SNAP-25 gene family members differentially support secretory vesicle fusion

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Summary statement: SNAP-25 family members differentially support neuron survival, dense-core and synaptic vesicle fusion in Snap-25 null mutant neurons.
Abstract

Neuronal dense-core vesicles (DCVs) transport and secrete neuropeptides necessary for development, plasticity and survival, but little is known about their fusion mechanism. We show that Snap-25 null mutant (SNAP-25 KO) neurons, previously shown to degenerate after 4 days in vitro (DIV), contain fewer DCVs and have reduced DCV fusion probability in surviving neurons at DIV14. At DIV3, before degeneration, SNAP-25 KO neurons show normal DCV fusion, but one day later fusion is significantly reduced. To test if other SNAP homologs support DCV fusion, we expressed SNAP-23, -29 or -47 in SNAP-25 KO neurons. SNAP-23 and -29 rescued viability and supported DCV fusion in SNAP-25 KO neurons, but SNAP-23 more efficiently. SNAP-23 also rescued synaptic vesicle (SV) fusion while SNAP-29 did not. SNAP-47 failed to rescue viability and did not support DCV or SV fusion. These data demonstrate a developmental switch, in hippocampal neurons between DIV3-4, where DCV fusion becomes SNAP-25 dependent. Furthermore, SNAP-25 homologs support DCV and SV fusion and neuronal viability to a variable extent, SNAP-23 most effectively, SNAP-29 less so and SNAP-47 ineffective.
Introduction

Neuronal communication primarily relies on the calcium-dependent fusion of two secretory organelles, synaptic vesicles (SVs) and neuropeptide-filled dense-core vesicles (DCVs). Neuropeptides modulate many aspects of brain function including brain development, synaptogenesis, synaptic plasticity and survival (Huang and Reichardt, 2001; McAllister et al., 1999; Meyer-Lindenberg et al., 2011; Poo, 2001; Samson and Medcalf, 2015; van den Pol, 2012) and dysfunctional neuropeptide signalling is associated with several mood, anxiety and social disorders (reviewed in, Kormos and Gaszner, 2013). However, in contrast to our in-depth understanding of SV fusion principles (see for a review, Südhof, 2013), much less is known about DCV transport and fusion mechanisms.

Soluble NSF attachment receptor (SNARE) proteins mediate membrane fusion of secretory vesicles (Ferro-Novick and Jahn, 1994). In neurons, the vesicular SNARE synaptobrevin-2/VAMP2 and membrane SNAREs syntaxin-1 and SNAP-25 drive fusion of SVs for fast neurotransmission (Jahn and Fasshauer, 2012; Südhof, 2013; Südhof and Rothman, 2009). Like SV fusion, DCV fusion is triggered by calcium influx (Balkowiec and Katz, 2002; de Wit et al., 2009; Farina et al., 2015; Shimojo et al., 2015; van de Bospoort et al., 2012), although efficient DCV fusion typically requires more prolonged stimulation (Balkowiec and Katz, 2002; Bartfai et al., 1988; Hartmann et al., 2001; van de Bospoort et al., 2012). Neuronal DCVs are often highly mobile (de Wit et al., 2006; Wong et al., 2012), not pre-docked at their release sites (Hammarlund et al., 2008; van de Bospoort et al., 2012) and fuse at synaptic as well as extra-synaptic sites (de Wit et al., 2009; Hartmann et al., 2001;
Ludwig and Leng, 2006; Matsuda et al., 2009). DCV fusion is sensitive to clostridial neurotoxins, indicating that neuropeptide release is SNARE-dependent (de Wit et al., 2009; Hammarlund et al., 2008; McMahon et al., 1992; Shimojo et al., 2015). However, the composition of SNARE complexes that drive efficient DCV fusion in neurons is not known.

The SNAP protein family consists of several homologues proteins of which SNAP-25 is essential for SV fusion (Delgado-Martínez et al., 2007; Washbourne et al., 2002). Deletion of SNAP-25 leads to reduced neuronal survival and impaired arborisation, reduced spontaneous release, and arrest of evoked release in the surviving neurons (Delgado-Martínez et al., 2007).

SNAP-23, SNAP-25’s closest homolog, is ubiquitously expressed and is involved in NMDA receptor cycling in post-synaptic spines (Suh et al., 2010). SNAP-23 over-expression rescues SV fusion in Snap-25 null mutant (SNAP-25 KO) neurons and secretory granule fusion in chromaffin cells, albeit with reduced efficiency (Sørensen et al., 2003; Delgado-Martínez et al., 2007).

Two other SNAP family members, SNAP-29 and SNAP-47, are also present in neurons (Holt et al., 2006; Pan et al., 2005) and co-purify in synaptic vesicle purifications (Takamori et al., 2006). Overexpression of SNAP-29 inhibits synaptic vesicle fusion possibly via inhibiting SNARE complex disassembly (Pan et al., 2005; Su et al., 2001). SNAP-47 binds to plasma membrane SNAREs in vitro, but is predominantly located on intracellular membranes (Holt et al., 2006). Recent data has shown that shRNA-mediated knockdown of SNAP-25 or SNAP-47 expression interferes with calcium-dependent release of BDNF in cortical neurons (Shimojo et al., 2015).
Here, we investigated the role of SNAP-25 in DCV fusion in cultured hippocampal neurons at 3-4 days in vitro (DIV), prior to synapse formation, and at DIV14 after synapses are formed. We find that neuronal DCV fusion is strongly impaired in SNAP-25 KO neurons at DIV14 but that calcium-evoked fusion at DIV3, but not DIV4, is SNAP-25-independent. In addition, we show a differential ability of SNAP-25 family members to support neuronal viability, and DCV and SV fusion in SNAP25 KO neurons at DIV14.
Results

**SNAP-25 is required for efficient DCV fusion in DIV14 neurons**

To investigate the role of SNAP-25 in neuronal DCV fusion, we first analysed the effect of SNAP-25 deletion on survival and morphology of single isolated hippocampal neurons of SNAP-25 KO mice (Washbourne et al., 2002) and wild type (WT) littermates (Fig. S1A). In line with previous reports (Delgado-Martínez et al., 2007; Washbourne et al., 2002), the majority (approximately 98%) of SNAP-25 KO neurons did not survive beyond DIV4 in culture (Fig. S1B). At DIV14, the surviving SNAP-25 KO neurons had smaller dendrites and axons, contained fewer VGLUT1 or Synaptophysin-labelled synapses and DCVs compared with WT littermates (Fig. 1A and Fig. S1A-M for quantification). In our culture system the majority of neurons is glutamatergic and SNAP-25 deletion did not affect the ratio of glutamatergic (VGLUT1-positive) over GABAergic (VGAT-positive) neurons at DIV14 (WT: 12.4% GABAergic; SNAP-25 KO 15.1% GABAergic, MW p = 0.32). Next, we expressed pHluorin-tagged neuropeptide Y (NPY-pHluorin), a DCV cargo reporter that allows analysis of DCV fusion events with single vesicle resolution, reported by the rapid increase of fluorescence upon pHluorin de-quenching when the fusion pore opens followed by rapid decline of fluorescence (transient event, representing full release of cargo or endocytosis and DCV re-acidification) or prolonged fluorescence (persistent event, representing prolonged fusion pore opening or stable deposits of NPY-pHluorin at the membrane), typical for neuronal DCV fusion (Figure 1B and, de Wit et al., 2009; Farina et al., 2015; van de Bospoort et al., 2012). In WT
cells, bursts of action potentials (16 x 50 AP at 50 Hz, Farina et al., 2015; Hartmann et al., 2001; van de Bospoort et al., 2012) triggered robust DCV fusion (28.6 ± 9.2 events/cell, Fig. 1C,F). In contrast, SNAP-25 KO neurons showed a more than 9-fold reduction in DCV fusion events upon stimulation (3.1 ± 1.3 events/cell, Fig. 1C,F). Deletion of SNAP-25 equally affected DCV fusion at synaptic and extra-synaptic sites (Fig. S1 N,O). WT cells showed a sharp increase in DCV fusion events after the first burst of 50 APs (Fig. 1D). In contrast, DCV fusion in SNAP-25 KO neurons required more prolonged stimulation and never reached fusion rates as in WT cells (Fig. 1D,E).

