Pool size estimations for dense-core vesicles in mammalian CNS neurons

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Abstract

Neuropeptides are essential signaling molecules transported and secreted by dense-core vesicles (DCVs), but the number of DCVs available for secretion, their subcellular distribution, and release probability are unknown. Here, we quantified DCV pool sizes in three types of mammalian CNS neurons in vitro and in vivo. Super-resolution and electron microscopy reveal a total pool of 1,400–18,000 DCVs, correlating with neurite length. Excitatory hippocampal and inhibitory striatal neurons in vitro have a similar DCV density, and thalamo-cortical axons in vivo have a slightly higher density. Synapses contain on average two to three DCVs, at the periphery of synaptic vesicle clusters. DCVs distribute equally in axons and dendrites, but the vast majority (80%) of DCV fusion events occur at axons. The release probability of DCVs is 1–6%, depending on the stimulation. Thus, mammalian CNS neurons contain a large pool of DCVs of which only a small fraction can fuse, preferentially at axons.

Keywords dense-core vesicles; pool sizes; release probability; secretion

Subject Categories Neuroscience

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Introduction

Regulated secretion of chemical signals in the nervous system occurs principally from two organelles, synaptic vesicles and dense-core vesicles (DCVs). In neurons, synaptic vesicles release neurotransmitters at synapses, whereas neuronal DCVs transport and secrete neuropeptides and neurotrophins that regulate brain development, neurogenesis, and synaptic plasticity (Park & Poo, 2013; van den Pol, 2012; Zaben & Gray, 2013; Pang et al., 2004; Malva et al., 2012). Alterations in neuropeptide levels in the brain have been associated with many pathological states, such as cognitive, anxiety, stress, and addiction related disorders (Morales-Medina et al., 2010; McGinty et al., 2010; Meyer-Lindenberg et al., 2011). DCVs are filled with cargo at the trans-Golgi network, undergo maturation steps (Kim et al., 2006), and are transported throughout the neuron by microtubule-based motor proteins of the kinesin and dynein protein families (Zahn et al., 2004; Lo et al., 2011), and upon action potential-triggered calcium influx, DCVs can fuse with the plasma membrane (Gärtner & Staiger, 2002; Frischknecht et al., 2008; van de Bospoort et al., 2012; de Wit et al., 2009; Hartmann et al., 2001; Matsuda et al., 2009; Farina et al., 2015).

Dense-core vesicle fusion requires repetitive and more prolonged stimulation compared to synaptic vesicle fusion (Lundberg et al., 1986; Hartmann et al., 2001; Balkowiec & Katz, 2002; Gärtner & Staiger, 2002; Frischknecht et al., 2008). Synaptic vesicles in neurons and DCVs in endocrine cells have been assigned to different pools depending on their ability to undergo exocytosis. Estimations of synaptic vesicle pool sizes in cultured hippocampal neurons show a total population of 100–200 synaptic vesicles per bouton, containing a fast ready releasable pool (RRP) of approximately five vesicles that are immediately available for secretion upon stimulation, which can be resupplied by a recycling pool of 10–20 vesicles (Neher, 2015; Rizzoli & Betz, 2005; Fernández-Alfonso & Ryan, 2006). Pool sizes for neuronal DCVs have not been characterized. We have previously shown that cultured mouse hippocampal neurons and human induced pluripotent stem cell (iPSC)-derived neurons release approximately 10–100 DCVs upon repetitive stimulation (Arora et al., 2017; Farina et al., 2015; van de Bospoort et al., 2012; Emperador Melero et al., 2017). However, the number of DCVs present in mammalian neurons and probability of their release upon stimulation are currently unknown.

Dense-core vesicles are transported to axons and dendrites by KIF1 motors (Lipka et al., 2016), but it is unclear whether DCVs have a preference for axonal or dendritic release. Previous studies have reported fusion of brain-derived neurotrophic factor (BDNF)-containing DCVs only at dendritic (Hartmann et al., 2001), axonal (Dieni et al., 2012), or both locations (Dean et al., 2009; Matsuda et al., 2009). Furthermore, BDNF-containing DCVs have different fusion properties depending on their location of fusion (Matsuda et al., 2009; Dean et al., 2009). Incomplete cargo release was observed at axons, while full fusion events occurred at dendrites (Matsuda et al., 2009; Dean et al., 2009), indicating more quantal...
BDNF release at dendrites. However, differences in release probability of DCVs at axons or dendrites have not been studied.

In this study, we characterized the total DCV pool in mammalian neurons using immunostainings, confocal, electron, and super-resolution microscopy. The DCV pool in cultured neurons ranged from 1,400 to 18,000 DCVs per neuron and highly correlated with neuron size. The number of DCVs per μm neurite was consistent between different culture systems, but slightly increased in labeled axons in in vivo sections of mouse cortex. Measurements of DCV fusion events showed a releasable pool of 20–400 vesicles, corresponding to 1–6% of the total pool, depending on the type of stimulation. Furthermore, we found that approximately 80% of DCV fusion events occurred at the axon. These data provide the first characterization of total and releasable pools of DCVs in mammalian neurons as well as insights into the subcellular location of DCVs and their release.

Results

The total DCV number per neuron correlates with neurite length and is comparable in vivo and in vitro

To estimate the total number of DCVs per neuron, we first analyzed single neurons cultured on glia micro-islands, sampled from two brain areas, hippocampus and striatum. These neurons were immunostained for the endogenous DCV cargo chromogranin B (ChgB), a secretory granule matrix protein involved in vesicular accumulation and storage of peptides and calcium (Bartolomucci et al., 2011; Fig 1A and B). Quantification of vesicular glutamate transporter 1 (VGLUT1)- or vesicular GABA transporter (VGAT)-positive neurons showed that hippocampal cultures predominantly contain glutamatergic neurons (Fig 1C), while GABAergic neurons predominate in striatal cultures (Fig 1G). To estimate the total number of DCVs per neuron, ChgB puncta were retrieved from 3D confocal image stacks and counted using automated analysis software SynD (Schmitz et al., 2011). Hippocampal neurons contained on average 4,000 ChgB puncta ranging from 750 to 10,000 puncta per neuron (Fig 1D). The total neurite length, quantified using β3-tubulin immunostaining, showed similar variation and was on average 7.35 mm (Fig 1E), resulting in a distribution of approximately 0.54 ChgB puncta per μm neurite (Fig 1F). Striatal neurons contained fewer DCVs, on average 1,360 ChgB puncta (Fig 1H), were smaller than hippocampal neurons (approximately 2.2 mm, Fig 1I), resulting in 0.64 ChgB puncta per μm neurite (Fig 1J). For hippocampal as well as striatal neurons, the number of ChgB puncta per neuron correlated with neurite length (Fig 1K).

