VAMP4 Maintains a Ca^{2+} -Sensitive Pool of Spontaneously Recycling Synaptic Vesicles

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Spontaneous neurotransmitter release is a fundamental property of synapses in which neurotransmitter filled vesicles release their content independent of presynaptic action potentials (APs). Despite their seemingly random nature, these spontaneous fusion events can be regulated by Ca^{2+} signaling pathways. Here, we probed the mechanisms that maintain Ca^{2+} sensitivity of spontaneous release events in synapses formed between hippocampal neurons cultured from rats of both sexes. In this setting, we examined the potential role of vesicle-associated membrane protein 4 (VAMP4), a vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein in spontaneous neurotransmission. Our results show that VAMP4 is required for Ca^{2+} -dependent spontaneous excitatory neurotransmission, with a limited role in spontaneous inhibitory neurotransmission. Key residues in VAMP4 that regulate its retrieval as well as functional clathrin-mediated vesicle trafficking were essential for the maintenance of VAMP4-mediated spontaneous release. Moreover, high-frequency stimulation (HFS) that typically triggers asynchronous release and retrieval of VAMP4 from the plasma membrane also augments Ca^{2+} -sensitive spontaneous release for up to 30 min in a VAMP4-dependent manner. This VAMP4-mediated link between asynchronous and spontaneous excitatory neurotransmission might serve as a presynaptic substrate for synaptic plasticity coupling distinct forms of release.

Key words: asynchronous release; ikarugamycin; spontaneous neurotransmission; spontaneous release; synaptic vesicle recycling; VAMP4

Significance Statement

Spontaneous neurotransmitter release that occurs independent of presynaptic action potentials (APs) shows significant sensitivity to intracellular Ca^{2+} levels. In this study, we identify the vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) molecule vesicle-associated membrane protein 4 (VAMP4) as a key component of the machinery that maintains these Ca^{2+} -sensitive fraction of spontaneous release events. Following brief intense activity, VAMP4-dependent synaptic vesicle retrieval supports a pool of vesicles that fuse spontaneously in the long term. We propose that this vesicle trafficking pathway acts to shape spontaneous release and associated signaling based on previous activity history of synapses.

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Introduction

Studies to date have subdivided modes of neurotransmitter release into three categories based on their timing and dependency on neuronal activity. Evoked synchronous and asynchronous forms of neurotransmission are triggered by the arrival of action potentials (APs) while spontaneous neurotransmission occurs in the absence of activity (Chanaday and Kavalali, 2018). Recent studies have shown exocytosis (fusion/release) and endocytosis (retrieval) of synaptic vesicles are coupled, and each mode of release is associated with a specific recycling pathway (Chanaday and Kavalali, 2017; Li et al., 2017; Silm et al., 2019). The fusion machinery, which is comprised of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins and Ca^{2+} -sensing proteins known as synaptotagmins

(Sudhof, 2013), are ideally poised to couple fusion and retrieval. As an example, during evoked asynchronous neurotransmission, in which release occurs with a delay with respect to AP arrival, the non-canonical SNARE vesicle-associated membrane protein 4 (VAMP4) and the calcium sensor synaptotagmin-7 mediate the delayed exocytosis of synaptic vesicles as well as their subsequent retrieval (Raingo et al., 2012; Bacaj et al., 2013; Li et al., 2017; Volynski and Krishnakumar, 2018). VAMP4 can direct synaptic vesicle retrieval to a slow mode of endocytosis primarily activated on repetitive neuronal firing (Raingo et al., 2012). Interestingly, the N-terminal dileucine motif of VAMP4, a domain that recruits clathrin adaptor proteins, is necessary for the regulation of both asynchronous release and VAMP4 trafficking (Raingo et al., 2012).

Several SNAREs have been associated with the spontaneous fusion of synaptic vesicles. The canonical SNARE synaptobrevin-2 (syb2; or VAMP2), while essential for evoked neurotransmission is only partially involved in spontaneous release (Schoch et al., 2001; Zimmermann et al., 2014; Liu et al., 2018, 2019). Other non-canonical SNAREs, including Vti1a (Vps10p-tail-interactor-1a) and VAMP7, have been shown to specifically modulate spontaneous neurotransmission (Ramirez et al., 2012; Bal et al., 2013; Crawford et al., 2017). Spontaneous fusion events are also known to be Ca^{2+} -dependent as increasing extracellular Ca^{2+} concentration or release of Ca^{2+} from internal stores can augment their propensity (Williams and Smith, 2018). Thus, from a molecular perspective, multiple mechanisms and populations of synaptic vesicles support the spontaneously recycling pool of synaptic vesicles (Chanaday and Kavalali, 2018). A better understanding of spontaneous release is important as spontaneous release events have been shown to regulate a number of developmental and signaling processes in neurons (Kavalali, 2015, 2018; Andrae and Burrone, 2018).

Here, we explore the potential role of VAMP4 in spontaneous neurotransmission and show that VAMP4 regulates Ca^{2+} -dependent spontaneous excitatory neurotransmitter release with limited impact on spontaneous inhibitory neurotransmission. We demonstrate that the dileucine motif in VAMP4 and functional clathrin-mediated vesicle trafficking are both essential for VAMP4-mediated spontaneous release. Moreover, augmentation of spontaneous neurotransmission that is seen after strong stimulation requires VAMP4, suggesting a trafficking pathway where asynchronously released synaptic vesicles may contribute to Ca^{2+} -sensitive spontaneous neurotransmission following their retrieval. Taken together, these data identify a novel VAMP4-mediated cross talk between asynchronous and spontaneous excitatory neurotransmission which may serve as a presynaptic substrate for synaptic plasticity to link distinct forms of release.

Materials and Methods

Primary hippocampal neuron culture and lentiviral transfection

Dissociated hippocampal cultures were prepared as previously described (Kavalali et al., 1999). Both hippocampi were dissected from postnatal day (P)2–P4 Sprague Dawley rats or embryonic day (E)18–E19 syb2 knock-out (KO) mice (Schoch et al., 2001). Hippocampi were trypsinized (10 mg/ml trypsin) for 10 min at 37°C, mechanically dissociated by pipetting, and plated on Matrigel (Corning Biosciences) or in the case of syb2 KO growth-D-lysine (Sigma-Aldrich)-coated 12-mm glass coverslips. Neuron growth medium contained MEM (no phenol red), 5 g/l D-glucose, 0.2 g/l NaHCO_3 , 100 mg/l transferrin, 5% of heat inactivated fetal bovine serum, 0.5 mM L-glutamine, and 2% B-27 supplement. At DIV1, 4 μM cytosine arabinoside (ARAC; Sigma) was added. At DIV4, the ARAC concentration was decreased to 2 μM , and the lentiviral vectors were added to the culture media. Lentivirus constructs and virus

preparation from HEK cells were done as previously described (Li et al., 2017). HEK-293T cells (ATCC) were co-transfected with pFUGW/L307 transfer vectors and three packaging plasmids (pCMV-VSV-G, pMDLg/pRRE, pRSV-Rev) using the FuGENE 6 transfection reagent (Promega), and supernatants containing the lentiviruses were harvested 48–72 h after transfection. Lentivirus containing the empty vector L-307 was used to infect the control group. VAMP4 short hairpin RNA, VAMP4 overexpression (OE) and VAMP4 L25A constructs were described before (Raingo et al., 2012). Superecliptic pHluorin-fused human transferrin receptor construct was a generous gift of Matthew Kennedy (Department of Pharmacology, University of Colorado Denver; Kennedy et al., 2010), and it was used as previously described (Ramirez et al., 2012). Cultures were kept in humidified incubators at 37°C and gassed with 95% air and 5% CO_2 until DIV17–DIV20 when experiments were performed.

