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Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1

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A hallmark of neuronal communication is the rapid millisecond time scale for synaptic information transfer. The speed of synaptic transmission encompasses a sequence of molecular events, including Ca2+-influx into the presynaptic terminal, Ca2+-triggering of synaptic vesicle fusion, and diffusion and binding of neurotransmitters to postsynaptic receptors. The Ca2+-triggering step for synaptic vesicle fusion is regulated by the synaptic vesicle protein Synaptotagmin 1 (Syt 1). Synaptotagmins are a large family of single-pass transmembrane proteins found on diverse populations of intracellular vesicles, with the Syt 1 subfamily localized to synaptic vesicles (1–3). Synaptotagmins consist of a N-terminal transmembrane segment followed by a cytoplasmic domain with two Ca2+-binding C2 domains. Although C2A and C2B share similar topology, distinct effectors for each domain have been identified (4).

Genetic perturbation studies of Syt 1 have demonstrated an essential role in neurotransmitter release (5–8). Our previous analysis at *Drosophila* embryonic neuromuscular junctions (NMJs) indicated Syt 1 functions to synchronize rapid fusion of synaptic vesicles to action potentials while reducing a slower asynchronous component of fusion (9). Together with studies at mammalian synapses (10–13), current data support a two-Ca2+-sensor model for neurotransmitter release (14), with Syt 1 functioning as the fast synchronous Ca2+-sensor and a second unknown Ca2+-sensor mediating the slower asynchronous phase of release. The synchronizing function of synaptotagmin has also been observed at mammalian systems, with enhanced asynchronous release found at cultured autaptic synapses from Syt 1-KO mice (11, 15) and calyx of Held synapses from Syt 2-KO mice (13). Although the mechanism by which Syt 1 synchronizes release is still being elucidated, it may directly trigger fast vesicle fusion while inhibiting asynchronous release mediated by a second Ca2+-sensor (9). Syt 1 could also suppress asynchronous release by directly competing with the asynchronous Ca2+-sensor for the release machinery (11, 16). Whether Ca2+-binding to Syt 1 is required for these distinct roles in synchronous versus asynchronous release is unclear. To date, Ca2+-binding mutations in the Syt 1 C2A domain have displayed mild phenotypes, whereas C2B Ca2+-binding mutations show profound defects in release (9, 16–18). Here, we compare synchronous and asynchronous release in C2A and C2B Ca2+-binding mutants and find striking contrasts in their effects on neurotransmission. These results indicate that Ca2+-binding to the Syt 1 C2A domain is required for suppression of the asynchronous component of release, whereas Ca2+-binding to C2B triggers synchronous fusion.

Results

To determine the physiological consequences on synaptic vesicle fusion caused by disrupting Ca2+-binding to the C2A or C2B domain of Syt 1, we generated transgenic *Drosophila* expressing Syt 1 with mutations in essential Ca2+-binding residues. The C2 domains of Syt 1 form a compact β-sandwich with two Ca2+-binding loops at the apex that penetrate membrane bilayers containing negatively charged lipids. Five highly conserved acidic aspartate residues are ordered within the loops emerging from the C2 domain and directly participate in coordinating Ca2+-binding to Syt 1 (18, 19). For this analysis, Ca2+-binding aspartates were neutralized to asparagines at the key D3 and D4 position of each C2 domain of Syt 1, we generated transgenic *Drosophila* expressing Syt 1 with mutations in essential Ca2+-binding residues. The C2 domains of Syt 1 form a compact β-sandwich with two Ca2+-binding loops at the apex that penetrate membrane bilayers containing negatively charged lipids. Five highly conserved acidic aspartate residues are ordered within the loops emerging from the C2 domain and directly participate in coordinating Ca2+-binding to Syt 1 (18, 19). For this analysis, Ca2+-binding aspartates were neutralized to asparagines at the key D3 and D4 position of each C2 domain. D282N and D284N in C2A and D416N and D418N in C2B (Fig. 1A). These mutations disrupt Ca2+-binding to the C2A (20) or C2B (17) domains of Syt 1. We expressed the UAS transgenes using GAL 4 under the control of the promoter of the pan-neuronal gene, elav (21), in a Syt 1 KO/Syt 1N13–null background. Immunostaining with anti-Syt1 antisera (22) demonstrated that the transgenic WT Syt 1 protein, as well as the transgenic C2A and C2B mutant proteins, localized properly at NMJs (Fig. 1B) and expressed at similar levels (Fig. S1). To examine evoked release properties in Syt 1 rescued animals, we stimulated motor nerves in 4 mM extracellular Ca2+, in which the asynchronous component is obvious, and recorded

