an increase in the size of the presynaptic bouton and a lengthening of the PSD, reminiscent of what is observed in invertebrates (Kaufmann et al., 2002; Zhen and Jin, 1999). Our data suggest that Liprin-α proteins are involved in recruiting RIM and ELKS to vertebrate synapses, as well as synapse assembly.

Altogether, my thesis work contributes to a working model for active zone assembly at vertebrate synapses, where RIM and ELKS are core components of the presynaptic active zone holding its protein scaffold together, while Liprin-α’s interact at a level above in recruiting RIM and ELKS to synapses, as well as mediating synapse formation possibly through the transsynaptic protein LAR. The results that I have presented here provide the
groundwork for additional avenues of investigation, which I will briefly introduce and
discuss in the following sections.

**The balance between redundancy and diversity at the presynaptic active zone.**

The redundant scaffolding mechanisms of the active zone protein complex make the
active zone structure resilient to loss of individual active zone protein families. In Chapter
2, we demonstrate that it is necessary to target multiple active zone protein families, here
RIM and ELKS, to disassemble the active zone. Compellingly, our results were
complemented by a study published at the same time which employed a similar strategy,
targeting both RIM and RIM-BP to generate a strong active zone disruption (Acuna et al.,
2016). Loss of RIM and RIM-BP proteins here resulted in a milder disruption of the
biochemical active zone complex, as ELKS, piccolo, and Liprin-α levels remained
unchanged. However, both studies establish that there is no single “master organizer” for
the active zone, but rather it is likely that multiple protein interactions nucleate the
recruitment of active zone components and in a heterogeneous manner (Tang et al., 2016).
One biochemical process that may support this heterogeneous nucleation is liquid-liquid
phase separation, which has previously been shown to mediate the organization of the
synaptic vesicle cluster and postsynaptic densities (Milovanovic et al., 2018; Zeng et al.,
2016). Indeed, a recent study established *in vitro* that the interactions between RIM and
RIM-BP can induce liquid-liquid phase separation and recruit Ca^{2+} channels to RIM/RIM-BP
condensates (Wu et al., 2019). Taken together, these findings establish a mechanism for
redundant scaffolding mechanisms to drive dynamic active zone protein interactions and
functions.
In such a system, it’s possible that these redundant scaffolding mechanisms also provide the molecular blueprint necessary to support active zone assembly, synaptic plasticity, and functional diversity of different synapse types. For instance, it’s well established that the same active zone protein family can have differential functional roles. Specifically, previous work studying ELKS proteins has demonstrated that while ELKS enhances Ca2+ influx and release probability at inhibitory synapses, ELKS controls the size of the RRP at excitatory synapses (Held et al., 2016; Liu et al., 2014). Thus it is likely that the composition of the active zone protein scaffold at excitatory vs inhibitory synapses and the diversity of their protein-protein interactions between multiple isoforms confers this functional diversity without sacrificing structural integrity, perhaps as a liquid-liquid phase. A rigorous analysis of the interplay between the structure and function of different active zone protein isoforms is necessary to understand both their redundant and diverse roles. For example, in our active zone disrupted mutants, ELKS1α is able to localize to synapses independent of RIM and recruits Bassoon. However, lentiviral expression of ELKS2α was not able to target to the active zone (data not shown). Understanding the differential molecular mechanisms by which ELKS isoforms target to the active zone may provide key insight on the functional diversity ELKS has at different synapse types.

Towards constructing a “minimal” active zone

As a whole, the active zone is an evolutionarily conserved protein scaffold that sits along the presynaptic plasma membrane, exactly opposite to postsynaptic receptors and specializations. Synaptic vesicles are spatially restricted at the active zone and brought into close proximity with the SNARE molecular release machinery and Ca2+ channels that
trigger release. Our work from Chapter 2 reveals that while the active zone is necessary to spatially restrict synaptic vesicles and for setting RRP and P, it is not necessary for fusion. Furthermore, our findings from Chapter 3 identify that the Zn finger of RIM is essential to recruit the priming factor Munc13, and attaching the Zn finger to the rest of RIM is essential for docking synaptic vesicles to the target membrane. It remains unclear, however, what the minimal components necessary to generate a functional active zone are. While it has been previously established that Munc13s are the priming factor that generate the RRP and Ca2+ channels enhance P, our work here further suggests that the Zn finger of RIM is an essential mechanism that links docking and priming together, and that the rest of RIM attaches these functions to the target membrane close to Ca2+ channels.

