Chapter 2
Live imaging of secretory vesicles trafficking and fusion in hippocampal neurons

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Abstract

Dense core vesicles (DCVs) contain a diverse range of neuromodulators, neurotrophic factors and neuropeptides that promote synaptogenesis, cell survival, proliferation and synaptic plasticity. Defects in DCV trafficking and fusion have been linked to pathological conditions as obesity, diabetes and cognitive disorders. Little is known about the mechanisms that recruit DCVs to their fusion sites at the plasma membrane and modulate fusion of DCVs in hippocampal neurons. We developed a quantitative method to study DCV trafficking and fusion in isolated hippocampal neurons at single vesicle resolution. We tested several stimulation paradigms of which high frequency electrical stimulation was most efficient in triggering DCV fusion. We found that DCVs preferentially fuse at synapses. Analysis of DCV behavior prior to fusion using dual color imaging of pHluorin- and mCherry-tagged cargo revealed that Ca$^{2+}$ influx triggers fusion of both stationary and mobile DCVs and that the majority of fusion events originate from stationary DCVs. Hence, our novel assay has the spatial and temporal resolution needed to characterize the process of DCV trafficking and release.
Introduction

DCVs transport and secrete a wide variety of neuropeptides and growth factors that are important in crucial brain processes as synaptic plasticity, development and neuronal connectivity (see reviews Guan & Rao, 2003; Lessmann et al. 2003; Malva et al. 2012; Pang et al. 2004). They owe their name to the fact that they appear as dark organelles in electron micrographs, because their cargos are densely aggregated in an osmotically inert form (see review Burgess & Kelly, 1987). DCVs are filled with cargo at the Golgi apparatus (see review Zupanc, 1996), undergo a maturation process (see review Tooze, 1991) and are transported along microtubules by kinesin and dynein motor proteins (Kapitein & Hoogenraad, 2011; Lo et al. 2011; Zahn et al. 2004) to their fusion sites. Upon Ca\textsuperscript{2+} influx, DCVs can fuse with the plasma membrane of the soma, neurites, synapses and extra-synaptic sites to release their content (see review Gondre-Lewis et al. 2012). Unlike synaptic vesicles (SVs) that can fuse upon a single action potential (AP), DCVs require repetitive stimulation for efficient fusion (de Wit et al. 2009b; Verhage et al. 1991). DCVs also lack a local recycling mechanism at fusion sites, which may explain their dynamic behavior in neurites to ensure constant supply of vesicles at fusion sites (de Wit et al. 2006; Sobota et al. 2010; Wong et al. 2012).

While the disruption of DCV exocytosis has been associated with many pathological states like cognitive disorders, stress and addiction problems, obesity and anorexia (Meyer-Lindenberg et al. 2011; Valentino & Aston-Jones 2010; Broberger & Hökfelt 2001), a detailed description of the dynamics of DCV recruitment and fusion is missing. A deep insight in DCVs transport and cargo release is crucial to gain mechanistic understanding of disorders related to neuropeptide deficits.

In this study, using simultaneous imaging of DCV cargos labeled with two fluorescent markers and with a novel single neuron assay, we investigate the fusion of DCVs, localization of DCV fusion sites and steps prior to fusion at single vesicle resolution, in hippocampal neurons. We show that repetitive, high frequency stimulation is the most reliable paradigm to elicit DCV fusion. Moreover we found that DCVs preferentially fuse at synapses and our data demonstrates that DCVs are either stationary or moving prior to fusion with the PM and that stationary DCVs are more likely to fuse compared to moving DCVs.
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**Results**

