Heterodimerization of UNC-13/RIM regulates synaptic vesicle release probability but not priming

Haowen Liu^{1§}, Lei Li^{1§}, Daniel Nedelcu^{2,3¶}, Qi Hall^{2,3¶}, Lijun Zhou⁴, Wei Wang¹, Yi Yu¹,
Joshua M. Kaplan^{2,3,5*}, Zhitao Hu^{1*}

¹Queensland Brain Institute, Clem Jones Centre for Ageing Dementia Research (CJCADR), The University of Queensland, Brisbane, 4072, Australia

²Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA

³Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA

⁴Howard Hughes Medical Institute, Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA.

⁵Program in Neuroscience, Harvard Medical School

[§]These authors contributed equally to this work

[¶]These authors contributed equally to this work

*Correspondence to: kaplan@molbio.mgh.harvard.edu
z.hu1@uq.edu.au

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Abstract

UNC-13 proteins play an essential role in synaptic transmission by recruiting synaptic vesicles (SVs) to become available for release, which is termed SV priming. Here we show that the C2A domain of UNC-13L, like the corresponding domain in mammalian Munc13-1, displays two conserved binding modes: forming C2A/C2A homodimers, or forming a heterodimer with the zinc finger domain of UNC-10/RIM (C2A/RIM). Functional analysis revealed that UNC-13L's C2A promotes synaptic transmission by regulating a post-priming process. Stimulus-evoked release but not SV priming, was impaired in *unc-10* mutants deficient for C2A/RIM heterodimerization, leading to decreased release probability. Disrupting C2A/C2A homodimerization in UNC-13L-rescued animals had no effect on synaptic transmission, but fully restored the evoked release and the release probability of *unc-10*/RIM mutants deficient for C2A/RIM heterodimerization. Thus, our results support the model that RIM binding C2A releases UNC-13L from an autoinhibitory

homodimeric complex to become fusion-competent by functioning as a switch only.

Introduction

Neurotransmitters are released by Ca^{2+} -triggered synaptic vesicle (SV) exocytosis, a process that is mediated by the SNARE complex, and regulated by synaptic proteins such as Munc18s, Munc13s, RIMs, and synaptotagmins (Sudhof and Rizo, 2011; Jahn and Fasshauer, 2012). Prior to their final fusion with the plasma membrane, SVs undergo two crucial steps, docking and priming. These processes ensure that the SVs are translocated to the plasma membrane and become fusion-competent at the active zone (Sudhof, 2004). Defects in docking or priming produce corresponding decreases in SV exocytosis (Voets et al., 2001; Hammarlund et al., 2007; Han et al., 2011a; Kaeser et al., 2011; Lai et al., 2017). However, post-priming regulation of SV release have also been reported (Pang et al., 2006; Bacaj et al., 2015; Luo et al., 2015). Various studies have demonstrated that Munc13-1, a protein with multiple functional domains, is required for the docking and priming (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999), as well as post-priming Ca^{2+} -triggered SV release (Chen et al., 2013; Hu et al., 2013). The functions of Munc13-1 in these processes are believed to be mediated by its central MUN domain which directly interacts with syntaxin and promotes SNARE assembly (Ma et al., 2011; Yang et al., 2015).

Multiple domains of Munc13-1 have been implicated in regulating MUN domain's SV priming activity (Michelassi et al., 2017; Xu et al., 2017). In mammals, four Munc13 isoforms have been identified, two of which contain an N-terminal C2A domain (Munc13-1 and ubMunc13-2) (Rosenmund et al., 2002; Chen et al., 2013). Betz et al. first found that the C2A domain binds to the zinc finger (ZF) domain or

RIM, an active zone protein that is required for SV priming (Betz et al., 2001). A double mutation in mouse RIM's ZF domain (K144/146E) disrupts C2A/RIM heterodimerization (Dulubova et al., 2005) and was subsequently shown to produce a large reduction in the readily releasable pool (RRP) of SVs following (Deng et al., 2011; Camacho et al., 2017), indicating that C2A binding to RIM plays an important role in SV priming. Apart from forming a heterodimer with RIM, the C2A domain also forms a homodimer, thereby promoting formation of MUNC13-1 homodimers. This homodimerization, however, is not necessary for SV priming and post-priming SV release (Camacho et al., 2017).

In contrast, studies in chromaffin cells have shown that a truncated Munc13-1 lacking the N-terminal C2A domain is fully able to mediate vesicle priming (Betz et al., 2001), suggesting that the role of Munc13-1 in priming is independent of the C2A domain and its binding with RIM. Similarly, a recent study in *C. elegans* found that removing the N-terminal C2A domain from UNC-13L, a Munc13-1 homolog, does not cause a change in the size of the RRP (Zhou et al., 2013a). These conflicting findings have called into doubt whether binding of the C2A domain with RIM is indeed playing a role in SV priming, prompting us to re-analyze the function of both C2A/RIM heterodimerization and C2A/C2A homodimerization using an *in vivo* system.

