Chapter 5
Munc13 controls the location and efficiency of dense-core vesicle release in neurons

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Abstract

Neuronal dense-core vesicles (DCVs) contain diverse cargo crucial for brain development and function, but the mechanisms that control their release are largely unknown. We quantified activity-dependent DCV release in hippocampal neurons at single vesicle resolution. DCVs fused preferentially at synaptic terminals. DCVs also fused at extra-synaptic sites but only after prolonged stimulation. In munc13-1/2 null mutant neurons, synaptic DCV release was reduced but not abolished and synaptic preference was lost. Remaining fusion required prolonged stimulation, similar to extra-synaptic fusion in wild type neurons. Conversely, Munc13-1 overexpression promoted extra-synaptic DCV release, also without prolonged stimulation. Thus, Munc13-1/2 facilitate DCV fusion but, unlike for synaptic vesicles, are not essential for DCV release and Munc13-1 overexpression is sufficient to produce efficient DCV release extra-synaptically.

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Introduction

Neuronal dense-core vesicles (DCVs) contain neuropeptides, neurotrophic factors, monoamines and other modulatory substances that are essential during brain development and regulate synaptic plasticity in the adult brain (see reviews Huang & Reichardt, 2001; McAllister et al. 1999; Poo, 2001; Samson & Medcalf, 2006). To date, more than 50 biologically active peptides have been identified that have profound effects on brain and body function. Not surprisingly, dysregulation of DCV signaling is associated with many diseases such as mood and cognitive disorders, obesity and diabetes (see review Meyer-Lindenberg et al. 2011). DCVs are filled with cargo at the Golgi network (see review Kim et al. 2006) and transported through axons and dendrites via microtubule-based motors of the kinesin and dynein families (see reviews Hirokawa et al. 2009; Schlager & Hoogenraad, 2009). Ca\(^{2+}\) influx triggers DCV release from synaptic and extra-synaptic regions on axons and dendrites (de Wit et al. 2009b; Hartmann et al. 2001; Knobloch et al. 2012; Ludwig & Leng, 2006; Matsuda et al. 2009) and previous work identified several stimulation paradigms that trigger DCV fusion (Bartfai et al. 1988; Hartmann et al. 2001; Matsuda et al. 2009; Verhage et al. 1991). Yet, little is known about the molecules that control the location and kinetics of DCV secretion in mammalian neurons.

In contrast to the relative lack of understanding of DCV fusion, synaptic vesicle (SV) fusion mechanisms are much better understood (see reviews Jahn & Scheller, 2006; Sudhof & Rothman, 2009). SVs cluster at active zones, fuse upon Ca\(^{2+}\) influx and locally recycle at the presynaptic terminal (see review Sudhof, 2004). DCVs are generally more dynamic (de Wit et al. 2006; Matsuda et al. 2009; Shakiryanova et al. 2006) and are present in synaptic terminals as well as neurites and perikarya but it is not clear whether DCVs specifically cluster at synaptic terminals or use activity-dependent recruitment mechanisms as seen in Drosophila neuromuscular junctions (Shakiryanova et al. 2006; Wong et al. 2012). Hence, transport and release characteristics of SVs and DCVs differ to some extent, but they also share properties such as the requirement for functional SNARE complexes and Ca\(^{2+}\) influx to initiate fusion (see review Xu & Xu, 2008). Therefore, proteins that function in synaptic vesicle release (de Wit et al. 2009a; Garner et al. 2000; Rosenmund et al. 2003; Sorensen, 2009; Verhage & Toonen, 2007) may have similar functions in DCV
release in neurons. Yet, no candidates have been investigated to date.

To assess the spatial and temporal characteristics of neuronal DCV release in a quantitative way, we used an optical probe to visualize single DCV release events in hippocampal neurons. We found that DCVs are preferentially released from synaptic terminals. Release onset is faster and the rate of release is higher at synapses compared to extra-synaptic sites. To unravel underlying molecular mechanisms we investigated the role of the Munc13 family members Munc13-1 and Munc13-2, SV priming proteins that are essential for SV release (Augustin et al. 1999; Basu et al. 2007; Rhee et al. 2002; Varoqueaux et al. 2002). We show that deletion of Munc13-1/2 strongly decreased but did not abolish DCV release, while Munc13-1 overexpression resulted in increased DCV release. Strikingly, both the absence and overexpression of Munc13 changed the synaptic preference of DCV release: Munc13 deletion specifically reduced synaptic release events, while overexpression increased release only from extra-synaptic sites. Thus in addition to its fusion promoting role, Munc13 also affects the localization of DCV release and Munc13 overexpression is sufficient to promote efficient DCV release at extra-synaptic sites along the plasma membrane.
Results and discussion