**DCV distribution in isolated hippocampal neurons**

We developed an assay to measure DCV fusion in single cells using isolated hippocampal neurons grown on micro islands of rat astrocytes (island diameter = 377.3 ± 87.75 μm, n=14. Figure 1A, B). To address their sub-cellular localization, DCVs were immune-stained with the endogenous DCV marker Chromogranin B (ChgB), the dendritic marker MAP2 and the synaptic vesicle marker VGLUT1 (Figure 1C). DCVs resided mainly in the axon with very little co-localization with the dendritic marker MAP2 (Pearson’s coefficient: 0.20 ± 0.01. Figure 1C, D). We found low co-localization of ChgB with VGLUT1 (Pearson’s coefficient: 0.30 ± 0.02. Figure 1C, D). We verified the co-localization of the exogenous fluorescent DCV marker (NPY-Venus) used for live cell imaging experiments with the endogenous DCV marker ChgB. (ChgB) and NPY-Venus showed a high degree of co-localization (Mander’s coefficients: proportion of endogenous ChgB immuno-reactivity in NPY-Venus puncta: 0.97 ± 0.02, n=14 neurons; proportion of NPY-Venus immuno-reactivity in ChgB puncta: 0.84 ± 0.01. Figure 1 E, F, and G). The punctate nature of both markers makes Mander’s coefficients more suitable to describe co-localization in this case. Hence, DCVs mainly resided in the axon and nearly 30% co-localized with synaptic markers. Furthermore, exogenous NPY-Venus (and NPY labeled with other fluorophores: NPY-pHluorin and NPY-mCherry) show high co-localization with endogenous DCV marker and will therefore be employed as live marker for DCV transport and fusion.
Figure 1. DCV fusion in isolated hippocampal neurons

(A) Schematic drawing of a glass coverslip with isolated neurons grown on micro islands of astrocytes (left). Insert shows a zoom of a single isolated neuron (right), expressing fluorescently tagged morphology marker (blue) and synapse marker (red) in addition to either NPY-pHluorin or NPY-mCherry (not shown). Average island diameter is 377.3 ± 87.75 µm; n=14. (B) Typical example of an isolated neuron stained for the dendrite marker MAP2 (green), grown on a micro island of astrocytes (as in A) stained for the glia marker GFAP (red). Scale bar: 20 µm. (C) Wild type hippocampal neuron (DIV 15) immuno-stained for ChgB, MAP2 and VGLUT1. Whole field of view (left). Scale bar: 20 µm. Zoom off white box (right). Scale bar: 5 µm. White arrows point to ChgB puncta in axons (MAP2 negative). (D) Level of co-localization of ChgB and MAP2 immunoreactivity (Pearson’s coefficient: 0.20 ± 0.01; n=9) and ChgB and VGLUT (Pearson’s coefficient: 0.30 ± 0.02; n=9). (E) Example image of a neurite from hippocampal neurons (DIV 14) infected with Lentivirus encoding NPY-Venus (green) and stained for ChgB (red). Scale bar: 2 µm. (F) Mander’s coefficients for the proportion of endogenous ChgB immuno-reactivity in NPY-Venus puncta: 0.97 ± 0.02; n=14 neurons or proportion of NPY-Venus immuno-reactivity in ChgB puncta: 0.84 ± 0.01; n=14 neurons. (G) Confocal image showing co-localization between NPY-Venus (green) and ChgB (red) and corresponding linescan of intensity for both signals. Scale bar: 1 µm.
High frequency electrical stimulation is most efficient in triggering DCVs fusion

To test which stimulation paradigms were most efficient to trigger DCV fusion, we used NPY fused to pHluorin, a pH sensitive variant of GFP. NPY-pHluorin is quenched at the low pH in the lumen of the DCVs (pH=5.5). Consequently, NPY-pHluorin fluorescence is quenched until vesicles fuse and the lumen comes into contact with the extracellular medium (pH=7.4), leading to a sudden increase of fluorescence (Figure 2A). We tested high potassium K\(^+\) superfusion (45mM KCl), ionomycin application (a Ca\(^{2+}\) ionophore that bypasses voltage-gated Ca\(^{2+}\) channels), single and repetitive electrical stimulation (1AP and 16x50AP@50 Hz) to elicit DCV secretion. 1AP was very inefficient in triggering DCV fusion and failed to elicit fusion in almost 80 % of the cells (Figure 2B, C, fusion events/cell: 0.3 ± 0.2, n=9). High K\(^+\) and ionomycin did trigger DCV fusion (Figure 2B). The majority of the fusion events triggered by high K\(^+\) (fusion events/cell: 22.0 ± 9.7; n=6) and ionomycin (fusion events/cell: 27.6 ± 9.5; n=5) started after the beginning of the stimulation and continued after the end of the paradigm (Figure 2B, D, E). Repetitive, high frequency stimulation of 16 times 50 AP bursts at 50 Hz with 0.5 s interval (16x50AP@50 Hz) triggered fusion synchronous to the stimulation (fusion events/cell: 26.4 ± 7.8; n=10. Figure 2 B and F). We therefore used this paradigm for further experiments.
Figure 2. Different paradigms to elicit DCV fusion

