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PICK1 controls activity-dependent synaptic vesicle cargo retrieval

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Yong et al. identify PICK1 as a key regulator of presynaptic vesicle recycling in central synapses. They find that the loss of PICK1 function causes surface stranding and mislocalization of SV cargoes, as well as reducing the speed of SV endocytosis and glutamate release efficacy during high-frequency stimulation.

Highlights

- PICK1 is required for efficient SV endocytosis during high-frequency stimulation.
- PICK1 controls activity-dependent SV cargo clustering and retrieval.
- PICK1-AP-2 complex is required for SV endocytosis and sustained glutamate release.
- PICK1 PDZ domain is required for SV cargo targeting and surface expression.

SUMMARY

Efficient retrieval of synaptic vesicles (SVs) is crucial to sustain synaptic transmission. PICK1 is a unique PDZ- and BAR-domain-containing protein that regulates the trafficking of postsynaptic glutamate receptors. It is also expressed in presynaptic terminals and is associated with the SVs; however, its role in regulating SV recycling remains unknown. Here we show that PICK1 loss of function selectively slows the kinetics of SV endocytosis in primary hippocampal neurons during high-frequency stimulation. PICK1 knockdown also causes surface stranding and mislocalization of major SV proteins, synaptophysin and vGlut1, along the axon. A functional PDZ domain of PICK1 and its interaction with the core endocytic protein AP-2 are required for the proper targeting and clustering of synaptophysin. Furthermore, PICK1, and its interaction with AP-2, are required for efficient SV endocytosis and sustained glutamate release. Our findings therefore identify PICK1 as a key regulator of presynaptic vesicle recycling in central synapses.

Keywords: PICK1, synaptic vesicles, glutamate release, endocytosis, synaptic vesicle recycling

INTRODUCTION

Efficient communication between neurons relies on the release of neurotransmitters through the fusion of synaptic vesicles (SVs) with the presynaptic plasma membrane (Sudhof, 2013). Following exocytosis, synaptic membranes and cargo proteins must be retrieved through endocytosis and rapidly refilled with neurotransmitter in order to maintain the fidelity of neurotransmission. There are multiple modes of SV endocytosis, which are activated by different forms of neuronal activity, specific temperatures, and have differences in their molecular requirements. These include clathrin-mediated endocytosis (CME), kiss-and-run, activity-dependent bulk endocytosis and ultrafast endocytosis (Chanaday et al., 2019; Cousin, 2017; Soykan et al., 2016). In general, SVs can be generated directly from the plasma membrane through CME, or indirectly from endosomes that are formed via ultrafast or activity-dependent bulk endocytosis during low or high neuronal activity, respectively (Cheung and Cousin, 2012; Granseth et al., 2006; Soykan et al., 2017; Watanabe et al., 2014). The reformation of SVs relies on the precise sorting and clustering of lipids and integral membrane proteins by adaptor proteins (APs) such as AP-2, AP-1/AP-3, stonin, AP-180 and CALM, all of which coordinate cargo selection into clathrin-coated pits (Kononenko and Haucke, 2015; Mori and Takamori, 2017). AP-2 is required for the fast retrieval of SV cargo from the plasma membrane, whereas AP-1/AP-3 mediate the slower generation of vesicles from the endosomes (Cousin, 2017; Kononenko and Haucke, 2015; Mori and Takamori, 2017). The SV cycle is therefore highly dependent on many endocytic proteins working in synchrony, the perturbation of which has been linked to the etiology of several neurodevelopmental and neurodegenerative diseases (Bonnycastle et al., 2020; Waites and Garner, 2011). However, despite its essential role in synaptic transmission, the molecular mechanisms underlying SV recycling remain enigmatic.

Protein interacting with C-kinase 1 (PICK1) is a unique peripheral membrane protein that contains an N-terminal PDZ (postsynaptic density-95/disc-large/zona-occluden-1) protein interaction domain and a central BAR (Bin-Amphiphysin-Rvs) lipid-binding/oligomerization domain (Hanley, 2008; Xu and Xia, 2006). It is well established that PICK1 plays an important role in regulating the membrane trafficking of many transporters and receptors with which it interacts. PICK1 performs a key role in the regulation of postsynaptic AMPA-type glutamate receptor trafficking, a process that is crucial for multiple forms of synaptic plasticity (Anggono et al., 2011; Anggono et al., 2013; Fiuza et al., 2017; Steinberg et al., 2006; Terashima et al., 2008; Volk et al., 2010; Widagdo et al., 2016). It is also known to regulate the biogenesis and trafficking of secretory vesicles in endocrine cells (Cao et al., 2013; Holst et al., 2013). In addition, PICK1 is expressed in presynaptic nerve terminals (Haglerod et al., 2009), but whether it plays any role in SV recycling is unknown. The finding that PICK1 is associated with

SVs (Haglerod et al., 2009), coupled with the fact that it interacts with several components of the endocytic machinery, including the α -adaptin subunit of the AP-2 heteromer, dynamin and syndapin/PACSIN (Anggono et al., 2013; Fiuza et al., 2017), led us to investigate whether PICK1 is a regulator of SV recycling in mammalian central neurons.

RESULTS AND DISCUSSION

PICK1 is required for efficient SV endocytosis during high-frequency stimulation

To monitor SV recycling, we took advantage of the pH-sensitive green fluorescent protein (pHluorin) fused to the intravesicular loop of SV resident proteins, synaptophysin (syphHy) or vesicular glutamate transporter 1 (vGlut1-pH), both of which interact with PICK1 in heterologous HEK293T cells (Figure S1A and S1B). They serve as reporters for SV exo-/endocytosis and reacidification due to the quenching of their fluorescence intensity within the acidic SV lumen and subsequent dequenching following SV fusion (Granseth et al., 2006; Wang et al., 2014). To investigate the function of PICK1 in SV recycling, we adopted a loss of function approach by using a previously characterized specific shRNA against PICK1 (Anggono et al., 2011) (Figure S1C, see also Figure 2G) and probed rat primary hippocampal neurons with identical numbers of action potentials at either mild (10 Hz, 30 s) or high (50 Hz, 6 s) frequency stimulation. When challenged with a 10 Hz train, PICK1-depleted neurons remained exocytosis competent and showed no endocytosis defects compared to neurons that were transfected with the vector-only control or shRNA-resistant PICK1 cDNA (Figure 1A, 1B and S2A–S2C). However, when neurons were stimulated at 50 Hz, loss of PICK1 function significantly slowed the endocytosis kinetics of syphHy (Figure 1C, 1D and S2D). The reduction in the endocytosis time-constant (τ) could be rescued by the overexpression of a shRNA-resistant PICK1 construct in PICK1 knockdown hippocampal neurons (Figure 1C, 1D and S2D), indicating that the slowing in SV endocytosis was due to PICK1 depletion and not an off-target effect of the shRNA. We did not observe any significant changes in the level of syphHy exocytosis between the control and PICK1 knockdown neurons when it was expressed as a percentage of the total syphHy pool. However, exocytosis was augmented slightly in neurons overexpressing the PICK1 shRNA-resistant construct (Figure S2D–S2F). This gain of function phenotype is likely to be dependent on the interaction of PICK1 with NSF (*N*-ethylmaleimide-sensitive factor) and the SNARE (soluble NSF attachment protein receptor) proteins, including syntaxin1, SNAP-25 and VAMP2 (Figure S1D–S1F), all of which play crucial roles in SV exocytosis (Hanley et al., 2002; Sudhof, 2013). Similar results were obtained with vGlut1-pH, another major SV cargo protein in excitatory synapses (Figure 1E, 1F and S3A–S3C), indicating that PICK1 controls SV endocytosis, rather than the retrieval of individual SV cargo.