To test if the smaller size of SNAP-25 KO neurons resulted in a reduced total number of DCVs, we quantified the number of DCV puncta upon instant de-quenching of intravesicular NPY-pHluorin using NH\textsubscript{4}Cl superfusion (de Wit et al., 2009). The total number of NPY-pHluorin puncta was >50% lower in SNAP-25 KO neurons compared with WT (Fig. 1G, WT: 974.6 ± 157.6, n = 20; SNAP-25 KO: 399.2 ± 78.2, n = 13). The DCV fusion probability, defined as the number of fusion events/total DCV number per cell, was strongly reduced in SNAP-25 KO neurons (Fig. 1H). Hence, SNAP-25 is critical for efficient stimulation-dependent DCV fusion in DIV14 hippocampal neurons. Its absence results in reduced DCV numbers and fusion probability, also after correcting for the smaller size of SNAP-25 KO neurons.

**DCV fusion is calcium dependent at DIV3 but becomes SNAP-25 dependent only at DIV4**
SNAP-25 KO neurons show signs of degeneration at 3-4 DIV and >98% of these neurons do not survive beyond DIV8 (Fig. S1B and Delgado-Martínez
et al., 2007; Washbourne et al., 2002). To test if reduced release of neuropeptides and neurotrophic factors may help to explain this phenotype, we first assessed whether our functional DCV probe NPY-pHluorin also labels DCVs in developing neurons by analysing its co-localization with the endogenous DCV cargo Secretogranin II (SecgrII, Bartolomucci et al., 2011) at DIV4. Both Pearson’s and Manders’ coefficients confirmed robust co-localization of NPY-pHluorin and SecgrII at DIV4 similar to co-localization of NPY-pHluorin with SecgrII or ChgB at DIV14 (Fig. 2B,C) indicating that our probe is correctly targeted to DCVs in developing neurons. Next, using NPY-pHluorin, we examined DCV fusion in neurons at DIV3 and DIV4, at which time neurons develop extensive axonal and dendritic arborizations in vitro and expression levels of SNAP-25 strongly increase (Fig. 2A and Fig S2J,K). At both time points, SNAP-25 KO neurons had smaller neurites with less complex arborisation compared with WT neurons (Fig. S2A-H). Action potential stimulation resulted in a robust increase in intracellular calcium (Fig. 2D,E, insets). In DIV3 neurons, stimulation failed to elicit fusion in approximately 60% of WT and SNAP-25 KO neurons (Fig. S2I), but events were observed in the other 40%. These events were stimulus-dependent in both genotypes (Fig. 2D) and similar between WT and SNAP-25 KO neurons (Fig. 2D,F and G). The number of DCV fusion events at DIV3 was almost 10-fold lower than at DIV14 (compare Fig. 2F with Fig. 1F). At DIV4, a larger fraction of neurons responded to AP-stimulation (Fig. S2I) and more DCV fusion events were observed in WT neurons than at DIV3 (Fig. 2E,F and H). However, fusion events were less frequent and more asynchronous to the stimulation in SNAP-25 KO neurons (Fig. 2E,H). Hence, while at DIV3 WT
and SNAP-25 KO neurons responded similarly to stimulation, at DIV4 SNAP-25 KO neurons were clearly impaired.

The total number of NPY-pHluorin labelled DCVs, assessed upon brief superfusion with NH\(_4^+\), in SNAP-25 KO neurons was somewhat lower but not statistically different than in WT neurons both at DIV3 and DIV4 (Fig. 2I). To correct for the total vesicle pool between the two genotypes, we computed the DCV fusion probability, i.e., the number of fusion events divided by the total number of vesicles per neuron. The fusion probability was similar between the genotypes at DIV3 (Fig. 2J), but lower in SNAP-25 KO neurons at DIV4 (Fig. 2K). In conclusion, activity-dependent DCV fusion is reduced at DIV4, but not DIV3, in SNAP-25 KO neurons. Hence, DCV fusion appears to become SNAP-25-dependent between DIV3 and DIV4 coinciding with a reduction of SNAP-23 expression in SNAP-25 KO neurons (Fig. S2L) and the occurrence of massive cell death.

**SNAP-23 and -25, but not SNAP-29, rescue synaptic vesicle fusion capacity in SNAP-25 KO neurons at DIV14**

Re-expression of SNAP-25 rescues neuronal viability and neurotransmission in SNAP-25 KO hippocampal neurons (Delgado-Martínez et al., 2007). To test if, and to what extent, other SNAP-25 related genes can rescue neuronal viability and SV fusion we expressed SNAP-23, -29 or -47 using lenti viral infections in SNAP-25 KO neurons (Fig. S3A-C). Viral expression of SNAP-23 or SNAP-29 resulted in an approximately 2-fold increase in cellular protein levels (Fig. S3S-U). Expression of these proteins rescued neuronal viability, similar to SNAP-25 expression. Dendrite morphology and synapse number
were comparable between SNAP-25 KO neurons expressing SNAP-23 or SNAP-25, but SNAP-29 expressing SNAP-25 KO neurons were smaller and had less synapses (Fig. S3D-R). The synapses of the few SNAP-25 KO neurons that survived until DIV14 showed a higher expression level of endogenous SNAP-23, but not SNAP-29, as compared to WT neurons (Fig. 3A). Viral expression at DIV1 further increased average synaptic levels of SNAP-23 or SNAP-29 in SNAP-25 KO neurons, whereas SNAP-25 expression produced synaptic protein levels almost similar to WT neurons (Fig. 3A). SNAP-47 did not rescue viability of SNAP-25 KO neurons despite the fact that viral expression of SNAP-47 resulted in a 2-fold increase compared to endogenous SNAP-47 expression levels (see Fig. 5 below).

Next, we assessed SV fusion using the synaptic vesicle protein synaptophysin fused to a pH-sensitive fluorophore (pHluorin) in a luminal domain (SypHy, Granseth et al., 2006). As expected, APs (200 AP at 10 Hz) did not elicit SV fusion in SNAP-25 KO neurons (Fig. 3C-F). Expression of SNAP-23 or -25 restored SV fusion capacity in 90-100% of SNAP-25 KO neurons. In contrast, although expression of SNAP-29 rescued survival, AP-triggered fusion events were not detected in these cells (Fig. 3C). The total SV pool, assessed by brief superfusion of NH$_4^+$, was smaller in SNAP-29 rescued neurons compared with WT neurons, but larger than in SNAP-25 KO neurons (Fig. 3D). SV fusion probability in SNAP-25 KO neurons expressing SNAP-23 was not significantly different from those expressing SNAP-25 and WT neurons (Fig. 3E) as reported earlier by (Delgado-Martínez et al., 2007). In addition, SNAP-25 KO neurons expressing SNAP-23 showed a higher amplitude and more prolonged increase, or slower decline, in SypHy fluorescence after
stimulation than WT and SNAP-25 KO neurons expressing SNAP-25 (Fig. 3F). SyPhy fluorescence in SNAP-25 KO neurons expressing SNAP-29 showed a small and gradual increase over time (Fig. 3E,F). Hence, expression of SNAP-25 or -23 restores SV fusion in SNAP-25 KO neurons while SNAP-29 rescues cell survival but not AP-triggered SV fusion.