Western blotting

Hippocampal cultures were harvested using Laemmli sample buffer (Bio-Rad) and subjected to SDS-PAGE followed by western blot as previously described (Nosyreva et al., 2013). The primary antibodies and dilutions were used as follows: VAMP4 1:1000 (Synaptic Systems, 136002), GAPDH 1:50,000 (Cell Signaling, 2118), Vti1a 1:500 (Synaptic Systems, 165002), synaptotagmin 1 (Syt1; 1:5000; Synaptic Systems, 105011, CL41.1), complexin1/2 (cpx1/2) 1:1000 (Synaptic Systems, 122002), VAMP7 1:1000 (Synaptic Systems, 232003), Syb2 1:5000 (Synaptic Systems, 104211), syntaxin1a (Stx1a; Synaptic Systems, 110302), Syt7 1:1000 (Synaptic Systems 105173), and SNAP25 1:2000 (Synaptic Systems 111111). Immunoreactive bands were revealed by enhanced chemiluminescence (ECL), captured on autoradiography film (Eastman Kodak), and analyzed using ImageJ software (NIH). VAMP4 protein levels were normalized to GAPDH loading control.

Immunofluorescence and colocalization

Neuron cultures were fixed with 1% paraformaldehyde and permeabilized using 0.0075% digitonin in PBS. Samples were blocked with 1% bovine serum albumin (BSA) and 3% goat serum in PBS for 1 h at room temperature. All antibodies were from Synaptic Systems. Antibodies against microtubule-associated protein 2 (MAP2; 188004), Syb2 (104318), vesicular glutamate transporter-1 (VGLUT1; 135302), and vesicular GABA transporter (VGAT; 131011) were used at 1:1000 dilution and anti-postsynaptic density protein (PSD)-95 (124011) at 1:100. Alexa Fluor-conjugated secondary antibodies (1:1000) were used to label the cells and then coverslips were imaged using an LSM 510 META confocal microscope (Carl Zeiss) with a 63 $\times$ (NA1.4) objective. Object-based colocalization was analyzed using Fiji (NIH). 3D objects in each channel were segmented and positive colocalization was defined as an overlap of >50 voxels of two colors in the same object (calibration: 1 voxel = $0.143 \times 0.143 \times 1 \mu\text{m}$).

Whole-cell voltage clamp recordings

At DIV17–DIV20, pyramidal neurons were voltage clamped at -70 mV using a Molecular Devices Axopatch 200B amplifier. Postsynaptic currents were recorded at room temperature using Clampex 9.0 software (Molecular Devices). Signals were filtered at 1 kHz and sampled at 10 kHz. The access resistances were around 10 M Ω for each recording. The external Tyrode's solution contained the following: 150 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 1.25 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES (pH 7.4) at $\sim 310 \text{ mOsm}$. The pipette internal solution contained the following: 110 mM K-gluconate, 20 mM KCl, 10 mM NaCl, 10 mM HEPES, 0.6 mM EGTA (ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid), 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM lidocaine N-ethyl bromide (pH 7.4) at $\sim 300 \text{ mOsm}$. The AP blocker tetrodotoxin (TTX, 1 μM ; Tocris) and the NMDA receptor blocker D-AP5 (50 μM ; Sigma-Aldrich) were added to the bath solution. To isolate inhibitory or excitatory (AMPA receptor-mediated) currents, CNQX (10 μM ; Sigma-Aldrich) or PTX (50 μM ; Sigma-Aldrich) were added, respectively. To test the Ca^{2+} dependence of spontaneous neurotransmission, hippocampal cultures were preincubated with 100 μM the slow Ca^{2+} -chelator EGTA-AM in 0 mM Ca^{2+} for 15 min at room temperature (final DMSO concentration in the media was 0.1%). For hypertonic sucrose

experiments, 500 mM sucrose was added to Tyrode's solution (final extracellular media osmolarity: 820 mOsm) and rapidly perfused using a continuous flux system.

Fluorescence imaging

We used 18–20 DIV cultured hippocampal neurons expressing VAMP4-pHluorin or VAMP4(L25A)-pHluorin for monitoring spontaneous fusion of VAMP4-containing synaptic vesicles. The modified Tyrode's solution from above containing 1 μ M TTX and 200 nM the vacuolar H^+ -ATPase inhibitor folimycin (Sigma-Aldrich) was used. Experiments were performed at room temperature using an Andor iXon+ back-illuminated EMCCD camera (Model no. DU-897E-CSO-#BV; Andor Technology) collected on a Nikon Eclipse TE2000-U microscope with a 100 $\times$ Plan Fluor objective (Nikon). For illumination, we used a λ -DG4 (Sutter Instruments) with a FITC excitation/emission filter. Images were acquired at $\sim$ 8–9 Hz with an exposure time of 120 ms and binning of four by four to optimize the signal-to-noise ratio. Data were collected using Nikon Elements software, 2- μ m diameter round regions of interest (ROIs) were drawn on presynaptic boutons and the resulting fluorescence measurements were exported to Microsoft Excel for analysis. To detect all presynaptic boutons, an isosmotic modified Tyrode's solution containing 50 mM NH_4Cl was perfused at the end of each experiment.

To monitor transferrin receptor endocytosis, neurons expressing TfR-pHluorin were imaged on a Nikon TE2000-U inverted microscope using a Cascade 512 cooled CCD camera (Roper Scientific) and Metafluor 7.6 software (Molecular Dynamics). Experiments were performed using the modified Tyrode's solution mentioned above containing 10 μ M CNQX and 50 μ M D-AP5. All solutions were applied onto the chamber holding the coverslip by an automatic perfusion system (ValveBank, Automate Scientific). After 100 s of baseline recording, 25 μ g/ml of prewarmed (37°C) transferrin freshly diluted in Tyrode's solution from 1 mg/ml stock was added to the chamber. Fluorescence changes were monitored for 10 min, followed by 50 mM NH_4Cl treatment to estimate total protein expression. Images were collected once every 10 s. Between 14 and 61 ROIs on three to seven neuronal cell bodies and proximal dendrites were selected from each coverslip to obtain the time courses of fluorescence changes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Student's *t* test (two-tailed, unpaired) or Kolmogorov–Smirnov test were used to analyze all pairwise datasets obtained from synapses under distinct conditions. For analysis of multiple comparisons, two-way ANOVA with Sidak's *post hoc* test was used. Number of experiments and detailed statistical information are provided on the corresponding figure legends. In general, mean $\pm$ SEM is informed and plotted. A statistically significant difference was defined as $p \leq 0.05$.