Optical sensor for DCV release in neurons

To study neuronal DCV release, we used reporters generated by fusing the canonical DCV cargo proteins Neuropeptide Y (NPY) or Semaphorin-3A to pH-sensitive EGFP (pHluorin) (Fig. 1A and Fig. S1). We have previously shown that these cargo, when overexpressed in mouse hippocampal neurons, are co-expressed in the majority of DCVs with an almost 90% overlap with endogenous DCV cargo Chromogranin A (Fig 1B and de Wit et al. 2009b). DCV-pHluorin release events become visible as bright diffraction limited spots (Fig. 1C). To test whether these spots represent single or multiple DCV release events, the fluorescence intensity increase ($\Delta F$) of individual release events was compared to the $\Delta F$ of DCV puncta upon NH$_4^+$ superfusion (to de-quench intra-vesicular pHluorin, Fig. 1D). The $\Delta F$ intensity plot upon NH$_4^+$ application showed a skewed distribution with a major population at $\pm$ 6 a.u. that overlapped with the $\Delta F$ of individual release events, suggesting that the latter are generally single vesicle fusion events (Fig. 1D). To initiate release of DCVs, neurons were stimulated with 16 bursts of 50 action potentials (AP) at 50 Hz. This stimulus is optimal for release of brain-derived neurotrophic factor (BDNF) from hippocampal neurons (Hartmann et al. 2001). DCV release was strongly coupled to this stimulation and resulting Ca$^{2+}$ influx (Fig. 1E and Fig. S1). Only a few DCVs were released before or after the stimulus train (Fig. 1E-F and Fig. S1). Other stimulation paradigms were not as effective in releasing DCVs (Fig. S1).
Figure 1. Optical sensor for DCV release in neuron

(A) Schematic representation of an optical reporter for dense-core vesicle release that allows visualization of single DCV fusion events. (B) Confocal image of a neuron transfected with sema3A-pHluorin (cargo-pH) and stained for endogenous DCV cargo chromograninA showing complete overlap (arrowheads). Scale bar, 2 µm. (C) Image series showing a cargo-pH release event (arrow) and the NH$_4^+$ response to reveal all vesicles in the neurite (arrowheads). (D) Normalized frequency distribution of ΔF for DCV release events measured with Sema3A-pHluorin (red bars) and ΔF upon NH$_4^+$ perfusion (grey bars) (NH$_4^+$: 395 puncta, 7 cells; release: 293 puncta, 30 cells, median ΔF release = 5.4 a.u.; median ΔF NH$_4^+$ = 6.0 a.u.). (E) Frequency distribution of DCV release events measured with Sema3a-pHluorin (570 release events in 53 cells; blue bars, 16 bursts of 50 AP at 50 Hz). (F) DCV release events during 60 seconds before stimulation, during stimulation and during 70 seconds after stimulation (before: 1 ± 0.6; during: 16 ± 3.4; after: 5 ± 1.3 vesicles/cell, n = 21 cells, N = 3).
DCVs do not accumulate at synapses but DCV release is enriched and more efficient at these sites

Many proteins involved in SV fusion are enriched in synaptic terminals (Sudhof, 2004). If these proteins were also involved in DCV release, DCV release should be enriched at synaptic sites. To test this, we quantified synaptic and extra-synaptic release (Fig. 2A-B) using the SV protein Synapsin to visualize synapses in living neurons (Gitler et al. 2004). Synapsin-mCherry fusion proteins formed discrete puncta along the dendrite that co-localized with endogenous VAMP2, confirming that Synapsin-mCherry is correctly localized to synapses (Fig. S1F and our previous results, de Wit et al. 2006).

We first tested whether DCVs accumulate at synapses. In fixed neurons, 18.7 ± 3.1 % of Synapsin-labeled terminals overlapped with DCV-pHluorin puncta (n = 616 synapses measured in 4 cells). Similar results were obtained for the endogenous cargo Secretogranin II (19.9 ± 3.7 %, n = 493 synapses in 5 cells). Hence, in hippocampal neurons most synapses do not contain DCVs.