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synaptic currents using whole-cell voltage clamp from body wall muscle number 6 in Drosophila embryos (Fig. 2). As previously observed, Syt 1-null mutants eliminated the synchronous component of release. In contrast, a slower asynchronous release component was uncovered, and it displayed a 100-msec time constant when the latency histogram was fit by an exponential curve. Asynchronous release following an action potential was not confounded from spontaneous miniature synaptic currents, as their frequency is significantly less than 0.1 Hz at these synapses, which have only 20 to 30 active zones at this stage of development. Syt 1-null mutants rescued with a WT Syt 1 transgene showed indistinguishable evoked synaptic currents from WT animals with endogenous Syt 1 (9). Consequently, we used the WT rescued strain as a control for further experiments. Evoked release was fully synchronous, and few asynchronous release events were observed in rescued animals (Fig. 2). To compare the total amount of release observed at Syt 1-null mutant synapses with the control, we measured cumulative charge transfer following stimulation. Total charge, reflecting the number of released vesicles, was reduced by 97.5% in Syt 1-null mutants compared with WT rescues. This finding contrasts with results from mammalian Syt 1 mutant autapses, in which total charge transfer is similar to WT (11, 23). The data more closely matches studies from the calyx of Held synapse in Syt 2-KO mice (13) and from dissociated cultures from Syt 1-KO mice (23), in which total charge is reduced compared with controls. These differences may reflect distinct releasable pool sizes at the various synapses. In summary, we find that Syt 1-null synapses show asynchronous release with only 2.5% of the total amount of release compared with WT rescues.

To analyze the respective contributions of Ca\(^{2+}\) binding to C2A and C2B for Syt 1 function, we compared release properties in transgenic animals expressing a C2A (Syt 1\(D^{22R}N, D^{22S}N\)) or C2B (Syt 1\(D^{416}N, D^{418}N\)) mutant in the Syt 1-null background. Striking differences in the effects on the number and kinetics of synaptic vesicle fusion were observed in C2A versus C2B Ca\(^{2+}\) binding mutants. Synapses expressing the C2A domain mutation displayed similar levels of evoked release as WT rescues at 4 mM Ca\(^{2+}\). However, asynchronous release (arrowheads in Fig. 2A) was even more prominent than at Syt 1-null synapses (Fig. 2B and C). Total charge transfer at C2A mutant synapses was similar to WT rescues (Fig. 2D). In contrast, synapses expressing a Syt 1 transgenic protein with C2B domain mutations largely suppressed the asynchronous component of release (Fig. 2A–C), similar to synapses expressing WT Syt 1. In contrast, only a small amount of residual synchronous release was observed, and total charge transfer was dramatically reduced, similar to Syt 1-null synapses (Fig. 2B and D). To precisely analyze the timing of synaptic vesicle fusion in C2A and C2B mutants, we performed a detailed latency analyses (Fig. 3). The latency histogram revealed a large asynchronous release component in C2A mutant rescued animals, which is more enhanced than that observed in null mutants (Fig. 3B). We next assayed spontaneous release rates during the 0.5- to 1-s interval when asynchronous release has subsided. Similar to studies of Syt 1 function at mammalian
synapses (24), mutations in C2A or C2B Ca\(^{2+}\)-binding sites caused enhanced rates of spontaneous release compared with control or null mutants (Fig. 3C). The presence of robust asynchronous release in the C2A mutant rescue suggests an important role for Ca\(^{2+}\)–C2A interactions in suppressing this slower component of fusion. The rescue of the synchronous phase of release by C2A mutants indicates that Ca\(^{2+}\)–C2A interactions are not essential for Ca\(^{2+}\)-dependent fast neurotransmitter release at Drosophila NMJ synapses. In contrast, the reduction of evoked release by 97% in C2B mutant rescue animals suggests that the Syt 1 C2B domain is the major Ca\(^{2+}\) sensor for synchronous neurotransmitter release. These results separate the requirement for the two C2 domains in the different components of release and indicate that Ca\(^{2+}\)–C2B interactions are not essential for suppression of asynchronous fusion.