It was possible that the observed frequency-dependent requirement of PICK1 in SV recycling was due to differences in the SV pool size, given that PICK1 controls the biogenesis and size of secretory vesicles. To address this, we estimated the relative size of the readily releasable, reserve and resting SV pools by measuring sypHy responses to various stimuli in the presence of a vesicular proton ATPase blocker, bafilomycin A1, which prevents the reacidification of internalized membranes (Burrone et al., 2006). Our data revealed no significant alterations in the relative SV pool size in the absence of PICK1 (Figure 1G, 1H). These data also confirmed that the evoked sypHy exocytosis was fully functional in PICK1 knockdown neurons. Taken together, these results demonstrate that PICK1 is required for efficient SV retrieval during intense neuronal stimulation in hippocampal neurons.

Loss of PICK1 function leads to mislocalization and surface stranding of synaptophysin and vGlut1 along the axon

A reduction in the rate of SV endocytosis can lead to the accumulation of cargo proteins on the neuronal plasma membrane over multiple SV cycles (Foss et al., 2013; Kim and Ryan, 2009; Koo et al., 2015). Accordingly, loss of PICK1 function would be expected to cause the stranding of SV cargo on the plasma membrane. To investigate this, we measured the surface fraction of sypHy by perfusing neurons with an acidic buffer to quench the sypHy fluorescence that was present on the plasma membrane. The total amount of sypHy expression at the steady state was then determined by switching to an alkaline buffer. This revealed a significant elevation in the fraction of unretrieved sypHy molecules that remained on the plasma membrane in PICK1 knockdown neurons (Figure 2A and 2B). Expression of the shRNA-resistant PICK1 construct in these neurons restored the plasma membrane expression of sypHy back to control levels (Figure 2A and 2B). Similar results were obtained in the acid-quenching experiments using an independent probe, vGlut1-pH (Figure 2D and 2E). To corroborate this finding, we performed a biochemical surface biotinylation assay and measured the level of endogenous synaptophysin expression on the plasma membrane. In agreement with our imaging data, we found that hippocampal neurons which were transduced with lentiviral particles expressing PICK1 shRNA exhibited a significant increase in the level of synaptophysin expression on the neuronal membrane (Figure 2G–2I). This was fully rescued by restoring PICK1 expression (Figure 2G–2I). No significant changes in the total protein levels of synaptophysin were observed between groups, indicating that the increase in surface synaptophysin was not due to an aberrant alteration in synaptophysin expression (Figure 2G–2I).

A defect in the targeting or clustering of SV-associated proteins at nerve terminals could also affect the efficient retrieval and surface stranding of these cargo molecules (Baker et al., 2018; Gordon et al., 2011). Given that PICK1 interacts with AP-2 and regulates the synaptic targeting of glutamate receptors (Boudin et al., 2000; Fiuza et al., 2017; Jin et al., 2006; Xia et al., 1999), we examined the distribution of sypHy and vGlut1-pH, and measured their coefficient of variation (CV) as a proxy for the diffuseness of their fluorescence. As expected, control neurons displayed punctate distributions of sypHy and vGlut1-pH reflected by high CV values, indicating the proper targeting of sypHy and vGlut1-pH to the nerve terminals (Figure 2A, 2C, 2D and 2F). In contrast, both sypHy and vGlut1-pH exhibited more diffuse distributions along the axon in the absence of PICK1, with significantly lower CV values than that of the control neurons (Figure 2A, 2C, 2D and 2F). The expression of the PICK1 rescue construct restored the targeting of sypHy and vGlut1-pH to the nerve terminals (Figure 2A, 2C, 2D and 2F).

The apparent mistargeting and accumulation of synaptophysin on the plasma membrane are likely to be consequences of impaired endocytic sorting post-exocytosis, arising from multiple SV cycles driven by resting neuronal activity. To address this hypothesis, we incubated neurons with a voltage-gated sodium channel blocker, tetrodotoxin (TTX), to block action potential generation, and performed the same experiments in the presence of TTX. Inhibition of action potential firing essentially normalized the surface expression and localization of sypHy in PICK1-depleted hippocampal neurons (Figure 2J–2L). Collectively, these results highlight a critical role for PICK1 in controlling activity-dependent SV cargo clustering and retrieval in mammalian central neurons.

The interaction between PICK1 and α -adaptin is required for efficient SV endocytosis

Given the profound effects of PICK1 knockdown on sypHy localization and retrieval, we sought to determine the underlying mechanism of action by performing structure and function analyses of PICK1. We focused on three mutants, namely K27A/D28A (KDAA) in the PDZ domain that blocks the PICK1-PDZ-dependent protein-protein interaction (Xia et al., 1999), K251,252,257E (3KE) in the BAR domain that significantly reduces PICK1 binding to lipid (Jin et al., 2006), and D189,356A (DDAA) that inhibits PICK1 binding to α -adaptin (Fiuza et al., 2017). We utilized an overexpression strategy for these experiments, since these mutants oligomerise with endogenous PICK1, resulting in a dominant negative effect (Jin et al., 2006; Karlsen et al., 2015; Pan et al., 2007). When these mutants were overexpressed individually, the PICK1 DDAA mutant caused a significant reduction in the rate of sypHy endocytosis in response to stimulation at 50 Hz, compared to neurons that overexpressed wild-type PICK1 (Figure 3A, 3B and S4A). Expression of the DDAA mutant did not affect sypHy exocytosis

(Figure S4A–S4C), essentially phenocopying the effects observed in the PICK1 knockdown neurons.