As DCVs are smaller than our resolution limit, individual DCV puncta may contain multiple DCVs. To assess this, we compared fluorescence intensity of individual puncta upon NH₄⁺ application at synapses and extra-synaptic regions. DCV-pHluorin puncta showed a similar intensity distribution at synapses as outside synapses with a major population at 6 a.u. (Fig. 2C-D, synaptic: median 6.0, average 7.4 ± 0.8, n = 589 puncta; extra-synaptic: median 5.7, average 7.0 ± 0.8, n = 612 puncta, N=8 cells, p > 0.05). Thus, the majority of single puncta are indeed single DCVs (compare Fig. 1D with Fig. 2C-D), while some puncta comprise multiple DCVs (between 1-4 with 6 a.u. representing a single DCV, see Fig. 1D). These DCV clusters are present at extra-synaptic as well as synaptic sites with no specific enrichment in synapses. Electron micrographs corroborated these findings and revealed both single DCVs and DCV clusters outside synaptic areas (Fig. S2D). On average 1.4 DCV was present per synapse section (average DCV diameter: 68.9 ± 1.0 nm, Fig. 2E-F) and almost half of the sections did not contain DCVs (Fig. 2G). In sections with DCVs, most DCVs were randomly distributed in the terminal, while about 22 % were physically attached (docked) to the active zone (Fig. 2G, inset). Together, these data show that most Synapsin-labeled terminals do not contain DCVs and that DCV clusters are not enriched in terminals at rest.
Yet, the majority of DCV release events occurred at Synapsin positive regions (synaptic: 66 %, 315 events; extra-synaptic: 34 %, 165 events, n = 34 cells, N = 3, Fig. 2H) and DCV release rates were much slower at extra-synaptic sites than at synapses (synaptic: 12.6 ± 0.3 vesicles/sec; extra-synaptic: 7.8 ± 0.2 vesicles/sec, p < 0.05, Fig. 2I). In line with this observation, synaptic DCV release required less intense stimulation compared to extra-synaptic release. Already after the first 50 APs, DCVs were released at synapses whereas extra-synaptic release started only after 3 bursts of 50 APs (Fig. 2I-J). Apart from the kinetic difference, no differences were observed in the size and shape of the individual release events (Fig. 2K). Hence, DCV release probability at synapses is higher than outside these regions.
Munc13 controls the location and efficiency of dense-core vesicle release in neurons

Deletion of Munc13-1 reduces and delays synaptic DCV release

To gain insight into the molecular priming mechanisms of neuronal DCV release, we tested the role of the SV priming proteins of the Munc13 family in munc13-1/2 null mutant (Munc13 DKO) mice. In Munc13 DKO neurons, the number of DCVs per cell was not changed (Fig. S2A) but DCV release was reduced by more than 60% compared to WT (WT: 11.8 ± 2.3, n = 41 cells; Munc13 DKO: 4.7 ± 1.0, n = 23 cells, p < 0.01, Fig. 3A). In addition, DCV release rates were slower (WT: 0.73 ± 0.12 vesicles/s; Munc13 DKO: 0.32 ± 0.10 vesicles/s, p < 0.05, Fig. 3B) and release required more prolonged stimulation as Munc13 DKO neurons released DCVs only after the third burst of 50 APs (Fig. 3B-C). Hence, deletion of Munc13-1/2 strongly reduces the number and rate of DCV release events. In contrast to SV release however, it does not totally abolish DCV release. Munc13-1 single null mutant neurons showed a similar strong reduction of DCV release, indicating that Munc13-1 is the dominant isoform supporting DCV release (Fig. S3).

Munc13-1 is highly enriched in presynaptic terminals (Kalla et al. 2006) and could thus influence the localization of DCV release. We first excluded that deletion of Munc13-1 affected DCV localization in the synapse using electron microscopy (Fig. S3C-D). Next, using Synapsin-mCherry as a synaptic marker we found that

Figure 2. DCV release is enriched and more efficient at synapses although DCVs do not accumulate at synaptic regions