To further characterize the role of the Syt 1 C2A domain in release, we extended the analysis of the synchronous and asynchronous release components at C2A mutant synapses. In addition, we examined the Ca\(^{2+}\) dependence of synaptic transmission in the C2A mutant rescues compared with WT rescues (Fig. 4). Surprisingly, synapses expressing the C2A domain mutation displayed larger synaptic currents at lower Ca\(^{2+}\) concentrations, not only in the asynchronous component (Fig. 4B), but also in synchronous release (Fig. 4A). In high extracellular Ca\(^{2+}\) (4 mM), synchronous release in WT rescued animals was comparable to that observed in C2A mutant rescued strains. These findings are consistent with the hypothesis that reduced suppression of synaptic vesicle fusion via the C2A domain enhances vesicle release.

Previous studies in Drosophila demonstrated that Syt 1-null mutants have a smaller releasable vesicle pool as revealed by hypertonic stimulation (9, 25). This reduction in the releasable vesicle pool is hypothesized to result from a lack of Syt 1 binding to the soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptor (SNARE) complex, the fusion machinery required for synaptic vesicle release (26). The loss of SNARE binding by Syt 1 may disrupt intimate docking of synaptic vesicles with the plasma membrane, resulting in loss of synchronous fusion and reduction in hypertonic-induced release.

To determine whether the phenotypes observed in C2A and C2B mutations are caused by abnormalities in synaptic vesicle pool size, we performed hypertonic stimulation to measure fusion-competent vesicles. As shown in Fig. 5, synapses expressing ei-
a second Ca\(^{2+}\) sensor supports a slower asynchronous phase of the rapid synchronous component of transmitter release, whereas fuse by Ca\(^{2+}\) in maily docked and fusion-competent, but cannot be triggered to genotype were: the null and each rescue strain. The numbers of samples analyzed for each Discussion

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mutant transgene, which shows only residual levels of synchro-

there transgenic Syt 1 protein with C2A or C2B mutations rescued hypertonic-induced release to the same level as that observed in WT Syt 1 rescued animals. These results indicate that pheno-
types observed at C2A and C2B mutant synapses are not secon-
dary to abnormal vesicle pool sizes. In the case of the C2B mutant transgene, which shows only residual levels of synchro-
nouse, the findings imply that synaptic vesicles are nor-
mally docked and fusion-competent, but cannot be triggered to fuse by Ca\(^{2+}\) influx. In summary, the defects in evoked trans-
mission at synapses expressing C2A and C2B mutations reflect changes in release properties, as opposed to alterations in syn-
aptic vesicle pool size.

Discussion

Neurotransmitter release at synapses is regulated by two kine-
tically distinct Ca\(^{2+}\) sensors. A low-affinity Ca\(^{2+}\) sensor mediates the rapid synchronous component of transmitter release, whereas a second Ca\(^{2+}\) sensor supports a slower asynchronous phase of fusion. Syt 1 has emerged as the primary candidate for the low-affinity Ca\(^{2+}\) sensor promoting synchronous neurotransmitter release (reviewed in refs. 27–29). How Syt 1 and the asynchronous Ca\(^{2+}\) sensor regu-
late the two distinct phases of release, and whether there are competitive interactions between the two fusion pathways, are unclear. To examine the requirements of Ca\(^{2+}\) binding to the C2A or C2B domain of Syt 1 in regulating the kinetics of vesicle fusion, we rescued Syt 1-null mutants with transgenes disrupting Ca\(^{2+}\) binding to either domain. Both C2A and C2B Ca\(^{2+}\) binding mutants fully restored the releasable synaptic vesicle pool trig-
gered by hypertonic stimulation, suggesting Ca\(^{2+}\) binding to Syt 1 is not required for its role in supplying the readily releasable pool through vesicle docking. Alternatively, Ca\(^{2+}\) binding to either C2 domain may be sufficient for supplying the readily releasable pool. Further studies with mutations disrupting Ca\(^{2+}\) binding to both C2 domains will be required to test these alternative models. Record-
ings of nerve-evoked synaptic currents revealed contrasting roles for the two C2 domains in triggering fusion. Transgenic rescue animals expressing Syt 1 with C2A mutations displayed enhanced asynchronous release and retained robust synchronous fusion, whereas Syt 1 with C2B mutations largely suppressed the asyn-
chronous component, but limited ability to rescue synchronous fusion. These observations suggest distinct roles for Syt 1 during the fusion process. First, the Syt 1 C2B domain is the major Ca\(^{2+}\) sensor for fast synchronous neurotransmitter release, whereas C2A Ca\(^{2+}\) binding is dispensable for triggering synaptic vesicle fusion. Second, Ca\(^{2+}\) binding to the C2A domain is critical for suppressing asynchronous release, whereas C2B Ca\(^{2+}\) binding is not essential in this role. Third, the enhanced asynchronous re-
lease observed in the absence of Syt 1 does not require reduction of the synchronous component, as observed in the C2A mutant rescue experiments. These data indicate that Syt 1 and the asynchronous Ca\(^{2+}\) sensor independently regulate synaptic vesicle fusion without mutually competing as previously proposed (16).