Here, we examined the functional importance of the C2A domain by generating a transgenic line of UNC-13L with a point mutation in the C2A domain (K49E) that blocks the formation of C2A/C2A homodimers, and a mutant carrying two mutations in the *unc-10*/RIM ZF domain (K77/79E) that disrupts C2A/RIM heterodimer formation. We found that tonic neurotransmitter release at a low Ca^{2+} concentration and stimulus-evoked release were significantly reduced in the *unc-*

10(nu487 K77/79E) mutants, whereas SV release was normal in the UNC-13L(K49E)-rescued *unc-13* mutants. Moreover, the hypertonic sucrose-evoked responses were unaltered following disruption of homodimerization and heterodimerization, suggesting that SV priming is not determined by the state of the C2A domain. Interestingly, simultaneously disrupting C2A/C2A homodimerization in the *unc-10(nu487 K77/79E)* mutant fully restored both tonic and evoked release. These results suggest that the synaptic transmission defect in the *unc-10(nu487 K77/79E)* mutant is most likely caused by formation of more C2A/C2A homodimers, and that the role of UNC-10/RIM binding UNC-13 C2A domain is to release the C2A from an autoinhibitory state, allowing it to become a fusion-competent monomeric form. Finally, we show that UNC-13's C2A domain increases release probability but has little effect on SV priming.

Results

C2A/C2A homodimerization and C2A/RIM heterodimerization are conserved in *C. elegans*

The UNC-13L C2A domain and the UNC-10/RIM ZF domain share high sequence identity with the corresponding domains of mouse Munc13-1 (50%) and RIM (48%) (Figure 1A, B), suggesting that UNC-13's C2A would form homo- and hetero-dimers in a manner similar to mouse Munc13-1. To test this idea, we directly assessed homo- and heterodimer formation by the UNC-13 C2A domain. Gel filtration analysis indicates that recombinant C2A forms a homodimer, and that this homodimerization was disrupted by a mutation (K49E) analogous to that which disrupts Munc13-1 homodimerization (Figure 1A, C) (Deng et al., 2011). To determine if UNC-13L forms a heterodimer with UNC-10/RIM, we performed isothermal titration

calorimetry (ITC) and co-immunoprecipitation experiments. Our results showed that the UNC-13L C2A domain tightly binds the UNC-10/RIM ZF domain ($K_d=1.29\mu\text{M}$, Figures 1D, E), and that this interaction was eliminated by a double mutation in the ZF domain (K77/79E), which is analogous to mutations that disrupt binding of the corresponding mammalian Munc13-1 and RIM domains (Figure 1D-F) (Dulubova et al., 2005). Thus, our findings confirmed that UNC-13L C2A has the same two binding modes exhibited by mouse Munc13-1.

Disrupting C2A/RIM heterodimerization reduces mEPSCs/mIPSCs in low Ca^{2+}

We next examined the functional roles of the C2A/C2A homodimerization and C2A/RIM heterodimerization in tonic neurotransmitter release by measuring miniature excitatory/inhibitory postsynaptic currents (mEPSCs/mIPSCs) at the neuromuscular junction. Both the mEPSCs and the mIPSCs were recorded in the presence of 0mM and 1mM Ca^{2+} , as we have previously found that the mIPSCs are nearly arrested in 0mM Ca^{2+} but are normal 1mM Ca^{2+} in some mutants (Liu et al., 2018), suggesting that different Ca^{2+} concentrations should be tested to provide an accurate and thorough evaluation of tonic release. Our results showed that the mEPSCs and mIPSCs were completely abolished in the *unc-13(s69)* null mutant, and were fully restored by neuronal expression of full-length UNC-13L (Figure 2).

Deleting the C2A domain (UNC-13L Δ C2A) significantly decreased mEPSC frequency in both 0mM and 1mM Ca^{2+} (Figure 2B, E, Table 1), demonstrating an important role of the C2A domain in tonic acetylcholine (ACh) release. The mIPSC frequency was significantly decreased in the UNC-13L Δ C2A rescue animals in 0mM Ca^{2+} (Figure 2C, Table 1), but was unchanged in 1mM Ca^{2+} , compared to that in the UNC-13L rescue animals (Figure 2F). Thus, the UNC-13L C2A domain is important

for tonic release of both ACh and GABA; however, the requirement for C2A in GABA release can be bypassed by the presence of extracellular calcium, presumably due to increased release probability. The differential impact of deleting C2A on ACh and GABA release in 1mM Ca^{2+} could reflect differences in release probability at these two types of synapses (Liu et al., 2018). In fact, SV release from inhibitory synapses displays a higher release probability than excitatory synapses in the mammalian central nervous system (Zhou et al., 2013b; Zhou et al., 2013c).