Owing to their ability to sense, induce and stabilize lipid membrane curvature, many BAR domain-containing proteins at nerve terminals, such as amphiphysin and endophilin, are involved in SV recycling (Di Paolo et al., 2002; Ringstad et al., 1999). Through its BAR domain, PICK1 also directly binds to lipids, particularly phosphoinositides, and is capable of tubulating liposomes (Holst et al., 2013; Jin et al., 2006). Strikingly, the PICK1 3KE lipid-binding-deficient mutant had no effects on sypHy exo-/endocytosis (Figure 3A, 3B and S4A–S4C). Similar results were obtained with another PICK1 lipid-binding mutant 2KE (K266,268E, data not shown) (Jin et al., 2006; Steinberg et al., 2006). These data suggest that BAR-dependent lipid binding of PICK1 is dispensable for SV recycling, despite its importance in regulating other membrane trafficking events (Jin et al., 2006; Madsen et al., 2012; Pinheiro et al., 2014; Steinberg et al., 2006). However, the functional relevance of the interaction of PICK1 with lipid in SV retrieval cannot be completely ruled out as the PDZ domain of PICK1 has also been shown to bind negatively charged phospholipids, including phosphatidylinositol 4,5-bisphosphate, thereby stimulating endocytic cargo binding to the AP-2 complex and clathrin-coated pit formation (Jackson et al., 2010; Kelly et al., 2014).

Interestingly, overexpression of the PICK1 KDAA mutant resulted in a statistically insignificant acceleration of sypHy endocytosis in hippocampal neurons which were stimulated at a high frequency (Figure 3A, 3B and S4A). It is also worth noting that the amount of sypHy exocytosis was slightly reduced in the PICK1 KDAA neurons compared to those overexpressing wild-type PICK1 (Figure S4A–S4C). These data indicate that the ectopic enhancement of sypHy exocytosis due to PICK1 gain of function requires an intact PDZ-dependent protein-protein interaction. Taken together, our data demonstrate the critical role of the interaction between PICK1 and AP-2 in facilitating efficient recycling of sypHy under high-frequency stimulation, consistent with its role in promoting activity-dependent internalization of AMPA receptors via CME on the postsynaptic membrane (Fiuza et al., 2017).

A functional PICK1 PDZ domain is necessary for the correct targeting and surface expression of sypHy

We next investigated the effects of the same set of PICK1 mutants on sypHy localization and surface expression by performing acid quenching-dequenching experiments. Neurons expressing the PDZ domain-defective KDAA mutant and the AP-2 binding DDAA mutant exhibited a significantly lower CV value than those overexpressing wild-type PICK1, indicating an impairment in the targeting and/or clustering of sypHy at nerve terminals (Figure 3C and

3E). These data demonstrate that PICK1, through its interactions with the α -adaptin subunit of AP-2 and an unknown PDZ-dependent interacting presynaptic molecule, is required for the clustering of sybHy on the plasma membrane. Interestingly, only the expression of the PICK1 KDAA mutant, and not the AP-2 binding-defective mutant, caused an increase in the ratio between surface-stranded and total sybHy in hippocampal neurons at rest (Figure 3C and 3D). The observed effects on sybHy surface stranding and clustering were eliminated by silencing neuronal activity at rest in the presence of TTX (Figure 3F–3H). Again, we did not observe any significant changes in the surface expression and synaptic targeting of sybHy in neurons expressing the 3KE mutant (Figure 3C–E), suggesting that PICK1 lipid binding, at least by the BAR domain, is not essential for activity-dependent sybHy retrieval.

The accumulation of SV cargo proteins on the plasma membrane is generally assumed to be a result of defective cargo retrieval mechanisms. In our study, neurons overexpressing the PICK1 AP-2 binding-defective DDAA mutant displayed slower endocytosis kinetics without an increase in surface-stranded sybHy. In contrast, those that overexpressed the PICK1 PDZ-defective KDAA mutant displayed the opposite phenotype. These data add to emerging evidence that demonstrates a disconnection between SV cargo retrieval and plasma membrane localization, suggesting that mechanisms which control the surface abundance of SV cargo are sometimes distinct from those that drive SV endocytosis. We propose that PICK1 regulates the post-exocytosis sorting and clustering of sybHy in an activity-dependent manner in response to a wide range of stimuli, from resting neuronal activity to high-frequency stimulation. This requires an intact PDZ domain and a functional interaction with AP-2. However, the latter is only required and recruited to facilitate efficient SV endocytosis via an AP-2-mediated pathway during high-frequency stimulation. We speculate that the sybHy which is mislocalized outside the perisynaptic zone is eventually retrieved via an alternative recycling pathway, possibly involving AP-1/AP-3. In support of this, PICK1 is an effector of the GTPase ADP-ribosylation factor 1 (Arf1) (Rocca et al., 2013), which promotes the recruitment and association of AP-3 to the plasma membrane (Faundez et al., 1998; Ooi et al., 1998). The binding of PICK1 to activated, GTP-bound Arf1 requires its PDZ domain and is blocked by the KDAA mutations (Rocca et al., 2013). We speculate that the AP-1/AP-3-dependent mechanism is blocked in neurons overexpressing the PICK1 KDAA mutant, resulting in the accumulation of sybHy on the plasma membrane. It is also plausible that the recycling of SVs at rest uses a different endocytic pathway from those that are evoked by neuronal activity (Chanaday and Kavalali, 2018; Fredj and Burrone, 2009; Kavalali, 2015; Sara et al., 2005).

PICK1 is required for sustained glutamate release

The missorting of SV cargo to the plasma membrane coupled with the slower kinetics of endocytosis in PICK1-depleted neurons should impact the efficacy of neurotransmitter release, particularly during intense neuronal stimulation. To address this, we measured changes in the fluorescence intensity of a genetically encoded glutamate-sensitive optical reporter iGluSnFR (Marvin et al., 2018) as a proxy for evoked glutamate release. Neurons were challenged with a total of five repetitive trains of 50 Hz stimulation with a 2 min inter-train recovery interval. Glutamate release evoked by each train of stimuli was measured and normalized to the fluorescence peak generated from the first stimulus train. A reduction in glutamate release following repetitive 50 Hz stimulation was observed in neurons transfected with an empty vector alone (Figure 4A and 4B). This occurred because continuous stimulation above 10 Hz depletes the releasable pool due to the rate of exocytosis exceeding the rate of endocytosis (Fernandez-Alfonso and Ryan, 2004). However, stimulus-dependent depression of glutamate release was significantly exacerbated in the PICK1-depleted neurons, which could be fully rescued by expression of the PICK1 shRNA-resistant construct (Figure 4A and 4B). Hippocampal neurons expressing the PICK1 AP-2 binding-deficient DDAA mutant exhibited a significantly greater stimulus-dependent depression of glutamate release than those expressing wild-type PICK1, consistent with the role of the interaction between PICK1 and AP-2 in maintaining efficient SV retrieval during high-frequency stimulation (Figure 4C and 4D). These data underscore the functional importance of PICK1 in SV recycling, and the maintenance of the fidelity of glutamate release during intense neuronal stimulation.