(A) Typical example of fusion events (left) of NPY-pHluorin labeled DCV in a neurite (blue) with the corresponding schematic drawing (right). Fusion pore opening causes a sudden increase of fluorescence intensity corresponding to the increase in intravesicular pH. Bottom panel: Example traces of NPY-pHluorin labeled DCV fusion events. (B) Fusion events measured during 180 s image acquisition at 1 Hz interval in isolated hippocampal neurons (DIV 14-17) infected with NPY-pHluorin (DIV 10) stimulated with: 1AP (fusion events/cell: 0.33 ± 0.2; n=9), 30 s superfusion with 45 mM KCl (fusion events/cell: 22.0 ± 9.6; n=6), 30 s superfusion with 1 µM Ionomycin (fusion events/cell: 27.6 ± 9.55; n=5) and 16 x 50AP at 50 Hz (fusion events/cell: 26.4 ± 7.8; n=10). (C) Normalized cumulative fusion events elicited by electrical stimulation (1AP). Stimulation period is indicated by blue bar. Note that release is not synchronous with the AP stimulation. (D) Normalized cumulative fusion events elicited by 45mM KCl application. Stimulation period is indicated by blue bar. (E) Normalized cumulative fusion events elicited by ionomycin application. Stimulation period is indicated by blue bar. (F) Normalized cumulative fusion events elicited by electrical stimulation (16x50AP@50 Hz). Stimulation period is indicated by blue bar.
DCVs preferentially fuse at synapses in isolated hippocampal neurons

We studied the localization of DCV fusion events in isolated hippocampal neurons using a live-marker for synapses (Synapsin-mCherry, Cijsouw et al. 2014) and NPY-pHluorin as DCV marker (Figure 3A, top panel, C). Repetitive high frequency stimulation (16x50AP@50 Hz) elicited robust Ca$^{2+}$ influx measured with the Ca$^{2+}$ dye Fluo4 (Figure 3A, B). Stimulation dependent Ca$^{2+}$ influx dynamics were similar in soma, synapses and extra-synaptic sites (Figure 3B). We next measured DCV fusion upon this stimulation paradigm in neurites of isolated hippocampal neurons. DCV fusion occurred at Synapsin-mCherry labeled synapses and extra-synaptically (Figure 3C, D). More than 80% of all fusion events occurred at synapses (Figure 3D, synaptic fusion events: 82.6 ± 6.1%, extra-synaptic 17.4 ± 6.1%, p<0.01) and started at the first burst of the stimulation (Figure 3E, F). Extra-synaptic fusion only started at the 2nd burst with the highest increase at the 3rd burst (Figure 3E, F). Hence, fusion at synapses occurred more often than extra-synaptic fusion and fusion at synapses required less stimulation.
Figure 3. DCVs preferentially fuse at synapses

(A) Stills from a dendrite labeled with the synaptic marker Synapsin-mCherry (top) and the Ca²⁺ dye Fluo4 (1 µM) before, during and after electrical stimulation. White arrowheads highlight synapses. (B) Intensity profile of Fluo4 fluorescence intensity changes in soma, synapses and extra-synaptic sites over time upon electrical stimulation (acquisition frequency=1 Hz). Insert: Intensity profile of Fluo4 taken at higher acquisition frequency (2 Hz) shows a peak corresponding to each 50AP burst. (C) Example of a synaptic fusion event (filled arrowhead) and an extra-synaptic fusion event (open arrowhead), Synapsin-mCherry shown in red and cargo-pHluorin fusion events in green (scale bar represents 1 µm). Right panel: Cartoon showing a synaptic (bottom green dot) and extra-synaptic (top green dot) fusion event. A cargo-pHluorin fusion event is scored as synaptic when the fluorescence center of such a fusion event lies within 200 nm (the approximate minimal point spread function of our system) of the Synapsin-mCherry fluorescence (red ellipse). (D) The percentage of synaptic and extra-synaptic DCV fusion events in neurites of WT cells measured with NPY-pHluorin (synaptic fusion events: 82.6 ± 6.1%, extra-synaptic 17.4 ± 6.1%). (E) Frequency distribution of the number of fusion events (blue bars represent 16 bursts of 50 AP at 50 Hz) for synaptic and extra-synaptic events (n=5 cells). (F) Cumulative frequency distribution of the number of fusion events (blue bars represent 16 bursts of 50 AP at 50 Hz) for synaptic and extra-synaptic events, n=5 cells.
Stationary DCVs are more likely to fuse than moving DCVs