(A) Example of a synaptic (filled arrowhead) and an extra-synaptic release event (open arrowhead), Synapsin-mCherry shown in red and release events in green (scale bar, 1 µm). (B) Cartoon showing a synaptic (left green dot) and extra-synaptic (right green dot) release event. (C) Overlay of frequency distribution of ΔF upon NH4+ application for synaptic DCV puncta (red bars, synaptic 589 puncta, syn) and extra-synaptic DCV puncta (black bars, extra-synaptic 612 puncta, e-syn). (D) Individual frequency distributions of ΔF upon NH4+ application. (E) Electron-micrographs of synaptic terminals that harbor DCVs. Arrowheads point to DCVs, scale bar, 100 nm. (F) Diameter of DCVs in electron micrographs (average diameter: 68.9 ± 1.0 nm, 146 DCVs in 7 cells). (G) Number of DCVs per synaptic profile (102 synapses in 7 cells). Inset, ± 22% of synaptic DCVs is docked to the plasma membrane (undocked 113, docked 33, total 146). (H) Percentage of synaptic and extra-synaptic DCV release events measured with Sema3a-pHluorin (235 synaptic release events, 131 extra-synaptic release events, n = 34 cells, N = 3, p < 0.05). (I) Cumulative number of release events per cell for synaptic and extra-synaptic events. (Synaptic release rate: 0.26 ± 0.03 vesicles/s; extra-synaptic: 0.13 ± 0.02 vesicles/s). (J) Cumulative number of events during the first four bursts (blue bars 1, 2, 3 and 4 represent 50 APs at 50 Hz) for synaptic and extra-synaptic events. (K) Example traces of synaptic (red) and extra-synaptic (black) release events. Arrows mark start of the stimulation.
Munc13-1/2 deletion did not change the localization of DCVs prior to release (Fig. S2C and Fig. 3D) but strongly affected the ratio of synaptic versus extra-synaptic release (Fig. 3E) by reducing the number of synaptic release events (Fig. 3F). This suggests that Munc13-1 specifically promotes synaptic DCV release. Consistent with this observation, Munc13-1/2 deletion affected synaptic DCV release rates much more than extra-synaptic release (WT synaptic: 0.49 ± 0.09 vesicles/s; Munc13 DKO synaptic: 0.17 ± 0.10 vesicles/s, p < 0.05; WT extra-synaptic: 0.24 ± 0.08 vesicles/s; Munc13 DKO extra-synaptic 0.16 ± 0.13 vesicles/s, Fig. 3G-H). Hence, Munc13 deletion does not alter DCV localization or mobility at steady state. It does, however, specifically affect the amount and rate of synaptic DCV release.

Figure 3. Munc13 specifically reduces DCV release at synapses

(A) Average number of DCV release events per cell for WT and Munc13-1/2 DKO (M13DKO) neurons measured with Sema3a-pHluorin (WT: 482 events, n = 41 cells; M13 DKO: 108 events, n = 23 cells, N = 4, p < 0.01). (B) Cumulative number of release events per cell. (C) Cumulative number of release events during the first four bursts of 50 AP at 50 Hz. (D) Percentage of synaptic-located DCVs that remain synaptic during 10 s acquisition (WT: 221 vesicles, n = 6 cells; M13 DKO: 146 vesicles, n = 5 cells, N = 2). (E) Percentage of synaptic and extra-synaptic DCV release events in WT and Munc13 DKO neurons (WT: 21 cells and M13 DKO: 23 cells, N = 4, p < 0.01). (F) Total numbers of synaptic and extra-synaptic events show a specific loss of synaptic events (total events WT: 255; M13 DKO: 151). (G) Cumulative number of synaptic DCV release events. (H) Cumulative number of extra-synaptic DCV release events.
Munc13-1 overexpression increases the number of extra-synaptic DCV release events

Munc13-1 overexpression in bovine chromaffin cells enhances DCV release (Ashery et al. 2000). To test if similar principles apply for DCV release in neurons, we tested the effect of Munc13-1 overexpression in WT hippocampal neurons. Munc13-1 overexpression (M13OE) resulted in a more than 4-fold increase in protein levels compared to endogenous levels (Fig. 4A). Endogenous Munc13-1 showed a clear punctate localization, which overlapped with Synapsin-mCherry. In contrast, overexpressed Munc13-1 was not restricted to synapses, but distributed homogenously throughout the neurites (Fig. 4B). Similar ectopic expression of Munc13-1 has been observed before (Deng et al. 2011).

Munc13-1 overexpression did not change the total number or localization of DCVs (Fig. S2B, C and Fig. 4C), but significantly increased DCV release events compared to WT (WT: 10.1 ± 1.8, n = 36; M13OE: 18.4 ± 3.2, n = 32, p < 0.05, Fig. 4D). Release started earlier in M13OE neurons (Fig. 4E-F), although this effect was rather subtle when measured regardless of the spatial localization of the events. However, as in Munc13 DKO neurons, the preference for DCV release at synaptic sites was lost in M13OE neurons (Fig. 4G). In contrast to Munc13 deletion, this was now caused by a specific increase in DCV release events at extra-synaptic sites (Fig. 4H) with significantly increased vesicle release rates at these sites (WT extra-synaptic: 0.24 ± 0.04 vesicles/sec; M13OE extra-synaptic: 0.36 ± 0.01 vesicles/sec, p < 0.05, Fig. 4I). In M13OE neurons, extra-synaptic release events occurred already during the first four stimuli. During this time window WT neurons did not show release (Fig. 4J). Munc13 overexpression hardly, if at all, affected synaptic release events (Fig. 4K-L). Together, this shows that overexpression of Munc13-1 is sufficient to produce efficient DCV release at extra-synaptic sites.
Figure 4 Munc13-1 overexpression increases DCV release at extra-synaptic sites