We next asked whether C2A/C2A homodimerization or C2A/RIM heterodimerization accounts for the role of the C2A domain in tonic release. To investigate this, we first examined the mEPSCs and mIPSCs in the *unc-13* mutants rescued by UNC-13L carrying the K49E mutation, which blocks C2A/C2A homodimerization (Figure 1B). The mEPSC and mIPSC frequencies in the UNC-13L(K49E) rescue animals were comparable to those in the UNC-13L rescue animals in both 0mM and 1mM Ca^{2+} conditions (Figure 2A-F, Table 1), indicating that homodimerization does not affect tonic ACh or GABA release. To determine the role of C2A/RIM heterodimerization, we used crispr to introduce a K77/79E double mutation in the UNC-10 ZF domain, *unc-10(nu487 K77/79E)*. Compared to wild-type animals, *unc-10(nu487 K77/79E)* mutants display a slightly uncoordinated behaviour (data not shown). Analysis of tonic release showed that the mEPSCs and mIPSCs were markedly reduced in *unc-10(nu487 K77/79E)* mutants in 0mM Ca^{2+} , whereas both were unaltered in 1mM Ca^{2+} (Figure 2A-F), suggesting that C2A/RIM heterodimerization is only required for tonic release in low Ca^{2+} when release probability is reduced. To confirm this, we measured mEPSCs and mIPSCs in two additional Ca^{2+} concentrations (0.05mM and 0.1mM). As shown in Figure 2G, increasing the Ca^{2+} concentration from 0mM to 0.05mM and 0.1mM caused a robust

enhancement of the mEPSC and mIPSC frequencies in wild-type animals. In *unc-10(nu487 K77/79E)* mutants, a significantly lower mIPSC frequency was observed in 0.05mM Ca^{2+} but not in 0.1mM Ca^{2+} , whereas mEPSCs were unaltered in both 0.05mM and 0.1mM Ca^{2+} (Figure 2G). Our results therefore demonstrate that C2A/RIM heterodimerization promotes tonic release but that this effect is bypassed when release probability is increased by raising external Ca^{2+} concentrations.

A monomeric C2A domain is fully functional for tonic release

The decreased mEPSC frequency observed in the UNC-13L Δ C2A rescue animals in 1mM Ca^{2+} indicates that the C2A domain may have an additional function in regulating tonic release, particularly in high Ca^{2+} . We therefore examined whether simultaneously blocking the homo- and heterodimerization of the C2A domain could account for this additional function. In 0mM Ca^{2+} recordings, UNC-13L(K49E); *unc-10(nu487 K77/79E)* double mutant mIPSC rate was significantly higher than that observed in *unc-10(nu487 K77/79E)* single mutants, suggesting that blocking homo-dimer formation was sufficient to prevent the decreased tonic GABA release caused by the *unc-10(nu487 K77/79E)* mutation (Figure 2C). By contrast, blocking homo-dimerization did not significantly improve mEPSC rates in 0mM Ca^{2+} in *unc-10(nu487 K77/79E)* mutants (Figure 2B). Thus, hetero-dimerization increases tonic GABA release by preventing homo-dimer formation whereas hetero-dimerization promotes tonic ACh release by a distinct mechanism. In addition, the tonic ACh and GABA release were in a wild-type level in the UNC-13L(K49E); *unc-10(nu487 K77/79E)* double mutant in 1mM Ca^{2+} , similar to those in the UNC-13L(K49E) rescue and the *unc-10(nu487 K77/79E)* mutant (Figure 2E, F). Taken together, these results demonstrate that blocking C2A homo- and hetero-dimerization does not

decrease tonic ACh and GABA release. These results differ from those obtained in the UNC-13LΔC2A rescue animals, suggesting that the effect of deleting the C2A domain on the tonic release is not due to a lack of homo- and hetero-dimerization.

Stimulus-evoked release is reduced when C2A/RIM hetero-dimerization is blocked

Next we analysed the impact of C2A interactions on stimulus-evoked ACh release. Compared to UNC-13L, UNC-13LΔC2A exhibited a smaller evoked EPSC (Figure 3A, Table 1), consistent with previous findings (Hu et al., 2013; Zhou et al., 2013a). The evoked EPSCs were significantly smaller in *unc-10(nu487 K77/79E)* mutants, exhibiting decreased amplitude and total charge transfer compared to wild-type animals (Figure 3A-C). The percentage decrease of the evoked charge transfer in the *unc-10(nu487 K77/79E)* mutant (44%) was similar to that observed in the UNC-13LΔC2A rescue animals (46%), suggesting that C2A/RIM heterodimerization accounts for the role of the C2A domain in evoked release. Thus, our results demonstrate that C2A/RIM heterodimerization plays an essential role in evoked neurotransmitter release.

Next we asked if C2A homo-dimers regulate evoked responses. The evoked EPSCs mediated by UNC-13L(K49E) were indistinguishable from those rescued by wild type UNC-13L, indicating C2A homo-dimers are not required for evoked responses (Figure 3A-C). To determine if homo-dimers inhibit evoked ACh release, we analyzed UNC-13L(K49E); *unc-10(nu487 K77/79E)* double mutants. We found that preventing C2A/C2A homodimerization in the *unc-10(nu487 K77/79E)* mutant fully restored the evoked EPSC to a wild-type level (Figure 3A-C). These results suggest that the reduced evoked EPSC caused by blocking C2A/RIM hetero-

dimerization is mediated by increased formation of C2A homo-dimers. These results are consistent with prior analysis of mouse synapses, which suggest that Munc13-1 homo-dimers inhibit SV release (Deng et al., 2011). These results also demonstrate that the monomeric C2A domain is sufficient to support evoked release, consistent with the observation of tonic release (Figure 2, Table 1). Thus, the RIM ZF domain appears to function only as a switch which releases the C2A domain from the inhibitory homomeric complex.