Thus, it is possible that by tethering just the Zn finger of RIM near a Ca2+ channels along the presynaptic plasma membrane, this would be sufficient to restore fast synaptic transmission without the need of extensive scaffolding provided by RIM, ELKS, and piccolo/bassoon. One way to address this hypothesis would be to generate chimeric proteins that fuse the Zn finger of RIM either to Ca2+ channels directly or to a protein that associates with Ca2+ channels at active zone disrupted synapses. If such a Zn finger fusion protein is sufficient to recruit soluble Munc13 and dock synaptic vesicles near Ca2+ channels, one would hypothesize that this would enhance both RRP and P, essentially restoring most active zone functions. By localizing the docking and priming function of the RIM Zn finger close to Ca2+ channels, this experiment would establish a “minimal” active zone without the need for extensive scaffolding domains. We would further hypothesize that all the domains that are not needed for these “minimal” active zones may by used for regulation of specific properties.
Mechanisms targeting the active zone to the presynaptic plasma membrane

A fundamental question that remains unanswered central to active zone assembly is: what tethers the active zone protein complex to specific sites along the presynaptic plasma membrane? There are multiple candidate mechanisms that would serve such a function. On the one hand, it is possible that certain lipids asymmetrically distributed at the presynaptic plasma membrane play a direct role in targeting the active zone machinery to specific membrane areas. One such lipid is phosphatidylinositol 4,5-bisphosphate (PIP$_2$), which is essential for synaptic vesicle exocytosis and hypothesized to cluster at vesicle release sites (Di Paolo et al., 2004; Lauwers et al., 2016; van den Bogaart et al., 2011).

Interestingly, PIP$_2$ interacts with C2 domains, which multiple active zone proteins contain, including RIM. In a recent study, PIP2 was shown to bind directly to the C2B domain of RIM and disrupting this interaction greatly impaired synaptic vesicle exocytosis (de Jong et al., 2018), thus supporting the hypothesis that PIP2 maybe provide a mechanisms to directly target the active zone machinery to specific sites along the presynaptic plasma membrane.

Another possibility is that various transmembrane proteins provide a link between the active zone complex and the presynaptic plasma membrane. Two attractive candidates enriched at synapses are the Liprin binding partner LAR and Ca$^{2+}$ channels. LAR is a transsynaptic type IIa receptor-type protein tyrosine phosphatase (RPTP) involved in pre- and postsynaptic cell adhesion (Takahashi and Craig, 2013) and Ca$^{2+}$ channels are necessary components of the presynaptic active zone that interact with both RIM and RIM-BPs. In Chapter 4 we show that loss of Liprin-α2/3, which has a function upstream of RIM and ELKS, increases the synaptic enrichment of these transmembrane proteins. It is
possible that the interactions between LAR or Ca$^{2+}$ channels and core active zone protein families provide a nucleation point for active zone assembly, and our preliminary data on Liprin-α, together with published work, support that these proteins may be one candidate.

**Closing Remarks**

The work presented in this dissertation leverages mouse genetics to provide new insight on the redundant structural mechanisms for active zone assembly at vertebrate synapses. Here we address what the active zone as a complex macromolecular structure is necessary for, and we identify specific protein interactions contributing to different layers of its assembly. Future studies building upon the mechanisms identified in this work will contribute to unanswered questions regarding the diversity of synapse function and assembly.
Supplemental Figures and Tables
Supplemental Figure 1. Genetic disruption of the active zone. (A) Schematic of the RIM and ELKS proteins targeted in the conditional RIM1 (Kaeser et al., 2008), RIM2 (Kaeser et al., 2011), ELKS1 (Liu et al., 2014), and ELKS2 (Kaeser et al., 2009) knockout mouse lines crossed to quadruple homozygosity. Arrowheads indicate transcription start sites of all known α-, β- and γ- isoforms of each gene (Kaeser et al., 2009, 2008; Liu et al., 2014, p. 2; Wang et al., 2002; Wang and Südhof, 2003), the red bars indicate the protein sequence that is encoded by the conditionally targeted exon. The zinc finger domain (Zn), PDZ domains (PDZ), C2 domains (C2A, C2B), coiled-coil regions (CC) and the ELKS C-terminal PDZ binding domain (B) are indicated. (B) Additional sample images for Figure 2.1 B, for the assessment of synaptic protein levels within Synaptophysin-1 (Syp-1) defined ROIs, and example images for stainings excluding the primary antibodies in control neurons. (C) Synaptophysin-1 staining was used to determine synapse density and size. The synapse density is expressed as synaptophysin-1 positive puncta per 30 μm of MAP2 positive dendrite, and synaptophysin-1 levels are measured as the average arbitrary fluorescence within the puncta (controlR+E n = 3 independent cultures, cKO R+E n = 3, 10 images per culture). (D) Quantitation of immunofluorescent stainings of wild type neurons incubated with or without RIM primary antibodies (with primary n = 3 independent cultures, without primary n = 3). The same fluorescent secondary antibodies were used in both conditions and in all test proteins in Figure 2.1 A, and acquisition settings were identical for the two groups. This experiment measures the background signal from the secondary antibody alone, and it does not account for background signal contributed by the primary antibody. (E) Quantification of the fraction of Synaptophysin-1 puncta containing active zone proteins in cKO R+E and control R+E synapses (controlR+E n = 6 independent cultures, cKO R+E
Supplemental Figure 1 (Continued)