(A) Normalized Munc13-1 intensity in WT neurons compared to neurons overexpressing Munc13-1 (M13OE, N = 3, p < 0.001). (B) Confocal image of a WT neuron with endogenous Munc13-1 and a WT neuron overexpressing Munc13-1 (arrowheads show synaptic localization of Munc13, scale bars, 2 µm). (C) Percentage of synaptically localized DCVs that stay synaptic for 10 s (WT: 199 vesicles, n = 7 cells; M13OE: 170 vesicles, n = 7 cells). (D) Average number of DCV release events per cell (WT: 361 events in 36 cells; M13OE: 588 events in 32 cells, N = 3, p < 0.05). (E) Cumulative DCV release events per cell. (F) Cumulative number of release events during the first four bursts of 50 AP at 50 Hz. (G) Percentage of synaptic and extra-synaptic DCV release events (p < 0.01, p < 0.05). (H) Total numbers of synaptic and extra-synaptic DCV release events. Loss of synaptic preference is due to an increase of extra-synaptic release events in M13OE. (I) Cumulative extra-synaptic DCV release events. (J) Cumulative number of events during the first four bursts at extra-synaptic sites. (K) Cumulative synaptic DCV release events. (L) Cumulative synaptic events per cell.
A model for DCV release in neurons

The present study characterized mechanisms that control synaptic DCV release in hippocampal neurons. We found that although DCVs are not enriched in synaptic terminals, they preferentially fuse at synapses. Release at extra-synaptic sites only occurs upon more prolonged stimulation compared to synaptic DCV release. We provide evidence that the synaptic priming proteins Munc13-1 and Munc13-2 are, unlike in synaptic vesicle secretion (Varoqueaux et al. 2002), not essential for DCV release, but primarily control the localization and efficiency of DCV release. In their absence, synaptic preference of DCV fusion is lost and remaining release events require stimulation intensities similar to extra-synaptic release events in wild type neurons, while overexpression promotes extra-synaptic release events that do not require prolonged stimulation.

Co-localization with Synapsin-mCherry and post-hoc confirmation using immune-detection indicated that only a small proportion (± 20 %) of the 100-150 DCV (-clusters) present in WT, M13DKO and M13OE neurons localizes to synaptic sites. Random sections of presynaptic profiles contained on average 1.4 DCV with few postsynaptic DCVs and almost half of the profiles were devoid of DCVs. Hence, our data suggest that there is no strong accumulation of DCVs in hippocampal synapses. Based on the observed DCV distribution, we expected ± 20 % of the DCV release events to occur at synaptic sites. Yet, we found that more than 65 % of all events did. Release at synapses started earlier upon stimulation and reached higher release rates than extra-synaptic release. As we did not find evidence for synaptic accumulation of DCVs prior to release, we conclude that DCV release probability at synaptic terminals is higher than at extra-synaptic sites. As most DCVs in mammalian neurons are mobile (Ramamoorthy et al. 2011; Silverman et al. 2005), on average only one in 5 synapses harbors stationary DCVs (Fig 2 and de Wit et al. 2006) which are often not pre-docked (de Wit et al. 2006; de Wit et al. 2009b; Matsuda et al. 2009; Silverman et al. 2005; Verhage et al. 1991). These findings suggest that DCVs are recruited to synapses during activity and consequently, dock and fuse from an undocked, mobile state during stimulation.

We found that loss of Munc13-1/2 strongly reduced DCV release and that Munc13-
1 is the dominant isoform supporting DCV release. Hence, Munc13 emerges as a general priming factor for regulated secretion of different types of secretory vesicles (synaptic vesicles and DCVs). However, almost 40% of the fusion events remained in M13DKO neurons, while synaptic vesicle fusion is abolished completely in such neurons (Varoqueaux et al. 2002). In fact, our data show that impaired release efficiency is the main effect of Munc13-1/2 loss: in their absence, the first fusion events occur only after 3-4 episodes of 50 APs at 50 Hz. This is the same type of drastic stimulation, which may be very rare in vivo, required to trigger extra-synaptic DCV fusion in wild type neurons. So, DCV fusion appears to lose its synaptic advantage upon Munc13 loss. This is consistent with the highly localized distribution of Munc13-1/2 to active zones (Kalla et al. 2006).