To analyze the steps prior to DCV fusion, we expressed NPY-pHluorin together with NPY-mCherry, which is not quenched in acidic vesicles and therefore can be used to track DCVs before fusion. NPY-pHluorin and NPY-mCherry showed a high degree of co-localization in DCVs (Figure 4A, B). When DCVs fused with the plasma membrane, the fluorescent intensity of NPY-pHluorin increased because of fusion pore opening, while the intensity of NPY-mCherry either remained constant, indicating fusion pore opening without release of cargo (Figure 4C), or decreased in case of cargo release (fusion pore opening without release: 21 events; fusion pore opening with cargo release: 14 events; total events: 35, in 5 cells, Figure 4D). Hence, 40% of the fusion events resulted in full release of DCV cargo (Figure 4E).

Prior to fusion, DCVs were stationary or moving (more than 200 nm displacement from their original position before fusion, Figure 4F). In isolated hippocampal neurons, 75% of DCVs were stationary prior to fusion, while approximately 25% moved during the 90 s recording time prior to fusion, which appeared to be the case for the general DCV population, also those that do not fuse (Figure 4G). Moving DCVs required more prolonged stimulation to fuse (Figure 4H, I). Taken together, these data show that we developed methodology to analyze DCV fusion and movement prior to fusion at single vesicle resolution. We found that the majority of DCVs are stationary before fusion. Moreover, fusion of moving DCVs requires more prolonged stimulation.
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**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**
Figure 4. DCVs steps prior to fusion

(A) Cartoon illustrating a fusion event of a DCV labeled with NPY-mCherry (red dots) and NPY-pHluorin (grey and green dots). NPY-mCherry signal reports vesicle trafficking and release of cargo while NPY-pHluorin reports fusion pore opening. (B) Stills (left panel) and kymographs (right panel) from simultaneous imaging on NPY-pHluorin and NPY-mCherry co-localizing in the same DCV. The electrical stimulation (16x50AP@50 Hz) is represented by the blue bars and the superfusion of NH₄⁺ (to un-quench NPY-pHluorin) is represented by the purple box. In this example, DCV fusion does not trigger full release of the cargo. The kymograph shows the dynamic behavior of the DCV (using NPY-mCherry signal) prior to and during fusion pore opening (depicted by the increase in pHluorin signal). (C) Intensity profile of a double-labelled DCV showing a fusion event via the increase in fluorescence of NPY-pHluorin (representing fusion pore opening) while NPY-mCherry stays constant during the increase and decrease of pHluorin intensity, indicating fusion pore opening and closure without substantial cargo release. (D) Intensity profile of a double-labelled DCV showing a fusion event via the increase in fluorescence of NPY-pHluorin and the drop in fluorescence of NPY-mCherry representing cargo release. (E) Percentage of ‘fusion pore opening’ events and ‘cargo-release’ events (fusion pore opening: 14 events, cargo-release events: 21; total events: 35; n=5). (F) Typical examples of kymographs from DCVs labeled with NPY-mCherry and NPY-pHluorin (Red: NPY-mCherry. Green: NPY-pHluorin signal upon fusion pore opening). Based on the mCherry signal, stationary DCVs did not show more than 200 nm displacement from the start of the recording to the onset of fusion. Moving DCVs changed position from the start of the recording to the onset of fusion. Blue bars represent the stimulation (16x50AP@50 Hz). Scale bar, 500nm. (G) Non-fusing and fusing DCVs classified as stationary or moving prior to fusion (Non-fusing: stationary: 66 ± 2.4%, moving: 34 ± 2.4%; n=5, p<0.05; Fusing: stationary prior to fusion: 75 ± 12%, moving: 25 ± 12%; n=10, p<0.05). (H) Cumulative frequency plot examples of 2 neurons showing the onset time of fusion of DCVs classified as stationary (black trace) or moving (blue trace) prior to fusion. (I) Average onset time of fusion for stationary DCVs and moving DCVs (Stationary: 37.8 ± 1.4s; n=20 vesicles. Moving: 43.7 ± 2.1 s, n=9 vesicles; neurons = 2, p<0.05).
Discussion