n = 6, 10 images per culture). (F) Quantification of the fraction of Bassoon pixels containing active zone proteins at cKO^{R+E} and control^{R+E} synapses (control^{R+E} n = 6 independent cultures, cKO^{R+E} n = 6, 10 images per culture). (G) Plot of all individual synaptic fluorescent intensity levels normalized to the average control (control^{R+E}) synaptic fluorescent intensity level. Red lines indicate mean \pm SEM, data shown corresponds to Figure 2.1 C. All data are means \pm SEM; \*p \leq 0.05, \**p \leq 0.01, \***p \leq 0.001 as determined by Student’s t test.
Supplemental Figure 2. Analysis of synapse ultrastructure in glutaraldehyde fixed neurons. (A, B) Sample images (A) and quantification (B) from glutaraldehyde fixed electron microscopic analyses of cKO<sup>R+E</sup> and control<sup>R+E</sup> synapses; postsynaptic densities are marked with arrowheads. (control<sup>R+E</sup> n = 50 synapses/3 independent cultures, cKO<sup>R+E</sup> n = 48/3). (C) Distribution of synaptic vesicles relative to the presynaptic plasma membrane area opposed to the PSD. Vesicle distribution is shown in 100 nm bins (left) in cKO<sup>R+E</sup> and control<sup>R+E</sup> synapses within 1 μm. Gaussian fits were used to model the vesicle distribution. The two genotypes were significantly different (*) and could not be fit with a single distribution, requiring individual fits. Distribution of synaptic vesicles within the first 100 nm in 10 nm bins and the number of tethered vesicles (defined as vesicles within 100 nm of the presynaptic plasma membrane) are shown in the middle and on the right, respectively. In each bin within 100 nm we observed a reduced vesicle number in cKO<sup>R+E</sup> synapses (control<sup>R+E</sup> n = 50/3, cKO<sup>R+E</sup> n = 48/3). All data are means ± SEM; *p ≤ 0.05, ***p ≤ 0.001 as determined by Student’s t test (B) or by extra sum of squares F test (C).
Supplemental Figure 2 (Continued)
Supplemental Figure 3. Additional electrophysiological analyses of stimulus trains and mPSCs. (A) Plot of EPSC amplitude during a 10 Hz, 50 stimulus train normalized to the amplitude of the first response (controlR+E n = 17 cells/3 independent cultures, cKO'R+E n = 19/3). (B) Analyses of total (0 - 6.5 s), tonic (0 - 5 s) and delayed (5 - 6.5 s) charge transfer from NMDAR-EPSCs during stimulation trains in cKO'R+E and controlR+E neurons (controlR+E n = 17/3, cKO'R+E n = 19/3). (C, D) Same as A, B, but for IPSCs (controlR+E n = 19/3 independent cultures, cKO'R+E n = 19/3). (E) Expanded traces showing mEPSCs from the experiment presented in Figure 3.5 L. For each condition (control, cKO, controlE, cKOE, controlR+E, and cKO'R+E) three consecutive seconds from a single cell are shown. (F) Statistical comparison of mEPSC frequencies between all genotypes (normalized to their respective controls). Statistical comparison between genotypes was done using one-way ANOVA (***, p<0.001) followed by Holm-Sidak post-hoc tests (cKO'R+E vs. cKO: ***, p < 0.001; cKO'R+E vs. cKOE: n.s., p = 0.474; cKOE vs. cKO: ***, p < 0.001; cKO'R+E n = 31/5, cKO n = 21/3, cKOE n = 24/3). (G, H) Sample traces (G) and quantification (H) of mIPSCs in controlR+E and cKO'R+E neurons. Quantitative analysis of mIPSC frequencies (H, left) and amplitudes (H, right) are shown (controlR+E n = 20/3, cKO'R+E n = 21/3). (I) Expanded traces showing mIPSCs from the experiment presented in G, H. Three consecutive seconds from a single cell are shown for each condition. All data are means ± SEM unless otherwise specified **p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test.
Supplemental Figure 4. Additional analysis for heterogeneity in cKO\textsuperscript{R+E} of synapses.