Conversely, Munc13-1 overexpression leads to many more DCV fusion events at extra-synaptic sites, which also required less intense stimulation. Upon overexpression, non-synaptic DCV fusion events appeared to gain synaptic-like properties and overexpression of Munc13-1 appears to be sufficient to specify extra-synaptic DCV release sites. Munc13 interacts with the tSNARE protein syntaxin and the S/M protein Munc18 (Ramamoorthy et al. 2011; Richmond et al. 2001). These proteins serve as essential and minimal components for membrane fusion in many systems (Rizo & Sudhof, 2002; Toonen & Verhage, 2007; Verhage et al. 2000), and are not exclusively present at synapses (Galli et al. 1995). Munc13 could organize/activate extra-synaptic tSNARE/Munc18 complexes to increase DCV release probability at these sites.
**Materials and Methods**

*Plasmids.* Semaphluorin was generated by replacing EGFP in Sema3A-EGFP (De Wit et al. 2005) with the super-ecliptic pHluorin (SpH) coding sequence (de Wit et al. 2009b). Neuropeptide Y-Venus was previously described (Nagai et al. 2002) and Neuropeptide Y (NPY)-SpH was generated by replacing Venus with SpH (de Wit et al. 2009b). Synapsin-mCherry was a kind gift of Dr. A. Jeromin (Allen Brain Institute, Seattle, USA).

*Laboratory animals.* Munc13-1/2 double knockout (M13DKO) mice have been described before (Varoqueaux et al. 2002). E18 embryos were obtained by caesarean section of pregnant females from timed mating of Munc13-2 homozygous and Munc13-1 heterozygous mice. M13DKO embryos are still born and M13DKO neurons in culture (DIV14) show neither evoked nor spontaneous release events, yet form normal numbers of synapses with typical ultra-structural features. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

*Primary neuronal cell culture and transfection.* Dissociated hippocampal neurons were prepared from embryonic day 18 mice as described (de Wit et al. 2009b). Hippocampi were dissected in Hanks Buffered Salts Solution (HBSS, Sigma) and digested with 0.25 % trypsin (Invitrogen) for 20 min. at 37°C. Hippocampi were washed and triturated with fire-polished Pasteur pipettes, counted and plated in Neurobasal medium (Invitrogen) supplemented with 2 % B-27 (Invitrogen), 1.8 % HEPES, 1 % glutamax (Invitrogen) and 1% Pen-Strep (Invitrogen). High-density cultures (25,000 neurons/well) were plated on pre-grown cultures of rat glia cells (37,500 cells/well) on 18mm glass coverslips in 12-well plates. At DIV10 neuronal cultures were transfected with calcium phosphate with DCV-pHluorin together with Synapsin-mCherry (to identify synapses) and ECFP (as neuronal morphology marker). Neurons were imaged at DIV14-DIV15.

*Imaging.* Coverslips were placed in an imaging chamber and perfused with Tyrodes (2 mM CaCl2, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl2, 20 mM glucose and 25 mM HEPES, pH 7.4) and imaged on an Axio Observer.Z1 microscope (Zeiss).
equipped with a Coolsnap HQ camera (Photometrics) and a Polychrome IV illumination unit (TILL Photonics). Images were acquired at 2 Hz with Metamorph 6.2 software (Universal Imaging) using a 40x objective (NA 1.3). Intracellular pH was neutralized with normal Tyrode’s solution containing 50 mM NH₄Cl, which replaced NaCl on an equimolar basis in the solution. A barrel pipette was used to apply NH₄⁺ solution to the cells. Electrical field stimulation by parallel platinum electrodes was applied by a Master 8 system (AMPI) and a stimulus generator (A385RC, World Precision Instruments) delivering 30 mA, 1 ms pulses. The stimulus used was 16 trains of 50 action potentials at 50 Hz with 0.5 s interval. All imaging experiments were performed at room temperature (21-24 °C) in the presence of 50 µM APV (Tocris) and 10 µM DNQX (Tocris) to block glutamatergic transmission. The imaging protocol consisted of a 30 s time-lapse, applying NH₄⁺ Tyrodes solution after 10 s for 10 s, then a 5-minute recovery period, in which spontaneous release was assessed during the last 60 s of this period. After this, a 100 s time-lapse with electrical field stimulation was recorded. Field stimulation started after 3 s and lasted 24 s. Spontaneous events after the stimulation were counted during the last 70 s. Furthermore, ECFP and Synapsin-mCherry images were acquired before and after the recording for further analysis. Neurons in which ECFP or Synapsin-mCherry masks before and after stimulation shifted more than 1 pixel (0.165 µm) were discarded from analysis.