Results

VAMP4 regulates Ca^{2+} -dependent spontaneous neurotransmission at excitatory but not inhibitory synapses

To examine a role for the non-canonical vesicular SNARE VAMP4 (Fig. 1A) in the regulation of excitatory spontaneous neurotransmission, we infected cultured hippocampal neurons with either a lentivirus containing a short hairpin RNA targeting VAMP4, VAMP4 knock-down (KD), or an empty vector control. The VAMP4 KD virus decreased VAMP4 protein expression by 85% compared with control neurons (Fig. 1B,C). Knock-down of VAMP4 caused a $\sim$ 55% reduction in the frequency of miniature EPSCs (mEPSCs) compared with control neurons (Fig. 1D–F), with no impact on mEPSC amplitudes (Fig. 1G). Other non-canonical SNAREs previously known to mediate spontaneous neurotransmission, Vti1a and VAMP7, selectively drive a high-frequency component of spontaneous release at low interevent intervals (Ramirez et al., 2012; Crawford et al., 2017). However, knocking down VAMP4 decreases all spontaneous neurotransmission regardless of event frequency as indicated by the

right shift in the histogram of interevent intervals (Fig. 1F). These results suggest VAMP4 as a new non-canonical SNARE that participates in spontaneous release at glutamatergic synapses.

VAMP4 has been previously shown to mediate asynchronous release in a Ca^{2+} -dependent manner, since it is blocked by the slow Ca^{2+} buffer EGTA (Raingo et al., 2012). To assess whether spontaneous release driven by VAMP4 is also Ca^{2+} sensitive, we pretreated control and VAMP4 KD neurons with EGTA-AM (EGTA, Tetra(acetoxymethyl Ester)). EGTA-AM treatment led to a 65% decrease in the frequency of mEPSCs in control neurons (Fig. 1E), indicating that slow and diffuse increases in intracellular Ca^{2+} might be mediating spontaneous glutamate release (see Courtney et al., 2018; Williams and Smith, 2018). Notably, the extent of reduction in spontaneous release by slow Ca^{2+} buffering was similar to the effect of VAMP4 KD (compare Fig. 1E, straight and dotted gray lines). In fact, the effect of EGTA-AM treatment on mEPSC frequency was occluded by VAMP4 KD (Fig. 1E,F), suggesting that VAMP4 mediates Ca^{2+} -dependent excitatory spontaneous neurotransmission. EGTA-AM effects were not due to synaptic fatigue or run down since mEPSC frequency was stable for tens of minutes in treated neurons (Fig. 1H), indicating that EGTA-AM may block transient Ca^{2+} changes but not resting Ca^{2+} levels.

The canonical vesicular SNARE syb2 is one of the most abundant presynaptic proteins, and it mediates the majority of synaptic vesicle release (Schoch et al., 2001). For evoked neurotransmission, syb2 and VAMP4 have been shown to recycle independently (Raingo et al., 2012). To evaluate whether VAMP4-driven miniature events are also independent of syb2, we knocked down VAMP4 in syb2 KO neurons (Fig. 1I,J). Eliminating VAMP4 in syb2 KO neurons did not further decrease mEPSC frequency (Fig. 1I), suggesting that VAMP4 cannot maintain spontaneous excitatory neurotransmission in the absence of syb2. Neurons lacking both, syb2 and VAMP4, also showed a small increase in mEPSC amplitudes (Fig. 1J).

To explore the role of VAMP4 in inhibitory spontaneous neurotransmission we examined miniature IPSCs (mIPSCs; Fig. 1K,N). VAMP4 KD did not lead to a statistically significant change in the frequency of mIPSCs (Fig. 1L,M), in agreement with previous findings from our group (Raingo et al., 2012). There was no effect of VAMP4 KD on mIPSC amplitudes (Fig. 1N). Moreover, mIPSCs were unaffected by EGTA-AM treatment in control and VAMP4 KD neurons (Fig. 1K–N), in agreement with the higher dependency of inhibitory spontaneous release on fast extracellular Ca^{2+} influx (Williams and Smith, 2018). Taken together, our results show that VAMP4 mediates spontaneous fusion of synaptic vesicles in a Ca^{2+} -dependent manner at excitatory, but not inhibitory, synapses.

In hippocampal neurons, axon outgrowth and the establishment of cell polarity during development relies on fusion of plasmalemmal precursor vesicles, a process also partially driven by VAMP4 (Grassi et al., 2015; Oksdath et al., 2017). Thus, we next examined if VAMP4 KD impacts synaptic vesicle protein levels or synapse numbers in mature (18 DIV) hippocampal cultures (Fig. 2). The total levels of the canonical vesicular SNAREs syb2, SNAP25, and Stx1a are not altered in VAMP4 KD neurons compared with control (Fig. 2A,B). The levels of other important regulators of neurotransmitter release are also unaffected, including the non-canonical SNAREs involved in spontaneous neurotransmission (VAMP7 and Vti1a) and SNARE complex associated proteins that modulate zippering and Ca^{2+} dependency of release (cpx1/2 and Syt1 and Syt7; Fig. 2A,B). Excitatory (glutamatergic) and inhibitory (GABAergic) presynaptic terminals