SNAP-25 homologs rescue DCV fusion in DIV14 SNAP-25 KO neurons to varying extents

We next tested the capacity of SNAP-25 related proteins to support DCV fusion at DIV14 in the absence of SNAP-25. Lenti viral expression of SNAP-25 in SNAP-25 KO neurons, using a similar approach as described above, fully rescued DCV fusion capacity with a similar number of fusion events and fusion probability as in WT neurons (Fig. 4A, C-D). SNAP-23 or SNAP-29 expression in SNAP-25 KO neurons did rescue DCV fusion capacity, albeit with different efficiencies. DCV fusion was 1.5-fold lower in SNAP-23 and 6-fold lower in SNAP-29 rescued neurons compared to rescue with SNAP-25 (SNAP-25: 294.1 ± 68.1 fusion events/ cell, SNAP-23: 172.3 ± 53.4 events/ cell, SNAP-29: 45.1 ± 19.2 events/ cell, Fig. 4A-C). Total DCV numbers in SNAP-25 KO neurons rescued with SNAP-25, -23 or -29 were similar and comparable to WT (Fig. 4D). DCV fusion probabilities, expressed as number of fusion events divided by the total DCV pool per cell, were not significantly different between SNAP-23 and SNAP-25 rescued neurons and almost 6-fold lower in SNAP-29 rescued neurons (Fig. 4D). The DCV fusion rate (number of fusion events over time) was similar between WT and SNAP-25 KO neurons expressing SNAP-25 (Fig. 4E), while fusion rates in SNAP-25 KO neurons
expressing SNAP-23 or SNAP-29 were significantly lower (Fig. 4F-G). In WT and SNAP-25 KO neurons expressing SNAP-25 the majority of DCV fusion events occurred between the 1\textsuperscript{st} and 2\textsuperscript{nd} burst of 50 APs, whereas in SNAP-25 KO neurons expressing SNAP-23 and SNAP-29 the majority of fusion events occurred in between the 6\textsuperscript{th} and 10\textsuperscript{th} stimulation burst (Fig. 4H). Hence, SNAP-25 related genes rescue DCV fusion capacity to a different extent when expressed in SNAP-25 KO neurons and the fusion kinetics are different between expression of SNAP-25 versus SNAP-23/-29.

**SNAP-47 expression in SNAP-25 KO neurons does not rescue cell survival, SV or DCV fusion**

To examine our observation that SNAP-47 expression does not rescue cell survival of SNAP-25 KO neurons in more depth, we tested SNAP-47 expression levels in DIV14 WT neurons and in the surviving SNAP-25 KO neurons, also upon lenti viral over-expression of SNAP-47. Endogenous and over-expressed SNAP-47 was readily detected in WT and SNAP-25 KO neuronal lysates and lenti viral expression led to higher SNAP-47 levels (Fig. 5A). However, despite over-expression of SNAP-47, these neurons showed a similar survival profile as SNAP-25 KO neurons: Neurons developed normally until DIV2-3 after which degeneration occurred leading to survival of less than 2\% of the neurons at DIV8 (Fig. 5B), as reported previously (Delgado-Martínez et al., 2007; Washbourne et al., 2002), and surviving SNAP-25 KO neurons overexpressing SNAP-47 showed similar reduced synapse numbers and dendrite morphology as SNAP-25 KO neurons (Fig. S4). Further examination using semi-quantitative immunofluorescence analysis showed
robust expression of SNAP-47 in synapses of WT, surviving SNAP-25 KO neurons and SNAP-25 KO neurons over-expressing SNAP-47 (Fig. 5C,D). SNAP-47 expression levels were similar in WT and SNAP-25 KO neurons and lenti viral expression increased SNAP-47 levels approximately 3-fold in synapses (Fig. 5C,D and E). Hence, despite high cellular expression levels, SNAP-47 did not rescue survival in SNAP-25 KO neurons.

We next tested if these neurons support SV fusion. To this end, we infected WT, surviving SNAP-25 KO neurons and SNAP-25 KO neurons over-expressing SNAP-25 or -47 with SypHy. Stimulation with 200 APs at 10 Hz triggered SV fusion in WT and SNAP-25 KO neurons expressing SNAP-25, as expected. However, SV fusion was absent in SNAP-25 KO neurons over-expressing SNAP-47, i.e., similar to SNAP-25 KO neurons (Fig. 5F). Also, SNAP-47 over-expression did not increase DCV fusion in SNAP-25-KO neurons and DCV fusion was still >95% reduced when compared to WT and SNAP-25-KO neurons expressing SNAP-25 (Fig. 5G and H). Hence, in contrast to SNAP-23 and -29, SNAP-47 does not rescue survival of SNAP-25 KO neurons and does not support SV or DCV fusion in the absence of SNAP-25.
Discussion

In this study we show that DCV exocytosis during early development in vitro (DIV3) was calcium-dependent and became SNAP-25 dependent at DIV4, prior to the onset of massive cell death of SNAP-25 KO neurons. Expression of SNAP-25 family members in SNAP-25 KO neurons rescued neuronal survival, DCV and SV fusion to different extents: SNAP-23 supported efficient SV and DCV fusion in the absence of SNAP-25, while expression of SNAP-29 rescued neuronal viability and DCV fusion but not SV fusion. SNAP-47 failed to rescue viability of SNAP-25 KO neurons and neither supported DCV nor SV fusion in the surviving SNAP-25 KO neurons.

We used the DCV cargo protein NPY coupled to pHluorin to assess activity-dependent fusion of DCVs prior to synapse formation in developing (DIV3-4) and mature (DIV14) neurons. NPY-pHluorin puncta showed strong co-localization with the endogenous DCV markers Secretogranin II and Chromogranin B (Fig. 2). Hence, the DCV cargo reporter NPY-pHluorin is properly sorted to SecrII/ChrB positive DCVs both in developing and mature neurons. The fact that DCV fusion is virtually abolished in the absence of SNAP-25 indicates that sorting of this reporter to the regulated secretory pathway, as opposed to constitutive secretion, is almost perfect.

**SNAP-25 regulates neuronal morphology and viability**

DCV fusion became SNAP-25 dependent at DIV4 prior to the massive cell loss in SNAP-25 KO neurons (Fig. S1 and Delgado-Martínez et al., 2007; Washbourne et al., 2002). DCV fusion in the remaining ± 2% of SNAP-25 KO
neurons at DIV14 was strongly reduced compared to WT neurons. Both at DIV4 and DIV14, SNAP-25 KO neurons were smaller, with reduced neurite arborisation and, at DIV14, less synaptic connections. This suggests that a lack of trophic support, due to impaired DCV fusion might limit survival of SNAP-25 KO neurons and affect cell morphology in the surviving neurons. However, additional, cell intrinsic mechanisms likely also play a role as SNAP-25 KO neurons showed reduced dendrite length already at DIV3 when DCV fusion was not affected by SNAP-25 deletion (Fig. S2) and other models with blocked DCV fusion, such as synaptobrevin-2/VAMP2 KO neurons and WT neurons treated with Tetanus toxin (which cleaves synaptobrevin/VAMP-1/2/3), do not degenerate (Peng et al., 2013; Schoch et al., 2001).