(A, B) Sample images (A) and quantification (B) of the number of neurons (left) and the fraction of infected neurons (right) with EGFP tagged cre (cKO\textsuperscript{R+E}) or an inactive form of cre (control\textsuperscript{R+E}). Consistent with Figures 2.1 and Supplemental Figure 1, no differences in neuronal density were detected and all neurons were infected with cre viruses (control\textsuperscript{R+E} n = 752 cells/9 frames/3 independent cultures, cKO\textsuperscript{R+E} n = 731/9/3). (C) Frequency distribution of synaptic protein fluorescence levels for all individual cKO\textsuperscript{R+E} and control\textsuperscript{R+E} synapses (normalized to the average of control\textsuperscript{R+E} synapses), related to Figure 2.6 A. (D-F) Quantification of SypHy infected cultures related to Figures 2.6 B-H. SypHy puncta density and the fraction of total SypHy puncta responsive to NH\textsubscript{4}Cl (%(\Delta F_{\text{NH4Cl}}/F_0) >200%) (D), SypHy fluorescence distribution of all synapses at baseline (F\textsubscript{0}) and upon NH\textsubscript{4}Cl application (%(\Delta F_{\text{NH4Cl}}/F_0)) (E), the fraction of SypHy puncta responsive to 40 and 200 APs stimulation (defined as %((\Delta F_{\text{during stimulation}})/\Delta F_{\text{NH4Cl}}) > 0) (F). The change in SypHy density in D suggests a change in synapse/cell density in cKO\textsuperscript{R+E} neurons, which was not seen in other experiments (Supplemental Figures 1 C, 4 A, B). We attribute this change to the potential toxicity of triple lentivirus infection (FSW-cre or control, FSW-SypHy, FSW-SV2-TdTomato) and suspect that the cKO\textsuperscript{R+E} neurons are more sensitive to multi-virus infection because of the strong genetic manipulation and loss of activity. All data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test.
Supplemental Figure 4 (Continued)
Supplemental Figure 5. Electron microscopic analyses of cKO<sub>R</sub>, cKO<sub>E</sub> and cKO<sub>R+E</sub> synapses. (A, C, E) Quantitation of vesicles per bouton, bouton size, and PSD length in cKO<sub>R</sub> (A), cKO<sub>E</sub> (C), cKO<sub>R+E</sub> (E), and respective control synapses. (B, D, F) Distribution of synaptic vesicles relative to the presynaptic plasma membrane area opposed to the PSD was analyzed in 100 nm bins for cKO<sub>R</sub> (B), cKO<sub>E</sub> (D), cKO<sub>R+E</sub> (F), and respective control synapses. Gaussian fits were used to model vesicle distribution (left); one Gaussian fit (orange line) was used if mutant and control best-fit distributions were not significantly different, and two Gaussian fits were used if the data could not be fit with a single Gaussian (*, p < 0.05 in F). Synaptic vesicle distribution within the first 100 nm in 10 nm bins and the number of tethered vesicles (defined as vesicles within 100 nm of the presynaptic plasma membrane) are shown in the middle and on the right, respectively. We observed a reduction of vesicles in the first 100 nm in cKO<sub>R+E</sub> synapses, but not in cKO<sub>R</sub> synapses or cKO<sub>E</sub> synapses (control<sub>R</sub> n = 25 synapses, cKO<sub>R</sub> n = 25; control<sub>E</sub> n = 25, cKO<sub>E</sub> n = 25; control<sub>R+E</sub> n = 25, cKO<sub>R+E</sub> n = 25 in A-F). (G) Statistical comparison of docking between all genotypes, normalized to their respective controls, using one-way ANOVA (***, p < 0.001) followed by Holm-Sidak post-hoc tests (cKO<sub>R+E</sub> vs. cKO<sub>R</sub>: **, p < 0.01; cKO<sub>R+E</sub> vs. cKO<sub>E</sub>: ***, p < 0.001; cKO<sub>E</sub> vs. cKO<sub>R</sub>: n.s., p = 0.0673; cKO<sub>R+E</sub> n = 25 synapses, cKO<sub>R</sub> n = 25, cKO<sub>E</sub> n = 25). (H) Statistical comparison of 0 - 10 s sucrose response between all genotypes, normalized to their respective controls, using one-way ANOVA (***, p < 0.001) followed by Holm-Sidak post-hoc tests (cKO<sub>R+E</sub> vs. cKO<sub>R</sub>: *, p < 0.05; cKO<sub>R+E</sub> vs. cKO<sub>E</sub>: *, p < 0.05; cKO<sub>E</sub> vs. cKO<sub>R</sub>: ***, p < 0.001; cKO<sub>R+E</sub> n = 20 cells/3 cultures, cKO<sub>R</sub> n = 20/3, cKO<sub>E</sub> n = 17/3). Data are means ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as determined by Student’s t test (E), by extra sum of squares F test (F), or by one-way ANOVA with Holm-Sidak post-hoc test (G, H)
Supplemental Figure 5 (Continued)
Supplemental Figure 6. Additional STED imaging of rescued active zone components. 

(A, B) Sample images (left) and line profiles (right) of ‘side view’ synapses stained for ELKS2 (A) and RIM-BP2 (B) (green channel) along with PSD-95 (red channel) and the synapse marker synaptophysin (blue channel). Scale bar is 500nm. All line profiles were taken from ~200 nm wide ROIs drawn perpendicular to the center point of PSD-95 ‘bars’ and aligned to the peak of PSD-95. Values were normalized to the max average of the control<sup>R+E</sup> condition (see experimental methods for details). Abscissa indicates the distance from the peak of PSD-95 (marked with a dashed line). For all conditions n = 60 synapses/ 3 independent cultures.
**Supplemental Table 1**, related to experimental procedures. Antibodies used in this work.

Target proteins of antibodies, the antibody source, RRID and dilutions are shown.