In conclusion, our study defines a presynaptic role for PICK1 in the activity-dependent targeting, sorting and retrieval of the SV cargo during endocytosis. PICK1 can interact with both activated Arf1 and α -adaptin, and could potentially be engaged in cargo sorting and endocytosis through different pathways controlled by specific adaptor protein complexes. We postulate that the interaction between PICK1 and AP-2 is specifically required for the efficient, activity-dependent sorting and endocytosis of SV protein cargoes under intense neuronal stimulation. Furthermore, the presence of PICK1 may promote SV endocytosis by stimulating dynamin polymerization and inhibiting Arp2/3-dependent actin polymerization, consequently enhancing formin-dependent actin polymerization (Fiuza et al., 2017; Rocca et al., 2008; Soykan et al., 2017). On a broader scale, our findings open avenues for studying the presynaptic function of PICK1, and its role in presynaptic plasticity and the maintenance of the fidelity of neurotransmission at central synapses.

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AUTHOR CONTRIBUTIONS

VA conceived the project. XLHY performed experiments. XLHY, MAC and VA designed the study and analyzed the data. XLHY and VA wrote the manuscript. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

FIGURE LEGENDS

Figure 1. PICK1 is required for efficient SV endocytosis during high-frequency stimulation

(A and B) SyHy endocytosis is unaltered in PICK1 knockdown neurons when challenged with a train of 300 action potentials at 10 Hz for 30 s. (A) Averaged normalized syHy traces ($\Delta F/F_0$) from control, knockdown and rescue neurons. (B) Comparison of endocytosis time-constant (τ) values between control ($n = 9$ coverslips), knockdown ($n = 7$) and rescue ($n = 9$) neurons from 2 independent cultures. No significant differences were found among groups as determined by one-way ANOVA. Data are presented as mean $\pm$ SEM.

(C and D) Depletion of PICK1 slows the kinetics of syHy endocytosis when challenged with a train of 300 action potentials at 50 Hz for 6 s. (C) Averaged normalized syHy traces ($\Delta F/F_0$) from control, knockdown and rescue neurons. (D) Comparison of endocytosis time-constant (τ) values between control ($n = 20$ coverslips), PICK1-shRNA ($n = 12$) and rescue ($n = 13$) in neurons from 3 independent cultures. Data are presented as mean $\pm$ SEM. ** $P < 0.01$, **** $P < 0.0001$ (One-way ANOVA with Tukey's multiple comparisons test).

(E and F) Depletion of PICK1 slows the kinetics of vGlut1-pHluorin endocytosis when challenged with a train of 300 action potentials at 50 Hz for 6 s. (E) Averaged normalized vGlut1-pHluorin traces ($\Delta F/F_0$) from control, knockdown and rescue neurons. (F) Comparison of endocytosis time-constant (τ) values between control ($n = 15$ coverslips), knockdown ($n = 13$) and rescue ($n = 15$) neurons from 3 independent cultures. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, (One-way ANOVA with Tukey's multiple comparisons test).

(G and H) PICK1 knockdown does not affect the size of SV pools. (G) A series of averaged normalized sypHy responses to two consecutive stimuli (40 action potentials, 20 Hz), followed by a train of 900 action potentials (20 Hz) in the control and PICK1 knockdown hippocampal neurons in the presence of bafilomycin A1. The amplitude of the first response corresponds to the readily releasable pool, while the sum of the second and third responses provides an estimation of the reserve pool. NH_4Cl was used to reveal the size of the resting pool. (H) Quantification of the relative percentage of each SV pool in control ($n = 14$ coverslips) and PICK1 knockdown ($n = 15$) neurons from 3 independent cultures. Data are presented as mean $\pm$ SEM. No statistical significance was observed between control and PICK1 knockdown neurons as determined using a two-tailed Student's t-test.

Figure 2. PICK1 regulates activity-dependent synaptic targeting and surface expression of sypHy and vGlut1-pH in hippocampal neurons

(A–C) PICK1 knockdown causes mislocalisation and surface stranding of sypHy in neurons. (A) Representative images of hippocampal neurons co-expressing sypHy with either empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs during acid quenching/dequenching experiments. Upon exposure to an acidic buffer, the fluorescence of sypHy localized on the plasma membrane will be quenched. The distribution of total sypHy was revealed by exposure to NH_4Cl . (B) Averaged surface expression of sypHy as a percentage of total sypHy. (C) Coefficient of variation (CV) analysis for control, knockdown or rescue neurons. Control $n = 13$ coverslips, shRNA $n = 17$, rescue $n = 12$ from 4 independent cultures. Data are presented as mean $\pm$ SEM. ** $P < 0.01$, **** $P < 0.0001$ (One-way ANOVA with Tukey's multiple comparisons test).

(D–F) PICK1 knockdown causes mislocalisation and surface stranding of vGlut1-pH in neurons. (D) Representative images of hippocampal neurons co-expressing vGlut1-pH with either empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs during acid quenching/dequenching experiments. Upon exposure to an acidic buffer, the fluorescence of vGlut1-pH localized on the plasma membrane will be quenched. The distribution of total vGlut1-pH was revealed by exposure to NH_4Cl . (E) Averaged surface expression of vGlut1-pH as a percentage of total vGlut1-pH. (F) Coefficient of variation (CV)

analysis for control, knockdown or rescue neurons. Control $n = 17$ coverslips, shRNA $n = 14$, rescue $n = 17$ from 4 independent cultures. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (One-way ANOVA with Tukey's multiple comparisons test).

(G–I) Depletion of PICK1 leads to surface stranding of endogenous of synaptophysin in neurons. (G) Cultured hippocampal neurons were transduced with lentiviral particles expressing GFP alone (control), PICK1 shRNA (knockdown) and PICK1-rescue cDNA and subjected to surface biotinylation assay. Samples were resolved by SDS-PAGE and probed with specific antibodies against synaptophysin, PICK1 and α -tubulin. Endogenous PICK1 is represented with an asterisk while overexpressed myc-tagged PICK1 is noted with an arrowhead. (H) Quantification of the endogenous surface to total synaptophysin ratio, normalized to control. (I) Quantification of the total synaptophysin to α -tubulin ratio, normalized to control. Control $n = 20$ wells, shRNA $n = 20$, rescue $n = 16$ from 7 independent cultures. Data are presented as mean $\pm$ SEM. ** $P < 0.01$ (One-way ANOVA with Tukey's multiple comparisons test).