**SNAP-25 is the major SNAP homolog for DCV fusion in DIV14 hippocampal neurons**

DCV fusion in SNAP-25 KO neurons at 14 DIV was almost abolished (Fig. 1). These findings are in line with previous studies demonstrating reduced BDNF release in hippocampal neurons upon shRNA-mediated knockdown of SNAP-25 (Shimojo et al., 2015) and secretory granule release in SNAP-25 KO chromaffin cells (Sørensen et al., 2003). As SNAP-25 deletion also blocks SV fusion (Delgado-Martínez et al., 2007; Washbourne et al., 2002), these findings show that SNAP-25 dependent SNARE machinery drives fusion of the two major secretory pathways in parallel and that endogenous expression of other SNAP-25 protein family members is not sufficient to support secretion in the absence of SNAP-25. Viral expression of SNAP-23, SNAP-25's closest homolog, in SNAP-25 KO neurons, rescued cell survival and calcium-
dependent SV and DCV fusion almost as efficiently as expression of SNAP-25 in SNAP-25 KO neurons (Figs 3 and 4). This shows that in principle SNAP-23 is able to replace SNAP-25 in a SNARE-complex that couples calcium influx to vesicle fusion. However, DCV fusion in SNAP-25 KO neurons rescued with SNAP-23 required prolonged stimulation and did not fully reach WT levels (Fig. 4E-H). In addition, SV fusion was more a-synchronous to the 200 AP at 10 Hz stimulation (Fig. 3F). This can be explained by the fact that, in contrast to SNAP-25, SNAP-23 does not bind synaptotagmin-1, the calcium sensor for fast synchronous fusion, but instead binds synaptotagmin-7 (Chieregatti et al., 2004), implicated in exocytosis in neuroendocrine cells (Schonn et al., 2008; Sugita et al., 2001) and asynchronous SV fusion in neurons (Bacaj et al., 2013; Weber et al., 2014). Delayed vesicle fusion upon calcium entry of synaptotagmin-7 labelled secretory granules compared to synaptotagmin-1 labelled vesicles has also been observed in adrenal chromaffin cells (Rao et al., 2014). In these cells synaptotagmin-1 and -7 appear to label different secretory granule populations. Although we cannot rule out the existence of different DCV pools in neurons, the finding that in absence of SNAP-25 evoked DCV fusion is largely abolished does not support a major role for SNAP-23/synaptotagmin-7 complexes in driving calcium-dependent DCV fusion in SNAP-25 KO neurons at DIV14. However, a SNAP-23/synaptotagmin-7 complex may be involved in DCV fusion during early development prior to the developmental switch to SNAP-25 dependent fusion (see below).
SNAP-29 rescues neuronal viability and DCV but not SV fusion

Like SNAP-23, SNAP-29 rescued the lethal phenotype of SNAP-25 KO neurons and completely restored total DCV numbers in SNAP-25 KO neurons. It also supported DCV fusion, albeit much less efficiently compared to SNAP-25 or SNAP-23 rescued neurons, but not SV fusion. As SNAP-29, in contrast to SNAP-25 and SNAP-23, lacks the cysteine-domains that are palmitoylated in SNAP-25 and -23 (Steegmaier et al., 1998) this argues that to support neuronal survival, DCV biogenesis and to lesser extent DCV fusion, protein palmitoylation is not required. It also suggests that SNAP-29 is able to engage in a plasma membrane SNARE-complex via a palmitoylation independent process to support DCV fusion but that such a complex cannot support SV fusion.

SNAP-29 functions in constitutive release in non-neuronal cells, interacting with syntaxin-19 (Gordon et al., 2010) and in intracellular fusion of autophagosomes with endo-lysosomes in a SNARE-complex with syntaxin-17 (Itakura et al., 2012) but also interacts with other plasma membrane syntaxins (Steegmaier et al., 1998) and has recently been implicated in secretory autophagy in combination with syntaxins-3 and -4 (Kimura et al., 2017). Overexpressed SNAP-29 does not efficiently replace SNAP-25 in the SNARE-complex driving SV fusion (Fig. 3). This is in line with previous findings that SNAP-29 inhibits SV fusion when overexpressed on WT background, probably via hindering canonical SNARE-complex disassembly and synaptic vesicle turnover (Pan et al., 2005; Su et al., 2001). Our results show that in neurons, SNAP-29 is able to support regulated release of DCV cargo to some extent (Fig. 4) but not SV fusion, which indicates that an alternative SNARE-
complex supports SNAP-29-dependent DCV fusion in the absence of SNAP-25 albeit much less efficiently than SNAP-25. The incomplete restoration of DCV fusion may also explain why expression of SNAP-29 does not rescue all morphological defects of SNAP-25 KO neurons (Fig. S3N-R).

**SNAP-47 does not rescue neuronal viability, nor support DCV- or SV fusion, suggesting a function upstream of SNAP-25.**

In contrast to SNAP-23, and -29, SNAP-47 did not rescue cell viability of SNAP-25 KO neurons (Fig. 5), nor supported SV and DCV fusion in the surviving SNAP-25 KO neurons (Fig. 5). Although initially discovered in subcellular fractionation studies in the fraction enriched in small synaptic vesicles (Holt et al., 2006), SNAP-47 appears to have a widespread intracellular localization: In neurons, SNAP-47 is present in cytosol and neurites but in contrast to SNAP-25 and -23, not selectively localized to the plasma membrane or to presynaptic nerve terminals (Figure 5D,E and Holt et al., 2006). In Hela cells, SNAP-47 localizes to ER and ERGIC where it interacts with VAMP-4, -7, and -8 (Kuster et al., 2015). SNAP-47 substitutes for SNAP-25 in a complex of syntaxin1 and VAMP-2 in SNARE-driven fusion of liposomes, but it does so with strongly reduced efficiency (Holt et al., 2006) and it only weakly interacts with VAMP-2, the VAMP isoform involved in SV and DCV fusion (Kuster et al., 2015). However, shRNA-mediated depletion of SNAP-47 blocks axonal release of brain-derived neurotrophic factor (BDNF) in neurons (Shimojo et al., 2015) and also affects fusion of AMPA receptor containing organelles (Arendt et al., 2015; Jurado et al., 2013). Hence, these observations suggest a role for SNAP-47 in fusion of neurotrophic factor
vesicles (DCVs) and AMPAR vesicles with the plasma membrane. In contrast, our data show that for DCV and SV fusion, SNAP-47 cannot execute this role in the absence of SNAP-25. Hence, based on its subcellular localization, the interaction with ER-resident VAMP isoforms in heterologous cells and the lack of synaptic enrichment, it is conceivable that SNAP-47 functions upstream of SNAP-25 in the secretory pathway, possibly contributing to proper subcellular localization and function of VAMP-4, -7, and -8, rather than at the plasma membrane controlling fusion of secretory vesicles. As SNAP-47 dependent BDNF release was tested in the presence of SNAP-25 (Shimojo et al., 2015), it is plausible that both proteins function in a similar pathway and that efficient BDNF release requires the orchestrated action of both SNAP isoforms with SNAP-47 most likely acting upstream of SNAP-25.

Already during early development, NPY-pHluorin fusion events were highly synchronous to the calcium influx (Fig. 2) suggesting that a regulated secretory pathway, most likely exploiting SNAREs and Ca^{2+}-sensors, becomes operational at an early developmental phase. Neurons gradually acquire this capacity during the first days in vitro as the number of cells unresponsive to calcium stimulation reduced from ± 60% at DIV3 to ± 40% at DIV4 and ± 5% at DIV14 (Fig. 2). It has been proposed that prior to synapse formation, specialized vesicles referred to as piccolo–bassoon transport vesicles, PTVs, ship active zone components to nascent synapses. Like the DCVs studied here, PTVs also have a dense core and are Chromogranin B positive (Zhai et al., 2001). Based on the high co-localization of NPY-pHluorin with Chromogranin B and Secretogranin II at DIV4 we conclude that NPY-
pHluorin also labels PTVs, which may suggest that PTVs can be delivered to the membrane in an activity-dependent manner from DIV3-4 onwards. Such a mechanism may operate to supply nascent, active synapses with additional presynaptic release machinery components to increase efficient synaptic transmission.