The C2A domain is not required for SV priming

A decrease in evoked EPSCs may arise from a priming defect or a change in release probability. We therefore examined whether SV priming is regulated by the C2A domain and its two different states (homodimer and heterodimer). SV priming was measured by the pulsed application of hypertonic sucrose solution (1M, 2s) onto the ventral nerve cord, and the size of the RRP was calculated by integrating the sucrose-evoked current. The current was nearly eliminated in the *unc-13* null mutants, and was fully restored by expressing a wild type UNC-13L transgene in all neurons (Figure 3E). Strikingly, UNC-13L lacking the C2A domain could still restore the sucrose-evoked current to a wild-type level (Figure 3E, F, Table 1), indicating that the C2A domain is not required for SV priming. These results differ from those reported in mouse hippocampal neurons, in which the RRP was largely reduced when the Munc13-1 C2A domain was removed (Camacho et al., 2017). Despite these differences, our results, together with those obtained in chromaffin cells in which Munc13-1 lacking the C2A domain enhances secretion as high a level as Munc13-1 (Betz et al., 2001), support the notion that truncated UNC-13 lacking the C2A domain is still able to prime SVs.

The fact that the C2A deletion had no effect on SV priming suggests that C2A/C2A homodimerization and C2A/RIM heterodimerization are also not required for priming. Nevertheless, we assessed the RRP size in the UNC-13L(K49E) rescue animals and the *unc-10*(K77/79E) mutant. Our data showed that the sucrose-evoked currents in these animals were comparable to those observed in UNC-13L rescue and wild-type animals (Figure 3E, F), demonstrating that homodimerization or heterodimerization is not playing a role in SV priming. Consequently, the probability of synaptic vesicle release (P_{vr}) calculated by the ratio of the charge transfer of the evoked EPSCs to the charge transfer of the sucrose-evoked currents was significantly reduced in the Δ C2A rescue and the *unc-10*(K77/79E) mutant (Figure 3G). Together, our results demonstrate that the UNC-13L C2A domain regulates release probability by interacting with the UNC-10/RIM, rather than by regulating SV priming.

Disrupting heterodimerization does not affect synaptic depression and recovery

Due to the reduced P_{vr} in the *unc-10*(K77/79E) mutant, we asked whether the synaptic depression and recovery would be altered by the disruption of C2A/RIM heterodimerization. An integrated line expressing channelrhodopsin-2 (ChR2) in the cholinergic motor neurons (*zxIs6*) was used to record the EPSCs evoked by a train or a paired light stimulus (Liu et al., 2009). Both the control animals and the *unc-10*(K77/79E) mutants exhibited synaptic depression following a 1Hz train stimulus (Figure 4A). The depression rate was described by normalizing the amplitudes of all the EPSCs ($EPSC_i$) to the amplitude of the first EPSC ($EPSC_1$). The *unc-10*(K77/79E) mutants displayed a similar depression rate to that of the control animals (Figure 4B), indicating that RIM binding to the C2A domain is not required for synaptic depression. To assess synaptic recovery, we applied a paired stimulus by changing the

intervals between the two stimuli (500ms, 1s, 5s, 15s) (Figure 4C). The recovery rate was quantified by the ratio of the second EPSC amplitude (EPSC₂) to the first EPSC amplitude (EPSC₁). As shown in Figure 4D, no difference in the recovery rate was observed between the control animals and the *unc-10(K77/79E)* mutants. This indicates that disrupting C2A/RIM binding does not affect the refilling of the SVs at synapses.

Discussion

In this study, we analyzed the functional importance of the C2A domain of UNC-13L, a Munc13-1 homolog in *C. elegans*, for SV priming and release probability. Prior studies in mouse and *C. elegans* have reported contradictory results regarding the role of the C2A domain in priming (Betz et al., 2001; Zhou et al., 2013a; Camacho et al., 2017). This motivated us to investigate the function of this domain, with a particular focus on its two bindings modes, in SV priming and post-priming Ca²⁺-triggered exocytosis. Below we discuss our findings.