The role of the C2B domain of Syt 1 as a Ca\(^{2+}\) sensing module was suggested by its structure (18) and biochemical properties (30). Genetic manipulations of Syt 1 in Drosophila confirmed that Ca\(^{2+}\)–C2B domain interactions were essential for synaptic neurotransmitter release (17), whereas Ca\(^{2+}\)–C2A interactions were not (31). Critical roles for the C2B domain of Syt 1 were also demonstrated using transfection of C2B Ca\(^{2+}\) binding mutants into Syt 1-deficient hippocampal cultured neurons. This approach indicated that Ca\(^{2+}\) binding into the slot formed by the D2 and D3 aspartates of C2B were required for triggering vesicle fusion (16). Our C2B domain mutation analysis, which used disruptions of both the D3 and D4 aspartates, is consistent with these observations and support a critical role for the C2B domain of Syt 1 in Ca\(^{2+}\)-triggered synaptic vesicle fusion. We previously demonstrated that vesicle fusion mediated by application of external salines containing high K\(^{+}\) or Ca\(^{2+}\) ion-
ophores is larger in Syt 1-null mutants than WT animals (9), indicating an inhibitory function of Syt 1 in addition to its posi-
tive role in driving synchronous release. Our current studies in-
dicate that Ca\(^{2+}\) binding to the C2B domain is dispensable for the suppression of asynchronous release, suggesting Ca\(^{2+}\)–C2A–
dependent interactions can negatively regulate the slow com-
ponent of fusion. These observations are consistent with studies at mammalian synapses, as Syt 1 C2B Ca\(^{2+}\)–binding mutants also suppress asynchronous release at hippocampal synapses (16). A prior model for the synchronization role of Syt 1 postulated that it directly competes with the asynchronous Ca\(^{2+}\) sensor for binding sites on the SNARE complex (16). Based on this model, asynchronous release is eliminated by binding of Syt 1 to SNARE complexes, preventing access of the asynchronous sensor to the fusion machinery. The data from our analysis of the C2A Ca\(^{2+}\)–binding mutant suggest a simple competition model cannot account for the effects observed. As the enhancement of asyn-
chronous release observed in the C2A mutant rescue occurs.
without a corresponding reduction in synchronous fusion, it does not require a competitive reduction of the synchronous component. Support for the competition model was based on observations that total release between Syt 1 and WT neurons was unchanged at autapses (11), but this is not observed at the calyx of Held (13), in dissociated hippocampal cultures (23), and our present study. Rather, the data suggest an inhibitory role in preventing asynchronous release mediated by Ca2+-binding to the C2A domain. Previous studies in hippocampal neurons support an inhibitory role for the C2A domain of Syt 1 in exocytosis. Stevens and Sullivan (32) found that neuronal Syt 1 cultures rescued with C2A mutated at the D3 and D4 Ca2+-binding residues showed larger EPSCs at lower Ca2+ concentrations when EPSC size is normalized to maximal response, generating a similar Ca2+-dependence curve to that observed in Drosophila (Fig. 4). Although distinct from the enhanced asynchronous release we describe, an inhibitory function for Ca2+-C2A interactions (and to a lesser extent, Ca2+-C2B) in spontaneous release has also been reported at mammalian cortical synapses (24). Similarly, we observed that transgenic rescue with mutations in C2A or C2B Ca2+-binding sites results in an elevation of spontaneous release, suggesting a conserved role for Syt 1 in this process. The lack of an increase in spontaneous release in Syt 1-null mutant embryonic synapses is likely a result of the known role of the protein in docking (33), and the corresponding reduction in the readily releasable pool revealed by hypertonic sucrose application (9). Our data indicate that C2A and C2B Ca2+-binding sites are not required for the “docking” function of Syt 1 (or that either can suffice), resulting in more docked vesicles than in the null case, and thus a greater chance to reveal a role in suppressing spontaneous release. This larger readily releasable pool of vesicles may also contribute to the enhanced asynchronous release observed in the C2A Ca2+-binding mutant rescue compared with that in Syt 1-null mutant. Consistent with the in vivo observations, in vitro reconstituted membrane assays also suggest a potential inhibitory role for Syt 1, which can be released by addition of Ca2+ (34).