Our data (Fig. 2) show that calcium-dependent DCV fusion prior to DIV4 appears to be regulated by a SNAP-25 independent mechanism (Fig. 2). Based on expression profiles of SNAP homologs and their capacity to support DCV fusion the most plausible SNAP to operate at this early time point is SNAP-23. SNAP-23 is expressed during early brain development (Prescott and Chamberlain, 2011; Suh et al., 2010) and its deletion leads to embryonic lethality during early embryo development (Suh et al., 2011). SNAP-25 expression levels are initially low but strongly increase during late pre- and early postnatal development (Prescott and Chamberlain, 2011; Suh et al., 2010). Also in our culture system, SNAP-23 levels are significantly higher at DIV3 than at DIV4 in WT and SNAP-25 KO neurons (Suppl. Fig. 2J-L). Hence, it is conceivable that prior to DIV4, DCV fusion is regulated by a SNAP-23 dependent SNARE-complex, which is gradually replaced by the canonical SNAP-25-dependent complex from DIV4 onwards. In adrenal chromaffin cells, SNAP-23/synaptotagmin-7 SNARE-complexes drive efficient secretory granule exocytosis upon mild stimuli, which lead to moderate elevation of intracellular calcium levels (Rao et al., 2014). Hence, prior to efficient clustering of calcium channels in presynaptic compartments a SNAP-23 dependent SNARE complex may operate more efficiently to fuse DCVs in developing neurons. When synapses become functional, SNAP-25 dependent
fusion machinery, with stricter coupling between calcium influx and DCV fusion (Fig. 4H), would then ensure properly timed calcium-dependent fusion of DCVs.

In conclusion, we have shown that the SNAP-25 is the canonical SNAP homolog to drive efficient DCV fusion in neurons suggesting that DCV and SV fusion is governed by similar SNARE machinery in mature neurons. Different SNARE complexes and Ca2+-sensors may be able to replace each other for DCV fusion, but less so for SV fusion. Finally, we identified a developmental switch, in cultured hippocampal neurons between DIV3-4, where evoked DCV fusion becomes SNAP-25 dependent.
Materials and Methods

Laboratory animals

E18 SNAP-25 KO embryos were obtained by cesarean section of time mated SNAP-25 heterozygous mice (Washbourne et al., 2002). WT littermates were used as controls. All animal experiments were performed in compliance with the guidelines for welfare of experimental animals issued by the Dutch government and approved by the ethical committee of the Vrije Universiteit, Amsterdam.

Primary neuronal culture

Single neuron suspensions were prepared from hippocampi of E18 mice according to (de Wit et al., 2009). Continental cultures with 25,000 neurons/well (WT) or 300,000 neurons/well (SNAP-25 KO) were plated on pre-made cultures of rat glia cells (37,500 cells/well) on 18 mm glass coverslips in 12-well plates and cultured in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 1% Glutamax (Gibco, UK) and 1% Pen-Strep (Invitrogen). For single isolated neuronal island cultures as in (Toonen et al., 2006; Wierda et al., 2007), neurons were plated at a density of 1400 (WT) or 8000 neurons/well (SNAP 25KO) on 18 mm glass coverslips in 12 well plates on rat glia micro-islands. Rat glia micro-islands were prepared by plating 8000 glia cells/well on 18 mm glass coverslips coated with agarose and stamped with solution consisting of 0.1 mg/ml poly-d-lysine (Sigma) and 0.2 mg/ml rat tail collagen (BD Biosciences). Neuron survival curves were generated by plating 300,000 WT, SNAP-25 KO neurons or SNAP-25 KO
expressing the different SNAP isoforms without glia. Neurons were manually counted at each DIV. Graphs were normalized to density at plating (DIV 0).

**Plasmids and Lenti viral infection**

NPY-pHluorin and Synapsin-mCherry have been previously described (Farina et al., 2015; van de Bospoort et al., 2012). Synapsin-ECFP was generated by replacing mCherry with ECFP. Synaptophysin-pHluorin has been described before (Granseth et al., 2006). cDNAs for mouse SNAP-25, -23, -29 and -47 were generated from a mouse brain cDNA library (Invitrogen) using standard PCR techniques and sequence verified. SNAP-25 isoform b was used for all rescue experiments. cDNAs were cloned into lenti viral vectors (as in, Farina et al., 2015; van de Bospoort et al., 2012) under control of a human synapsin promoter that co-expressed nuclear-targeted mCherry (Cre-mCherry) using an IRES. Lentiviral production was according to (Farina et al., 2015; van de Bospoort et al., 2012). Synaptophysin-pHluorin and Synapsin-ECFP infections were performed at DIV7 and neurons were imaged between DIV14-18. NPY-pHluorin infections were performed at DIV10 for neurons imaged between DIV14-18 and on DIV0 for neurons imaged at DIV3 and -4. SNAP-25 KO neurons were rescued with SNAP-23, -25, -29 or -47- Cre-mCherry constructs on DIV1 as in (Delgado-Martínez et al., 2007). Rescued neurons were subsequently infected with NPY-pHluorin as stated above and imaged between DIV14–DIV18.
Imaging

Imaging was performed on an inverted fluorescence microscope (IX81; Olympus) equipped with a MT20 light source (Olympus), appropriate filter sets (Semrock, Rochester, NY), 40x oil objective (NA 1.3) and an EM charge-coupled device (C9100-02; Hamamatsu Photonics, Japan) driven by Xcellence RT imaging software (Olympus). Coverslips were placed in an imaging chamber and perfused with Tyrode’s buffer (in mM: 2 CaCl2, 2.5 KCl, 119 NaCl, 2 MgCl2, 20 Glucose, 25 HEPES, pH 7.4). During SypHy and NPY-pHluorin experiments, intracellular pH was neutralized with Tyrode’s solution, in which 50mM NaCl was replaced by 50mM NH4Cl, applied by gravity flow through a glass capillary placed between two platinum electrodes that were used to deliver 30 mA 1 ms electrical stimulations via a stimulus generator (A385RC, World Precision Instruments, Germany). The stimulus used for DCV fusion consisted of 16 trains of 50 APs at 50 Hz with 500 ms interval as in (de Wit et al., 2009; van de Bospoort et al., 2012). The stimulus used for SyPhy measurements was 200 APs at 10 Hz. Experiments were performed at room temperature (21- 24°C). DCV and Synaptophysin assays were acquired at sampling rates of 2 Hz and 1 Hz, respectively. Synapsin masks and Cre-mCherry signals were acquired at 1 Hz before stimulating neurons for DCV assays.

Image analysis

Time-lapse images of DCV fusion were analyzed by placing a 3×3 pixel region (0.45 µm × 0.45 µm). Differences between fluorescence changes were expressed as ∆F and compared to baseline fluorescence (F0), which was the
average of the first four frames. A DCV fusion event was detected as a sudden rise in fluorescence at least two-fold above baseline. Fusion events were scored as synaptic when the fluorescence center of a release event was within 200 nm (±1 pixel) of the Synapsin-ECFP fluorescence centroid. We only measured fusion events from neurites and excluded somatic fusion events as these 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 manually analyzed by counting fluorescent DCV puncta from the frames of the NH₄⁺ application.

Time-lapse images of Synaptophysin-pHluorin assays were analyzed by placing a 3x3 pixel region (0.45 µm x 0.45 µm). Differences between fluorescence changes were expressed as ∆F and compared to the baseline fluorescence (F₀), which was the average of the first five frames. ∆Fmax (total vesicle pool) was calculated as the highest ∆F value during NH₄⁺ application. ∆F/∆Fmax indicates the total fusion pool.

Analysis of confocal images was done with SynD software (Schmitz et al., 2011) using default settings. The ratio of glutamatergic versus GABAergic neurons was assessed by immunofluorescence labeling of DIV14 WT or SNAP-25 KO neurons with VGLUT1 and VGAT antibodies and manual counting of 20 fields of view of 3 independent cultures using a 40x objective. Colocalization (Mander’s and Pearson’s) of NPY-pHluorin and endogenous DCV cargo was analyzed with ImageJ plugin JACoP using default settings. NPY-pHluorin signal was amplified using EGFP antibody (mouse monoclonal, Clontech, 632569).
Statistics

Shapiro and Levene’s tests were used to assess distribution normality and homogeneity of variances, respectively. When assumptions of normality or homogeneity of variances were met, parametric tests were used: t-test or one-way ANOVA (Tukey as post-hoc test). Otherwise, non-parametric tests were used: Mann-Whitney (MW) or Kruskal-Wallis with Dunn’s correction. Data plotted represent mean and standard error of the mean. n indicates the number of neurons while N is the number of independent experiments.