(J–L) Surface accumulation and mislocalisation of sypHy in PICK1 knockdown neurons are rescued by tetrodotoxin (TTX)-mediated neuronal silencing. (J) Representative images of control and PICK1 knockdown hippocampal neurons expressing sypHy during acid quenching/dequenching in the presence of 1 μ M TTX to suppress resting neuronal activity. (K) The level of sypHy surface expression as a percentage of total sypHy. (L) CV values of sypHy distribution in the presence of 1 μ M TTX. Control $n = 10$ coverslips, shRNA $n = 12$ from 3 independent cultures. Data are presented as mean $\pm$ SEM. No statistical significance was observed between control and PICK1 knockdown neurons as determined using a two-tailed Student's t-test.

Figure 3. A functional PDZ domain of PICK1 and its interaction with AP-2 is required for sypHy retrieval in hippocampal neurons

(A and B) PICK1 interaction with α -adaptin is required for efficient endocytosis of sypHy during high-frequency stimulation. (A) Averaged normalized sypHy traces ($\Delta F/F_0$) from neurons expressing wild-type PICK1 or KDAA, 3KE or DDAA mutants when stimulated a train of 300 action potentials (50 Hz, 6 s). (B) Comparison of endocytosis time-constant (τ) values between neurons overexpressing wild-type PICK1 ($n = 21$ coverslips), KDAA ($n = 13$), 3KE ($n = 15$) and DDAA mutants ($n = 16$) from 3 independent cultures. Data are presented as mean $\pm$ SEM. ** $P < 0.01$ (One-way ANOVA with Dunnett's multiple comparisons test).

(C–E) A functional PDZ domain of PICK1 is required for the proper clustering and surface expression of sypHy in hippocampal neurons. (C) Representative images of hippocampal neurons expressing sypHy with myc-PICK1 constructs, either wild-type or mutants as

indicated, during acid quenching/dequenching. (D) The level of sypHy surface expression as a percentage of total sypHy. (E) Coefficient of variation (CV) analysis of the sypHy distribution in hippocampal neurons expressing myc-PICK1, either wild-type or mutants. Wild-type $n = 29$ coverslips, KDAA $n = 21$, 3KE $n = 13$, DDAA $n = 18$, from 3 independent cultures. Data are presented as mean $\pm$ SEM. ** $P < 0.01$, **** $P < 0.0001$ (One-way ANOVA with Dunnett's multiple comparisons test).

(F–G) Surface accumulation and mislocalisation of sypHy in neurons overexpressing PICK1 KDAA and DDAA mutants are rescued by tetrodotoxin (TTX)-mediated neuronal silencing. (F) Representative images of hippocampal neurons expressing sypHy with myc-PICK1 constructs during acid quenching/dequenching in the presence of 1 μ M TTX. (G) The level of sypHy surface expression as a percentage of total sypHy. (H) CV values of sypHy distribution in the presence of 1 μ M TTX. Wild-type $n = 16$ coverslips, KDAA $n = 11$, DDAA $n = 9$, from 3 independent cultures. Data are presented as mean $\pm$ SEM. No statistical significance was observed between groups of neurons as determined using one-way ANOVA.

Figure 4. PICK1, and its interaction with α -adaptin, are required to maintain glutamate release following repetitive electrical stimulation

(A and B) Depletion of PICK1 exacerbates depression of glutamate release following 5 trains of 300 action potentials (50 Hz, 2 min interstimulus intervals). (A) Averaged traces of iGluSnFR responses normalized to the peak of the first response in hippocampal neurons expressing empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs. The origins of the shRNA and rescue traces have been shifted horizontally for presentation purposes. Dark lines and shaded regions denote mean and SEM, respectively. (B) Summary data of normalized iGluSnFR peak responses over 5 repetitive stimulations. Control $n = 13$ coverslips, shRNA $n = 17$, rescue $n = 10$, from 3 independent cultures. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ control vs shRNA, ##### $P < 0.0001$ rescue vs shRNA (Two-way ANOVA with Sidak multiple comparisons test).

(C and D) The interaction between PICK1 and AP-2 is required to maintain glutamate release following 5 trains of 300 action potentials (50 Hz, 2 min interstimulus intervals). (C) Averaged traces of iGluSnFR responses normalized to the peak of the first response in hippocampal neurons expressing wild-type PICK1 or the DDAA mutant. The origin of the DDAA trace has been shifted horizontally for presentation purposes. Dark lines and shaded regions denote mean and SEM, respectively. (D) Normalized changes iGluSnFR responses over 5 repetitive stimulations. Wild-type $n = 8$ coverslips, DDAA $n = 9$ from 3 independent cultures. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ wild-type vs DDAA (Two-way ANOVA with Sidak multiple comparisons test).

STAR METHODS

KEY RESOURCES TABLES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
β -actin (rabbit monoclonal, clone 13E5, used 1:10,000 in WB)	Cell Signaling Technology	Cat# 4970, RRID:AB_2223172
GFP (chicken polyclonal, used 1:5,000 in WB)	Aves Labs	Cat# GFP-1020, RRID:AB_10000240
GST (mouse monoclonal, clone 3G12B10, used 1:5,000 in WB)	Proteintech	Cat# 66001-2-Ig
HA (rabbit polyclonal, used 1:5,000 in WB)	Proteintech	Cat# 51064-2-AP, RRID:AB_11042321
Myc (mouse monoclonal, clone 9E10, used 1:5,000 in WB)	Santa Cruz Biotechnology	Cat# sc-40, RRID:AB_627268
Peroxidase-conjugated donkey anti-rabbit IgG antibody	GE Healthcare	Cat# NA934, RRID:AB_772206
Peroxidase-conjugated sheep anti-mouse IgG antibody	GE Healthcare	Cat# NA931, RRID:AB_772210
PICK1 (rabbit polyclonal, used 1:500 in WB)	R. Huganir (Anggono et al., 2011)	N/A
Synaptophysin (mouse monoclonal, used 1:10,000 in WB)	Synaptic Systems	Cat# 101 011, RRID:AB_887824
α -tubulin (mouse monoclonal, clone 1E4C11, used 1:10,000 in WB)	Proteintech	Cat# 66031-1-Ig, RRID:AB_2687491
Chemicals, Peptides, and Recombinant Proteins		
Bafilomycin A1	Calbiochem	Cat# 196000
DL-AP5 sodium salt	Abcam	Cat# ab120271
cOmplete, EDTA-free protease inhibitor cocktail	Sigma-Aldrich	Cat# 11836170001
EZ-Link Sulfo-NHS-SS-biotin	Thermo Scientific	Cat# 21331
Glutathione agarose	Thermo Scientific	Cat# 16100
Lipofectamine 2000	Thermo Scientific	Cat# 11668019
Neutravidin agarose	Thermo Scientific	Cat# 29204
NBQX disodium salt	Abcam	Cat# ab120046
Papain suspension	Worthington	Cat# LS003126
Phosphatase inhibitor cocktail	AG Scientific	Cat# P-1517
Tetrodotoxin citrate	Abcam	Cat# ab120055
Experimental Models: Cell Lines		
Human: HEK293T	ATCC	Cat# CRL-3216, RRID:CVCL_0063
Experimental Models: Organisms/Strains		
Rat: Sprague-Dawley	University of Queensland Biological Resources	RRID:RGD_70508
Recombinant DNA		
FuGW	Addgene	Plasmid #14883
FuGW-PICK1-shRNA	(Anggono et al., 2011)	N/A
FUW-H1-PICK1-shRNA-Ub-myc-PICK1-shRNA resistant	This paper	N/A
pAAV.CAG.SF-iGluSnFR.A184V	Addgene (Marvin et al., 2018)	Plasmid #106199