UNC-13L primes SVs independently of the C2A domain

The fact that the UNC-13/Munc13 is required for SV priming has been reported in both invertebrates and vertebrates (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999). Sucrose-evoked responses are nearly eliminated in neurons lacking UNC-13 or Munc13. Thus far, however, it is still unclear how the priming activity of UNC-13/Munc13 is achieved and regulated. It is believed that the MUN domain plays an essential role in SV priming. Removing the C2A domain from Munc13-1 leads to a marked decrease in the RRP of SVs in cultured hippocampal neurons, suggesting a role for C2A in priming (Camacho et al., 2017). However, we

found that *unc-13* mutants lacking a C2A domain (UNC-13L Δ C2A rescued animals) exhibit a normal RRP, arguing that the C2A domain is not required for priming (Figure 3E). Zhou et al. showed that an isolated *unc-13* mutant (*n2609*) with a point mutation in the C2A domain of UNC-13L exhibits a similar reduction in evoked EPSCs to UNC-13L Δ C2A, whereas docked SV numbers are normal in the *unc-13(n2609)* mutant. This suggests that the C2A domain is not required for docking (Zhou et al., 2013a), consistent with our observations in relation to priming.

Our results imply that UNC-13L regulates SV priming via a different mechanism, and that this mechanism is independent of the C2A domain. Prior studies have shown that a point mutation in the C1 domain of Munc13-1 accelerates hypertonicity-induced SV release without changing the size of the RRP (Basu et al., 2007). Transgenic *C. elegans* rescued by a truncated UNC-13L lacking its C1 or C2B domain also exhibit a normal RRP (Michelassi et al., 2017). Expression of a mutated Munc13-1 (P827L, located in the linker domain between C2B and MUN) leads to a significant increase in release probability without causing an increase in the size of the RPP (Lipstein et al., 2017). These observations appear to support the notion that the regulatory domains in UNC-13L (e.g. C2A, C1 and C2B) alter release probability but are not required for SV priming. Thus, our results, together with the findings from other studies in *C. elegans* and mouse, suggest that UNC-13 has multiple regulatory effects on synaptic strength and plasticity, and which are achieved by differential control of release probability by its various functional domains.

State of the C2A domain and release probability

The reduced evoked EPSCs and unchanged RRP in the UNC-13L Δ C2A rescue animals led to a significant decrease in release probability (P_{vr}). Similar to

these observations, the *unc-10*(K77/79E) mutant also exhibits an impairment in evoked EPSCs but a normal RRP, thereby leading to a decreased P_{vr} (Figure 3). These results demonstrate that deleting the C2A domain or blocking C2A/RIM heterodimerization decreases release probability. It should be noted that, release probability remains unchanged in the hippocampal neurons when C2A/RIM heterodimerization is disrupted, due to a similar decrease in the evoked EPSCs and the RRP (Camacho et al., 2017), different from our findings in this study.

The function of the C2A domain in regulating the release probability appears to be determined by its state. It has been proposed that the Munc13 C2A domain has three binding states, homodimer, heterodimer, and monomer, and that these three forms may co-exist in synaptic terminals. The decrease in evoked EPSC and release probability in the *unc-10*(*nu487* K77/79E) mutant was blocked by disrupting C2A/C2A homodimerization (Figure 3). This indicates that the synaptic transmission defect caused by disrupting C2A/RIM hetero-dimerization occurs because of an increase in C2A/C2A homo-dimerization, which is proposed to comprise an autoinhibitory state. Again, this is post-priming regulation, as SV priming is normal in both the UNC-13L(K49E) rescue and UNC-13L(K49E); *unc-10*(*nu487* K77/79E) animals (Figure 3E). Although our findings are similar to the observations reported in cultured hippocampal neurons in which the reduced evoked EPSCs caused by the disruption of heterodimerization were partially recovered when homodimerization and heterodimerization were disrupted simultaneously (Camacho et al., 2017), we found that UNC-13L with a monomeric C2A domain is fully functional for both the priming and post-priming Ca^{2+} -triggered tonic and evoked release. Thus, our results support the notion that the homodimerization of the C2A domain inhibits SV release, and that RIM releases the C2A domain from the autoinhibitory homodimeric complex

by competitively binding the C2A domain to form a heterodimer, thereby allowing the C2A domain to become fusion-competent. The heterodimer itself, however, does not produce an additional effect on synaptic transmission.

Distinct priming mechanisms between worm and mouse?

RIM binding to Munc13-1 is a key determinant in SV priming in the mouse central nervous system. In fact, deleting Munc13-1 C2A or disrupting C2A/RIM heterodimerization leads to a similar reduction in the size of the RRP with that observed in RIM knockout neurons (Deng et al., 2011; Camacho et al., 2017). Whereas SV priming in worm does not rely on the UNC-13 C2A and the heterodimerization of UNC-13/RIM, although this interaction is highly conserved between worm and mouse. This raises a question of whether the UNC-10/RIM is required for priming in worm. Electron microscopy results have revealed that the number of docked SVs is normal in the *unc-10* mutant (Koushika et al., 2001; Weimer et al., 2006; Gracheva et al., 2008), demonstrating a post-docking role of RIM in the active zone. Consistent with these relatively minor docking defects, we found that the size of the RRP in *unc-10* mutants (assessed by sucrose evoked currents) is comparable to that in the wild-type animals (data not shown), demonstrating that UNC-10/RIM is not required for SV priming. These results suggest that UNC-10/RIM primarily regulates SV fusion by increasing release probability and not by promoting SV priming. UNC-10/RIM increases release probability via its ability to bind UNC-13 but also through its requirement for recruiting CaV2 calcium channels to pre-synaptic terminals (Han et al., 2011b; Kaeser et al., 2011; Muller et al., 2012).