Immunocytochemistry and confocal imaging

Neurons were fixed in 4% formaldehyde (Electron Microscopies Sciences, Germany) in phosphate-buffered saline (PBS, Gibco, The Netherlands), pH 7.4, for 20 min at RT. Cells were permeabilized for 5 min in PBS containing 0.5% Triton X-100 (Sigma-Aldrich) and incubated for 30 min with PBS containing 2% normal goat serum and 0.1% Triton X-100. Incubations with primary antibodies and secondary antibodies were done for 1.5 hr and 1 hr at RT. Primary antibodies used were: polyclonal MAP2 (Abcam, United Kingdom, 1:500), polyclonal ChromograninB (SySy, Germany, 1:500), polyclonal synaptophysin (SySy, Germany, 1:250), polyclonal VGLUT1 (Millipore 1:5000), polyclonal SecretograninII (Biodesign International 1:500), monoclonal smi312 (Biolegend, 1:1000), monoclonal SNAP-25 (Sternberger, 1:1000), polyclonal SNAP-23 (SySy, Germany, 1:50), polyclonal SNAP-29 (SySy, Germany, 1:500), polyclonal SNAP-47 (SySy, Germany, 1:500). Alexa Fluor conjugated secondary antibodies were purchased from Invitrogen.
Coverslips were mounted in Mowiol and examined on a Zeiss LSM 510 confocal microscope with a 40× (NA 1.3) or 60× objective (NA 1.4).

**Western blot analysis**

To characterize protein expression levels of SNAP isoforms, cultured SNAP-25 KO and WT cortical neurons uninfected or expressing SNAP isoforms were washed in PBS at DIV14 and homogenized in Laemmli sample buffer consisting of 2% SDS, 10% glycerol, 0.26 M β-mercaptoethanol, 60 mM Tris-HCl pH 6.8, and 0.01% Bromophenol blue. E18 brain lysates were made by grinding brain tissue in PBS. After spinning down, pellets were resuspended in Laemmli sample buffer (weight to volume: 0.01 gram in 0.1 ml). Samples were separated on 12% SDS-Polyacrylamide gels and transferred to PVDF membranes using standard techniques. Blots were incubated in 2% milk and 0.5% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 for 1 hr at 4°C, and incubated with primary antibodies: monoclonal SNAP-25 (Sternberger, 1:500), polyclonal SNAP-23 (SySy, Germany, 1:500), polyclonal SNAP-29 (SySy, Germany, 1:500), polyclonal SNAP-47 (SySy, Germany, 1:500) and actin (Chemicon, 1:2000) in PBS-tween for 16 hrs at 4°C. After washing, blots were incubated with anti-rabbit or anti-mouse secondary antibodies (Jackson Immuno Research Laboratories company) in PBS-tween for 1 hr at RT. Blots were scanned using a Fuji Film FLA 5000. Results were analyzed using GelAnalyzer plugin in ImageJ (NIH, Bethesda, USA).
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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.A., R.F.T. and M.V. designed the experiments. S.A. collected and analyzed live and confocal imaging data. I.S. performed western blot experiments. R.K. performed and analyzed co-localization experiments. R. vd B. performed and analyzed confocal imaging data. S.A., M.V. and R.F.T. wrote the manuscript.
Fig. 1. Impaired DCV fusion in DIV14-21 SNAP-25 KO neurons

(A) Representative images of single isolated SNAP-25 KO and WT neurons stained for the dendritic marker MAP2 (blue), endogenous DCV marker Chromogranin B (red) and glutamatergic synapse marker VGLUT1 (green). Scale bar, 25 μm. See for quantification of morphology Fig. S1.

(B) Schematic diagram of a DCV labeled with NPY-pHluorin. NPY-pHluorin fluorescence is quenched in the acidic lumen of a DCV. Upon fusion, pH instantly rises and de-quenches NPY-pHluorin. Representative examples of calcium-dependent NPY-pHluorin fusion events categorized as transient and persistent based upon duration of the fluorescence signal. Stills show sudden increase and rapid decrease of NPY-pHluorin fluorescence (upper panels, transient event) and sudden increase followed by prolonged fluorescence (lower panels, persistent event). Panels on the right show ΔF/F0 traces of transient or persistent events. Both types of events were included in the analysis of DCV fusion.

(C) Histogram of fusion events from control (WT) and SNAP-25 KO (S25KO) neurons before, during and after stimulation. Stimulation consisted of 16 bursts of 50 APs at 50 Hz (16x50AP@50Hz) represented by blue bars. WT neurons showed robust DCV fusion after the first AP burst while fusion in SNAP-25 KO neurons was strongly impaired.

(D) Average cumulative frequency of DCV fusion events per cell before, during and after stimulation.
Average cumulative frequency of fusion events during the first four bursts of 50 APs at 50 Hz shows that DCV fusion starts after the first AP burst in WT but not in SNAP-25 KO neurons.

Average number of DCV fusion events per cell for WT and S25KO neurons (WT: n = 20 cells, N = 3 independent experiments, 657 events; S25KO: n = 13 cells, N = 3, 47 events; MW *** p < 0.001).

Total DCV number, measured upon dequenching of NPY-pHluorin using 50mM NH$_4^+$ superfusion, and fusion probability are strongly reduced in SNAP-25 KO neurons.

Average total DCV number per cell in WT and S25KO neurons (WT: n = 20 cells, 18929 vesicles; S25KO: n = 13 cells, 5147 vesicles; MW ** p = 0.009).

Normalized DCV fusion probability (NFP) during stimulation in WT and S25KO neurons. (WT: n = 20 cells, NFP = 1.0; S25KO: n = 13 cells, NFP = 0.2692; MW * p = 0.0192).

Data is plotted as mean ± SEM. Dots in 1F,G represent individual neurons.
Fig. 2. Calcium and SNAP-25 dependent DCV fusion in DIV3 and DIV4 neurons

(A) Representative images of single isolated SNAP-25 KO and WT neurons at DIV3 (left) and DIV4 (right) stained for dendritic MAP2, axonal smi312 and endogenous DCV marker Secretogranin II (SecgrII). Scale bar: DIV3: 15 µm, zoom: 4 µm; DIV4: 15 µm, zoom: 3 µm.

(B) Representative image of DIV4 neurite showing colocalization of NPY-pHluorin (stained with EGFP antibody) and endogenous SecgrII. Colocalization analysis of overexpressed NPY-pHluorin with SecgrII at DIV14 and DIV4, and with Chromogranin B (ChgB) at DIV14 in neurons quantified by Pearson’s coefficient.

(C) Manders’ coefficient for the overlap of overexpressed NPY-pHluorin with endogenous SecgrII or ChgB (left panel), or overlap of endogenous SecgrII or ChgB with NPY-pHluorin immunoreactivity (right panel).

(D-K) Stimulation dependent fusion of NPY-pHluorin in DIV3 and DIV4 WT and SNAP-25 KO neurons.

(D, E) Frequency distribution of DCV fusion events measured with NPY-pHluorin in WT and S25KO neurons before, during and after stimulation in DIV3 and DIV4 neurons, respectively (DIV3 WT: n = 7 cells, 26 events; DIV3 S25KO: n = 5 cells, 12 events; N = 3 independent experiments; DIV4 WT: n = 13 cells, 69 events; DIV4 S25KO: n = 8 cells, 13 events; N = 4 independent experiments). Blue bars represent stimulation of 16 bursts of 50 APs at 50 Hz; inset: typical calcium traces in WT (black) and S25KO (red) neurons measured with Fluo5-AM show similar calcium dynamics before, during and
after stimulation in both genotypes. Calcium influx is more robust at DIV4 compared to DIV3, note different scale bars for calcium measurements in DIV3 and DIV4 neurons.