pCIS	R. Huganir	N/A
pCIS-PICK1 WT	R. Huganir	N/A
pMD2.G	Addgene	Plasmid #12259
pMDLg/pRRE	Addgene	Plasmid #12251
pRK5-myc-PICK1 WT	(Xia et al., 1999)	N/A
pRK5-myc-PICK1 KDAA (K27A/D28A)	(Xia et al., 1999)	N/A
pRK5-myc-PICK1 3KE (K251,252,257E)	This paper	N/A
pRK5-myc-PICK1 DDAA (D189,356A)	This paper	N/A
pRK5-H1-shRNA-CMV-myc-PICK1-shRNA resistant	This paper	N/A
pRK5-myc-SNAP-25	(Bademosi et al., 2018)	N/A
pRK5-HA-syntaxin1A (<i>Drosophila</i>)	(Bademosi et al., 2018)	N/A
pSuper	Oligoengine	VEC-PBS-0001/0002
pSuper-PICK1-shRNA	(Anggono et al., 2011)	N/A
Synaptophysin-pHluorin (CMV-sypHy-A4)	Addgene (Granseth et al., 2006)	Plasmid #24478
VAMP2-pHluorin	(Miesenbock et al., 1998)	N/A
vGlut1-pHluorin-mCherry	(Wang et al., 2014)	N/A
Software and Algorithms		
Fiji	http://fiji.sc	RRID:SCR_002285
Image Studio Lite	https://www.licor.com/bio/image-studio-lite/	RRID:SCR_015795
GraphPad Prism 8	https://www.graphpad.com/	RRID:SCR_002798

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by the Lead contact, Victor Anggono (v.anggono@uq.edu.au).

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and Code Availability

This study did not generate datasets/code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rats

Adult female Sprague-Dawley rats and their embryos (males and females, embryonic day 18) were used for the preparation of primary hippocampal neurons. All research procedures involving the use of animals were conducted in accordance with the Australian code of practice

for the care and use of animals for scientific purposes and were approved by the University of Queensland Animal Ethics Committee.

HEK293T cells

HEK293T cells were obtained from ATCC (CRL-3216). Cells were grown in DMEM with 4.5g/L glucose (GIBCO) supplemented with 10% FBS (Invitrogen) and 50 U/ml penicillin, 50 µg/ml streptomycin (GIBCO) in a humidified 5% CO₂ incubator at 37°C.

METHOD DETAILS

DNA constructs

The PICK1 shRNA targeting sequence (5'-GCCTCACCATCAAGAAGTACC-3') was cloned into the pSuper vector system (Oligoengine, Seattle, WA) or the FuGW lentiviral vector, both of which have been validated previously (Anggono et al., 2011). The shRNA-resistant rescue construct was generated by introducing five silent mutations, namely 5'-GCCTCACGATAAAAAATATC-3', into pRK5-myc-PICK1 using the overlapping PCR protocol (boldface and underlining represent mutated bases in the sequences). A cassette containing the H1 RNA polymerase III promoter and the PICK1 shRNA sequence was amplified from the pSuper construct and cloned into the unique *PciI* restriction site within the pRK5-myc-PICK1 shRNA resistant construct to create a bicistronic pRK5-H1-shRNA-CMV-myc-PICK1 rescue construct. A similar cloning strategy was employed to produce a bicistronic FUW-H1-shRNA-ubiquitin promoter-myc-PICK1 lentiviral rescue construct. Plasmids encoding the PICK1 3KE (K251,252,257E) lipid binding mutant and the DDAA (D189,356A) α-adaptin binding mutant were generated using the overlapping PCR protocol and were subcloned into the pRK5-myc-PICK1 vector. The pRK5-myc-PICK1 wild-type and KDAA (K27A/D28A) PDZ-defective mutant, as well as the pCIS-PICK1 and pCIS vectors were gifts from Dr. Richard Huganir (Johns Hopkins University) (Xia et al., 1999). The vGlut1-pHluorin-mCherry and VAMP2-pHluorin constructs were gifts from Dr. Subhojit Roy (University of California San Diego) and Dr. James Rothman, respectively (Miesenbock et al., 1998; Wang et al., 2014). The pRK5-HA-syntaxin1A (*Drosophila*) and pRK5-myc-SNAP25 were constructed as previously described (Bademosi et al., 2018). Other plasmids were obtained from Addgene, including CMV-syHy-A4 (#24478, synaptophysin-pHluorin) (Granseth et al., 2006) and pAAV.CAG.SF-iGluSnFR.A184V (#106199, medium affinity glutamate sensor) (Marvin et al., 2018).

Primary hippocampal neuronal cultures and transfection

Hippocampi derived from embryonic day 18 Sprague Dawley rat pups were used to generate neuronal cultures (Widagdo et al., 2016). Hippocampi were isolated and dissociated with 30U

of papain suspension (Worthington, Lakewood, NJ) for 20 min in a 37°C water bath. A single-cell suspension was obtained by triturating tissues with fire-polished glass Pasteur pipettes and then plated at a density of 3.5×10^4 cells on poly-L-lysine- and laminin-coated coverslips in Neurobasal growth medium supplemented with 2 mM Glutamax, 1% penicillin/streptomycin, and 2% B27. Neurons were kept in a humidified 5% CO₂ tissue culture incubator at 37°C. Transfection was carried out at days *in vitro* (DIV) 9-12 with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were imaged at DIV 12-15.