To test whether these estimates of DCV density in cultured neurons are comparable to mouse neurons in vivo, we labeled thalamo-cortical axonal projections by stereotaxic injections in the right medio-dorsal thalamus of adeno-associated virus (AAV) expressing mCherry driven by the CAG promoter (Fig 1L). Cortical slices were stained with the canonical DCV cargo ChgA. ChgA puncta were quantified in mCherry-positive axons (Fig 1M–O), and 0.53 ChgA puncta per μm axon were detected (Fig 1P). These data show that different populations of neurons show a similar distribution of DCVs, correlating with neurite length, and similar to DCV density in axonal projections in vivo.

Unitary cargo intensity and super-resolution imaging reveal total DCV pool

The total DCV pool is likely underestimated when counting ChgB puncta in confocal microscopy, due to accumulation of multiple DCVs in resolution-limited space (Fig 2A). To address this, we took two approaches: We analyzed ChgB staining intensity as a proxy for the number of DCVs per punctum and performed super-resolution fluorescence imaging by direct stochastic optical reconstruction microscopy (dSTORM) of ChgB to correlate ChgB intensity measurements at the confocal microscopy level with the number of DCVs resolved with dSTORM (Fig 2B–D). We assume that densely clustered reconstructed dSTORM localizations of ChgB represent one...
**Figure 1.**

*In vitro* and *In vivo* images of hippocampal and striatal neurons showing ChgB puncta per neuron and total neurite length.

**Hippocampal neurons**
- **A** MAP2 GFAP
- **B** ChgB β3-tubulin

**Striatal neurons**
- **C** VGLUT1+; VGAT+; Und.

**Graphs**
- **D, E, F** ChgB puncta/neuron (x1000), Total neurite length (mm), ChgB puncta per μm neurite
- **G, H, I** VGLUT1+; VGAT+; Und.

**K** $r^2 = 0.7876$, $p < 0.0001$

**L, M, N, O, P** Merged images showing ChgB puncta per μm axon, ChgA puncta per μm neurite, and **P** in vivo vs in vitro comparisons.

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Figure 2.
single DCV and each DCV is loaded with a similar amount of ChgB and labeled with a similar number of antibodies. Imaging of Alexa 647-labeled ChgB using dSTORM showed puncta of 60–200 nm in size (Fig 2D). A linear positive correlation between fluorescence intensity of ChgB and the number of dSTORM puncta was observed in both hippocampal and striatal neurons (Fig 2E). The fluorescence intensity distribution of the total pool of ChgB puncta (Fig 2F) was divided in bins corresponding to the fluorescence representing the number of resolved dSTORM puncta (Fig 2E and F, dotted lines). On average, per hippocampal neuron, 47% of the ChgB puncta had a fluorescence intensity representing one dSTORM punctum, 27% an intensity representing two dSTORM puncta, 22% representing three puncta, and 5.3% representing four puncta (Fig 2G, green). Per striatal neuron, on average, 15% of the ChgB puncta had a fluorescence intensity representing one dSTORM punctum, 34% an intensity representing two dSTORM puncta, 11% representing three puncta, and 39% representing four puncta (Fig 2G, red). From these findings, we conclude that the average 4,000 ChgB puncta in hippocampal neurons (Fig 1D) represent a total DCV pool of approximately 7,400 DCVs per neuron and the average 1,360 ChgB puncta in striatal neurons (Fig 1H) represent a total DCV pool of approximately 3,730 DCVs per neuron.

To resolve the number of DCVs in mouse neurons in vivo, we performed stimulated emission depletion (STED) microscopy on cortical brain slices with labeled thalamo-cortical axonal projections and stained for ChgA (Fig 1L–O). STED microscopy of ChgA resolved the confocal signal to single DCV localizations (Fig 2H). Within the axonal mask, on average, 67% of ChgA puncta overlaid with one STED punctum, 29% with two STED puncta, and 4% with three STED puncta (Fig 2I). We conclude that the average 0.53 ChgA puncta per µm axon (Fig 1P) represent approximately 0.72 DCVs per µm axon in vivo.

DCVs are present in axonal, dendritic, and synaptic compartments and concentrate in dendrites

To study the subcellular location of DCVs, single mouse hippocampal neurons were cultured on glia islands and immunostained for ChgB, β3-tubulin as neurite marker (axons and dendrites) and MAP2 as dendritic marker (Fig 3A). ChgB staining showed a punctate expression in MAP2-positive neurites (dendrites) and β3-tubulin-positive, MAP2-negative neurites (axons; Fig 3A–C). Co-staining of ChgB with VGLUT1 as a synapse marker showed DCVs in synapses but also at extra-synaptic sites (Fig 3D). ChgB staining also colocalized with β3-tubulin and showed approximately 50% overlap with MAP2 staining, indicating that ChgB staining originates from dendrites and axons (Fig 3F). VGLUT1 staining highly colocalized with ChgB (Fig 3G, Manders’ coefficient 0.81), while ChgB staining overlapped for approximately 52% with VGLUT1 (Fig 3G, Manders’ coefficient 0.52). This indicates that the majority of synapses contain DCVs. Colocalization of the staining for synaptic vesicle markers VGLUT1 and Synapsin was used as a positive control (Fig 3E and G) and VGLUT1 and Synapsin rotated 90° as negative control (Fig 3G). These data show that DCVs are present in axons and dendrites and in the majority of synapses of cultured hippocampal neurons.

To further study the distribution of DCVs in axonal and dendritic compartments, the number of ChgB puncta was calculated in all β3-tubulin-positive neurites and in MAP2-positive dendrites. On average, 4,000 ChgB puncta were present in all neurites per neuron, of which 50% was present in MAP2-positive dendrites (Fig 3H and I). When subtracting the ChgB puncta in MAP2-labeled dendrites from all β3-tubulin-positive neurites, approximately 50% of ChgB puncta were in β3-tubulin-positive, MAP2-negative axons (Fig 3H and I). The number of ChgB puncta per µm neurite was higher in dendrites than in axons (Fig 3K), because dendritic length per neuron was significantly shorter than axonal length (Fig 3J). The mean intensity per ChgB punctum was higher in dendritic areas (Fig 3L), and the intensity histogram showed a shift toward higher peak intensity for the dendritic population of ChgB puncta compared to the total pool (Fig 3M). To validate the distribution of ChgB puncta in axons and dendrites in single neurons, ChgB puncta were measured in SM1312-positive axons and MAP2-positive dendrites (Fig 3N and O). The number of ChgB puncta per µm neurite (Fig 3N) and the mean intensity per ChgB punctum were higher in dendrites compared to axons (Fig 3O). This indicates that dendrites, on average, contain...
more intense ChgB puncta, suggesting that multiple DCVs cluster at specific locations in dendrites.