(F) Average number of DCV fusion events per cell during stimulation in WT and S25KO neurons (DIV3 WT: n = 7 cells, 26 events; S25KO: n = 5 cells, 12 events; N = 3 independent experiments; ns, MW p = 0.9178; DIV 4 WT: n = 13 cells, 69 events; S25KO: n = 8 cells, 13 events; N = 4 independent experiments; ** MW p = 0.013, ns p = 0.342).

(G, H) Average cumulative DCV fusion events during stimulation in DIV3 and DIV4 WT and S25KO neurons. Blue bars represent stimulation period.

(I) Average total number of DCVs, visualized by de-quenching of NPY-pHluorin upon application of NH$_4^+$ in S25KO and WT neurons at DIV3 and DIV4 measured in F. (DIV3 WT: n = 7 cells, 1835 vesicles; DIV3 S25KO: n = 5 cells, 782 vesicles; N = 3 independent experiments; MW p = 0.559; DIV 4 WT: n = 13 cells, 3937 vesicles; DIV 4 S25KO: n = 8 cells, 1673 vesicles; N = 4 independent experiments; ns, MW p = 0.152 and 0.146 for DIV3 and DIV4).

(J, K) Normalized fusion probability measured as ratio of vesicles fused during stimulation per cell to their total pools in DIV3 and DIV4 WT and S25KO neurons (DIV 3 WT: n = 7 cells; S25KO: n = 5 cells; N = 3 independent experiments; ns, MW p = 0.845; DIV 4 WT: n = 13 cells; S25KO: n = 8 cells; N = 4 independent experiments; ns = 0.635, ** MW p = 0.011).

Data is plotted as mean ± SEM. Dots in 2F,I represent individual neurons.
Fig. 3. SNAP-25 family members rescue SV fusion in SNAP-25 KO neurons at DIV14 to varying extent
(A) Mean synaptic intensity of endogenous SNAP-25, -23, and -29 and in WT, and surviving S25KO neurons and of exogenous SNAP-25, -23, and -29 in S25KO neurons upon lenti viral mediated overexpression (+S25, +S23, or +S29. Comparisons to KO were significantly different with *** p < 0.01 or ** p = 0.013, Dunn’s multiple comparisons test.

(B) Typical examples of SyPhy fluorescence pre-stimulation, during stimulation and during NH$_4^+$ application for DIV14 SNAP-25 KO neurons expressing SNAP-25 (+S25), SNAP-29 (+S29) or SNAP-23 (+S23).

(C) Percentage of neurons from WT, S25KO and S25KO neurons expressing SNAP homologs that show stimulus dependent SV fusion.

(D) Maximum SyPhy fluorescence intensity upon stimulation with 200 AP at 10 Hz (Release pool) and upon NH$_4^+$ superfusion (Total pool) in WT, S25KO and S25KO neurons expressing SNAP homologs. Number of cells and independent experiments are depicted under x-axis. * p = 0.02, ** p = 0.008, ns not-significant.

(E) Fusion probability expressed as ratio of fused to total pool in WT, S25KO and S25KO neurons expressing SNAP homologs. MW WT vs S29 *** p = 0.001. ns not-significant.

(F) Normalized ΔF/F0 SyPhy fluorescence profiles upon stimulation with 200 AP at 10 Hz (blue bar) in WT, S25KO and S25KO neurons expressing SNAP homologs normalized to total pool (NH$_4^+$, green bar).
Fig. 4. SNAP-25 family members differentially rescue DCV fusion in SNAP-25 KO neurons at DIV14
(A) Frequency distribution of DCV fusion events from WT, S25KO and S25KO neurons expressing SNAP-25 (+S25). Blue bars represent 16 bursts of 50 APs at 50 Hz.

(B) Frequency distribution of DCV fusion events from S25KO and S25KO neurons expressing SNAP-23 (+S23) or SNAP-29 (+S29).

(C) Average number of DCV fusion events in WT, S25KO and S25KO neurons expressing SNAP homologs (+S23: n = 18 cells, N = 4, 2791 events; +S25: n = 22 cells, N = 4, 5538 events; +S29: n = 16 cells, N = 4, 541 events; S25KO: n = 19 cells, N = 3, 44 events; WT: n = 18 cells, N = 4, 4286 events; MW test to compare to KO levels, vs S23 *** p < 0.001, vs S25 ***p < 0.001, vs S29 * p = 0.0123, vs WT *** p < 0.001.). Dots represent individual cells.

(D) Total pool and normalized fusion probability (NFP; measured as ratio of vesicles fused per cell to their total pools) in WT, S25KO and S25KO neurons expressing SNAP homologs (+S23: n = 15 cells, N = 4, 14146 vesicles, NFP = 0.63; +S25: n = 21 cells, N = 4, 21714 vesicles, NFP = 0.90; +S29: n = 15 cells, N = 4, 16567 vesicles, NFP = 0.11; S25KO: n = 12 cells, N = 3, 6009 vesicles, NFP = 0.03; WT: n = 18 cells, N = 4, 17321 vesicles, NFP = 1.00). Total vesicle pool KO vs WT * p = 0.024, NFP of KO vs WT *** p < 0.001, NFP of S29 vs WT *** p < 0.001.

(E) Average cumulative DCV fusion events before, during and after stimulation in S25KO and S25KO neurons expressing SNAP-25 (+S25) or SNAP-23 (+S23).

(F) Average cumulative DCV fusion events before, during and after stimulation in S25KO and S25KO neurons expressing SNAP-23 (+S23, same trace as in E) or SNAP-29 (+S29).
(G) Average cumulative frequency during the first four bursts of 50 APs at 50 Hz.

(H) Average and mode (most frequent) time of fusion upon AP-stimulation in S25KO, WT and S25KO expressing SNAP-25, -23 or -29 neurons. Horizontal blue bars represent 50 AP bursts of a 16 x 50 APs at 50 Hz stimulus train (Average time of fusion: +S23: 43.68 ± 0.21 s, mode: 38.5 s; +S25: 40.76 ± 0.12 s, mode: 31 s; +S29: 43.71 ± 0.51 s, mode: 41 s; S25KO: 46.45 ± 1.75 s, mode: 53 s; WT: 40.30 ± 0.11 s, mode: 31 s).
Fig. 5 SNAP-47 does not rescue survival, synaptic transmission or DCV fusion in SNAP-25 KO neurons at DIV14

(A) Representative western blot of cultured WT, surviving S25KO neurons and S25KO neurons overexpressing SNAP-47 (+S47) stained for SNAP-47 and actin as loading control. Note the overexpression of SNAP-47 in lane 3. TCE, 2,2,2-Trichloroethanol incorporated into gels to assess protein loading.

(B) Survival curve comparing percentage of surviving S25KO neurons and S25KO neurons overexpressing SNAP-47 (+47) from the day of plating (DIV0). SNAP-47 fails to rescue survival of SNAP-25 KO neurons.

(C) Mean overall intensity of SNAP-47 at synapses of WT, surviving S25KO neurons and S25KO neurons expressing SNAP-47 (+S47). *** MW p < 0.001.

(D) Representative images of WT, surviving S25KO neurons and S25KO overexpressing SNAP-47 stained for dendrites (MAP2, blue), synapses (Synaptophysin, red) and SNAP-47 (green). Scale bar, 10 µm.

(E) Zooms of D, scale bar, 3 µm in all panels.

(F) Synaptophysin-pHluorin responses to 200 action potentials at 10 Hz in WT, surviving S25KO neurons and S25 KO neurons overexpressing SNAP-25 or -47 normalized to the total pool upon NH₄⁺ superfusion.