Figure 1. VAMP4 regulates Ca^{2+} -dependent excitatory spontaneous neurotransmission. **A**, Schematic representation of VAMP4 protein and its functional domains. **B**, Representative immunoblot of hippocampal neuron cultures infected with L-307 lentivirus (control) or VAMP4 KD lentivirus. **C**, VAMP4 protein expression in neuronal cultures infected with VAMP4 KD virus was $15.4 \pm 4.3\%$ of that in control culture ($n = 7$ for control, $n = 6$ for VAMP4 KD). **D**, Representative traces of mEPSCs in control or VAMP4 KD neurons preincubated with 0.1% Dimethyl Sulfoxide (DMSO) (vehicle) or 100 μ M EGTA-AM for 15 min. **E**, Average frequency of mEPSCs. EGTA-AM significantly reduced the frequency of mEPSCs in control neurons ($n = 10$), compared with DMSO treated ($n = 10$). Knock-down of VAMP4 decreased frequency of mEPSCs ($n = 9$), EGTA-AM did not produce further reduction of frequency of mEPSCs in VAMP4 KD neurons [$n = 9$; two-way ANOVA: $F_{(1,34)} = 7.719$, $p = 0.0088$, Sidak's multiple comparisons test: control-DMSO vs control-EGTA-AM $p = 0.0009$, control-DMSO vs VAMP4 KD-DMSO $p = 0.0073$, VAMP4 KD-DMSO vs VAMP KD-EGTA-AM $p = 0.9999$]. **F**, Cumulative histogram of interevent intervals of mEPSCs. EGTA-AM treatment (straight gray line) and VAMP4 KD (dotted black line) led to a similar increase in the interevent intervals compared with control neurons (straight black line). EGTA-AM treatment is occluded by VAMP4 KD (dotted gray line; two-way ANOVA: $F_{(1,21583)} = 466.4$, $p < 0.0001$; Sidak's multiple comparisons test: control-DMSO vs VAMP4 KD-DMSO $p = 0.0009$, VAMP4 KD-DMSO vs VAMP KD-EGTA-AM $p = 0.5929$). **G**, mEPSC amplitudes were not impacted by EGTA-AM or VAMP4 KD (two-way ANOVA: $F_{(1,34)} = 0.2833$, $p = 0.5980$). **H**, Prolonged mEPSC recordings for control ($n = 7$) and EGTA-AM ($n = 7$) pretreated cultures showing stability of miniature event frequency over time for both groups. mEPSC frequency was calculated over 30-s windows (two-way ANOVA: time factor $F = 1.132$, $p = 0.2558$; group factor $F = 6.789$).

were immunofluorescently labeled using antibodies against VGlut1 and VGAT, respectively (Fig. 2C,D). Reduction of VAMP4 levels in neurons had no impact on the number of glutamatergic (~70%) or GABAergic (~30%) presynaptic boutons (Fig. 2C,D). Given the specific effect of VAMP4 KD on excitatory spontaneous neurotransmission, we measured the number of excitatory synapses in more detail (Fig. 2E,F). The number presynaptic VGlut1-positive boutons opposed to PSD-95 were counted relative to dendrite length (labeled using MAP2). The linear density of excitatory synapses was similar between control and VAMP4 KD cultures (Fig. 2E,F), indicating that VAMP4 has no effect on synapse numbers in mature hippocampal neurons. Finally, we asked whether VAMP4 impact on spontaneous release is a consequence of alterations in synaptic vesicle priming. To answer this question, we measured the size of the readily releasable pool using hypertonic sucrose (+500 mOsm; Fig. 2G,H) following a previously described method (Rosenmund and Stevens, 1996). The total charge transfer was similar for control and VAMP4 KD neurons (Fig. 2G,H), implying that VAMP4 does not regulate the number of primed synaptic vesicles at presynaptic terminals. In conclusion, despite its putative role during axonal development, VAMP4 does not regulate synaptic vesicle composition, release site number or synapse density in mature neuronal networks. Overall, these results support that our VAMP4 loss-of-function manipulations are rather specific and the effects we observe are attributable to a role for VAMP4 in acute synaptic vesicle trafficking dynamics.

Our results so far demonstrate a specific role of VAMP4 in driving Ca^{2+} -dependent excitatory spontaneous neurotransmission. To further test the Ca^{2+} dependence of VAMP4 trafficking, we directly monitored spontaneous VAMP4 recycling in live neurons using VAMP4 fused to the pH sensitive GFP, pHluorin (Fig. 3A; Raingo et al., 2012). We took advantage of the V-ATPase blocker folimycin which abolishes re-acidification of vesicles after endocytosis and allows us to measure spontaneous fusion as an accumulation of fluorescence over time in the presence of the AP blocker TTX (Fig. 3B,C). To control for possible variability in the expression levels of the probe among different synapses and cultures, we measured the maximal pHluorin response by perfusing 50 mM NH_4^+ and normalized the fluorescence to this value (Fig. 3C). We found that VAMP4-pHluorin fluorescence accumulates over time in the presence of folimycin and TTX, corroborating our electrophysiological studies and indicating that VAMP4 can traffic at rest (Fig. 3B,C). This accumulation is the result of successive fusion events of individual synaptic vesicles since the increase occurs in a step-wise manner (Fig. 3B) and amplitudes show a quantal distribution (Fig. 3B), analogous to previous measurements of spontaneous release

using VGlut1-pHluorin (Leitz and Kavalali, 2014). Moreover, treating neurons with the Ca^{2+} chelator EGTA-AM results in 50% decrease in the rate (slope) of accumulation of VAMP4-pHluorin fluorescence (Fig. 3C,D), similar to the extent of reduction of mEPSC frequency by EGTA-AM (compare with Fig. 1E). This decrease was accompanied by a significant reduction in the total change in fluorescence ($\Delta F/F_{\text{NH}_4}$) after 5 min of recording (Fig. 3E). These findings support our electrophysiological results and indicate that a considerable proportion of VAMP4-driven spontaneous synaptic vesicle fusion is Ca^{2+} dependent.

VAMP4 trafficking determines the rate of Ca^{2+} -dependent spontaneous neurotransmission at excitatory synapses

The roles of VAMP4 in asynchronous release, endocytosis, as well as its subcellular localization, but not its role in fusion per se, are critically dependent on VAMP4 N-terminal dileucine motif (Peden et al., 2001; Raingo et al., 2012; Nicholson-Fish et al., 2015). To explore the importance of the dileucine motif in VAMP4 spontaneous trafficking, we expressed a dominant negative form of VAMP4 with a mutated dileucine motif (VAMP4 L25A) fused to pHluorin (Fig. 3F). Although trafficking patterns specific to VAMP4 are impaired in L25A mutant (Raingo et al., 2012), VAMP4(L25A)-pHluorin can still be transported to presynaptic terminals and traffic akin to syb2 (Fig. 3F; also see Raingo et al., 2012). However, this mutant cannot effectively recycle spontaneously as there is no accumulation of fluorescence over time in the presence of TTX and folimycin (only a decay likely due to photobleaching is detectable; Fig. 3G–I). Moreover, there is no further effect of EGTA-AM on VAMP4 (L25A)-pHluorin fluorescence (Fig. 3G–I). To further investigate the involvement of the dileucine motif in VAMP4-driven spontaneous neurotransmission, we measured mEPSCs in cultured hippocampal neurons (Fig. 4). In control neurons, OE of wild-type VAMP4 had no effect on the frequency, amplitude, or Ca^{2+} dependency of excitatory spontaneous neurotransmission (Fig. 4A–E). In contrast, although VAMP4(L25A) expression levels were modest (Fig. 4A,B), there was a dominant negative effect on the frequency of mEPSCs (50% reduction; Fig. 4F,G) with no effect on their amplitudes (Fig. 4H). Moreover, the reduction of mEPSC frequency induced by EGTA-AM treatment was occluded in neurons expressing VAMP4(L25A) (Fig. 4F,G), suggesting that VAMP4's dileucine motif and proper endocytosis are necessary for the Ca^{2+} -dependent spontaneous release triggered by VAMP4 at excitatory presynaptic terminals.