Axonal and dendritic markers showed high overlap in single cultured neurons (Fig EV1). Therefore, we assessed the distribution of ChgB puncta in a culture with sparse fluorescently labeled neurons (Fig EV2), which are not limited in space by a glial island. This offers better separation between axons and dendrites. The number of ChgB puncta per μm was similar to single neuron cultures (compare Fig 3K and P) and significantly higher in dendrites compared to axons, also similar to single neuron cultures (Fig 3P, mean dendrites: 0.87, mean axons: 0.31, *P < 0.0001*). The mean intensity per ChgB puncta was also higher in dendrites compared to axons (Fig 3Q). Together, these data show that DCVs are more enriched in dendrites compared to axons in cultured hippocampal neurons.

To test whether these estimates of DCV distribution in cultured neurons are comparable to mouse neurons in vivo, we labeled dentate gyrus (DG) granule neurons by stereotaxic injections of AAV expressing mCherry driven by the CAG promoter (Fig 3R). Hippocampal slices were prepared using the “magic cut” to visualize the mossy fibers (Bischofberger et al, 2006), and ChgA puncta were quantified in mCherry-positive axons and dendrites (Fig 3R). 0.42 ChgA puncta per μm dendrite and 0.46 ChgA puncta per μm axon were detected (Fig 3S). These data show that in DG granule neurons in vivo, DCVs are equally distributed between axons and dendrites.

**Average synapses contain two to three DCVs, at the periphery of the synaptic vesicle cluster**

Dense-core vesicles are smaller than the resolution limit of confocal microscopy (Fig 2A, van de Bospoort et al, 2012). To study the synaptic localization of DCVs, we therefore used dSTORM imaging of ChgB (Fig 4A–C) and electron microscopy (Fig 4D and E). dSTORM imaging showed Alexa 647-labeled ChgB puncta at synapses, which localized to the border of VGLUT1-positive synaptic vesicle clusters (Fig 4C, zooms a–c). dSTORM ChgB puncta were also found in axons and dendrites outside synapses (Fig 4C, middle panel, arrow).

Electron microscopy (EM) confirmed that DCVs are present at the border of synaptic vesicle clusters (Fig 4D and E). Fifty-four DCVs were found in 110 random presynaptic sections, approximately 0.45 DCV per section (Fig 4F, inset). Seventy percent of synaptic sections did not contain DCVs, 20% contained 1 DCV, and the remaining 10% contained multiple DCVs (Fig 4F). From the 110 random synaptic sections, only five showed a DCV in postsynaptic compartments. Using the volume of an average hippocampal bouton (Schikorski & Stevens, 1997) and the volume of synaptic EM sections, hippocampal synapses are predicted to contain on average 2.6 DCVs, with approximately 30% chance to find a DCV in a synaptic section (Fig 4F and see Materials and Methods). The average DCV diameter was 70 nm (Fig 4G), comparable with previous observations (Richardson, 1962; van de Bospoort et al, 2012). The distance to the active zone (AZ), measured from the center of the DCV to the center of the AZ, was on average 0.34 μm (Fig 4H and I), while the shortest distance from the center of the DCV to the AZ was on average 0.25 μm (Fig 4I; inset histogram). Compared to synaptic vesicles, DCVs were located further away from the AZ (Fig 4J), although we cannot exclude the presence of part of the active zone at a closer distance in another plane of the section. Approximately 80% of DCVs resided outside the synaptic vesicle cluster (Fig 4K and L), at close proximity to the border of the synaptic vesicle cluster (Fig 4M). Together, these results show that the
Figure 3.
DCV pool sizes in mammalian neurons
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average synapse/bouton contains two to three DCVs and that these DCVs are typically located at the periphery of the synaptic vesicle cluster (Figs 3D and 4).

**DCVs have a release probability of 1–6%, depending on the type of stimulation**

To quantify DCV fusion events, we expressed the DCV reporter neuropeptide Y (NPY) fused to pH-sensitive EGFP (pHluorin; Palareti et al, 2016; Arora et al, 2017; van Keimpema et al, 2017; Emperador Melero et al, 2017; Farina et al, 2015). NPY-pHluorin is a reliable marker for neuropeptide release, irrespective of which type of neuron or which neuropeptide is endogenously expressed in cultured mouse hippocampal neurons (Arora et al, 2017), mouse cortical neurons (van Keimpema et al, 2017), or human IPSC-derived neuronal micro-networks (Emperador Melero et al, 2017). This reporter was targeted to DCVs in all neurons and co-localized with the vast majority (90%) of ChgB-labeled DCVs (Fig 5A–C). The number of ChgB puncta and their mean intensity did not differ between infected and non-infected neurons (Fig 5D and E). NPY-pHluorin-infected neurons showed a strong correlation between number of ChgB puncta and total neurite length (Fig 5F), similar to non-infected neurons (see Fig 1K). These data show NPY-pHluorin expression did not affect the total number of DCVs per neuron.

Dense-core vesicle fusion is triggered by depolarization-induced Ca^{2+}-influx (Matsuda et al, 2009; de Wit et al, 2009; van de Bospoort et al, 2012; Hartmann et al, 2001; Balkowiec & Katz, 2002; Gätter & Staiger, 2002). To assess which fraction of the total DCV pool can be released upon stimulation, DCV fusion was measured by expressing NPY-pHluorin in single hippocampal neurons. In the low pH environment of the DCV lumen, NPY-pHluorin is quenched (Fig 5A–a). A rapid increase in fluorescence is detected upon fusion pore opening when pHluorin de-quenches (P 5A-b), followed by a rapid decline in fluorescence once the cargo is fully released or upon fusion pore closure and reacidification of the vesicle lumen (Fig 5A-c). Different stimulation paradigms to trigger DCV fusion were compared: single action potentials (AP), a repetitive electrical stimulation train (16 bursts of 50 AP at 50 Hz) known to be efficient in triggering DCV fusion (van de Bospoort et al, 2012; Farina et al, 2015; Arora et al, 2017; van Keimpema et al, 2017; Emperador Melero et al, 2017; Hartmann et al, 2001), superfusion with 60 mM KCl, or the calcium ionophore Ionomycin (5 μM; Fig 5G). Single AP stimulation resulted in a transient calcium influx (Fig 5G inset) but did not elicit DCV fusion (Fig 5H and I). Repetitive electrical stimulation (16 × 50 AP, 50 Hz) resulted in prolonged calcium influx and on average 120 DCV fusion events per cell (Fig 5G–I). Application of 60 mM KCl resulted in on average 80 DCV fusion events per cell during the stimulation (Fig 5G–I). Ionomycin application led to a slower calcium influx and delayed DCV fusion of approximately 30 DCVs per neuron (Fig 5G and H). These data show that DCV fusion occurs exclusively upon a repetitive or prolonged rise in intracellular calcium levels, either by entry through voltage-gated calcium channels during depolarization (electrical stimulation, 60 mM KCl) or by increasing the permeability of the membrane by application of a calcium ionophore.