Abbreviations: IS: Immunostaining, IB: Immunoblotting, n.a.: information not available, KO: knockout, KD: knockdown

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—APPENDIX TWO—

Supplemental Experimental Procedures
Mice. Previously generated conditional RIM1 (Kaeser et al., 2008) (RRID:IMSR_JAX:015832), RIM2 (Kaeser et al., 2011) (RRID:IMSR_JAX:015833), ELKS1 (Liu et al., 2014) (RRID:IMSR_JAX:015830) and ELKS2 (Kaeser et al., 2009) (RRID:IMSR_JAX:015831) mice were crossed and maintained as quadruple homozygous floxed mice. The RIM1/2 double floxed mice (Kaeser et al., 2011) and the ELKS1/2 double floxed mice (Liu et al., 2014) were described previously. All animal experiments were performed according to institutional guidelines at Harvard University.

Cell culture and lentiviral infection. Primary mouse hippocampal cultures were generated from newborn conditional quadruple or double floxed pups as described before (Kaeser et al., 2011, 2008; Liu et al., 2014; Maximov et al., 2007) within 24 h after birth. For the biochemical solubility experiments, neurons were plated directly on 12-well cell culture plates, for all other experiments, chemically stripped glass coverslips were used. Lentiviruses expressing EGFP-tagged cre recombinase (to generate cKO neurons) or a truncated, enzymatically inactive EGFP-tagged cre protein (to generate control neurons) were produced in HEK293T cells by Ca\textsuperscript{2+}-phosphate transfection and neuron-specific expression was driven by a synapsin promoter (Liu et al., 2014). Neurons were infected with HEK cell supernatant at 5 days in vitro (DIV) as described before (Kaeser et al., 2008; Liu et al., 2014). All subsequent experiments were performed at DIV 15-19. In the SypHy experiments, additional lentiviruses expressing SypHy or SV2-TdTomato under a human synapsin promoter were produced in HEK293T cells and were applied to the cultured neurons at DIV3.
Immunofluorescence stainings and confocal imaging of cultured neurons. Neurons were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 20 minutes, permeabilized in 0.1% Triton X-100/3% bovine serum albumin/PBS for 1 h, and incubated in primary antibodies at 4°C overnight. Following overnight primary antibody incubation, neurons were incubated in AlexaFluor-488 (for detection of the protein of interest), AlexaFluor-546 (for detection of MAP2), and AlexaFluor-633 (for detection of Synaptophysin-1) conjugated secondary antibodies (1:500) for 2 h at room temperature and mounted in Fluoromount-G for imaging. Images were taken on an Olympus FV1200 confocal microscope using identical settings for each condition in a given experiment with a 60X oil-immersion objective and single confocal sections were analyzed in ImageJ software. For quantitative analyses of synaptic protein levels, the Synaptophysin-1 signal was used to define synaptic puncta as ROI, and the average intensity within the ROI was quantified. For each image, the “rolling ball” ImageJ plugin was set to a diameter of 1.4 μm to calculate a local background value for background subtraction (Sternberg 1983). The quantitative data for each protein per condition were derived from n ≥ 3 independent cultures, and ≥ 10 fields of view per culture. A control experiment in which wild type neurons were incubated either with primary (mouse anti- RIM) or without primary antibodies in the AlexaFluor-488 channel was done to determine the level of non-specific staining by secondary antibodies alone for comparison (Supplemental Figures 1B, D). The average intensity per protein in cKO<sup>R+E</sup> neurons was normalized to the respective staining in the control. For co-localization of active zone proteins, the ImageJ plugin BioVoxxel was used in the puncta to puncta comparison (The BioVoxxel Image Processing and Analysis Toolbox. Brocher, 2015, EuBIAS-Conference, 2015, Jan 5) and a custom MATLAB script was
used for the pixel to pixel comparison. Student’s t-test was used to determine whether experimental and control conditions were significantly different. All experiments and analyses were performed by an experimenter blind to the genotype.

**Biochemical solubility assay.** At DIV 15 the neurons were harvested in 400 μl ice-cold buffer (25 mM HEPES, 5 mM EDTA, 0.32 M Sucrose, 7.2 pH) and homogenized with a glass-Teflon homogenizer. The homogenate was solubilized with 1% Triton X-100 for 1 h rotating at 4°C and then centrifuged at 100,000 x g for 1 h. The pellet and supernatant were collected in 1X sodium dodecyl sulfate (SDS) sample buffer and quantitative Western blotting was performed on the pellet and the supernatant as described below. In each fraction and for each protein, the signal was normalized to β-actin as an internal loading control resulting in values for the pellet (P) and the supernatant (S), and the experiment was expressed as the cKO*R+E to controlR+E ratio. Solubility was calculated as S/(S+P). Student’s t-test was used to determine whether experimental and control conditions were significantly different.