The dileucine motif of VAMP4 mediates its interaction with clathrin adaptor proteins and subsequent clathrin-mediated trafficking of VAMP4 (Peden et al., 2001). To examine whether clathrin-mediated trafficking is required for VAMP4-dependent spontaneous neurotransmission, we used the clathrin inhibitor ikarugamycin (IKA; Elkin et al., 2016). We first tested the efficacy of IKA by measuring prototypical transferrin receptor trafficking. Transferrin receptor uptake is the most classical and widely studied clathrin-mediated trafficking pathway (Mayle et al., 2012). We expressed pHluorin-tagged human transferrin receptor (TfR-pHluorin; Fig. 5A; Kennedy et al., 2010) in hippocampal neurons and triggered its uptake by the addition of transferrin. TfR-pHluorin endocytosis was revealed by a decrease in fluorescence in control neurons (Fig. 5B,C). We observed significant block of clathrin-mediated trafficking of TfR-pHluorin by 10 μM IKA, indicating that IKA can effectively block clathrin-mediated trafficking in cultured hippocampal neurons (Fig. 5A–C). We then applied IKA (15-min pretreatment) to control and

←

$p = 0.0230$). **I**, Average mEPSC frequency for syb2 KO ($n = 27$) and syb2 KO + VAMP4 KD ($n = 23$) hippocampal neurons (unpaired t test with Welch's correction: $p = 0.8765$). **J**, Average mEPSC amplitude for syb2 KO ($n = 27$) and syb2 KO + VAMP4 KD ($n = 23$) hippocampal neurons (unpaired t test with Welch's correction: $p = 0.0102$). **K**, Representative traces of mIPSCs in control or VAMP4 KD [neurons preincubated with DMSO (vehicle) or 100 μM EGTA-AM for 15 min]. **L**, Average frequency of mIPSC for control ($n = 22$) and VAMP4 KD ($n = 17$) hippocampal neurons pretreated with vehicle (0.1% DMSO) or EGTA-AM (control w/ EGTA-AM $n = 15$; VAMP4 KD w/EGTA-AM $n = 14$). mIPSC frequency was not impacted by EGTA-AM or VAMP4 KD (two-way ANOVA: $F_{(1,64)} = 0.08307$, $p = 0.7741$). **M**, Cumulative histogram of interevent intervals of mIPSCs (two-way ANOVA: $F_{(1,4818)} = 1.545$, $p = 0.2235$). **N**, EGTA-AM or VAMP4 KD had no effect on mIPSC amplitudes (two-way ANOVA: $F_{(1,30)} = 1.426$, $p = 0.2384$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, nonsignificant.

Figure 2. Elimination of VAMP4 has no impact on structural presynaptic properties. **A**, Representative immunoblot of hippocampal neuron cultures infected with L-307 lentivirus (control) or VAMP4 KD lentivirus. **B**, Protein expression of synaptic proteins in neuronal cultures with VAMP4 KD is unaltered compared with expression control cultures (Syb2: $n = 6$ for control, $n = 6$ for VAMP4 KD, t test₍₁₀₎ = 0.5161, $p = 0.6170$; SNAP25: $n = 6$ for control, $n = 5$ for VAMP4 KD, t test₍₉₎ = 1.076, $p = 0.3099$; Stx1a: $n = 6$ for control, $n = 5$ for VAMP4 KD, t test₍₉₎ = 0.5593, $p = 0.5896$; Cplx1/2: $n = 6$ for control, $n = 6$ for VAMP4 KD, t test₍₁₀₎ = 1.188, $p = 0.2623$; VAMP7: $n = 6$ for control, $n = 6$ for VAMP4 KD, t test₍₁₀₎ = 1.287, $p = 0.2272$; Vti1a: $n = 6$ for control, $n = 5$ for VAMP4 KD, t test₍₉₎ = 0.6977, $p = 0.5030$; Syt7: $n = 6$ for control, $n = 6$ for VAMP4 KD, t test₍₁₀₎ = 0.3804, $p = 0.7116$; Syt1: $n = 6$ for control, $n = 6$ for VAMP4 KD, t test₍₁₀₎ = 0.5468, $p = 0.5965$). **C**, Representative confocal images from control (top) and VAMP4 KD (bottom) cultures showing presynaptic terminals labeled with syb2 (blue), VGLuT1 (green), and VGAT (red). White bar = 5 μm . **D**, Quantification of the percentage of glutamatergic and GABAergic presynaptic boutons in control ($n = 15$) and VAMP4 KD ($n = 15$) neurons (two-way ANOVA: $F_{(1,28)} = 0.4621$, $p = 0.5022$). **E**, Representative confocal images from control (top) and VAMP4 KD (bottom) cultures showing excitatory synapses labeled with VGLuT1 (red) and PSD-95 (green). Dendrites are labeled with MAP2 (blue). White bar = 5 μm . **F**, Quantification of the linear density of excitatory synapses (# of synapses per μm of dendrite) in control ($n = 12$) and VAMP4 KD ($n = 12$) neurons (unpaired t test with Welch's correction: $p = 0.0527$). **G**, Representative electrophysiology recordings showing hypertonic (+500 mOsm) sucrose response in control and VAMP4 KD pyramidal neurons. **H**, Average sucrose charge transfer in control ($n = 16$) and VAMP4 KD ($n = 16$) neurons (unpaired t test with Welch's correction: $p = 0.6924$). NS, nonsignificant.