The fraction of fusing DCVs over the total DCV pool (release probability) was calculated for the different types of stimulation. When computing the total DCV pool, a similar correlation was observed for NPY-pHluorin intensity and the number of ChgB-labeled DCVs resolved at the super-resolution level (Fig EV3), and was used to correct the total DCV pool (as in Fig 2). Upon repetitive electrical stimulation, a release probability of approximately 6% was measured (Fig 5J). Application of 60 mM KCl resulted in a release probability of approximately 4%, while Ionomycin elicited fusion of approximately 1% (Fig 5J).

To test possible differences in release capacity between excitatory and inhibitory neurons, viral infection of mKate2 driven by the CaMKII promoter was used as a marker for glutamatergic neurons (Benson et al, 1992; Wang et al, 2013). Upon repetitive electrical stimulation, both CaMKII-positive and CaMKII-negative neurons showed abundant DCV fusion with a release probability that was not significantly different between the two groups, although strong variation between neurons occurred (Fig 5K). Together, these data show that DCVs have a release probability of 1–6%, depending on the type of stimulation, which is similar in excitatory and inhibitory hippocampal neurons.

**DCVs fuse predominantly at axons**

To test whether DCVs fuse preferentially at axons or dendrites, DCVs in network cultures were labeled with NPY-mCherry, which reports full cargo release events as complete disappearance of
Figure 5.

**A**
Puncta distribution of NPY-pHluorin and ChgB at pH 5.5 and pH 7.4. 

**B**
Images showing the fluorescence of NPY-pHluorin and ChgB puncta in a neuron.

**C**
Bar graph showing the mean ChgB intensity per puncta (a.u.) for Control and NPY-pH conditions.

**D**
Graph displaying the number of ChgB puncta per neuron (x1000) for Control and NPY-pH conditions.

**E**
Graph showing the mean ChgB intensity per puncta (a.u.) for Control and NPY-pH conditions.

**F**
Graph illustrating the relationship between total neurite length (mm) and the number of ChgB puncta per neuron (x1000) for Control and NPY-pH conditions.

**G**
Diagram showing DCV fusion events over time for different stimuli: 1 AP, 5 μM Ionomycin, 60 mM KCl, 16x50AP, 50 Hz.

**H**
Graph illustrating cumulative fusion events per neuron for different stimuli: 1 AP, 5 μM Ionomycin, 60 mM KCl, 16x50AP, 50 Hz.

**I**
Bar graph showing the number of fusion events per neuron for different stimuli: 1 AP, 16x50AP, 50 Hz, 60 mM KCl, 5 μM Ionomycin.

**J**
Graph illustrating release probability for different stimuli: 1 AP, 16x50AP, 50 Hz, 60 mM KCl, 5 μM Ionomycin.

**K**
Graph showing release probability for different stimuli: CaMKII+, CaMKII-, DCV pool sizes in mammalian neurons.
fluorescence signal upon fusion (Fig 6A), and neurons were sparse-labeled with membrane-bound mEGFP (Fig 6B). Axons were defined as small, spineless neurites positive for the axonal marker SMI312 in post hoc stainings, and dendrites as thick, spine-containing MAP2-positive neurites (Fig EV2). Upon maximal stimulation (16 bursts of 50 AP at 50 Hz, see above), DCV fusion events were observed in both axons and dendrites (Fig 6C and D). DCV fusion occurred predominantly at axons (Fig 6C and D). Axonal release was observed during the whole stimulation period, while dendritic release occurred predominantly in the first 10 s of stimulation with only a few events in the last bursts of stimulation (Fig 6C and D). Fusion onset was slightly earlier in axons compared to dendrites (Fig 6E; normalized cumulative plot). Per neuron, approximately 80% of the DCV fusion events occurred at axons (Fig 6F). Together, these data show that DCVs are fourfold more likely to fuse at axons.

Discussion

This study characterized the number of DCVs present in individual mouse CNS neurons, their distribution, and release probability. Cultured hippocampal and striatal neurons contained a large pool of DCVs ranging from 1,400 to 18,000 DCVs per neuron, correlating with neurite length. DCVs were present in synapses, axons, and dendrites but were enriched in dendrites. Their distribution was slightly higher in axonal in vivo sections of mouse cortex compared to cultured neurons. DCVs showed a release probability of 1–6% depending on the stimulation, with repetitive electrical stimulation being most efficient, and fused predominantly at axons.

Total DCV pool and DCV fusion quantified at single vesicle resolution indicate a release probability of 1–6%

We used immunostainings and dSTORM super-resolution imaging of ChgB and NPY-pHluorin on single cultured neurons to provide accurate estimations of the total DCV pool size in mammalian neurons (Figs 2 and EV3). Using this method, a XY resolution of 20–50 nm was reached, but only 100 nm in the Z-direction and partial overlapping vesicles in the Z-dimension might not be fully reconstructed. Hence, although analysis of the intensity profiles confirmed that estimations of DCV numbers are generally accurate, some underestimation of the absolute number of DCVs cannot be excluded. Given these considerations, we conclude that cultured hippocampal neurons contain a total pool of 1,400–18,000 DCVs (Figs 1 and 2). Upon stimulation, 20–400 DCVs fuse, corresponding to 1–6% of the total pool (Fig 5). This secretion efficiency is similar to sporadic observations in axons and whole neurons (Shimoo et al, 2015; Xia et al, 2009; van de Bospoort et al, 2012; Farina et al, 2015; Emperador Melero et al, 2017).