Fluorescence imaging of syHy and vGlut1-pHluorin trafficking

Live hippocampal neurons expressing syHy or vGlut1-pHluorin were mounted in an imaging chamber with field stimulation electrodes (RC-21BRFS; Warner Instruments, Hamden, CT) and continuously perfused with imaging buffer (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES, 30 mM D-glucose, pH 7.4) supplemented with 10 μM NBQX (ab120046, Abcam, Cambridge, UK) and 50 μM DL-APV (ab120271, Abcam). All experiments were performed at 35°C. The imaging solution was kept constant at 35°C using an inline solution heater (SH-27B; Warner Instruments). Neurons expressing syHy or vGlut1-pHluorin were challenged with a train of 300 action potentials delivered at either 10Hz or 50Hz (100 mA and 1ms pulse width) and imaged at 0.5 Hz (2 x 2 binning) through a 40X (1.4 NA) oil objective using an inverted Zeiss Axio Observer Z1 epifluorescence microscope equipped with an Andor Luca R EMCCD camera. pHluorin fluorescence was imaged at 488 nm excitation and collected through a 500-550 nm emission filter. At the end of the imaging session, neurons were perfused with an alkaline imaging buffer (50 mM NH₄Cl substituted for 50mM NaCl) to reveal total pHluorin expression. Equal-sized regions of interest were placed over nerve terminals to measure the pHluorin fluorescence elicited by stimulation over time using the Time Series Analyser plugin in FIJI software (NIH). Activity-dependent changes in fluorescence ($\Delta F/F_0$) were normalized to the respective peak heights from the train of stimuli (to calculate the rate of endocytosis) or to the total amount of fluorescence present after alkaline treatment (to calculate the exocytosis amplitude). The endocytosis time constant (τ value) was calculated by fitting the decay phase for each trace to a single exponential function.

Estimation of the surface fraction of syHy and vGlut1-pH

Surface-localized syHy and vGlut1-pH were revealed by perfusing cells in imaging buffer, followed by an acidic buffer (2-(*N*-morpholino)-ethanesulfonic acid substituted for HEPES, pH 5.5) to quench the fluorescence of syHy or vGlut1-pH on the plasma membrane. Neurons were washed with imaging buffer and subsequently exposed to an alkaline buffer to reveal total syHy or vGlut1-pH fluorescence. The surface fraction of syHy or vGlut1-pH was

expressed as a percentage of the total sypHy or vGlut1-pH expression using the following equation: $[\text{baseline fluorescence} - \text{acidic fluorescence}] / [\text{alkaline fluorescence} - \text{acidic fluorescence}] \times 100$ (Harper et al., 2017). To silence resting neuronal activity, hippocampal neurons were treated with 1 μM of tetrodotoxin (ab120055, Abcam) 24 h prior to and during the entire imaging session.

Distribution of sypHy and vGlut1-pH in axons

The diffuseness of sypHy and or vGlut1-pH distributed along axons were determined by calculating the CV value. Five freehand lines that were larger than 100 pixels each were drawn over several axonal segments expressing sypHy or vGlut1-pH reporter as neurons were bathed in alkaline imaging buffer. The ratio of the standard deviation over the mean fluorescence intensity of each line was calculated from each line profile (Harper et al., 2017).

Measuring the size of vesicle pools

To estimate the size of the SV pools in hippocampal neurons, changes in sypHy fluorescence intensity were measured following the delivery of a series of electrical stimuli of varying intensity in imaging buffer containing 1 μM bafilomycin A1 (196000, Calbiochem, Darmstadt, Germany) (Burrone et al., 2006). The first stimulus of 40 action potentials was delivered at 20 Hz to reveal the readily releasable pool of SVs. One minute later, a second train of stimuli (40 action potentials, 20 Hz) was delivered, releasing a further subset of vesicles. Neurons were then challenged with a prolonged stimulus (900 action potentials, 20Hz) one minute after the second stimulus. The response from the second and third stimulations revealed the reserve pool of SVs in hippocampal neurons. The remaining fluorescence, after bathing neurons in alkaline imaging buffer, represented the resting pool.

Glutamate release assay

A total of five electrical train of stimuli (300 action potentials, 50Hz) was delivered to hippocampal neurons expressing iGluSnFR fluorescence (Marvin et al., 2018). Each stimulus was delivered at a 2 min interval from the preceding stimulation. Transient glutamate release was quantified by placing identically sized regions of interest placed over nerve terminals and measuring iGluSnFR fluorescence intensity over time using the Time Series Analyser plugin in FIJI software (NIH). Activity-dependent glutamate release was calculated as $\Delta F/F_0$ normalized to the first train of stimuli $[(\Delta F/F_0) / (\Delta F/F_0)_{\text{max}}]$.

Lentivirus preparation

HEK293T cells were transfected by the calcium-phosphate co-precipitation method using 10 μg of the plasmid of interest and 5 μg each of pMD2.G envelope plasmid, pRSV-Rev encoding

plasmid and pMDLg/pRRE packaging construct (Anggono et al., 2013). Forty-eight hours after transfection, culture supernatants were collected and passed through a 0.45 μ m cellulose acetate low binding protein membrane filter. Lentiviral particles were harvested by ultracentrifugation at 106,559 *g* for 2 h at 4°C using a Beckman SW 32 Ti rotor. Concentrated virus particles were resuspended in Neurobasal medium and flash frozen with liquid nitrogen. Virus particles were stored at -80°C until further use.

Cell surface biotinylation assay

Hippocampal neuronal cultures were transduced with lentiviral particles expressing either GFP (FuGW-empty), PICK1 shRNA (FuGW-PICK1 shRNA) or PICK1 shRNA with myc-PICK1 shRNA resistant cDNA (FuW-PICK1 rescue) at DIV 9. The surface biotinylation assay was carried out at DIV 14 to measure the amount of endogenous synaptophysin protein on the plasma membrane (Tan et al., 2017). Briefly, live neurons were washed once in ice-cold artificial cerebrospinal fluid (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 30 mM D-glucose, pH 7.4), followed by a 30 min incubation with 0.5 mg/ml of EZ-Link Sulfo-NHS-SS-biotin (21331; Thermo Scientific, Rockford, IL) at 4°C. Free biotin was quenched by washing the cells three times with ice-cold Tris-buffered saline (TBS). Neurons were lysed in RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (11836170001; Sigma, St. Louis, MO) and phosphatase inhibitor cocktail I (P-1517; AG Scientific, San Diego, CA) at 4°C. Lysates were centrifuged at 20,627 *g* for 20 min at 4°C and incubated with Neutravidin agarose beads (29204; Thermo Scientific) overnight at 4°C. Beads were washed three times with ice-cold RIPA buffer and bound proteins were eluted with 2X SDS sample buffer at 95°C and analyzed by western blotting. The absence of PICK1 and α -tubulin on the surface fraction confirmed the specificity of the assay.