**Western blotting.** For non-quantitative assessment of cre-recombination in cultured neurons, chemiluminescence was used to detect RIM and ELKS removal in all experiments. At DIV15, cultured neurons were harvested in 25 μl 1X SDS buffer and run on standard SDS-Page gels followed by transfer on a nitrocellulose membrane. Membranes were blocked in filtered 10% nonfat milk/5% goat serum for 1 h at room temperature and incubated with primary antibodies in 5% nonfat milk/2.5% goat serum for 2 hours at room temperature to overnight at 4°C, and HRP- conjugated secondary antibodies (1:10,000)
were used. *For quantitative assessment* of protein levels in cultured neurons, fluoresceinly
tagged secondary antibodies were used. Cultured neurons were either directly harvested in
1X SDS buffer or processed as described in the biochemical solubility assay section.
Membranes were blocked in filtered 5% nonfat milk/5% goat serum for 1 h at room
temperature and incubated with primary antibodies in 5% BSA overnight at 4°C. Each
membrane was incubated with primary antibodies against the protein of interest and
Synapsin or β-actin antibodies as a loading control. Blots were scanned on a fluorescent
scanner and all images were analyzed with ImageJ. The fluorescent signal in each
experimental condition was normalized first to either Synapsin (total culture
homogenates) or β-actin (biochemical solubility experiments) to account for differences in
loading and then to the respective control R+E signal. All quantitative analyses were
performed in three independent cultures. For the plot of total protein levels, the
experiments from total culture homogenates (n = 3) and the total protein levels from the
solubility assay (n = 3, sum of the soluble and insoluble fraction) were pooled. Student’s t-
test was used to determine whether experimental and control conditions were significantly
different.

**Electron microscopy and analysis of synaptic vesicle distribution.** *For high-pressure
freezing:* neurons cultured on 6 mm carbon-coated sapphire coverslips were frozen using
the HPM 100 high-pressure freezer in extracellular solution containing (in mM): 140 NaCl,
5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES-NaOH (pH 7.4), 10 Glucose (~310 mOsm) with
picrotoxin (50 μM), AP5 (50 μM), and CNQX (20 μM) added to block synaptic transmission.
After freezing, samples were stored in liquid nitrogen and processed by the Electron
Microscopy Facility at Harvard Medical School. Samples were first freeze-substituted in 1\% glutaraldehyde, 1\% osmium tetroxide, 1\% water, and anhydrous acetone with the following protocol: -90\(^{\circ}\)C for 5 h, 5\(^{\circ}\)C per hour to -20\(^{\circ}\)C, -20\(^{\circ}\)C for 12 h, and 10\(^{\circ}\)C per hour to 20\(^{\circ}\)C (AFS2, Leica). Following freeze substitution, samples were Epon infiltrated, and baked for 24 h at 60\(^{\circ}\)C before sectioning at 50 nm and imaging. For glutaraldehyde fixation: neurons cultured on glass coverslips were fixed with 2\% glutaraldehyde in 0.1 M sodium cacodylate buffer at 37\(^{\circ}\)C for 15 minutes and immediately processed by the Electron Microscopy Facility at Harvard Medical School. Glutaraldehyde fixed samples were first stained with 1\% osmium tetroxide/1.5\% potassium ferrocyanide for 1 h at room temperature, washed in water and then maleate buffer (pH 5.15) 3X, and then stained with 1\% uranyl acetate for 1 h. Post-staining, samples were dehydrated through a series of EtOH treatments (70\% for 15 min, 90\% for 15 min, and 100\% for 15 min 2X) and propylene oxide (1 h). Samples were resin infiltrated and prepared for embedding with a 1:1 Epon, propylene oxide mixture for 2 h at room temperature. Samples were then polymerized for 24 h at 60\(^{\circ}\)C and sectioned at 50 nm for transmission electron microscopy. Imaging and quantification: Images of both high-pressure frozen and glutaraldehyde fixed samples were taken with a transmission electron microscope (JEOL 1200 EX at 80 kV accelerating voltage) and processed with ImageJ. The total number of vesicles, the number of docked vesicles, the length of the PSD, the area of the presynaptic bouton, and the distance of each vesicle from the active zone were analyzed with SynapseEM, a MATLAB macro provided by Drs. M. Verhage and J. Broeke. Bouton size was calculated from the perimeter of each synapse. Docked vesicles were defined as vesicles touching the presynaptic plasma membrane opposed to the PSD. The nearest distance of the vesicle membrane to the
presynaptic plasma membrane area opposed to the PSD was measured and plotted as frequency distribution over the total number of vesicles in 100 or 10 nm bins (Figures 2.2 C, Supplemental Figure 2 C and Supplemental Figures 5 B, D, and F). To test whether there was a genotype effect on vesicle distribution, Gaussian fits were performed. The extra sum-of-squares F test was used to compare whether the best-fit values of mutant and control Gaussian distributions were significantly different, and if they were not different a single Gaussian fit for all data is shown for the mutant and control data set. Student’s t-test was used to determine whether all other experimental and control conditions were significantly different. All experiments and analyses were performed by an experimenter blind to the genotype.