The DCV release probability is comparable to secretory granule fusion in adrenal chromaffin cells and β-cells: Adrenal chromaffin cells that release catecholamines, adrenaline, and noradrenaline from secretory granules have an estimated total pool of approximately 2,000 vesicles with an RRP of 140, approximately 7% of the total pool (Sorensen, 2004; Voets et al, 1999) and murine pancreatic β-cells, which secrete insulin-containing granules, have a total pool of 10,000 insulin-containing vesicles, with an RRP of 200–300, 2–3% of the total pool (Dean, 1973; Olofsson et al, 2002). In cultured hippocampal synapses, a total pool of 100–200 synaptic vesicles per bouton has been estimated, with an RRP of approximately five vesicles and a recycling pool of 10–20 vesicles (Schikorski & Stevens, 1997; Neher, 2015; Rizzoli & Betz, 2005). The Calyx of Held contains approximately 500 release sites with each containing 250 synaptic vesicles, a recycling pool of 20,000–42,000 vesicles, and an RRP of 2,000–4,800 vesicles (De Lange et al, 2003; Yamashita et al, 2005; Fernández-Alfonso & Ryan, 2006). For synaptic vesicles, the release probability is defined as the reliability of an action potential to trigger synaptic vesicle fusion and is on average < 0.3, but ranging from < 0.05 to 0.9 for different nerve terminals (Zucker, 1973; Rosenmund et al, 1993; Murthy et al, 1997). When release probability is defined in the same way for DCVs, the release probability in the neurons studied here is zero (Fig 5), indicating a marked difference in the likelihood...
that the two secretory organelles will fuse and release their content upon mild stimulation. However, the release probability for neuropeptide secretion has been typically defined as the fraction of fusing DCVs during extensive stimulation over the total DCV pool, i.e., the equivalent of the “release pool” for synaptic vesicles (Shimojo et al., 2015; Xia et al., 2009; Emperador Melero et al., 2017). Similar repetitive stimulation depletes approximately 40% of the total synaptic vesicle pool [i.e., the release pool, (Fernández-Alfonso & Ryan, 2006; van Keimpema et al., 2017)]. Hence, also for stronger stimulation, synaptic vesicles are on average much more likely to fuse than DCVs, probably indicating an evolutionary adaptation to faster, more efficient secretion.

For the different secretion pathways, different phases of release have been characterized. Chromaffin cells have a first initial burst, followed by a sustained phase (Voets et al., 1999) and glucose-stimulated insulin secretion consists of an early transient first phase and a sustained secondary phase (Olofsson et al., 2002). Neuronal DCV fusion upon repetitive electrical stimulation shows a peak in fusion only after several bursts of stimulation (Figs 5G and H, and 6C–E) and therefore does not seem to have a fast...
component. Only upon many action potentials, more than 50 in the first burst, the first DCV starts to fuse. This observation confirms original findings in the submaxillary gland of the cat and the pig spleen in vivo (Andersson et al., 1982; Lundberg & Hökfelt, 1983; Lundberg et al., 1989), proposing “chemical frequency coding”, i.e., the release of different chemical signals at different stimulation intensities.

**Several factors may explain why DCV fusion requires prolonged stimulation**

The stimulation-secretion coupling for DCV fusion is remarkably different compared to other secretory pathways. The requirement for prolonged stimulation may be explained by (i) the translocation of DCVs to release sites, (ii) different calcium sensing principles for DCVs, and/or (iii) different calcium signals/sources required for DCV fusion.

(i) Unlike synaptic vesicles, DCVs are often highly mobile and generally not preloaded at a release site. Hence, DCVs often need to translocate to a release site, which may require dissociation from the microtubule tracks that transport mobile DCVs (Zahn et al., 2004; Lo et al., 2011), and engage with the release machinery before exocytosis can occur. Synaptic DCVs are predominantly located at the border of the synaptic vesicle cluster (Fig 4), and although DCVs do not accumulate at synapses, approximately 60% of fusion events occur at the synapse (van de Bospoort et al., 2012; Farina et al., 2015). This preferential synaptic fusion is much slower than synaptic vesicle fusion, consistent with the observation that synaptic DCVs are not docked and therefore not in close contact with the release machinery. The synaptic vesicle cluster may act as a barrier for DCVs to reach their release site.

(ii) Tight spatial coupling between synaptic vesicles and voltage-gated calcium channels regulates fast exocytosis (Zucker & Fogelson, 1986; Llinás et al., 1992). High levels of prolonged calcium influx are probably required before calcium reaches DCVs and can activate DCV calcium sensors (Fig 5G). Although it is well established that DCV fusion critically depends on Ca²⁺ (Hartmann et al., 2001; Balkowiec & Katz, 2002; de Wit et al., 2009), it remains unclear how Ca²⁺ actually activates fusion of vesicles that are not yet docked and are therefore not yet engaged with the components of the fusion machinery known to reside at the plasma membrane.

(iii) Presynaptic functions are controlled by the calcium content of the axonal endoplasmic reticulum (ER) which regulates changes in synaptic cytosolic calcium levels during prolonged activity (de Juan-Sanz et al., 2017). In *Drosophila* motor neurons, it was shown that ryanodine receptor (RyR)-mediated calcium release from presynaptic ER and resulting calmodulin kinase II activation are required for post-tetanic potentiation of neuropeptide secretion (Shakiryanova et al., 2007). Furthermore, the activation of cAMP-dependent protein kinase may be required for DCV fusion (Shakiryanova et al., 2011). Therefore, calcium-induced calcium influx from internal stores, which requires prolonged activity, may be a unique aspect of DCV fusion, which discriminates this type of chemical signaling from synaptic transmission (Nizami et al., 2010; Shakiryanova et al., 2007, 2011).

**Mammalian DCVs do not accumulate at synapses**

In *Drosophila* motor neurons, bidirectional transported DCVs are captured at nerve terminals upon activity, providing a constant availability of synaptic DCVs (Wong et al., 2012). In this study, electron microscopy and dSTORM imaging showed that DCVs are localized at the perimeter of the synaptic vesicle cluster in resting mammalian CNS neurons, but do not accumulate at synapses (Fig 4). In chronic experiments (chronic disinhibition) and upon 90-s depolarization with high K⁺, synaptic capture has also been described for neurotyspin containing DCVs in mammalian neurons (Frischknecht et al., 2008). Hence, synaptic DCV capture may also exist in mammalian neurons, but occurs mostly during/after stimulation and requires strong/permanent stimulation, and in resting neurons, DCVs are apparently not accumulated.

**DCVs are equally distributed over axons and dendrites but fuse predominantly in axons**

Dense-core vesicles are present in axons and dendrites in cultured hippocampal neurons (Fig 3). This is in line with previous studies, which have shown that KIF1 motors transport DCVs to axons and dendrites (Lipka et al., 2016; Zahn et al., 2004). The current study suggests that a similar number of DCVs ends up in axons and dendrites (Fig 3). The intensity of ChgB puncta was on average higher in dendrites, suggesting that multiple DCVs may cluster at certain locations in dendrites and the total population of dendritic DCVs may be somewhat underestimated. Furthermore, super-resolution microscopy showed a linear relation between intensity of ChgB puncta and the number of DCVs (Fig 2), indicating that most likely dendrites contain more DCVs than axons. Conversely, in cultured neurons, axons tend to run along and wrap around dendrites (Fig EV1) and some ChgB signal may therefore be incorrectly labeled as dendritic. Analysis of sparse-labeled networks cultures provided better separation of axons from dendrites and led to the same conclusion as in single cultured neurons (Fig 3) that DCVs have a higher density in dendrites compared to axons.