**Image analysis.** Stacks from time-lapse recordings acquired with 0.5 s intervals were used to analyze DCV release. A 4x4 pixel region (0.6 x 0.6 µm) of interest was centered on each event, and the average intensity of the fluorescence was measured with Metamorph software (Universal Imaging). Fluorescent traces were expressed as the fluorescence change (∆F) compared to the initial fluorescence (F₀), obtained by averaging the first 4 frames of the time-lapse movie. Onset of exocytosis was defined as the first frame with an increase of fluorescence of two standard deviations above F₀. DCV release rates were measured from linear fits of the cumulative plots. Co-localization with Synapsin was measured by overlaying both images in Metamorph. A cargo-pHl release event or punctum was scored as synaptic when the fluorescence center of such a release event/punctum was within 200nm (±1 pixel, the approximate minimal point spread function of our system) of the Synapsin-mCherry fluorescence centroid. Extra-synaptic events were all events that did not meet this criterion. We only measured release events.
Munc13 controls the location and efficiency of dense-core vesicle release in neurons

from neurites and excluded somatic release events. Somatic release events cannot be reliably measured using wide-field fluorescence microscopy due to the bright fluorescence from vesicles in/near the Golgi apparatus in which the intraluminal pH is not yet acidic. The total number of vesicles was automatically analyzed from the NH$_4^+$ application time lapse using SynD software (Schmitz et al. 2011). In figures 3D and 4C, the localization and dynamics of DCVs was determined by analyzing cargo-pHluorin labeled vesicles during NH$_4^+$ application. Per neuron, four 50µm regions containing synapses were selected for line scan measurements. DCV fluorescence peaks 1 SD above average were scored for overlap with Synapsin-mCherry fluorescence peaks at the first frame of a 10 s time window. During these 10 seconds, even the slowest moving DCVs in our system (0.29 µm/s, de Wit et al. 2006) would have traveled ± 3µm (or >18 pixels). Stable DCVs are therefore defined as DCVs that do not leave the synaptic area in 10 seconds.

*Immunocytochemistry.* After imaging, cells were fixed in 4 % formaldehyde (Electron Microscopies Sciences) in PBS, pH 7.4 for 20 min at room temperature (RT). Cells were washed in PBS and first permeabilized for 5 min in PBS containing 0.5 % Triton X-100 (Sigma-Aldrich) then incubated for 30 min with PBS (Gibco) containing 2 % normal goat serum and 0.1% Triton X-100. Incubations with primary and secondary antibodies were done for 1 hour at RT. Primary antibodies used were; polyclonal MAP2 (Abcam), monoclonal VAMP2 (SySy) and polyclonal Munc13 (SySy), polyclonal chromogranin A and polyclonal secretogranin II (kind gifts from P. Rosa, Institute of Neuroscience, Milan, Italy). Alexa Fluor conjugated secondary antibodies were from Invitrogen. Coverslips were mounted in Mowiol and examined on a Zeiss LSM 510 confocal laser-scanning microscope with a 40x objective (NA 1.3).

*Electron microscopy.* Neurons were fixed at DIV14-16 for 1-2 hours at room temperature with 0.1 M cacodylate buffer/0.25mM CaCl$_2$/0.5mM MgCl$_2$ (pH 7.4) and processed as described (Meijer et al. 2012; Wierda et al. 2007). Briefly, cells were post-fixed for 2 hr at room temperature with 1% Osmium tetroxide/1 % Potassium ferro-cyanide, washed and stained with 1% uranyl acetate for 40 min in the dark. Following dehydration, cells were embedded in Epon. Cells of interest were selected using the light microscope, and mounted on pre-polymerized Epon blocks for thin sectioning. Ultrathin sections (~ 90 nm) were cut parallel to the cell mono-
layer, collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate. Synapses with a recognizable pre- and postsynaptic density were randomly selected using a JEOL 1010 electron microscope and imaged at 100,000 x magnification using analysis software (Soft Imaging System, Gmbh, Germany). The observer was blinded for the genotype. DCV distribution was measured with Image J (National Institute of Health, USA). Docked DCVs were 0 nm from the vesicle membrane to the plasma membrane.