Figure 3. VAMP4-containing synaptic vesicles recycle spontaneously in a Ca^{2+} -dependent manner. **A**, top, Schematic representation of a synaptic vesicle containing pHluorin fused to VAMP4 (VAMP4-pHluorin). Bottom, Representative image of VAMP4-pHluorin fluorescence after 50 mM NH_4Cl . White arrowheads point to presynaptic boutons. White bar = 2 μm . **B**, Expanded view of VAMP4-pHluorin fluorescence time course in the presence of folimycin (200 nM) showing a step-wise increase in fluorescence (left). Each “step” corresponds to the fusion of one synaptic vesicle since the amplitude shows a quantal distribution (right). **C**, Average fluorescence traces of VAMP4-pHluorin from individual presynaptic puncta in the presence of TTX and folimycin showing spontaneous release of VAMP4-containing synaptic vesicles. Fluorescence is normalized to the maximal signal obtained after perfusion of 50 mM NH_4Cl ($\Delta F/F_{\text{NH}_4}$). VAMP4-pHluorin spontaneous fusion is partially inhibited by EGTA-AM pretreatment (gray) compared with control (black). **D**, **E**, EGTA-AM treatment reduces the rate of spontaneous fusion of synaptic vesicles containing VAMP4-pHluorin. Mean slope (**D**) and total amplitude (**E**) achieved after 5 min of recording in TTX and folimycin. There is a significant reduction of both parameters in neurons pretreated with 100 μM EGTA-AM (white bars, $n = 423$ puncta from six coverslips) compared with control (black bars, $n = 361$ puncta from six coverslips; * $p = 0.0428$ Kolmogorov–Smirnov test, *** $p = 0.0004$ Kolmogorov–Smirnov test). **F**, top, Schematic representation of a synaptic vesicle containing VAMP4(L25A)-pHluorin. Bottom, Representative image of VAMP4(L25A)-pHluorin fluorescence after 50 mM NH_4Cl . White arrowheads point to putative presynaptic boutons. White bar = 2 μm . **G**, Average fluorescence traces showing that VAMP4(L25A)-pHluorin does not traffic at rest (black trace) and is insensitive to EGTA-AM pretreatment (gray trace). **H**, **I**, EGTA-AM treatment reduces the rate of spontaneous fusion of synaptic vesicles containing VAMP4-pHluorin. Mean slope (**H**) and total amplitude (**I**) achieved after 5 min of recording in TTX and folimycin show a small decay in fluorescence (probably resulting from photobleaching), but no accumulation of fluorescence over time, indicating that VAMP4(L25A)-pHluorin does not recycle spontaneously (black bars, $n = 92$ puncta from six coverslips). There is no further effect of 100 μM EGTA-AM pretreatment (white bars, $n = 154$ puncta from six coverslips; Kolmogorov–Smirnov test: $p = 0.3534$ for **H**, $p = 0.8411$ for **I**).

Figure 4. Clathrin-mediated trafficking is required for VAMP4-mediated Ca^{2+} -dependent excitatory spontaneous neurotransmission. **A**, Representative immunoblot of hippocampal neuron cultures infected with L-307 lentivirus (control), wild-type VAMP4 OE lentivirus or VAMP4(L25A) mutant lentivirus. **B**, VAMP4 protein levels in hippocampal neuron cultures infected with VAMP4 OE or VAMP4(L25A) expression lentivirus [$n = 5$ for control, $n = 4$ for VAMP4 OE, $n = 4$ for VAMP4(L25A)]. **C**, Representative traces of mEPSCs in control or VAMP4 OE neurons preincubated with 0.1% DMSO or 100 μ M EGTA-AM for 15 min. **D**, Average frequency of mEPSCs. EGTA-AM significantly reduced mEPSC frequency in control ($n = 7$) and VAMP4 OE ($n = 8$) neurons, compared with DMSO (control, $n = 8$; VAMP4 OE, $n = 10$; two-way ANOVA: $F_{(1,29)} = 33.88$, $p < 0.0001$, Sidak's multiple comparisons test: control-DMSO vs control-EGTA-AM $p = 0.0007$, VAMP4 OE-DMSO vs VAMP4 OE-EGTA-AM $p = 0.0045$). **E**, mEPSC amplitude was not impacted by EGTA-AM or VAMP4 OE (one-way ANOVA: $F = 0.4017$, $p = 0.7528$). **F**, Representative traces of mEPSCs in control or VAMP4(L25A) neurons preincubated with 0.1% DMSO or 100 μ M EGTA-AM for 15 min. **G**, Average frequency of mEPSCs. EGTA-AM significantly reduced mEPSC frequency in control neurons ($n = 7$), compared with DMSO ($n = 12$). VAMP4(L25A) dominant negative mutant decreased mEPSC frequency ($n = 8$), and EGTA-AM did not further reduce mEPSC frequency in VAMP4 L25A neurons [$n = 8$; two-way ANOVA: $F_{(1,31)} = 4.416$, $p = 0.0438$, Sidak's multiple comparisons test: control-DMSO vs VAMP4(L25A)-DMSO $p = 0.039$, control-DMSO vs control-EGTA-AM $p = 0.003$, VAMP4(L25A)-DMSO vs VAMP4(L25A)-EGTA-AM $p = 0.8571$]. **H**, mEPSC amplitude was not impacted by EGTA-AM treatment or expression of the VAMP4(L25A) mutant protein (one-way ANOVA: $F = 0.2852$, $p = 0.8357$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, nonsignificant.

VAMP4 KD neurons and measured mEPSCs. We found that IKA treatment reduced the frequency of mEPSCs in control neurons but had no effect on mEPSC amplitudes (Fig. 5D–F). However, the reduction of spontaneous release induced by IKA

was occluded by VAMP4 KD (Fig. 5D–F), suggesting that clathrin-mediated trafficking is involved in the maintenance of a pool of VAMP4-containing synaptic vesicles that support spontaneous neurotransmission at excitatory synapses.

Figure 5. VAMP4 spontaneous recycling is clathrin dependent, and it regulates the rate of spontaneous release of excitatory neurotransmitters. **A**, top, Schematic representation of the pHluorin-tagged human transferrin receptor (TFR-pHluorin) probe used. Bottom, Representative images of neurons expressing TFR-pHluorin before (baseline) and after 10-min incubation with exogenous transferrin (25 μ g/ml) to induce clathrin-mediated endocytosis of TFR-pHluorin. Maximal signal was obtained by perfusion of 50 mM NH_4Cl at the end of the experiment. Cultured hippocampal neurons were pretreated with 0 (control) or 10 μ M IKA for 15 min. Control neurons show a decrease in fluorescence of TFR-pHluorin after 10 min of incubation in exogenous transferrin, consistent with stimulation of TFR endocytosis, which is ablated in neurons treated with 10 μ M IKA. TFR-pHluorin fluorescence after treatment with NH_4Cl was relatively equal in neurons treated with IKA or left untreated. White bars = 20 μ m. **B**, Average time courses of TFR-pHluorin signal in neuronal cell bodies. Treatment of control cultured neurons (light gray circles) with exogenous transferrin produces a robust time-dependent decrease in TFR-pHluorin fluorescence due to endocytosis. IKA pretreatment at 10 μ M (black circles) blocks this endocytosis and uncovers a constitutive exocytic component of TFR trafficking as shown by a time-dependent increase in TFR-pHluorin fluorescence. Transferrin was added to the bath after 120 s of baseline recording. **C**, Comprehensive analysis from multiple experiments (control: $n = 227$ puncta from eight coverslips; 10 μ M IKA: 172 puncta from five coverslips) of the peak fluorescence generated after 10 min of exogenous transferrin treatment (ΔF) relative to the peak fluorescence generated after NH_4Cl treatment (F_{NH_4} ; $**p = 0.001$ by unpaired two-tailed t test). Recordings were collected from two independent cultures of neurons. **D**, Representative traces of mEPSCs in control or VAMP4 KD cultured hippocampal neurons preincubated with DMSO or 10 μ M IKA for 15 min. **E**, Average mEPSC frequency. IKA pretreatment significantly reduced mEPSC frequency in control neurons ($n = 10$) compared with 0.1% DMSO (vehicle)-treated neurons ($n = 7$). In VAMP4 KD neurons ($n = 9$), where mEPSC frequency is already decreased, pretreatment with IKA had no further effect ($n = 10$; two-way ANOVA: $F_{(1,32)} = 5684$, $p < 0.0232$, Sidak's multiple comparisons test: control-DMSO vs control-IKA $p = 0.047$; control-DMSO vs VAMP4 KD-DMSO $p = 0.0159$; VAMP4 KD-DMSO vs VAMP4 KD-IKA $p = 0.9981$). **F**, mEPSC amplitude was not impacted by IKA or VAMP4 KD (one-way ANOVA: $F = 1.248$, $p = 0.3088$). * $p < 0.05$; NS, nonsignificant.