Why does RIM play different roles at worm and mouse synapses? SV priming is thought to consist of two sequential steps, termed molecular and positional priming (Neher and Sakaba, 2008). Molecular priming is proposed to account for a slow component of release, which consists of fusion of SVs that are docked far from calcium channels driving release. Positional priming is proposed to mediate a faster form of release, which comprises fusion of SVs that are docked very close to calcium channels. One possibility is that worms rely less on positional priming, which presumably requires UNC-13/RIM interaction, and instead rely more on molecular priming. In this scenario, worm *unc-10* RIM mutants would have decreased release probability but relatively normal RRP size. By contrast, mice may require positional priming to maintain the RRP, and thus RIM mutants would have significant priming defects.

Additional functions of the C2A domain

Despite the above findings, the actual mechanism whereby the UNC-13L with a monomeric C2A regulates SV release, however, remains unclear. Based on the normal synaptic transmission in the UNC-13L(K49E); *unc-10(nu487 K77/79E)* double mutants, RIM binding to the UNC-13L C2A domain only appears to be playing a switch role. The C2A domain must therefore have additional functions for the regulation of SV release. This is confirmed by our observations in relation to tonic release, which has been proposed to have a different regulatory mechanism from evoked release (Ramirez and Kavalali, 2011; Melom et al., 2013). The mEPSC frequency was reduced by 50% in the UNC-13L Δ C2A rescue animals at both 0mM and 1mM Ca²⁺, similar to the evoked release findings (Figure 2). These results demonstrate that the C2A domain is essential for tonic release at cholinergic synapses.

However, this role cannot be explained by the state of the C2A domain (homodimer, heterodimer, or monomer), as the mEPSC frequencies were normal in the UNC-13L(K49E) rescue, *unc-10(nu487 K77/79E)* mutant, and UNC-13L(K49E); *unc-10(nu487 K77/79E)* double mutants in 1mM Ca²⁺. We only observed a change in the Ca²⁺ sensitivity in very low Ca²⁺ at GABAergic synapses, and this phenotype disappeared when the Ca²⁺ levels were increased (Figure 2G). Our observations at cholinergic synapses therefore demonstrate that, although the C2A/C2A homodimer inhibits evoked release, it does not affect tonic release. There must still be an unknown mechanism by which the monomeric C2A domain regulates post-priming SV exocytosis.

Materials and Methods

Strains

Strain maintenance and genetic manipulation were performed as previously described (Brenner, 1974). Animals were cultivated at room temperature on nematode growth medium (NGM) agar plates seeded with OP50 bacteria. On the day before experiments L4 larval stage animals were transferred to fresh plates seeded with OP50 bacteria for all the electrophysiological recordings. The following strains were used:

Wild type, N2 bristol

KP6901 *unc-13(s69)* I

KP7503 *nu487 unc-10(nu487 K77/79E)*, X

KP6893 *nuEx1515 [Psnb-1::UNC-13L];unc-13(s69)*

ZTH6 *unc-13(s69);hztEx106 [Psnb-1::UNC-13LΔC2A]*

ZTH454 *unc-13(s69);hztEx91 [Psnb-1::UNC-13L(K49E)]*

ZTH7 *unc-10(nu487 K77/79E);hztEx91 [Psnb-1::UNC-13L(K49E); unc-13(s69)]*

ZX460 *zxIs6 [Punc-17::ChR2(H134R)::YFP + lin-15(+)]* V

ZTH71 *unc-10(nu487 K77/79E);zxIs6*

Constructs, transgenes and germline transformation

A 5.451 kb cDNA corresponding to UNC-13L was amplified by PCR from the N2 cDNA library and inserted into MCSII of the JB6 vector between the KpnI and NotI sites. The *snb-1* promoter (3kb) was inserted into MCSI between the SphI and BamHI sites. The C2A domain deletion removed the first 150 amino acids. The 5' primer (GCTGCAGACTGTAGAATCAACAACTGT) and 3' primer (ACAGTTGTTGATTCTACAGTCTGCAGC) were used to create the *Psnb-1::UNC-*

13L(K49E) construct.

Transgenic strains were isolated by microinjection of various plasmids using either Pmyo-2::NLS-GFP (KP#1106) or Pmyo-2::NLS-mCherry (KP#1480) as the co-injection marker. Integrated transgenes were obtained by UV irradiation of strains carrying extrachromosomal arrays. All integrated transgenes were outcrossed at least seven times.

Electrophysiology

Electrophysiology was conducted on dissected *C. elegans* as previously described (Hu et al., 2012). Worms were superfused in an extracellular solution containing 127 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 20 mM glucose, 1 mM CaCl₂, and 4 mM MgCl₂, bubbled with 5% CO₂, 95% O₂ at 22°C. The 1mM CaCl₂ was replaced by 1mM MgCl₂ to record mEPSCs and mIPSCs in 0mM of Ca²⁺.