Interestingly, the majority of DCV fusion events occurred at axons in the neurons studied here (Fig 6). Hence, axonal DCVs have a much higher release probability compared to dendritic DCVs. This is markedly different from a few specialized neurons in the mammalian brain, such as paraventricular and supraoptic nuclei (PVN/SON; Ludwig & Leng, 2006) or raphe nucleus neurons. However, for the majority of neurons, this might be explained by the availability of SNARE proteins, and other proteins involved in membrane fusion in axons. Furthermore, it has previously been shown that BDNF-containing DCVs show different fusion properties at axons, where incomplete cargo release was observed, while full fusion events occurred at dendrites (Dean et al., 2009; Matsuda et al., 2009). However, the NPY-mCherry reporter we used in this study to distinguish between axonal and dendritic release (Fig 6) did not show differences in fusion characteristics between axons and dendrites. NPY-mCherry detects full cargo release and does not discriminate between different types of release. In conclusion, in the neurons studied here, DCV fusion events occur predominantly at the axon, while DCVs accumulate more in dendrites, indicating strong differences in the probability of release based on the cellular localization of a DCV.
Materials and Methods

Animals

Embryonic day (E) 18.5 C57BL/6 mouse embryos were used for neuronal cultures. Animals were housed and bred according to institutional and Dutch governmental guidelines (DEC-FGA 11-03 and AVD112002017824).

Primary neuronal cultures

Dissociated hippocampal or striatal neuron cultures were prepared from E18.5 C57BL/6 mouse embryos. Cerebral cortices were dissected free of meninges in Hanks’ balanced salt solution (Sigma, H9394) supplemented with 10 mM HEPES (Gibco, 15630-056). The hippocampi or striata were isolated from the tissue and digested with 0.25% trypsin (Gibco, 15090-046) in Hanks’ HEPES for 20 min at 37°C. Hippocampi were washed three times with Hanks’ HEPES and triturated with fire-polished glass pipettes. Dissociated neurons were counted and plated in neurobasal medium (Gibco, 21103-049) supplemented with 2% B-27 (Gibco, 17504-044), 1.8% HEPES, 0.25% Glutamax (Gibco, 35050-038), and 0.1% penicillin–streptomycin (Gibco, 15140-122). To obtain single neuron micro-islnd cultures, hippocampal neurons were plated in 12-well plates at a density of 1,400–1,500 cells/well on 18-mm glass coverslips containing micro-islnds of rat glia. For glia preparations, newborn pups from female Wistar rats were used. Micro-islnds were generated as described previously (Meijer et al., 2015) by plating 8,000/well rat glia on UV-sterilized agarose (Type II-A; Sigma, A9918)-coated etched glass coverslips stamped with a mixture of 0.1 mg/ml poly-D-lysine (Sigma, P6407), 0.7 mg/ml rat tail collagen (BD Biosciences, 354236), and 10 mM acetic acid (Sigma, 45731).

For super-resolution microscopy experiments, coverslips were coated with 0.01% poly-L-ornithine (Sigma, P4957) and 2.5 μg/ml laminin (Sigma, L2020) diluted in Dulbecco’s phosphate-buffered saline (DPBS; Gibco, 14190-250) overnight at room temperature (RT). Dissociated hippocampal neurons or striatum neurons were plated in low density (1,000–3,000 neurons) on coated coverslip and placed in close proximity to astrocytes previously grown on the bottom of 12-well plates to provide glia support (Kaech & Banker, 2006).

Constructs

NPY-pHluorin or NPY-mCherry were generated by replacing Venus in NPY-Venus (Nagai et al., 2002) with super-ecliptic pHluorin or mCherry. CaMKII-mKate2 was a kind gift of Oleksandr Shcheglovtov (University of Utah School of Medicine, Salt Lake City, USA). Synapsin-mCherry was a kind gift of Dr. A. Jeromin (Allen Brain Institute, Seattle, USA) and Synapsin-ECFP was obtained by replacing mCherry with ECFP.

A tetracycline-controlled Tet-On gene expression system containing NPY-mCherry and membrane-bound mEGFP was used to determine the axonal or dendritic localization of DCV fusion. Membrane-bound mEGFP was generated by the addition of the last 20 amino acids (KMSKDGGKKKKSSTTKCVIM) of K-Ras4B, representing a canonical palmitoylation signal (Welman et al., 2000).

All constructs were sequence-verified and subcloned into pLenti vectors, and viral particles were produced (Naldini et al., 1996).

Adeno-associated virus particles of CAG-mCherry were used for stereotoxic injections of mice.

Infection

For DCV fusion experiments neuronal cultures were infected with lentiviral particles encoding for NPY-pHluorin at DIV 9–10. CaMKII-mKate2 was added at DIV7.

To determine axonal or dendritic localization of release, WT hippocampal neurons were infected in suspension with TRE(pr) hNPYmCherry-ires2-KrasGFP-pSyn(pr)rtTA2 and incubated for 2 h at 37°C, 5% CO2. Cells were centrifuged for 5 min at 800 rpm, washed three times in DMEM, resuspended in neurobasal medium supplemented with 2% B-27, and plated (5,000 neurons/well) on coverslips containing 25,000 WT hippocampal neurons per well. 2 μg/ml doxycycline hyclate (Sigma, D9891) was added at DIV 10–11, and cells were imaged at DIV 16–17.

Immunohistochemistry of mouse brain tissue

Stereotoxic injection

For viral injection (Wimmer et al., 2004), mice were injected s.c. with 0.1 mg/kg buprenorphine, anaeasthetized with a constant flow of 1–2% isofluorane, and placed in a stereotoxic head holder (Kopf). Before cutting of the skin, xylocain solution (2%) was injected subcutaneously. A small hole in the skull was made with a dental drill over the injection site. Then, for thalamic injections, 1 μl AAV particles was slowly injected into the right medio-dorsal thalamus (Coordinates from bregma: x = 1.13, y = –0.82, z = –3.28). For stainings in the dentate gyrus, animals were injected with 100 nl AAV particles (coordinates: AP: –3.16 ML + –2.2, DV: 2.3). After surgery, mice received carprofen (Rimadyl, 5 mg/kg; Pfizer). Mice were single-housed after surgery.