VAMP4-mediated spontaneous synaptic vesicle fusion is increased after high-frequency stimulation (HFS)

Our findings support a mechanism of synaptic vesicle recycling that requires VAMP4 and specifically regulates asynchronous and spontaneous release (Raingo et al., 2012). Both functions are critically dependent on the interaction of VAMP4 with clathrin adaptors, implying that segregated recycling of VAMP4 is

essential for the maintenance of this pool of synaptic vesicles. If endocytic structures, i.e., endosomes, generated after asynchronous release are enriched in VAMP4, synaptic vesicles formed from these endosomes may have a higher content of VAMP4 which, considering the results shown in the previous section, would then direct these vesicles to fuse spontaneously under resting conditions. Based on this premise, we would expect that,

synaptic vesicles generated during compensatory endocytosis that follows strong neuronal activity may increase the frequency of spontaneous neurotransmitter release. To test this hypothesis, we used a HFS protocol that was previously shown to induce VAMP4 retrieval (40 Hz, 400 AP; Raingo et al., 2012; Nicholson-Fish et al., 2015) and subsequently measured mEPSCs for 30 min in the presence of TTX (Fig. 6A). Asynchronous release after HFS was measured as the total charge transfer following the last AP of the train (Fig. 6A, left, Q asyn). As previously shown, knocking down VAMP4 lead to a ~50% reduction in asynchronous release after HFS (Fig. 6B). Low-frequency stimulation (1 Hz, 10 AP) did not trigger a significant amount of asynchronous release (Fig. 6B). When compared with the milder control stimulation (1 Hz, 10 AP), the frequency of mEPSCs was increased after HFS (40 Hz, 400 AP) and this increase was sustained up to 30 min (Fig. 6C). In contrast, in VAMP4 KD neurons a smaller acute increase in mEPSC frequency was observed in the initial a few minutes, but this increase was not maintained (Fig. 6C). After 40-Hz stimulation, mEPSC frequency decayed to half of its initial value after 20 min of recording in control neurons, while in VAMP4 KD neurons the half-decay time was 8 min (Fig. 6C). Although both control and VAMP4 KD neurons had increased mEPSC frequency following 40-Hz stimulation (compared with 1 Hz) after 30 min, the magnitude of mEPSC frequency increase was much larger in control neurons. These results suggest that VAMP4 recycling after prolonged neuronal activity can refill a pool of synaptic vesicles that, in the absence of neuronal activity, is prone to fuse spontaneously (Fig. 6D).

Discussion

Here, we demonstrate that VAMP4 drives spontaneous release of synaptic vesicles at glutamatergic synapses in a Ca^{2+} -dependent manner in addition to its role in the regulation of asynchronous neurotransmitter release. We propose a model in which VAMP4, depending on the level of neuronal activity, directs synaptic vesicles to fuse either spontaneously or asynchronously to then be recycled in a clathrin-dependent manner (presumably from synaptic endosomes), thus regenerating and maintaining a specific VAMP4-containing synaptic vesicle pool (Fig. 6D). Our findings reveal a possible mechanism of cross talk between synaptic vesicles that fuse spontaneously and those fusing asynchronously following strong activity, providing a means for synaptic activity to influence spontaneous neurotransmission.

VAMP4 has been shown to selectively maintain asynchronous fusion of synaptic vesicles after strong neuronal activity (Raingo et al., 2012). VAMP4 also regulates activity-dependent bulk endocytosis after HFS (Nicholson-Fish et al., 2015). These pathways of synaptic vesicle release and retrieval are segregated from the recycling of other synaptic vesicle proteins, including syb2 and syt1 (Raingo et al., 2012; Nicholson-Fish et al., 2015), revealing an exquisite mechanism to exclusively maintain VAMP4-mediated neurotransmission. For spontaneous release, however, we found that VAMP4 function requires syb2 expression suggesting that VAMP4 and syb2 may act jointly or in tandem to maintain Ca^{2+} -sensitive spontaneous neurotransmission. Our results also show that VAMP4-dependent fusion of synaptic vesicles in the absence of APs occurs via a pathway that is dependent on Ca^{2+} and the interaction with clathrin adaptors likely through VAMP4's dileucine motif. This latter finding agrees with previous reports on the role of the dileucine motif in VAMP4-driven evoked asynchronous release and bulk endocytosis (Raingo et al., 2012; Nicholson-Fish et al., 2015), pointing

to a shared trafficking mechanism for VAMP4 regardless of the mode of fusion. Taken together with our results, these earlier findings imply that VAMP4 can dynamically be incorporated into distinct pools of synaptic vesicles, suggesting that the segregation between vesicles maintaining asynchronous release versus spontaneous release is altered depending on prior neuronal activity.

VAMP4-driven spontaneous release appears to arise from the same pool of vesicles that is sensitive to intracellular Ca^{2+} buffering by EGTA. While inhibitory spontaneous neurotransmission can be triggered by stochastic opening of voltage-gated calcium channels (VGCC), thus depending on influx of extracellular Ca^{2+} , excitatory spontaneous release seems to be more dependent on intracellular Ca^{2+} sources (Courtney et al., 2018; Williams and Smith, 2018; but also see Eggermann et al., 2011). These differences may in part arise from variations among intrinsic Ca^{2+} buffering capacities of different neuronal and synapse types (Lee et al., 2000; Eggermann et al., 2011; Matthews and Dietrich, 2015). Ca^{2+} release from intracellular stores can generate slower and more diffuse Ca^{2+} waves explaining the susceptibility to EGTA-AM buffering. Taken together with our results, these earlier findings point to an intracellular source as the origin of the Ca^{2+} that triggers VAMP4-dependent spontaneous release and it may also explain the stronger dependence of excitatory spontaneous neurotransmission on VAMP4 compared with inhibitory spontaneous release. Spontaneous release occurs in a stochastic manner, giving rise to a random distribution of interevent intervals. Fast and concentrated Ca^{2+} increases, due to random opening of VGCCs, for example, can trigger a short-lasting spontaneous release phase with short interevent intervals (i.e., high frequency). Slow and diffuse Ca^{2+} increases, on the other hand, may modulate a broader range of spontaneous events. Together with our current notions of the molecular underpinnings of spontaneous release, we can hypothesize that the high-frequency component of spontaneous release at low interevent intervals driven by Vti1a and VAMP7 (Ramirez et al., 2012; Crawford et al., 2017) is triggered by a different Ca^{2+} source than VAMP4-dependent spontaneous fusion, which occurs with a more random and broader distribution of frequencies.