Whole-cell recordings were carried out at -60mV for all EPSCs, including mEPSCs, evoked EPSCs, and sucrose-evoked responses. The holding potential was switched to 0mV to record mIPSCs. The internal solution contained 105 mM CH₃O₃SCs, 10 mM CsCl, 15 mM CsF, 4mM MgCl₂, 5mM EGTA, 0.25mM CaCl₂, 10mM HEPES, and 4mM Na₂ATP, adjusted to pH 7.2 using CsOH.

Isothermal titration calorimetry (ITC)

ITC experiments were performed using a Nano ITC (TA Instruments). ITC titrations were performed in degassed 20 mM HEPES pH 7.5, 150 mM NaCl at 25°C. After baseline subtraction, the area under each peak was integrated and data were fit to a one site Independent binding model using the NanoAnalyze software (TA

Instruments) to determine the binding affinity (K), enthalpy (dH) and stoichiometry (n); binding was not observed for *unc-10* 62-121 K77/79E.

Protein expression and purification (for ITC experiments): *C. elegans* UNC-13 long isoform residues 1-258 bearing the K49E mutation (which abolishes homodimer formation), *C. elegans* UNC-10 residues 62-121 WT or *C. elegans* UNC-10 K77/79E were expressed from pGEX6p1 as a GST fusion in Rosetta2 (DE3) cells. Cells were grown at 37°C in 6L 2xyt with Carbenicillin (100µg/mL) Chloramphenicol (35µg/mL) and ZnSO₄ (200µM, Unc-10 constructs only), to O.D.₆₀₀ ~0.5, then allowed to cool down to 18°C for an hour before induction with 1mM IPTG overnight at 18°C. The cells were harvested the next day and lysed in 20mM HEPES (pH 7.5), 150mM NaCl, 1mM PMSF, 1% Triton X-100, 1mM DTT, 200µM ZnSO₄ (UNC-10 constructs only); the cells were sonicated 3x40sec using a midi tip, and lysates clarified 45mins @ 40,000rpm, 4°C in a Sorvall Type 45 Ti rotor. Clarified lysates were bound to 1.5mL (bed volume) glutathione sepharose beads tumbling overnight at 4°C. The next day, the beads were washed extensively, and HRV3C protease added for on-beads cleavage overnight at 4°C. The eluted soluble constructs were concentrated and subjected to gel filtration on a Superdex 75 10/300 against 20mM HEPES pH 7.5 150mM NaCl at 4°C. Single peaks corresponding to the monomeric proteins were concentrated to 21µM (UNC-13L residues 1-258), to 140µM (UNC-10 62-121 WT) and to 116µM (UNC-10 62-121 K77/79E mutant) and used in the ITC experiment.

Data acquisition and statistical analysis

All recordings were obtained using a HEKA EPC10 double amplifier (HEKA Elektronik) filtered at 2 kHz, and analyzed by built-in programs in Igor 7 software

(WaveMetrics). Each set of data represents the mean $\pm$ SEM of an indicated number (n) of animals. Statistical significance was determined using student's-t test or one-way ANOVA followed by Dunnett's test to control for multiple comparisons.

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Figure Legends

Figure 1. UNC-13L exhibits conserved homodimerization and heterodimerization in *C. elegans*.

(A, B) Sequence alignment of the C2A domain between worm UNC-13L and rat Munc13-1, and the zinc finger (ZF) domain between worm UNC-10 and mouse RIM1. The conserved residues in the C2A domain (K49) and the ZF domain (K77, K79) that disrupt homodimer and heterodimer formation are indicated by stars. (C) Separation of purified UNC-13L C2A monomer and homodimer by gel filtration. The wild type C2A forms a homodimer that runs at 12.57mL (blue trace, corresponding to an apparent weight of 89kDa); this homodimer is disrupted in the monomeric UNC-13L(K49E) mutant which runs at 13.88mL (apparent weight 52kDa). Elution volumes of gel filtration standards are indicated underneath the plot, as follows: 11.62mL for alcohol dehydrogenase (150 kDa), 12.85mL for bovine serum albumin (66 kDa), and 15.45mL for carbonic anhydrase (29 kDa), with the column void volume being 10.78mL. (D) Co-immunoprecipitation (co-IP) shows that HA-LNTD (aa 1-605) binds wild type FLAG-UNC-10 (aa 1-601) but not UNC-10(K77/79E) or GST. These results are representative of three independent experiments. (E, F) Isothermal titration calorimetry (ITC) analysis of binding of wild-type or mutated (K77/79E) UNC-10 with UNC-13L.