Glutathione S-transferase (GST) pulldown assay

GST pulldown assays were carried out as previously described (Clairfeuille et al., 2016). Briefly, HEK293T cells were transfected co-transfected with either pCIS empty (GST) or pCIS-PICK1 wild-type (GST-PICK1) and plasmids encoding sypHy, vGlut1-pH, myc-SNAP-25, VAMP2-pH or HA-syntaxin-1A by the calcium-phosphate precipitation method. Cells were lysed 48 h post-transfection with ice-cold cell lysis buffer (1% Triton X-100, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM Na-pyrophosphate in PBS) supplemented with EDTA-free protease inhibitor cocktail. Lysates were centrifuged at 20,627 *g* for 20 min at 4°C and incubated with Glutathione agarose beads (16100; Thermo Scientific) overnight at 4°C. Beads were washed three times with ice-cold cell lysis buffer and bound proteins were eluted with 2X SDS sample buffer at 50°C for 30 min and analyzed by western blotting.

Western blot

Both total and eluted proteins were separated by SDS-PAGE and transferred onto 0.45 μ m PVDF membranes at a constant voltage of 100V for 2 h. Membranes were blocked in 5% skim milk (in TBS containing 0.1% Tween-20) for 1 h at room temperature, washed with TBS buffer and incubated with primary antibodies overnight at 4°C. Membranes were washed with 1% skim milk and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Following extensive washes, blots were analyzed using the enhanced chemiluminescence method. Images were acquired on the Odyssey Fc imaging system (LI-COR) and band intensities were quantified using Image Studio Lite software (LI-COR, Lincoln, NE).

Quantification and statistical analysis

The sample size (n) reported in figure legends represents the number of individual coverslips or wells generated from at least three independent cultures (N), unless otherwise stated. Statistical analysis was performed using Graph Pad Prism 8.0. One-way analysis of variance (ANOVA) with the Tukey's or Dunnett's post-hoc multiple comparisons tests were used to compare the parameters for more than two groups. Two-way ANOVA with the Sidak's multiple comparisons test was used to compare the efficacy of glutamate release following repetitive stimulations among groups. For comparison between two groups, a two-tailed Student's t-test was employed. All data are reported as the mean $\pm$ standard error of the mean (SEM).

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Figure S1 (related to Figure 1). Biochemical experiments performed in HEK293T cells

(A and B) PICK1 interacts with syphHy and vGlut1-pH in HEK293T cells. Cells were co-transfected with pCIS empty vector or pCIS-PICK1 with plasmids encoding syphHy (A) or vGlut1-pH (B). Lysates were subjected to GST pulldown experiments, followed by western blotting analyses with specific antibodies against GFP and GST.

(C) Biochemical validation of the PICK1 shRNA and shRNA-resistant rescue constructs in HEK293T cells. Cells were transiently co-transfected with myc-PICK1 wild-type or shRNA#1-resistant constructs with either pSuper empty vector control or PICK1 shRNA#1 (used in this study). Lysates were resolved on SDS-PAGE and subjected to western blotting with specific antibodies against myc and actin.

(D–F) PICK1 interacts with members of the SNARE complex in HEK293T cells. Cells were co-transfected with pCIS empty vector or pCIS-PICK1 with plasmids encoding VAMP2-pH (C), myc-SNAP-25 (E) or HA-syntaxin1A (F). Lysates were subjected to GST pulldown experiments, followed by western blotting analyses with specific antibodies against GFP, myc, HA and GST.

A **B** **C** **D** **E** **F**

Figure S2 (related to Figure 1). PICK1 overexpression enhances sypHy exocytosis during high-frequency stimulation

(A) Representative images of hippocampal neurons co-expressing sypHy with either empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs, challenged with a train of 300 action potentials (10 Hz, 30 s). Images were acquired at rest ($t = 0$ s), during the stimulation ($t = 38$ s), post-stimulation ($t = 70$ s) and following NH_4Cl treatment.

(B) Averaged sypHy traces normalized to total sypHy expression traces from control, knockdown and rescue neurons.

(C) Peak height of sypHy responses normalized to NH_4Cl . Control ($n = 9$ coverslips), knockdown ($n = 7$) and rescue ($n = 9$) neurons from 2 independent cultures. Data are presented as mean $\pm$ SEM. No significant differences were found among groups as determined by one-way ANOVA.

(D) Representative images of hippocampal neurons co-expressing sypHy with either empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs. Neurons were challenged with a train of 300 action potentials (50 Hz, 6 s). Images were acquired at rest ($t = 0$ s), during the stimulation ($t = 38$ s), post-stimulation ($t = 70$ s) and following NH_4Cl treatment.

(E) Averaged sypHy traces normalized to total sypHy expression from control, knockdown and rescue neurons.

(F) Peak height of sypHy responses normalized to NH_4Cl . Control ($n = 20$ coverslips), PICK1-shRNA ($n = 12$) and rescue ($n = 13$) in neurons from 3 independent cultures. Data are presented as mean $\pm$ SEM. * $P < 0.05$ (One-way ANOVA with Tukey's multiple comparisons test).

Figure S3 (related to Figure 1). PICK1 depletion does not affect vGlut1-pH exocytosis at high-frequency stimulation

(A) Representative images of hippocampal neurons co-expressing vGlut1-pH with either empty vector (control), PICK1 shRNA (knockdown) or PICK1 shRNA-resistant (rescue) constructs, challenged with a train of 300 action potentials (50 Hz, 6 s). Images were acquired at rest ($t = 0$ s), during the stimulation ($t = 38$ s), post-stimulation ($t = 70$ s) and following NH_4Cl treatment.

(B) Averaged vGlut1-pH traces normalized to total vGlut1-pH expression from control, knockdown and rescue neurons.

(C) Peak height of vGlut1-pH responses normalized to NH_4Cl . Control ($n = 15$ coverslips), knockdown ($n = 13$) and rescue ($n = 15$) neurons from 3 independent cultures. Data are presented as mean $\pm$ SEM. No significant differences were found among groups as determined by one-way ANOVA.

Figure S4 (related to Figure 3). Overexpression of PICK1 mutants has no effect on sypHy exocytosis at high-frequency stimulation

(A) Representative images of hippocampal neurons co-expressing sypHy with myc-PICK1 constructs, either wild-type or mutants as indicated, challenged with a train of 300 action potentials (50 Hz, 6 s). Images were acquired at rest ($t = 0$ s), during the stimulation ($t = 38$ s), post-stimulation ($t = 70$ s) and following NH_4Cl treatment.

(B) Averaged sypHy traces normalized to total sypHy expression from neurons expressing wild-type PICK1 or KDAA, 3KE or DDAA mutants.

(C) Peak height of sypHy responses normalized to NH_4Cl . Wild-type PICK1 ($n = 21$ coverslips), KDAA ($n = 13$), 3KE ($n = 15$) and DDAA mutants ($n = 16$) from 3 independent cultures. Data are presented as mean $\pm$ SEM. No significant differences were found among groups as determined by one-way ANOVA.