Besides the source of Ca^{2+} for spontaneous neurotransmission, the identity of the Ca^{2+} sensor for spontaneous release is also a matter of debate (Williams and Smith, 2018). One possible Ca^{2+} sensor for the VAMP4-mediated spontaneous fusion may be Syt7, since this protein supports asynchronous release (Bacaj et al., 2013), in a manner similar to VAMP4 function (Li et al., 2017). However, this possibility seems unlikely given that hippocampal neurons lacking Syt7 have no change in the frequency of either excitatory or inhibitory miniature postsynaptic currents (Bacaj et al., 2013). Another candidate is the soluble calcium-binding double C2 domain (Doc2)-like family of proteins, since they have been implicated in promoting both asynchronous and spontaneous release (Groffen et al., 2010; Pang et al., 2011; Yao et al., 2011; Ramirez et al., 2017), similarly to VAMP4. A recent study has shown that Doc2 α specifically mediates spontaneous glutamatergic neurotransmission (Courtney et al., 2018). Whether Doc2 α and VAMP4 act together as part of the same complex for spontaneous release remains to be demonstrated.

Our experiments using the clathrin inhibitor IKA and the L25A mutant form of VAMP4 demonstrated that the regeneration and maintenance of VAMP4-containing synaptic vesicles proceeds through a clathrin-dependent recycling pathway. The strong impact of the VAMP4 L25A mutant on spontaneous

Figure 6. HFS leads to sustained increase in spontaneous release in a VAMP4-dependent manner. **A**, left, Representative traces of (evoked) EPSCs from control and VAMP4 KD hippocampal pyramidal neurons after stimulation at low (1 Hz, 10 AP, 10 s) or high (40 Hz, 400 AP, 10 s) frequency (extracellular recording solution contained 50 μ M PTX and 50 μ M D-AP5). Asynchronous charge transfer (Q asyn) was measured as the area under the curve ranging from the last AP of the stimulation train to the recovery back to baseline. Right, After the stimulation, same extracellular solution containing 1 μ M TTX was perfused for 1 min, and (spontaneous) mEPSCs were recorded for up to 30 min. Time courses of representative recording for the following 29 min are presented for control and VAMP4 KD groups. **B**, Average asynchronous charge transfer (EPSC – Q asyn) for control (black bars, $n=8$) and VAMP4 KD (white bars, $n=7$). There is a $\sim 50\%$ reduction in asynchronous release in VAMP4 KD (unpaired t test with Welch's correction: control, 40 Hz vs VAMP4 KD, 40 Hz $p=0.0339$; 40 vs 10 Hz $p=0.0214$). **C**, Time course of average mEPSC frequency. After HFS (40 Hz, white symbols) both control ($n=8$) and VAMP4 KD ($n=7$) neurons show an acute increase in mEPSC frequency compared with low-frequency stimulation (1 Hz, black symbols; $n=6$ for CTL, $n=5$ for VAMP4 KD). High rate of spontaneous fusion is maintained for up to 30 min in the CTL group (white circles) but not in the VAMP4 KD group (white triangles). Overall, the effect of HFS is dampened in pyramidal neurons lacking VAMP4, indicating that VAMP4 recycling after HFS can determine the subsequent rate of spontaneous release [two-way ANOVA: $F_{(3,428)} = 95.7$, $p < 0.0001$; Tukey's multiple comparisons test: CTL (40 Hz) vs VAMP4 KD (40 Hz) $p < 0.0001$, CTL (1 Hz) vs VAMP4 KD (1 Hz) $p = 0.0006$, CTL (40 Hz) vs CTL (1 Hz) $p < 0.0001$, VAMP4 KD (40 Hz) vs VAMP4 KD (1 Hz) $p < 0.0001$]. **D**, Schematic representation of the proposed cycle of VAMP4 in excitatory presynaptic terminals. VAMP4 directs synaptic vesicles to fuse spontaneously in the absence of neuronal activity. Upon strong stimulation (high levels of neuronal activity), VAMP4 mediates a delayed release of synaptic vesicles (asynchronous). Both functions of VAMP4 occur in manner that is dependent on kinetically slow increases in Ca^{2+} (EGTA-AM sensitive). Regeneration of VAMP4-containing synaptic vesicles is clathrin dependent and necessary to maintain VAMP4-mediated spontaneous and asynchronous neurotransmission. * $p < 0.05$.

release despite its modest expression levels is consistent with the proposal that a small number of vesicular SNARE proteins are sufficient to mediate fusion (Sinha et al., 2011). Whether clathrin-mediated budding of VAMP4 vesicles occurs directly from the presynaptic plasma membrane or from synaptic endosomes remains an open question. Both forms of recycling have been proposed to occur at presynaptic terminals (Milosevic, 2018). We also cannot rule out the possibility that VAMP4's dileucine motif can be important for the interaction of VAMP4 with other proteins involved in synaptic vesicle trafficking through a clathrin-independent pathway. Nevertheless, the increase in VAMP4-mediated spontaneous release after strong stimulation in hippocampal neurons reveals that synaptic vesicles generated from compensatory endocytosis can be enriched in VAMP4 and thus directed to fuse spontaneously. This cross talk between the evoked and spontaneous pool is reminiscent of the long-lasting high-frequency-induced miniature release (HFMR) observed at the *Drosophila* neuromuscular junction (Yoshihara et al., 2005), although whether the underlying molecular mechanisms are shared or not needs further investigation.

The VAMP4-mediated vesicle trafficking pathway discussed here could be a substrate for synaptic plasticity, where previous levels of neuronal activity can shape spontaneous release in the long-term as part of a mechanism to regulate spontaneous release-mediated signaling based on synapses' activity history (see Chung et al., 2010). Accordingly, spontaneous neurotransmission has been implicated in the regulation of neuronal excitability and can trigger different forms of homeostatic synaptic plasticity (Sutton et al., 2006; Kavalali, 2015; Crawford et al., 2017; Andreae and Burrone, 2018; Gonzalez-Islas et al., 2018). Considering that VAMP4 containing vesicles are also important for axonal specification and growth (Meldolesi, 2011; Grassi et al., 2015; Oksdath et al., 2017), one attractive hypothesis to explore would be if VAMP4-mediated fusion and its downstream signaling processes are involved in axonal structural changes and synaptic plasticity in mature synapses.

Collectively, our findings reveal a central role for VAMP4 in the maintenance of Ca^{2+} -dependent spontaneous neurotransmission at excitatory synapses. The identification of VAMP4 as a putative player in mediating cross talk between asynchronous and spontaneous excitatory neurotransmission uncovers a previously overlooked link between the two forms of release.

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