The underlying molecular interactions that mediate Syt 1’s activity in suppressing asynchronous fusion and triggering synchronous release are still unclear. Although Syt 1 can bind to SNARE complexes in a Ca2+-independent manner (35), Ca2+-binding to Syt 1 causes conformational changes (36, 37). Ca2+-C2A (but not C2B) binding is important for such high-affinity SNARE binding (36), suggesting a reduction in tight coupling between Syt 1 and SNAREs may be a physical substrate for the loss of asynchronous release suppression in the C2A Ca2+-binding mutant. We hypothesize that Ca2+-independent SNARE binding would contribute to synaptic vesicle docking and intimate association between the two merging membranes. Ca2+-independent SNARE binding would enhance hypertonic-induced release by positioning synaptic vesicles adjacent to the plasma membrane. Consistent with this model, a reduction in synaptic vesicle docking has been observed by EM (23, 33) and functionally demonstrated by loss of hypertonic-induced release (9, 23, 25) in Syt 1-null mutants. A Ca2+-independent docking function is also supported by the observation that Ca2+-binding to C2A or C2B is dispensable for hypertonic-induced release (Fig. 5). Similarly, work at the calyx of Held synapse demonstrates that the Syt 2 R399, 400Q mutant, which retains normal Ca2+-sensing, reduces the tight coupling between Ca2+-influx and release probably as a result of incorrect positioning of synaptic vesicles, suggesting Ca2+-independent docking of synaptic vesicles is promoted by Syt 1 (38).

Ca2+-C2B interactions have recently been demonstrated to drive bending of target membranes via lipid tubulation, a property not associated with C2A activity (39). The efficiency of Ca2+-C2A binding as a release trigger would likely be weaker than C2B, as a result of its inability to bend the plasma membrane into a fusion-promoting conformation. When Ca2+-binding to the C2A domain is disrupted, an enhanced synchronous release triggered by Ca2+-binding to the C2B domain might be expected if C2A–Ca2+ functions as a clamp, as shown in Fig. 4. How Ca2+-binding to C2A normally suppresses asynchronous release is unclear, but we speculate that higher-affinity C2A Ca2+-binding would allow this domain to hold Ca2+ until its concentration subsides to resting levels, driving a conformational shift of Syt 1 that would suppress the slow phase of release until C2A–Ca2+-disengages. Further studies will be required to define precise molecular interactions required for the function of each C2 domain during vesicle fusion.

The dual roles of the Syt 1 C2 domains is consistent with observations that synaptotagmins have universally evolved with both a C2A and C2B domain throughout the animal kingdom (40). Systematic comparison between C2A and C2B Ca2+-binding mutations in inhibitory transmission in mammalian cortical cultures also supports unique roles for both C2 domains in regulating fusion (41), suggesting C2A and C2B have important functions in neurotransmission from invertebrates to mammals. In summary, we conclude that the C2A domain of Syt 1 suppresses asynchronous release, whereas the C2B domain acts as a synchronous fusion trigger. This dual role for Syt 1 allows neurotransmitter release to be tightly regulated, with vesicle fusion occurring only at high Ca2+ concentrations, thus synchronizing release to the action potential.

Materials and Methods

Drosophila Strains and Mutagenesis. Drosophila melanogaster were cultured on standard medium at 22°C. DNA constructs for UAS-Syt C2A-D418N encoding Syt 1 C2ASYD418 were obtained from N. E. Reist (Colorado State University, Fort Collins, CO). DNA for UAS-Syt 1 C2B-D416 encoding Syt 1 C2BSYD416 was generated using the QuikChange multi site-directed mutagenesis kit (Stratagene) with the primer ctcggtgtgccatcttcaatgc. All transgenic strains were generated using standard microinjection into white embryos. UAS transgenes were expressed using the GAL4 driver lines, and neuronal elav promoter (21) on the third chromosome in the Syt 1-null background. Null mutants lacking endogenous Syt 1 were generated by crossing Syt 1fl17, an intragenic Syt 1 deficiency (7), with Syt 1D4f, which truncates Syt 1 before the transmembrane domain (8). These null alleles were recombined with a chromosome containing the muscle-specific myosin heavy chain (Mhc) null mutant, Mhc 2, to inhibit muscle contraction and facilitate stable recordings as previously described (42). The Mhc 2 mutant had no observed effect on synapse formation, neurotransmitter release, or postsynaptic glutamate receptor clustering (9, 42). Mutant chromosomes were placed over a CyO balancer containing actin-driven GFP to allow unambiguous identification of embryos with Syt 1 null and Mhc 2 backgrounds.