We describe a single neuron assay to study DCV transport and fusion at single vesicle resolution in a quantitative manner. For this, DCVs were co-labeled with NPY-pHluorin to report DCV fusion pore opening and NPY-mCherry to monitor vesicle dynamics prior to fusion and full cargo release. Using this paradigm we made several observations: first, single AP stimulation was inefficient to elicit DCV fusion in isolated neurons. Stronger stimuli like superfusion with 45mM KCl or with the Ca$^{2+}$ ionophore ionomycin resulted in robust release, while high frequency repetitive stimulation (16x50AP@50 Hz) was the most effective. Second, DCVs fuse at synapses and at extra-synaptic sites with a preference for fusion at synapses. Fusion at synapses was more efficient than extra-synaptic fusion. Third, analysis of DCV dynamics prior to fusion identified DCVs, stationary and mobile before fusion. Stationary vesicles require fewer stimuli to fuse compared to mobile vesicles. Finally, DCV fusion can lead to partial and complete cargo release. We conclude that our novel paradigm is ideally suited to identify the DCV release machinery and vesicle fusion characteristics in a quantitative manner.

Quantitative single neuron assay for DCV fusion

Several studies have addressed the mechanisms of DCV fusion in primary neuronal cultures using live cell imaging of fluorescent cargo (de Wit et al. 2009b; Hartmann et al. 2001; Matsuda et al. 2009). Our novel paradigm extents these findings measuring DCV fusion at single vesicle resolution from an entire neuron. In contrast to previous analyses on mass cultures, in which only a portion of the neurite arbor can be visualized (de Wit et al. 2009b; van de Bospoort et al. 2012), our paradigm allows for quantitative measurements of total DCV pool size and vesicle release probability calculations. Another advantage of our assay is the possibility to simultaneously visualize DCV fusion and trafficking prior to cargo release thanks to the co-expression of NPY-pHluorin and NPY-mCherry in the same DCV. This novel assay will therefore allow in-depth investigation of the proteins that play a role in DCV trafficking, capture and fusion.

Prolonged high frequency stimulation is required for efficient DCV fusion

A single AP is capable of eliciting SV fusion but it is very inefficient to trigger DCV
fusion (Figure 2B). Trains of high frequency stimulation (16x50AP@50 Hz) were more efficient to elicit DCV fusion (Figure 2B and F, in line with Matsuda et al. 2009). Hence, DCV requires robust and repetitive stimulation, triggering prolonged influx of Ca$^{2+}$ through voltage-gated Ca$^{2+}$ channels (VGCC) and probably also Ca$^{2+}$ release from internal stores (Wong et al. 2012). Repetitive stimulation was as effective as prolonged membrane depolarization by superfusion of 45mM KCl or by bypassing Ca$^{2+}$ channels via superfusion of the Ca$^{2+}$ ionophore ionomycin (Figures 2B-F)(de Wit et al. 2009b; Lessmann et al. 2003). These stimulations paradigms all result in robust and prolonged Ca$^{2+}$ influx. Why does DCV fusion require such a strong stimulus? Several plausible explanations may account for this phenomenon: first, many DCVs are mobile and hence connected to the microtubule network. It is conceivable that these vesicles need to be released from the microtubule tracks prior to fusion. Vesicle detachment may involve the release of the (as yet unknown) vesicle adaptor protein from the propelling kinesin (see review Maday et al. 2014) or the release of the DCV from the adaptor protein or release of the kinesin-adaptor protein-DCV complex from the microtubules. Although we lack insight into these mechanisms they are likely to be rate-limiting steps. Second, the majority of DCVs are not pre-docked at the plasma membrane (See chapter 5). These vesicles are therefore further away from Ca$^{2+}$ channels than SVs comprising the RRP and not yet engaged with the vesicle release machinery. This holds even stronger for vesicles that fuse outside synapses, which likely accounts for the fact that extra-synaptic fusion requires even more prolonged stimulation (Figure 3F). These assumptions will be tested in the following Chapters of this thesis.

**DCVs preferentially fuse at synapses**

We found that DCVs can fuse at synaptic and extra-synaptic regions (Figure 3C-D). However, the majority of fusion events occurred at Synapsin-mCherry labeled synapses in line with previous findings from our lab (de Wit et al. 2009b; van de Bospoort et al. 2012) and others (Hartmann et al. 2001; Zhu et al. 1986). Extra-synaptic fusion required prolonged stimulation and showed at slower release rate (Figure 3E-F).