(G) Average DCV fusion events in WT, surviving S25KO neurons and S25KO neurons overexpressing SNAP-25 or -47. (+S47: n = 8 cells, N = 2, 7 events; +S25: n = 8 cells, N = 2, 1392 events; S25KO: n = 4 cells, N = 2, 6 events; WT: n = 5 cells, N = 2, 732 events; * p = 0.0159, ** p = 0.004). Dots represent individual neurons.
(H) Total pool (orange bars) and normalized fusion probability (green bars, NFP; ratio of vesicles fused per cell to their total pools) in WT, S25KO and S25KO neurons expressing SNAP isoforms (+S47: n = 2 cells, N = 2, 1441 vesicles, NFP= 0.03; +S25: n = 6 cells, N = 2, 6354 vesicles, NFP= 1.29; S25KO: n = 3 cells, N = 2, 1792 vesicles, NFP= 0.04; WT: n = 3 cells, N = 2, 3646 vesicles, NFP = 1.00).
References


Supplementary Figures

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Supplementary Figures
Supplementary Figure 1 Deletion of SNAP-25 reduces neuronal survival, dendrite length and arborization at DIV14 but does not affect localization of remaining DCV fusion events

(A) Representative images of DIV14 WT and SNAP-25 KO (S25KO) neurons stained for dendritic marker MAP2 (blue), axonal marker smi312 (red) and synapse marker synaptophysin (green). Scale bar, 10 µm.

(B) Survival curve showing percentage of surviving S25KO neurons from the day of plating (DIV0) over the subsequent 8 days in vitro. On average ±1% of S25KO neurons survives till DIV8.

(C) Mean dendritic length of WT and S25KO neurons (MW * p = 0.025).

(D) Mean synapse number of WT and S25KO neurons (MW * p = 0.018).

(E) Mean synapse number per µm dendrite of WT and S25KO neurons (MW * p = 0.117).

(F) Mean synapse area of WT and S25KO neurons (MW p = 0.121).

(G) Mean soma area of WT and S25KO neurons (MW *** p < 0.0001).

(H) Soma major axis of WT and S25KO neurons (MW *** p < 0.0001).

(I) Sholl analysis of mean number of dendrites with increasing distance from the soma of WT and S25KO neurons.

(J) Sholl analysis of mean number of synapses with increasing distance from the soma of WT and S25KO neurons.

(K) Mean MAP2 intensity of WT and S25KO neurons (MW p = 0.749).

(L) Mean synaptophysin intensity of WT and S25KO neurons (MW p = 0.267).

(M) Mean smi312 intensity of WT and S25KO neurons (MW p = 0.536).
n/N for C-M: WT: 49/3 and S25KO: 44/3

(N) Extra-synaptic and synaptic DCV fusion events in WT and S25KO neurons (WT: n = 43 cells, N = 2, 1882 events, MW * p = 0.013; S25KO: n = 30 cells, N = 2, 89 events).

(O) Percentage of extra-synaptic and synaptic fusion events in WT and S25KO neurons (WT: MW *** p < 0.0001; S25KO: MW * p = 0.04).

Data is plotted as mean ± SEM.
Supplementary Figure 2 SNAP-25 KO neurons are smaller compared with control at DIV3 and DIV4

(A) Mean axonal length of DIV3 and -4 WT and S25KO neurons (DIV3 MW p = 0.3148, DIV4 MW p = 0.086).

(B) Mean dendritic length of WT and S25KO neurons (DIV3 MW *** p = 0.0008, DIV4 MW *** p = 0.0003).

(C) Mean dendritic and axonal length of WT and S25KO neurons (DIV3 MW p = 0.723, DIV4 MW * p = 0.014).

(D) Mean soma area of WT and S25KO neurons (DIV3 MW p = 0.0739, DIV4 MW * p = 0.012).

(E) Soma major axis of WT and S25KO neurons (DIV3 MW p = 0.1756, DIV4 MW p = 0.055).

(F) Soma minor axis of WT and S25KO neurons (DIV3 MW p = 0.184, DIV4 MW * p = 0.017).

(G) Sholl analysis of mean number of dendrites with increasing distance from the soma of DIV3 WT and S25KO neurons.

(H) Sholl analysis of mean number of dendrites with increasing distance from the soma of DIV4 WT and S25KO neurons.

n/N for (A- H): DIV3 WT: 64/3 and S25KO: 57/3. DIV4 WT: 42/3 and S25KO: 42/3

(I) Percentage of S25KO and WT neurons unresponsive to electrical stimulation (silent neurons) (DIV3 MW p = 0.90, DIV4 MW p = 0.77). n/N: DIV3 WT: 23/3 and S25KO: 16/3. DIV4 WT: 19/4 and S25KO: 19/4.

(J) Typical confocal images of DIV4 WT neuron stained for SNAP23 (red), SNAP25 (green) and the dendritic marker MAP2. Scale bar represents 10 µm.
(K) Fluorescence intensity of endogenous SNAP-23 or SNAP-25 in soma of WT neurons measured at DIV2, 3, 4, 7 and DIV14 shows increase in expression levels of both proteins and indicates a sharp increase in SNAP-25 expression between DIV3-4 (n = 10 cells per DIV). ** p < 0.001.

(L) Fluorescence intensity of SNAP-23 in SNAP-25 KO neurons shows significant reduction of SNAP-23 expression between DIV3 and DIV4, coinciding with massive cell loss. SNAP-23 expression in surviving (<1%) SNAP-25 KO neurons remains stable from DIV4 onwards (DIV7 and DIV15). Individual cells are plotted (n = 10 cells per DIV). ** p = 0.0012 MW.

Data is plotted as mean ± SEM.
Supplementary Figure 3 Rescue of morphological features and localization of endogenous and overexpressed SNAP-23 and -29 in SNAP-25 KO and wild type neurons.

(A) MAP2 (blue), Synaptophysin (red) and SNAP-23 (green) in wild type WT, S25KO and S25KO neurons expressing SNAP-23.
(B) MAP2 (blue), Synaptophysin (red) and SNAP-25 (green) in wild type WT, S25KO and S25KO neurons expressing SNAP-25.
(C) MAP2 (blue), Synaptophysin (red) and SNAP-29 (green) in wild type WT, S25KO and S25KO neurons expressing SNAP-29.
(D,I,N) Dendritic lengths of SNAP-23, 25 and 29 rescued neurons compared to WT and S25KO neurons.
(E,J,O) Number of synapses of SNAP-23, 25 and 29 rescued neurons compared to WT and S25KO neurons.
(F,K,P) Synaptophysin intensity of SNAP-23, 25 and 29 rescued neurons compared to WT and S25KO neurons.
(G,L,Q) Mean soma area of SNAP-23, 25 and 29 rescued neurons compared to WT and S25KO neurons.
(H,M,R) Sholl analysis of mean number of dendrite branches of SNAP-23, 25 and 29 rescued neurons compared to WT and S25KO neurons.

Data is plotted as mean ± SEM.

(S) Western blot of cultured WT, surviving S25KO neurons and S25KO neurons expressing SNAP-23 stained for SNAP-23 and actin.

(T) Western blot of cultured WT, surviving S25KO neurons and S25KO neurons expressing SNAP-25 stained for SNAP-25 and actin.

(U) Western blot of cultured WT, surviving S25KO neurons and S25KO neurons expressing SNAP-29 stained for SNAP-29 and actin.
Supplementary Figure 4 Viral expression of SNAP-47 does not rescue morphological defects of SNAP-25 KO neurons

(A) Dendritic lengths of WT, S25KO and S25KO neurons rescued with SNAP-47.

(B) Number of synapses of WT, S25KO and S25KO rescued with SNAP-47.

(C) Synaptophysin intensity of WT, S25KO and S25KO rescued with SNAP-47.

(D) Mean synapse area of WT, S25KO and S25KO rescued with SNAP-47.

(E) Mean soma area of WT, S25KO and S25KO rescued with SNAP-47.

(F) Sholl analysis of mean number of dendrite branches of WT, S25KO and S25KO rescued with SNAP-47.

(G) Sholl analysis of mean number of synapses of WT, S25KO and S25KO rescued with SNAP-47.

Data is plotted as mean ± SEM.