**Electrophysiology.** Electrophysiological recordings in cultured hippocampal neurons were performed as described (Kaeser et al., 2011, 2009, 2008; Liu et al., 2014; Maximov et al., 2007) at DIV 15-19. The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES-NaOH (pH 7.4), 10 Glucose (~310 mOsm). To assess excitatory transmission in response to action potentials, NMDAR excitatory postsynaptic currents (EPSCs) were pharmacologically isolated with picrotoxin (PTX, 50μM) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM). NMDAR-EPSCs were recorded because analyses of AMPA-receptor mediated EPSCs in cultured neurons are limited by recurrent activity in response to action potentials. Action potential evoked inhibitory postsynaptic currents (eIPSCs) were isolated with D-amino-5-phosphonopentanoic acid (D-APV, 50 μM) and CNQX (20 μM). For miniature recordings, tetrodotoxin (TTX, 1 μM) was added to block action potentials, and in addition PTX (50 μM) and APV (50 μM) were added for mEPSCs or
APV (50 μM) and CNQX (20 μM) for mIPSCs, respectively. For sucrose EPSC recordings, TTX (1 μM), PTX (50 μM), and APV (50 μM) were added. All recordings were performed in whole cell patch clamp configuration at 20 – 23°C. Glass pipettes were pulled at 2-4 MΩ and filled with intracellular solutions containing (in mM) for EPSC recordings: 120 Cs-methanesulfonate, 10 EGTA, 2 MgCl₂, 10 HEPES-CsOH (pH 7.4), 4 Na₂-ATP, 1 Na-GTP, 4 QX314-Cl (~300 mOsm) and for IPSC recordings: 40 CsCl, 90 K-Gluconate, 1.8 NaCl, 1.7 MgCl₂, 3.5 KCl, 0.05 EGTA, 10 HEPES-CsOH (pH 7.4), 2 MgATP, 0.4 Na₂-GTP, 10 Phosphocreatine, 4 QX314-Cl (~300 mOsm). Cells were held at +40 mV for NMDAR-EPSC recordings and at -70 mV for mEPSC, mIPSC, eIPSC, and sucrose EPSC recordings. Access resistance was monitored during recording and cells were discarded if access exceeded 15 MΩ or 20 MΩ during recording of evoked or spontaneous synaptic currents, respectively. Action potentials were elicited with a bipolar focal stimulation electrode fabricated from nichrome wire. For analysis of action potential trains, 50 stimuli were provided at a frequency of 10 Hz. Individual PSCs within the train were aligned using the peak of the stimulus artifact and the baseline value set to the negative peak immediately following the artifact. Peak amplitudes and the synchronous component of charge transfer during the train were determined using these aligned events. To determine tonic charge transfer, the cumulative charge during the synchronous component was subtracted from the total charge transfer during the first 5 seconds of the train. Delayed charge transfer was taken as the area under the curve for 1.5 seconds after the end of the train (i.e. 5 – 6.5 seconds). Paired-pulse ratios were calculated as the amplitude of the second PSC divided by the amplitude of the first PSC. The baseline value for the second PSC was taken as the negative peak immediately following the second stimulus artifact, as in the train analysis, and the
amplitude of second PSC was measured relative to this baseline. The RRP was measured by application of 500 mM sucrose in extracellular solution applied via a microinjector syringe pump for 10 s at a rate of 10 μL/min through a tip with an inner diameter of 250 μm. For calcium titration experiments, the following \([\text{Ca}^{2+}]_{\text{ex}} / [\text{Mg}^{2+}]_{\text{ex}}\) ratios were used (in mM): 0.5/3.5, 1/3, 2/2, 5/0.25, 7/0.25. Recordings began at 0.5 mM \([\text{Ca}^{2+}]_{\text{ex}} / 3.5 \text{ mM } [\text{Mg}^{2+}]_{\text{ex}}\) and with each solution exchange five chamber volumes of solution were exchanged. After each exchange there was delay of at least one minute to ensure equilibration of the new solution. During this delay action-potential evoked responses were recorded at 0.2 Hz and monitored for stability at each \([\text{Ca}^{2+}]_{\text{ex}}\). Afterward, at least 10 sweeps were recorded and the average amplitude was measured at each \([\text{Ca}^{2+}]_{\text{ex}}\). Only cells in which all five \([\text{Ca}^{2+}]_{\text{ex}}\) were recorded were included in the analysis. For the normalized data, IPSC amplitudes for each cell were normalized to the same cell’s amplitude at 7 mM \([\text{Ca}^{2+}]_{\text{ex}}/ 0.25 \text{ mM } [\text{Mg}^{2+}]_{\text{ex}}\).

The absolute and normalized data was fit to the model \(I = I_{\text{min}} + (I_{\text{max}} - I_{\text{min}}) / (1+10^{((\text{LogEC}_{50} - [\text{Ca}^{2+}]_{\text{ex}}) \cdot n)})\). For comparing \(\text{EC}_{50}\) values, normalized data from individual cells was fit to the above model and the averaged \(\text{EC}_{50}\) values were compared using a Student’s t-test. Data were acquired with an Axon 700B Multiclamp amplifier and digitized with a Digidata 1440A digitizer. For action potential and sucrose-evoked responses, data were acquired at 5 kHz and low-pass filtered at 2 kHz. For miniature recordings data were acquired at 10 kHz. All data acquisition and analysis was done using pClamp10. For all electrophysiological experiments, the experimenter was blind to the genotype throughout data acquisition and analysis.