Statistics. Throughout the paper, Student’s t tests for unpaired data were used unless otherwise specified. If F-tests showed significantly different standard deviations, t tests were Welch corrected. Mann-Whitney tests were used to compare groups when one or both did not pass the normality test. To test more than 2 groups, Kruskal-Wallis one-way analysis of variance with post-hoc Dunn’s multiple comparisons test was used. Kolmogorov-Smirnov test was used to test whether distributions were normally distributed and for testing frequency distributions. Data are plotted as average with SEM.
Supplemental figures

Supplemental Figure 1

(A) Normalized cumulative frequency plot of DCV release measured with NPY-pHluorin in WT cells (left y-axis, black line, 159 release events in 16 cells, N = 3), together with the frequency distribution of the release events (right y-axis, red bars). (B) Percentage of DCV release events 30 s before, during and 90 s after the stimulation period (p < 0.01) shows that DCV release is strongly coupled to Ca\(^{2+}\) influx during the stimulation period. (C) Percentage of synaptic and extra-synaptic DCV release events in WT cells (56 synaptic release events, 15 extra-synaptic release events, n = 5, p < 0.01). Shows that DCVs are preferentially released from synaptic regions. (D) Average Ca\(^{2+}\) response measured with Fluo4 upon electrical field stimulation (16 bursts of 50 AP at 50 Hz, n = 56 synaptic regions in 4 cells). (E) Number of release events in response to 16 bursts of 50 AP at 50 Hz compared to other stimulation paradigms (1 AP, 40 AP at 20 Hz, 5 x 40 AP at 100 Hz, n = 5 cells for each condition). (F) Confocal images of a WT neuron transfected with Synapsin-mCherry and post-hoc stained for endogenous VAMP2 and MAP2. Synapsin-mCherry strongly overlaps with VAMP2. Scale bar, 10 µm.

(G) Zoom of boxed area in (F). Scale bar, 5 µm. (H) Typical example of a hippocampal dendrite stained for VAMP and MAP2. Scale bar, 20 µm. (I) Histogram of synapse density per µm dendrite (130 cells, 559 ± 28 synapses per cell, 1729.0 ± 83.1 µm dendrite per cell analyzed, 5 independent experiments). With ± 0.3 Synapsin-labeled terminals per µm dendrite and an average synapse size of 1 µm, the ± 20 % overlap we and others (Matsuda et al. 2009) observed suggests that the overlap between synapses and DCVs is random.
Supplemental Figure 2

(A) Average number of DCVs per neuron upon NH$_4^+$ superfusion to instantly dequench all pHluorin molecules and visualize all DCVs. The total number of DCVs per neuron is similar between Munc13 DKO and WT neurons (WT: 112 ± 8.4 vesicles/cell, n = 22; M13 DKO: 102 ± 10.1 vesicles/cell, n = 13).

(B) Average number of DCVs per neuron upon NH$_4^+$ superfusion to instantly dequench all pHluorin molecules. The total number of DCVs per neuron is similar between Munc13 overexpressing (M13 OE) and WT neurons (WT: 101 ± 7.9 vesicles/cell, n = 37; M13 OE: 114 ± 6.1 vesicles/cell, n = 34).

(C) Average percentage of synapses containing DCVs measured upon NH$_4^+$ superfusion to instantly dequench all pHluorin molecules and visualize all DCVs. Synapses were identified by Synapsin-mCherry co-transfection. In each neuron, 50 random synapses were selected and assessed for co-localization with DCVs. The number of synapses with DCVs does not differ between WT, Munc13 DKO and Munc13 OE neurons (WT: 15 ± 3 %, n = 50 synapses in 4 cells; M13 DKO: 13.4 ± 5 %, n = 50 synapses in 5 cells; M13 OE: 14.9 ± 2 %, n = 50 synapses in 4 cells).

(D) Representative electron micrographs of DCVs in neurites of WT neurons. DCVs do appear as single vesicles but are also frequently clustered in rows of 2 to 5 vesicles. Note that when clustered, DCVs appear to be aligned to tubulin tracks. M = mitochondrion, scale bar, 100 nm.
Supplemental Figure 3

(A) Average number of DCV release events per cell for WT and munc13-1 null mutant (M13KO) neurons measured with NPY-pHluorin (WT: 108 events, n = 9 cells; M13KO: 9 events, n = 6 cells, N = 2, p < 0.01). (B) Cumulative number of release events during the total acquisition time. Blue bar represents 16 x 50 AP at 50 Hz stimulation. (C) Electron micrographs of synaptic terminals from wild type (WT) and Munc13-1 null mutant (M13KO) neurons. Scale Bar, 100 nm. (D) Average distance of DCVs present in the presynaptic terminal to the active zone (AZ) is similar between WT and M13 KO neurons (WT: 317 ± 13 nm, n = 54 synapses from 3 independent cultures; M13KO: 302 ± 15 nm, n = 42 synapses from 3 independent cultures). (F) Average number of DCVs per synaptic profile is similar between WT and M13 KO neurons (WT: 1.46 ± 0.04, n = 54 synapses from 3 independent cultures; M13KO: 1.61 ± 0.03, n = 42 synapses from 3 independent cultures).