Immunohistochemistry

For immunohistochemistry, mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brains were postfixed overnight in 4% paraformaldehyde (PFA) and then sliced into 70-μm-thick sections and stored in PBS. To preserve dentate gyrus granule cells with dendrites and mossy fibers in the same slice, brains were sliced horizontally under an angel as described elsewhere (Bischoffberger et al., 2006). For increased permeabilization, the slices were treated with 1% sodium dodecyl sulfate (SDS) in PBS for 5 min before antibody staining as described previously (Knabbe et al., 2018). For antibody staining, slices were incubated for 1 h in PBS containing 5% normal goat serum (NGS), 1% bovine serum albumin (BSA), 1% cold fish gelatin, and 0.5% Triton X-100. Incubations with primary antibody were done overnight at 4°C. The secondary antibody was incubated for 1–2 h at room temperature. Primary antibody was chromosome A (Sysy, 259003; 1:500). Alexa Fluor antibody from Invitrogen was used as secondary antibody in a dilution of 1:500. The slices were mounted in SlowFade Gold (Life Technologies) and imaged on a Leica SP8 inverted confocal microscope with a 63× oil immersion objective (NA = 1.4) and maximal resolution in x, y and z (0.08 × 0.08 × 0.3 μm).
**Primary antibodies used were as follows**

Polyclonal rabbit chromogranin B (1:500; SySy 25103), monoclonal mouse β3-tubulin (1:500; Millipore MAB1637), polyclonal chicken MAP2 (1:10,000; Abcam ab5392), monoclonal mouse GFAP (1:1,000; Sigma G3893), monoclonal mouse SMI-312 (1:5,000; Covance), polyclonal guinea pig VGLUT1 (1:1,000; Millipore ABS905), polyclonal rabbit VGAT (1:500; SySy 131002), and polyclonal rabbit Synapsin I&II (1:1,000; Sigma G3893), monoclonal mouse SMI-312 (1:5,000; SySy 131002), and polyclonal rabbit Synapsin I&II (1:1,000; E028).

Alexa Fluor conjugated secondary antibodies were from Invitrogen (1:1,000). Cover slips were washed again and mounted with Mowiol 4-88 (Sigma, 81381) and examined on a confocal microscope [Nikon A1R with LU4A laser unit or Zeiss LSM 510 confocal microscope using a 40× oil immersion objective (NA = 1.3)]. Z-stacks (5 steps of 0.5 μm) were acquired, and resulting maximum projection images were used for analysis. A 60× oil immersion objective (NA = 1.4) was used for detailed zooms. To count the number of VGLUT1+ or VGAT1+ neurons in hippocampal or striatal cultures, cover slips were scanned to ensure every neuron was captured. Confocal settings were kept constant for all scans within an experiment.

**Electron microscopy**

Hippocampal autaptic cultured neurons were fixed for 60 min at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed for 1 h at room temperature with 1% OsO₄/1% K₂Ru(CN)₅ in double distilled water. Following dehydration through a series of increasing ethanol concentrations, cells were embedded in Epon and polymerized for 24 h at 65°C. After polymerization of the Epon, the coverslip was removed by alternately dipping in liquid nitrogen and hot water. Cells were selected by observing the Epon-embedded culture under the light microscope, and mounted on prepolymerized Epon blocks for thin sectioning. Ultrathin sections (approximately 70 nm) were cut parallel to the cell monolayer and collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate.

Synapses were selected at low magnification using a JEOL 1010 electron microscope. All analyses were performed on single ultrathin sections of randomly selected synapses. Digital images of synapses were taken at 100,000× magnification using iTEM software (EMISYS, Germany). For all morphological analyses, we selected only synapses with intact synaptic plasma membranes with a recognizable pre- and postsynaptic density and clear synaptic vesicle membranes. DCVs were recognized as an electron dense core surrounded by a vesicular membrane. Measurements were performed using ImageJ software.

To calculate the average number of DCVs per synapse, the volume of a typical synapse (assumed to be a sphere) was divided by volume of the synaptic area of a typical slice (assumed to be a cylinder) and was calculated as follows:

\[ \text{Volume cultured hippocampal synapse} = \frac{4}{3} \pi r^3 = 0.122 \mu m^3 \]

(Schikorsi & Stevens, 1997), \( r = 0.307 \)

\[ \text{Volume synaptic slice} = r^2 \pi \times d = 0.02073 \mu m^3, \]

\( d = \text{thickness slice} = 70 \text{ nm} \)

\[ \text{DCVs per synapse} = \frac{\text{Volume synapse}}{\text{Volume synaptic slice} \times \text{average DCV per synapse}} \]

\[ = \frac{5.8851 \times 0.45}{2.633} \]

\[ \text{Chance of finding DCV in synaptic section} : \]

\[ \frac{70 \text{ nm}}{\text{Thickness synaptic slice}} \times \frac{1.3}{\text{Diameter/thickness synapse}} \]

\[ = 0.304 \]

**Direct stochastic optical reconstruction microscopy (dSTORM) acquisition**

PFA fixation and staining protocol for dSTORM imaging were optimized according to (Whelan & Bell, 2015) and included 12 min fixation with 3.7% PFA-PBS (37°C followed by several PBS washes lasting 30 s, 1, 5, 10, and 15 min). Normal immunocytochemistry protocol was followed using 5% BSA-PBS as blocking buffer. Cover slips were stored in PBS until image acquisition. Coverslips were incubated with fluorescent microspheres (Invitrogen constellation microspheres c14837) and placed in a closed imaging chamber containing photo-switchable buffers with oxygen scavengers consisting of 50 mM MEA-HCl (Sigma M6500), enzyme solution (40 μg/ml catalase (Sigma C1345), 4 mM TCEP (Sigma C4706), 25 mM KCl, 0.5 mg/ml glucose oxidase (Sigma G2133), 20 mM Tris–HCl pH 7.5, 50% glycerin), and a glucose–glycerin base solution (10% glycerin, 10% glucose).

Imaging was performed on a Nikon Ti-E Eclipse inverted microscope system with LU4A Laser unit and an EMCCD Camera (Andor DU-897) equipped with 647-nm laser, a perfect focus system and an astigmatic lens for 3D localizations (Nikon). A 100× oil objective lens (NA 1.49) and appropriate filtersets were used for dSTORM acquisition. NS elements software (version 4.30) was used to control acquisition in streaming mode of 256 × 256 pixel region of interest, 8,000 frames per region were acquired. After transferring the fluorophores to the OFF state, laser power was adjusted to keep the number of the stochastically activated molecules constant and well separated during the acquisition.