Why do DCVs fuse preferentially at synapses? First, synapses are more densely packed with Ca$^{2+}$ channels resulting in higher local Ca$^{2+}$ levels in synapses than at
extra-synaptic sites. Second, priming proteins that likely are part of the DCV fusion machinery strictly localize at synapses (see Chapter 5). In Chapter 5 we show that changing the sub-cellular localization of the priming protein Munc13, from synaptic to extra-synaptic sites leads to an increase of extra-synaptic DCV fusion efficiency. Thus, the presence of a single DCV priming protein is sufficient to increase DCV fusion at that specific extra-synaptic location. Extra-synaptic fusion in the presence of Munc13-1 is as efficient as synaptic fusion, which argues that the vicinity to Ca\textsuperscript{2+} channels does not significantly increase DCV release probability, at least not with the stimulation paradigm used in our studies. In vivo, DCV fusion is likely triggered by less robust stimulation. In this case the distance from Ca\textsuperscript{2+} channels may play a more important role in determining DCV release probability.

Why is synaptic DCV fusion important for brain function? Many of the molecules contained in DCVs act as modulators of neurotransmitter release (Luikart et al. 2008). Thus, local synaptic release of cargo from a single DCV may be sufficient to affect synaptic transmission. Recent data on the effect of BDNF release upon theta-burst stimulation from post-synaptic sites on timing-dependent LTP is in support of this notion (Edelmann et al. 2015).

**DCVs fuse from stationary and mobile vesicle pools**

Levitan and co-workers reported the high degree of mobility of DCVs in Drosophila neuromuscular junction (NMJ) (Wong et al. 2012). In murine hippocampal neurons we also observed that DCVs move anterogradely, retrogradely and bidirectionally in axons and dendrites even though the majority of DCVs that we observed is stationary. As already proposed by Levitan (Wong et al. 2012) the dynamic behavior of DCVs could be the strategy that the neurons use to ensure DCVs supply to the most distal neurites. Interestingly we observed that also the moving DCVs can fuse to the PM upon Ca\textsuperscript{2+} influx during the 90s of our imaging experiments (around 25% of the total fusion events), hence moving DCVs are fusion competent. Our data show is that stationary DCVs are the predominant population of fusing DCVs probably due to the fact that they are already properly located in the vicinity of the PM and for the same reason they need fewer stimuli to fuse compared to moving DCVs. Nevertheless, neurons do not empty their stationary pool before they start fusing moving DCVs.
DCV fusion does not always lead to full release of cargo

The kiss-and-run fusion mechanism that only leads to partial release of the cargo and reseal of the vesicle has been thoroughly described for DCVs (Xia et al. 2009). This process allows DCVs to fuse again, after the first time, in a different location. It was also shown that the amount of Ca\(^{2+}\) flowing in the neuron affects the probability of a DCV to fuse in one or the other mode (Xia et al. 2009). Here we show that in isolated hippocampal neurons kiss and run is the fusing mode that the majority of the DCVs undergoes when stimulated with trains of AP at high frequency (16x50AP@50 Hz). We did not investigate other paradigm but it is possible that changing paradigm will change this ratio.
Materials and Methods

Plasmids. Neuropeptide Y-Venus was previously described (Nagai et al. 2002) and Neuropeptide Y NPY-pHluorin and –mCherry were generated replacing Venus (de Wit et al. 2006) with pHluorin or mCherry. Synapsin-mCherry was a kind gift of Dr. A. Jeromin (Allen Brain Institute, Seattle, USA). All constructs were sub-cloned into lentiviral vectors produced as described (Naldini et al. 1996), and NPY-mCherry-2ANPY-pHluorin was cloned in Semliki-forest virus backbone. Transduction efficiencies were tested on HEK cells.

Laboratory animals. Mouse embryos were obtained by caesarean section of pregnant females from timed mating. Animals were housed and bred according to institutional and Dutch governmental guidelines.

Primary neuron cultures. 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 seeded on pre-grown cultures of rat astrocytes cells (37,500 cells/well) on 18mm glass coverslips in 12-well plates. For micro-island culture, originally described by (Mennerick et al. 1995), hippocampal neurons were plated at a density of 2,000 neurons/well of a 12-well plate on micro-islands of rat glia as in Wierda et al. 2007. These micro-islands were generated by plating 8,000/well rat glia on UV-sterilized agarose-coated etched glass coverslips stamped with a 0.1mg/ml poly-D-lysine (Sigma) and 0.2 mg/ml rat tail collagen (BD Biosciences) solution.