Figure 2. Tonic release is reduced by disrupting C2A/RIM heterodimerization in low Ca^{2+} . (A) Representative traces of mEPSCs and mIPSCs recorded at 0mM Ca^{2+} from indicated genotypes. (B, C) Averaged frequency and amplitude of the mEPSCs and mIPSCs. (D) Representative traces of mEPSCs and mIPSCs recorded at 1mM Ca^{2+} from the same genotypes in A. (E, F) Quantification of the mEPSC and mIPSC

frequency and amplitude from the genotypes in D. Data are mean $\pm$ SEM (##, $p < 0.01$, ###, $p < 0.001$, n.s., non-significant when compared to UNC-13L rescue; ***, $p < 0.001$ when compared to wild type; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar graphs. (G) Quantification of the mEPSC and mIPSC frequencies at various Ca^{2+} levels (0, 0.05, 0.1, and 1mM) from wild type (black) and *unc-10(nu487 K77/79E)* mutants (red). Data are mean $\pm$ SEM (***, $p < 0.001$, n.s., non-significant when compared to wild type; student's t-test).

Figure 3. Effects of homodimerization and heterodimerization on evoked neurotransmitter release. Electronic stimulus and hypertonic sucrose-evoked EPSCs were recorded from the body wall muscle of adult animals in 1mM Ca^{2+} . (A) Example traces of the evoked EPSCs from indicated genotypes. (B-D) Quantification of the amplitude, charge transfer and 20-80% risetime of the evoked EPSCs in A. Example traces and averaged charge transfer of the sucrose-evoked EPSCs from the same genotypes in A are shown in (E) and (F). (G) Quantification of the probability of SV release (P_{vr}). Data are mean $\pm$ SEM (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ when compared to wild type; #, $p < 0.05$, n.s., non-significant when compared to UNC-13L rescue; one-way ANOVA).

Figure 4. Synaptic depression and recovery are unaltered by disrupting C2A/RIM heterodimerization. Synaptic depression and recovery were investigated by applying a train (1Hz) or a paired light stimulus onto the ventral nerve cord of the adult worms with expression of ChR2 in cholinergic motor neurons. (A) Example traces of 1Hz light train stimulus-evoked EPSCs (black, wild type; red, *unc-10(K77/79E)* mutants). (B) Quantification of synaptic depression by normalizing the

EPSC amplitude (EPSC_i) to the first EPSC amplitude (EPSC₁) (n=7 for wild type, and n=7 for *unc-10(nu487 K77/79E)* mutant). (C) Evoked EPSCs triggered by a paired light stimulus with various intervals ranging from 0.5s to 5s. (D) Synaptic recovery calculated by the ratio of EPSC₂ to EPSC₁ (wild type, 500ms n=6, 1s n=6, 5s n=6, 15s, n=6; *unc-10(nu487 K77/79E)* mutant, 500ms n=7, 1s n=7, 5s n=7, 15s, n=6). Data are mean ± SEM (n.s., non-significant when compared to wild type; student's t-test).

[illegible]

Figure 2

Figure 3

Figure 4

Table 1. Quantification of tonic release, evoked release, RRP, and release probability

	mEPSC frequency (Hz)		mIPSC frequency (Hz)		Amplitude (-nA)	Evoked EPSC		Sucrose Charge (-pC)	P_{vr}
	0 Ca^{2+}	1 Ca^{2+}	0 Ca^{2+}	1 Ca^{2+}		Charge (-pC)	Risetime (ms)		
Wild type	1.87 ± 0.16	43.5 ± 4.3	10.9 ± 1.1	42.1 ± 4.1	2.2 ± 0.2	20.6 ± 2.1	2.51 ± 0.2	221 ± 23.1	0.12 ± 0.01
<i>unc-13 (s69)</i>	0.1 ± 0.01	0.5 ± 0.02	0.2 ± 0.01	0.4 ± 0.03	0.05 ± 0.01	0.1 ± 0.02	N/A	25.5 ± 7.1	0
UNC-13L	1.51 ± 0.45	43.4 ± 3.7	10.1 ± 0.9	38.1 ± 3.2	1.7 ± 0.2	15.4 ± 2.6	1.86 ± 0.2	237 ± 25.4	0.06 ± 0.01
UNC-13LΔC2A	0.64 ± 0.07	20.2 ± 3.1	5.4 ± 0.5	34.4 ± 3.3	1.1 ± 0.1	8.4 ± 1.6	2.1 ± 0.3	248 ± 66.1	0.034 ± 0.006
UNC-13L(K49E)	1.91 ± 0.48	43.3 ± 4.3	9.8 ± 1.2	40.3 ± 5.3	2.1 ± 0.3	17.3 ± 2.3	1.88 ± 0.2	243 ± 38.3	0.07 ± 0.01
<i>unc-10 (K77/79E)</i>	0.57 ± 0.11 (***)	45.4 ± 3.7	3.5 ± 0.5 (***)	39.2 ± 4.1	1.2 ± 0.1 (***)	11.5 ± 1.5 (***)	2.48 ± 0.2	240 ± 17.5	0.046 ± 0.006 (***)
K49E;K77/79E	0.94 ± 0.18	40.8 ± 4.4	11.1 ± 1.4 (###)	43.7 ± 6.3	1.81 ± 0.2 (##)	18.1 ± 2.2 (†)	2.47 ± 0.3	280 ± 40.5	0.07 ± 0.008 (†)

***, $p < 0.001$, compared to wild type; #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$, compared to *unc-10 (K77/79E)* mutant