**Stimulated emission depletion microscopy (STED) acquisition**

Stimulated emission depletion microscopy (STED) was performed on a Leica TCS SP8 STED 3× microscope, Leica Microsystems (Wetzlar, Germany). Samples were irradiated with a pulsed white light laser at wavelengths 499 and 590 nm. A continuous wave STED laser line at a wavelength of 592 nm, Leica Microsystems, was used for depletion of the 488 nm fluorophore, reaching a lateral resolution of ~70 nm. The signal was detected using a gated hybrid
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detector (HyD), Leica Microsystems, in photon-counting mode. STED images were acquired using a dedicated oil objective with 100× magnification and a numerical aperture of 1.4 (Leica Microsystems). A Z-stack was made with a step size of 150 nm and pixel size of 19 × 19 nm, optimized using Nyquist Calculator from SVI (Scientific Volume Imaging, Hilversum, the Netherlands). Finally, deconvolution was performed with Huygens Professional (SVI).

Live imaging

Live imaging experiments were performed on a Nikon Ti-E Eclipse inverted microscope system fitted with a Confocal A1R (LU4A Laser) unit and an EMCCD (Andor DU-897). The inverted microscope together with the EMCCD was used for live imaging using the LU4A laser unit with a 40× oil objective (NA 1.3) and appropriate filtersets. NIS elements software (version 4.30) controlled the microscope and image acquisition.

Coverslips were placed in an imaging chamber and perfused with Tyrode’s solution (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl2-2H2O, 2 mM MgCl2-6H2O, 25 mM HEPES, and 30 mM glucose·H2O, pH 7.4, mOsmol 280). Isolated single neurons on glial islands were selected for acquisition. Time-lapse (2 Hz) recordings consisted of 30 s baseline recordings followed by stimulation. Electrical field stimulation was applied through parallel platinum electrodes powered by a stimulus isolator (WPI A385) delivering 30-mA, 1-ms pulses, regulated by a Master-8 pulse generator (A.M.P.I.) providing 1 action potential (AP) or 16 trains of 50 APs at 50 Hz with a 0.5-s interval. Chemical stimulations were applied through glass capillaries placed in close proximity the cell by gravity flow and included 60 mM KCl. Tyrode’s solution (61.5 mM NaCl, 60 mM KCl, 2 mM CaCl2-2H2O, 2 mM MgCl2-6H2O, 25 mM HEPES and 30 mM glucose·H2O, pH 7.4, mOsmol 280) or 5 μM lonomycin (Fisher BioReagent) dissolved in normal Tyrode’s solution. Intracellular pH was neutralized by barrel application of normal Tyrode’s solution containing 50 mM NH4Cl, which replaced NaCl on an equimolar basis in the solution. To define calcium influx profiles upon stimulation, neurons were incubated for 15 min with 1 μM Fluo-5F-AM (Molecular Probes, F14222; stock in DMSO). All experiments were performed at RT (20–24°C), and no NMDA receptor or AMPA receptor antagonists were added.

Imaging analysis

DCV total pool size

Neuronal morphology and DCV numbers were analyzed using automated image analysis software SynD (Schmitz et al., 2011). Synapse detection settings were optimized to measure ChgA, ChgB puncta, or NPY-pHluorin signal and kept constant for the corresponding dataset.

Colocalization

For colocalization analysis of different markers, morphological analysis masks were drawn using SynD (Schmitz et al., 2011) and imported in ImageJ to remove background fluorescence. Colocalization was measured in ImageJ with JACoP (Bolte & Cordelieres, 2006). Thresholds were set manually to correct for background.

DCV fusion

Dense-core vesicle fusion events were analyzed in stacks of time-lapse recordings (2 Hz, 512 × 512 pixels). In ImageJ DCV, fusion events were manually selected and fluorescence traces were measured in a circular 4 × 4 pixel ROI (1.56 × 1.56 μm). NPY-pHluorin events were defined by a sudden increase in fluorescence, and NPY-mCherry events were defined as a sudden decrease in fluorescence. Resulting fluorescence traces were loaded in a custom-built Matlab program where the traces were expressed as fluorescence change (ΔF) compared to initial fluorescence (F0) obtained by averaging the first 10 frames of the time-lapse recording. Fusion events were automatically detected and included when fluorescence showed a sudden increase (NPY-pHluorin) or a sudden decrease (NPY-mCherry) two standard deviations above or below F0. Start of a fusion event was defined as the first frame above 2 × SD of F0 and end of the fusion event as the first frame below this threshold.

Calcium imaging

Calcium measurements were performed in ImageJ. Five neurite-located ROIs (4 × 4 pixels) and a background ROI were measured per neuron. Normalized ΔF/F0 data were calculated per cell after background subtraction.

dSTORM

dSTORM images were reconstructed using the open-source ImageJ plugin ThunderSTORM (Ovesny et al., 2014). 3D calibration was performed on recordings of fluorescence microspheres, and single molecule localizations were reconstructed using Wavelet B-Spline filtering and 3D astigmatism. Postprocessing was performed to remove localizations with unrealistic parameters, and drift correction was performed using fiducial markers.

To estimate the number of DCVs per fluorescence puncta measured with confocal microscopy, an overlay was reconstructed between dSTORM regions and corresponding confocal images (40×). Intensity of ChgB puncta was measured in confocal images, and the number of reconstructed ChgB clusters with dSTORM was counted in the corresponding area. SynD analysis was performed on confocal recordings (40×) of the corresponding neurons used for dSTORM reconstruction to evaluate, based on intensity of ChgB puncta, the underrepresentation of pool size measurements using SynD. The fluorescence intensity distribution of the total pool of ChgB puncta was divided based on the number of resolved dSTORM puncta. Cutoff measurements were set as the intermediate between the average fluorescence intensity of 1–2, 2–3, and 3–4 dSTORM puncta.

STED

To estimate the number of DCVs per fluorescence punctum measured with confocal microscopy, an overlay was reconstructed between the deconvoluted STED image of ChgA and corresponding deconvoluted confocal image (100× magnification). The number of STED puncta within a single ChgA confocal puncta was counted.

The soma was always excluded from analysis.

Statistics

Shapiro–Wilk test was used to assess distribution normality. When assumptions of normality or homogeneity of variances were met, parametric tests were used: Student’s t-test or one-way ANOVA (Tukey as post hoc test). Otherwise, non-parametric tests used were Mann–Whitney U-test for two independent groups, or Kruskal–Wallis with Dunn’s correction for multiple groups. Wilcoxon
expanded-pairs signed rank test was used for paired data, and slopes of linear regressions were tested using ANCOVA. Data are plotted as mean with standard error of the mean; N represents number of independent experiments, and n represents the number of cells.

**Expanded View** for this article is available online.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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