Lentiviral infection. At DIV10, neuronal cultures were infected with a combination of lentiviruses encoding NPY-pHluorin, NPY-Venus, NPY-mCherry, Synapsin-mCherry.

Imaging. Coverslips were placed in an imaging chamber perfused with Tyrode’s solution (2 mM CaCl₂, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl₂, 20 mM glucose
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and 25 mM HEPES, pH 7.4). All live imaging experiments were performed on a custom-built tandem illumination microscope (TIM; Olympus) consisting of an inverted imaging microscope (IX81; Olympus) and an upright laser-scanning microscope. The inverted microscope part was used for imaging fluorescence using an MT20 light source (Olympus), appropriate filter sets (Semrock), and a 40x oil objective (NA 1.3), or 60x (NA 1.49) for experiments in Figure 7, on an EM charge-coupled device (CCD) camera (C9100-02; Hamamatsu Photonics). Xcellence RT imaging software (Olympus) was used to control the microscope and record the images. In pHluorin experiments intracellular pH was neutralized with Tyrode’s solution containing 50 mM NH₄Cl, which replaced NaCl on an equimolar basis. NH₄⁺ solution was delivered by gravity flow through a capillary placed onto the cells. Solution of 45mM KCl and 1mM ionomycin (1 µM dissolved in DMSO, Sigma) were delivered by gravity flow through a capillary placed close to the cells. The electrical stimuli, 1AP and 16 trains of 50 APs at 50 Hz with 0.5 s interval were delivered using a parallel platinum electrodes placed close to the cell soma delivering 30 mA, 1 ms pulses controlled by a Master 8 system (AMPI) and a stimulus generator (A385RC, World Precision Instruments). Ca²⁺ imaging was performed by incubation the coverslips with 1 µM Fluo4-AM for 10 minutes at 37 °C after which they were placed in the imaging chamber and illuminated with 488 nm light. The acquisition frequency was either 1 or 2 Hz (as noted in the figures). All imaging experiments were performed at room temperature (21-24°C). For DCV fusion assays of single labeled DCVs imaging frequency used was 2 Hz, while for double labeled DCVs the frequency was 1Hz.

Image analysis. Stacks from time-lapse recordings were used to analyze DCV fusion. A 2x2 pixel region (0.4 x 0.4 µm) was analyzed according to the experiment as follows. NPY-pHluorin: fluorescent traces were expressed as fluorescence change (∆F) compared to initial fluorescence (F₀), obtained by averaging the first 4 frames of the time-lapse recording. A fusion event was counted when fluorescence showed a sudden increase of two standard deviations above F₀. Onset of fusion was defined as the first frame with an increase of fluorescence of two standard deviations above F₀. An NPY-pHluorin fusion event, or punctum, was scored as synaptic when the fluorescence center of such a fusion 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 fusion events from neurites and excluded somatic fusion events. Somatic fusion 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 \( \text{NH}_4^+ \) application time lapse using SynD software (Schmitz et al. 2011). When analyzing double labeled DCVs, the number of fusion events was scored as disappearing NPY-mCherry fluorescence. DCVs were categorized as stationary or moving based on the slope of the Kymograph (ImageJ, MultipleKymograph) of NPY-mCherry signal, if the slope of the line over the kymograph was different from 0 at any point of the movie, the DCV was considered moving.

**Fixation and Immunocytochemistry.** Cells were fixed in 4 % formaldehyde (Electron Microscopies Sciences) in PBS, pH 7.4 for 20 min at room temperature (RT) and washed in PBS. First cells were 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 to 2 hours at RT. Primary antibodies used were: polyclonal MAP2 (Abcam, 1:500), polyclonal Chromogranin B (SySy, 1:500), VGLUT1 (SySy, 1:5000), monoclonal GFAP (Sigma, 1:1000). 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) or 60x (NA 1.4).

**Statistics.** Student’s t tests for unpaired data were used throughout the paper, unless otherwise specified. If deviations differed significantly, t tests were Welch corrected. The Mann-Whitney test was used to compare two groups when one or both groups did not pass the normality test. Kolmogorov-Smirnov test was used to verify the whether the data were normally distributed.