**Ca\(^{2+}\) Imaging.** All \(\text{Ca}^{2+}\) imaging experiments were done with hippocampal cultures infected
with lentiviruses (expressing active cre or inactive cre) at DIV 5. Neurons were recorded at DIV 15-18 in whole-cell patch-clamp configuration at 20 – 23°C. The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 Glucose, 0.05 APV, 0.02 CNQX, 0.05 PTX, 10 HEPES-NaOH (pH 7.4, ~310 mOsm). Glass pipettes were filled with intracellular solution containing (in mM) 140 K Gluconate, 0.1 EGTA, 2 MgCl2, 4 Na2ATP, 1 NaGTP, 0.3 Fluo5F, 0.03 Alexa Fluor 594, 10 HEPES-KOH (pH 7.4, ~300 mOsm). Only neurons with membrane potentials between -55 and -65 mV (junction potential uncorrected) were used. After filling for 7 min, axons and dendrites were identified in the red channel. Presynaptic boutons were identified by their typical bead-like morphology. Neurons in which the distinction between axons and dendrites was unclear were discarded. 10 min after break-in, a holding current was injected to keep the membrane potential at ~ -60 mV, and Ca2+-transients were induced by a single action potential evoked via brief somatic current injection (5 ms, 500-1500 pA). Images were acquired using an upright microscope with a 60X, 1.0 numerical aperture water immersion objective. Fluorescence signals were excited by a light-emitting diode at 470 nm, and were collected with a scientific complementary metal–oxide–semiconductor camera (sCMOS) at 100 frames/s. Images were collected for 0.2s before and 1s after the action potential initiation. An extra 10 frames of images were acquired for each neuron without excitation and these images were used to estimate the dark current level of the camera. Ca2+ transients were quantified using ImageJ. 7-20 boutons and 5-10 areas in the second order dendrites were randomly selected from each neuron. After background subtraction (removing the dark current and rolling ball (Sternberg 1983) with a radius of 4 μm), the (F-F0)/F0 was calculated (F = average green emission at a given time point, F0 = average fluorescent intensity in frames 0 to 20 before
action potential induction). For all Ca$^{2+}$ imaging experiments, the experimenter was blind to the genotype throughout data acquisition and analysis.

**Synaptophysin-pHluorin Imaging.** The SypHy A4 open reading frame (Granseth et al., 2006) was obtained from Addgene (Plasmid #24478), and was subcloned into the synapsin driven lentiviral vector FSW. FSW-SypHy A4- and FSW-SV2-tdTomato- expressing viruses were produced in transfected HEK293T cells and were applied to dissociated hippocampal cultures at DIV3. Cultures were subsequently infected with lentiviruses to express cre or a control virus at DIV5. Cultures were imaged at DIV14-17 with an upright microscope at 20-23°C in extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 Glucose, 10 HEPES-NaOH, 0.05 APV and 0.025 CNQX (pH 7.4, ~310 mOsm). Synapse rich areas were identified in the red channel. Images were acquired at 0.5 Hz with 2x2 binning using a sCMOS camera with a 60X, 1.0 numerical aperture water immersion objective. SypHy and SV2-tdTomato were excited with at 488 nm or 550 nm with a light-emitting diode, respectively. Light paths were split and filtered with a multiband filter set. Neurons were stimulated with a bipolar electrode made from nichrome wire at 20 Hz. For each experiment, 5 s of baseline image acquisition preceded stimulation. NH$_4$Cl solution (extracellular solution substituted with NH$_4$Cl for 50 mM NaCl, adjusted to pH 7.4) was applied at the end of experiments to visualize all SypHy puncta. We did not correct for photobleaching. Images were analyzed in ImageJ, a rolling ball of 5 μm was used for background subtraction for all images (Sternberg 1983). NH$_4$Cl responsive puncta defined as %$(\Delta F_{NH4Cl}/F_0) > 200\%$ were included in the analysis, and were used to define ROIs. Active puncta in response to action potential stimulation (40 or 200 action potentials) were
defined as those puncta in which the %($\Delta F_{\text{during stimulation}}/F_0$) during stimulations (2s for 40Hz, 10s for 200Hz) > 0, where $\Delta F_{\text{during stimulation}}$ is defined as the mean fluorescence of $F-F_0$ during stimulation. SypHy $F_0$ was defined as the mean fluorescence during 5 s prior to stimulation. SypHy $\Delta F$ was quantified as fluorescence intensity $F$ at each time point minus $F_0$. Peak fluorescence ($\Delta F_{\text{peak}}$) is defined as the average $\Delta F$ during the first four imaging frames immediately following the end of the stimulation. Data were normalized to the total pool as defined by SypHy response to NH$_4$Cl application ($\Delta F_{\text{NH}_4}$). All experiments and analyses were performed by an experimenter blind to the genotype.
References


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