Electrophysiological Analysis. Synaptic currents were recorded with the patch-clamp technique in whole-cell configuration from embryonic muscle fiber 6 in segments A2 to A5 that were maintained at a holding potential of −60 mV. Embryos were aged 21 to 24 h after fertilization and recorded in HL3.1 saline solution (43) (in mM: NaCl, 70; KCl, 5; MgCl2, 5.5; NaHCO3, 10; trehalose, 5; sucrose, 115; Hepes-NaOH, 5; pH 7.2) as described (42), using an Axopatch 2008 amplifier (Axon Instrument) at 23 °C to 24 °C. External saline solutions with various concentrations of Ca2+ were prepared by replacing MgCl2 with CaCl2. The internal solution in patch pipettes contained: (in mM) CsCl, 158; ATP, 2; EGTA, 5; Hepes-NaOH, 10, pH 7.1. Motor nerves were positioned in a suction electrode at their site of emergence from the CNS for nerve stimulation. Before recording, embryos were treated for 1 min with 0.4 mg/mL collagenase (type IV; Sigma) in 0.1 mM Ca2+ saline solution. For nerve stimulation, 2 to 5 μA of positive current was passed for 1 ms through a suction electrode, which contained HL3.1 saline solution and was tightly attached to the nerve. For hypertonic-stimulated release, 500 mM sucrose dissolved in Ca2+-free HL3.1 saline solution was included in a puff pipette with a 1-μm tip, and placed in close vicinity of the boundary between muscles 6 and 7, where the nerve terminal is ending. Hypertonic solution was puffed using positive pressure for 3 s. Slow responses originating from electrically coupled muscle fibers were excluded in subsequent analysis in all genotypes. We performed three independent experiments in Ca2+-free saline solution. The slow phase of presynaptic release mediated by retrograde signaling downstream of postsynaptic Ca2+-influx (44, 45). Statistical analysis was performed using Prism 5 software (GraphPad). The variation of data between animals (av-
erage of five animals) was small compared with the larger variation in data from one animal. Thus, results were combined and the number of events recorded shown as N.

**Immunostaining.** Immunostaining on filleted embryos was performed as previously described (46). FITC-conjugated IgG against HRP, which labels neuronal cell membranes, was purchased from Cappel and used at 1:1,000. DSYT2 against Syt 1 (22) was used at 1:1,000. Immunoreactive proteins were visualized on a Zeiss Pascal confocal microscope using fluorescent secondary antibodies (Molecular Probes/Chemicon).

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Supporting Information

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SI Materials and Methods

Western Analysis. First instar larvae were collected 24 h after egg laying and five first instar larvae were homogenized in sample buffer and loaded on a 10% SDS/PAGE gel. DSYT2 antiserum against Syt 1 (1) was used at 1:500 dilution; anti-complexin antiserum (2) was used at 1:1,000 dilution and detected using a goat anti-rabbit antibody conjugated to Alexa Fluor 680 (A21076; Invitrogen). Anti-Dlg antiserum (3) and anti-syntaxin monoclonal antibody (4) were used at 1:1,000 and detected using a goat antimouse antibody conjugated to IRDye800 (no. 610-132-121; Rockland). Detection of immunoblots was accomplished using an Odyssey infrared scanner (LI-COR).


Fig. S1. Western analysis of Syt 1 transgenic protein levels. Western analysis of first instar larval extracts for Syt 1 expression level in WT (white, a genetic background commonly used for making transgenic flies), Syt 1-null mutants (Syt 1^{N13}/Syt 1^{AD4}), and transgenic rescue animals with elav^{G115}_{GAL4} driving UAS-Syt 1 WT (WT), UAS-Syt 1^{C2A,D3,4N} (C2A D > N) or UAS-Syt 1^{C2B,D3,4N} (C2B D > N). C2A and C2B transgenic lines overexpress similar levels of Syt 1 protein compared with the WT overexpression strain used as a control for physiology experiments. Null mutants lack Syt 1 expression. Larval extracts were probed with anti-Dlg, anti-syntaxin (Syx), and anti-complexin (Cpx) antibodies as loading controls. Extracts from five first instar larvae were loaded per lane.